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BACTERIAL FUNGAL AND VIRUS MOLECULAR BIOLOGY - SHORT COMMUNICATION

Trend of clinical vancomycin-resistant enterococci isolated in a regional Italian hospital from 2001 to 2018

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Abstract

A retrospective study of the epidemiology of vancomycin-resistant enterococci (VRE) in a regional hospital of central Italy in 2001–2018 demonstrated an increased VRE prevalence since 2016. A total of 113 VRE isolates, 89 *E. faecium* (VREfm) and 24 *E. faecalis* (VREfs), were collected in the study period. All strains showed high-level resistance to vancomycin; 107 also showed teicoplanin resistance. Altogether, 84 VREfm and 20 VREfs carried *vanA*, whereas 5 VREfm and 1 VREfs carried *vanB*. MLST analysis documented that 89 VREfm isolates mainly belonged to ST78, ST80, and ST117. Most strains were isolated from 2001 to 2007, ST78 being the predominant clone. VREfm re-emerged in 2016 with a prevalence of the ST80 lineage. Most VREfs were isolated from 2001 to 2006; although they belonged to 7 different STs, there was a prevalence of ST88 and ST6. Notably, ST88 was sporadically recovered throughout the study period. The increasing rate of VREfm isolation from 2016 to 2018 may be related to the influx of new successful clones and to the renewed and widespread use of vancomycin. Improved infection control measures in hospital wards should be adopted to limit the spread of new epidemic VRE strains.

Keywords *Enterococcus faecium* · *Enterococcus faecalis* · Vancomycin resistance · *vanA* gene · *vanB* gene · MLST

Introduction

Among human gut commensals, enterococci have a special ability to colonize healthy carriers and hospital patients, to adapt to adverse environmental conditions, and to evolve and transmit antimicrobial resistance determinants. Enterococci are opportunistic pathogens commonly associated to urinary tract infections, sepsis, and infective endocarditis [1], *Enterococcus faecalis* and *Enterococcus faecium* being the most clinically relevant species. Whereas *E. faecalis* is the more virulent, *E. faecium* rapidly acquires multidrug resistance determinants, a feature that has led to the marked increase of this pathogen as a cause of human infections [1, 2]. In recent decades, the gradual evolution of hospital-associated lineages of *E. faecium* has made it a major nosocomial pathogen worldwide [1].

Vancomycin was largely used in the 1980s as the drug of choice to treat *Clostridioides difficile* enterocolitis as well as severe hospital-acquired infections caused by aminoglycoside- and ampicillin-resistant enterococci and methicillin-resistant *Staphylococcus aureus* (MRSA) [3]. Its heavy clinical use induced a steady increase in vancomycin-resistant enterococci (VRE) until

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the early 2000s. In Europe, their emergence was also ascribed to the administration of the glycopeptide avoparcin as a growth promoter in farm animals, which in the EU was banned in 1997 (Commission Directive 97/6 EC) [4]. The failure of antibiotic treatments against VRE is a serious and growing problem that is associated to increased mortality and rising hospital costs [2]. Moreover, patients infected with or colonized by VRE constitute a reservoir of resistant hospital clones [5, 6]. In hospital patients, VRE bacteremia is often due to the predominance of VRE in the gastrointestinal microbiota after vancomycin treatment [1].

In Italy, VRE recovery declined from 2003 to 2013 as a consequence of the reduced use of glycopeptides in humans and animals [7]. A decline was also detected in 2013–2016 in some European countries and in North America, likely due to regional and national recommendations to use vancomycin more sparingly and to efforts aimed at preventing the spread of MRSA and VRE infections (https://www.ecdc.europa.eu/sites/default/files/documents/AMR-surveillance-Europe-2016) [8].

Recently, VRE incidence has been showing an upward trend in several European countries, including Italy (www.ecdc.europa.eu/en/publications-data/surveillance-antimicrobial-resistance-europe-2017) [9–12]. Although nine vancomycin resistance gene clusters have been described to date, *vanA* and *vanB* (both transferable) are still those most commonly found. The VanA phenotype is characterized by high-level resistance to both vancomycin and teicoplanin, whereas the VanB phenotype is associated to resistance to vancomycin alone [13].

