Detection of a Novel G2603T Mutation in cfr Harboring Linezolid-Resistant *Staphylococcus haemolyticus*: First Report from India

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Abstract

Background *Staphylococcus haemolyticus* has emerged as an important multidrug-resistant nosocomial pathogen. Linezolid is useful in the treatment of severe infections caused by methicillin-resistant *Staphylococci*. Resistance to linezolid in *Staphylococci* is due to one or more of the following mechanisms: acquisition of the *cfr* (chloramphenicol florfenicol resistance) gene, mutation in the central loop of domain V of the 23S rRNA, and mutation in the *rplC* and *rplD* genes. This study was carried out to detect and characterize resistance to linezolid among the clinical isolates of *Staphylococcus haemolyticus*.

Materials and Methods The study included 84 clinical isolates of *Staphylococcus haemolyticus*. Susceptibility to various antibiotics was determined by disc diffusion method. Minimum inhibitory concentration (MIC) was determined by agar dilution method for linezolid. Methicillin resistance was screened using oxacillin and cefoxitin disc. Polymerase chain reaction was done to detect *mecA, cfr* and mutations in the V domain of the 23S rRNA gene.

Results Resistance to linezolid was exhibited by 3 of the 84 study isolates with MIC more than 128 µg/mL. The *cfr* gene was detected in all the three isolates. The G2603T mutation was observed in the domain V of the 23S rRNA among two isolates, whereas one isolate lacked any mutation.

Conclusion The emergence and spread of linezolid-resistant *Staphylococcus haemolyticus* isolates carrying G2603T mutation in the domain V of the 23S rRNA and harboring the *cfr* gene pose a threat in clinical practice.

Keywords
- linezolid resistance
- *cfr* gene
- 23S rRNA gene
- *Staphylococcus haemolyticus*

Introduction

Staphylococcus haemolyticus is an opportunistic bacterial pathogen that colonizes human skin and mucous membrane. It is the second most common coagulase-negative *Staphylococci* (CONS) isolated from clinical specimens and is associated with bloodstream infections related to intravascular catheters, skin and soft tissue infection, meningitis, endocarditis, and a variety of device-associated infections.[1] It has an inherent ability to acquire and maintain exogenous

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genetic material or mobile genetic elements that encode for antimicrobial resistance.[2] Hence, they are often multidrug resistant exhibiting resistance to antimicrobial classes such as beta lactams, macrolides, lincosamides, and streptogramins and more recently display reduced susceptibility to glycopeptides and oxazolidinones.[3]

Linezolid is a synthetic bacteriostatic drug, belonging to oxazolidinone class of antibiotics and is active against various multidrug-resistant gram-positive pathogens, such as methicillin-resistant *Staphylococci* and vancomycin-resistant *Enterococci*. Linezolid inhibits protein synthesis by interacting with the 23S rRNA in the 50S ribosomal subunit.[4,5] It is effective in the treatment of bacteremia, nosocomial pneumonia, and severe skin and soft tissue infections.[6]

A year after its introduction, the first clinical linezolid-resistant *Staphylococcus aureus* strain appeared in 2001 and thereafter few reports were published from the United States and Europe.[7] The first linezolid-resistant *Staphylococcus haemolyticus* (LRSH) was reported in 2009, since then a few strains have been reported from countries such as India, China, Brazil, Italy, and Spain.[2] More recently due to its extensive use, linezolid resistance is on the rise. This resistance is mediated by the mutations in the domain V of 23S rRNA, presence of the *cfr* gene, or the mutations in the ribosomal proteins.[4,8,9] There are only very few Indian studies describing the mechanism of resistance to linezolid in *Staphylococcus haemolyticus*. Resistance mediated by *cfr* and mutation in 23S rRNA have been reported with G2576T mutation as the most common.[6,9]

This study was undertaken to detect and characterize resistance to linezolid among clinical isolates of *Staphylococcus haemolyticus*.

