Host Specificity of Vancomycin-Resistant Enterococcus faecium

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Amplified-fragment length polymorphism (AFLP) analysis was used to investigate the genetic relationships among 255 vancomycin-resistant Enterococcus faecium (VREF) strains isolated from hospitalized patients, nonhospitalized persons, and various animal sources. Four major AFLP genogroups (A–D) were discriminated. The strains of each taxon shared ≥65% of the restriction fragments. Most isolates recovered from nonhospitalized persons (75%) were grouped together with all pig isolates in genogroup A. Most isolates from hospitalized patients (84%), a subset of veal calf isolates (25%), and all isolates from cats and dogs clustered in genogroup C. Most isolates from chickens (97%) and turkeys (86%) were grouped in genogroup B, whereas most veal calf isolates (70%) clustered in genogroup D. Therefore, VREF strains are predominantly host-specific, and strains isolated from hospitalized patients are genetically different from the prevailing VREF strains present in the fecal flora of nonhospitalized persons.

During the last 20 years, an increase in antimicrobial resistance among enterococci has been observed. In particular, vancomycin-resistant Enterococcus faecium (VREF) strains are often multidrug-resistant and pose a serious threat in hospital infections, because infections with VREF strains are difficult to treat. In the United States, many hospitals reported a high prevalence of vancomycin resistance, but vancomycin-resistant enterococci seemed to be virtually absent in the community. This is in contrast to Europe, where VREF strains can easily be detected outside hospitals, in nonhospitalized persons and in farm animals. The high prevalence of VREF strains in farm animals in Europe is thought to be the result of the use of the glycopeptide antibiotic avoparcin as an antimicrobial growth promoter. Consequently, VREF strains from animal husbandry may enter the food chain and subsequently spread to humans.

The most prevalent and best-studied vancomycin resistance transposon is Tn1546, which confers high-level resistance to vancomycin and teicoplanin. Detailed molecular analysis of Tn1546 isolated from different animal and human strains has shown considerable DNA polymorphism in Tn1546 and has revealed the presence of common Tn1546 types among animal- and human-derived VREF strains, irrespective of the host strain. In addition, VREF strains from pigs predominantly carried a particular Tn1546 type with a specific point mutation at position 8234, whereas enterococci isolated from poultry predominantly contained Tn1546 subtypes harboring an IS1216V insertion in the vanX-vanY intergenic region. Whether an animal reservoir of VREF actually poses a threat to humans depends on the ability of animal strains to colonize the human gut. In several studies, genetically indistinguishable enterococci have been found in animals and humans, suggesting that animal-derived enterococci may colonize the human gut. Recently, Berchieri et al. showed that ingestion of a VREF strain isolated from a chicken resulted in colonization of his own gut for 20 days.
strains are associated with dogs, which suggests that certain enterococci are host-specific.

Amplified-fragment length polymorphism analysis (AFLP) is a novel technique that allows for the analysis of polymorphism among small restriction fragments [43]. An advantage of AFLP typing is that these small fragments originate from both variable and conserved DNA sequences, thus establishing a degree of genetic relatedness between strains that, by PFGE, would show no similarity at all. AFLP combines restriction enzyme analysis with polymerase chain reaction (PCR) and has been proven successful in studying the molecular epidemiology of various microorganisms [44–52]. In this study, we used AFLP to obtain insight in the genetic relationships among VREF strains isolated from infected patients, nonhospitalized persons, pets, and various farm animals.

Materials and Methods

**Bacterial strains.** Two hundred fifty-five vanA-containing *E. faecium* isolates were analyzed. Eighty-seven were from hospitalized patients from 9 countries (United States: 38, two hospitals; United Kingdom: 24, two hospitals; the Netherlands: 11, two hospitals; France: 6, three hospitals; Israel: 3, two hospitals; Italy: 2, two hospitals; Czech Republic: 1; Germany: 1, Slovak Republic: 1) [7, 53–57], 24 were from nonhospitalized persons from 3 countries (United Kingdom: 3; Germany: 1; the Netherlands: 20) and 11 different cities [4, 9, 14], 12 were from pigs from 2 countries (United Kingdom: 3; the Netherlands: 9) and 12 different farms [4, 11, 58], 10 were from poultry farmers (10 different farms; provided by A. E. J. M. van den Bogaard, University of Maastricht, the Netherlands) and poultry slaughterers (the Netherlands, 1 processing plant, provided by A. E. J. M. van den Bogaard), 31 were from chickens from 2 countries (United Kingdom: 4; the Netherlands: 27) and 29 different farms (22 isolates were provided by A. E. J. M. van den Bogaard) [4, 12]. 10 were from turkey farmers (the Netherlands: 10 different farms) and turkey slaughterers (the Netherlands: 1 processing plant) [9], 7 were from turkeys (the Netherlands: 7 different farms) [9], 9 were from veal calf farmers (the Netherlands: 4 different farms), 60 were from veal calves (the Netherlands: 56 different farms), 5 were from dogs, and 2 were from cats (the Netherlands) [28].

