Polish Journal of Microbiology
2007, Vol. 56, No 2, 65–70

ORIGINAL PAPER

PCR Melting Profile Method for Genotyping Analysis of Vancomycin-resistant *Enterococcus faecium* Isolates from Hematological Unit Patients

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Received 16 February 2007, accepted 10 April 2007

Abstract

A number of *Enterococcus* strains with high-level inducible resistance to vancomycin have been identified, and the relative incidence of these strains has increased significantly in the last years. The first outbreak caused by vancomycin-resistant enterococci in Poland was reported in 1999. Vancomycin-resistant *Enterococcus faecium* is known for its propensity to cause infections which are difficult to eradicate. In this study, we determined the genetic similarities between vancomycin-resistant *E. faecium* isolates consecutively recovered from single patients to assess the duration of infection or colonization. The isolates taken in the study were identified by the conventional methods as *E. faecium*. PCR melting profile (PCR-MP) and pulsed-field gel electrophoresis (PFGE) typing revealed that the isolates belonged to six distinct genotypes and that two of them were predominant. Consecutive *E. faecium* isolates with identical genotypes were found in 7 of 12 (58.0%) patients. The delay between the times of recovery of the first and last isolates of identical genotypes from each patient was from 9 days to about 1 year. In six patients, paired blood and non-blood isolates showed identical genotypes. Data presented here demonstrate the complexity of the epidemiological situation concerning vancomycin-resistant enterococci that may occur in a single medical ward. We also show for the first time the evaluation of PCR-MP technique in enterococci strains differentiation and we revealed that there is at least a similar power of discrimination between the present gold-standard REA-PFGE and a PCR-MP method.

Key words: genotyping; PCR fingerprinting; LM PCR; PFGE; vancomycin-resistant enterococci

Introduction

Vancomycin-resistant enterococci (VRE) belong nowadays to the most important nosocomial pathogens worldwide (Bonten and Hayden, 1996; Jarvis and Martone, 1992; Moellering, 1992; Murray, 1998), and they usually cause infections in severely debilitated, immunocompromised patients who undergo prolonged and intensive antibiotic therapy (Banerjee *et al.*, 1991; Maki and Agger, 1988; Murray, 1990; Schaberg *et al.*, 1991). In some countries VRE may significantly contribute to enterococcal populations circulating in hospitals. The first reported identification of VRE in Poland occurred at the end of 1996 in a hospital in Gdańsk (Hematological Unit) with the isolation of *E. faecium* of the phenotype VanA, and it was followed by a large VRE outbreak in this center (Hryniewicz *et al.*, 1999; Samet *et al.*, 1999; Krawczyk *et al.*, 2003). To determine whether the isolates were epidemiologically related, isolated strains were differentiated by PCR fingerprinting method (Samet *et al*., 1999). The PCR fingerprinting of VRE from the Hematological Unit demonstrated only small genetic heterogeneity among the isolates over 11 months, with two main genotypes being identified. These strains were genetically closely related. In the next study, 100 VRE strains within a duration of 36 months (between January 1997 and December 1999) taken from 100 patients were examined using ADSRRS-fingerprinting and pulsed field gel electrophoresis (PFGE) methods (Krawczyk *et al*., 2003). Several lines of evidence obtained in this study with a large number of isolates suggested that the VanA phenotype was selected by most likely by one or two independent events within the ward enterococcal

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population. This was supported by the observation of the dominant existence of the two closely related ADSRRS-fingerprinting groups. Only minor differences in ADSRRS-fingerprinting patterns observed in both groups revealed the ongoing evolutionary diversification process within their populations. These results confirmed the results described previously (Samet *et al*., 1999) with also two main closely related genotypes. Up till now, a number of *E. faecium* strains with high-level inducible resistance to vancomycin still have been isolated from patients of Haematological Unit of an University Hospital in Gdańsk. Analysis of microbiological data based on our collection of clinical *E. faecium* VRE isolates revealed successive isolates from patients with persistent or recurrent infections. Although biochemical and serological data pointed to a close similarity of at least some of the strains isolated from single patients, information on relatedness at the genetic level was lacking. Here, to study the genetic similarities between these successive *E. faecium* VRE isolates the PCR-MP and PFGE methods were used.

