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Molecular Strain Typing of Vancomycin Resistant *Enterococcus* Isolates Using DiversiLab and PFGE

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BACKGROUND

Vancomycin-resistant enterococci (VRE) has become a common pathogen responsible for nosocomial infections, with incidence rates increasing significantly over the past decade and continuing to rise steadily. [1] Over the last 2 decades, enterococci, formerly viewed as organisms of minimal clinical impact, have emerged as important hospital-acquired pathogens in immunosuppressed patients and intensive care units (ICUs). Enterococci are normal colonizers of the intestinal tract, where it is possible to acquire resistance from other intestinal pathogens through transduction of resistance genes via plasmids or transposons.[2] In fact, VRE composed 26% of nosocomial enterococci in 1999, a 47% increase from 1994 to 1998. The use of antimicrobial agents in the treatment of multiple bacterial infections has acted to catalyze the occurrence of VRE outbreaks.

Nosocomial outbreaks have a tremendous impact on the morbidity, mortality, and cost associated with hospital-acquired infections. An estimated $5 billion per year is spent as a result of infections that patients acquire while being treated for another illness. The ability to rapidly identify and screen for resistance is essential in limiting the spread of VRE through early intervention measures, such as the implementation of patient isolation and contact decontamination procedures[3]. The most widely used molecular method for bacterial strain discrimination is pulsed-field gel electrophoresis; however, this technology is time-consuming (**Figure 1**) and is not often directly accessible for use in clinical laboratories. Another method, repetitive sequence-based PCR (rep-PCR), has proven useful in strain typing.[4-6] This study reports the performance of automated rep-PCR,[7] the DiversiLab System, and PFGE analysis of enterococci clinical isolates from the SENTRY collection

METHOD

Twenty-two clinical enterocci isolates were collected from four different healthcare centers as part of an antibiotic resistance surveillance study (SENTRY). The isolates were tested using the DiversiLab System (Bacterial Barcodes, Inc., Houston, TX). The isolates were cultured on tryptic soy agar with 5% sheep blood and incubated for 24 hours at 37ºC. Genomic DNA was extracted from each culture using the UltraClean™ Microbial DNA Isolation Kit and the DNA was amplified by rep-PCR methodology using the DiversiLab *Enterococcus* Kit for DNA Fingerprinting. Briefly, 50ng of genomic DNA, the rep-PCR primer mix and master mix provided in the Kit, 2.5 units Ampli*Taq* and 1.5 uL 10X PCR Buffer (Applied Biosystems, Foster City, CA) were added for a total of 25 uL per reaction. The thermal cycling parameters were; initial denaturation of 94ºC for 2 min; followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 50ºC for 30 sec, extension at 70ºC for 90 sec; and a final extension at 70ºC for 3 min. The DNA amplicons were analyzed using the DiversiLab System which includes fragment separation using microfluidics lab-on-a-chip technology and the 2100 Bioanalyzer. Analysis was performed with the web-based software (beta v. 3.0) using the Pearson correlation coefficient to determine distance matrices and Unweighted Pair Group Method with Arithmatic Mean (UPGMA) to create dendrograms. Reports were automatically generated, including the dendrogram, similarity matrix, electropherograms, gel-like images, scatter plot and selectable demographic fields to aid with interpretation of the data. Sample relationships were designated as follows: indistinguishable – no band differences, similar – one band difference, and different – two and greater band differences.

PFGE was carried out using an in-house protocol and the CHEF-DR™II (Bio-Rad Laboratories, CA) electrophoresis system. The agarose plug of each of the twenty-two VRE isolates was restricted with the *Sma*I restriction enzyme. PFGE of DNA was carried out as follows: initial switching time, 2.0s; final switching time, 40.0s; run time, 16.5h; 6V/cm; 14°C. Pulsed-field gels were imaged using the AlphaImager (Alpha Innotech, CA). PFGE relationships were defined using Tenover's criteria.[10]

RESULTS

Rep-PCR fingerprints and PFGE patterns were obtained for enterococci isolates from four cities including New York City, Milwaukee, Barcelona, and Sao Paulo. Rep-PCR and PFGE fingerprint patterns from nine isolates collected in New York City are shown in **Figure 2a** and **2c**, respectively. The interpretations obtained from each technique are listed in **Table 1**. Briefly, two clusters can be seen in the data: samples 1-4 and 5-7. Samples 1-2 and 5-6 showed the same results with both rep-PCR and PFGE. When compared to samples 1 and 2, sample 3 was indistinguishable by rep-PCR and highly related using PFGE. Sample 4 was different according to both methods. The rep-PCR overlay (**Fig 2b**) highlights the differences between samples 1 & 4. Samples 8 & 9 were outliers using both methods. Sample 9 was the only *E. faecalis* in this study, and it clearly showed a distinct fingerprint pattern from the *E. faecium* in both rep-PCR and PFGE.

