

Emerging Linezolid-Resistant, Vancomycin Resistant *Enterococcus faecium* from a Patient of a Haematological Unit in Poland

BEATA KRAWCZYK, ALFRED SAMET¹, MAREK BRONK¹,
ANDRZEJ HELLMANN² and JÓZEF KUR*

Department of Microbiology, Gdańsk University of Technology
ul. G. Narutowicza 11/12, 80-952 Gdańsk, Poland

¹Department of Clinical Bacteriology, State Hospital No. 1
ul. Dębinki 7, 80-211 Gdańsk, Poland

²Department of Haematology, Medical University of Gdańsk
ul. Dębinki 1, 80-211 Gdańsk, Poland

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Abstract

The oxazolidinone antimicrobial, linezolid, has been approved for the treatment of infections caused by various gram-positive bacteria, including vancomycin-resistant enterococci (VRE). This is the first report on isolation of a linezolid resistant, vancomycin-resistant *Enterococcus faecium* (LRVREF) strain in Poland, from a Haematological Unit patient in the Clinical Hospital in Gdańsk. PCR-RFLP analysis of rDNA and allele-specific PCR of the domain V region of the 23S ribosomal RNA gene demonstrated the presence of the G2576U mutation previously reported to be associated with linezolid resistance. Both assays detected heterozygous in this position.

Key words: *Enterococcus*, PCR, oxazolidinone, allele-specific PCR, PFGE

The oxazolidinones are a new antibiotic class that displays excellent activity against gram-positive organisms; linezolid was the first member licensed for clinical use (Livermore, 2000). It is a synthetic antimicrobial that inhibits bacterial protein synthesis by binding to the central loop of domain V of the 23S rRNA subunit. Resistance to linezolid has been observed in clinical isolates of *E. faecium* (Zurenko *et al.*, 1999; Gonzales *et al.*, 2001) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Tsiodras *et al.*, 2001), mostly selected during prolonged courses of therapy. Nosocomial transmission of linezolid- and vancomycin-resistant *E. faecium* (LRVREF) from a linezolid-treated patient to several untreated patients, resulting in asymptomatic colonization, has also been documented (Herrero *et al.*, 2002). Infection with LRVREF was also recently described in a patient without prior oxazolidinone exposure (Jones *et al.*, 2002). Clinical strains consistently have a G→T change at the position equivalent to nucleotide 2576 of the *Escherichia coli* sequence (GenBank accession no. AF053964), giving a G→U change in the corresponding rRNA (Kloss *et al.*, 1999; Prystowsky *et al.*, 2001). Rapid methods for detecting G2576T are therefore desirable to ascertain whether this mutation maintains its dominance; moreover, a facility for rapid confirmation of oxazolidinone resistance will influence the management of cases of a serious gram-positive infection, where linezolid is likely to be used.

The aim of this work was to report the first case of LRVREF isolation in Poland and to develop and evaluate an allele-specific PCR assay for detecting the G2576T mutation in linezolid-resistant enterococci.

A 26 year old male patient with a diagnosis of acute myeloid leukaemia (AML), since June 2003, was hospitalized in a Haematological Unit of the Medical University of Gdańsk. Blood cultures on admission

* Address correspondence to: Józef Kur, Gdańsk University of Technology, Department of Microbiology, ul. G. Narutowicza 11/12, 80-952 Gdańsk, Poland, e-mail: kur@chem.pg.gda.pl.; tel/fax: + 48 58 3471822

were negative. Vancomycin resistant *E. faecium* (VREF) was isolated from his stool. During the remainder of his hospital stay, the patient received amikacin for 7 days 2×0.5 g IV, metronidazole for 20 days 3×0.5 g IV, meropenem for 19 days 3×2.0 g IV, imipenem for 7 days 3×1.0 g IV, ciprofloxacin for 12 days 2×2.0 g IV and doxycycline for 3 days 1×0.1 g IV. Next, after 77 days VREF was isolated from the blood. Due to VREF bacteremia he was given linezolid, 2×0.6 g IV for the period of 26 days. On the 23rd day of treatment a blood culture grew VREF resistant to linezolid (LRVREF). Positive LRVREF blood cultures were accompanied by a high fever reaching 40°C. The patient was next successfully treated with quinupristin-dalfopristin (3×0.5 g IV) for 14 days. Blood cultures became negative but the LRVREF carrier state in gastrointestinal tract persisted.

