Analysis of methods commonly used for glycopeptide and oxazolidinone susceptibility testing in *Enterococcus faecium* isolates

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The susceptibility to teicoplanin, vancomycin and linezolid of 30 clinical isolates of *Enterococcus faecium* was tested by Vitek 2, Phoenix, Etest, broth microdilution and disc diffusion tests. The *vanA* and *vanB* resistance genes and the 23S rRNA gene G2576T mutation were detected by PCR and PCR-RFLP, respectively. Resistance rates to teicoplanin ranged from 3 % for Vitek 2 to 57.6 % for the Phoenix test, and those to vancomycin ranged from 56.7 % for Vitek 2 to 86.7 % for the Phoenix test. Only two out of 25 strains carrying the *vanA* gene were univocally recognized as the VanA phenotype. The only strain with the G2576T mutation did not carry the *vanA* gene and showed resistance to linezolid by the disc diffusion, Vitek 2 and broth dilution methods (MIC >8 µg ml⁻¹), but was susceptible when tested with the Phoenix test and Etest (MIC ≤4 µg ml⁻¹). Therefore, the resistance to glycopeptides and linezolid was not univocally detected by the susceptibility testing methods used in this study.

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INTRODUCTION

Enterococcus species are part of the normal flora of the human gut and other animals. In recent years, enterococci have become important nosocomial pathogens, as they are among the leading causes of bacteraemia in hospitalized patients (Biedenbach *et al.*, 2004). One of the characteristics of these organisms is their resistance to several antimicrobial agents that represent the widely used therapeutic options, such as β -lactams, macrolides, aminoglycosides and, of particular clinical relevance, glycopeptides. The resistance of enterococci to vancomycin has increased progressively in western countries in recent years (NNIS, 2001; Rice *et al.*, 2004). As reported by the SENTRY surveillance program, resistance to vancomycin is commonly recovered for *Enterococcus faecium*, being observed in North America and Europe with resistance rates of 93 and 74.1 %, respectively (Deshpande *et al.*, 2007). The introduction of linezolid into clinical practice has offered an alternative therapeutic option for the successful treatment of glycopeptide-resistant enterococci, especially *E. faecium* (Falagas & Vardakas, 2008; Linden, 2002; Scheetz *et al.*, 2006). However, the prompt emergence of linezolid-resistant strains of *E. faecium* has been reported (Gonzales *et al.*, 2001; Jones *et al.*, 2007). Therefore, correct identification of strains and determination of their susceptibility to glycopeptides and oxazolidinone are fundamental for the treatment of infections caused by multiresistant strains of *E. faecium*.

The purpose of this study was to assess the performance of methods commonly used in susceptibility testing for teicoplanin, vancomycin and linezolid. Antimicrobial susceptibilities obtained from the bioMérieux Vitek 2 and BD Phoenix systems were compared with those obtained by a disc diffusion test, Etest and broth microdilution determination of MIC against clinical isolates of *E. faecium*. Resistance to glycopeptides was then correlated with the carriage of *vanA* and *vanB* genes, and resistance to linezolid with the presence of the G2576T mutation in domain V of the 23S rRNA gene by molecular methods.

METHODS

Strain collection. Thirty strains of *E. faecium* were collected during 2007 from clinical specimens from patients hospitalized in different wards of the 'Azienda Policlinico Umberto I' academic hospital of Rome, Italy. The strains were representative of a single infective episode and were isolated from blood and central venous catheters (ten strains), the urogenital tract (seven strains), wound infections (six strains), bile (two strains) and swabs of various anatomical sites (five strains). The strains were collected if they displayed an MIC for teicoplanin and/or vancomycin of ≥ 2 µg ml⁻¹ during routine screening, performed in the diagnostic laboratory by the Vitek 2 test (bioMérieux; software version 4.01). Only one strain (L228) was found to be resistant to linezolid. Bacterial strains were adhered to porous beads (Microbank; Pro-lab Diagnostics) and stored at -80 °C until further analysis.