Vancomycin resistance has also been associated to hospital-adapted lineages of *E. faecium* (such as those belonging to Sequence Types ST17, ST18 and ST78) and *E. faecalis* (ST6). Notably, vancomycin resistance is much more common in *E. faecium* [8]. Horizontal propagation of mobile genetic elements plays a greater role than clonal diffusion in resistance spread [14].

A comprehensive picture of VRE epidemiology in Italy is not available. A retrospective study was conducted to characterize the VRE strains isolated at the Marche regional hospital in Ancona (central Italy) from 2001 to 2018.

Material and methods

Bacterial strains

We investigated 113 VRE, 89 *E. faecium* (VREfm), and 24 *E. faecalis* (VREfs), isolated from clinical specimens (mostly urine and blood) collected in 2001–2018 in different hospital wards of a regional teaching hospital in central Italy with almost 1000 beds. Only one isolate per patient was included in the study. Isolates were cultured on brain-heart infusion agar and VRE screening plates containing 6 mg/L vancomycin (both from Oxoid, Basingstoke, UK). Strains were identified by matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF/MS) (Bruker Daltonik GmbH, Bremen, Germany).

Antimicrobial susceptibility testing

Antibiotic susceptibility patterns were demonstrated by the Vitek-2 system (bioMèrieux, Marcy-l'Etoile, France). Vancomycin and teicoplanin minimal inhibitory concentrations (MICs) were determined by the agar microdilution method [15]; the results were interpreted according to the EUCAST MIC breakpoints (www.eucast.org). *E. faecalis* ATCC 29212 was used for quality control.

vanA and *vanB* gene detection by polymerase chain reaction (PCR)

Two primer pairs were used to detect the *vanA* (FW 5'-GGGAAAACGACAATTGC-3′/ RV 5′-GTAC AATGCGGCCGTTA–3′) and *vanB* genes (FW 5′-ATGGGAAGCCGATAGTC-3′/RV 5′- GATTTCGT TCCTCGACC-3′) [16].

Bacterial DNA was obtained by resuspending some colonies collected from a Slanetz Bartley agar plate in 200 μl sterile distilled water and by boiling them for 10 min in a water bath. Then, 5 μl of suspension was added in a final volume of 25 μl of mastermix containing 0.2 μM of each primer for *vanA* and *vanB*, 500 mM dNTP mix, 7 mM $MgCl_2$, and 2 U Dream Taq DNA polymerase (ThermoFisher Scientific, Waltham, MA, USA). PCR conditions were as follows: 94 °C for 3 min; 30 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min; and 72 °C for 5 min. PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems System 9700 GeneAmp PCR Thermal Cycler). PCR products were resolved by electrophoresis on 1.5% agarose gel [16]. *E. faecium* BM4147 (*vanA*) and *E. faecalis* ATCC 51299 (*vanB*) were the positive controls.

Typing assays

Multilocus sequence typing (MLST) was performed as recommended in the MLST database (www.pubmlst.org).

Isolates with identical STs were considered as members of a single lineage, those that differed in only one locus were considered as single-locus variants (SLV), and those that

differed in two loci were considered as double-locus variants (DLV). Using eBURST analysis, STs sharing 5 or 6 of the 7 loci were clustered into a clonal complex (CC). The goeBURST 1.2.1 algorithm was used to cluster the STs (http://www.phyloviz.net/).

Pulsed-field gel electrophoresis (PFGE) typing was performed as described previously [17]. Briefly, genomic DNA extracted from cells embedded in agarose plugs was digested with SmaI endonuclease (New England Biolabs, Beverly, MA), and the resulting fragments were separated by PFGE. DNA patterns were analyzed with BioNumerics software version 7.0 (Applied Maths Scientific Software Development, Sint-Martens-Latem, Belgium). The dendrogram was built by applying the Dice similarity coefficient, with 1.5% optimization and 2.0% tolerance. Clustering was obtained using the unweighted pair group method with arithmetic mean. Strains showing a pattern similarity > 80% were considered closely related and grouped into clusters.