Materials and Methods

Bacterial Isolates

The study was conducted in a 1,600-bedded, university teaching hospital in South India. A total of 84 clinically significant, consecutive, nonrepetitive *Staphylococcus haemolyticus* isolated during the period 2019 to 2021 were included in the study. The study was approved by Institutional Ethics Committee (REF: IEC-NI/19/FEB/68/12)

The source of the isolates was blood ($n = 36$), exudative specimens ($n = 38$), and urine ($n = 10$). The isolates were identified up to species level by standard biochemical tests and automated systems: VITEK2 GP-card (bioMerieux, Marcy l'Etoile, France) and MALDI-TOF MS (bioMerieux, Marcy l'Etoile, France).

Antimicrobial Susceptibility Testing

Antibiotic susceptibility testing was done by Kirby Bauer disc diffusion method for different classes of antimicrobial agents such as ampicillin (10 µg), cefuroxime (30 µg), erythromycin (30 µg), clindamycin (2 µg), amikacin (30 µg), ciprofloxacin (5 µg), linezolid (30 µg), and teicoplanin (30 µg). Methicillin resistance was detected by cefoxitin (30µg) and oxacillin (1µg) disc (Himedia, Mumbai, Maharashtra, India) as per Clinical and Laboratory Standards Institute (CLSI 2019) guidelines (CLSI-M100-S29).[10] Minimum inhibitory concentration (MICs) of linezolid (MicroExpress, Goa, India) and vancomycin were determined by agar dilution method in accordance to CLSI 2019 guidelines.

Molecular Methods

DNA Extraction

Colonies of clinical strains were transferred to sterile distilled water. The samples were then boiled to prepare the DNA template. This was used as template for polymerase chain reaction (PCR).

Polymerase Chain Reaction

All the isolates were subjected to molecular confirmation using the species specific *nuc* gene.[11] *MecA* gene was amplified to detect methicillin resistance.[12] PCR was done to detect *cfr* gene and the amplification of 23S rRNA gene was done to determine mutations in the V domain. All the PCR reactions were caried out with a final volume of 25 µL reaction. Each reaction contained 10 pmol of each primer (Eurofins, India) and 23 µL of master mix (Takara, India) and 2 µL of template DNA. The amplicons were separated in a 1% agarose gel containing ethidium bromide.

The primers used are described in ►**Table 1**. Previously, characterized strains were used as positive controls. Sterile Mili Q water was used as negative controls.

The obtained sequences were compared to the reference 23S rRNA gene sequences of *Staphylococcus haemolyticus* (JCSC1435). The sequences were submitted to GenBank database with the following accession numbers OL691912, OL691913, OL743221, OL743222, ON249039, ON249040.

The medical records were perused to collect the clinical details of the patients from whom the LRSH was isolated.

Results

Among the 84 isolates, the *nuc* gene was present in all the isolates, confirming the identification as *Staphylococcus haemolyticus*. The resistance exhibited to various classes of antimicrobials is as follows: ampicillin 95.2% (80/84), cefuroxime 79% (66/84), cefoxitin 79% (66/84), cefotaxime 79% (66/84), erythromycin 88% (74/84), clindamycin 57% (48/84), ciprofloxacin 72.6% (61/84), and linezolid 3.5% (3/84). All isolates were susceptible to vancomycin and teicoplanin.

Methicillin resistance was detected by oxacillin and cefoxitin disc diffusion method. Resistance to oxacillin was observed in 67/84 and resistance to cefoxitin was detected in 66/84 of the study isolates. The *mecA* gene was present in 98.5% (66/67) of the oxacillin-resistant isolates.

The linezolid-resistant isolates ($n = 3$) had MIC of greater than 128 µg/mL. *Cfr* gene was detected in all the three linezolid-resistant isolates. The domain V of the 23S rRNA gene was amplified by PCR. The obtained sequences were compared to the reference 23S rRNA gene sequences of *Staphylococcus haemolyticus* (JCSC1435). The BLAST alignment revealed G2603T point mutation in the domain V of 23S rRNA gene in two of the linezolid-resistant isolates.

