Clonal dissemination of linezolid-resistant *Staphylococcus haemolyticus* harbouring a G2576T mutation and the *cfr* gene in an Indian hospital

Linezolid, an oxazolidinone drug available in both parenteral and oral formulations, has emerged as a novel alternative to vancomycin and other second-generation drugs for treatment of infections from Gram-positive cocci (Gu *et al.*, 2013). Mutation at domain V of 23S rRNA can result in modification of the ribosomal peptidyl transferase centre region and resistance in isolates. A naturally occurring resistance gene *cfr*, encoding a cfr methyltransferase, has also been reported in clinical isolates. Moreover, resistance has also been associated with mutations in the genes for the ribosomal proteins L3 and L4, which interact closely with the linezolid binding site in the peptidyl transferase centre region (Long & Vester, 2012). Linezolid resistance in clinical *Staphylococcus* isolates is increasingly being reported worldwide (Jones *et al.*, 2009). Here, we describe four linezolid-resistant clinical isolates of *Staphylococcus haemolyticus* with a dual mechanism of resistance.

A pure culture of linezolid-resistant *S. haemolyticus* was isolated from pus samples from four patients. Isolates 1 and 2 were from patients with chronic osteomyelitis from an orthopaedic unit, while isolates 3 and 4 were from cases of pemphigus vulgaris from a dermatology unit. All patients had received multiple antibiotics before referral, including linezolid, and the duration of linezolid therapy varied from 10 days to 5 weeks. Bacterial identification was performed using a biochemical test panel adapted from the scheme of Kloos & Schleifer (1975). Linezolid resistance was assessed by screening with linezolid (30 µg) discs and MIC determination by the E-test method (bioMérieux). Also, *mecA*-mediated methicillin resistance was evaluated using cefoxitin (30 µg) discs and was confirmed by detection of the *mecA* gene using PCR, as described by Roth *et al.* (2001). Potential co-resistance to other antimicrobial agents (erythromycin, clindamycin, chloramphenicol, rifampin, tetracycline, gentamicin, amikacin, ciprofloxacin and streptogramin) was determined by the disc diffusion method according to Clinical and Laboratory Standards Institute guidelines (2015). MIC estimation of vancomycin and teicoplanin was performed by the E-test method.

To determine the molecular mechanism of resistance to linezolid, a point mutation in domain V of the 23S rRNA gene was identified by sequencing following amplification of a 746 bp region, using the primers described by Bonora *et al.* (2006). Sequences were aligned with the corresponding nucleotide sequences from reference strains of *Escherichia coli* and *S. haemolyticus* (GenBank accession nos V00331, AP006716). PCR for the *cfr* gene was also performed using the protocol given by Keidenberg *et al.* (2005). The clonal relatedness of the isolates was examined by PFGE of *Smal*-digested genomic DNA according to the protocol described by Goering & Winters (1992), with some modifications. Genomic DNA was prepared in agarose blocks and digested with *Smal* (Promega). The DNA fragments were separated in a 1 % agarose gel using CHEF Mapper System III (Bio-Rad) for 20 h at 6 V cm\(^{-1}\) at 14 °C, with a pulse angle of 120 ° and a ramped pulse time of 1–40 s. *Staphylococcus aureus* NCTC 8325 was used as a reference marker. Comparison and grouping of PFGE patterns were performed with InfoQuest FP Software v.5.4 (Bio-Rad).

MIC testing by the E-test method confirmed linezolid MICs of 96, ≥256, ≥256 and ≥256 mg l\(^{-1}\), respectively, for the four isolates. The isolates were cefoxitin-resistant and harboured the *mecA* gene for methicillin resistance. The strains showed similar multidrug-resistant phenotype, exhibiting resistance to chloramphenicol, clindamycin, streptomycin and ciprofloxacin. Additionally, these isolates were susceptible to erythromycin, rifampin, tetracycline and amikacin, and showed MICs of ≤2 mg l\(^{-1}\) for teicoplanin and vancomycin. The two isolates from the orthopaedic unit showed similar susceptibility profiles while those from the dermatology unit differed only in their susceptibilities to gentamicin and cotrimoxazole.