Results and discussion

We investigated 113 VRE, 89 VREfm, and 24 VREfs, isolated in 2001–2018 in a regional hospital in central Italy for their vancomycin and teicoplanin resistance genotype and phenotype and clonal relatedness. All isolates showed high-level resistance to vancomycin (MIC range, 32; > 256 mg/L) and 107 also to teicoplanin (MIC range, 4; > 256 mg/L). The remaining 6 strains (3 VREfm and 3 VREfs) showed teicoplanin MICs < 0.5 mg/L.

PCR screening demonstrated *vanA* in 92% of isolates (84 VREfm and 20 VREfs) and *vanB* in 5.3% (5 VREfm and 1 VREfs). The remaining 3 (2.7%) isolates (all VREfs) were negative for both determinants. Although 9 *van* operons related to vancomycin resistance have been described, the *vanA* and *vanB* gene clusters are still prevalent among clinical VRE isolates, with varying frequencies in different countries [13]. In recent surveys, a higher proportion of VREfm and VREfs showing the VanA phenotype has been described in Europe and North America, whereas in Australia *vanB*-positive VREfm strains were involved in more than 50% of bacteremias caused by VRE in 2015 [8, 18].

In this study, *vanA* was the most frequent vancomycin resistance determinant among VREfm and VREfs, in line with previous Italian studies [7, 19].

MLST analysis assigned the 89 VREfm isolates to 9 STs (Fig. 1a). The most common were ST78 (54%), ST80 (15%), and ST117 (10%) and were followed by ST1590 and ST1115 ($n = 4$), ST17 ($n = 3$), ST18 and ST1666 ($n = 2$), and ST145, ST202, and ST1626 ($n = 1$). Interestingly, more that 60% of the VREfm isolates belonged to ST17, ST18, and ST78, which are well-known members of CC17, one of the most widespread nosocomial clones of *E. faecium*. The *vanA* strains were distributed in all STs, whereas *vanB* isolates were found only in ST18, ST117, and ST80. eBURST analysis grouped all STs in a single CC, since each ST was a SLV (ST17, ST18, ST117, ST145, ST1115, ST1590, ST1626, and ST1666) or a DLV (ST80 and ST202) of the putative founder ST78.

The analysis of VREfm distribution revealed that most ($n = 59$) were isolated from 2001 to 2007, the largest number ($n = 17$) being isolated in 2006, and that ST78 (accounting for 74% of the isolates) was predominant throughout this period. No VREfm was recovered in 2008–2015 with the exception of a single ST117 strain isolated in 2013. VREfm re-emerged in 2016 ($n = 8$) and was also isolated in 2017 ($n = 9$) and 2018 ($n = 12$). The most common STs were ST117 in 2016 and ST80 in 2017 and 2018. Although ST78 was again recovered in 2018, ST80 is currently the dominant lineage in the hospital (Fig. 1c and Fig. 2a). The replacement of ST78 by ST117 and ST80 may be related to a greater adaptability of these new VRE clones to the clinical setting.

The ST78 strains circulating until 2006 clustered in two main, closely related pulsotypes and were collected in different wards, whereas a new ST78 clonal group with a different pulsotype (similarity < 80%) was established in 2018. The strains isolated in 2017–2018 belonging to ST117 and ST80 show no clonal relationship, as documented by their pulsotypes (Fig. 2a).

The 24 VREfs belong to 7 STs (Fig. 1b), which according to eBURST analysis were unrelated. The most represented was ST88 ($n = 13$) followed by ST6 ($n = 6$) and by ST159, ST170, ST952, ST44, and ST282. The *vanA* VREfs ($n = 20$) were distributed among all the STs, whereas the single *vanB* VREfs belonged to ST6. The 79% of VREfs belonged to ST88 and ST6 which include nosocomial multidrug-resistant enterococcal strains [20]. Moreover, ST6 (CC2) has frequently been reported as a cause of invasive infection [20]. Although a limited number of VREfs were recovered during the study period, most (58%) were isolated in 2001–2006; notably, the dominant clone (ST88) continued to be sporadically recovered throughout the study period. Altogether, the VREfs are genetically diverse, except for a small ST88 cluster that encompassed isolates recovered from different wards and sample types in 2004–2006 and in 2012

