Chapter 4

Comparison of Multiple-Locus Variable-Number Tandem Repeat Analysis and Pulsed-Field Gel Electrophoresis in a Setting of Polyclonal Endemicity of Vancomycin-Resistant *Enterococcus faecium*.

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Abstract

Genotyping of vancomycin-resistant *Enterococcus faecium* (VREF) isolates is important to study its epidemiology in hospitals. Although pulsed-field gel electrophoresis (PFGE) is considered the "gold standard" genotyping method for VREF, it is labor intensive and lacks standardized methods and interpretative criteria, thereby hampering interlaboratory data exchange. Multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA) is a rapid, PCR-based typing scheme that yields an unambiguous numerical result suitable for data exchange between laboratories. In order to assess whether MLVA could replace PFGE, we compared the typeability, discriminatory power, concordance and costs of these methods for VREF isolates obtained from patients, environmental samples and health care worker (HCW) hands in a medical ICU where VREF are endemic. Over a 58-day period, 393 VREF isolates were collected from cultures of patient rectal swabs (77), the environment (268) and HCW hands (48). For PFGE, 358 (91.1%) isolates were typeable, yielding 19 PFGE types (> 6 bands different) and 24 subtypes (1-3 bands different). MLVA typed 391 (99.5%) isolates, generating 11 genotypes. The discriminatory power of PFGE subtypes was 83%, compared to 68% for MLVA. Concordance between the 2 methods based on matched or mismatched MLVA-types and PFGE-types or -subtypes was 67.5% and 82.8%, respectively. By PFGE, 13 isolates could be genotyped in 3 days at an estimated cost of $10 per isolate; MLVA genotyped 94 isolates in 2 days at $11 per isolate. PFGE and MLVA were highly concordant in assigning genotypes to nosocomial VREF isolates. MLVA was faster but PFGE subtyping was more discriminatory.

Introduction

Since 1989, vancomycin-resistant *Enterococcus faecium* (VREF) have emerged as nosocomial pathogens in the United States (U.S.) (3), especially in immunocompromised patients (14). VREF are now endemic in many hospitals (3). Recently multilocus sequence typing (MLST)-based molecular epidemiological studies of both human and animal derived vancomycin-resistant and vancomycin-susceptible *E. faecium* isolates revealed the existence of host-specific genogroups. A specific genogroup, labeled clonal complex-17 (CC17), is associated with nosocomial outbreaks and infections on five continents and characterized by ampicillin- and quinolone-resistance and the presence of the *esp* virulence gene (10,11,20,21).

Genotyping of isolates is important to study the epidemiology of VREF in hospitals. So far, pulsed-field gel electrophoresis (PFGE) has been considered the

"gold standard" method for genotyping of enterococci (9,13,15,17,19). However, PFGE is labor-intensive, and lacks standardized methods and strict criteria for interpretation of banding patterns, thereby hampering interlaboratory data exchange. Multiple-locus variable-number tandem repeat analysis (MLVA) is based on differences in the variable number of tandem repeats (VNTR) on multiple loci on the chromosomes of bacteria, which can rapidly be detected by PCRs with specific primers based on the flanking regions of the tandem repeats. Because MLVA types are discriminated by gain or loss of discrete repeats, this method yields an unambiguous numerical result suitable for data exchange between laboratories via internet (18).

To determine whether PFGE can be replaced by MLVA, we compared typeability, discriminatory power, concordance, cost and turn around time of these two methods for VREF isolates obtained from patients, environmental samples and health care worker (HCW) hands in a medical ICU where VREF colonization is endemic.

Materials and methods

Bacterial isolates

Between March and May 2001 VREF were isolated from patient rectal swab specimens, samples of healthcare worker hands and of swab samples of environmental sites in the 21-bed medical intensive care unit (MICU) at Rush University Medical Center, Chicago, Illinois, USA. This 58 day period was part of a larger intervention study to reduce the spread of VREF and of other species of vancomycin-resistant enterococci (5).