Experimental

Materials and Methods

Isolates and patients. Clinical samples obtained between April 2003 and April 2005 from patients of the single Hematological Unit at the University Hospital in Gdańsk were examined for the presence of *E. faecium* isolates. These isolates were tested for the resistance to vancomycin and teicoplanin. A 36 *E. faecium* VRE isolates (from 12 patients) were chosen for further examination by molecular typing methods. A PCR assay for identification of *E. faecium* and primers used were the same as in Cheng *et al*. (1997), with some modifications as described by Samet *et al*. (1999). A multiplex PCR-restriction fragment length polymorphism (MPCR/RFLP) assay for Van-type identification of *E. faecium* isolates were carried out according to Patel *et al*. (1997). Amplification products obtained with Van-specific primers were further analysed by digestion with MspI restriction endonuclease (RFLP). VRE isolates were recovered from blood (21 isolates), stool (12 isolates), urine (1 isolate), sputum (1 isolate) and abscesses (1 isolate). The medical records of the patients from whom these isolates were recovered were examined for information on sex, age, and clinical history (Table I).

Genotyping methods. PFGE was performed according to the method described previously (Krawczyk *et al*., 2003). DNA was digested overnight with 25 U of XbaI (Sigma) at 37°C. DNA was separated on an agarose gel using the GenePath instrument (based on the pulsed field method of contoured clamped homogenous electric field). The band patterns obtained from the gels were converted and analyzed using the Quantity One software, version 4.3.1 (Bio-Rad).

PCR-MP procedure was carried out according to the method described for *E. coli* isolates (Krawczyk *et al*., 2006) with slight modifications. The DNA concentration range was about 100–200 ng per microliter. Denaturation temperature was calculated during the optimization experiments for several *E. faecium* isolates using a gradient thermal cycler (Biometra Tgradient Engine) with a gradient range of 79.6–82.5°C for denaturation step. PCRs were performed as follows: 7 min at 72°C to release unligated oligo-

Table I
Patients' clinical histories and results of genotyping of the vancomycin-resistant *E. faecium* isolates

Patient no.	Sex[a]	Age (yr)	Patient clinical history	Isolate source	PCR MP/ PFGE genotype	Day(s) of isolation[b]
1	M	63	myeloid leukaemia	Blood Blood	A A	1 153
2	M	57	myeloid leukaemia	Blood Blood	B B	1 31
3	M	47	myeloid leukaemia	Blood Blood Blood Blood Sputum Abscess	A A A A A D	1 2 4 8 24 150
4	M	38	myeloid leukaemia	Blood Blood Stool Stool	B B C C	1 3 37 67
5	F	49	non-Hodgkin lymphoma	Blood Urine Blood	A A A	1 36 290
6	M	24	multiple myeloma	Blood Stool Stool	B B B	1 48 199
7	F	56	myeloid leukaemia	Stool Blood Blood Blood Stool Stool	A B B B E A	1 148 149 150 162 342
8	F	43	myeloid leukaemia	Blood Stool	F B	1 7
9	M	25	lymphoid leukaemia	Blood Stool	A A	1 9
10	F	63	lymphoid leukaemia	Stool Blood	A A	1 33
11	F	26	myeloid leukaemia	Stool Blood	B A	1 146
12	M	64	myeloid leukaemia	Stool Blood	A A	1 12

[a] M, male; F, female.
[b] Day(s) of isolation refers to the day of the first day of isolation.

nucleotides and to fill in the single-stranded ends and create amplicons, followed by initial denaturation at 81.2°C for 90 s and 24 cycles of denaturation at 81.2°C for 1 min, annealing and elongation at 72°C for 2 min. After the last cycle, samples were incubated for 5 min at 72°C. PCR products, 15 µl out of 50 µl, were electrophoresed on 6% polyacrylamide gels with TBE buffer, stained in ethidium bromide (0.5 µg/ml aqueous solution) for 10–15 min. Images of the gels were analyzed using a Versa Doc Imaging System version 1000 (BioRad). Similarities between fingerprints were calculated by use the Dice band-based similarity coefficient (S_D). The patterns with the Dice coefficient of 0.85 were assigned to the same type.

