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Phenotypic and genotypic characterization of vancomycin-resistant enterococci in a university hospital of southern Italy

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Abstract

Background: In the last decades vancomycin-resistant enterococci (VRE) have emerged as important pathogens responsible for hospital-acquired infections. To analyze the spread and clonal relatedness of VRE, a two-year study of isolates was carried out in the hospital of the University "Federico II" in Naples.
Methods: Enterococcus species were identified by using API-20 Strep and antibiotic susceptibility was determined through the use of four tests: disk diffusion, broth dilution methods, Etest and Vitek 2. Polymerase Chain Reaction (PCR) was used to analyse glycopeptide resistance. Pulsed-field gel electrophoresis (PFGE) and arbitrarily primed (AP)-polymerase chain reaction were used for molecular typing of the strains.
Results: Thirty-two isolates of enterococci (18 E. faecium and 14 E. faecalis) showed resistance to vancomycin and teicoplanin and all the strains were vanA-positive. AP-PCR showed a unique clone of E. faecium, as well as for E. faecalis isolates. Identical results were obtained by PFGE for E. faecalis isolates, while three different PFGE patterns emerged for E. faecium.
Conclusions: The low degree of genetic diversity among the isolates strongly suggests a clonal spread of antibiotic-resistant strains among hospitalized patients in high-risk wards.
This report represents the first step to understanding VRE spread in our hospital as well as contributing to the comparison among different antibiotic susceptibility tests and molecular typing methods.
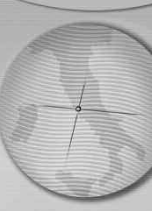
Keywords: vancomycin-resistant enterococci, antibiotic susceptibility, genotyping, PFGE, AP-PCR.

Introduction

Enterococci are normal inhabitant bacteria of human and animal gastrointestinal tracts, but in the past decade *Enterococcus faecalis* and *Enterococcus faecium* have emerged as important pathogens responsible for hospital-acquired infections [1, 2]. The importance of these bacteria has increased with the occurrence of high-level resistance to multiple antimicrobial drugs, such as vancomycin [3, 4]. The first clinical strains of Vancomycin-Resistant Enterococci (VRE) were isolated in England and France in 1986, and one year later in the US. After these outbreaks the genetic mechanism for vancomycin resistance was studied and associated to mobile elements, which can potentially be transmitted to other Gram-positive bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) [1, 5].

The antibiotic resistance is determined by different genotypes, which lead to the production of cell-wall precursors with low affinity for glycopeptides. Six molecular mechanisms, labelled as VanA, VanB, VanC, VanD, VanE and VanG were identified in enterococci. VanA and VanB phenotypes are dominant in Europe and in America and are more clinically relevant. Both phenotypes derive from acquisition of genetic determinants of resistance carried on transposon Tn 1546, while other types of resistance are constitutive. VanA-positive enterococci show high-level resistance to

vancomycin and teicoplanin; VanB-positive enterococci are resistant to vancomycin and susceptible to teicoplanin [1, 2, 4, 5].

Valid and rapid methods to detect vancomycin resistance and to establish the clonality of isolates are fundamental in order to set up effective infection control strategies. Several laboratory methods for the detection of glycopeptides resistance are available today. Some authors, however, report the failure of certain automated susceptibility tests [6], while other studies account for the sensitivity of new automated systems [7]. Although Pulsed-Field Gel Electrophoresis (PFGE) is generally acknowledged as the method of choice for typing isolates in most epidemiological investigations, Arbitrarily Primed – Polymerase Chain Reaction (AP-PCR) is much faster, but it is much more susceptible to technical variations and should be carefully evaluated before its introduction for surveillance purposes [8].

The aim of this study was to investigate the spread and the clonal relatedness of vancomycin-resistant enterococci isolated in an Italian University hospital during the years 2003 – 2005, by employing phenotypic and genotypic tests. Vancomycin resistance phenotype was detected using three currently available commercial methods, and compared to a disk diffusion screen method. Furthermore, PCR for the Van genotype was performed and compared with phenotypic tests. At last, all VRE strains were genotyped with two molecular methods to determine the relationships among them.