Antimicrobial susceptibility testing was performed on all blood isolates recovered from the patient using an automated microtube dilution method (bioMérieux Vitek), the standard Kirby-Bauer disk diffusion technique (for doxycycline, rifampin, chloramphenicol, and quinupristin-dalfopristin), and the Etest (AB Biodisk; for linezolid).

For the detection of the G2576T mutation, previously reported to be associated with linezolid resistance, PCR amplification of the domain V region of the 23S rRNA gene was performed on culture isolates using primers: 5'-TGGGCACTGTCTCAACGA (forward primer) and 5'-GGATAGGGACCGAACTGTCTC (reverse primer) and PCR reactions as described previously (Tsiodras *et al.*, 2001). The amplification products were analyzed by electrophoresis on 6% polyacrylamide gels stained with ethidium bromide. Small amounts (5 µl) of PCR products were digested with 5 u of *NheI* (Fermentas, Lithuania) for 2 h at 37°C, and the fragments were separated by electrophoresis through 6% polyacrylamide gels.

Primers: 5'-GGTGGTTC CGCATGG (23S forward primer), 5'-CGTTCTGAACCCAGCTA (23SM reverse primer) and 5'-CGTTCTGAACCCAGCTC (23SW reverse primer), were designed based on *E. faecium* sequence (GenBank accession no. AF432914). For allele-specific method, DNA samples were subjected to two differential amplifications in two separate tubes. Both reactions were performed with the same 23S forward primer, and one of the two discriminator primers, 23SM or 23SW for mutated or wild sequences, respectively. The reaction solution consisted of: 100 ng of the bacterial DNA, 1 µl (10 µM) of each primer, 5 µl (10 mM) dNTP's, 5 µl 10×PCR buffer (100 mM Tris-HCl, pH 8.9, 500 mM KCl, 25 mM MgCl₂, 1% Triton X-100), 1 u of native DNA polymerase *Taq* (Fermentas, Lithuania). The cycling conditions were as follows: initial denaturation at 94°C for 5 min; 30 cycles of amplification at 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s; and final synthesis at 72°C for 5 min. The amplicons were separated by electrophoresis through 8% polyacrylamide gels.

All VREF and LRVREF isolates were susceptible to chloramphenicol and quinupristin-dalfopristin, and resistant to tetracycline, high levels of gentamicin and streptomycin, rifampin, penicillin and ampicillin. The LRVREF isolates demonstrated resistance to linezolid with MIC 32 µg/mL. The susceptibility breakpoint for linezolid, as recommended by the NCCLS is ≤ 2 mg/mL (Herrero *et al.*, 2002).

For the detection of the G2576T mutation, previously reported to be associated with linezolid resistance, PCR amplification of the domain V region of the 23S rRNA gene was performed and specific, 633-bp, PCR products were obtained and next digested with *NheI* restriction enzyme. The results of *NheI* PCR-RFLP analysis of 633-bp rDNA amplicons (Fig. 1A) showed that amplicons from the linezolid-resistant isolate was partially digested with a *NheI* enzyme to give products of 633-bp, 591-bp and 42-bp, with the latter running off the gel. This assay detected heterozygous in the position of G2576T.

To confirm the above results the assay based on allele-specific PCR for detecting the G2576T mutation in linezolid-resistant enterococci was designed and evaluated. 197-bp amplification products were visualized in both reactions in the case of LRVREF isolates and in one reaction (only with 23SW primer) in the case of VREF isolates (Fig. 1B). The allele-specific PCR assay described here can be suggested as a simple and rapid tool for detecting clinical linezolid-resistant strains. The assay is easy to perform and to interpret; it is based on a simple PCR and minigel electrophoresis without any further extensions.

The linezolid-resistant and -susceptible *E. faecium* isolates recovered from the patient's stool and blood were shown to be indistinguishable by pulsed-field gel electrophoresis (PFGE) of *SmaI* digested DNA (results not shown), thereby indicating that linezolid resistance had arisen during therapy.