Results

Patient characteristics and PCR identification of *E. faecium* and Van-type. Table I shows the characteristics of the patients, the sites and times of recovery of the VRE isolates, and the genotyping analysis. The mean age for the patients was 46.2 ± 22.2 years (age range, 24 to 64 years). All patients had been hospitalized several times, and the mean hospital stay for the patients was 42.9 ± 24.5 days. Ten out of twelve of them died during hospitalization. To confirm the phenotypic identification, the PCR identification of *E. faecium* and Van-type of antibiotic resistance was carried out. Using the EM1A and EM1B primers (Cheng *et al*., 1997), a specific 658-bp DNA product, upon PCR amplification of DNA from all isolates identified as *E. faecium* by standard biochemical assays, was identified (results not shown). These results confirmed that examined isolates in fact belong to *E. faecium*. Next, the convenient multiplex PCR-restriction fragment length polymorphism (MPCR/RFLP) assay to detect and discriminate *vanA*, *vanB* and *vanC-1* genes was applied (Patel *et al*., 1997). All examined clinical isolates, phenotypically identified as vancomycin-resistant VanA-type of the *E. faecium*, yielded the 885-bp amplicon, which is characteristic for *vanA* and *vanB* genes. The amplified DNAs from all isolates digested with MspI restriction enzyme gave distinct electrophoretic patterns for *vanA* gene (231, 184, 163, 133 and 131 bp restriction fragments) (results not shown). These experiments confirmed that the isolates belong in fact to VanA-type of the vancomycin resistance.

PFGE and PCR MP analysis. PFGE and PCR-MP fingerprinting patterns of the 36 VRE found only six unique profiles represented by A to F groups (Figs 1 and 2, Tab. I). Each PFGE (XbaI) pattern consists of approximately 12–17 fragments. PCR-MP profile consists of approximately 20–25 fragments in the size range of 100–1200 bp. Two groups, A and B, were markedly predominant, as these were represented by 53% and 33%, respectively. Successive isolates