Methods

Phenotypic characterization

During the period May 2003 – Jan 2005, all *Enterococcus* spp. isolated from patients admitted to the University hospital "Federico II" in Naples were collected and typed with phenotypic and genotypic methods. Only the first isolate from each patient was included in the analysis. The strains were identified by API 20 Strep system (bioMérieux, France) and their susceptibility to vancomycin (Van) and teicoplanin (Tei) was tested through the use of four methods. Screening was performed using the Kirby Bauer test on Mueller Hinton agar with disks containing 30 μg of Van and 30 μg of Tei (Oxoid SpA, Italy). Resistance was revealed by a diameter of inhibition zone ≤ 14 mm for Van and ≤ 10 mm for Tei [9]. The Etest (AB Biodisk, Sweden/Biolife Italiana srl) was carried out on Brain Heart Infusion agar, with a 0.016-256 μg/mL concentration range for both antibiotics. The Sensititre test (Biomedical Service srl, Venice, Italy) was performed in Mueller Hinton broth on

multi-well plates containing Van (0.5-16 g/mL) and Tei (0.5-32 g/mL). Finally, the resistance to Van and Tei was evaluated by the Vitek 2 system (bioMérieux, France), with AST-P516 susceptibility cards. The MIC breakpoints were considered as follows: Van, ≤ 4 mg/mL for susceptible, 8 to 16 mg/mL for intermediate, and ≥ 32 mg/mL for resistant; Tei, ≤ 8 mg/mL for susceptible, 16 mg/mL for intermediate, and ≥ 32 mg/mL for resistant [9].

Molecular characterization

For each enterococcal strain which showed resistance to vancomycin and teicoplanin, two PCRs were set up. The first one contained primers VanABF, VanAR, and VanBR, which allow the amplification of 231 and 330 bp fragments from the vanA and vanB genes, respectively. The second reaction was performed with primers VanC1F, VanC1R, VanC23F, and VanC23R, which allow for the amplification of 447 and 597 bp fragments; both fragments are amplified from the vanC1 gene or from the vanC2 and vanC3 gene, respectively. All the reactions were carried out with 200 M dNTP, 1 M each primer and 1 U of Taq polymerase (Boehringer Mannheim). The amplification protocol included 35 cycles, as following: 1 min at 94°C; 2 min at 60°C; 2 min at 72°C. Electrophoresis was carried out in a 1.5% agarose gel. The VRE genotype was determined on the basis of the size of amplification product.

All the VRE strains were characterized through AP-PCR by using the 8F primer, as previously published [10]. The preparation of genomic DNA for PFGE was performed as previously described [11]. DNA restriction was done with SmaI according to the manufacturer's recommendations (New England Biolabs). PFGE gels were run in a CHEF-DR II apparatus (BioRad) using the following conditions: run time, 26 h; temperature, 7 °C; voltage, 200 V; initial forward time, 5 sec; final forward time, 35 sec.

Results

Between May 2003 and January 2005, 488 clinical isolates of *Enterococcus* spp. were collected as part of the routine diagnostic microbiology services in the University hospital "Federico II", Naples. Thirty-two (6.5%) of 488 isolates showed resistance to vancomycin and were included in the analysis. In particular, 18 (12.5%) of the *E. faecium* isolates and 14 (4%) of the *E. faecalis* isolates were VRE. The epidemiological and molecular features of the 32 VRE isolates are showed in Table I. All VRE were isolated in three wards: the Intensive Care Unit (ICU) (15 isolates of 32, 46.9%), the haematology unit (9 of 32, 28.1%) and the nephrology unit (8 of 32, 25%). VRE isolates originated from urine
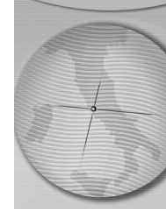
Table 1. source and date of isolation, species identification, genotype, AP-PCR and PFGE patterns of the 32 VRE isolates.