In conclusion, PCR-RFLP analysis of rDNA and allele-specific PCR of the domain V region of the 23S ribosomal RNA gene demonstrated the presence of the G2576U mutation previously reported to be associated with linezolid resistance and showed that examined strain carried in addition to mutation copies the wild-type sequence, indicating heterozygosity at this position. All of the clinical isolates of LRVREF reported to date, including those described here, demonstrate a G2576U mutation (Livermore, 2000; Gonzales *et al.*, 2001; Zurenko *et al.*, 1999). The molecular assays used in this study could be also used to detect the G2576T

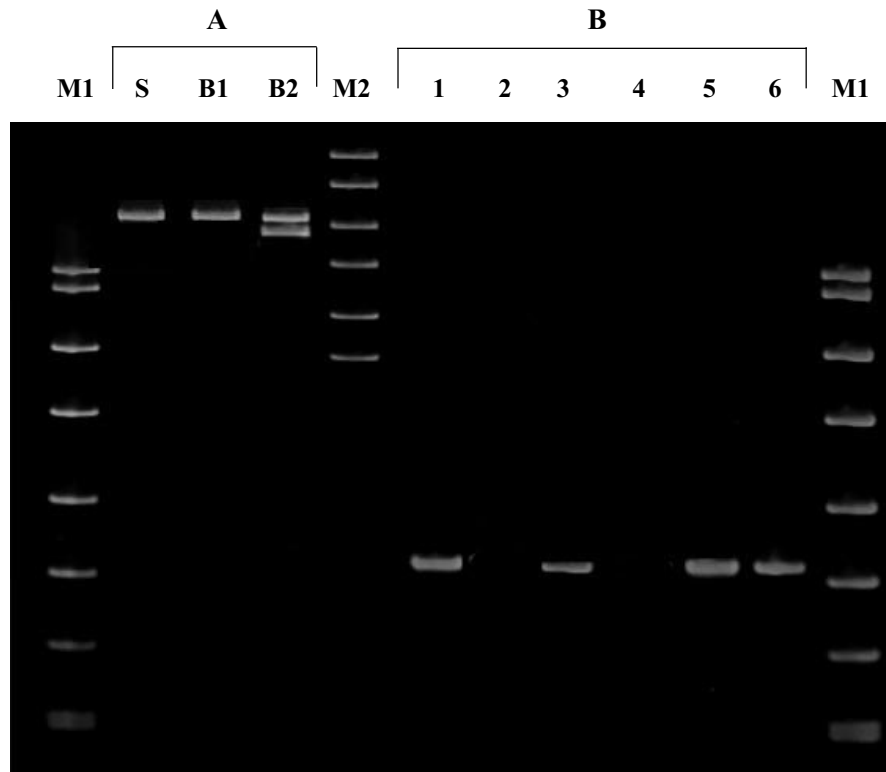


Fig. 1. **A:** Representative results of PCR-RFLP analysis of rDNA amplicons with *NheI*. Lanes: S, the isolate of VREF from stool; B1, the isolate of VREF from blood; B2, the isolate of LRVREF from blood. **B:** Representative results of allele-specific PCR. Lanes: 1 and 2, the isolate of VREF from stool, amplification with 23SW and 23SM primer, respectively; 3 and 4, the isolate of VREF from blood, amplification with 23SW and 23SM primer, respectively; 5 and 6, the isolate of LRVREF from blood, amplification with 23SW and 23SM primer, respectively. The lane designated M1 contained molecular mass marker: 501, 489, 404, 331, 242 and 190 bp; and the lane designated M2 contained molecular mass marker: 1008, 883, 615, 517, 466 and 396 bp. The DNA fragments were electrophoresed in 8% polyacrylamide gel by using 1xTris-borate EDTA running buffer at field strength of 8 V cm⁻¹.

mutation in oxazolidinone-resistant isolates of MRSA, such as the strain described by Tsiodras *et al.* (2001). The *NheI* PCR-RFLP assay provided a slower means of detecting the G2576T mutation potentially suitable for laboratories without access to sequencing in comparison to allele-specific PCR assay. Both assays could be used for the detection of heterozygous strains with G2576 and T2576 in different rDNA copies. Both methods will be useful for molecular characterization and surveillance of emergent resistance, and the speed of allele-specific PCR (linezolid resistance detection in less than 3 h) may also be an advantage in the case of management for individual patients.

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