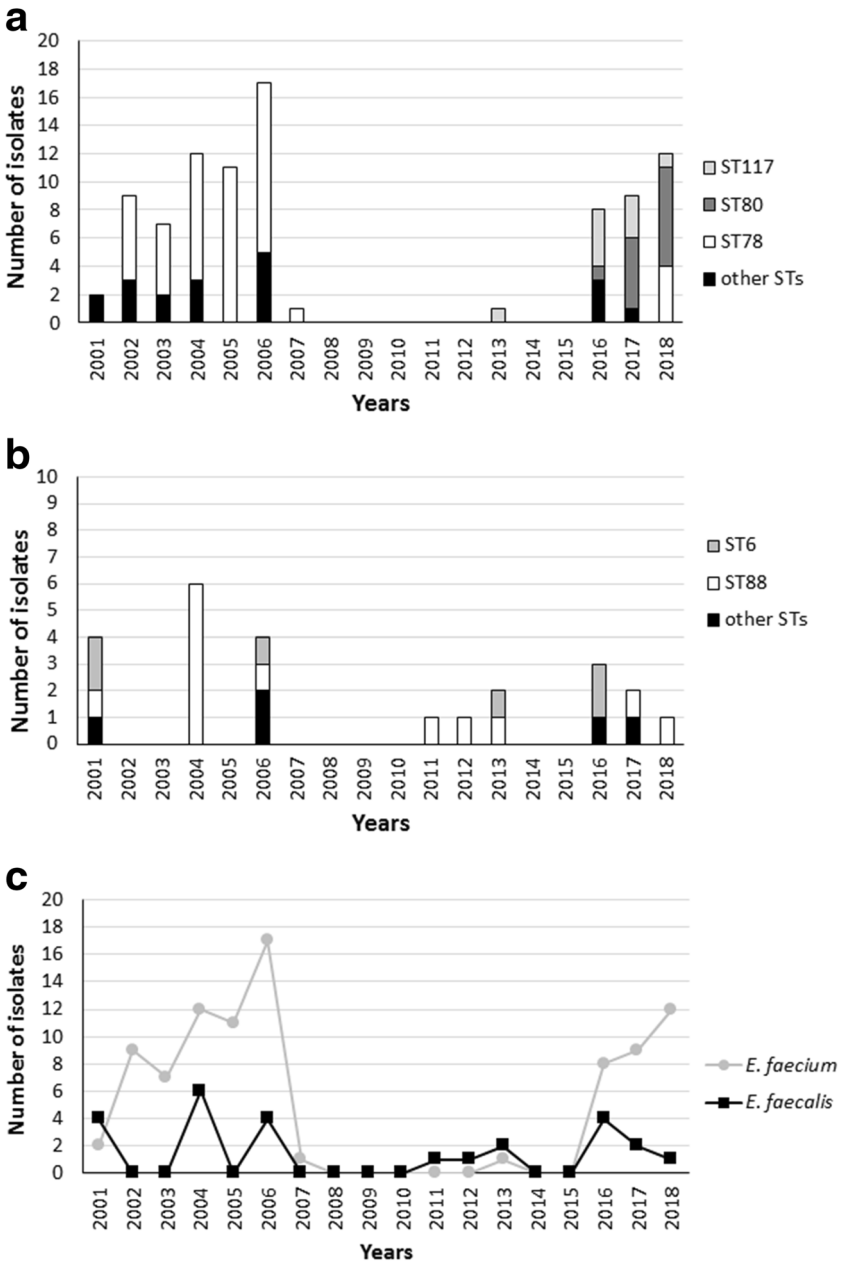


Fig. 1 ST distribution of VREfm isolates from 2001 to 2018 (**a**). ST distribution of VREfs isolates from 2001 to 2018 (**b**). Total distribution of VRE isolates from 2001 to 2018 (**c**)

(Fig. 2b). The overall distribution of VREfm and VREfs in the study period is shown in Fig. 1c.

