Stability of Vancomycin-Resistant Enterococcal Genotypes Isolated from Long-Term–Colonized Patients

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Genotypic variation and stability of isolates of vancomycin-resistant enterococci (VRE) were studied to determine genetic diversity and whether strain definition based on pulsed-field gel electrophoresis (PFGE) is applicable to an endemic setting. Twenty-two PFGE types were identified among 455 VRE isolates. One-on-one comparisons of 10 *van*A *Enterococcus faecium* strain types all yielded >10 band differences. Variations among *van*A and *van*B *E. faecium* isolates from individual long-term–colonized (4–160 days) patients yielded <3 band differences for >85% of comparisons. Comparison of all strains without grouping by vancomycin resistance types yielded two peaks of band differences: one with <3 and one with >10 band differences. These data show that VRE isolates were genetically closely related or very different; demonstrate that within individual patients, VRE isolates show little genetic variation; and provide empirical evidence that PFGE can be used to study the epidemiology of VRE endemicity.

The prevalence of colonization and infection with vancomycin-resistant enterococci (VRE) has increased dramatically in recent years [1], and VRE are now endemic in many hospitals in the United States [2–4]. Epidemiologic studies that include genotyping are essential for understanding the epidemiology of VRE and for designing interventions to prevent further spread of VRE. Pulsed-field gel electrophoresis (PFGE) is one of the most widely used techniques for genotyping VRE. Recently, recommendations were made for interpreting PFGE patterns of bacterial isolates obtained during nosocomial outbreaks [5]. Based on theoretical constructs about the number of possible genetic events, classes of identity were defined according to the number of band differences between isolates. However, since these recommendations were designed specifically to analyze small sets of isolates (typically ≤30) from putative monoclonal outbreaks, it is unclear how they apply to the interpretation of isolates from wards where study species, such as VRE, are endemic. In addition, it is unknown to what extent individual strains of VRE show genetic diversity over time. To address these questions, we studied the genotypic variation of multiple isolates of VRE and genotypic stability of related strains in long-term–colonized patients over a period of 26 weeks in a medical intensive care unit (MICU) where colonization with VRE was endemic.

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Material and Methods

Setting and study design. Cook County Hospital is a 900-bed public teaching hospital with a 16-bed MICU. As part of ongoing infection control efforts, rectal cultures were obtained daily from all patients who had been admitted for at least 48 h during two periods totaling 26 weeks (26 October 1994–7 March 1995 and 11 April 1995–29 May 1995). During the latter period, daily cultures also were obtained from multiple body sites (groin, arm, oropharynx, trachea, and stomach). Infection control and epidemiologic data derived from these patients have been published elsewhere [2, 4].

Surveillance culturing. Cultures obtained by swabs (Culturette System; Becton Dickinson Microbiology Systems, Cockeysville, MD) were inoculated onto agar (Enterococcosel; Becton Dickinson) supplemented with 6 μg/mL vancomycin. Plates were examined after 48 h of incubation at 35°C. One isolate in the first study period and all morphotypes (or up to 3 randomly chosen colonies when only 1 or 2 morphotypes were observed) in the second study period were isolated from each culture for further analysis. Isolates were identified to the species level (API 20 STREP system; bioMerieux Vitek, Hazelwood, MO) and by motility and pigmentation. Antibiotic susceptibility testing was done with standard disk diffusion and agar dilution methods [6–8].

Genotype analysis. Total genomic DNA was digested with *Sma*I (GIBCO BRL, Grand Island, NY) or *Apa*I (Sigma, St. Louis) and subjected to PFGE in a 1% agarose–0.5× Tris-borate-EDTA gel (CHEF-DRIII apparatus; Bio-Rad Laboratories, Richmond, CA) as previously described [9]. The pulse time was ramped from 1 to 20 s. Restriction digestion profiles were analyzed by two independent observers' visual inspection of photographs of ethidium bromide–stained gels; gels were also digitized and compared (Paperport 3.0.1 for Windows software; Visioneer, Woodland Hills, CA) to facilitate visual inspection. *Enterococcus faecalis, Enterococcus faecium, Enterococcus gallinarum,* and *Enterococcus avium* were screened directly for *van*A and *van*B by the polymerase chain reaction (PCR) by use of intragenic primers derived from the published gene sequences [10, 11]. *E. gallinarum* strains were also screened for *van*C1 by PCR [12].