Code	Epidemiological data				Genotype	AP-PCR pattern	PFGE pattern
	Date (yr/mo/day)	Patient location	Specimen	Species			
30	03.05.23	ICU*	Urine	*E. faecalis*	Van A	X	A
16	03.10.01	ICU*	Pleuric fluid	*E. faecalis*	Van A	X	A
17	03.10.08	ICU*	Catheter	*E. faecalis*	Van A	X	A
1	03.11.02	ICU*	Urine	*E. faecalis*	Van A	X	A
7	04.01.25	ICU*	Urine	*E. faecalis*	Van A	X	A
9	04.02.01	ICU*	Urine	*E. faecalis*	Van A	X	A
27	03.05.30	ICU*	Urine	*E. faecium*	Van A	Y	C
26	03.06.20	ICU*	Urine	*E. faecium*	Van A	Y	C
2	03.08.08	ICU*	Urine	*E. faecium*	Van A	Y	C
12	03.09.02	ICU*	Sputum	*E. faecium*	Van A	Y	B
13	03.10.04	ICU*	Catheter	*E. faecium*	Van A	Y	B
29	03.10.07	ICU*	Urine	*E. faecium*	Van A	Y	C
25	03.11.05	ICU*	Urine	*E. faecium*	Van A	Y	D_1
3	03.11.17	ICU*	Blood	*E. faecium*	Van A	Y	C
10	04.02.07	ICU*	Urine	*E. faecium*	Van A	Y	B
11	03.09.02	Nephrology	Urine	*E. faecalis*	Van A	X	A
6	03.09.21	Nephrology	Sputum	*E. faecalis*	Van A	X	A
4	03.07.24	Nephrology	Throat swab	*E. faecalis*	Van A	X	A
19	03.10.25	Nephrology	Urine	*E. faecalis*	Van A	X	A
18	03.10.26	Nephrology	Urine	*E. faecalis*	Van A	X	A
22	03.11.22	Nephrology	Catheter	*E. faecalis*	Van A	X	A
24	03.11.16	Nephrology	Sputum	*E. faecium*	Van A	Y	D_1
31	03.11.18	Nephrology	Catheter	*E. faecium*	Van A	Y	E
20	04.10.26	Haematology	Throat swab	*E. faecalis*	Van A	X	A
28	04.12.28	Haematology	Urine	*E. faecalis*	Van A	X	A
32	04.07.05	Haematology	Blood	*E. faecium*	Van A	Y	D_2
21	04.09.05	Haematology	Urine	*E. faecium*	Van A	Y	C
15	04.09.28	Haematology	Throat swab	*E. faecium*	Van A	Y	B
14	04.10.05	Haematology	Throat swab	*E. faecium*	Van A	Y	B
23	04.12.17	Haematology	Urine	*E. faecium*	Van A	Y	B
5	05.01.11	Haematology	Urine	*E. faecium*	Van A	Y	B
8	05.01.25	Haematology	Throat swab	*E. faecium*	Van A	Y	B

cultures (53.1%), respiratory materials, such as throat swabs, sputum or pleuric fluid (28.1%), as well as blood and vascular catheters (18.8%).

All isolates were shown to be resistant to high doses of vancomycin (MIC > 128 mg/mL) as well as being resistant to teicoplanin in at least three of four used susceptibility tests, always with MIC > 16 mg/mL (data not shown). All VRE isolates belonged to the vanA genotype.

AP-PCR typing showed a clonal relatedness within *E. faecium* and *E. faecalis* isolates, respectively. All 18 *E. faecium* isolates showed an identical pattern (Y), while a different pattern (X) was shown by all the 14 *E. faecalis* isolates (Fig. 1). PFGE after restriction with SmaI resolved the genomic DNA of the 18 *E. faecium* isolates into four distinct PFGE patterns, that differed in migration for at least four DNA fragments. PFGE pattern B represented the main clone, since it was shown by eight isolates from ICU [3] and the haematology

unit [5], followed by PFGE pattern C, which was found in five isolates from IUC and in one isolate from the haematology unit. PFGE pattern D, which could be further classified into two sub-types (D1 and D2) was represented by three isolates from the three wards, whereas PFGE pattern E was shown in a single isolate from the nephrology unit. All 14 *E. faecalis* isolates belonged to the same PFGE pattern (A) (Figure 2). It is noteworthy that both in the ICU and in haematology unit it was possible to isolate all the four major VRE clones found in this study, whereas the nephrology unit was characterized by the clonal spread of *E. faecalis*, that occurred together with two sporadic isolations of *E. faecium*.