PFGE

PFGE typing of *Sma*I-digested DNA was performed as described by Kim et al. (7). The assignment of PFGE-types (PT) was adapted from the Tenover criteria (17). Isolates that differed by ≤ 6 bands were assigned the same PT and isolates that differed by ≥ 7 bands were considered different types. Within each PT, isolates that differed by 1-3 bands were assigned the same subtype (PST).

MLVA typing

MLVA typing was performed as described previously (18), with the following minor modifications. PCR on *ddl*, VNTR-1, -7, -8, -9 and -10 was performed using HotStarTaq master mix (Qiagen inc., Valencia, CA, USA), while PuRe Taq Ready-To-Go PCR beads (GE healthcare Bio-sciences AB, Uppsala, Sweden) were used for VNTR-2. The PuRe Taq Ready-To-Go PCR beads were dissolved in 20 µl MilliQ water including 10 pmol of both the forward and reverse primer for VNTR-2 and finally 5 µl of lysate was added. Furthermore, PCR programs for

VNTR-8 and VNTR-9 were similar to VNTR-7 and VNTR-10, while for VNTR-2 the extension time was prolonged to 2 minutes, instead of 30 s, at 72°C.

Identification of CC17 specific MLVA types was performed by comparing the MLVA profiles obtained with the previously described different repeat combinations for VNTR-7, -8 and -10, which have been shown to have a positive predictive value of 87% and a specificity of 90% for CC17, formerly designated MLST-C1 (18).

Esp PCR

All strains were screened for the *esp* gene (as a marker gene for the presence of the putative pathogenicity island (10,20)) by PCR with the primers *esp*$_{fm}$.14F and *esp*$_{fm}$.12R as described previously (12).

Statistics

Difference in typeability for MLVA and PFGE was analyzed by Chi-square test. To compare the discriminatory abilities of PFGE and MLVA, Simpson's index of diversity (*D*) and 95% confidence intervals (CI) were calculated for the number of isolates typed by both methods according to formulas previously described (4,6) using EpiCompare (version 0.99; Ridom GmbH, Würzburg, Germany). The concordance between MLVA type and PFGE types and subtypes was calculated using EpiCompare (version 0.99; Ridom GmbH). For this analysis all possible pairs of isolates were cross-classified on the basis of matched or mismatched types according to the method described by Robinson *et al.* (16). The significance of the concordance was not estimated because the pairs on which the analysis was based are not independent (16).

Table 1. Characteristics of PFGE and MLVA

Characteristic	PFGE n=393	PFGE-Subtype	MLVA n=393
Typeability	358 (91.1%)		391 (99.5%)
Discriminatory power	0.4	0.83	0.68
95% Confidence interval	0.33- 0.46	0.81 - 0.85	0.65 - 0.71
Duration of procedure[a]	3 days		2 days
Duration of type assignment[a]	Hours		Hours
Estimated cost per isolate[b]	$10		$11

[a]Duration of procedure and duration of type assignment are estimates for a single PFGE gel on which 13 isolates and 2 controls are analyzed, and a single MLVA plate on which 94 isolates and 2 controls are analyzed

[b]Cost per isolate tested, including materials, labor and equipment depreciation, in 2005 U.S. dollars.

Abbreviations: PFGE, pulsed-field gel electrophoresis; MLVA, multiple-locus variable-number tandem repeat analysis.

Results

Patients and bacterial data

The VREF isolates analyzed were from a larger data set of vancomycin-resistant *E. faecium* and *E. faecalis* isolates identified during the baseline, non-intervention period of a previously published study (5). A total of 146 patients were admitted to the MICU during the baseline study period and 393 VREF isolates, all ampicillin-resistant, were identified in 77 rectal swabs from 33 patients, 268 environmental samples and 48 samples of HCW hands. Four patients in the MICU were colonized with vancomycin-resistant enterococci at the start of the study period, while 29 (19.9%) patients were colonized on admission. Of the remaining 113 patients, 16 (14.2%) acquired vancomycin-resistant enterococcal colonization during their stay in MICU (5).

