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Sequential Linezolid-Resistant *Staphylococcus epidermidis* Isolates with G2576T Mutation▽

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We report on an analysis of sequential isolates of *Staphylococcus epidermidis* from cultures of blood obtained from a patient with acute myeloid leukemia while the patient was receiving linezolid treatment. All 12 isolates had a linezolid MIC of 32 μg/ml. A 420-bp domain V region of the 23S rRNA gene from all isolates was amplified, and their nucleotide sequences were determined. A G2576T mutation was identified in all isolates. It was estimated that 67% of the 23S rRNA genes carried this mutation. This is the first report of the failure of linezolid treatment for *Staphylococcus epidermidis* bacteremia associated with a G2576T mutation in an immunocompromised patient.

Linezolid is an important antibacterial agent with extended activity against gram-positive organisms, including resistant organisms, such as methicillin-resistant *Staphylococcus aureus*, methicillin-resistant coagulase-negative staphylococci (CoNS), vancomycin-resistant enterococci, and multidrug-resistant *Streptococcus pneumoniae*. The mechanism of action is the inhibition of protein synthesis through binding to the domain V region of the 23S rRNA. Since its introduction in 2000, resistance to linezolid has been reported in isolates of vancomycin-resistant enterococci, methicillin-resistant staphylococci, and CoNS. Linezolid resistance has been associated with mutations in the central loop of the domain V region of the 23S rRNA gene, with the G2576T mutation (the substitution of thymine for guanine at position 2576) most frequently reported in *Enterococcus faecium*, *Enterococcus faecalis*, and *Staphylococcus aureus* isolates (4, 6, 8).

Linezolid resistance has been reported to occur in *Staphylococcus epidermidis* isolates, and recently, the specific gene mutation G2576T was also reported to be associated with linezolid resistance in *S. epidermidis* (14). Here we report on an analysis of sequential linezolid-resistant *Staphylococcus epidermidis* isolates from blood cultures in a case of apparent linezolid treatment failure, with identification of the G2576T mutation in 67% of the domain V regions of the 23S rRNA genes.

CASE REPORT

A 66-year-old man with acute myeloid leukemia in second relapse was admitted with neutropenic fever. He became afebrile while he was receiving empirical piperacillin-tazobactam and underwent placement of a subclavian Hickman catheter for salvage chemotherapy. Six days later he developed a Hickman catheter-associated tunnel infection, with cultures of blood being positive for viridans group streptococcus and *Staphylococcus epidermidis*. Because of a history of intolerance to vancomycin, he was started on linezolid at 600 mg intravenously every 12 h, and the catheter was removed. Blood cultures remained positive for *Staphylococcus epidermidis* for another 7 days. All isolates were found to be resistant to linezolid, with MICs of 32 μg/ml for all isolates by E-test. The patient became afebrile with negative blood cultures 5 days after the treatment was changed to daptomycin. The possibility of endocarditis was raised. A transthoracic echocardiogram was negative. The patient had never had a documented infection with any CoNS isolate, nor had he ever received linezolid.

MATERIALS AND METHODS

Bacterial isolates. Twelve sequential *Staphylococcus epidermidis* isolates were obtained from blood cultures (standard aerobic and anaerobic media; BacT/ALERT 3D; bioMerieux, Inc., Durham, NC) from the source patient. Clonal relationships were determined by pulsed-field gel electrophoresis with SmaI-macrorestricted genomic DNA.

Antimicrobial susceptibility testing. Susceptibility testing was performed by using a Vitek GNS card and E-test (AB Biodisk, Solna, Sweden). Broth macrodilution was performed as recommended by the CLSI (1a).

16S rRNA gene sequencing. Primers 0008F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 0532R (5′-TACCGCGGCTGCTGGCAC-3′) (4) were used to amplify the first 500 bp of the 16S rRNA gene. The PCR mixture, which was made up to 50 μl with sterile water (Sigma), contained 1× GeneAmp PCR buffer, $MgCl_2$ (2.5 mM), 200 μM each deoxynucleoside triphosphate, 500 nM each primer, 5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), and 10 μl purified sample DNA (range, 50 to 250 ng). PCR conditions were 94°C 12 min and 40 cycles of 94°C for 60 s, 59°C for 20 s, and 72°C for 20 s, with a final extension at 72°C for 5 min. The mixture was then held at 4°C. The success of amplification was determined by electrophoresis of the PCR products in a 1% agarose gel and staining with ethidium bromide. Amplicons of between 500 and 550 bp were excised from the agarose gels (1% agarose gel in Tris-borate EDTA) and purified with a QIAquick gel extraction kit (QIAGEN Inc., Valencia, CA).