Discussion

The epidemiology of VRE infection differs between Europe and the United States. In the United States, nosocomial VRE infections and
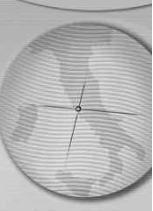
Figure 1. results of AP-PCR performed on the 32 VRE isolates with primer 8F. *Enterococcus faecalis* isolates (n. 1, 4, 6, 7, 9, 11, 16-20, 22, 28 and 30) show a similar pattern, while *Enterococcus faecium* isolates (n. 2, 3, 5, 8, 10, 12-15, 21, 23-27, 29, 31, 32) show another pattern. M: molecular weight marker.
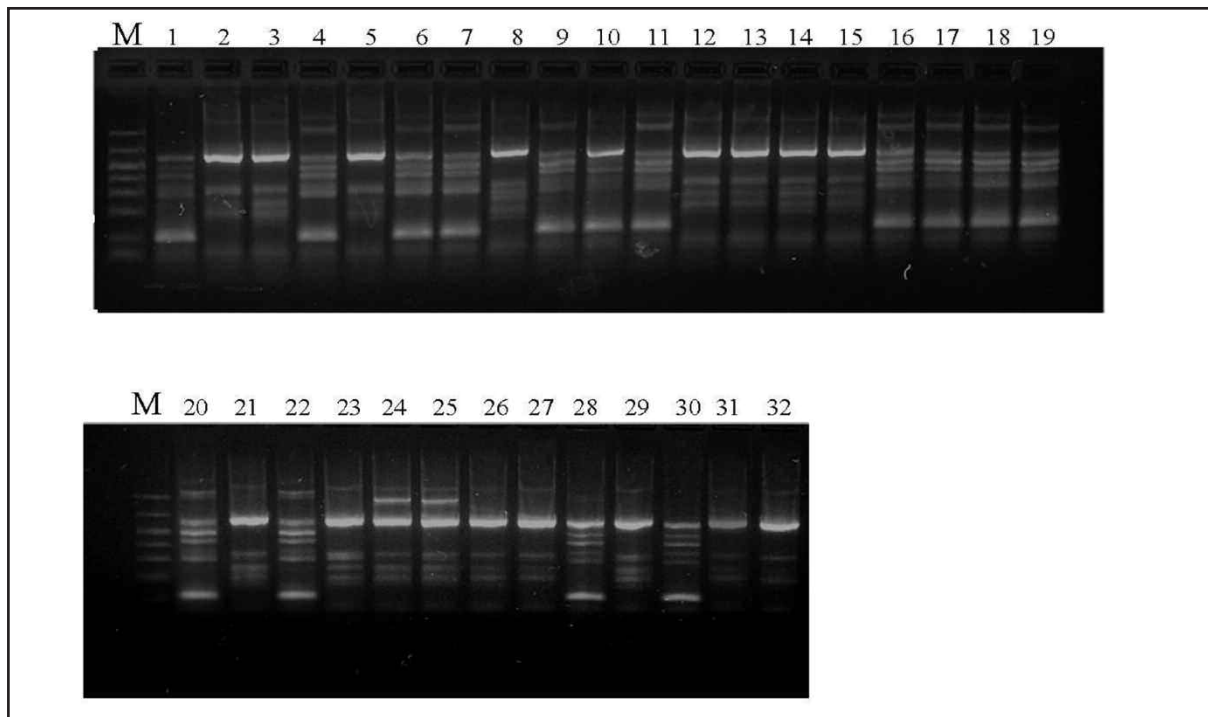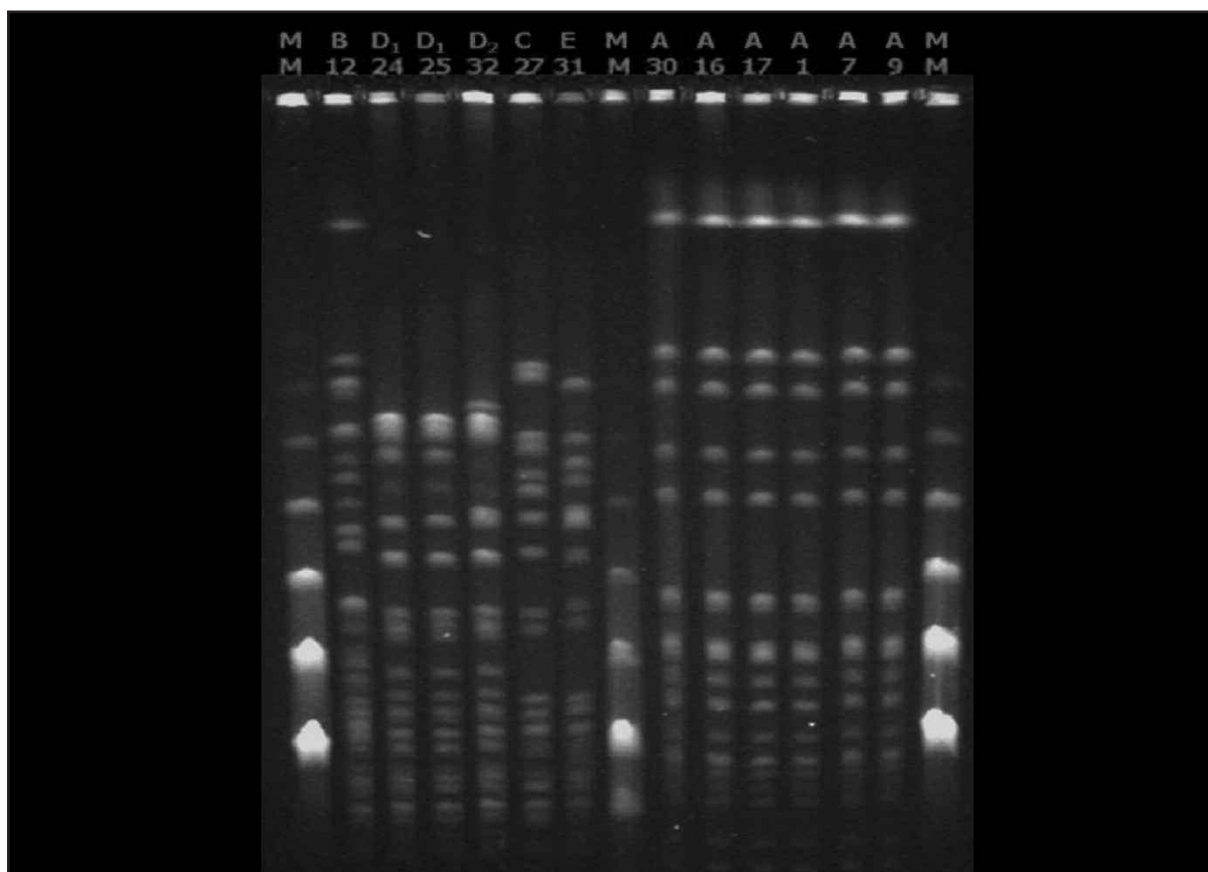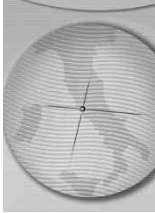

