Indian J Med Res 149, June 2019, pp 795-798
DOI: 10.4103/ijmr.IJMR_283_18

Quick Response Code:

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Linezolid-resistant *Staphylococcus haemolyticus*: Emergence of G2447U & C2534U mutations at the domain V of 23S ribosomal RNA gene in a tertiary care hospital in India

Sir,

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 the treatment of infections from Gram-positive cocci. The first clinical isolates of linezolid-resistant staphylococci and enterococci were reported in 2001[1]. Since then, linezolid-resistant strains have become an increasing problem worldwide. The most frequently reported mechanisms of linezolid resistance include the mutation in 23S ribosomal RNA (23S rRNA) and presence of *cfr* gene.

At our hospital, a tertiary care hospital in north India, methicillin-resistant coagulase-negative staphylococci (CoNS) and vancomycin-resistant enterococci have become a worrisome clinical problem[2,3]. This situation brings about new challenges for the treatment of these infections and patient safety. This study was aimed to determine the distribution of linezolid-resistant isolates in an inpatient setting of the All India Institute of Medical Sciences (AIIMS), New Delhi, India, and to evaluate the resistance mechanisms among these isolates. In addition, the clonal diversity of the isolates was determined by pulsed-field gel electrophoresis (PFGE). The study included linezolid resistance *Staphylococcus haemolyticus* (LR-SH) isolates [linezolid resistance screening was assessed by linezolid (30 µg) discs] recovered from pus specimens of patients with chronic osteomyelitis and pemphigus vulgaris hospitalized in the departments of Orthopaedics and Dermatology & Venereology of the AIIMS, New Delhi, respectively, from June 2015 to December 2016. The study was approved by the Institutional Ethics Committee.

Bacterial identification was performed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)[4]. Antimicrobial susceptibility testing was performed by disc diffusion method according to Clinical and Laboratory Standards Guidelines (2015)[5] and minimum inhibitory concentration (MICs) of linezolid, vancomycin and teicoplanin by *E*-test method (bioMérieux, USA).

Isolates were screened for the presence of *cfr* (chloramphenicol - florfenicol resistance) gene and mutations in the 23S rRNA gene by PCR and DNA sequencing as described previously[6,7]. Amplicons were sequenced on both strands and were compared with *S. aureus* ATCC 29213 (bioMérieux).

The clonal relatedness of the LR-SH isolates was examined by PFGE of *Sma*-I-digested genomic DNA according to the protocol described by Goering and Winters[8], with some modifications. Genomic DNA was prepared in agarose blocks and digested with *Sma*I (Promega, USA). The DNA fragments were separated on one per cent agarose gel using CHEF Mapper System III (Bio-Rad, USA) for 20 h at 6 V/cm at 14°C, with a pulse angle of 120° and a ramped pulse time of 1-40 sec. *S. 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).

A total of 13 LR-SH isolates were recovered from 16 pus specimens. The rate of linezolid resistance among *S. haemolyticus* isolates was 81.3 per cent. All patients had received multiple antibiotics before referral. Three patients had received linezolid, the duration of which varied from 10 days to two weeks. The characteristics of the patients and their isolates are presented in the Table.

MIC testing by *E*-test confirmed linezolid MIC of ≥256 µg/ml in all the isolates of *S. haemolyticus*

Table. Clinical characteristics of patients with linezolid-resistant *Staphylococcus haemolyticus* (n=13)

Isolates	Date of isolation	Age/ Sex	Clinical diagnosis	LZ MIC (µg/ml)	VAN MIC (µg/ml)	TEICO MIC (µg/ml)	LZ exposure	23 S ribosomal RNA gene mutation	*Cfr* gene	PFGE types
SHLR 248	07/03/2015	26/M	Chronic osteomyelitis	≥ 256	≥ 2	1.5	No	G2576T	Positive	Clone III
SHLR 225	29/06/2015	54/M	Chronic osteomyelitis	≥ 256	1.5	≥ 2	2 wk	G2576T	Positive	Clone I
SHLR 229	12/08/2015	32/M	Chronic osteomyelitis	≥ 256	≥ 2	1.5	10 days	G2447U	Positive	Clone IV
SHLR 237	16/09/2015	45/M	Chronic osteomyelitis	≥ 256	2	1.5	4 wk	G2447U	Positive	Clone V
SHLR 220	06/10/2015	45/F	Chronic osteomyelitis	≥ 256	2	2	5 wk	G2447U	Positive	Clone VI
SHLR 224	28/07/2016	44/F	Chronic osteomyelitis	≥ 256	2	2	No	C2534U	Positive	Clone VII
SHLR 227	05/08/2016	54/M	Chronic osteomyelitis	≥ 256	1.5	2	No	G2576T, G2447U	Positive	Clone VIII
SHLR 240	15/08/2016	29/F	Chronic osteomyelitis	≥ 256	≥ 2	≥ 2	No	G2576T	Positive	Clone I
SHLR 204	28/08/2016	49/F	Chronic osteomyelitis	≥ 256	2.5	2	No	G2447U	Positive	Clone IX
SHLR 773	16/09/2015	42/F	Pemphigus vulgaris	≥ 256	1.5	1.5	No	G2576T	Positive	Clone X
SHLR 213	03/10/2015	42/F	Pemphigus vulgaris	≥ 256	1.5	1.5	10 days	G2576T	Positive	Clone XI
SHLR 230	03/10/2015	52/M	Pemphigus vulgaris	≥ 256	2	2	No	G2576T	Positive	Clone II
SHLR 247	07/03/2016	26/M	Pemphigus vulgaris	≥ 256	2	2	2 wk	G2576T	Positive	Clone II

LZ, linezolid; VAN, vancomycin; TEICO, teicoplanin; PFGE, pulsed-field gel electrophoresis; MIC, minimum inhibitoryconcentration; M, male; F, female

including susceptibility to teicoplanin and vancomycin. All the isolates were cefoxitin resistant and showed similar multidrug-resistant phenotype, exhibiting uniform resistance to chloramphenicol, clindamycin, ciprofloxacin and rifampicin. However, variable susceptibility to erythromycin (84.6%) and amikacin (92.3%) was observed in all the isolates irrespective of prior linezolid exposure.

