

## Emergence of Linezolid Resistance in the Vancomycin-Resistant *Enterococcus faecium* Multilocus Sequence Typing C1 Epidemic Lineage

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**A relatively high rate of vancomycin-resistant *Enterococcus faecium* not susceptible to linezolid was observed in intensive care unit patients. Linezolid-resistant isolates carried the G2576T mutation in the 23S rRNA gene, belonged to different clones, and shared the same allelic profile, which clusters in the C1 multilocus sequence typing epidemic lineage.**

Linezolid, an oxazolidinone antibiotic, was introduced early in 2000 as a new therapeutic option against gram-positive cocci, including vancomycin-resistant enterococci (VRE). Over the 4-year period since the beginning of linezolid clinical use, resistance began to emerge, albeit at a very low rate, in clinical isolates of enterococci (4, 9) and was dependent on prior linezolid exposure and duration of therapy (10). The molecular basis of linezolid resistance has been identified as a single G2576T nucleotide polymorphism in multiple alleles encoding 23S rRNA (7, 8, 11–14).

From January to December 2004, active surveillance of VRE colonization (stool or rectal swab cultures) was performed on 127 patients admitted to different intensive care units (ICUs) at the General Hospital of Verona, Verona, Italy (hospital-wide ICU, neurosurgery ICU, and transplant ICU), as part of a series of measures to control nosocomial infection by VRE. All VRE isolated from both colonized and infected patients were stored for antibiotic susceptibility testing and molecular characterization.

A total of 35 VRE isolated from the 127 patients were used in this study. Identification to the species level was performed by biochemical tests (API; bioMérieux). The resistance genotype of VRE was identified by PCR using primers specific for *van* genes, as described previously (1). Antimicrobial susceptibility was determined by agar diffusion according to the CLSI (formerly NCCLS) guidelines (2). *Enterococcus faecalis* ATCC 29212 was used for quality control. The linezolid minimum inhibitory concentration (MIC) was determined by Etest (Oxoid). CLSI MIC interpretative standards for *Enterococcus* spp. tested against linezolid were as follows:  $\leq 2$   $\mu\text{g/ml}$  for the susceptible category, 4  $\mu\text{g/ml}$  for the intermediate category, and  $\geq 8$   $\mu\text{g/ml}$  for the resistant category. Strain clonality was analyzed by pulsed-field gel electrophoresis (PFGE), as de-

scribed previously (1). Strains representative of each distinct PFGE pattern were analyzed by multilocus sequence typing (MLST), as proposed by Homan et al. (6). Both linezolid-susceptible (LS) and linezolid-nonsusceptible (LNS) isolates were analyzed for the presence of the G2576T mutation (according to the *Escherichia coli* numbering system) in the V domain of the 23S rRNA gene, which generates a new restriction site for NheI (14). An internal fragment (746 bp) of the 23S rRNA gene, including the V domain, was amplified by PCR in both LS and LNS isolates. Primers designed on the basis of the sequence of 23S rRNA for *Enterococcus faecium* (GenBank accession number AF4329149) were 23Sfor (5'-TAG TAC CTG TGA AGA TGC AGG-3') and 23Srev (5'-CAC ACT TAG ATG CTT TCA GCG-3'). The cycling conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 30 cycles consisting of 94°C for 1 min, 50°C for 30 s, 72°C for 1 min, and a final extension step at 72°C for 5 min. Small amounts (5  $\mu\text{l}$ ) of PCR 23S rRNA were digested with 10 units of NheI (Invitrogen Life Technologies) at 37°C for 4 h and separated on 2% agarose gel in 1 $\times$  Tris-acetate-EDTA buffer. To confirm the G2576T point mutation, an allele-specific PCR assay was used in which total DNA was subjected to two distinct PCRs with primers specific for the wild type and mutant (7). The specificities of both reactions were assessed by testing 35 LS vancomycin-resistant *E. faecium* (VREF) strains with the same procedures. Sequencing of the internal fragment of the 23S rRNA gene obtained by PCR was also done to confirm the G2576T mutation.

Of the 127 patients admitted to different ICUs at the General Hospital of Verona, 29 (22.8% colonization rate) were positive for VRE (24 for *E. faecium*, 3 for *Enterococcus gallinarum*, 1 for *E. faecalis*, and 1 for *Enterococcus avium*). Ten patients developed clinically significant infections caused by VRE (7.4% infection rate); six of these did not present concurrent stool colonization.