**PFGE analysis of VREF strains.** PFGE typing was done, as described elsewhere [12]. The DNA banding patterns were analyzed with BioNumerics, version 1.5 (Applied Maths, Kortrijk, Belgium). The Dice coefficient of similarity was calculated, and the unweighted pair group method with arithmetic averages was used for cluster analysis.

**Molecular characterization of Tn1546 derivatives.** Characterization of the *vanA* gene-containing transposons was done by a combination of restriction-fragment length polymorphism and DNA sequencing, as described elsewhere [14]. The Tn1546 types and subtypes—A1, A2, A3, B, C, D, E, and F—are characteristic of the *vanA*-containing transposon, Tn1546 [16]. Types A2 and A3 are characterized by the G→T point mutation at position 8234 and the T→C point mutation at position 4847, respectively. Type B transposons are characterized by an IS1216V insertion in the *vanX-vanY* intergenic region, and type C is characterized by a left-end deletion. Types D and E combine the features of type B and C—that is, a left-end deletion and the IS1216V insertion. In addition, type D contains a deletion of the *vanY* gene. Some of the E subtypes contain a deletion of the *vanZ* gene. The F types are characterized by an IS251 insertion in the *vanS-vanH* intergenic region and the G→T point mutation at position 8234. Furthermore, in some, but not all, F types, point mutations at the positions 7658 (T→C) and 9692 (C→T) are found. In this study, the B, D, E, and F types were not subdivided into the different subtypes that have been described elsewhere.

**AFLP analysis of VREF strains.** AFLP, as originally described by Vos et al. [43], is based on the ligation of 2 adapters to genomic restriction fragments, followed by a PCR-based amplification with adapter-specific primers. In this study, we used a single adapter instead of 2, resulting in self-ligation of the digested DNA, because the adapter will ligate to the cohesive ends generated by the 2 restriction enzymes. The main advantages of using a single adapter are less variation in peak intensities and improved reproducibility [44].

DNA was isolated, as described elsewhere [14], with the addition of a final ethanol precipitation step to further purify the DNA. The EcoRI-CfoI adapter used in this study was prepared by mixing 2 oligonucleotide solutions (2 µM each), heating for 5 min at 95°C, and allowing the mixture to cool for 10 min at room temperature. The structure of the EcoRI-CfoI adapter was as follows: 5′-AATTGTAAAAACGCGCCAGTAACG and 5′-CTTTTCGCGCCTGGTCATT-5′ (complementary sequence is underlined). For restriction ligation, a 5-µL mixture consisting of 2× One-Phor-All buffer (Amersham-Pharmacia Biotech, Uppsala, Sweden), 2 µM ATP, 5 µL of EcoRI, 1 µL of CfoI, 1 µL of T4 DNA ligase, and 0.8 µM adapter was prepared. After addition of 5 µL (10 ng) of genomic *E. faecium* DNA, the mixture was incubated for 2 h at 37°C, to allow simultaneous restriction and ligation. This resulted in the formation of circularized DNA molecules. For amplification, 90 µL of TE (20 mM Tris, 0.1 mM EDTA, pH 8.0) was added to the restriction ligation mixture, and, subsequently, 2 µL of this mixture was mixed with 0.25 µL (10 µM) of each AFLP primer (primer 1 [CfoI-G]: 5′-CGACGCGCCGTTAAACGCAG and primer 2 [EcoRI-A]: 5′-GGCCGCTGTTTTACAATTCA-3′) and 7.5 µL of AFLP amplification core mix (PE Biosystems, Foster City, CA). Primer 1 contained an extra selective base, G, and was labeled with the blue fluorescent dye 5-carboxyfluorescein. Primer 2 contained an extra selective base, A. PCR was done on a thermal cycler (model 9600; PE Biosystems). After the PCR mixture had been heated for 2 min at 94°C, it was used for amplification by means of a “touchdown” PCR program as follows: 30 cycles of a 20-s denaturing step at 94°C, a 30-s annealing step (see below), and a 2-min extension step at 72°C, followed by incubation at 60°C for 30 min. The annealing temperature during the first cycle was 66°C and decreased 1°C at each cycle during the next 9 cycles. During the remaining 20 cycles, an annealing temperature of 56°C was used.