Identification and antimicrobial susceptibility testing. The strains were analysed by the Vitek 2 system, using ID-GPC cards

for identification and by both P535 and P534 cards for susceptibility testing, and by the Phoenix system (BD Diagnostic Systems) using PMIC panels, following the manufacturer's recommendations. Strain identification was verified by the biochemical reactions of the API 20 STREP strips (bioMérieux). Etest (AB Biodisk), broth microdilution determination of MIC and disc diffusion test were also used to check susceptibility to teicoplanin, vancomycin and linezolid, following the manufacturer's instructions and Clinical and Laboratory Standards Institute guidelines. *Staphylococcus aureus* ATCC 25923 was routinely used as a quality control strain for the disc diffusion tests, and *Enterococcus faecalis* ATCC 29212 was used for broth microdilution and the automated methods (CLSI 2006, 2007). The susceptibility of *E. faecium* ATCC 19434 to the test drugs was also tested as a control for all the susceptibility tests studied. Each isolate resistant to glycopeptides was assigned to a resistance phenotype as follows: strains resistant to both vancomycin and teicoplanin were ascribed to the VanA phenotype; strains resistant to vancomycin but susceptible to teicoplanin were assigned to the VanB phenotype (Deshpande *et al.*, 2007). The results of susceptibility tests for vancomycin were considered to be false-positives for those strains non-susceptible to the drug (either resistant or with intermediate susceptibility) in the absence of *vanA* gene carriage, whilst false-negative test results were defined for those strains that were susceptible to vancomycin in the presence of *vanA* gene carriage.

Amplification of *vanA* and *vanB* genes and detection of the G2576T mutation in the 23S rRNA gene. PCR was used to detect the presence of the *vanA* and *vanB* resistance genes, using a PCR Reagent System (Gibco-BRL). Reactions were carried out in a Perkin-Elmer 480 thermal cycler in a 50 µl volume containing: 1.25 U *Taq* polymerase, 0.2 mM each dNTP, 1.5 mM MgCl₂, 1 × PCR buffer, 1 µg each primer and 0.1 µg template DNA. PCR was performed for 35 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C, followed by a final extension step at 72 °C for 10 min. Primers specific for regions flanking Tn1546, *vanRS*, *vanSH*, *vanHaX*, *vanXY* and *vanYZ* were used to amplify this area, as described previously (Alam *et al.*, 2001; Clark *et al.*, 1993; Donabedian *et al.*, 2000; Thal *et al.*, 1998).

The molecular basis of linezolid resistance has been identified as a single G2576T nucleotide substitution in multiple alleles encoding the 23S rRNA gene, which generates a new restriction site for the *NheI* restriction enzyme. The presence of the G2576T mutation (*Escherichia coli* numbering scheme) in domain V of the 23S rRNA gene was detected as described previously (Bonora *et al.*, 2006). An internal fragment (746 bp) of the 23S rRNA gene, including the V domain, was amplified by PCR. Primers designed on the basis of the sequence of the 23S rRNA gene for *E. faecium* (GenBank accession no. AF432914) were 23Sfor (5'-TAGTACCTGTGAAGATGCAGG-3') and 23Srev (5'-CACACTTAGATGCTTTCAGCG-3'). The cycling conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 30 s and 72 °C for 1 min, with a final extension step at 72 °C for 5 min. Samples (5 µl) of the PCR product were digested with 10 U *NheI* enzyme (Invitrogen Life Technologies) at 37 °C for 4 h and separated on a 2 % agarose gel in 1 × Tris/acetate/EDTA buffer. The 746 bp band corresponding to the undigested amplification product represented the wild-type, and the presence of two bands of 557 and 189 bp corresponded to the products of *NheI* digestion and indicated the mutant alleles responsible for linezolid resistance.

RESULTS AND DISCUSSION

The results of Vitek 2 and Phoenix agreed with the API 20 STREP system in identifying the collected strains as *E.*

faecium. All strains were susceptible to ampicillin and quinupristin/dalfopristin by all of the methods tested.

The Vitek 2 results indicated that only strain L117 was resistant to teicoplanin (Table 1), and the results obtained with P535 cards differed from those of P534 cards for two strains (L103 and L210) recognized as intermediate. The MIC values obtained by Vitek 2 fully agreed with those observed by the broth microdilution test for two strains only (L228 and L259), whilst the MICs for the remaining strains were at least a twofold dilution lower than those obtained by the broth microdilution test. Furthermore, 12 teicoplanin-resistant strains with MICs $\geq 16 \mu\text{g ml}^{-1}$ in the broth microdilution test were reported by Vitek 2 to be in the susceptibility range with MICs $\leq 2 \mu\text{g ml}^{-1}$.

