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Clonal Spread of a Vancomycin-Resistant *Enterococcus faecium* Strain among Bloodstream-Infecting Isolates in Italy

Lucia Stampone,[1] Maria Del Grosso,[1] Delia Boccia,[2]† and Annalisa Pantosti[1]*

Dipartimento di Malattie Infettive, Parassitarie ed Immunomediate[1] and Centro Nazionale di Epidemiologia, Sorveglienza e Promozione della Salute,[2] Istituto Superiore di Sanità, Rome, Italy

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Recent data indicated that the rate of vancomycin resistance in bloodstream-infecting enterococcal isolates in Italy is one of the highest in Europe. The aims of this study were to characterize bloodstream-infecting vancomycin-resistant enterococci (VRE) obtained from various Italian hospitals and to establish whether the isolates were clonally related. During the years 2001 to 2003, a total of 39 VRE isolates were obtained from 19 hospital laboratories in various areas of Italy. Species identification and resistance genotypes of the isolates were obtained by multiplex PCR. Further characterization included antibiotic susceptibility testing, pulsed-field gel electrophoresis (PFGE) of SmaI-digested genomic DNA, detection of virulence genes (*esp* and *hyl*), and multilocus sequence typing (MLST) of selected isolates. VRE were identified as 31 *Enterococcus faecium* (VREfm) isolates and 8 *E. faecalis* isolates. All but one isolate carried the *vanA* gene; one VREfm isolate carried the *vanB* gene. Analysis of the PFGE profiles showed that 28 VREfm isolates shared a similar electrophoretic profile, designed type 1, and were clonally related. All type 1 isolates were resistant to ampicillin, streptomycin, gentamicin, and rifampin and were positive for the *esp* gene. MLST identified an allelic profile (ST78) comprising *purK* allele 1, belonging to the C1 clonal lineage, characteristic of human infection and hospital outbreak isolates. The *vanB*-carrying VREfm isolate, of PFGE type 2, was shown to be a single-locus variant of ST78. Our data indicate that the recent increase in the number of bloodstream infections caused by VRE in Italy is due to the spread of a hospital-adapted, multidrug-resistant VREfm clone belonging to an internationally disseminated lineage.

Since the first report of vancomycin-resistant enterococci (VRE) in 1988, VRE have emerged as an important cause of hospital-acquired infections, particularly in the United States. According to the National Nosocomial Infection Surveillance System of the Centers for Disease Control and Prevention, over a 10-year time period the percentage of VRE has increased from 0.4 to 25% among enterococcal isolates from patients in intensive care units (ICUs) (17). Conversely, infections with VRE are still relatively uncommon in European hospitals, although a large pool of VRE strains is present in Europe, especially among the intestinal flora of farm animals and healthy humans. This large pool is likely due to the use of the glycopeptide avoparcin as a growth promoter in animal husbandry until its ban in 1997 (17).

In European countries, including Italy, VRE have been demonstrated in the feces of farm animals and in animal-derived foods, such as raw meat products (7). After discontinuation of the use of avoparcin, the frequency of VRE in these reservoirs decreased significantly (1; A. Pantosti, M. Del Grosso, S. Tagliabue, A. Macrì, and A. Caprioli, Letter, Lancet **354**:741–742, 1999).

In the past decade, two hospital outbreaks were reported in Italy; one was due to VRE belonging to the species *Enterococcus faecalis* (VREfl) in a neurosurgical ICU (E. Manso, G. De Sio, F. Biavasco, P. E. Varaldo, G. Sambo, and C. Maffei, Letter, Lancet **342:**616–617, 1993), and another was due to VRE belonging to the species *E. faecium* (VREfm) in a hematology department (21). The latter outbreak was followed by a long period of endemic circulation of genetically related strains in the same hospital. In addition, sporadic cases of infections caused by VRE were observed. In a multicenter study carried out from 1993 to 1995, the proportion of VRE among enterococcal isolates from infected sites varied between 0 and 36% in the 20 hospitals surveyed. The highest proportion was associated with the VREfm outbreak described above (10).