PCR amplification of the domain V region and DNA sequence. The domain V region spanning 2,280 to 2,699 bp (*Escherichia coli* numbering) was amplified. The primers used were 5′-GCGGTCGCCTCCTAAAAG-3′ (upper primer, corresponding to bases 2280 to 2297 of the *S. aureus* 23S rRNA gene; GenBank accession no. X68425) and 5′-ATCCCGGTCCTCTCGTACTA-3′ (lower primer, consisting of the strand complementary to the upper primer and corresponding to bases 2680 to 2699 of the *S. aureus* 23S rRNA gene; GenBank accession no. X68425) (6). PCR conditions were 5 min of lysis and denaturation at 94°C; 32 cycles of denaturation, annealing, and extension at 94°C (30 s), 55°C

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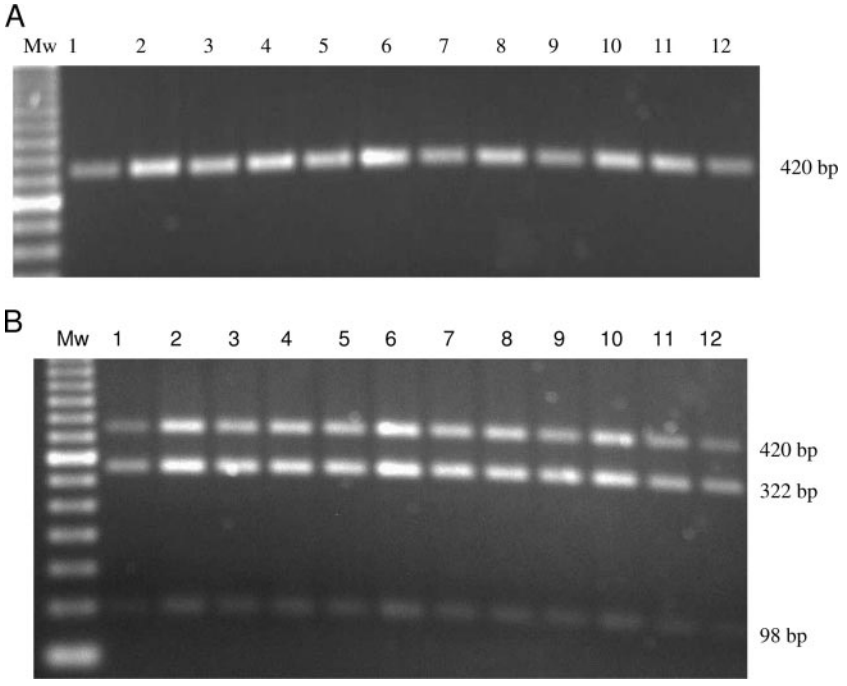

FIG. 1. NheI restriction enzyme digestion of domain V of 23S rRNA gene. (A) Lanes 1 to 12, serial isolates from the patient subjected to PCR amplification of a 420-bp fragment of domain V region of 23S rRNA gene; lane Mw, molecular size marker. The gel was stained with ethidium bromide. (B) Same as panel A, plus NheI restriction enzyme digestion. 420 bp, NheI-resistant fragment; 322 bp and 98 bp, NheI-digested fragments.

(30 s), and 72°C (1 min), respectively; and a final 10-min extension at 72°C. The products were 420 bp and were separated by agarose (1%) gel electrophoresis. The PCR products were gel extracted and purified (QIAGEN). They were then sequenced by use of the BigDye (version 3.1) sequencing kit and an Applied Biosystems 310 genetic analyzer.

Restriction enzyme digestion. The 420-bp domain V fragment was digested with the NheI restriction enzyme (Invitrogen) by using the conditions recommended by the manufacturer. After digestion, the DNA fragments were separated on a 3% agarose gel and stained with ethidium bromide.

Cloning. The PCR-amplified 420-bp domain V fragments from the *S. epidermidis* strain (including the wild type and the G2576T mutant) were cloned into the pGEM-T Easy vector, according to the manufacturer's instructions (Promega Corp., Madison, WI). The 420-bp domain V fragment was then reisolated from a single transformed bacterial colony. The fragment was analyzed by NheI restriction enzyme digestion and nucleotide sequencing.

RESULTS

Twelve CoNS blood culture isolates from the patient were identified as *Staphylococcus epidermidis* with the Vitek GPI card, and their identities were confirmed by 16S rRNA gene sequencing. The MICs of linezolid for all isolates were 32 μg/ml (resistant) by E-test, and these were confirmed by the CLSI macrodilution method. Pulsed-field gel electrophoresis analysis indicated that all isolates belonged to a single strain (data not shown).

The nucleotide sequence available from GenBank indicates that the domain V region of the 23S rRNA gene nucleotide sequence from both *Staphylococcus aureus* (GenBank accession no. NC_007795) and *Staphylococcus epidermidis* (GenBank accession no. NC_004461) are identical. A 420-bp fragment (Fig. 1A) was amplified from the domain V region of the 23S rRNA genes of all isolates by using the primer set originally designed for *Staphylococcus aureus* (6). The mutations associated with linezolid resistance reported from most other organisms were associated with this domain. Nucleotide sequence analysis of the amplified domain V region identified a G-to-T point mutation at position 2576 (*E. coli* numbering) from all 12 isolates.