The susceptibility tests performed by the Phoenix test showed that 12 strains were susceptible to teicoplanin, one strain (L89) was intermediate and the remaining 17 strains were resistant to teicoplanin (MIC $\geq 16 \mu\text{g ml}^{-1}$). The MIC values obtained by the Phoenix test fully agreed with those observed by the broth microdilution test for 21 strains, and differed for eight strains by one or two twofold dilutions, even though they were in the same range of susceptibility. A single strain (L118) was shown to be susceptible by the Phoenix test (MIC = $8 \mu\text{g ml}^{-1}$), whereas the broth microdilution test reported the strain to be resistant, with an MIC of $32 \mu\text{g ml}^{-1}$. The results of the Phoenix test agreed with those of Vitek 2 for 13/30 isolates (12 susceptible strains and one resistant strain), even though the MIC values differed.

In comparison with the broth microdilution test, both the Etest and disc diffusion test agreed in classifying 11 strains as susceptible, two strains as intermediate and 14 as resistant. However, MICs obtained by Etest differed from the values observed by broth microdilution (more than two twofold dilutions) in seven strains, even though they were in the same range of resistance. By comparing all the results in terms of resistance rates, it was found that 3–3.4 % of the strains were reported as resistant to teicoplanin by the Vitek 2 system, whereas the resistance rates of the other methods ranged from 50 % for the disc diffusion test to 57.6 % for the Phoenix test (Fig. 1).

The Vitek 2 P535 cards revealed that six strains were susceptible to vancomycin (MIC $\leq 1 \mu\text{g ml}^{-1}$) and 24 were resistant (MIC $\geq 32 \mu\text{g ml}^{-1}$; Table 1). Despite different MIC values, these results agreed with those obtained with the broth microdilution test for 28 strains in classifying the strains as susceptible or not susceptible. Two strains (L1 and L228) reported as having an MIC of $1 \mu\text{g ml}^{-1}$ by the Vitek 2 P535 cards showed different MICs by broth microdilution (32 and $16 \mu\text{g ml}^{-1}$, respectively).

The analysis performed with Vitek 2 P534 cards showed that 13 strains were susceptible and 17 were resistant to the drug. Once again, despite different MIC values, these results agreed with those obtained with the broth dilution test for 21 strains in classifying the strains as susceptible or

Table 1. Results of antimicrobial susceptibility testing for teicoplanin, vancomycin and linezolid and molecular analysis of the 30 clinical isolates of *Enterococcus faecium*

Study code	Teicoplanin						Vancomycin						Linezolid						Molecular tests	
	Vitek 2 P535*	Vitek 2 P534*	Phoenix PMIC*	Etest*	Broth micro-dilution*	Disc diffusion†	Vitek 2 P535*	Vitek 2 P534*	Phoenix PMIC*	Etest*	Broth micro-dilution*	Disc diffusion†	Vitek 2 P535*	Vitek 2 P534*	Phoenix PMIC*	Etest*	Broth micro-dilution*	Disc diffusion†	vanA‡	23S rRNA mutation‡
L1	2	1	4	4	4	S	1	1	>16	32	32	R	2	2	2	4	6	S	+	—
L23	2	1	>16	64	128	R	32	32	>16	256	256	R	2	2	2	2	3	S	+	—
L39	4	2	>16	48	128	R	32	32	>16	256	256	R	2	2	2	2	3	S	+	—
L45	1	1	>16	96	128	R	32	32	>16	256	256	R	2	2	2	3	3	S	+	—
L64	4	1	8	4	4	S	32	1	>16	256	256	R	2	2	2	3	3	S	+	—
L73	4	0.5	8	4	4	S	32	1	>16	256	256	R	2	2	2	3	3	S	+	—
L80	4	1	8	4	4	S	64	1	>16	32	32	R	2	2	2	3	3	S	+	—
L89	4	1	16	8	16	I	64	1	>16	32	32	R	2	2	2	3	3	S	+	—
L98	1	4	>16	64	128	R	32	32	>16	256	256	R	2	2	2	3	3	S	+	—
L99	1	1	>16	32	128	R	32	32	>16	256	256	R	2	2	2	2	3	S	+	—
L101	1	1	>16	32	128	R	32	32	>16	256	256	R	2	2	2	2	1.5	S	+	—
L103	16	4	>16	24	32	R	32	32	>16	256	256	R	2	2	2	3	3	S	+	—
L117	32	32	>16	32	128	R	32	32	>16	256	256	R	2	2	2	3	3	S	+	—
L118	4	2	8	16	32	I	64	1	>16	32	32	R	2	2	2	4	3	S	+	—
L121	4	1	8	1	8	S	1	1	>16	1.5	2	S	2	2	2	2	3	S	—	—
L129	16	16	>16	96	128	R	32	32	>16	256	256	R	2	2	2	2	1.5	S	+	—
L152	4	1	8	4	4	S	64	1	>16	8	32	R	2	2	2	2	3	S	+	—
L157	0.5	4	<1	4	4	S	1	1	<1	1	2	S	2	2	2	4	3	S	—	—
L158	1	4	<1	4	4	S	1	1	<1	1.5	2	S	2	2	2	3	3	S	—	—
L201	4	1	8	4	4	S	64	1	>16	8	32	R	2	2	2	3	3	S	+	—
L210	16	4	>16	64	128	R	32	32	>16	256	256	R	4	4	4	4	3	S	+	—
L212	4	1	>16	64	64	R	32	32	>16	32	32	R	2	2	2	2	3	S	+	—
L228	1	1	<1	6	1	S	1	1	<1	24	16	I	>8	>8	2	4	24	R	—	+
L235	4	1	>16	48	128	R	32	32	>16	256	256	R	2	2	2	2	1.5	S	+	—
L236	4	1	>16	48	128	R	32	32	>16	256	256	R	4	2	2	4	1.5	S	+	—
L246	1	1	>16	48	128	R	32	32	>16	256	256	R	2	2	2	2	3	S	+	—
L249	4	4	>16	24	16	I	32	64	>16	32	32	R	2	2	2	2	2	S	+	—
L251	4	4	>16	128	128	R	32	32	>16	256	256	R	2	2	2	2	3	S	+	—
L257	4	4	>16	12	128	I	32	64	>16	32	32	R	2	2	2	2	1.5	S	+	—
L259	4	4	<1	4	4	S	1	1	<1	1	1	S	2	2	2	2	3	S	—	—

