ORIGINAL ARTICLE

cfr and *optr*A gene based characterization of linezolid resistant *Staphylococcus haemolyticus* in a tertiary care hospital in western Uttar Pradesh, India

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Abstract

Background: Staphylococcus haemolyticus is a clinically significant Coagulase Negative *Staphylococci* (CoNS) accountable for various infections and is often multi-drug resistant along with resistance to linezolid. *Aim and Objectives:* This study was carried out to detect the presence of *cfr* and *optr*A gene in clinical isolates of linezolid resistant *S. haemolyticus* in a tertiary care hospital. *Material and Methods:* A total of 227 clinical isolates of *S. haemolyticus* recovered from various clinical samples were tested to determine susceptibility to linezolid by phenotypic method. Further, the presence of *cfr* & *optr*A gene was looked for, in clinical isolates of *S. haemolyticus*. *Results: S. haemolyticus* was isolated predominantly from inpatient department mainly from blood culture (85%). Linezolid resistance was confirmed in 9.2% isolates of *S. haemolyticus* phenotypically and they were also methicillin resistant. On genotypic characterization, *cfr* gene could be detected in 7.04% the isolates. However, all the isolates were negative for *optr*A gene. *Conclusion:* Emergence of resistance to linezolid in *S. haemolyticus* was reported for the first time. The presence of *cfr* gene is the most common mechanism of resistance to linezolid. Correct identification of these isolates and adherence to strict infection control protocols will help in better clinical outcomes.

Keywords: Coagulase negative *Staphylococci*, *Staphylococcus haemolyticus,* Linezolid Resistance, *cfr*, optrA gene

Introduction

Coagulase negative *Staphylococci* (CoNS) are normal commensal of the skin and mucous membranes and have emerged as the important cause of hospital acquired infection [1-2]. CoNS are common cause of hospital mortality and morbidity world-wide. *Staphylococcus haemolyticus* is a clinically significant CoNS species accountable for various infections and is often Multi-Drug Resistant (MDR) [3]. Linezolid Resistant *S. haemolyticus* (LRSH) was first reported by Rodriguez-Aranda *et al*. in 2009 [4] and thereafter by other workers worldwide [5]. Linezolid was granted a license for clinical use in China in the year 2007. Since then, linezolid resistant Methicillin-Resistant *S. aureus* (MRSA) and CoNS have emerged in China [6-7]. Treatment of infections due to CoNS has become a challenge for clinicians.

Dissemination of drug resistance among these strains has left few options left for management of infections caused by them. Acquired drug resistance associated with methicillin resistance is a major problem not only in hospital but also in community acquired infection [8].

Linezolid is the first drug and the only oxazolidinone antibiotic for clinical use. Emergence of resistance to linezolid is on the rise and is a cause of concern in antimicrobial chemotherapy [9]. The first case of linezolid-resistant *Staphylococci*

appeared within one year after linezolid was approved for use in treatment [10]. However, the first report of linezolid resistance in CoNS was reported by Peer *et al.* in 2011 from India [11]. The *cfr* (chloramphenicol-florfenicol resistance) gene is not only responsible for resistance to oxazolidinones but also mediates cross- resistance to other antibiotics, such as phenicols and lincosamides. Recently, the linezolid resistance was also associated with the novel transferable oxazolidinone resistance gene, namely *optr*A (oxazolidinone phenicol resistance) which was first identified in enterococci and later also found in a single species *S. sciuri* [12]. Recently there has been an emergence of linezolid resistance in clinical isolates of *S. haemolyticus* which is a matter of concern. Both *cfr* and *optr*A genes play decisive role in linezolid resistance. Lack of data from this geographical area prompted us to carry out this study to detect the presence of *cfr* and *optr*A gene in *S. haemolyticus* isolated from various clinical samples in a tertiary care hospital.

Material and Methods

Study design

This cross-sectional study was conducted in the Department of Microbiology, Santosh Medical College and Hospital, Ghaziabad and Subharti Medical and Hospital, Meerut for a period of one year. This study was approved by University Ethics Committee, SMC (Ref. No. SMC/UECM/2022.155). All the paired blood samples were collected by the trained phlebotomists and all other clinical samples were collected following standard precautions as per inclusion and exclusion criteria.

Inclusion Criteria: Patient of all age groups and genders were included with clinically significant isolates of *S. haemolyticus* recovered from various clinical samples received in Microbiology Laboratory.

Exclusion Criteria: All other bacteria except *S. haemolyticus* isolated from various clinical samples were excluded along with patients with history of antimicrobial therapy.

