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A *cfr*-positive clinical staphylococcal isolate from India with multiple mechanisms of linezolid-resistance

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***Background & objectives*: Linezolid, a member of the oxazolidinone class of antibiotics, has been an effective therapeutic option to treat severe infections caused by multidrug resistant Gram positive bacteria. Emergence of linezolid resistant clinical strains is a serious issue in the healthcare settings worldwide. We report here the molecular characterization of a linezolid resistant clinical isolate of *Staphylococcus haemolyticus* from India.**

***Methods*: The species of the clinical isolate was identified by 16S rRNA gene sequencing. The minimum inhibitory concentrations (MICs) of linezolid, clindamycin, chloramphenicol and oxacillin were determined by E-test method. To elucidate the mechanism of linezolid-resistance, presence of *cfr* gene (chloramphenicol florfenicol resistance) and mutations in 23S rRNA and ribosomal proteins (L3, L4 and L22) were investigated. Staphylococcal Cassette Chromosome *mec* (SCC*mec*) typing was performed by multiplex PCR.**

***Results*: The study documented a rare clinical *S. haemolyticus* strain with three independent mechanisms of linezolid-resistance. The strain carried *cfr* gene, the only known transmissible mechanism of linezolid-resistance. The strain also possessed resistance-conferring mutations such as $G_{2576}T$ in domain V of 23S rRNA gene and $Met_{156}Thr$ in L3 ribosomal protein. The other ribosomal proteins (L4 and L22) did not exhibit mutations accountable for linezolid-resistance. Restriction digestion by *Nhe*I revealed that all the alleles of 23S rRNA gene were mutated. The isolate showed elevated MIC values (>256 µg ml^{-1}) of linezolid, clindamycin, chloramphenicol and oxacillin. Methicillin resistance was conferred by type I SCC*mec* element. The strain also harboured *lsa*(B) gene which encodes an ABC transporter that can efflux clindamycin.**

***Interpretation & conclusions*: The present study reports the first clinical strain from India with transmissible and multiple mechanisms of linezolid-resistance. Judicious use of linezolid in clinical practice and proper surveillance of *cfr*-positive strains are of utmost importance to safeguard the efficacy of linezolid.**

Key words *cfr* gene - linezolid - mutations - ribosomal proteins

Linezolid is a synthetic drug and the only oxazolidinone antibiotic licensed for clinical use. It is widely used to treat severe infections in adults caused by various drug resistant Gram positive bacteria such as streptococci, methicillin resistant staphylococci and vancomycin resistant enterococci. Although rare (<1% in *Staphylococcus aureus*, and <2% in coagulase negative staphylococci), linezolid resistance is currently on the rise and emergence of bacterial strains with multiple mechanisms of linezolid-resistance is a cause of concern in antimicrobial chemotherapy[1]. The first linezolid resistant clinical strain appeared in 2001, a year after the introduction of linezolid as a drug with unique mechanism of action[2]. Linezolid remains efficient in treating complicated cases of bacteremia, endocarditis, osteomyelitis, nosocomial pneumonia and severe soft tissue infections[3]. This bacteriostatic antibiotic blocks protein synthesis by interfering the positioning of A-site tRNA in the peptidyl transferase centre of 23S rRNA. Resistance to linezolid is primarily caused by mutations in the domain V of 23S rRNA gene, mutations in the ribosomal proteins L3, L4 and L22 or methylation at C-8 position of A2503 of the 23S rRNA by a methyl transferase encoded by the gene *cfr* (chloramphenicol florfenicol resistance)[4]. Co-occurrence of *cfr*-mediated resistance and mutational resistance has also been documented[1]. The low occurrence of linezolid resistance is mainly attributed to the absolute synthetic nature of this antibiotic for which natural resistance genes are not widely distributed. Moreover, the presence of multiple copies of 23S rRNA gene in majority of the bacteria (5-6 alleles in staphylococci) reduces the probability of mutational resistance[5]. Resistance mediated by *cfr* gene is of great concern as it is usually plasmid or transposon borne and can be disseminated to susceptible population[6]. *cfr* also encodes resistance to a group of chemically distinct antibiotics: phenicols, lincosamides, pleuromutilins and streptogramin A[7]. There have been a few reports of linezolid resistant staphylococci from India[8-10]. But so far no work has been done on the molecular analysis of the isolates from this geographical area.

We undertook this study to investigate the mechanism of resistance in a linezolid resistant clinical isolate of *S. haemolyticus*.