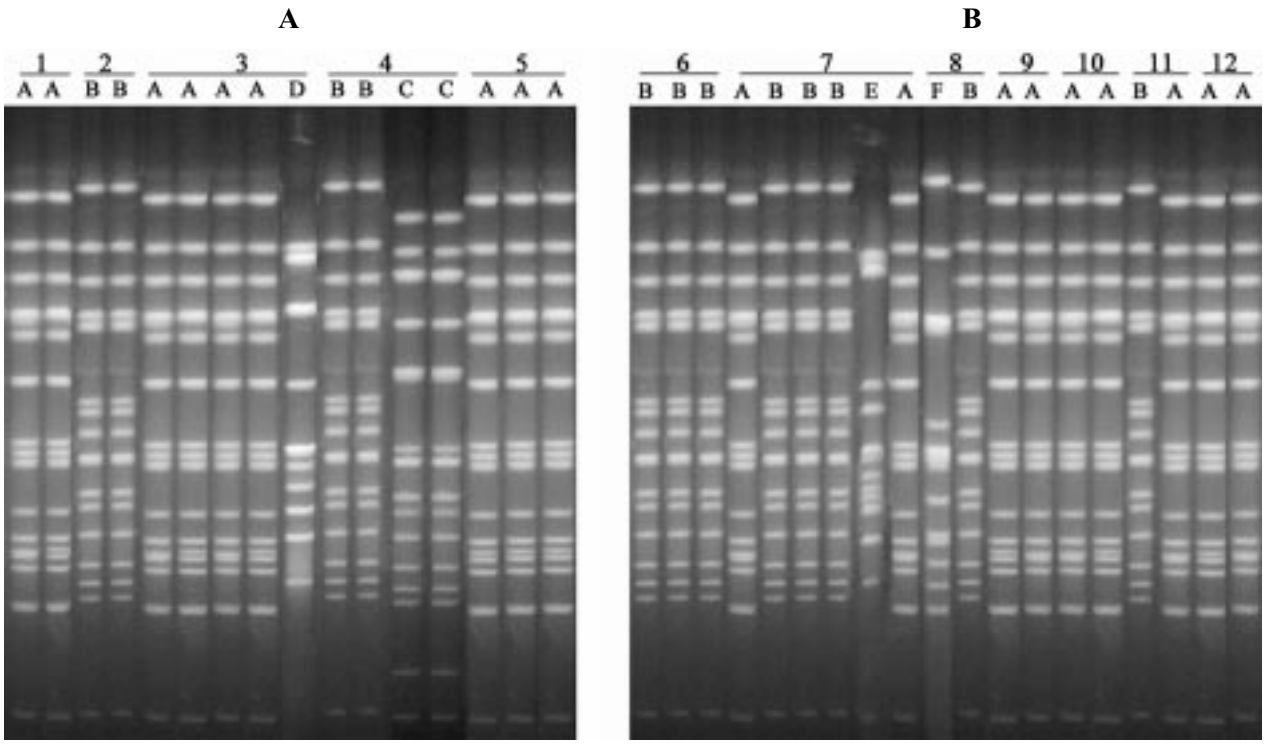

Fig. 1. PFGE profiles of the vancomycin-resistant *E. faecium* strains.

Chromosomal DNA was digested with XbaI, and the fragments were fractionated on an 0.9% agarose gel. Numbers 1–12 refer to patients shown in Table I. PFGE genotypes are given above each lane.

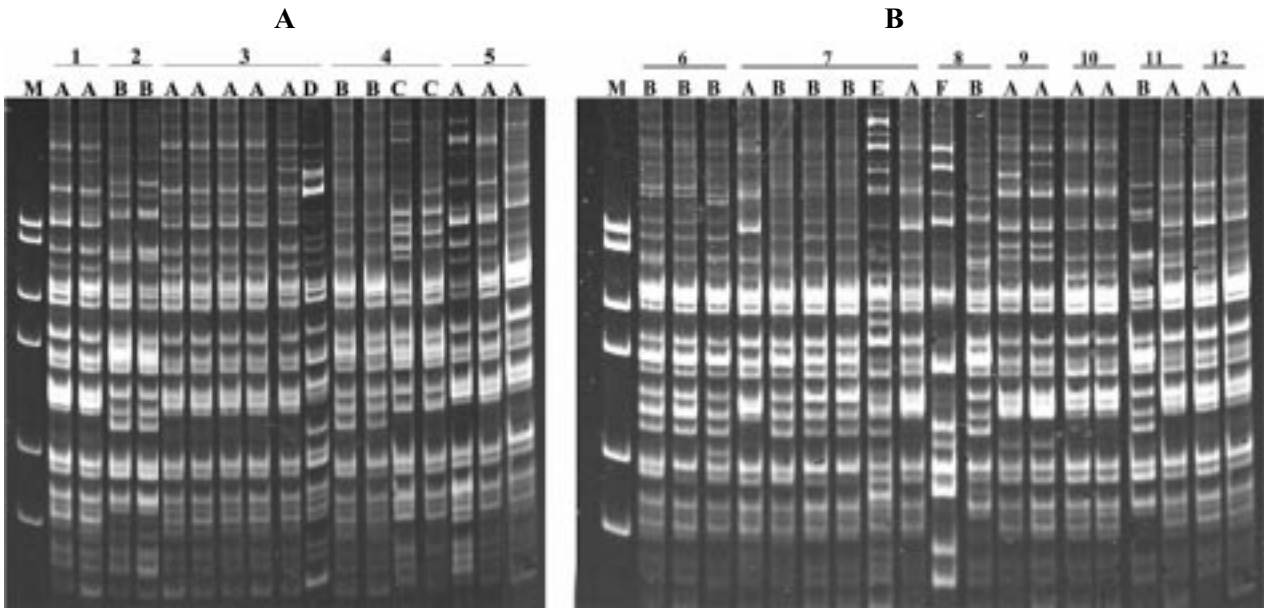

Fig. 2. PCR MP fingerprints of the vancomycin-resistant *E. faecium* strains.