According to the European Antibiotic Resistance Surveillance System, an international network which collects data on antibiotic resistance of bloodstream-infecting isolates in 28 European countries (http://www.earss.rivm.nl/), in Italy the proportion of VRE of higher than 10% in 2001 and 2002. In particular, the proportion of VRE among bloodstream-infecting *E. faecium* isolates in 2002 (19%) was one of the highest in Europe. In contrast, the proportion of VRE among bloodstream-infecting *E. faecalis* isolates (4%) was within the average for the other European countries. As in Italy, in the United Kingdom the vancomycin resistance rate among *E. faecium* from bacteremia increased sharply, from 6.3% in 1995 to 24% in 1998 (18). The high prevalence of VRE in Italy and the United Kingdom was also confirmed by another European study carried out in 2001 in ICUs and other at-risk hospital wards; in that study, the United Kingdom and Italy were the countries with the highest rates of VRE, 10.4 and 19.6%,

* Corresponding author. Mailing address: Dipartimento di Malattie Infettive, Parassitarie ed Immunomediate, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. Phone: (39) 064990 2852. Fax: (39) 0649387112. E-mail: pantosti@iss.it.

† Present address: Health Protection Agency (HPA), Communicable Disease Surveillance Centre, 61 Colindale Ave., London NW9 5EQ, United Kingdom. Phone: (44) 208 2006868. Fax: (44) 208 2007868. E-mail: delia.boccia@HPA.org.uk.

TABLE 1. PFGE profiles of bloodstream-infecting VRE isolates

Species	PFGE type	No. of isolates	Yr	No. of hospitals	Area of Italy[a]	Presence (+) or absence (−) of *esp* gene	Resistance pattern[b]
E. faecium	1	28	2001–2003	18	N, C, S	+	AMP, STR, GEN, ERY, RIF[c]
	2	1[d]	2002	1	N	+	AMP, ERY, TET, RIF
	3	1	2001	1	N	−	AMP, STR, ERY, TET, RIF
	4	1	2002	1	N	−	AMP, STR, GEN, ERY, TET, RIF, CHL
E. faecalis	A	1	2001	1	S	−	GEN, ERY, TET, CHL
	B	1	2001	1	S	+	STR, TET, CHL
	C	1	2001	1	S	−	STR, GEN, ERY, TET
	D	1	2002	1	C	−	STR, GEN, ERY, TET, CHL
	E	2	2002, 2003	1	C, S	−	GEN, ERY, TET
	F	1	2003	1	N	+	STR, GEN, ERY, TET
	G	1	2003	1	S	−	STR, GEN, ERY, TET, CHL

[a] N, northern; C, central; S, southern.
[b] AMP, ampicillin; STR, streptomycin; GEN, gentamicin; ERY, erythromycin; RIF, rifampin; TET, tetracycline; CHL, chloramphenicol.
[c] One isolate was also resistant to CHL, and one isolate was also resistant to TET, CHL, and quinupristin-dalfopristin.
[d] *vanB*-carrying isolate.

respectively (11). The high rate in Italy was ascribed to the circulation of a multidrug-resistant VREfm clone in two hospital centers.

The aims of this study were to characterize bloodstream-infecting VRE isolates obtained from various Italian hospitals during the years 2001 to 2003 and to establish whether the isolates were clonally related.

MATERIALS AND METHODS

The laboratories collaborating in the Italian nationwide antibiotic resistance surveillance system (AR-ISS), started in May 2001 (http://www.simi.iss.it/antibiotico_resistenza.htm), were asked to send data on the antibiotic susceptibilities of all bloodstream-infecting *E. faecalis* and *E. faecium* isolates and to send VRE (the first isolate from each patient) for further testing.

The species identification and the genotypes of glycopeptide resistance were obtained by a previously described multiplex PCR assay (7).