The G-to-T point mutation introduces an NheI site into the mutant domain V region. NheI restriction enzyme digestion of the PCR-amplified domain V region generates a 322-bp fragment and a 98-bp fragment. The result shown in Fig. 1 indicated incomplete digestion, even after prolonged incubation (overnight) with NheI. This result suggested that the PCR-amplified fragments were a mixture of fragments with both G2576T mutant and wild-type sequences. On the basis of visual observation on an ethidium bromide-stained agarose gel, we estimated that approximately 50% or a little more of the fragment was digested by the NheI restriction enzyme (Fig. 1B).

To confirm our estimation that 50% or a little more of the domain V fragment carries the G2576T mutation, we cloned the PCR-amplified fragments (a mixture of G2576T mutant and wild-type sequences) into the pGEM-T Easy Vector and transformed *E. coli* cells. Thirty-six transformed bacterial colonies were analyzed by purifying the plasmid, reisolating the 420-bp fragment, and identifying the G2576T mutation by NheI restriction enzyme digestion and nucleotide sequencing. The result indicated that 67% (24/36) of the clones carried the G2576T mutation. This result correlates well with our estimation of partial restriction enzyme digestion.

All the isolates were resistant to oxacillin and susceptible to vancomycin, quinupristin-dalfopristin, and daptomycin.

DISCUSSION

Staphylococcus epidermidis has been documented as a pathogen in many cases of bacteremia; native and prosthetic valve endocarditis; surgical wounds; ophthalmologic infections; and prosthetic joint-, ventricular shunt-, peritoneal dialysis-, and intravascular catheter-related infections. Linezolid is an important alternative agent for the treatment of severe infections caused by *S. epidermidis*. Our report describes the first case of clinical failure of linezolid treatment due to linezolid-resistant *Staphylococcus epidermidis* in an immunocompromised patient.

Among a number of domain V mutations associated with linezolid resistance (9, 10, 12), the G2576T mutation is the only one reported from clinical isolates of *Enterococcus faecium* (9), *Staphylococcus aureus* (6, 7), and *Staphylococcus epidermidis* (14). Our report confirms that this mutation is associated with linezolid resistance in *Staphylococcus epidermidis*. The domain V regions of the 23S rRNA genes of both *S. aureus* and *S. epidermidis* are identical, and we speculate that homologous recombination between the wild-type and mutant genes could be the mechanisms by which this mutation is spreading among genetically similar organisms. Alternatively, *S. epidermidis* may also become resistant to linezolid when it generates its own G2576T mutation in one of the five to six alleles of the domain V region of the 23S rRNA gene and then homologous recombination occurs within the same cell.

Most organisms carry multiple copies of rRNA genes. According to GenBank data (accession nos. NC_002976 and NC_004461) (2, 13), *Staphylococcus epidermidis* carries five or six copies of the 23S rRNA gene. Not all genes from our isolates carried the G2576T mutation. It was estimated, on the basis of the NheI digestion and cloning data, that approximately 67% of the genes carried this mutation (Fig. 1B); therefore, three of the five gene copies or four of the six gene copies may carry this mutation. This is different from the case for the linezolid-resistant *Staphylococcus aureus* isolates characterized by Pillai et al. (7), who reported that all five 23S rRNA genes carried the G2576T mutation. The MICs for all of our isolates were 32 μg/ml, which is not as high as that reported by Potoski et al. (MIC > 256 μg/ml) (8). We believe that there might be a correlation between the number of genes carrying the G2576T mutation and the MIC of linezolid for *S. epidermidis*, as has been described for enterococci (5) and *S. aureus* (11). It would be interesting to determine whether most or all of the 23S rRNA genes in isolates with much higher linezolid MICs (>256 μg/ml) carry the mutation. The important message is that our strain with a linezolid MIC of 32 μg/ml was capable of causing the clinical failure of linezolid therapy in an immunocompromised patient.

Linezolid was added to our hospital formulary in January 2001, with usage requiring prior approval by an infectious disease physician. Despite a significant increase in the rate of consumption since its introduction, with the numbers of defined daily doses of linezolid rising from 0.31 per 100 patient-days in 2001 to 0.82 per 100 patient-days in 2005, this was our first case of a microbiologically documented treatment failure related to linezolid resistance. We have been performing routine linezolid susceptibility testing of all gram-positive isolates in our microbiology laboratory since 2004. We detected linezolid resistance in only 1 other CoNS isolate of 1,771 isolates tested through August 2006, resulting in a resistance rate of <0.1%. This is in keeping with the low rates from the 2002 and 2003 surveillance programs (1).

The previous administration of linezolid has been reported to be an independent predictor of linezolid resistance in CoNS (8). This was not the case in our patient, who had never received linezolid prior to becoming infected with the resistant strain of *Staphylococcus epidermidis*. Cases of patients developing infections with linezolid-resistant CoNS in the absence of prior exposure to linezolid have also been reported (8). The source of the resistant strain remains undetermined, but the clonal spread of CoNS has been reported to occur within hospitals (8), and therefore, the possibility of nosocomial transmission from patients colonized with linezolid-resistant CoNS following linezolid exposure needs to be entertained.

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