Table 2. Distribution of MLVA types, including the MLVA profiles among the isolates

MLVA type	MLVA profile[a]						No. of isolates	CC17 specific MTs[b]	%
	VNTR-1	VNTR-2	VNTR-7	VNTR-8	VNTR-9	VNTR-10			
1	5	7	3	3	2	3	34	+	7.7
2	5	6	3	3	2	3	177	+	43.5
5	5	7	3	2	2	3	11	+	2.3
9	5	3	3	3	2	3	1	+	0.3
163	5	5	3	3	2	3	1	+	0.3
206	5	6	3	3	1	3	140	+	34.5
208	5	8	3	2	1	1	44	-	10.5
209	5	8	3	3	2	3	1	+	0.3
211	5	6	3	2	1	1	1	-	0.3
212	5	4	3	3	1	3	1	+	0.3
213	4	6	3	3	2	3	1	+	0.3

[a]Numeric values in the table refer to the number of repeats for each VNTR locus.

[b] +; MTs with the following repeat profile for VNTR-7, -8 and -10: 3-3-3; 3-2-3; 3-3-2; 4-3-3; 3-4-3; 4-2-3; 3-3-1 were identified as belonging to CC17 with a sensitivity of 97% and a specificity of 90% (18).

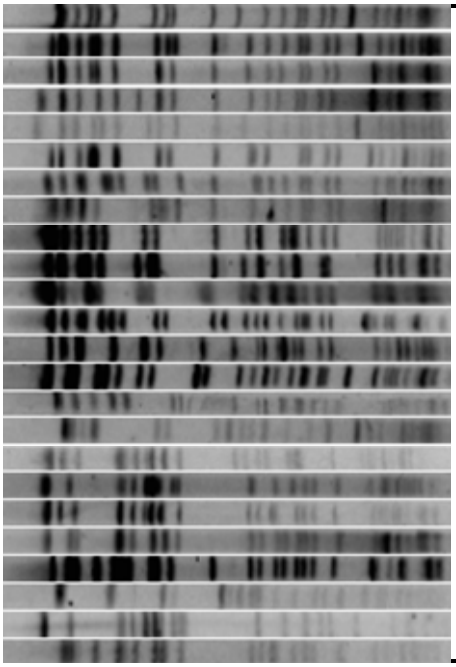
Abbreviations: MLVA, multiple-locus variable-number tandem repeat analysis; VNTR, variable number tandem repeat.

VREF genotyping

Of 393 VREF isolates, 358 (91.1%) yielded interpretable PFGE banding patterns the first time they were tested (Table 1), resulting in 19 PTs and 24 PSTs (Figure 1). PT-1 was the predominant type (275/358; 76.8%). Within PT-1, 4 PSTs were found of which PST-1B and PST-1C accounted for 36.0% (99/275) and 31.2% (86/275) of the PT-1 isolates, respectively. PT-6 revealed 3 subtypes.

MLVA generated complete MLVA profiles in 391 of 393 VREF (99.5%) (Table 1), representing 11 MLVA types (MTs) (Table 2). MT-2 was the predominant type, accounting for 43.5% of the isolates (170/391). MT-206 and MT-1, which are single locus variants of MT-2, were found among 135/391 (34.5%) and 30/391 (7.7%) of the isolates, respectively. MT-208 accounted for 10.5% of the isolates (41/391).

All but 19 MT-1 isolates contained the *esp* gene, representing 374/393 (95.2%) of the isolates.



PFGE subtype (PST)	No. of isolates	%
1A	47	13.1
1B	99	27.7
1C	86	24
1D	43	12
2	1	0.3
3	1	0.3
4	2	0.6
5	1	0.3
6A	30	8.4
6B	9	2.5
6C	1	0.3
7	4	1.1
8	6	1.7
9	3	0.8
10	1	0.3
11	3	0.8
12	1	0.3
13	4	1.1
14	2	0.6
15	9	2.5
16	2	0.6
17	1	0.3
18	1	0.3
19	1	0.3

Figure 1. Overview of representative banding patterns for 24 pulsed-field gel electrophoresis-subtypes, with corresponding numbers of isolates and the percentage of the total number of isolates. PFGE types 1 and 6 comprised 76.8% and 11.2%, respectively of all PFGE types identified.