*Results of antimicrobial testing ($\mu\text{g ml}^{-1}$).

†Results of disc diffusion expressed as resistant (R), intermediate (I) or susceptible (S). The zone diameter and equivalent MIC breakpoints for the interpretation were as follows. Teicoplanin: R: ≤ 10 mm, MIC $\geq 32 \mu\text{g ml}^{-1}$; I: 11–13 mm; S: ≥ 14 mm, MIC $\leq 8 \mu\text{g ml}^{-1}$. Vancomycin: R: ≤ 14 mm, MIC $\geq 32 \mu\text{g ml}^{-1}$; I: 15–16 mm; S: ≥ 17 mm, MIC $\leq 4 \mu\text{g ml}^{-1}$. Linezolid: R: ≤ 20 mm, MIC $\geq 8 \mu\text{g ml}^{-1}$; I: 21–22 mm; S: ≥ 23 mm, MIC $\leq 2 \mu\text{g ml}^{-1}$.

‡The presence of the *vanA* gene and/or the 23S rRNA gene mutation is indicated as positive (+) or negative (—).

Fig. 1. Susceptibility to teicoplanin of 30 *Enterococcus faecium* isolates. Results are expressed as the percentage of susceptible (S), intermediate (I) and resistant (R) strains.

not susceptible. However, nine strains, reported by the Vitek 2 P534 cards as having an MIC of $1 \mu\text{g ml}^{-1}$, showed MICs of $\geq 16 \mu\text{g ml}^{-1}$ when tested by the broth microdilution test.

The susceptibility tests performed by the Phoenix test reported four strains as susceptible and 26 as resistant. The MIC values agreed with those reported by the broth dilution test for all but two strains: strain L121 displayed an MIC of $>16 \mu\text{g ml}^{-1}$ by the Phoenix test whilst the broth dilution test gave an MIC of $2 \mu\text{g ml}^{-1}$, and strain L228 gave an MIC of $<1 \mu\text{g ml}^{-1}$ with the Phoenix test and $16 \mu\text{g ml}^{-1}$ with the broth microdilution test.

The Etest and broth microdilution method gave the same MIC values for all but two strains (L152 and L201), where the Etest showed an MIC in the range of intermediate susceptibility ($8 \mu\text{g ml}^{-1}$) whilst the broth microdilution test gave an MIC of $32 \mu\text{g ml}^{-1}$. In all instances, the broth microdilution method agreed with the disc diffusion test in defining the strains as susceptible, intermediate or resistant to vancomycin. Comparing the results in terms of resistance rates, it was evident that 56.7% of the strains showed resistance to vancomycin when tested by Vitek 2 P534 cards, whilst the other tests produced resistance rates ranging from 80% for the Vitek 2 (P535) test to 86.7% for the Phoenix test (Fig. 2).