Table 1 Primers and PCR conditions used for resistance genes

Gene	Primer	PCR conditions	Amplicon	References
23s rRNA	F- CGGCGGCCGTAACTATAACG R- CAGCACTTATCCCGTCCATAC	Initial denaturation: 95°C for 3 min Denaturation: 95°C for 30 s Annealing: 55°C for 30 s Extension: 72°C for 30 s for 30 cycles Final extension: 5 min at 72°C	846	2
nuc	F- TAGTGGTAGGCGTATTAGCC R- ACGATATTTGCCATTCGGTG	Initial denaturation: 94°C for 3 min Denaturation: 94°C for 30 s Annealing: 50°C for 30 s Extension: 72°C for 45 s for 30 cycles Final extension: 7 min at 72°C	434	10
mecA	F- GTAGAAATGACTGAACGTCCGATA R- CCAATTCCACATTGTTTCGGTCTAA	Initial denaturation: 94°C for 3 min Denaturation: 94°C for 30 s Annealing: 50°C for 30 s Extension: 72°C for 45 s for 30 cycles Final extension: 7 min at 72°C	310	11
cfr	F-TGAAGTATAAAGCAGGTTGGGAGT R- ACCATATAATTGACCACAAGCAGC	Initial denaturation: 94°C for 2 min Denaturation: 94°C for 10 s Annealing: 55°C for 30 s Extension: 72°C for 30 s for 30 cycles Final extension: 7 min at 72°C	746	12

Abbreviation: PCR, polymerase chain reaction.

The clinical details of the patient from whom LRSH was isolated are tabulated in ►**Table 2**.

Discussion

Staphylococcus haemolyticus is increasingly recognized as an important pathogen due to its ability to develop multiple drug resistance, its adaptability and ability to survive in the hospital environment, especially on medical devices.[3] Linezolid, an oxazolidinone, is indicated for the treatment for a variety of Gram-positive infections; it is often the last-resort antibiotic for the management of infections caused by methicillin-resistant *Staphylococci*.[4]

Resistance to linezolid in *Staphylococci* is due to one or more of the following mechanisms: acquisition of the *cfr* (chloramphenicol florfenicol resistance) gene, mutation in the central loop of domain V of the 23S rRNA, and mutation in the *rplC* and *rplD* genes, which encodes for the 50S ribosomal proteins L3 and L4, respectively.[4,8,9] Transferable plasmid mediated *cfr* gene encodes for a ribosomal methyltransferase conferring resistance to phenicol, lincosamide, oxazolidinone, pleuromutilin, and streptogramin A ($PhLOPS_A$).[9,13] Since it confers resistance to other classes of antimicrobial agents, attention should be paid to the fact that linezolid-resistant strains might be selected during treatment with any of these drugs.[14] Co-occurrence of *cfr* mediated resistance and mutational resistance has also been documented more frequently.[4,6,13] The ability of *cfr* gene to be transmitted between different bacterial strains and species is a cause for concern.

In this study, linezolid resistance was detected in three isolates and their MIC was greater than 128 µg/mL. All the three isolates carried the *cfr* gene and among them the mutation in the domain V of 23S rRNA was detected in two isolates. The BLAST alignment revealed a novel G2603T point mutation in the domain V of 23S rRNA gene in two of the linezolid-resistant isolates. The G2603T mutation has not been reported previously in India. The G2603T mutation has been reported in China among *Staphylococcus epidermidis*, *Staphylococcus capitis*[4,8] and in Brazil among *Staphylococcus hominis*, *Staphylococcus epidermidis*, and *Staphylococcus haemolyticus*.[15,16] Other mutations in domain V of 23S rRNA reported in literature include G2614T, C2384T, T2500A, C2534T, T2504T, G2447T, G2215A, C2190T, C2505A, and G2631T among other clinical *Staphylococci*.[9,13,15,16] However, none of the above mutations was observed in this study. ►**Table 3** represents the mutations reported in the domain V of 23S rRNA encoding for linezolid resistance in *Staphylococcus haemolyticus* since 2012.[5,6,13,15,17–22]

