Detection of the classical G2576U mutation in linezolid resistant *Staphylococcus aureus* along with isolation of linezolid resistant *Enterococcus faecium* from a patient on short-term linezolid therapy: First report from India

*S Rai, DK Niranjan, T Kaur, NP Singh, V Hada, IR Kaur*

**Abstract**

**Purpose:** Linezolid is an effective drug against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). We describe the emergence of linezolid resistance in MRSA and VRE from India. **Material and Methods:** One MRSA and two VRE strains were isolated from a patient on linezolid therapy of one week duration. All three isolates were resistant to linezolid with minimal inhibitory concentrations (MIC) ≥4 mg/L. The 746-bp region flanking the possible G2576U mutation on the corresponding DNA from the 23S rRNA was amplified by polymerase chain reaction (PCR) and amplicons were sequenced for all the three isolates. Conjugation experiments using the linezolid resistant MRSA (LRMRSA) and linezolid resistant VRE (LRVRE) isolates as donors and wild strains of corresponding genera as recipients were performed. **Results:** The MRSA isolate had the classical G2576U mutation. High quality value scores in the sequencing software validated the mutation. Conjugation studies did not indicate presence of transferable resistance for linezolid. Sequencing did not indicate presence of any mutation in the two LRVRE isolates. **Conclusions:** This is the first report from India citing resistance in *Staphylococcus* and *Enterococcus* against Linezolid.

**Key words:** *Enterococcus, G2576U, India, linezolid resistance, Staphylococcus*

**Introduction**

Linezolid is an oxazolidinone used as an effective therapeutic option against vancomycin-resistance enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA). Linezolid resistance in staphylococci and enterococci have been reported, but not from India.[1] However, two reports from the Health Protection Agency provide evidence of transferable linezolid resistance in enterococci from a patient who received treatment in India.[2,3] The most common cause of linezolid resistance in staphylococci and enterococci is due to a G2576U mutation in the 23S rRNA. Resistance may also be due to presence of a transferable plasmid-borne *cfr* gene encoding a methyl transferase enzyme responsible for methylation of A2503 in the 23S rRNA.[4,5] Many other mutation sites have been identified on the 23S rRNA among different bacteria corresponding to the peptidyl transferase centre (PTC) which confers resistance to linezolid.[6]

We report isolation of a linezolid-resistant MRSA (LRMRSA) and linezolid-resistant VRE (LRVRE) at a tertiary care hospital of East Delhi, India from a patient exposed to short-term linezolid therapy. Their phenotypic characterisation involved conjugation studies to observe presence of transferable linezolid resistance followed by sequencing of the target domain for linezolid resistance.

**Materials and Methods**

**Patient history**

An 80-year-old patient was seen in the emergency department with complaints of fever, vomiting, breathlessness and heaviness in the chest. He had myocardial infarction with left bundle branch block. Cerebellar infarct, and haemorrhage with mid-line shift were observed on radiological investigations. Haematological investigations revealed an elevated leucocyte count. The patient was given thrombolytic therapy and empirically treated for fever with piperacillin/tazobactam and metronidazole. The patient had a history of multiple hospitalisations due to development of abscess in the right arm in the past two months and intake of multiple antibiotics, but did not include linezolid. Before
hospitalisation at our institute he was given Linezolid 600 mg twice daily for seven days after incision and drainage of the abscess at another healthcare setup.

**Microbiological investigations and susceptibility testing**

Blood culture of the patient isolated pure growth of *E. faecium*. The isolate was subjected to antimicrobial susceptibility for ampicillin, chloramphenicol, erythromycin, vancomycin, linezolid, tetracycline, tigecycline and high level gentamicin [Hi–Media, India] by Kirby Bauer disc diffusion method. All breakpoint zones were interpreted as per Clinical and Laboratory Standards Institute breakpoints.[7] Minimal inhibitory concentrations (MIC) for vancomycin was determined by E-Test (AB BioMerieux, India). MIC’s for linezolid were determined using the commercial PBP20 (positive breakpoint combo) panel of MicroScan Walkaway 96 Plus (Siemens, India) and GP67 (Gram positive) panel of Vitek 2 Compact (BioMerieux, India). Stool sample was collected and cultured on Mac Conkey medium [Hi – Media, India] to detect colonisation with *Enterococcus*, which also isolated linezolid resistant *E. faecium*. The patient was screened for carriage of MRSA from the anterior nares, axilla and groin. Swabs collected from these sites were cultured on mannitol salt agar [Hi – Media, India]. Sample from the nares isolated an MRSA strain, which was resistant to linezolid.

**Conjugation studies**

To demonstrate the presence of transferable resistance, conjugation experiments were performed. The LRVRE isolates from blood and LRMRSA from nares were used as donor strains. Eighteen tetracycline resistant clinical isolates of genus Enterococcus ([*E. faecium* (4), *E. falcis* (12) and *E. raffinosus* (2)]) and 10 tetracycline resistant *S. aureus* isolates were included as prospective recipients for linezolid resistance. All recipient strains were used were wild clinical isolates. Fresh overnight growth of donor and recipient strains were mixed in a 1:10 ratio for broth mating experiments overnight in brain heart infusion (BHI) broth in presence and absence of sub-inhibitory concentrations of linezolid (≤0.25 mg/L) with intermittent shaking for upto 48 hours at 37°C in ambient air.[9] Sub-inhibitory concentration of linezolid was used to detect whether presence of the drug would act as a stimulus for possible transfer of resistance to susceptible wild recipients. Subcultures were made on 5% sheep blood agar containing 8 mg/L tetracycline and incubated for 24 hours to allow the growth of transconjugates. Pure growth of transconjugate was used to assess linezolid sensitivity by Kirby Bauer disc diffusion method as well as by the automated system (MicroScan Walkaway).