Susceptibilities to ampicillin, aminoglycosides (streptomycin and gentamicin for high-level aminoglycoside resistance), glycopeptides (vancomycin and teicoplanin), tetracycline, chloramphenicol, rifampin, quinupristin-dalfopristin, and linezolid were assayed by the microdilution method according to NCCLS guidelines (16) with prepared Sensititre panels (Biomedical s.r.l., Venice, Italy).

The presence of putative virulence genes, such as the enterococcal surface protein gene *esp* (29) and the hyaluronidase gene hyl_{Efm} (20), in VRE isolates was investigated by PCR with the primer pairs suggested by Vankerckhoven et al. (25).

To analyze clonal relatedness among the isolates, total genomic DNAs were digested with SmaI and separated by pulsed-field gel electrophoresis (PFGE) by previously described methods (7). Isolates whose profiles differed by one to six bands were assigned to different subtypes of the same type. Isolates that differed by more than six bands were considered unrelated and were assigned to different types (23). A computer-assisted dendrogram of fragment patterns was constructed by using Diversity Database Fingerprinting software, version 2 (Bio-Rad Laboratories). Clustering was obtained by the unweighted-pair group method using average linkages (UPGMA) with the Dice similarity coefficient.

To better characterize the principal VREfm clone, multilocus sequence typing (MLST) was carried out with isolates showing different PFGE subtype profiles. For each isolate, the sequences of seven housekeeping genes (*adk*, *atpA*, *ddl*, *gdh*, *gyd*, *purK*, and *pstS*) were obtained by previously published methods (13) and compared with those of the alleles recorded in the database available at the MLST website (http://efaecium.mlst.net).

The sequence of the *purK* gene was obtained for VREfm isolates not belonging to the main clone as well.

RESULTS

Identification and antibiotic susceptibility of VRE. In the years 2001 to 2003, 918 bloodstream-infecting *E. faecalis* isolates and 345 *E. faecium* isolates were reported by hospital laboratories participating to the AR-ISS project. In particular, 1.2% of the *E. faecalis* isolates and 20% of the *E. faecium* isolates were VRE. The isolation of VRE was reported by 26 of 62 hospital laboratories. Only 39 VRE isolates, approximately half of all those reported, from 19 laboratories throughout Italy were available for further testing: 13 isolates in 2001, 15 isolates in 2002, and 11 isolates in 2003. Each laboratory sent one to three isolates. All patients were adults (median age, 68.5 years). They were admitted to hematology (14 patients), ICU (10 patients), surgical (7 patients), medical (5 patients), and other (3 patients) wards, mainly in large hospitals (>600 beds).

A total of 31 isolates belonged to the species *E. faecium*, and 8 belonged to the species *E. faecalis*. The *vanA* gene was the glycopeptide resistance determinant found in all but one of the isolates; the exception was a VREfm isolate carrying the *vanB* gene.

The resistance patterns for the isolates are shown in Table 1. All VREfm isolates were resistant to ampicillin, erythromycin, and rifampin. All but two isolates showed high-level resistance to both streptomycin and gentamicin. In addition, four isolates were resistant to tetracycline, three isolates were resistant to chloramphenicol, and one isolate was resistant to quinupristin-dalfopristin. All VREfl isolates were susceptible to ampicillin and resistant to tetracycline. All but one isolate were resistant to erythromycin, four isolates were resistant to chloramphenicol, four isolates were resistant to both of the aminoglycosides tested, one isolate was resistant to streptomycin only, and three isolates were resistant to gentamicin only. All of the VRE isolates tested, both *E. faecium* and *E. faecalis*, were susceptible to linezolid.