Twenty-four strains were reported as susceptible to linezolid by all the methods used ($\text{MIC} \leq 3 \mu\text{g ml}^{-1}$; Table 1). Vitek 2 P535 cards revealed that 27 strains were susceptible to the drug, two strains were found with intermediate

susceptibility ($\text{MIC}=4 \mu\text{g ml}^{-1}$) and one strain was reported as resistant (L228, $\text{MIC} \geq 8 \mu\text{g ml}^{-1}$). The analysis performed with Vitek 2 P535 cards agreed with the P534 cards for the 27 susceptible strains, the single resistant strain (L228) and for one isolate with intermediate susceptibility (L210), but disagreed for strain L236 by finding the MIC to be $\leq 2 \mu\text{g ml}^{-1}$ by the P534 cards (intermediate by P535 cards; $\text{MIC}=4 \mu\text{g ml}^{-1}$). The Phoenix test agreed with the Vitek 2 P534 cards for 28 susceptible isolates and for one intermediate strain, but reported a susceptible result for strain L228, which was resistant by the two Vitek 2 P534 and P535 cards. The Etest and broth microdilution test agreed in defining strain L1 as intermediate, but four strains that were reported to be intermediate by the Etest were susceptible by broth microdilution. Strain L228 showed intermediate resistance by the Etest, but was resistant by broth microdilution ($\text{MIC}=24 \mu\text{g ml}^{-1}$) and disc diffusion. Comparing the results in terms of susceptibility rates, it appeared that most of the strains were reported as susceptible (ranging from 83.3% for the Etest to 96.6% for the Phoenix and disc diffusion tests; Fig. 3). Much higher variability was observed for the strains that were reported as having intermediate susceptibility, with values ranging from 0% (disc diffusion) to 16.7% (Etest). The resistance rate was attributable to strain L228, which was reported as resistant by the Vitek 2 (P534 and P535 cards), broth microdilution and disc diffusion tests.

The presence of the *vanA* and *vanB* genes in the *E. faecium* strains was analysed by PCR. Twenty-five strains carried

Fig. 2. Susceptibility to vancomycin of 30 *Enterococcus faecium* isolates. Results are expressed as the percentage of susceptible (S), intermediate (I) and resistant (R) strains.

Fig. 3. Susceptibility to linezolid of 30 *Enterococcus faecium* isolates. Results are expressed as the percentage of susceptible (S), intermediate (I) and resistant (R) strains.

the *vanA* gene whilst no strain carried *vanB* (Table 1). Only two strains carrying *vanA* (L117 and L129) were univocally assigned to the VanA phenotype by all the methods. However, the number of strains showing the VanA phenotype reached 19 when the results obtained by the Vitek 2 test were excluded. Six strains carrying *vanA* (L1, L64, L73, L80, L152 and L201) showed the VanB phenotype (resistant to vancomycin and susceptible to teicoplanin), but the Vitek 2 P534 cards reported these strains as susceptible to vancomycin. All strains carrying *vanA* were non-susceptible to vancomycin when tested by the Phoenix, Etest, disc diffusion and broth microdilution methods. However, false-positive results, indicating strains non-susceptible to vancomycin in the absence of *vanA* gene carriage, occurred for strain L121, resistant to vancomycin by the Phoenix test, and strain L228, which was reported as having intermediate susceptibility for vancomycin when tested by the Etest, broth microdilution and disc diffusion tests (Table 1). Furthermore, one false-negative result, indicating strains susceptible to vancomycin in the presence of the *vanA* gene, was observed with the Vitek 2 P535 cards, and Vitek 2 P534 cards reported eight strains carrying the *vanA* gene as susceptible, indicating 30% false-negative results.

Experiments were performed to detect whether the collected *E. faecium* strains carried the G2576T mutation in the V domain of the 23S rRNA gene responsible for linezolid resistance (Fig. 4). A band of 746 bp corresponding to the undigested amplification products was found in all the collected strains. A band of 746 bp together with two bands of 557 and 189 bp corresponding to the two

products of *NheI* digestion were found only in strain L228, indicating the presence of both wild-type and mutant alleles, thus confirming the linezolid resistance of this strain.

E. faecium has progressively changed from an innocent commensal organism to a feared multidrug-resistant hospital-adapted pathogen. Therefore, accurate identification and reliable antimicrobial susceptibility testing may be of paramount importance in the clinical management of infections caused by this micro-organism.