Performance of MLVA compared to PFGE

Typeability of MLVA (99.5%) was significantly higher than PFGE (91.1%) (p<0.01) (Table 1). The discriminatory ability of PFGE-types, -subtypes and MLVA was determined and compared by calculating the discriminatory power (D) with 95% CIs of 356 isolates typed with both methods. MLVA had a higher

level of discrimination compared to PFGE-types, but lower compared to PFGE-subtypes (Table 1). The total time required to complete one PFGE gel with 13 samples, including type assignment was approximately 3 days, while approximately 2 days was required to determine MLVA profiles of 94 samples. The estimated cost per isolate tested, including materials, labor and equipment depreciation, was similar for the 2 methods (Table 1).

Cross-classifying the isolates based on matched or mismatched MT and PT or PST showed that the concordance of the typing schemes was 67.5% and 82.8%, respectively (Table 3). When compared to PTs, MTs were more predictive for the PTs than vice versa. Ninety-four percent (18953/20211) of all isolate pairs sharing identical MTs also had identical PTs, while 50% (18953/38214) of the pairs with identical PTs shared identical MTs, illustrating that MLVA provided a more discriminatory dataset than PFGE patterns classified into PTs. In contrast, PSTs were more predictive for MTs, than vice versa. Ninety-three percent (10133/10906) of all pairs sharing identical PSTs also had identical MTs, while 50% (10133/20211) of the pairs with identical MTs shared identical PSTs (Table 3). This can be explained by the greater diversity in PFGE-subtype classification.

An advantage of MLVA typing is that it can be used to identify isolates belonging to CC17. Comparison of the obtained MLVA profiles with the previously described CC17 specific repeat combinations for VNTR-7, -8 and -10 (18) revealed that except for MT-208 and MT-211, all MLVA profiles belonged to CC17, representing 349/391 isolates (89.3%).

Table 3. Cross-classification of all possible pairs of isolates based on matched or mismatched MTs and PTs or PSTs

		PFGE-type			PFGE-subtype		
		Match	Mismatch	Sum	Match	Mismatch	Sum
MLVA	Match	18953	1258	20211	10133	10078	20211
	Mismatch	19261	23718	42979	773	42206	42979
	Sum	38214	24976	63190	10906	52284	63190
		Concordance = 67.5%			Concordance = 82.8%		

[a]All pairwise comparisons that were undistinguishable by both MLVA and PFGE (match-match) or were considered different by MLVA and PFGE (mismatch- mismatch) are in concordance.

Abbreviations: PFGE, pulsed-field gel electrophoresis; MLVA, multiple-locus variable-number tandem repeat analysis; MT, MLVA type; PT, PFGE type; PST, PFGE subtype.

Distribution of PSTs among MTs

The three most prevalent MTs (MT-2, MT-206, and MT-208) were predominantly linked to specific PTs: MT-2 and MT-206 to PT-1, 148/153 and 122/123, respectively, and MT-208 to PT-6 (36/37) (Table 4). Linkage to a specific PT was not found for MT-1. At the start of the study period MT-2 and MT-208

were represented by a single PST; PST-1A in MT-2 and PST-6A in MT-208, while MT-206 was represented by two PSTs, PST-1C and PST-1D, of which PST-1C clearly dominated. Over time the dominant PST in MT-2 changed from PST-1A to PST-1B. In MT-206 the proportion of PST-1C first decreased over time in favor of PST-1D, while at the end of the study period MT-206 almost disappeared. Finally, PST-6A was partly replaced by PST-6B among MT-208 isolates.

Discussion

This study reveals that MLVA typing may serve as a faster, more standardized, but less discriminatory alternative to PFGE typing to study the genetic relatedness of VREF within hospitals. Results of PFGE and MLVA typing were highly concordant. The typeability for MLVA, however, was significantly higher than for PFGE. Although PFGE has been described as a highly reproducible method for *E. faecium* (19) the feasibility of generating banding patterns may differ substantially among isolates, and it is not always clear what causes the diversity of banding pattern quality. Use of a band-based cluster tool, like the Dice algorithm found in some software packages, abrogates some of these difficulties, but assignment of bands is still subject to personal interpretation and an important cause of lack of reproducibility. Strict standardized procedures and techniques are necessary for interlaboratory comparison (8). In contrast, MLVA is a highly reproducible method (18) with the advantage that in the PCR single bands are produced. The translation from band size to number of repeats can easily be read from the MLVA website (www.mlva.umcutrecht.nl), where also the type assignment can be made and compared to a database, including isolates from different continents.