Of the 35 VRE infected/colonized patients, 14 carried LNS VREF. Eight of these patients harbored strains with intermediate susceptibility (MIC = 4  $\mu\text{g/ml}$ ), and six harbored resis-

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TABLE 1. Clinical data of patients infected or colonized by linezolid-resistant *E. faecium* and molecular typing of linezolid-resistant isolates

Patient (sex/age [yr])	Ward <sup>a</sup>	Underlying disease(s)	Risk/outcome <sup>b</sup>	Strain code	Type or site of infection (isolation date [mo/day/yr])	Linezolid MIC ( $\mu\text{g/ml}$ )	PFGE type <sup>c</sup>	MLST type	Detection of G2576 mutation	Detection of T2576 mutation
P1 (male/64)	TICU	Kidney transplant	Prolonged hospitalization (58 days), ICU care, linezolid use, discharged	SM902 SM912	Ascites (03/25/04) Ascites (05/05/04)	4 (LI) 8 (LR)	D <sub>04</sub> D <sub>04</sub>	78	Yes Yes	No Yes
P2 (female/71)	NICU	Communicans artery aneurysm	Prolonged hospitalization (81 days), ICU care, linezolid use, discharged	SM922 SM924 SM925	Urine (07/12/04) Rectal (07/13/04) Rectal (07/21/04)	8 (LR) 8 (LR) 8 (LR)	C <sub>04</sub> C <sub>1,04</sub> C <sub>04</sub>	78	Yes Yes	Yes Yes
P3 (female/64)	ICU	Ictus, diabetes	Died after 8 days of hospitalization	SM941	Rectal (10/19/04)	8 (LR)	F <sub>04</sub>	78	Yes	Yes
P4 (female/75)	NICU	Communicans artery aneurysm	Prolonged hospitalization (56 days), ICU care, discharged	SM934	Rectal (09/22/04)	64 (LR)	A* <sub>2n,04</sub>	78	Yes	Yes
P5 (male/74)	ICU	Necrotizing pancreatitis	Prolonged hospitalization (46 days), ICU care, linezolid use, death	SM943	Ascites (11/02/04)	64 (LR)	A* <sub>2,04</sub>	78	Yes	Yes
P6 (male/67)	ICU	Spontaneous esophageal rupture, mediastinitis, peritonitis	Hospitalization overlapping P5, ICU care, discharged	SM936 SM944	Rectal (10/01/04) Ascites (11/18/04)	64 (LR) 64 (LR)	A* <sub>2,04</sub> A* <sub>2,04</sub>	78	Yes Yes	Yes Yes

<sup>a</sup> ICU, hospital-wide intensive care unit; TICU, transplant ICU; NICU, neurosurgery ICU.

<sup>b</sup> Time elapsing from date of admission to date of isolation of first LR VREF in body samples is shown in parentheses.

<sup>c</sup> \* indicates the PFGE type of the index strain (SM685) of nosocomial outbreaks in northern Italy (1).

tant strains (MIC  $\geq 8 \mu\text{g/ml}$ ). Table 1 gives the clinical data for the six patients infected by linezolid-resistant (LR) VREF. The criteria for active infection as listed by the Centers for Disease Control and Prevention (3) were met for the patients from whom the strains were isolated. In all, one linezolid-intermediate (LI) isolate and nine LR isolates were recovered and analyzed (Table 1). Five of the six patients were infected by LR

VREF (MIC  $\geq 8 \mu\text{g/ml}$ ), and one was initially infected by a VRE with intermediate susceptibility to linezolid which developed resistance during linezolid therapy. All strains were resistant to vancomycin and teicoplanin, to ampicillin, and to high concentrations of gentamicin and streptomycin; all were susceptible to quinupristin-dalfopristin; and all carried the *vanA* gene.