A recent paper reported difficulties in the correct identification of *E. faecium* by Vitek 2, Phoenix and API Rapid ID 32 STREP (bioMérieux) in the case of strains with low-level inducible resistance to vancomycin (Pendle *et al.*, 2008). The strain collection in our study was mostly composed of susceptible or fully vancomycin-resistant strains, and even in the presence of low-level resistance to vancomycin (strain L228, MIC=16 µg ml⁻¹ by broth dilution), our results confirmed previous observations (Abele-Horn *et al.*, 2006) on the univocal identification accomplished by the Vitek 2, Phoenix and API 20 STREP systems. However, our study showed variability among the tests used for the determination of antimicrobial susceptibility, especially for the glycopeptides. The presence of *vanA* and/or *vanB* genes in the bacterial genome can be taken as a reference indicator for resistance to glycopeptides (Deshpande *et al.*, 2007). In our study, 25 strains carried *vanA*, whilst no strain harboured *vanB*. Therefore, it could be expected that these strains, expressing the VanA phenotype in the various antimicrobial susceptibility tests, were resistant to both teicoplanin and vancomycin

Fig. 4. PCR-RFLP analysis of the 23S rRNA gene of strain L228 and of 21 other strains randomly taken from the collection. *NheI* restriction enzyme digestion of the internal fragment of 746 bp of the 23S rRNA gene generated two bands of 557 and 189 bp (faint band) in strain L228, thus confirming linezolid resistance. Lane M, 100 bp ladder.

(Deshpande *et al.*, 2007). In our experiments, this hypothesis was confirmed only for two strains carrying the *vanA* gene, which were univocally reported as resistant to both teicoplanin and vancomycin by all methods used. This observation was due to the results obtained with Vitek 2, which showed the highest degree of *vanA*-positive teicoplanin-susceptible strains, a feature that was not confirmed by the other methods used. Indeed, excluding the results of Vitek 2, the number of strains carrying the *vanA* gene and displaying the VanA phenotype reached 19. Six strains carrying *vanA* were reported as susceptible to teicoplanin by all the methods used and were resistant to vancomycin when tested by the Phoenix, Etest, broth microdilution and disc diffusion tests. Therefore, again excluding the results of Vitek 2, these isolates could be assigned as displaying the VanB phenotype/*vanA* genotype, referring to strains susceptible to teicoplanin and resistant to vancomycin, despite *vanA* gene carriage (Deshpande *et al.*, 2007). VanB phenotype/*vanA* genotype *E. faecium* strains have been reported (Lee *et al.*, 2004), and a recent paper described similar strains of *E. faecium* which also had heterogeneous expression of teicoplanin resistance, as witnessed by the growth of colonies in the elliptic inhibition zone of the Etest susceptibility testing (Park *et al.*, 2008). However, this latter feature was not observed in our experiments, as none of the VanB phenotype/*vanA* genotype *E. faecium* strains showed heterogeneous resistance to teicoplanin in the Etest. Therefore, summarizing the teicoplanin susceptibility data observed with the various methods used and the correlation with *vanA* gene carriage, including the possibility of matching the VanB phenotype/*vanA* genotype, it was evident that the Etest and disc diffusion tests agreed with the broth microdilution test in indicating resistance, the Phoenix test displayed one false-negative result and the Vitek 2 test presented a high degree of false-negative results, regardless of the type of card used. Our observations in determining teicoplanin resistance agreed with those of other authors (Garcia-Garrote *et al.*, 2000; van den Braak *et al.*, 2001), even though the failure of Vitek 2 to detect teicoplanin resistance was reported at a much lower degree (Kobayashi *et al.*, 2004), and improved detection of teicoplanin resistance has been envisaged (Abele-Horn *et al.*, 2006). However, the susceptibility testing of vancomycin showed that comparable performances were provided by the Phoenix, Etest, broth dilution and disc diffusion tests (one false-positive result each), and that Vitek 2 generated several false-negative results, reporting susceptibility to vancomycin in strains carrying the *vanA* gene (one strain with P534 cards and eight strains with P535 cards). Our results were comparable to those obtained by Abele-Horn *et al.* (2006), who reported an overall sensitivity of 98.5 % for the detection of *vanA* enterococci with the same Vitek 2 P534 cards, but highly discordant results using P535 cards, when the sensitivity dropped to 68 %.