Table 1. Strain types of vancomycin-resistant enterococci and no. of patients and (patient-days) colonized with each type during study periods.

Strain type	Vancomycin resistance type*	No. of patients colonized (no. of patient-days)
E. faecium 1	A	13 (71)
E. faecium 2	B	5 (69)
E. faecium 3	B	4 (53)
E. faecium 4	A	14 (211)
E. faecium 5	A	1 (6)
E. faecium 7	B	13 (85)
E. faecium 11	B	1 (21)
E. faecium 12	B	1 (3)
E. faecium 13	A	24 (229)
E. faecium 14	A	9 (106)
E. faecium 15	A	10 (132)
E. faecium 16	A	3 (41)
E. faecium 18	A	2 (10)
E. faecium M11	B	1 (7)
E. faecium M14	A	1 (9)
E. faecium M15	A	1 (1)
E. faecium M3	B	1 (1)
E. faecalis 1	B	7 (87)
E. gallinarum 1	C1	1 (6)
E. gallinarum 2	C1 and A	1 (8)
E. gallinarum 3	C1	1 (3)
E. avium	A	3 (39)

NOTE. Isolates are listed in temporal order of appearance within each species. Isolates were considered unique strain types if they differed from all other isolates by >6 bands after *Sma*I digestion and PFGE.

* Vancomycin resistance type was determined by polymerase chain reaction and primers specific for *van*A, *van*B, *van*C1 genes.

Comparison of genotypes. Isolates were grouped according to species and the presence of the *van*A, *van*B, or *van*C1 genes (vancomycin resistance types). All available isolates of the same species and vancomycin resistance type from an individual patient were compared one on one, and for each comparison, the number of different bands in the PFGE pattern was calculated. We then grouped isolates (strain types) according to published guidelines for evaluation of epidemic isolates [5]. An isolate was considered a unique strain type if it differed from other isolates by >6 bands.

Endemic variation in time. The prevalence of VRE colonization was expressed as the number of days on which patients were colonized divided by the total number of patient-days in the unit. The number of days on which patients were colonized was calculated for each strain type of VRE, and the number of patients colonized with each different strain type per week was calculated.

Results

Patients. A total of 219 newly admitted patients were studied during the two periods (181 patients in the first and 38 patients in the second); 106 patients were colonized with VRE at some time during their stay and form the basis of the following analyses.

Strain type stability. In total, 455 isolates of VRE were typed by PFGE, and 22 different strain types were found (table 1): 17 strain types of *E. faecium,* 1 strain type of *E. faecalis,* 3 strain types of *E. gallinarum,* and 1 strain type of *E. avium. E. faecium* with the *van*A resistance type represented the majority of isolates. Representative PFGE profiles of each of the 10 *van*A *E. faecium* strain types (figure 1) from a total of 21 patients were compared one on one. All comparisons yielded >10 band differences.

The variations of *Sma*I-digested PFGE patterns among different *E. faecium* isolates with the *van*A resistance type from individual patients were analyzed for 67 isolates from 9 patients. To provide a second measure of genetic similarity of the isolates, a subset of 50 of these isolates digested with *Apa*I was also analyzed. Each series represented consecutive isolates from one or more body sites from a single patient, yielding 5–76 one-on-one PFGE profile comparisons for each of the patients and totals of 281 and 146 one-on-one comparisons for isolates digested with *Sma*I and *Apa*I, respectively. The time span over which each patient's isolates were recovered ranged from 4 to 160 days. For 7 patients, the series of isolates from rectal cultures was used, representing a mean time span of 53 (range, 21–160) days per patient. Persistent colonization of the groin and arm were studied in 5 and 3 patients, respectively; the mean time spans for these patients were 14 (range, 9–25) days and 7 (range, 4–9) days, respectively. The maximum number of band differences observed within a patient's series was 4, and for both restriction enzymes, >50% of comparisons showed no band differences and >85% of the comparisons showed <3 band differences.