Four prevalent PFGE types were identified among these

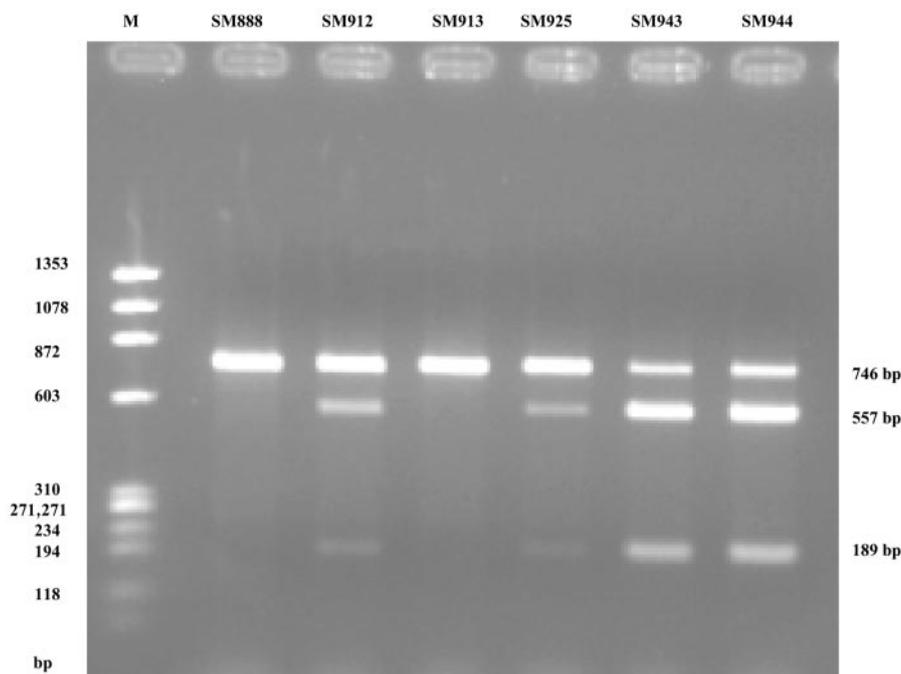


FIG. 1. Digestion of 746-bp 23S rRNA obtained by PCR with *NheI* and separated on 2% agarose gel in  $1\times$  Tris-acetate-EDTA buffer. Lane 1,  $\phi$ X174 replicative-form DNA/*HaeIII* molecular mass standard; lanes 2 and 4, linezolid-susceptible strains SM888 and SM913 (MIC, 2  $\mu\text{g/ml}$ ); lanes 3 and 5, strains SM912 and SM925 (MIC, 8  $\mu\text{g/ml}$ ); lanes 6 and 7, strains SM943 and SM944 (MIC, 64  $\mu\text{g/ml}$ ).

strains (Table 1). One strain representative of each distinct PFGE pattern was typed by MLST, and all were found to belong to the same sequence type, 78, as that of the reference strain *E. faecium* SM685, representative of strains which caused nosocomial outbreaks in several hospitals in northern Italy from 2000 to 2001 (1).

The G2576T point mutation was detected only in strains with linezolid MICs of  $\geq 8$   $\mu\text{g/ml}$ . Figure 1 shows the results of *NheI* digestion of the PCR amplicons from the V domain of the 23S rRNA gene. The 746-bp band corresponding to the undigested amplification products and the two bands of 557 bp and 189 bp corresponding to the two products of *NheI* digestion were found in all LR VRE, suggesting that both wild-type and mutant alleles were present in the same strain. The higher density of the 746-bp band in strains with MICs of 8  $\mu\text{g/ml}$  suggested a lower copy number of the mutated gene in these strains than that in strains with MICs of 64  $\mu\text{g/ml}$ . The presence of the G2576T point mutation in linezolid-resistant strains was confirmed by allele-specific PCR and by sequencing the internal fragment of the 23S rRNA gene obtained by PCR (not shown).

The emergence of LR VRE has been reported in several studies, but overall, it is considered to be uncommon (9). Thus, the finding of a high rate of linezolid nonsusceptibility in VREF isolated from patients in the ICUs of our institution is quite alarming. Our data, combined with the clinical descriptions, suggest different modalities of acquisition of LR VREF: (i) an independent event of de novo selection of resistant mutants in colonizing/infecting VREF (patients P1, P2, and P3, who carried genetically unrelated strains), (ii) possible patient-to-patient spread (patients P4, P5, P6, who carried genetically related strains), and (iii) emergence of linezolid-resistant mutants from LI VRE during linezolid therapy (patient P1).

In our patients, the risk factors for LNS VRE infections were the same as those described for LS VRE (10): prolonged hospitalization, serious underlying diseases, multiple cycles of antibiotic treatments, etc. However, in one case (patient P3), none of these risk factors were present. The patient, colonized by an LR VREF with a unique PFGE type, was a 64-year-old female living at home with no hospitalization over the previous 10 years and no antibiotic treatment during the past year. Patient P6 did not receive linezolid therapy but had other risk factors, including overlapping hospitalization with patient P5, who was treated with linezolid for 3 weeks.

Clinical microbiology laboratories should be aware of the emergence of resistance and should test appropriate isolates for susceptibility to linezolid. CLSI breakpoints for enterococci should be carefully applied, and strains with intermediate susceptibility (MIC = 4  $\mu\text{g/ml}$ ) should be considered at the risk of developing LR under antibiotic selective pressure. The case (patient P1) of selection of LR VREF during therapy in a patient infected by an LI VREF who apparently did not carry the point mutation strongly suggests the possibility that the molecular assay we used was not sufficiently sensitive to detect a low number of copies of the mutated allele. In a study based

on pyrosequencing of 23S rRNA genes, the occasional detection of the G2576T mutation in strains with an MIC of 4  $\mu\text{g/ml}$  has been reported (13).

The high rate of LNS VREF we found in our institution is suggestive of an increasing resistance rate trend that could limit the clinical use of linezolid in the near future. Also intriguing is the fact that all the LR VREF strains belonged to the MLST C1 sublineage that has been emerging recently worldwide and includes isolates mainly from human infections and hospital outbreaks (1, 6). Acquisition of linezolid resistance is the latest disturbing conquest of this lineage and would limit the range of effective therapies available for various life-threatening infections.

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