Figure 2. *Sma*I PFGE patterns of VRE isolates, representative gel. Lanes 1 to 6, *Enterococcus faecium* isolates. Lanes 7 to 12, *Enterococcus faecalis* isolates. M: molecular weight marker.

transmission has occurred much more frequently than in the Europe [1, 12]. From 1989 to 1999, the National Nosocomial Infection Surveillance (NNIS) System of the Centers for Disease Control and Prevention reported an increase in the percentage of VRE associated with nosocomial infections in ICU from 0.4% to 25.2% [13]. In Europe, hospital infections due to VRE are still relatively uncommon, although VRE strains are common in the intestinal flora of healthy humans and farm animals [1, 14].

Nevertheless, the European Antibiotic Resistance Surveillance System (EARSS) reported in Italy a percentage of VRE among enterococcal clinical isolates higher than 10% in 2001 and 2002. The proportion of VRE among bloodstream-infecting *E. faecium* isolates in 2002 (19%) was one of the highest in Europe, while the percentage of VRE among bloodstream-infecting *E. faecalis* isolates (4%) was similar to the average matched in the other European countries (http://www.earss.rivm.nl/). The high prevalence of VRE in Italy was also confirmed by another European study carried out in 2001 in at-risk hospital wards, where Italy and the United Kingdom reported the highest rates of VRE, 10.4 and 19.6%, respectively [15]. In the last decades, several hospital outbreaks and the endemic spread of genetically related VRE strains were reported in Italy [16-20].

The spread of VRE infections in health-care institutions involves person-to-person transmission and selective antibiotic pressure [2]. In 1995, the Hospital Infection Control Practices Advisory Committee (HICPAC) published recommendations for preventing the spread of vancomycin resistance, which include surveillance for colonization, identification of colonized and infected patients, isolation or cohorting of colonized patients, use of gloves and gowns, room cleaning after patient discharge, and limited use of vancomycin in therapy [2, 21].

