Research article

Molecular characterization of linezolid resistance in clinically significant isolates of coagulase negative Staphylococcus species, a hospital based study from Western Uttar Pradesh

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ABSTRACT

Introduction and aim: The emergence of Multidrug-resistant Coagulase-negative *Staphylococcus* species is a therapeutic problem. Linezolid-resistant CoNS (LRCoNS) are on a significant rise, with a global prevalence of around 2%. The acquisition of *cfr* (chloramphenicol-florfenicol resistance) gene is the commonest mechanism leading to resistance. This study aimed to determine the molecular characterization of linezolid resistance in clinically significant isolates of Coagulase Negative Staphylococci (CoNS).

Material and Methods: The 1061 clinical isolates of CoNS were identified by standard bacteriological technique. Antibiotic sensitivity test was performed to determine the susceptibility to linezolid and those isolates with zone diameter ≤ 20 mm (linezolid screen positive) were further confirmed by the automated Vitek 2 compact system and MIC ≥ 8 μg/ml was resistant . The *cfr* gene was detected in phenotypically confirmed LRCoNS.

Results: Resistant to Linezolid was seen in 2.5% of clinically important isolates of Coagulase Negative Staphylococci. The resistance was maximum in *S.hemolyticus* (70%), followed by *S.cohnii* (22.2%). Among the LRCoNS, the overall cfr gene was detected in 78% of isolates, predominantly in *S.hemolyticus* and *S. cohnii*. All the LRCoNs were also MRCoNS (methicillin resistance). However, all isolates were susceptible to glycopeptides.

Conclusion: There has been a surge of CoNS being reported from clinical samples with resistance to many important antimicrobials, including linezolid. The presence of *cfr* gene is the most common mechanism of resistance to linezolid. Early and correct identification of these isolates and adherence to infection control protocols will help for better clinical outcomes.

Keywords: Linezolid resistance; CoNS; *cfr* gene; molecular characterization.

INTRODUCTION

Genus Staphylococcus are Gram-positive cocci that are broadly divided into a group that produces coagulase (*Staphylococcus aureus*) and those that do not produce coagulase (Coagulase negative *Staphylococci*) (CoNS) (1). CoNS were earlier considered to be contaminants having little clinical significance. But in recent years, these organisms have been identified as harbouring significant pathogenic potential (2). There are numerous recognized species of CoNS; to name of few like *S.epidermidis, S.hemolyticus, S.cohnii, S.lugdunensis* etc., which are among the most common commensal flora of skin and mucous membranes.

They are significant opportunistic microorganisms and have been isolated from various human infections like bacteremia, purulent wounds, and UTIs etc., (3) Increased use of implants and devices, more elderly and sick patients, and more people with compromised immune systems has led to rising significance of CoNS (4). Isolation of multi-drug resistance (MDR) CoNS in a healthcare setting is a therapeutic concern. Recently several new antimicrobials with good activity against these MDR CoNS have been introduced into clinical practices such as linezolid, tigecycline, glycopeptides, and daptomycin (5).

Linezolid got therapeutic approval for clinical use in the U.S in 2000. (6) As it is the first agent of the oxazolidinone class, linezolid is an important antimicrobial agent to stay active against MDR Gram-positive cocci (7). It is an effective agent in treating complicated clinical conditions like bloodstream infections, skin soft tissue infections, etc. and it acts by inhibiting bacterial growth via inhibition of protein synthesis (8).

Linezolid resistance (LR) has been reported in *S.aureus* as well as in CoNS, and the first *S. hemolyticus* strain resistant to linezolid was reported in 2009 (9). The most common mechanism involved in LR is a naturally occurring resistant gene, *cfr* gene (chloramphenicol-florphenicol resistance), encoding a cfr methyltransferase that catalyzes methylation in 23S rRNA (10). Mutation in L3, L4, and L22 ribosomal proteins and transferable oxazolidinone resistance gene, *optr*A, discovered in a bovine *Staphylococcus sciuri* strain are other mechanisms associated with LR (11-12). Among all the associated mechanisms of LR, the resistance due to *cfr* gene is of therapeutic importance because it is generally

plasmid-mediated and spreads from one person to another (8). Limited study on molecular characterization of clinically significant LRCoNS from Western Uttar Pradesh prompted us to carry out this study.