The results of the glycopeptide susceptibility tests were in agreement with the resistance genotypes. For all of the isolates except the *vanB*-carrying isolate, the MIC of vancomycin was

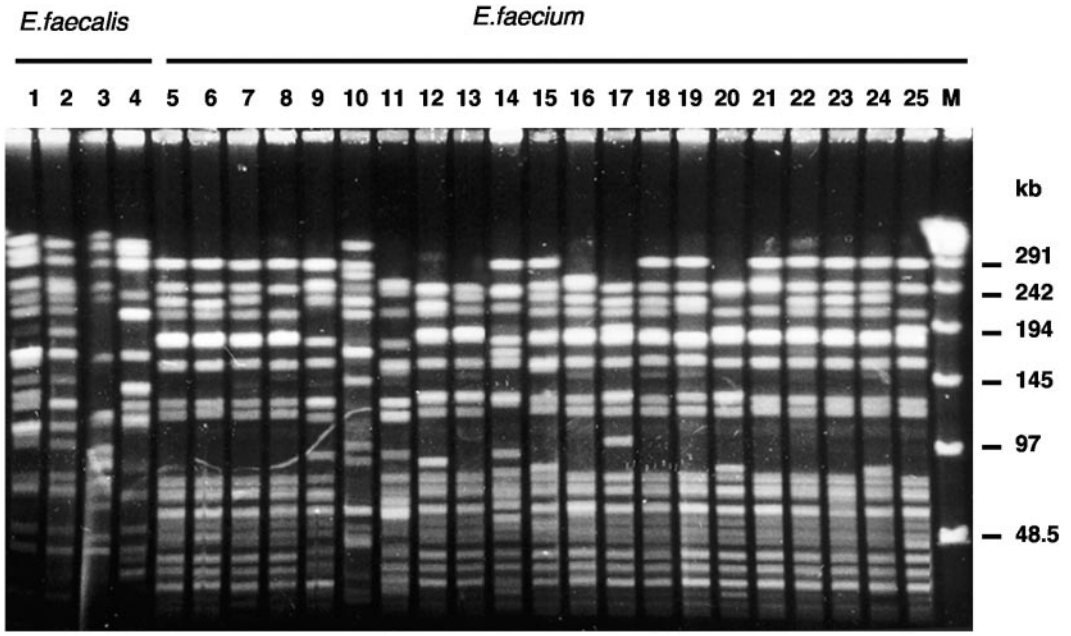

FIG. 1. PFGE profiles of bloodstream-infecting VRE isolates. Lanes 1 to 4, VREfl isolates of different PFGE types. Lanes 5 to 25, VREfm isolates; all of the profiles shown belong to different subtypes of type 1, with the exception of the profiles in lane 10 (PFGE type 4), lane 11 (PFGE type 3), and lane 14 (PFGE type 2). M, molecular size markers.

≥32 μg/ml, and the MIC of teicoplanin was ≥64 μg/ml. For the *vanB*-carrying isolate, the MIC of vancomycin was ≥32 μg/ml, and the MIC of teicoplanin was ≤0.5 μg/ml.

Molecular typing and clonal characteristics of VRE. Analysis of VREfm isolates by PFGE showed that 28 isolates had identical or similar profiles, differing by six or fewer bands; they were assigned to PFGE type 1 (Fig. 1). The computer-assisted clustering of VREfm based on the Dice similarity coefficient is shown in Fig. 2. PFGE type 1 encompassed 14 different subtypes (1.1 to 1.14). Subtype 1.1 contained nine isolates, subtypes 1.2 and 1.10 contained two isolates each, and subtypes 1.4 and 1.7 contained three isolates each. The other subtypes were represented by single isolates. Type 1 isolates were obtained over a span of 3 years from 18 hospitals in various geographical areas of Italy (Fig. 3). An apparent cluster of isolates was observed in hospitals in the Lombardia region (Milan area), but this finding could be a consequence of the number of large hospitals in the area participating in the surveillance.

The other three VREfm isolates had PFGE profiles that differed by seven or more bands from that of type 1 (Fig. 1) and were assigned to three different PFGE types; type 2 included the only *vanB*-carrying isolate. The dendrogram showed that the isolates belonging to types 2 and 3 were not genetically distant from type 1 isolates, as their coefficient of similarity was 0.8, at the limit for clonal relatedness (Fig. 2).