Sequencing results revealed a G2576T mutation in domain V of the 23S rRNA gene in all isolates compared with the *S. haemolyticus* reference sequence, which is the most common mutation at this site. Sequences were submitted to GenBank with accession nos KT277663, KT277664, KT277665 and KU379673. In addition, all strains carried the *cfr* gene as detected by PCR assay from a plasmid DNA extract. PFGE of *Smal*-digested genomic DNA (Fig. 1) demonstrated two pulsotypes. The two linezolid-resistant isolates from the orthopaedic unit were indistinguishable from each other, suggesting that they were derived from a similar clone. Similarly, isolates from the dermatology unit were derived from a common clone. Linezolid was immediately stopped in the patients and therapy was modified. One patient from the orthopaedic unit was given rifampin combination therapy while the other was lost to follow up. Patients from the dermatology unit were switched to teicoplanin. Samples from the patients' environment, including swabs from dressing trolleys, bed railing, mattresses and in-use antiseptic solutions, were collected. Swabs from the hands of attending hospital personnel were also taken. All the samples were found to be culture-negative. Nasal, axilla and groin
swabs collected from the patients showed growth of *S. haemolyticus* with the same linezolid-resistant phenotype as the clinical isolates. Follow-up cultures taken after a month from all the colonization sites were negative.

*S. haemolyticus* is an important causal agent of hospital-associated infections and characteristically demonstrates high multidrug resistance (Barros et al., 2012). Apart from a 23S rRNA mutation or *cfr* gene as a lone mechanism of linezolid resistance in *S. haemolyticus*, multiple mechanisms in a single isolate have also been described. Cui et al. (2013) reported a clinical isolate with a ribosomal L3 mutation and *cfr* gene, while Rajan et al. (2014) identified a linezolid-resistant *S. haemolyticus* clinical isolate with a G2576T mutation, L3 mutation and *cfr* gene. The four isolates in our study had the *cfr*-associated PhLOPS pattern, exhibiting resistance to chloramphenicol, clindamycin, linezolid and streptogramin A (Long & Vester, 2012). Acquisition of *cfr* in isolates thus further reduces the treatment options available. An alarming possibility is that these isolates harbouring the *cfr*-carrying plasmid could act as reservoirs for the *cfr* gene in the nosocomial environment. This ability for rapid spread warrants greater emphasis on infection control practices in our hospital.

All the isolates in our study demonstrated a dual mechanism of resistance with both the G2576T mutation and presence of the *cfr* gene. Target site mutation analysis for other ribosomal sites L3, L4 and L22 was not performed. A recent study in Italy reported a worrying trend of new multiple mechanisms of linezolid resistance in coagulase-negative staphylococcus. This points toward linezolid and multiple antibiotic use in co-selecting multiple mechanisms of linezolid resistance (Campanile et al., 2013). All patients in our study had prior exposure to linezolid with an average duration of therapy of about 3 weeks. Linezolid resistance is known to be associated with prolonged linezolid treatment (Pai et al., 2002), as also observed in our study. Targeted prevention and judicious antibiotic use are necessary to safeguard against emerging resistance and treatment failure.

Linezolid-resistant *S. haemolyticus* has been documented in both isolated case reports and institutional outbreaks, across healthcare settings. Clonal dissemination in intensive care units has been described in Spain and Italy, each involving a single PFGE clone with the G2576T mutation (Mazzariol et al., 2012; Rodríguez-Aranda et al., 2009). Seven *S. haemolyticus* isolates belonging to same pulsotype with the G2603T mutation were reported from a Rio de Janeiro hospital, Brazil (Chamon et al., 2014). Presence of the *cfr* gene in linezolid-resistant *S. haemolyticus* has been described in clinical case reports from China, Spain and India (Cui et al., 2013; Feßler et al., 2014; Rajan et al., 2014). This is the first study in India to use PFGE to confirm a clonal relationship between linezolid-resistant *S. haemolyticus* strains with both the G2576T mutation and *cfr* gene. PFGE analysis indicated clonal dissemination of linezolid-resistant *S. haemolyticus* in our hospital, suggesting accumulation of different resistance mechanisms probably due to linezolid exposure and their selection and spread through our healthcare facility. This study highlights the need for continuous surveillance to detect linezolid-resistant strains in our hospital. Furthermore, the finding of linezolid-resistant clones underlines the importance of implementation of infection control practices to prevent their further dissemination.

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**References**