In order to improve the role of surveillance in the control of VRE infections, the microbiology laboratories have to optimize their ability to rapidly detect VRE as well as clonal relatedness of isolates. Several studies have been done assessing the accuracy of various antimicrobial susceptibility methods in detecting vancomycin resistance in enterococci: some authors reported that automated methods (i. e. Vitek system) accounted for good sensitivity and specificity, while in other studies agar-diffusion or traditional broth microdilution methods showed higher reliability [6,7,22,23]. Comparative analyses of AP-PCR and PFGE for genetic typing of VRE are still relatively scarce:

while some studies reported that AP-PCR analysis is well-suited for the epidemiological typing of VRE, there are concerns about the reproducibility of the method [24-26].

This report was carried out in order to analyze vancomycin-resistant enterococci isolated in the University hospital "Federico II" in Naples, during a two-year period. A low prevalence of VRE strains was registered in this hospital, and the spread was limited to three wards: intensive care, haematology and nephrology units. The prevalence of vancomycin resistance among both *faecium* and *faecalis* species of 12.5% and 4% respectively, is similar to that reported in the national data [16]. However, the clonal relatedness detected among *E. faecium* isolates and especially among *E. faecalis* isolates which was shown by AP-PCR and PFGE, indicates the transmission of these multiresistant strains among hospitalized patients and the need of an improvement in infection control.

The application of HICPAC guidelines, through the limitations of vancomycin use, performance of surveillance cultures and improved infection control measures, has shown good efficacy in several epidemic outbreaks [27, 28]. In a more recent study carried out in the Netherlands, a program based on preemptive isolation, genotyping analyses, identification of VRE carriers and enhancement of hand-hygiene compliance was successful in the control of nosocomial spread of VRE infections [29].

Many epidemiological surveillance programs carried out with molecular genotyping have shown that sporadic spread introduction of VRE in the hospital through admission of colonized patients may lead to small outbreaks caused by single clones and thus to a polyclonal endemicity state. For each stage different infection control measures could be applied [1]. In order to prevent the development of endemicity in those units in which VRE strains were isolated in our institution, an improvement of hospital hygiene through educational programs for the personnel staff, a more responsible use of antibiotics, culture surveillance of high-risk patients and isolation of VRE-colonized patients should be adopted.

With regards to the methods used to check the antimicrobial susceptibility of strains, they produced similar results, confirming their usefulness. All of these methods, which are commonly used for routine analysis, showed a good level of sensitivity referring to the screening method and were easy to use. Accordance between results obtained with Vitek 2 and other methods may also enhance the use of this automated, more rapid test. Moreover, the
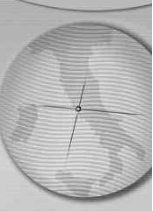
correlation between the results of genotypic and phenotypic characterization of glycopeptide resistance, demonstrated the usefulness of PCR to analyze the molecular mechanisms of susceptibility and the accuracy of Etest, Sensititre test and AST-P516 cards for detection of vanA-positive strains. However, due to the absence of other genotypes among our strains, these results are limited. The presence of one genotype may also account for a clonal diffusion of this resistance determinant.

PFGE is currently considered the "gold standard" for VRE typing, but it is time-consuming and requires specific equipment. PCR-based techniques, such as AP-PCR, are faster and easier to perform. However, the results of our comparative evaluation clearly show that the discriminatory power of PFGE is higher than that of AP-PCR, since it was able to screen four different patterns among *E. faecium* isolates. These different PFGE patterns were identified using the three bands difference criterion proposed by Tenover et al.; it is interesting to note that, even using a less strict six bands difference criterion, as recently proposed by some authors, the results would have been the same [30, 31]. The lower discriminatory power of AP-PCR compared to PFGE has been shown by other investigators [25, 26]

In conclusion, the results of this study clearly show that susceptibility tests, such as Etest, Sensitre test and Vitek 2 with AST-P5151 cards are useful to detect VanA-positive strains of *E. faecium* and *E. faecalis* and that PFGE, but not AP-PCR, could be used to identify clonal spread of isolates. Therefore, the combined use of these techniques is fundamental to identify the need for specific control measures against VRE.

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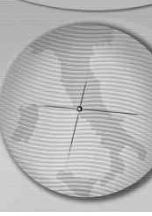
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