The stability of PFGE banding patterns in *E. faecium* has been studied by Morrison *et al*. (13). A single colony was subcultured repeatedly and a large degree of DNA banding pattern polymorphism was observed, which the authors hypothesized was due to mobile element-induced genomic rearrangement. In contrast, in a study by Bonten *et al.* little genetic variation was found among isolates cultured from long-term colonized patients (2). Within the patient group in the current study there are several examples where the environmental and rectal samples from one patient collected on one day showed different PSTs, but the same MT (data not shown). Among MT-2, -206 and -208 isolates a shift in PSTs was observed over time, which can be explained by genetic rearrangements. Therefore it could be argued that genetic diversity inferred from PFGE banding patterns, especially when stringent criteria like the PFGE subtype criteria in this study are used, is too high to recognize clones or events of cross-transmission in a setting of endemicity. MT-1 did not correlate to a single PT. It is possible that

Table 4. Distribution of PFGE-subtypes among MLVA types[a]

MLVA	PFGE-subtypes																							
MT	1A	1B	1C	1D	2	3	4	5	6A	6B	6C	7	8	9	10	11	12	13	14	15	16	17	18	19	Total
1						1							6			3	1	4	2	8		1	1	1	28
2	47	95	1	5				1	2											1	1				153
5							2					4		3											9
9					1																				1
163		1																							1
206		1	84	37																	1				123
209		1																							1
212				1																					1
213			1																						1
208									26	9	1				1										37
211									1																1
Total	47	98	86	43	1	1	2	1	29	9	1	4	6	3	1	3	1	4	2	9	2	1	1	1	356

[a] MLVA types predicted to belong to CC17 are indicated in bold. Numbers in the body of the table indicate the number of isolates belonging to each group.

Abbreviations: PFGE, pulsed-field gel electrophoresis; MLVA, multiple-locus variable-number tandem repeat analysis.

genome rearrangements account for this observation. However, previous MLST-based studies demonstrated that MT-1 is polyclonal (18). Our observation that, in contrast to the other MTs, *esp* is present in only a subset of MT-1 underscores polyclonality of MT-1.

Based on MLVA profiles, ampicillin resistance and the presence of *esp*, >85% of the isolates were identified to belong to CC17. However, two CC17 characteristic features, ampicillin resistance and the presence of the *esp* gene, were also found among the MT-208 and MT-211 isolates. This suggests that in addition to previously described MLVA profiles (18), other MLVA profiles found within this cluster may also be linked to CC17. Multilocus sequence typing of representatives of MT-208 and MT-211 will ultimately show whether or not isolates with these MLVA profiles group within CC17. To our knowledge there are no prior studies that describe PFGE banding patterns' linkage to CC17.

In the current study the discriminatory power of MLVA was lower (0.68) than reported previously (1,18). This is due to the fact that we analyzed isolates that were collected over a relatively short period from patients on a single hospital ward where VREF was endemic, so that highly related clones were to be expected. An earlier study in which the discriminatory power of MLVA was found to be 0.95 (18) evaluated a more genetically diverse strain collection that included isolates from various ecological niches such as the community, animals, the environment and hospitals, and from different geographic locations. A more recent investigation that found the discriminatory power of MLVA to be 0.846 analyzed VREF isolated over a 2 year period from patients housed on different

hospital wards (1); the genetic diversity of these isolates would also be expected to be greater than the diversity in the current strain set.

In conclusion, MLVA is highly congruent to PFGE but faster. It provides less ambiguous typing data that are easier to store in databases and to exchange between laboratories. MLVA is able to link isolates to CC17, which is not possible with PFGE. However, analysis of PFGE subtypes has a greater ability to discriminate among VREF strains than does MLVA. Both MLVA and PFGE are useful methods for determining clonal spread of VREF in an MICU setting of high-level, polyclonal endemicity.

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