The amplification products were separated on a 36-cm, 5% denaturing sequencing polyacrylamide gel (Long Ranger Singel Pack; FMC Bioproducts, Rockland, ME) on a DNA sequencer (ABI PRISM 377; PE Biosystems). For this, 1 µL of reaction mixture was mixed with 1.25 µL of formamide, 0.5 µL of loading buffer.
Figure 1. Amplified-fragment length polymorphism (AFLP) patterns and dendrogram of 25 vancomycin-resistant Enterococcus faecium strains, typed by AFLP and pulsed-field gel electrophoresis (PFGE). Numbers on horizontal axes indicate % similarity, as determined by Pearson product-moment correlation coefficient and unweighted pair group method, with arithmetic averages for AFLP typing, and by Dice and unweighted pair group method, with arithmetic averages for PFGE typing. HP1–12, hospitalized patients; NHP1–5, nonhospitalized persons; P1–3, pigs; C1–5, chickens. A, B, and C in left dendrogram represent 3 genogroups. Dotted line depicts 95% similarity coefficient, above which strains were considered to be of identical AFLP type.

Results

Comparison of AFLP analysis with PFGE. In a pilot experiment, 25 VREF strains isolated from human patients, nonhospitalized persons, pigs, and chickens were subjected to AFLP analysis and PFGE. The number of AFLP bands with sizes of 50–500 bp was 13–37, with an average of 27 bands (figure 1). AFLP typing was found to be highly reproducible. The degree of similarity between quadruplicates was 95%–99% (data not shown). The strains clustered into 3 distinct AFLP groups (figure 1). Group A contained strains from nonhospitalized persons and pigs, group B from chickens, and group C from hospitalized patients. This apparent host-specific grouping was less distinct by PFGE typing (figure 1). Furthermore, the strains originating from a given host were more dissimilar by PFGE than by AFLP analysis, with the exception of strains HP3–HP12, which were recovered from a hospital outbreak [55]. These strains showed highly similar or identical PFGE patterns (similarity >82%) and have been considered to belong to a single clone [55]. As with PFGE typing, the strains from the hospital outbreak also showed identical AFLP patterns (similarity >97%). In addition, 2 strains from nonhospitalized persons (NHP2 and NHP3) were indistinguishable by both PFGE and AFLP. The number of bands in PFGE patterns was 11–16, which is considerably less than in the AFLP patterns. Therefore, genomic changes in only a few restriction fragments may result in disproportionate differences in the PFGE banding patterns.

Genogrouping and association with source of isolation. Two hundred fifty-five VREF strains recovered from different human and animal sources were subjected to AFLP typing. The strains from hospitalized patients were isolated from different sites, such as stool, blood, pus, urine, and ascites (table 1).

The result of the grouping by AFLP of these VREF strains is shown in figure 2. Four main groups (groups A–D) were discernible, and the strains within each group shared ≥65% of
their restriction fragments. As described above, in the pilot experiment, grouping of the strains by AFLP was clearly associated with the source of the strains. Most of the 87 strains from hospitalized patients (84%) clustered in genogroup C (figures 2, 3). Within this genogroup, 2 subgroups, C1 and C2, were discerned, each containing strains with indistinguishable AFLP banding patterns (similarity >95%). Group C1 strains were isolated during a 32-month period at 6 Detroit metropolitan area hospitals [54], and group C2 strains were isolated during a 3-month period at the John Radcliffe Hospital in Oxford, United Kingdom [55]. Strains in subgroups C1 and C2 are most likely repetitive isolates of a single strain. The same is probably true for 2 sets of 2 strains in genogroup B and for 3 other sets of 2 strains in genogroup C, which have indistinguishable AFLP patterns (similarity >95%) and originated from the same hospital. When these repetitive isolates were counted only once, 74% of the strains from hospitalized patients clustered in genogroup C. Group C strains also comprised some isolates from veal calves and all 5 isolates from cats and dogs. No association was found between the body site of the hospitalized patients from which the strains were recovered and the grouping of the strains (table 1).