Comparable observations were made for *E. faecium* isolates with the *van*B resistance type; 33 isolates from 4 patients were digested with *Sma*I and 30 with *Apa*I. All comparisons for

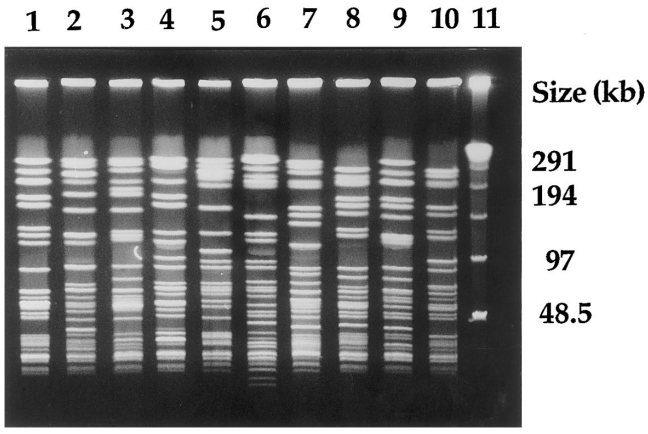

Figure 1. PFGE gel of representative examples of 10 different strain types of *Enterococcus faecium* with *van*A-type resistance following *Sma*I digestion of total genomic DNA. Examples of each unique strain type are shown in lanes 1–10. Lane 11: λ concatemers used as molecular DNA markers.

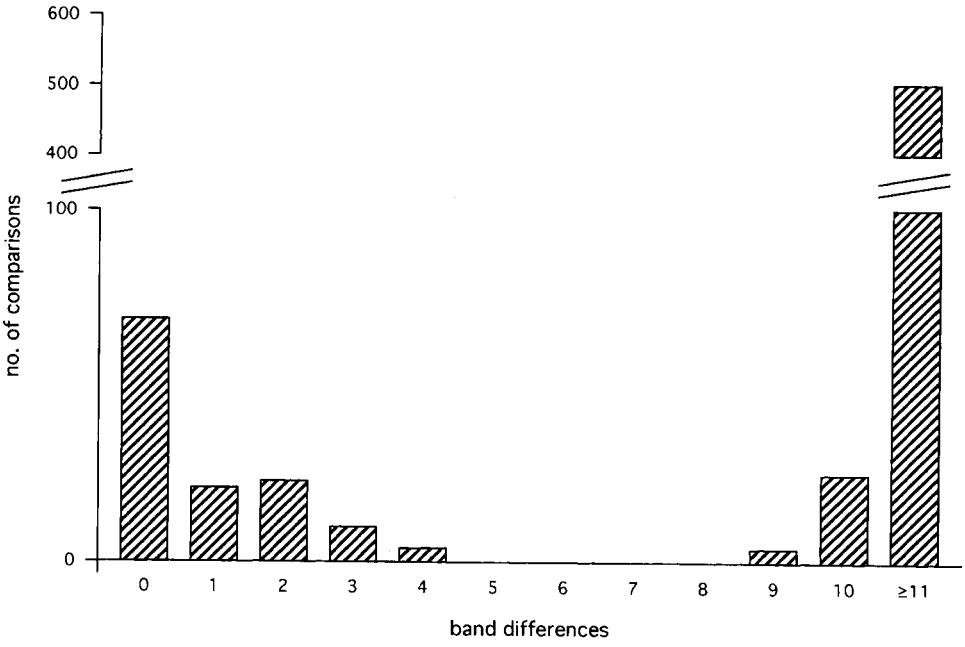

Figure 2. Number of band differences following *Sma*I digestion and PFGE in one-on-one comparisons of 455 vancomycin-resistant enterococcal isolates. Total numbers of comparisons with >10 band differences are grouped together.