MATERIALS AND METHODS

A hospital-based study from Western Uttar Pradesh

This hospital based prospective study was carried out for a period of two years. Approval from the Ethics Committee of Swami Vivekanand Subharti University, Meerut, was taken before starting this study. The samples were received in the microbiology laboratory from patients admitted in various inpatient units (IPD) or outdoors (OPD). In all 1061 isolates of CoNS were presumptively identified by standard bacteriological technique (13). Further, the species was identified using GP ID p628 card by Vitek 2 (Biomeriux, France). However, other bacterial pathogens isolated besides CoNS were not-included from this study.

Antimicrobial susceptibility testing (AST)

AST was done in accordance with the CLSI recommendations by Kirby Bauer method on Muller Hinton agar (MHA) plates using commercially available antibiotic discs from Hi media (14). Susceptibility to linezolid was interpreted using a disc (30µg) and marked resistant if the zone diameter is ≤20mm. The screen-positive isolates were further confirmed by automated Vitek 2 using GPAST cards (Biomeriux, France) to determine the MIC for linezolid and value of ≥ 8 µg/ml was considered as resistant. The *S.aureus* ATCC 25923 was taken for quality control. Further DNA extraction was done for isolates that were phenotypically confirmed as LRCoNS and 10% linezolid sensitive isolates (for quality control) to detect *cfr* gene.

DNA extraction

The DNA was extracted using Trueprep auto kit (MolbioDiagnostics Pvt.Ltd) (15).

Polymerase Chain Reaction (PCR)

Species-specific primers were used for final confirmation. Briefly, conventional PCR detecting *cfr* gene was carried out using 25µl of reaction volume, which included 2µg template DNA with a 23 µl of master mix to which two specific primer sets, forward 5′-TGAAGTATAAAGCAGGTTGGGAGTCA-3′ and reverse 5′-ACCATATAATTGACCACAAGCAGC-3′ was added. To amplify *cfr* gene, the initial denaturation was done at 94°C for 3 min; 94 °C for 15s, 55° C for 15s, and 72°C for 1 min successive 30 cycles. The final extension was done at 72°C for 5 min. The PCR product corresponding to 850 bp was confirmed by gel electrophoresis using 1.2% agarose gel; stained with ethidium bromide, which was visualized under ultraviolet trans- illumination. An A100-bp DNA ladder was used as a standard molecular weight marker (12).

RESULTS

Overall resistance to linezolid was seen in 27 (2.5%) isolates of CoNS. The majority of these isolates 16 (59.2%), were from indoor patients, predominantly from ICUs. (Fig.1) The LRCoNS species were most frequently isolated from individuals in the extreme of ages, i.e., in <10 and > 61 years (22.2% for each) and predominantly in males (70%), and the male: female ratio was 2.3:1. The LRCoNS were isolated mainly from blood 20 (74%), pus sample 5 (18.51%) (Table 1). Among the CoNS*;* highest isolation frequency was of *S.hemolyticus* 19 (70.4%) in our hospital then *S.cohnii* 6(22.2%) *S. lugdunensis* 1 (3.70%) and *S.xylosus* 1 (3.70%) (Table 2).

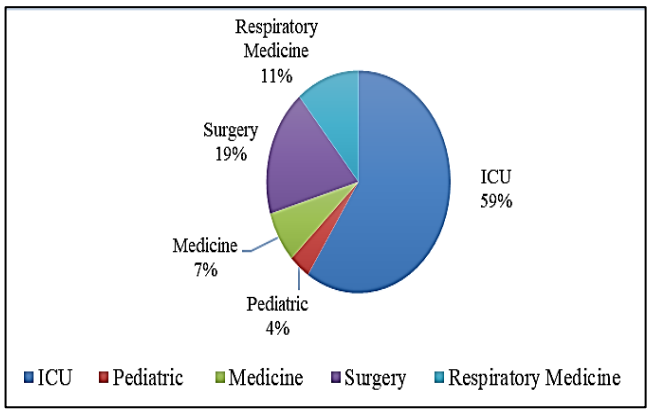

Fig. 1 Unit-wise distribution of LRCoNS

Table 1: Distribution of LRCoNS in various clinical samples (n=27)