The susceptibility testing of linezolid showed a lower degree of variability among all the methods used, as most

of the strains were reported as susceptible to the drug (22/30 strains with MIC values ranging from 1.5 to 3 µg ml⁻¹). However, among the methods used, differences emerged both in recognition of the resistant strain and in the determination of strains expressing intermediate susceptibility. These observations partly agreed with those of Tenover *et al.* (2007), who found variability in detecting resistance of *E. faecium* to linezolid among various methods including those reported in our work. Carriage of the G2576T mutation in domain V of the 23S rRNA gene of *Enterococcus* has been associated with resistance to linezolid (Marshall *et al.*, 2002; Ruggero *et al.*, 2003; Tenover *et al.*, 2007). In our experiments, the unique strain showing this mutation was recognized as resistant by Vitek 2 as well as by the broth dilution and disc diffusion tests, but not by the Phoenix test, which reported the strain as susceptible. The Etest reported the strain as having intermediate susceptibility, possibly due to the intrinsic difficulties in interpreting the 80 % growth inhibition end point by visual examination, with the Etest method rounding up the MIC. Indeed, Etest was the method that reported the highest number of strains with intermediate susceptibility to linezolid. It has been reported that the level of resistance to linezolid correlates with the number of 23S rRNA genes containing the mutation and that different alleles can be found in the same linezolid-resistant strain (Marshall *et al.*, 2002; Ruggero *et al.*, 2003). The density of the 746 bp band of the undigested amplification products correlated with the number of mutated copies: strains with lower MIC values for linezolid had a 746 bp band with a higher density, whilst strains with higher MICs for linezolid displayed a lower density band, depending on the number of copies of the mutated gene (Rice *et al.*, 2004). However, we did not observe any major difference in the density of the 746 bp bands of the test strains, especially in those strains reported as having intermediate susceptibility, and the definitive demonstration of linezolid resistance was observed in the strain that produced the two products of *NheI* digestion (L228). Finally, it is noteworthy that, although this strain bore the G2576T mutation, it did not carry the *vanA* gene.

In conclusion, the results of glycopeptide and linezolid susceptibility testing of *E. faecium* produced by some of the more widely used methods need careful interpretation, and integration of different methods should be implemented by the clinical microbiologist for the best therapeutic approach.

REFERENCES

- Abele-Horn, M., Hommers, L., Trabold, R. & Frosch, M. (2006). Validation of VITEK 2 version 4.01 software for detection, identification, and classification of glycopeptides-resistant enterococci. *J Clin Microbiol* **44**, 71–76.
- Alam, M. R., Donabedian, S., Brown, W., Gordon, J., Chow, J. W., Zervos, M. J. & Hershberger, E. (2001). Heteroresistance to vancomycin in *Enterococcus faecium*. *J Clin Microbiol* **39**, 3379–3381.