Unexpectedly, most isolates recovered from the feces of non-hospitalized persons (75%) were grouped in a different genogroup, group A. Furthermore, half the isolates from poultry farmers or slaughterers and 70% of the isolates from turkey farmers or slaughterers were in this group (figures 2, 3, A). All isolates from pigs were also grouped in genogroup A. Two subgroups, A1 and A2, were distinguished, and either subgroup comprised both pig and human isolates indistinguishable by AFLP (similarity >95%). Genogroup B comprised mainly strains originating from poultry; 97% of the chicken and 86% of the turkey isolates. Interestingly, a large percentage of isolates from poultry farmers or slaughterers were found in taxon B, as well (figures 2, 3, A). Finally, genogroup D comprised exclusively strains from veal calves (70% of the calf strains) and veal calf farmers (figures 2, 3, A). About half the genogroup D strains were similar, and the human isolates were identical to ≥1 of the calf strains.

**Table 1.** Isolation sites and genogrouping of vancomycin-resistant *Enterococcus faecium* strains recovered from hospitalized patients.

<table>
<thead>
<tr>
<th>Genogroup</th>
<th>Ascites</th>
<th>Bile</th>
<th>Blood</th>
<th>Catheter</th>
<th>Nasal</th>
<th>Skin</th>
<th>Urine</th>
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<td>4</td>
<td>2</td>
<td>29</td>
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Association between AFLP types and Tn1546 types. The Tn1546 types of all 255 VREF strains were determined (figure 3B). The Tn1546 (sub)types A1, A2, and B have been found elsewhere in strains from humans, pigs, and poultry [9, 12, 14, 17, 18, 20, 21, 25]. Strains with these Tn1546 types were found among 3 or among all 4 *E. faecium* genogroups, suggesting horizontal spread of the VanA transposon among genetically different enterococci (figure 3B). Strains with transposon types C and E were found in half the genogroups. In contrast, 3 transposon types, A3, D, and F, were confined mainly to a single AFLP genogroup, and these strains were also closely associated with specific hosts. Transposon types D and F were found mainly in genogroup C VREF strains from hospitalized patients, suggesting clonal expansion (figure 3B). Type A3 was restricted to isolates of genogroup D, and these originated from veal calves and veal calf farmers. Remarkably, no A3 transposon types were found in veal calves present in genogroup C. This suggests the existence of 2 separate VREF subtypes in veal calves.

**Discussion**

This study shows that particular *E. faecium* genogroups are associated with particular hosts and environments, such as farms and hospitals. We limited this study to vancomycin-resistant isolates. Therefore, the results may differ for drug-susceptible isolates.

Although many studies of the epidemiology of vancomycin-resistant enterococci have made use of molecular typing, only a few studies have suggested the existence of host-specific ecovars. On the basis of slight differences in *E. faecium* isolates, Devriese and colleagues [41, 42] have suggested that species-specific variants occur among dogs and poultry. To our knowledge, the study presented here is the first systematic study showing an association between host species and VREF strain genogroup. The disclosure of such ecovars has probably been hampered by the use of typing methods that were too limited in the degree of strain differentiation, such as ribotyping [32, 34], or by the use of methods, such as PFGE, that discriminate well but do not adequately establish the degree of genetic relatedness between dissimilar strains [34, 35, 38].

The AFLP method used in this study permits a high degree of strain differentiation, because of the large number of restriction fragments analyzed and the establishment of genetic relatedness among dissimilar, non–epidemiologically related strains made possible by the presence of shared restriction fragments of evolutionarily more-conserved DNA stretches. Comparison of AFLP with PFGE for 25 VREF isolates, including VREF strains isolated during a hospital outbreak of VREF.
Figure 2. Abridged dendrogram of all vancomycin-resistant Enterococcus faecium (VREF) strains and separate dendrograms of strains belonging to each of 4 genogroups. Symbols depict VREF strains from various sources. A1, A2, C1, and C2 depict subgroups of genogroups A and C. Numbers on horizontal axis indicate % similarities, as determined by Pearson product-moment correlation coefficient. Vertical dashed lines indicate 95% similarity coefficient, above which strains were considered to be of identical amplified-fragment length polymorphism type.
infections, revealed that the degree of strain differentiation and the identification of outbreak strains by AFLP typing is comparable with that of PFGE typing.