For the eight VREfl isolates, heterogeneity of PFGE profiles was observed (Fig. 1). Seven different types (A to G) were recognized, with only one type containing two isolates that had identical profiles but were obtained from different hospitals (Table 1).

The *esp* gene was amplified by PCR from 29 of the 31 VREfm isolates and from 2 of the 8 VREfl isolates. The two *esp*-negative VREfm isolates did not belong to PFGE type 1. The *hly* gene was not detected in any of the VRE isolates examined.

MLST was performed for four VREfm isolates belonging to PFGE type 1 and obtained from different hospitals. Strain AE1, subtype 1.1, was considered the reference strain for the group. Strain AE20 was chosen because it was resistant to quinupristin-dalfopristin, and strains AE10 and AE15 were chosen because their PFGE profiles showed a difference of six bands from the profile for the reference subtype 1.1 strain. In addition, strain AE12, PFGE type 2, was analyzed by MLST, as it was the only *vanB*-carrying isolate.

The four type 1 isolates were found to share an identical allelic profile, to which ST78 was assigned at the MLST website. The *vanB*-carrying isolate was found to be a single-locus variant of ST78 bearing a newly discovered allele of the *atpA* gene, to which ST209 was assigned (Table 2). These findings confirm the partial relatedness obtained by computer-assisted clustering of strain AE12 and type 1 isolates.

Both ST78 and ST209 were single-locus variants of ST17, found in isolates from hospital outbreaks in different countries, including the United Kingdom, Australia, and the United States, and comprising either vancomycin-susceptible isolates or those carrying *vanA* or *vanB* (Table 2). In addition, ST78 shared five or more alleles out of seven with 30 other STs retrievable from the MLST website (last accessed 1 December 2004).

The sequence of the *purK* gene was obtained for the two VREfm isolates belonging to PFGE types 3 and 4 as well; both carried *purK* allele 1.

DISCUSSION

The use of molecular methods such as AFLP and PFGE has led to major advancements in the understanding of the epide-