Material & Methods

The isolate was obtained from a 60 yr old male who was admitted in JSS Medical College, a tertiary care hospital in Mysore, India, following oedema and cellulitis of left lower limb. The patient had been undergoing dialysis in another hospital for the past six months. During the course of dialysis, he had received oral linezolid therapy (600 mg twice daily for a period of 28 days) for methicillin-resistant *S. aureus* (MRSA) bacteremia. He had hypertension and type II diabetes. His great toe had already been amputed. The patient presented with an open sore and redness in the infected area with pus filled bumps. The swab from the lesion was inoculated on 5 per cent sheep blood agar and MacConkey agar plates and colony was subjected to Gram staining. The culture was further inoculated onto Mannitol Salt Agar (MSA) and DNase plates (Hi-Media, India). Tube coagulase test was also performed using rabbit plasma (Hi-media, Mumbai, India). A second swab from the same site was taken on the following day for confirmation of the pathogen and also to rule out any nosocomial contamination of the wound.

Antimicrobial susceptibility test was performed by Kirby-Bauer disc diffusion method on Mueller Hinton Agar (MHA) plates according to Clinical and Laboratory Standards Institute (CLSI) guidelines[11]. Minimum inhibitory concentrations (MICs) of linezolid, clindamycin and chloramphenicol were determined by using E-test strips (Biomerieux SA, France) on MHA plates incubated at 37 ºC for 24 h. For determining oxacillin MIC, the medium was supplemented with 2 per cent NaCl for the expression of *mec*A gene and incubated at 35 ºC for 24 h.

The genomic DNA was isolated using phenol-chloroform extraction method[12] with the addition of lysostaphin (Sigma-aldrich, USA) to a final concentration of 15 µg/ml. The species was identified by 16S rRNA gene sequence analysis[13]. Staphylococcal Cassette Chromosome *mec* (SCC*mec*) typing of the isolate was performed by multiplex PCR using 10 sets of primers in different concentrations as described by Milheirico *et al*[14]. Amplification was performed using Accuprime Taq DNA polymerase system (Invitrogen, USA) with the following PCR conditions: denaturation at 95 ºC for 15 min followed by 35 cycles of 95 ºC for 25 sec, 53 ºC for 30 sec and 72 ºC for 1 min; and a final extension at 72 ºC for 7 min. The prototype strains used as controls for typing were *S. aureus* COL (type I), *S. aureus* BK2464 (type II), *S. aureus* ANS46 (type III), *S. aureus* MW2 (type IV), *S. aureus* WIS (type V) and *S. aureus* HDE288 (type VI)[14]. To elucidate the mechanism of linezolid resistance, screening for *cfr* gene was performed using primers described by Kehrenberg and colleagues[15]. Point mutation in domain

V of 23S rRNA gene was investigated by amplifying the locus using primers and PCR conditions described by Meka *et al*[16]. The sequence was aligned with the *Escherichia coli* reference strain (GenBank accession No. V00331) as well as the linezolid susceptible *S. haemolyticus* strain JCSC1435 (GenBank accession No. AP006716). The amplified fragment was digested with *Nhe*I (New England Biolab, UK) using conditions given by the manufacturer to find out the number of alleles of 23S rRNA gene with $G_{2576}T$ mutation. The target site mutations were also investigated in ribosomal proteins L3 (*rpl*C), L4 (*rpl*D) and L22 (*rpl*V) by amplifying the respective loci. *rpl*C and *rpl*V genes were amplified using the primers and PCR conditions described by Mendes *et al*[17]. The *rpl*D gene was amplified using primers 5'TATCCGAGCACCTCCTCAAC3' and 5'ACGGAACTAAATCAGGTTCA3′ (Deshpande LM, personal communication, November 21, 2012). The optimal cycling conditions for all the *rpl* loci were the following: 95 ºC for 5 min; 35 cycles of 94 ºC for 30 sec, 50 ºC for 30 sec, and 72 ºC for 1 min; and a final extension at 72 ºC for 7 min. All the amplicons were sequenced on both stands. Translated nucleotide sequences of *rpl*C, *rpl*D and *rpl*V genes were aligned with the corresponding L3, L4 and L22 proteins of *S. haemolyticus* strain JCSC1435 (GenBank accession numbers- L3: BAE04111, L4: BAE04112 and L22: BAE04116). As the isolate showed the unusual pattern of lincosamide resistance and macrolide sensitivity (L^R/M^S), mechanisms by which lincosamide alone is inactivated/effluxed were also investigated. For this, presence of genes which inactivate lincosamide [*lnu*(A), *lnu*(B), *lnu*(C) and *lnu*(D)] and genes which mediate efflux of the drug [*vga*(A), *vga*(B), *vga*(C), *vga*(E) and *lsa*(B)] were checked using primers and PCR conditions described by Lozano *et al*[18].

All the nucleotide sequences from this study have been submitted to GenBank database under the accession numbers KC544271-KC544273, KC572114, KC736551, KC736552 and KC809978.