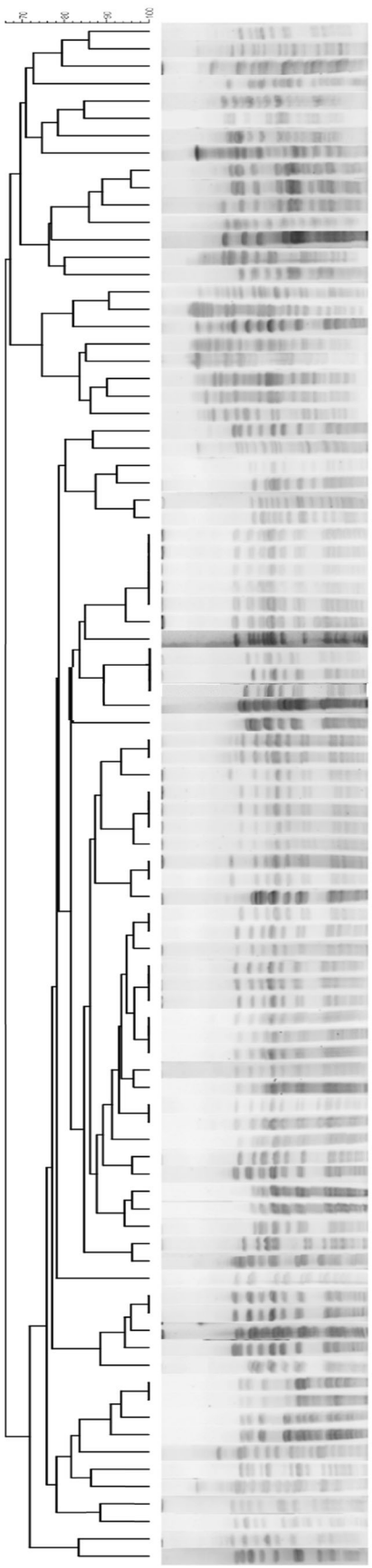
Although the prevalence of specific VREfm and VREfs clones was observed in the hospital in 2001–2018, PFGE analysis disclosed an overall genetic heterogeneity among strains within the same lineage. This finding suggests that the diffusion of *vanA* resistance could be due to the dissemination of Tn*1546*-like elements rather than to clonal diffusion of a single strain [14].

This study reports an increasing incidence of VRE infections in an Italian hospital from 2016 to 2018 that is in line with reports from other European countries [9–12]. It is also in line with the literature showing that VREfm has overtaken VREfs as agents of hospital-acquired infections [21].

As in Denmark [22], the increase in VRE infections in our hospital could be related to the influx of new successful VRE lineages such as ST80. The increasing tolerance of VRE to the biocides used in hospital settings [21] and

Fig. 2 SmaI-PFGE pattern and dendrogram of VREfm (**a**) and VREfs isolates (**b**). The main pulsotypes are highlighted in a square box