The rep-PCR dendrogram obtained from the five isolates collected in Milwaukee shows that all isolates clustered with greater than 95% similarity; however, the banding patterns show differences (**Figure 3a**). Samples 1 and 3 are indistinguishable using both methods; sample 2 is indistinguishable from these using rep-PCR and highly related (3 band difference) using PFGE (**Figure 3b**). PFGE interpretation considers sample 5 as indistinguishable from samples 1 and 3; but, rep-PCR shows a single band difference and designates it as similar (Table 2). Sample 4 is similar to 1-3 using rep-PCR; however, it is different using PFGE.

Figure 1. Rep-PCR and PFGE workflow comparison (**a**) and an example of the DiversiLab Software as an aid for data interpretation (**b**).

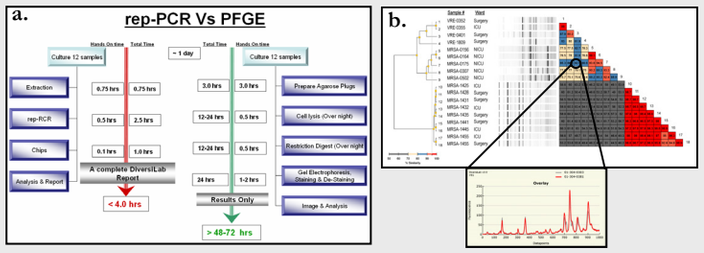

Figure 2. Rep-PCR Dendrogram (**a**) and overlay (**b**) and (**c**) PFGE agarose gel image of the 9 New York, NY isolates.

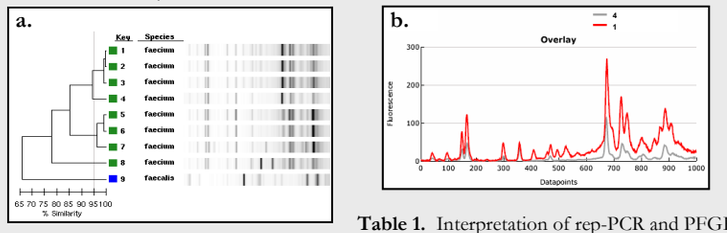

Table 1. Interpretation of rep-PCR and PFGE

key	# band differences		relationship	
	rep-PCR	PFGE	rep-PCR	PFGE
1-3	0	#1,2 = 0	I	I
		#3 = 1		HR
4	#1-3 = 3	>6	D	D
5-7	#5,6 =0	#5,6 = 4-5	I	PR
	#7= 1	#7 = 5-6	S	PR
8	>2	>6	D	D
9	>2	>6	D	D

I=indistinguishable, HR=highly related, PR=possibly related, S = similar D=different

Figure 3. Rep-PCR Dendrogram (**a**) and PFGE image (**b**) comparing 5 Milwaukee, WI isolates

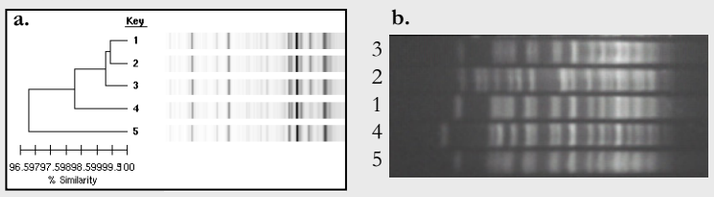

Table. 2 Interpretation of rep-PCR and PFGE

key	# band differences		relationship	
	rep-PCR	PFGE	rep-PCR	PFGE
1,3	0	0	I	I
2	0	3	I	HR
4	#1-3 = 1	>6	S	D
5	#1-3 = 1	0	S	I
	#4 = 2	>6	D	D