The amplicons were electrophoresed in 6% polyacrylamide gel by using 1 × Tris-borate EDTA running buffer at a field strength of 12 V/cm. The lane designated M contained molecular mass marker (1008, 883, 615, 517, 466 and 396 bp). PCR MP genotypes are given above each lane. Numbers 1–12 refer to patients shown in Table I.

with identical patterns were recovered from 7 patients. Different genotypes were found in the 5 patients. The delay between the time of recovery of the first *E. faecium* VRE isolate and the last isolate of the same genotype was in range from 9 days to about 1 year. For patients 1, 2 and 5, the same *E. faecium* VRE genotype could still be isolated from the blood after 5, 1 and 10 months, respectively. Long-term persis-

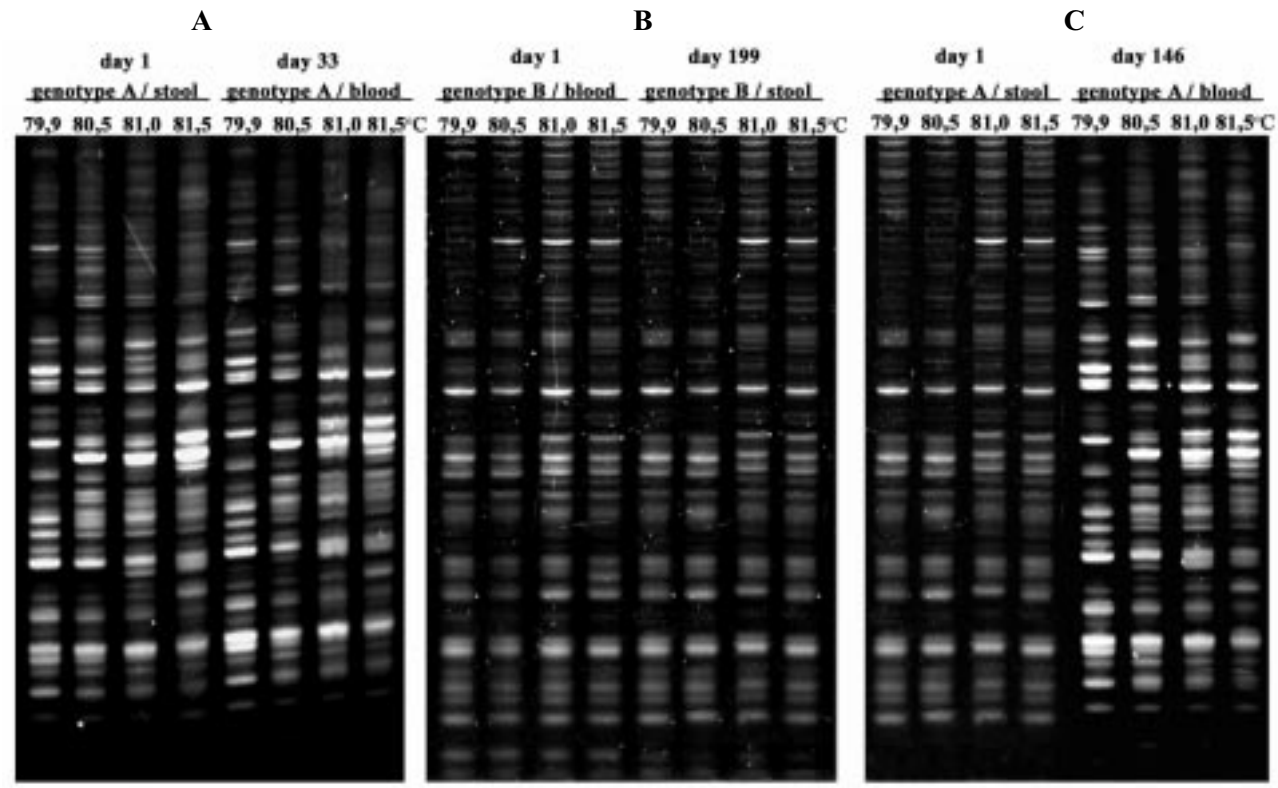

Fig. 3. PCR MP fingerprints of the vancomycin-resistant *E. faecium* strains at increasing denaturation temperatures (79.9°C, 80.5°C, 81.0°C, and 81.5°C) (representative results).

Isolates from patients 10 (panel A), 6 (panel B) and 11 (panel C) are compared. A steady increase in the number of amplified DNA fragments, which is dependent on *Td* increase, was observed and still produced identical profiles for isolates belonging to the same genotype (panels A and B).

tence (approximately 1 year) of *E. faecium* VRE was detected for patient 7. Isolates of that patient were typed into three groups (A, B, E). Genotype A was identified first and at the end (both from stool). Genotype B was detected only from blood (3 isolates). In six patients (patients 3, 5, 6, 9, 10, and 12), the paired blood- and not-blood- related isolates from each patient were of identical genotype.