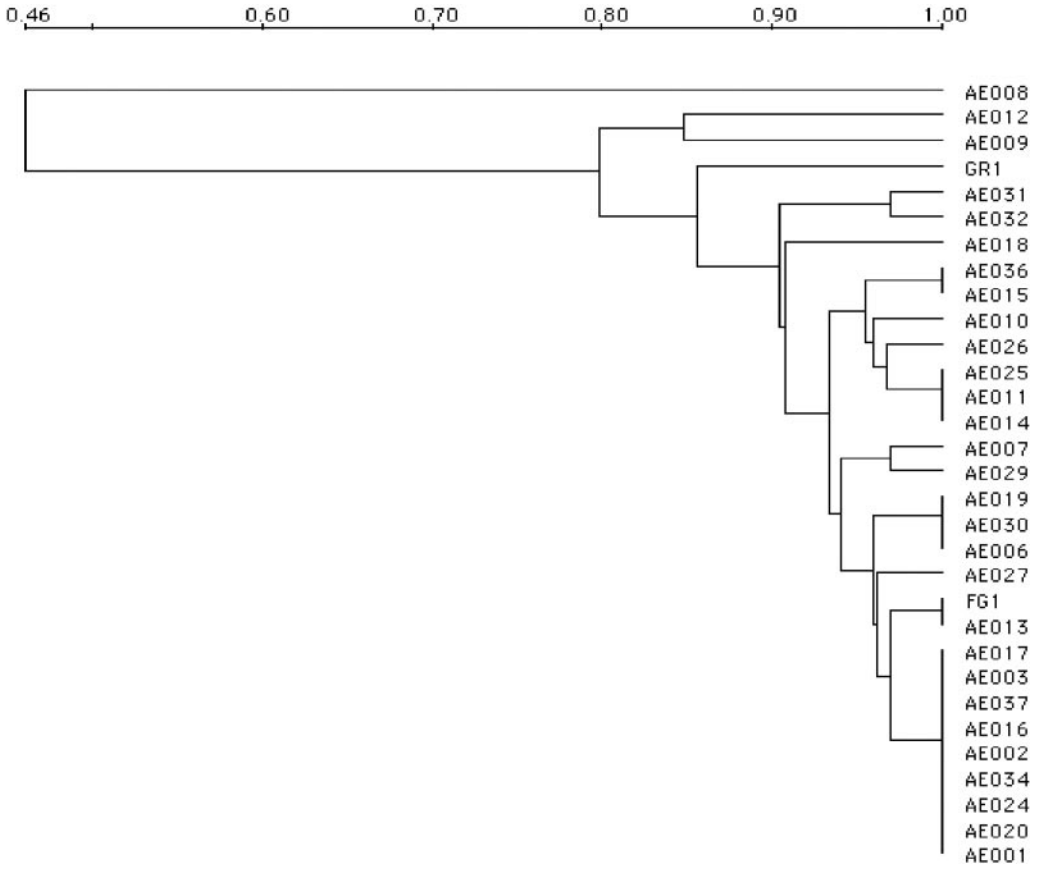

FIG. 2. Dendrogram based on PFGE SmaI restriction pattern analysis of 31 VREfm isolates. Similarity analysis was performed with the Dice coefficient, and clustering was performed by UPGMA.

FIG. 3. Map of Italy. Triangles indicate the sites of hospital laboratories at which PFGE type 1 VREfm isolates were obtained.

miology of VRE, allowing comparisons of isolates from different sources, especially animal and human isolates (3, 8, 30), and from hospital outbreaks (14, 19, 21, 24). However, these methods often have proven too discriminatory to establish genetic relationships in a broader setting and to gain general insight about isolates from diverse areas and typed by different laboratories. Recently, an MLST scheme originally devised for *Streptococcus pneumoniae* (9) was proposed for *E. faecium* (13). The different sequenced alleles of seven housekeeping genes constitute an unambiguous database that is available through the World Wide Web and that allows comparisons of isolates from different environments and different laboratories.

Although we studied a limited number of VRE isolates, they represent clinically significant isolates obtained from bloodstream infections. All VREfm isolates were multidrug resistant and carried *purK* allele 1, which has been demonstrated to be typical of isolates of human origin (13). While all of the VREfl isolates seemed to retain their susceptibility to ampicillin, therapeutic options for the VREfm isolates appeared to be very limited, with only quinupristin-dalfopristin and linezolid being active.

By using PFGE we demonstrated that the majority of the VREfm isolates are clonally related. We are not aware of epidemiological links among the patients infected, as the isolates were obtained from hospitals scattered throughout the country over a span of 3 years. VREfl isolates appeared to be

TABLE 2. Allelic profiles (MLST), PFGE types, and vancomycin-resistance genes for selected bloodstream-infecting *E. faecium* isolates from Italy

Strain	Country (area)[a]	PFGE subtype	Resistance gene	MLST type	Allele at:						
					atpA	*ddl*	*gdh*	*purK*	*gyd*	*pstS*	*adk*
AE1	IT (C)	1.1	*vanA*	ST78	15	1	1	1	1	1	1
AE20[b]	IT (S)	1.1	*vanA*	ST78	15	1	1	1	1	1	1
AE10	IT (N)	1.7	*vanA*	ST78	15	1	1	1	1	1	1
AE15	IT (N)	1.10	*vanA*	ST78	15	1	1	1	1	1	1
AE12	IT (N)	2.0	*vanB*	ST209	37	1	1	1	1	1	1
A0007	UK		*vanA*	ST17	1	1	1	1	1	1	1
R05	AU		*vanB*	ST17	1	1	1	1	1	1	1
A0008[c]	AU			ST17	1	1	1	1	1	1	1

[a] IT, Italy; UK, United Kingdom; AU, Australia; C, central; S, southern; N, northern.
[b] Resistant to quinupristin-dalfopristin.
[c] Susceptible to vancomycin.

more heterogeneous on the basis of their PFGE profiles and susceptibility patterns.