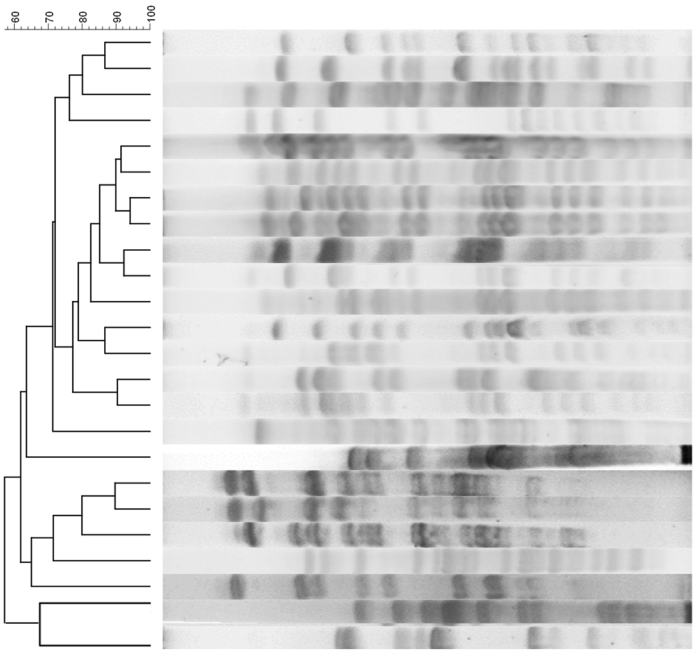
a



Strain	ST	*van* gene	Year	Ward
245392	ST78	*vanA*	2006	Clinic of Haematology
437423-2	ST117	*vanB*	2018	ICU
208644	ST78	*vanA*	2005	Medicine
384557	ST117	*vanB*	2017	Medicine
315994-3	ST117	*vanA*	2016	Medicine
386727	ST80	*vanA*	2017	Hepatic Transplant Surgery
296648	ST117	*vanA*	2016	ICU Lancisi
436329-3	ST80	*vanA*	2018	Emergency
394839	ST80	*vanA*	2017	Medical Clinic
395893	ST80	*vanA*	2017	Infectious Diseases Clinic
388951	ST117	*vanA*	2017	Infectious Diseases Clinic
414164	ST145	*vanA*	2017	Medicine
438354	ST80	*vanA*	2018	Clinic of Neurology
243543	ST78	*vanA*	2006	ICU Salesi
409688	ST80	*vanA*	2017	LTC
161065	ST1666	*vanA*	2004	Clinic of Gastroenterology
154596	ST1666	*vanA*	2004	Clinic of Neurorehabilitation
235395	ST78	*vanA*	2006	ICU Salesi
438173	ST80	*vanA*	2018	Infectious Diseases Clinic
297754	ST17	*vanA*	2016	Nefrology
432388	ST80	*vanA*	2018	Coronaric ICU
10140	ST80	*vanA*	2018	LTC
175688	ST78	*vanA*	2005	ICU
254696	ST78	*vanA*	2006	Gastroenterology
427997	ST80	*vanA*	2018	Clinic of Gastroenterology
219133	ST78	*vanA*	2005	Paediatric ICU
243779	ST78	*vanA*	2006	Clinic of Urology
312031	ST117	*vanA*	2016	Infectious Diseases Clinic
311921	ST117	*vanA*	2016	ICU Lancisi
86111	ST78	*vanA*	2003	NA
58100	ST78	*vanA*	2002	NA
50146	ST78	*vanA*	2002	NA
47979	ST78	*vanA*	2002	NA
58883	ST78	*vanA*	2002	NA
128764	ST78	*vanA*	2004	NA
272442	ST202	*vanA*	2006	Oncology
169304	ST78	*vanA*	2004	Infectious Disease Clinic
244584	ST78	*vanA*	2006	ICU
331861	ST78	*vanA*	2007	General Surgery
296234	ST17	*vanA*	2016	Nefrology
246721	ST1626	*vanA*	2006	Paediatric Infectious Diseases
59205	ST78	*vanA*	2002	NA
133453	ST78	*vanA*	2004	Haematology
115515	ST78	*vanA*	2003	NA
75580	ST1590	*vanA*	2003	NA
59199	ST1590	*vanA*	2002	NA
99531	ST1590	*vanA*	2001	NA
24117	ST1590	*vanA*	2001	NA
117338	ST78	*vanA*	2003	NA
119338	ST78	*vanA*	2003	NA
235741-2	ST78	*vanA*	2006	Paediatric ICU
50216	ST78	*vanA*	2002	NA
10369	ST78	*vanA*	2004	NA
12230-2	ST78	*vanA*	2005	NA
175676	ST78	*vanA*	2005	ICU
189897	ST78	*vanA*	2005	Clinic of Haematology
11872	ST78	*vanA*	2005	NA
185566	ST78	*vanA*	2005	Clinic of Haematology
142955	ST78	*vanA*	2004	Infectious Diseases Clinic
187829	ST78	*vanA*	2005	Clinic of Haematology
195241	ST78	*vanA*	2005	Gastroenterology
177683	ST78	*vanA*	2005	LTC
120623	ST78	*vanA*	2003	NA
132301	ST78	*vanA*	2004	Clinic of Urology
130652	ST78	*vanA*	2004	Clinic of Surgery
233651	ST78	*vanA*	2006	Gastroenterology
242995	ST78	*vanA*	2006	Clinic of Haematology
166532	ST78	*vanA*	2004	Sub-intensive Medicine
134843	ST78	*vanA*	2004	ICU Lancisi
247184	ST78	*vanA*	2006	Paediatric Neuropsichiatry
CQ6038	ST18	*vanB*	2002	NA
296342	ST18	*vanA*	2016	Nefrology
83389	ST17	*vanA*	2003	NA
258950	ST1115	*vanA*	2006	Clinic of Haematology
264157	ST1115	*vanA*	2006	Clinic of Neurosurgery
156598	ST1115	*vanA*	2004	ICU Lancisi
255151	ST1115	*vanA*	2006	Clinic oh Haematology
244471	ST78	*vanA*	2006	Paediatric Neuropsichiatry
430147-2	ST78	*vanA*	2018	Hepatic Transplant Surgery
432133	ST78	*vanA*	2018	Clinic of Dermatology
430277	ST78	*vanA*	2018	Nefrology
419715	ST78	*vanA*	2018	Nefrology
316632	ST80	*vanA*	2016	Medicine
391131	ST117	*vanB*	2017	Nefrology
423993	ST80	*vanA*	2018	Clinic of Surgery
CA805	ST17	*vanA*	2002	NA
81983-2	ST117	*vanB*	2013	Plastic Surgery
232695	ST78	*vanA*	2006	ICU Lancisi
396891	ST80	*vanA*	2017	ICU