Following its first detection in 2001, sporadic cases of linezolid resistance have been reported globally. In India, the first case report on LRSH from North India was published in 2011[23] followed by another report in 2012[24] and later from South India.[6] In the former, mechanism of resistance was not studied, while the latter described the mechanism of resistance was due to the presence of *cfr* and mutation in domain V of 23S rRNA. A recent study published from South India reported linezolid resistance in 3.7% (13/356) of *Staphylococcus haemolyticus* with 12 isolates harboring the *cfr* gene. Mutation in domain V of 23S rRNA was not looked for in their study.[3] In a hospital from Delhi, nine LRSH isolates were characterized and it was found that all of them carried the *cfr* gene along with mutation G2614T in domain V of 23S rRNA.[13]

A study from Vietnam carried out whole genome sequencing and demonstrated the transferability of the plasmid carrying the *cfr* gene.[14] However, in this study gene transfer experiments were not carried out.

Table 2 Clinical profile, molecular characterization among the LRSH

Characteristics	Patient I	Patient II	Patient III
Isolate number	E7710	MS7890	GE920
Age/Sex	71/Female	65/Male	69/Male
Hospital location	ICU	ICU	ICU
Admitting speciality	General surgery	General medicine	General surgery
Date of isolation	26/08/2019	26/11/2020	11/03/2021
Underlying disease/diagnosis	Diabetic foot Microcytic hypochromic anemia	Septic encephalopathy	Diabetic foot ulcer Acute pyelonephritis
Co-morbid conditions	Diabetes mellitus	Diabetes mellitus	Diabetes mellitus
Days in hospital	9 days	9 days	19 days
Surgical procedures	Ray amputation	Wound debridement	Ray amputation
Antimicrobials used prior to detection of linezolid resistance	Amoxicillin/clavulanate	Amoxicillin/clavulanate, azithromycin	Ciprofloxacin, levofloxacin, cefoperazone–sulbactam
Indwelling devices	Peripheral line	Peripheral line	Peripheral line
Outcome	Recovered	Discharged against medical advice	Recovered
Source specimen	Pus	Pus	Pus
MIC (μg/mL)	> 128	> 128	> 128
mecA	+	+	+
cfr	+	+	+
23s rRNA mutations in V domain	G2603T	Nil	G2603T

Abbreviations: ICU, intensive care unit; LRSH, linezolid-resistant *Staphylococcus haemolyticus*; MIC, minimum inhibitory concentration.

Table 3 Mutations reported in the domain V of 23S rRNA encoding for linezolid resistance in *Staphylococcus haemolyticus*

Mutation	Year	Country	Reference
G2576T	2012	Brazil	17
G2576T	2013	Spain	5
G2576T	2014	India	6
G2603T	2014	Brazil	15
G2576T	2014	USA	18
G2447U			
U2504A			
C2534U			
G2576T	2016	India	19
G2614T	2019	India	13
G2447U	2019	India	20
C2534U			
G2576T	2019	India	21
G2576T	2020	India	22
G2603T	2022	India	This study

This study reports dual mechanisms of resistance to linezolid. Although the mutational resistance to linezolid poses a threat in clinical practice, the acquisition of the *cfr* gene is threatening because of its potency for horizontal transmission between species. Only one isolate carried the *cfr* gene alone and lacked any mutations, which is similar to the observations made in previous studies.[2,13]

In this study, of the three patients who had infection with LRSH, two patients underwent wound debridement and ray amputation for removal of nidus of infection and source control. Though these patients were treated with beta lactam antibiotics and fluoroquinolone, they recovered and were discharged from the hospital. It may be reasonably assumed that *Staphylococcus haemolyticus* could have been a colonizer in the wound and the recovery may be attributed to source control. Follow-up was lost in one patient.

Since many CONS are usually considered as part of normal skin flora, most clinical laboratories do not test for antimicrobial susceptibility unless from a sterile site such as blood. These organisms have relatively low virulence but are now increasingly recognized as clinically significant. As the pathogenic significance becomes apparent, it becomes necessary to characterize them and study their antimicrobial susceptibility profile.[14]

Conclusion

The presence of *cfr* gene along with mutations is alarming. Prudent use of linezolid and strengthening implementation of infection control measures and screening of patients with

linezolid resistant-CONS should be mandated to curtail the spread of resistance and preserve the drug.

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Conflict of Interest
None declared.

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