Sample processing

Clinical samples received in Microbiology Laboratory from various Inpatient Units (IPD) and Outpatients Departments (OPD) of the Hospital were processed as per standard bacteriological technique [13] to isolate *S. haemolyticus*. The samples were collected as per the clinical condition. Specimen like blood, pus, urine, E.T (endotracheal tube) aspirate, tissue and ascitic fluid were cultured on blood agar, nutrient agar, and MacConkey agar plates. All agar plates were incubated for 24-48 hours at 37ºC. Out of the total 522 isolates of CoNS, 227 were confirmed as clinically significant *S. haemolyticus* because majority of our isolates were recovered from paired blood samples plus in other samples, the findings could be correlated with clinical condition of the patients. The isolates were identified using GP ID 628 card followed by antibiotics susceptibility test using GP AST cards in Vitek 2 compact system (Biomerieux France) to determine Minimum Inhibitory Concentration (MIC) of linezolid. MIC of ⩾ 8 µg/ml was taken as resistant for linezolid. The aliquots of isolates of *S. haemolyticus* were stored at – 80ºC till further molecular testing was performed.

Deoxyribonucleic Acid (DNA) extraction

Presence of *cfr* and *optr*A gene was looked for in the clinical isolates of *S. haemolyticus* through DNA extraction. The DNA was extracted using Trueprep auto Kit (Molbio Diagnostic Pvt.Ltd ®) as per manufacture's instruction.

cfr and _optr_A gene amplification using Polymerase Chain Reaction (PCR)

Specific primers were used for final confirmation of *cfr* and *optr*A gene which were detected using 25µg template DNA using two specific primers set, one forward '5 TGA AGT ATA AAG CAG GTT GGG AGT CA 3' and other reverse '5 ACC ATA TAA TTG ACC ACA AGC AGC 3' for *cfr* gene and another nucleotide sequence of primer set, one forward '5 AGG TGG TCA GCG AAC TAA 3' and other reverse 5' ATC AAC TGT TCC CAT TCA 3' for *optr*A gene [14, 6]. The PCR master mix kit and primer were purchased from GeNei Laboratories Private Limited®, Bengaluru. Briefly, to amplify *cfr* and *optr*A gene, the initial denaturation was done at 94ºC for 1 minute; annealing at 48ºC for 2 minutes; and final extension at 72ºC for 7 minutes; successively for 30 cycles. The PCR condition for *optr*A gene was similar to that of *cfr* gene except for the annealing temperature which was at 55ºC for 2 minutes. Finally, the PCR product corresponding to 746-bp for *cfr* gene and 1395 bp for *optr*A gene was detected using 1% agarose gel electrophoresis which was stained with ethidium bromide and visualized under ultra-trans illumination light.

Statistical analysis

The data analysis was done using the software STATA MP-17 and the Statistical Package for Social Sciences (SPSS). Proportion of linezolid resistance was calculated and presented as frequencies (%). The association between two categorical variables was analyzed by the Chi-squared test. At 95% confidence level, the value of *p* less than 0.05 was regarded as statistically significant.

Results

S. haemolyticus was isolated predominantly from IPD patients mainly from paired blood culture 193 (85%) followed by urine 12 (5.2%) and pus 11 (4.8%) (Table 1). The clinical isolates of *S. haemolyticus* were predominantly methicillin resistant (94%) showing complete resistance to penicillin and clindamycin (100% each) followed by high level of resistance against erythromycin (92%). Slightly lower level of resistance was observed toward ciprofloxacin (49.3%) followed by gentamicin (47.1%), tetracycline (33%) and moxifloxacin (24%). However, all our isolates were sensitive to vancomycin (MIC $\leqslant$ 2µg/ml) and nitrofurantoin (100% each) (Table 2).

Overall, linezolid resistance was seen in 21 (9.2%) of *S. haemolyticus* (MIC $\geqslant$ 8µg/ml) phenotypically. All the LRSH were also methicillin resistant (MRSH) (*p* value- 0.7430) (Table 3). However, the gene could be detected only in 16 (7.6%) of isolates of *S. haemolyticus* (Table 4). These LRSH and MRSH were isolated predominantly from blood culture (95.23%) followed by pus (4.7%) (Table 5) and predominantly from patients admitted in ICUs 19 (90.4 %) followed by those admitted in surgery ward 2 (9.5%).

Detection of _cfr_ and _optr_A gene

A total of 16/227 (7.04%) isolates of LRSH were positive for *cfr* gene at 746 bp [Figure 1]. However, 5 isolates of LRSH identified phenotypically, did not show the presence of *cfr* gene. None of the clinically significant isolates of LRSH harbored *optr*A gene. The *cfr* gene was also not detected in Linezolid Sensitive *S. haemolyticus* (LSSH) tested.