Abbreviations: ICU, intensive care unit; LTC, Long-term care; NA, not available.

b



Strain	ST	*van* gene	Year	Ward
580606-1	ST6	*vanA*	2001	LTC
83863-2	ST6	*vanA*	2013	Medicine
386813	ST88	-	2017	Paediatrics
268910	ST170	*vanA*	2006	Sub-intensive Medicine
159259	ST88	*vanA*	2004	Haematology
233913	ST88	*vanA*	2006	Medical Clinic
158657	ST88	*vanA*	2004	Neurosurgery
136029	ST88	*vanA*	2004	Sub-intensive Medicine
148606	ST88	*vanA*	2004	Sub-intensive Medicine
56870-3	ST88	*vanA*	2012	Clinic of Neurosurgery
276358	ST952	*vanA*	2006	Nefrology
559198	ST88	*vanA*	2001	LTC
73766-2	ST88	*vanA*	2013	Neurosurgery
151290	ST88	*vanA*	2004	NA
574829	ST88	*vanA*	2011	LTC
302548	ST44	-	2016	Emergency
436177-2	ST88	*vanA*	2018	LTC
252634	ST6	*vanB*	2006	ICU Lancisi
312729	ST6	*vanA*	2016	Clinic of Neurosurgery
303777-2	ST6	*vanA*	2016	Vascular Medicine
595391	ST6	*vanA*	2001	LTC
375834	ST282	-	2017	Oncology
154903	ST88	*vanA*	2004	Clinic of Orthopaedics
554722-2	ST159	*vanA*	2001	LTC

Abbreviations: LTC, Long-term care; NA, not available.

Fig. 2 (continued)

the renewed widespread use of vancomycin to treat severe infections caused by multidrug-resistant enterococci and MRSA may also be contributing factors. Furthermore, the use of broad-spectrum antibiotics (particularly carbapenems, third-generation cephalosporins) or antibiotic combinations (such as piperacillin/tazobactam) against Gram-negative bacteria in the hospital environment may favor the selection of intestinal VRE, thus increasing the risk of VRE infections [8].

An in-depth knowledge of the genotype of endemic nosocomial VRE and of the relatedness of the different isolates is critical to limit the fast dissemination of old and new VRE clones in clinical settings. The re-emergence of endemic VRE in European (and Italian) hospitals emphasizes the need for stringent control measures to reduce the risk of dissemination of resistant clones, such as the isolation of infected/colonized patients, more accurate disinfection procedures, and improved antimicrobial treatments.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

Ethics approval Ethical approval was waived by the institutional ethics committee due to the retrospective nature of the study and to the fact that all procedures were part of routine care.

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