S. No.	Sample	Number (n=27)	Percentage %
1.	Blood	20	74
2.	Pus	5	18.51
3.	CSF	1	3.70
4.	Umbilical line	1	3.70

Table 2: Species-wise distribution of LRCoNS (n=27)

S. No.	Species identified	Number (n=27)	Percentage %
1.	*S. haemolyticus*	19	70.4
2.	*S. cohnii*	6	22.2
3.	*S. lugdenensis*	1	3.7
4.	*S. xylosus*	1	3.7

All the isolates of LRCoNS were also MRCoNS (methicillin-resistant CoNS; Table 3). The LRCoNS were multi-drug resistant with complete resistance to penicillin, erythromycin, clindamycin, ciprofloxacin, and cotrimoxazole. Reduced susceptibility was seen towards moxifloxacin, gentamicin, and tetracycline. However, glycopeptides were effective against all the isolates of LRCoNS (Table 4).

Table 3: Relationship between LRCoNS and MRCoNS

	LRCoNS*	**LSCoNS****
MRCoNS# (n= 964)	27	937
MSCoNS##(n=97)	00	97

*LRCoNS -Linezolid resistant CoNS **LSCoNS - Linezolid sensitive
CoNS# MR CoNS – Methicillin-resistant CoNS ## MSCoNS Methicillin sensitive CoNS

Table 4: Susceptibility pattern of isolates of LRCoNS to other antibiotics (n=27)

Species	**P**	**E**	**CD**	**CIP**	**COT**	**TE**	**MO**	**C**	**GEN**	**VA**	**LZ**	**MR-CoNS**
S.haemolyticus (n=19)	100	100	100	100	100	27.7	88.8	89.4	84.2	0	100	100
S. cohnii (n=06)	100	100	100	100	100	0	83.3	83.3	16.6	0	100	100
S.lugdenensis (n=1)	100	100	100	100	100	0	100	100	100	0	100	100
S. xylosus (n=1)	100	100	100	100	100	0	100	100	100	0	100	100

P-Penicillin, E-Erythromycin, CD-Clindamycin, Cot-Cotrimoxazole, Te-Tetracycline, Mo-moxifloxacin, C-Chloramphenicol, Gen-Gentamicin, Va-Vancomycin, Lz-Linezolid

Linezolid resistance determinants (*cfr* gene)

On genotypic characterization, *cfr* gene with amplification of 850 bp was seen in 21 (77.8%) isolates. The species-wise distribution of LRCoNS in which *cfr* gene was detected is shown in Table 5. However, the six clinically significant isolates of LRCoNS identified phenotypically did not show the presence of *cfr* gene (Figs. 2 & 3). The reason for this resistance may be due to some other mechanism like the presence of optraA gene or mutation in the ribosomal proteins (16). The *cfr* gene was also not detected in the entire 10% linezolid-sensitive CoNS tested.

Table 5: Species-wise distribution of clinically significant LRCoNS isolates in which *cfr* gene was detected (n=27)

S.No.	**Species**	***cfr* gene Positive (%)**	***cfr* gene Negative (%)**
1.	*S. haemolyticus*(n=19)	15 (78.94%)	4(21.05%)
2.	*S. cohnii*(n=6)	5 (83.33%)	16.66%
3.	*S. lugdenensis*(n=1)	0	1(100%)
4.	*S. xylosus*(n=1)	1(100%)	0 ()

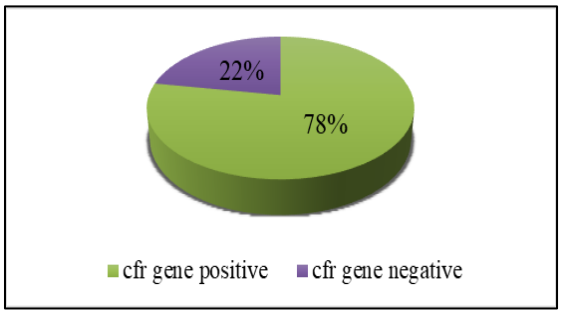

Fig. 2: Distribution of isolates of LRCoNS in which *cfr* gene was detected (n=27)