Table 1: Distribution of *S. haemolyticus* in various clinical samples (n=227)

Samples	Number (Percentage)
Blood	193 (85)
Urine	12 (5.2)
Pus	11 (4.8)
E.T. Aspirate	08 (3.5)
Tissue	02 (0.88)
Ascitic fluid	01 (0.44)

Table 2: Antibiotic susceptibility pattern of *S. haemolyticus* (n = 227)

Antibiotics	Number (Percentage)	Number (Percentage)
Penicillin	0 (0.0)	227 (100)
Erythromycin	18 (7.9)	209 (92)
Clindamycin	0 (0.0)	227 (100)
Ciprofloxacin	115 (50.6)	112 (49.3)
Co-trimoxazole	174 (76.6)	53 (23.4)
Tetracycline	152 (66.9)	75 (33)
Moxifloxacin	172 (75.7)	55 (24.2)
Nitrofurantoin	227 (100)	NA (0.0)
Chloramphenicol	177 (77.9)	50 (22)
Gentamycin	120 (52.8)	107 (47.1)
Vancomycin	227 (100)	0 (0.0)
Linezolid	206 (90.7)	21 (9.2)

Table 3: Correlation between LRSH and MRSH (n = 227)

	LRSH*	LSSH**	Chi-square	*p*
MRSH# (n= 214)	21 (9.2%)	193 (90%)	0.1075	0.7430
MSSH## (n=13)	0 (0%)	13 (100%)	0.0194	0.8891

*LRSH- Linezolid resistant *S.haemolyticus,* **LSSH= Linezolid sensitive *S. haemolyticus,* #MRSH-Methicillin resistant *S.haemolyticus* ##MSSH-methicillin sensitive *S.haemolyticus*

Table 4: Comparative analysis between phenotypic and genotypic detection of *S. haemolyticus* (n= 227)

LRSH	Number (Percentage)	Number (Percentage)
Positive	21 (9.2)	16 (7.04)
Negative	206 (90.7)	211 (92.9)

Table 5: Sample wise distribution of LRSH and MRSH (n=21)

Samples	Number (Percentage)	Number (Percentage)
Blood	20 (95.23)	20 (95.23)
Pus	1 (4.7)	1 (4.7)

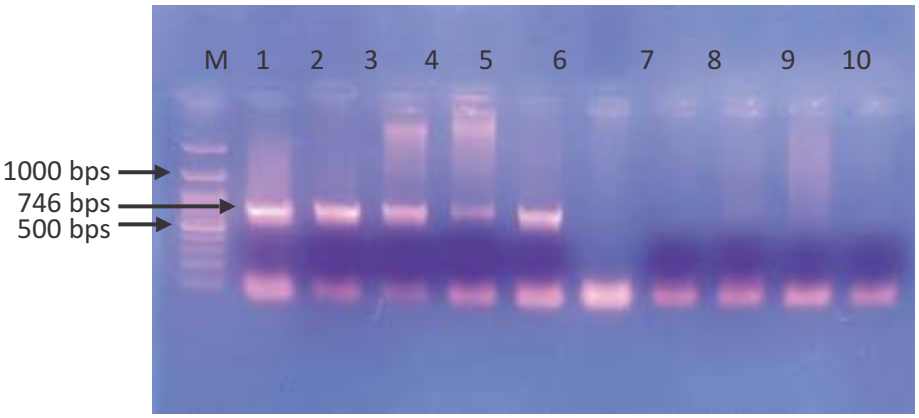

Figure 1: Agarose gel electrophoresis showing *cfr* gene amplification. M is the 100 bp molecular marker. Lane-1, 2, 3, 4, 5 isolates are positive for *cfr* gene (746 bps). Lane 6, 7, 8, 9 & 10 isolates are negative for *cfr* gene.

Discussion

CoNS species are common cause of hospital mortality and morbidity worldwide and frequently infect immunocompromised patients who have prostheses and other invasive devices in place. *S. haemolyticus* is a clinically significant CoNS species accountable for various infections and has emerged as a significant nosocomial pathogen because it is often multidrug resistant and has the ability to attain high level of resistance to many antibiotics including linezolid and glycopeptides [15].

Linezolid, the first class oxazolidinone agent is an effective treatment option for infection caused by Gram-positive organisms [16]. Nevertheless, the emerging resistance to linezolid in *S. haemolyticus* and other CoNS species is worrisome. The resistance to linezolid is usually due to misuse and/or overuse of the drug [17].