I=indistinguishable, HR=highly related, S=similar, D=different

Figure 4. Dendrogram (**a**) and PFGE images (**b**) comparing 4 Sao Paulo, Brazil isolates

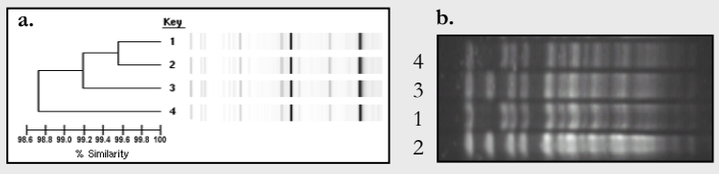

Table. 3 Interpretation of rep-PCR and PFGE

key	# band differences		relationship	
	rep-PCR	PFGE	rep-PCR	PFGE
1-4	0	#1,4 =0	I	I
		#2,3 =0		I
		(1,4) & (2,3) = 2		HR

I=indistinguishable, HR=highly related

Figure 5. Rep-PCR Dendrogram (**a**) and overlay (**b**) and PFGE agarose gel images (**c**) of the 5 Barcelona, Spain isolates

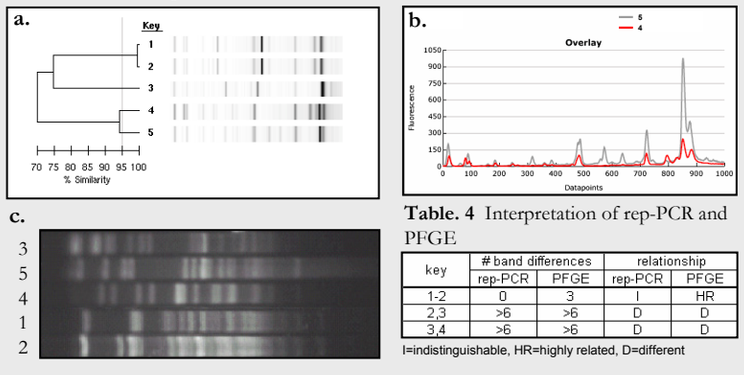

Table. 4 Interpretation of rep-PCR and PFGE

key	# band differences		relationship	
	rep-PCR	PFGE	rep-PCR	PFGE
1-2	0	3	I	HR
2,3	>6	>6	D	D
3,4	>6	>6	D	D

I=indistinguishable, HR=highly related, D=different

The four isolates from Sao Paulo were indistinguishable and showed greater than 98% similarity using rep-PCR (**Fig 4a**). Using PFGE, the samples showed two fingerprint patterns that clustered samples 1 & 4 and clustered samples 2 & 3 (**Fig 4b**). However, all samples were highly related, indicating that the results from the two methods were concordant.

Isolates from Barcelona showed a high level of diversity with rep-PCR and PFGE. Samples 1 and 2 were the only samples that showed similarity, and in fact were indistinguishable, using rep-PCR (**Fig 5a**). These samples were possibly related using PFGE (3 band difference) (**Fig 5c**). All other samples were different. Although the dendrogram shows approximately 95% similarity for samples 4 & 5, the rep-PCR graph overlay shows the many differences (**Fig 5b**).

Although there were minor differences between the interpretations of rep-PCR and PFGE, the overall outcome of the methods was concordant in most cases. In general, rep-PCR and PFGE were 93% concordant when comparing the interpretations of the two methods. In some cases, it appeared PFGE had a slightly higher level of discrimination; and in other cases, rep-PCR appeared more discriminating.