Four distinct genogroups among 255 VREF isolates were disclosed in this study, and strains within each group shared two-thirds or more of their restriction fragments. The use of restriction enzyme combinations other than EcoRI-CfoI led to a similar grouping (R.J.L.W., unpublished data). The strongest association between host and genogroup was found among strains of genogroup D: virtually all genogroup D strains were from calves and a few were from veal calf farmers. Therefore, the host range of strains of this type is restricted mainly to calves. Although the host range of strains from the remaining 3 genogroups seems broader, these also exhibited a strong association with the source. Strains from chickens and turkeys were found almost exclusively in genogroup B, all pig strains were found in genogroup A, and all 5 strains from cats and dogs were found in genogroup C. Recently, Van den Braak et al. [12] distinguished poultry-specific PFGE types that were not found in humans.

The most unexpected finding in this study is the apparent dichotomy between VREF strains isolated from nonhospitalized persons and those isolated from hospitalized patients. The tight genetic clustering of strains from hospitalized patients is even more surprising because these strains were collected from geographically diverse locations (Europe, Israel, and the United States). The strains isolated from hospitalized patients were clustered mainly in genogroup C, whereas those isolated from nonhospitalized persons without VREF infection were mainly of genogroup A. This difference cannot easily be explained by only a difference in the habitat in the human body, because we found no significant difference in the distribution among the genogroups of hospital strains from fecal origin or from other infected body sites, such as blood or urine (table 1). A possible explanation for the observed dichotomy is that, in the hospital environment, a subset of VREF strains is being selected that is normally present in low numbers in the human gut. Although the nature of this selective force is not known, it may involve the selection of strains that are more resistant to antibiotics or the selection of strains with specific virulence traits. Various studies suggest that bacteriocins, cytolsins, and hemolysins are more prevalent among enterococci from infected hospitalized patients than among fecal isolates from healthy persons [59–61]. Furthermore, most of the E. faecalis strains harboring the putative virulence gene esp, which encodes a surface antigen, are infection-derived [62]. It is unknown, however, whether esp-positive strains belong to a genetically well-defined taxon, such as the E. faecium genogroup C disclosed in this study. Interestingly, all isolates from cats and dogs and 25% of the veal calf isolates grouped in genogroup C, which may suggest that pet animals and veal calves are a potential source of VREF strains for hospitalized patients.

Molecular typing of Tn1546 in the VREF strains analyzed in this study shows that the various VanA transposon variants are not randomly distributed among the 4 main VREF genogroups identified. The transposon types A3, D, E, and F are predominantly found in only 1 genogroup, thus exhibiting a high degree of host specificity. Transposon types A1, A2, and B seem to be more promiscuous, because these are found in most VREF genotypes. This finding confirms our previous findings. In previous studies, types D and F transposons have been found only in hospitalized patients in the United Kingdom and United States, respectively [14], and the E type transposons were found predominantly in poultry [9, 14]. In contrast, types A1, A2, and B transposons were found in various animals, humans, and other sources, including nonhospitalized persons, hospitalized patients, pigs, veal calves, chickens, sewage, turkeys, turkey farmers or slaughterers, a duck, and a pony.

One of the aims of the present study and our previous studies [9, 14] was the assessment of the contribution of animal husbandry to the occurrence of vancomycin resistance in humans. In the fecal flora of poultry farmers and slaughterers and veal calf farmers, we found VREF genotypes that were specific for the animal hosts—poultry and veal calves, respectively. This suggests that VREF strains from animals are transmitted to humans. Because strains of genotypes B and D were not or only rarely found among the general population, these animal-specific strains may colonize humans only transiently. This is
in contrast to strains of genotype A. All pig strains were of this genegroup, and most strains from nonhospitalized persons were also of genegroup A. Furthermore, various pig strains were indistinguishable by AFLP from human strains. These data strongly suggest that, in the community, VREF strains in humans mainly originate from pigs in which a high level of glycopeptide resistance has been observed [2, 11, 15]. This idea is consistent with observations by others that humans and pigs may harbor VREF strains with identical PFGE types [17, 26]. The predominance of piglike VREF strains among humans in the community is the result of exposure and survival in the gut. It is presently unclear which of these factors is critical in the ecology of VREF.

Thus far, molecular comparison of human- and pig-derived enterococci has been done only on VREF strains. Therefore, further studies also including drug-susceptible enterococci are needed to analyze more extensively the populations of the predominant flora in humans, pigs, and other animals.

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References