This is the first study carried out in this geographical area which has investigated for the presence of linezolid resistance in clinically significant isolates of *S. haemolyticus*. Overall, resistance to linezolid in CoNS ranges from as low as 0% to as high as 16% in India [10] and abroad [18]. We reported linezolid resistance in 9.2% of our clinical isolates of *S. haemolyticus* which is higher as compared to the data from the global surveillance studies report of 2%. Such high level of resistance to linezolid is an alarm for the judicious use of this drug. Gupta *et al.,* [19] and Matlani *et al*., [20] have reported mucoid strain of *S. haemolyticus* showing resistance to linezolid from India but they have not mentioned the exact rate for comparison with our study.

To the best of our knowledge this is the first study reported from this geographical area of Western Uttar Pradesh which has looked for the presence of *cfr* and *optr*A gene in phenotypically confirmed LRSH. Our finding showed presence of *cfr* gene in 7.04% of isolates. Studies by Cui *et al*., [21] and Cai *et al*., [7] have also described presence of c*fr* gene in isolates of *S. haemolyticus* from clinical samples however; these studies have not mentioned the percentage of isolates in which the gene was present. Therefore, it is difficult to compare our finding with that of others. Overall presence of gene in such high number of isolates is a matter of therapeutic concern because linezolid is one of the high-end antibiotics reserved for treatment of Gram positives besides vancomycin.

However, all our phenotypically confirmed isolates of LRSH were negative for *optr*A gene. Moreover, both the *cfr* and *optr*A genes were also not detected in LSSH isolates tested. The *cfr* gene is well established gene responsible for linezolid resistance and has been studied worldwide. However, studies showing association of *optr*A gene with linezolid resistance is limited and only few Indian studies documented in literature highlight the genetic basis of resistance.

In our study, both *cfr* and *optr*A gene were not detected in 5/21(23.8%) phenotypically confirmed isolates of LRSH. The reason for this absence of gene may be because of other mechanisms or gene responsible for resistance. This bacteriostatic antibiotic block 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 23S rRNA by a methyl transferase encoded by the gene *cfr* [22]. Co-occurrence of *cfr* mediated resistance and mutational resistance has also been documented [9]. The various other genes such as *pox*A gene and

other mutations as mentioned were not looked for due to lack of resources which is the limitation of our study.

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. Resistance mediated by *cfr* gene is of great concern as it is usually plasmid or transposon borne and can be disseminated to susceptible population [23]. *cfr* gene also encodes resistance to a group of chemically distinct antibiotic: phenicols, lincosamides, pleuromutilins and streptogramin A [24]. There have been few reports of linezolid resistant *Staphylococci* from India [19, 25]. Linezolid has an acceptable safety profile for both intravenous and oral administration and has proven to be effective in the treatment of infections due to methicillin resistant *Staphylococcus* in critically ill patients [26], though linezolid therapy is associated with side effects such as pain in abdomen and hematological adverse events. Linezolid is also a good therapeutic option in patients with diabetes in case of failure of treatment with vancomycin. Thus linezolid is a promising therapeutic option in the era of rapidly growing antibiotic resistance due to its cost effectiveness and comparatively lower side effects [27].

The frequency of isolation of LRSH is especially in India is underestimated due to limited reports. The increase in infections with MDR *S. haemolyticus* is likely to become a serious problem in the future because of limited alternative therapeutic options. Thus understanding the epidemiologic profile of linezolid resistance is crucial to controlling this emerging problem. Continuous surveillance of antimicrobial susceptibility pattern is necessary to control the emergence of these MDR strains.

The presence of *cfr* gene is the most common mechanism of resistance to linezolid. Correct identification of these isolates, judicious use of linezolid and stringent infection control measures are important to prevent the horizontal spread of these *cfr* carrying strains in nosocomial environment. It is important to keep a close monitoring to track resistance to linezolid particularly when frequent and extended linezolid therapy is prescribed. Paucity of newer antimicrobials demands judicious use of linezolid. This study highlights the importance of continuous monitoring of linezolid resistance in staphylococci.

Conclusion

We report emergence of resistance to linezolid in clinical isolates of *S. haemolyticus* for the first time from Western Uttar Pradesh. All our clinical isolates of LRSH were methicillin resistant and also MDR and moreover all of them were recovered from indoor patient, thus identification, treatment and management of such resistant pathogen especially in a hospital setting is of utmost importance because such resistant strains may spread horizontally into various inpatients units in the hospital and will be a nosocomial threat if proper infection control measures are not followed.

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