*van*B *E. faecium* strains (57 for *Sma*I and 39 for *Apa*I) yielded ≤1 band difference.

When all VRE isolates were compared one on one, without grouping initially by vancomycin resistance types, the distribution of band differences demonstrated two peaks: the first, a large group of comparisons with <3 band differences, and the other peak, comparisons with ≥10 band differences (figure 2).

Prevalence of strain types in MICU. The number of different strain types present in the MICU per week varied from 3 to 10. VRE were isolated on 674 (36%) of 1856 patient-days in the first study period and on 384 (50%) of 764 patient-days in the second study period. Since several patients were colonized with >1 strain type simultaneously, the actual number of patient-days for the different strain types of VRE (n = 1206) was higher than the number of patient-days for VRE colonization (n = 1058). Seven strain types each were isolated from ≥6 patients, and these strains accounted for 914 (76%) of 1206 patient-days. The prevalence of these 7 types varied widely over time (figure 3). For example, Fm4 colonized 7 patients at study onset and remained present in the unit throughout the first study period but was less common during the second period. In contrast, Fm14 was not detected until the end of the first study period and then colonized up to 6 patients simultaneously in the second study period.

Discussion

In the present study, 455 isolates of VRE were genotyped, and multiple one-on-one comparisons of PFGE band patterns were made. In all, 22 different strain types were found. Strain types were either very similar (≤4 band differences), indicating that these isolates were genetically indistinguishable, closely related, or possibly related, or very different (≥10 band differences). Similar results were obtained whether or not isolates were initially categorized by species or vancomycin resistance type. We believe that the results of this study provide empiric evidence, at least for VRE, supporting the theoretical concept that unique strain types of nosocomial bacterial isolates can be distinguished on the basis of the number of intercurrent genetic events inferred from observed differences in PFGE band patterns [5]. In addition, our results demonstrate that these guidelines [5] can be used to study the epidemiology of large numbers of isolates in endemic settings.

Our data show that the endemic situation in our MICU was characterized by the occurrence of multiple genotypes with little genetic resemblance. These results complement those of Morris et al. [3] and Chow et al. [13], who also demonstrated endemicity with multiple genotypes of VRE in US hospitals, and the results from other investigators demonstrating intrahospital spread of VRE [14–17]. Moreover, interhospital spread of VRE has been reported as well [13, 15, 16, 18]. We have also demonstrated that within individual patients, strains of VRE showed little genetic variation. The time span for our comparisons was up to 160 days, which probably represents thousands of in vivo bacterial replications in the presence of selective or competing forces, such as other microorganisms and antibiotics. Persistence of rectal VRE colonization for months has been reported [19–21], but the question of genetic variation of VRE in these patients has not been studied extensively. In a smaller study, Montecalvo et al. [21] analyzed 11 perianal isolates and 3 bloodstream isolates collected from patients on an oncology ward over periods of 1 week to 14