Results & Discussion

The pathogen was identified as *Staphylococcus* based on the characteristic growth on blood agar followed by microscopic observation. The isolate showed negative results for mannitol fermentation, DNase and tube coagulase tests, thus confirming coagulase negative *Staphylococcus*. The 16S rRNA gene sequence revealed the isolate as *S. haemolyticus* and showed 99 per cent similarity with *S. haemolyticus* strain JCSC1435 in BLAST search. The strain was resistant to penicillin, cefoxitin, gentamicin, clindamycin, trimethoprim-sulphamethoxazole, rifampicin, chloramphenicol and linezolid. Susceptibility was recorded for the following antibiotics: erythromycin, tetracycline, levofloxacin, quinupristin-dalfopristin and teicoplanin. The isolate showed high MIC values (>256 µg/ml) of linezolid, clindamycin, chloramphenicol and oxacillin. The SCC*mec* element present in the isolate was found to be of type I, as evidenced by the presence of CIF locus in the cassette. The prototype strain for type I (*S. aureus* COL) worked as the positive control. *cfr* gene was detected and confirmed by sequencing the amplicon on both strands. This is perhaps the first report of a *cfr*-carrying clinical isolate from India. Recently Cui *et al*[19] and Cai *et al*[20] have described *cfr* carrying *S. haemolyticus* strains from clinical samples.

The BLAST alignment revealed $G_{2576}T$ (*E.coli* numbering) mutation in the domain V of 23S rRNA gene. This is the most frequent mutation in linezolid resistant strains and it has been previously reported in *S. haemolyticus*[21-23]. The mutation was confirmed by the restriction digestion of the 420 bp amplicon by *Nhe*I which can detect this transverion. Agarose gel pattern revealed new fragments of sizes 322 and 98 bp which indicated the presence of $G_{2576}T$ mutation. Moreover, the complete digestion of the PCR products with *Nhe*I indicated that all the five alleles of 23S rRNA gene were mutated.

The blastx analysis of *rpl*C gene which codes for L3 protein, revealed $Met_{156}Thr$ mutation. This substitution mutation has previously been reported in a few linezolid resistant *S. epidermidis* strains[24]. Until recently this mutation was not reported in the context of linezolid resistance in *S. haemolyticus*[25]. In L4 protein, no mutation was detected in the highly conserved region ($_{63}$KPWK/RQKGTGRAR$_{74}$), which is usually implicated in oxazolidinone resistance. The isolate did not possess any mutation in L22 ribosomal protein, and was negative for the tested *lnu* and *vga* genes. Only *lsa*(B) gene which encodes an ABC transporter was detected and confirmed by sequencing.

S. haemolyticus has emerged as a nosocomial pathogen on account of its ability to attain high level resistance to many antibiotics including glycopeptides[26]. Linezolid resistance in *S. haemolyticus* is considered rare; however, there have been recent reports from India, China, Brazil, Italy and Spain[8,19-23]. In the present

study, the ribosomal mutations in the isolate might have occurred under antibiotic pressure as the patient had undergone linezolid therapy. Moreover, the long-term linezolid usage by the patient could be the reason for $G_{2576}T$ mutations in all the copies of 23S rRNA gene. Previous *in vivo* studies have established a positive correlation between the number of rRNA alleles mutated and the dosage and duration of linezolid therapy[27]. $M_{156}T$ mutation and mutations in adjacent amino acids ($V_{154}L$, $G_{155}R$, $A_{157}R$, $S_{158}F$ and $D_{159}Y$) have been implicated in linezolid resistance owing to their proximity to the binding cleft of linezolid in L3 protein[4]. In the present study, the elevated MIC (>256 µg/ml) of linezolid can be attributed to the simultaneous occurrence of all the three mechanisms of resistance. More worrisome is the presence of *cfr* gene in the isolate, as its low fitness cost would enable the cells to retain it even in the absence of selection pressure imposed by the antibiotic. *cfr* is usually located in an unstable genetic environment either on the chromosome or on multidrug resistant plasmids[28]. This would facilitate the easy spread of *cfr* into susceptible population and other pathogenic bacteria. Furthermore, *cfr*-mediated resistance limits therapeutic options as it encodes resistance to an array of antibiotics.

In conclusion, the study emphasizes the need for proper surveillance of *cfr*-carrying strains in the healthcare settings in India. Linezolid resistant strains which exhibit the unusual pattern of erythromycin-sensitivity and clindamycin-resistance could be suspected for the presence of *cfr* gene. *cfr* can also be selected under the pressure imposed by other classes of antibiotics such as phenicols, lincosamides, pleuromutilins and Streptogramin A. Judicious use of these antibiotics and stringent infection control measures are important to prevent the spread of *cfr*-carrying strains in nosocomial environment.

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