Sequencing results revealed G2576T mutations in eight, G2447U in four and C2534U in one isolate of *S. haemolyticus*. All three isolates of *S. haemolyticus* from patients with prior linezolid exposure showed G2447U mutation. One isolate of *S. haemolyticus* showed two simultaneous mutations (G2576T and G2447U) in the domain V region of 23S rRNA gene. Sequences were submitted to GenBank with accession numbers- KT277663, KT277664, KT277666, KT277667, KT277668, KT277669, KT277670, KT277671, KT277672, KT277673, KT277674, KT277665 and KU379673. All the 13 isolates carried the *cfr* gene.

Eleven clones (I-XI) were identified on PFGE (Figure). Of these, clones I and II had two isolates each. Isolates of clone I exhibited identical band pattern with the previous isolates of LR-SH isolated from department of Orthopaedics. Similarly, isolates of clone II also shared same band pattern with the previous LR-SH isolates from department of Dermatology & Venereology of our centre[9].

In a hospital setting, knowledge of clonal spread and resistance patterns of LR isolates are important in patient management and formulation of infection control measures. Linezolid resistance was observed only in *S. haemolyticus.* Neither LR- *S. aureus* nor LR- enterococci were found during this study. Worldwide, the incidence of LR-CoNS is 28 times that of LR- *S. aureus*[10]. All the isolates exhibited high-level resistance to linezolid. Our results were similar to previous studies from China where high-level resistance (MIC values ≥256 µg/ml) was described in most strains of LR-CoNS[11]. On the contrary, reports from other parts of the world demonstrated a predominance of low to medium level LR-CoNS with a complete absence of high-level LR-CoNS strains[12,13]. The LR-SH isolates had the *cfr*-associated PhLOPS (phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin) pattern, thereby further reducing treatment options available.

Similar to our previous report[9], all the isolates demonstrated a dual mechanism of resistance with a

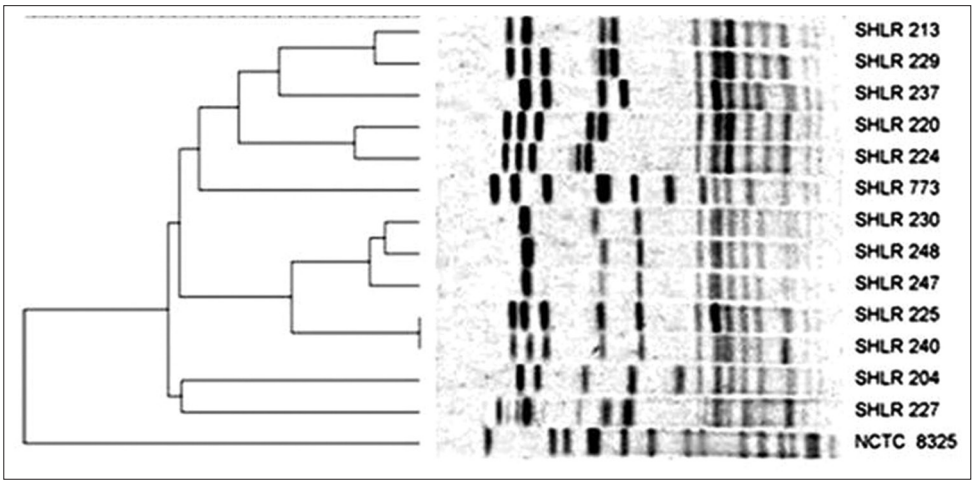

Figure. Dendrogram based on the similarities using InfoQuest FP software v5.4 (Bio-Rad). Pulsed-field gel electrophoresis patterns of *Sma*-I macrorestriction fragments of linezolid-resistant *S. haemolyticus* isolates are shown.

mutation at domain V of 23S rRNA gene and presence of *cfr* gene. However, contrary to our previous findings[9], in addition to G2576T mutation, several previously described mutations including G2447U and C2534U were identified[9,14]. The presence of mutations highlights excessive or inadequate exposure to linezolid, but their chromosomal location does not threaten rampant spread of such infections. In contrast to our previous report[9] where we had documented clonal dissemination, the present study documented the emergence of multiple clones of LR-SH. Linezolid resistance is known to be associated with prolonged linezolid treatment or inappropriate linezolid dosage. In our study, most of the patients had not received linezolid.

In conclusion**,** this study highlights the importance of continuous monitoring of linezolid resistance in staphylococci. Rationalizing the use of linezolid and implementing methods to control the spread of hospital clones are of paramount importance to prevent further dissemination of these strains.

***Financial support & sponsorship*:** None.

***Conflicts of Interest*:** None.

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Received February 8, 2018

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