Fig. 3: Agarose gel electrophoresis showing *cfr* gene amplification. M: 100bp molecular marker;
N: Negative control Lanes 3,5,6: Isolates positive for *cfr* gene; Lanes 1,2,4: Isolates negative for *cfr* gene

DISCUSSION

MDRCoNS has been reported in recent studies from India (17). If resistance develops to linezolid and vancomycin, which are the reserve drugs for such MDR organisms , we are left with no therapeutic choice. We found linezolid resistance in 2.5 % of clinical isolates of CoNS, which is comparable to the 2% found in the data from the global surveillance studies (11). Most of these LRCoNS (59.2%) were found in ICUs, primarily in cases of bloodstream infections. Similar results have recently been reported from India (18). High patients usually come to us or are referred to us after seeking medical advice from local doctors and taking multiple or incomplete courses of antibiotics, which might have resulted in a high isolation rate. Our centre is a tertiary care hospital.

Our study showed that the isolation was predominantly in males at extreme ages. This might be because people in this age group are more susceptible to infection due to weakened immune systems and sex-dependent genetic variables. Similar finding has been reported in a recent study by Nepal *et al.*,(19). *S.hemolyticus* (70.4%) and *S. cohnii* (22.2%) were the most resistant phenotypes in our hospital. Similarly, different researchers from India have documented species-specific susceptibility to linezolid; Kalawat *et al*., (20) reported LR in *S. lugdunensis* and *S.hominis*, Gupta *et al*., (21) in *S.hemolyticus* and Matlani *et al*., (22) reported LR *S.hemolyticus.* Resistance to reserve drugs like linezolid is a matter of therapeutic concern.

Linezolid which comes in both oral and parenteral formulations is one of the few treatments proven to be used as successful against MDR Staphylococcal infections. According to studies, LR in Staphylococci has developed due to prolonged drug exposure thus stressing the judicious use of reserve drugs in everyday practice. LR has been associated with the acquisition of a transmissible *cfr* ribosomal methyl-transferase gene or mutations in 23S rRNA (23). Although linezolid mutational resistance is problematic, acquiring the *cfr* gene is more concerning due to its quick dissemination. Studies have shown that the main mechanism of acquiring resistance against linezolid is due to presence of *cfr* gene. However, on the contrary to the above findings, Chamon *et al*.,(24) from Brazil reported mutation in 23S rRNA as the principal resistance mechanism in clinical isolates of LRCoNS and they were negative for *cfr* gene.

In our study, *cfr* gene could be detected only in (78 %) of isolates of CoNS which were phenotypically linezolid resistant. Among them, *cfr* gene was detected predominantly in isolates of *S.hemolyticus* (79%). Similarly high (92.4%) rate has been reported by Manoharan *et al*., (25). However, in 22.2% of phenotypically confirmed isolates in the present study, *cfr* gene could not be detected. The reason may be due to mutations or other genes (11).

Although presence of *cfr* gene is the most significant factor for LR, many studies have shown presence of *cfr* gene even in linezolid-susceptible isolates of CoNS. Thus, only the sole responsibility of *cfr* gene for LR is questionable, and maybe in these isolates, the expression of *cfr* gene may have been blocked by as yet unidentified factors (25). However, we did not detect the presence of *cfr* gene in the linezolid-sensitive isolates tested.

In this era of rising antimicrobial resistance, the detection of the most common mechanism behind the resistance as well as various methods to curtail the rise of these superbugs holds equal importance. The paucity of newer antimicrobials and strict implementation of antibiotic policy under the umbrella of antimicrobial stewardship is the need in this era of desperate antibiotic usage. Hence, judicious use of high-end antibiotics like linezolid should be the aim.

Limitations

Due to a lack of resources 1) the mutations in *optra*A gene was not done both in linezolid resistant and in few linezolid sensitive isolates 2) mutation was also not detected in *cfr* gene negative isolates.

CONCLUSION

Clinically significant LRCoNS is a therapeutic problem. The presence of *cfr* gene is the most common mechanism causing transferable plasmid-mediated resistance to linezolid. Early and correct identification of these isolates and adherence to stringent infection control protocol is essential.

CONFLICT OF INTEREST

The authors have no conflicts of interest.

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