The reproducibility of PFGE and PCR-MP methods was assessed by the duplicate analyses of the isolates. For these duplicates, DNA was extracted separately and proceeded in independent procedures. The resulting products were then electrophoresed on the same gel. All paired PFGE or PCR-MP patterns were found to be identical indicating that the methods were highly reproducible (data not shown).

To increase differentiation efficiency of the strain genotyping, isolates from particular patient were tested with PCR-MP at increasing denaturation temperatures. A steady increase in the number of amplified DNA fragments, which is dependent on T_d increase, was observed and still produced identical profiles for isolates belonging to the same genotype (for representative results see Fig. 3). Thus, the order of appearance of DNA bands in PCR performed in subsequent increasing temperatures is invariable for a given genomic DNA (genotype) (see Fig. 3, panels A and B).

Discussion

In the report presented here, we describe the genotypic similarities of multiple *E. faecium* VRE isolates consecutively recovered from 12 patients. The samples from the majority of the patients contained only a single PFGE/PCR-MP genotype. However, in some cases coinfection with other genotype was found. In addition, in some cases of bacteremic patients, the blood- and non-blood derived *E. faecium* VRE isolates from the same patient had identical *E. faecium* VRE genotype. The genotypic similarities of the consecutive isolates from the patients described here indicate the ability of *E. faecium* VRE isolates to persist as both infecting and colonizing flora for a long time. The present report confirmed the results described previously, where VRE isolates from patients of Hematological Unit between 1996 and 1997 analyzed using PCR fingerprinting (RAPD) technique (Samet *et al*., 1999) and between 1997 and 1999 analyzed using ADSRRS-fingerprinting and PFGE techniques (Krawczyk *et al*., 2003) revealed the presence of two main closely related genotypes (groups A and B). The epidemiological studies presented here revealed that those genotypes still exist in Hematological Unit of the hospital. Only minor differences in PCR MP fingerprinting patterns were observed in both groups (not shown). It is due to the ongoing evolutionary diversification process within their populations.

PCR-MP analysis of the *E. faecium* VRE isolates produced reliable, discriminatory, and reproducible typing results; and the existence of distinct PCR-MP types in single patients was supported by differences in PFGE identification. Although PFGE analysis of chromosomal macrorestriction fragments is one of the most commonly used method for the epidemiological typing of bacteria and it is generally recognized to be the method with the highest level of discrimination, we recommend the use of PCR MP technique for routine epidemiological study. The complexity of the PFGE and the costs involved in setting up and using the method may be beyond the capabilities of most laboratory. Besides, PFGE is the method probably not suitable for long-term epidemiology because the evolution of the strains might be too fast. Theoretically, the patterns of the larger bands should have a tendency to evolve into unrelated patterns at a significantly faster rate than that of the small bands. Indeed, the frequency of random genetic events increases with the size of the DNA fragment. These genetic events include point mutation generating a new restriction site, insertion/deletion of a sequence, and rearrangement. In this context, the PCR-MP typing method, in contrast to PFGE, permits the study of genetic relationships based on relatively small restriction fragments. Using PCR-MP method we also have the possibilities to increase the number of amplified DNA restriction fragments by increasing denaturation temperature during PCR. A steady increase in the number of amplified DNA fragments is dependent on denaturation temperature increase. Considering the lower costs and a high discriminatory power of PCR-MP method, we concluded that PCR-MP is a better choice for epidemiological studies (mainly long-term epidemiology analysis) than the use of PFGE analysis of chromosomal macrorestriction fragments obtained with several restriction enzymes.

In conclusion, the PCR-MP is generally a simple technique with high discriminatory power and low cost and may be most suitable for epidemiological studies. As shown in this report. a PCR-MP analysis proved to be a suitable technique for determination of the genetic similarities of consecutive *E. faecium* VRE isolates.

Acknowledgements

This work was supported by the Polish State Committee for Scientific Research Grant N404 078 31/3454 to B.K.

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