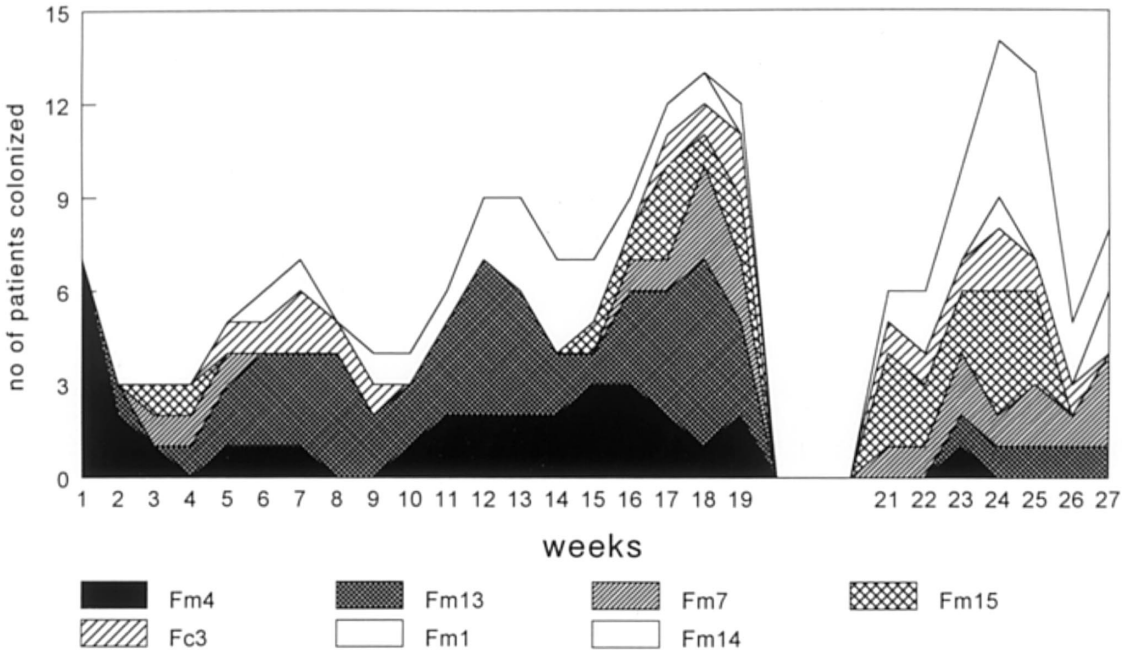

Figure 3. Prevalence over 25 weeks of 7 most common strain types of vancomycin-resistant enterococci.

months. Three patients had identical strains detected at intervals of 7 days, 77 days, and 5 months, and 1 patient was shown to be colonized at two time points with different strains. A fifth patient had 3 strains identified among 5 isolates obtained over 14 months. In another investigation, PFGE analysis of 8 VRE isolates obtained from 8 patients over a period of 5 months in Italy demonstrated minor differences in the endemic strain [22]. Whether individual strain types of VRE will remain stable over a period of years remains to be established.

Variations in PFGE patterns of 4–6 bands may hamper correct interpretation in epidemiologic studies (e.g., genetically linked strains may be interpreted as not linked epidemiologically and vice versa). We found that only 3 strains of *E. faecium* with *van*A resistance type and none of the isolates with the *van*B resistance type differed by 4 bands, suggesting that this degree of PFGE pattern variability is not an important problem when studying VRE. However, recently Thal et al. [23] demonstrated that in vitro introduction of a transposon at multiple sites in an *E. faecalis* chromosome resulted in a PFGE pattern that differed by as many as 7 bands from the original strain. Similarly, we [24] and others [25] have detected up to 6 band differences on PFGE gels between recipient and transconjugant strains of VRE after mating experiments that transfered *van*B and *van*A, respectively, to the chromosome of recipient *E. faecalis* strains. The results of the current study, that is, that few strains differ by 4–8 bands, suggests that such events may occur infrequently among clinical isolates in nature.

Our observations of nosocomial enterococci seem comparable to findings of genetic diversity of *Pseudomonas aeruginosa* persistently colonizing patients with chronic bronchiectasis [26] and cystic fibrosis [27]. Among 166 isolates of *P. aeruginosa* collected from 31 cystic fibrosis patients over a 1- to 20-month period, Struelens et al. [27] showed that the genomic PFGE patterns of sequential isolates from individual patients had a similarity of $\geqslant$80%. Furthermore, isolates with different genotypes differed by 14–45 bands. When comparing all isolates one on one, they also found two clusters of band differences; one cluster with 0–3 band differences had ''identical'' genotypes, and a large cluster with $>$10 band differences was considered to represent completely different and genetically unrelated genotypes.

There are potential shortcomings in PFGE analysis. Because bacterial DNA analyzed by PFGE is typically digested with a restriction endonuclease that recognizes a DNA sequence present infrequently in the bacterial genome, differences between strains in large portions of the genome that are not recognized by the enzyme will remain undetected. We repeated PFGE for a subset of strains while using a second restriction enzyme to improve discriminatory power. However, silent or cryptic genetic changes may have occurred over time that would have been detected only with a more sensitive method, such as direct DNA sequencing.

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