- Biedenbach, D. J., Moet, G. J. & Jones, R. N. (2004). Occurrence and antimicrobial resistance pattern comparisons among bloodstream infection isolates from the SENTRY Antimicrobial Surveillance Program (1997–2002). *Diagn Microbiol Infect Dis* **50**, 59–69.
- Bonora, M. G., Solbiati, M., Stepan, E., Zorzi, A., Luzzani, A., Catania, M. R. & Fontana, R. (2006). Emergence of linezolid resistance in the vancomycin-resistant *Enterococcus faecium* multilocus sequence typing C1 epidemic lineage. *J Clin Microbiol* **44**, 1153–1155.
- Clark, N. C., Cooksey, R. C., Hill, B. C., Swenson, J. M. & Tenover, F. C. (1993). Characterization of glycopeptide-resistant enterococci from U.S. hospitals. *Antimicrob Agents Chemother* **37**, 2311–2317.
- CLSI (2006). *Performance Standards for Antimicrobial Susceptibility Tests*; Approved Standard, 9th edn, M2-A9. Wayne, PA: Clinical and Laboratory Standards Institute.
- CLSI (2007). *Performance Standards for Antimicrobial Susceptibility Testing*; 17th Informational Supplement, M100-S17. Wayne, PA: Clinical and Laboratory Standards Institute.
- Deshpande, L. M., Fritsche, T. R., Moet, G. J., Biedenbach, D. J. & Jones, R. N. (2007). Antimicrobial resistance and molecular epidemiology of vancomycin-resistant enterococci from North America and Europe: a report from the SENTRY antimicrobial surveillance program. *Diagn Microbiol Infect Dis* **58**, 163–170.
- Donabedian, S., Hershberger, E., Thal, L. A., Chow, J. W., Clewell, D. B., Robinson-Dunn, B. & Zervos, M. J. (2000). PCR fragment length polymorphism analysis of vancomycin-resistant *Enterococcus faecium*. *J Clin Microbiol* **38**, 2885–2888.
- Falagas, M. E. & Vardakas, K. Z. (2008). Benefit–risk assessment of linezolid for serious Gram-positive bacterial infections. *Drug Saf* **31**, 753–768.
- Garcia-Garrote, F., Cercenado, E. & Bouza, E. (2000). Evaluation of a new system, VITEK 2, for identification and antimicrobial susceptibility testing of enterococci. *J Clin Microbiol* **38**, 2108–2111.
- Gonzales, R. D., Schreckenberger, P. C., Graham, M. B., Kelkar, S., DenBesten, K. & Quinn, J. P. (2001). Infections due to vancomycin-resistant *Enterococcus faecium* resistant to linezolid. *Lancet* **357**, 1179.
- Jones, R. N., Fritsche, T. R., Sader, H. S. & Ross, J. E. (2007). LEADER surveillance program results for 2006: an activity and spectrum analysis of linezolid using clinical isolates from the United States (50 medical centres). *Diagn Microbiol Infect Dis* **59**, 309–317.
- Kobayashi, I., Muraoka, H., Iyoda, T., Nishida, M., Hasegawa, M. & Yamaguchi, K. (2004). Antimicrobial susceptibility testing of vancomycin-resistant *Enterococcus* by the Vitek system, and comparison with two NCCLS reference methods. *J Med Microbiol* **53**, 1229–1232.
- Lee, W. G., Huh, J. Y., Cho, S. R. & Lim, Y. A. (2004). Reduction in glycopeptide resistance in vancomycin-resistant enterococci as a result of *vanA* cluster rearrangements. *Antimicrob Agents Chemother* **48**, 1379–1381.
- Linden, P. K. (2002). Treatment options for vancomycin-resistant enterococcal infections. *Drugs* **62**, 425–441.
- Marshall, S. H., Donskey, C. J., Hutton-Thomas, R., Salata, R. A. & Rice, L. B. (2002). Gene dosage and linezolid resistance in *Enterococcus faecium* and *Enterococcus faecalis*. *Antimicrob Agents Chemother* **46**, 3334–3336.
- NNIS (2001). National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992–June 2001, issued August 2001. *Am J Infect Control* **29**, 404–421.
- Park, I. J., Lee, W. G., Shin, J. H., Lee, K. W. & Woo, G. J. (2008). VanB phenotype-*vanA* genotype *Enterococcus faecium* with heterogeneous expression of teicoplanin resistance. *J Clin Microbiol* **46**, 3091–3093.
- Pendle, S., Jeffs, P., Olma, T., Su, Y., Gilroy, N. & Gilbert, G. L. (2008). Difficulties in detection and identification of *Enterococcus faecium* with low-level inducible resistance to vancomycin, during a hospital outbreak. *Clin Microbiol Infect* **14**, 853–857.
- Rice, L. B., Hutton-Thomas, R., Lakticova, V., Helfand, M. S. & Donskey, C. J. (2004). β -Lactam antibiotics and gastrointestinal colonization with vancomycin-resistant enterococci. *J Infect Dis* **189**, 1113–1118.
- Ruggero, K. A., Schroeder, L. K., Schreckenberger, P. C., Mankin, A. S. & Qiunn, J. P. (2003). Nosocomial superinfections due to linezolid-resistant *Enterococcus faecalis*: evidence for a gene dosage effect on linezolid MICs. *Diagn Microbiol Infect Dis* **47**, 511–513.
- Scheetz, M. H., Qi, C., Noskin, G. A., Warren, J. R., Postelnick, M. J., Malczynski, M., Huang, J. & Zembower, T. R. (2006). The clinical impact of linezolid susceptibility reporting in patients with vancomycin-resistant enterococci. *Diagn Microbiol Infect Dis* **56**, 407–413.
- Tenover, F. C., Williams, P. P., Stocker, S., Thompson, A., Clark, L. A., Limbago, B., Carey, R. B., Poppe, S. M., Shinabarger, D. & McGowan, J. E., Jr (2007). Accuracy of six antimicrobial susceptibility methods for testing linezolid against staphylococci and enterococci. *J Clin Microbiol* **45**, 2917–2922.
- Thal, L.-A., Donabedian, S., Robinson-Dunn, B., Chow, J. W., Dembry, L., Clewell, D. B., Alshab, D. & Zervos, M. J. (1998). Molecular analysis of glycopeptide-resistant *Enterococcus faecium* isolates collected from Michigan hospitals over a 6-year period. *J Clin Microbiol* **36**, 3303–3308.
- van den Braak, N., Goessens, W., van Belkum, A., Verbrugh, H. A. & Endtz, H. P. (2001). Accuracy of the VITEK 2 system to detect glycopeptides resistance in enterococci. *J Clin Microbiol* **39**, 351–353.