SUMMARY

- The rising incidences of hospital-acquired infections due to VRE[1] demonstrate the need for a rapid molecular typing method. This and other studies[4-7] show that rep-PCR is a useful and expeditious method for the epidemiologic characterization of VRE outbreaks.
- PFGE has higher setup costs and high labor costs per test due to several difficult, laborious and time-consuming steps.[11] While the procedural steps are straightforward, the time needed to complete the procedure is a minimum of 3 days (**Fig 1a**). This can reduce the laboratory's ability to analyze large numbers of samples (~15 samples in a given batch).
- Rep-PCR has been adapted to an automated format that provides a low-cost alternative. Analysis of over 24 samples can easily be completed by one technician in a single day .[4,5,7]
- Both in this and another study, interpretation of PFGE results by the criteria of Tenover et al.[10] and the interpretation of rep-PCR fingerprints were about 93% concordant.[4] While PFGE is complex and often difficult to interpret, the DiversiLab software offers interactive analysis tools to aid interpretation (**Fig 1b**).
- The DiversiLab software allows storage of the data in a database as digitized images of the fingerprint-patterns (**Fig 2-5**). The DNA fingerprint of an unknown microbial strain can be compared against a stored database for identification and typing purposes. This enables standardized-comparisons of strains isolated over time or in different laboratories.
- The ability of rep-PCR to distinguish VRE isolates at the clonal-level indicates that it can be a useful tool for source tracking of infections, especially by clinical laboratories. SHEA guidelines for preventing nosocomial transmission of multidrug-resistant strains of *Enterococcus* indicate active surveillance cultures are essential to track the spread of VRE infections and make control possible.
- When comparing rep-PCR and PFGE results, it is important to consider that they are two different methods. Rep-PCR takes advantage of the repetitive elements and surveys distances between those elements by PCR (but only visualize between ~150 and 5,000 base pairs), while PFGE digests the entire genome by restriction enzyme (but only visualizes about 50,000 to 500,000 base pairs). Therefore, it is important to compare the outcome or interpretation of the two methods. For example, by PFGE samples that are indistinguishable and highly related, and indistinguishable by rep-PCR, are generally assumed to be part of the outbreak by infection control. Samples that are probably and possibly related by PFGE, and similar by rep-PCR, need additional investigation by infection control to determine if there is other evidence to relate the samples to the outbreak and finally samples different by PFGE are generally different by rep-PCR and are not considered part of the outbreak.

REFERENCES

1. Mundy, L.M., Sahm, D.F., and Gilmore, M. 2000. Relationships between Enterococcal virulence and antimicrobial resistance. *Clin. Microbiol. Rev.*, 13:513-522.
2. Cetinkaya, Y., Falk, P., and Mayhall, C.G. 2000. Vancomycin resistant enterococci. *Clin. Microbiol. Rev.* 13:686-707.
3. Weber, D., Rutala, W. 1997. Role of environmental contamination in the transmission of vancomycin-resistant enterococci. *Infect. Control Hosp. Epidemiol.* 18:306-309.
4. Schutt CK, Pounder JI, Page SR, Schaecher BJ, Woods GI. 2005. Clinical Evaluation of the DiversiLab Microbial Typing System using Rep-PCR for Strain Characterization of *Staphylococcus aureus*. *J Clin Microbiol.* 43(3):1187-92.6
5. Healy M, Reece K, Walton D, Huong J, Shah K, Kontoyiannis DP. 2004. Identification to the Species Level and Differentiation between Strains of Aspergillus Clinical Isolates by Automated Repetitive-Sequence-Based PCR. *J Clin Microbiol.* 42(9):4016-24.
6. Versalovic J, de Bruijn FJ, and Lupski JR. 1998. Bacterial Genomes: Physical Structure and Analysis. Repetitive sequence-based PCR (rep-PCR) DNA Fingerprinting of Bacterial Genomes, Chapman and Hall, New York, NY. 34:437-53.
7. Healy M, Huong J, Bittner T, Lising M, Frye S, Raza S, Schrock R, Manry J, Renwick A, Nieto R, Woods C, Versalovic J, and Lupski J R. 2005. Microbial DNA Typing by Automated rep-PCR. *J Clin Microbiol.* 43(1):199-207.
8. Dahl, K., Simonsen, G., Olsvik, O., and Sundsfjord, A. 1999. Heterogeneity in the *van*B gene cluster of genomically diverse clinical strains of vancomycin-resistant Enterococci. Antibicrob. Agents Chemother., 43: 1105-1110.
9. Supola, J., Holho, E., Salmenlinna, S., Tarkka, E., Vuopio-Varkila, J., and Vaara, M. 1999. vanA and vanB incorporate into an endemic ampicillin-resistant vancomycin-sensitive *Enterococcus faecium* strain: effect on interpretation of clonality. J. Clin. Micro. 37:3934-3939.
10. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol.* 33(9):2233-9.
11. Olive DM, Bean P. 1999. Principles and applications of methods for DNA-based typing of microbial organisms. *J Clin Microbiol.* 37(6):1661-1669.