The findings obtained by PFGE regarding the clonality of VREfm isolates were confirmed by MLST typing. The five representative isolates analyzed by this method belonged to ST78 or to a single-locus variant of this ST. PFGE appears to be more discriminatory than MLST for the typing of *E. faecium*, since the *vanB*-positive strain, considered different from the main clonal group by the former method, was shown by MLST to be a single-locus variant of the same clone bearing a novel *atpA* allele. This discordance might be due to the contribution of genetic mobile elements, such as plasmids, to the number and size of DNA bands in PFGE profiles (28).

According to the published MLST-based classification (13), ST78 is encompassed by clonal group C1, a genetic lineage with a worldwide distribution and including mainly isolates from human infections and hospital outbreaks. Although specific information on isolates belonging to ST78 is not available to date at the MLST website, Bonora and coworkers reported that recent VRE outbreaks in various hospitals in northern Italy were due to VREfm isolates belonging to ST78 (3). Common features of the ST78 clone appear to be multidrug resistance and the presence of the *esp* gene, a marker of hospital-adapted isolates that may be linked to the ability to persist in the hospital environment (29).

Intrahospital transmission of the same clone of VREfm has been demonstrated frequently (5, 21, 27). Conversely, interhospital dissemination of isolates has been rarely studied, although it has been shown to occur when investigated (3, 6, 15, 19, 24). In this study, we found a widespread distribution of a single clone involving the entire country. Since only isolates originating from bloodstream infections were studied—likely representing only a small portion of all of the VRE isolates in health care facilities, which are usually recovered from less serious infections or from colonized patients (12)—the spread of the VREfm clone is likely to be wider and to involve additional hospitals.

In the past decade in Italy, most vancomycin-susceptible *E. faecium* isolates from clinical infections were found to be resistant to ampicillin (10) and to bear the *esp* gene (L. Baldassarri, L. Bertuccini, M. Ammendolia, G. Gherardi, and R. Creti, Letter, Lancet **357:**1802, 2001), representing a fit genetic background for the acquisition of vancomycin resistance determinants. An ampicillin- and streptomycin-resistant, vancomycin-susceptible, *esp*-positive *E. faecium* isolate from our collection, obtained in 1997 from a human infection in Rome, was found to belong to ST78 (data not shown). It can be speculated that a similar hospital-adapted isolate acquired the *vanA* determinant, generating the widespread ST78 VREfm clone. This occurrence has been demonstrated in outbreaks described in France, Poland, and Germany (14, 22, 27). We did not examine the *vanA* resistance determinants of the VREfm isolates to ascertain whether they have similar or different structures and reside on the chromosome or on a plasmid.

As for a possible role of the animal reservoir in the spread of VRE isolates in Italian hospitals, we recently demonstrated that animal VREfm isolates are different from VREfm isolates from human infections on the basis of their antibiotic susceptibility profiles; notably, they are generally susceptible to ampicillin, while the human isolates are not (4). Using various typing methods, several investigators have demonstrated that animal and human VREfm isolates are genetically distinct (2, 13, 30) and that animal isolates do not carry the *esp* gene (29). All of these observations suggest host specificity for VRE isolates, although the genetic elements carrying *vanA* can be shared between human and animal isolates (26). Therefore, it is possible that the animal VRE reservoir that is still present in Italy several years since the withdrawal of avoparcin (A. Ricci and A. Battisti, personal communication) can contribute to the spread of VRE through the sharing of *vanA* resistance determinants with human and hospital-adapted isolates.

In conclusion, our data indicate that the recent increase in the number of VRE infections in Italy might be due to the appearance and spread of a hospital-adapted, multidrug-resistant VREfm clone belonging to an internationally disseminated lineage. It is possible that both horizontal gene transfer and clonal spread have contributed to the high rate of VRE infections in Italy.

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