**PCR and amplicon sequencing**

DNA was extracted from fresh growth of the three isolates using the GeneiPureID DNA isolation kit (Merck, India). Control strains ATCC 29212 *E. faecium* and ATCC 25923 *S. aureus* were used as positive controls and ATCC 25922 *Escherichia coli* was used as negative control for the PTC region. Forward (5′-TAGTACCTGTGAAAGATGCAGG-3′) and reverse (5′-CACACTTAGATGCTTTCAGCG-3′) primers (Sigma, India) were used to amplify the 746-bp region of domain V of the 23S rRNA gene, which is the known hotspot harbouring the mutations responsible for linezolid resistance.[9] The PCR conditions were standardised at; heated lid at 112°C and initial denaturation at 95°C for 5 min, followed by 30 cycles consisting of denaturation at 95°C for one minute, annealing at 55°C for 30 seconds, extension at 72°C for one minute, and a final extension step at 72°C for seven minutes. The 746-bp amplicon was purified and direct sequencing was done on ABI 3730 DNA Analyser by using BigDye® Terminator v3.1 Cycle Sequencing Kit from ABI (Hitachi, Applied Biosystems, Japan). The 23S rRNA reference sequences were BLAST searched on the National Center for Biotechnology Information (NCBI) database and compared with the obtained sequence on the SeqSequence® software. Presence of a mutation was considered valid only if the quality value (QV) score for the site was >20 as generated by the software.[10]

**Results**

**Antimicrobial susceptibility testing**

For the *E. faecium* isolate from blood, a decreased breakpoint zone for linezolid (18mm) was observed by disc diffusion. It was susceptible to tetracycline and tigecycline but resistant to all other antibiotics. Antibiogram pattern of *E. faecium* isolated from stool was identical to the blood isolate. MIC for vancomycin determined by E-test in both isolates was 94 mg/L. MIC breakpoints for linezolid in the PBP20 panel of Microscan walkaway 96 plus GP67 panel of Vitek 2 for both isolates were >4 and ≥8 mg/L. From anterior nares we isolated an MRSA strain with linezolid MIC > 8 mg/L detected by both the automated methods. This isolate was susceptible to vancomycin and tetracycline and resistant to all other drugs on the PBP20 and GP67 panels.

**Conjugation studies**

Among the 18 donor-recipient combinations in enterococci, only six exhibited growth on sheep blood agar with tetracycline (four *E. falcis* and one each of *E. faecium* and *E. raffinosus*) in the presence as well as absence of sub-inhibitory dose of linezolid while only four did so in *S. aureus*. Subsequent antimicrobial susceptibility testing demonstrated that all transconjugates were susceptible to linezolid by disc diffusion as well as the automated system indicating presence of non-transferable linezolid resistance to homologous or heterologous wild enterococci or *S. aureus* isolates.
PCR and sequencing

PCR produced an approximately 746-bp amplicon for Gram positive isolates as depicted in Figure 1. Sequencing of the amplicon confirmed the presence of the classical G2576U mutation on the 23S rRNA in the LRMRSA isolate from the nares [Figure 2]. The QV scores for the forward and reverse sequences for this site were 44 and 56, respectively. Neither the classical G2576U mutation nor any other mutations were observed from positions 2017 to 2763 in either of the E. fecum isolates from blood and stool.[6] Detection of the cfr gene was not performed due to cost constraints. The forward segment of LRMRSA isolate was submitted in the BankIt facility of NCBI [BankIt1650323]. The GenBank accession number generated was KF 479352 and the isolate was labelled SA2606.

Discussion

Availability in an oral formulation has made linezolid an empirical outpatient drug in a country like India which has no stringent policies on antimicrobial prescribing practices. This is the first formal report from India describing linezolid resistance in patient harbouring both, LRVRE and LRMRSA isolates. What is alarming in this case is the presence of linezolid resistance in strains isolated from the infection as well as surveillance sites and death of the patient which may not be attributed totally to isolation of LRVRE from blood and failure of antimicrobial therapy given to the patient. The patient however should have been screened for presence or absence of these isolates prior to linezolid therapy. The role of the classical G2576U mutation in LRMRSA isolate could not be correlated clinically but we hypothesise that rapid development of linezolid resistance in S. aureus may have been due to presence of cryptic resistance in other alleles of the 23S rRNA PTC region.[11] This can be linked only if prior molecular and phenotypic screening for MRSA/VRE is performed before commencement of linezolid therapy. Sequencing of the LRVRE isolates also did not highlight any known or unknown mutations for linezolid resistance. It may be possible that cfr gene may have existed in these LRVRE isolates, and that the conjugations experiments may have failed to detect its transfer. More studies are required to prove the hypothesis of presence of cryptic resistance to linezolid. Presence of mutation in a single operon of multiple alleles of 23S rRNA may not exhibit phenotypic linezolid resistance and therefore it becomes imperative that such mutations with cryptic resistance need to be screened prior to linezolid therapy before development of frank resistance due to sequential mutations during therapy.[10,11] This report cites the result of irrational use of antimicrobial agents highlighting importance of implementation of antibiotic prescription audits and hospital antibiotic policies. Presence of the classical G2576 mutation highlights excessive or inadequate exposure to linezolid, but its chromosomal location does not threaten rampant spread and such infections can be controlled by infection control practices. Contrarily, presence of the cfr gene, poses a much greater threat due to its location on a transferable plasmid and may cause rapid dissemination of linezolid resistance in enterococci.[11,12]

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