Research Article

Molecular characterization of linezolid resistant *Staphylococcus epidermidis* from a tertiary care hospital.

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ABSTRACT

Introduction and Aim: *Staphylococcus* species are important bacterial pathogens implicated in invasive infections. *Staphylococcus aureus* and coagulase negative staphylococci including *Staphylococcus epidermidis* have been considered as multi drug resistant and emergence of linezolid resistance in staphylococci has been reported. The molecular characterization of linezolid resistance in *Staphylococcus epidermidis* isolated from clinical specimens of blood culture was planned in this study by targeting *cfr* and *optr*A genes associated with linezolid resistance.

Materials and Methods: A total of 120 clinical isolates of *Staphylococcus epidermidis* recovered from blood culture samples were tested for linezolid susceptibility by phenotypic methods including disk diffusion and minimum inhibitory concentration. Further, molecular characterization of linezolid resistance in phenotypically confirmed linezolid resistant *Staphylococcus epidermidis* was performed by detecting *cfr* and *optr*A genes by polymerase chain reaction.

Results: Out of total 120 isolates of *Staphylococcus epidermidis,* 3.3% were found linezolid resistant by phenotypic methods. On molecular testing, *cfr* gene was detected in 2.5% of *Staphylococcus epidermidis* isolates. However, all the isolates were negative for *optr*A gene.

Conclusion: The emergence of linezolid resistance in *Staphylococcus epidermidis* is a serious concern. The molecular characterization targeting *cfr* gene can serve as a rapid and reliable method for confirmation of linezolid resistance to guide antimicrobial treatment.

Keywords: *Staphylococcus epidermidis,* Coagulase negative *staphylococci*, Linezolid resistance, *cfr* gene, *optrA* gene

1. INTRODUCTION

Among staphylococci, *Staphylococcus aureus* is well established pathogen while coagulase negative staphylococci (CoNS) are usually considered as commensals on skin and mucous membrane. However, CoNS have been recognized as important agent of human disease especially causing opportunistic infections including blood stream infections (BSI) and other invasive and non-invasive conditions [1,2]. The most frequently isolated species of CoNS from human blood cultures are *Staphylococcus haemolyticus* and *Staphylococcus epidermidis* [2]. *Staphylococcus epidermidis* is crucial for the preservation of local homeostasis through regulation of the composition of skin microflora of the host [3,4]. The capability of *Staphylococcus epidermidis* to develop resistance to various antibiotics complicated the treatment of these infections. A significant proportion of *Staphylococcus epidermidis* strains has been identified as methicillin-resistant, known as methicillin-resistant *Staphylococcus epidermidis* (MRSE) especially in the hospital environments [5]. Currently, over 70% of healthcare-associated strains of *Staphylococcus epidermidis* are resistant to methicillin, limiting the available treatment options for effective

management of the clinical conditions [6]. Linezolid is an oxazolidinone antibiotic used to treat infections caused by multidrug resistant (MDR) Gram-positive cocci (GPC) including methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Staphylococcus epidermidis* [7]. The linezolid is a high-end antibiotic belonging to the oxazolidinone group and considered as a reserve drug [8]. The first reported case of linezolid resistant staphylococci was identified within one year after clinical approval of linezolid for treatment [9]. The first report from India on linezolid resistance was published in 2011 from clinical cases of sepsis from Kashmir [10]. The emergence of resistance to linezolid among CoNS has emerged as a significant therapeutic concern pertaining to the limitations of the treatment options against CoNS including *Staphylococcus epidermidis*. The chloramphenicol-florfenicol resistance (*cfr*) gene is mobile genetic element which is found responsible for resistance to oxazolidinones as well as mediator of cross-resistance to other antibiotics including phenicols and lincosamides. The oxazolidinone phenicol resistance (*optr*A) gene is another novel transferable oxazolidinone resistance genetic element has also been linked to linezolid resistance, which was first detected among enterococci and later on also identified in *Staphylococcus sciuri* as a mediator of linezolid resistance [11]. Recently, the emergence of linezolid resistance in clinical isolates of *Staphylococcus epidermidis* has become a significant concern due to transferable nature of the genes responsible. As the *cfr* and *optrA* genes play an important role in mediating linezolid resistance, this study was planned for molecular characterization of linezolid resistance in *Staphylococcus epidermidis* targeting *cfr* and *optrA* genes.

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2. MATERIALS & METHODS

This cross-sectional study was conducted in a tertiary care medical teaching hospital from North India for a period of one year from January 2023 to December 2023. This study was approved by university ethics committee with reference number SU/2022/175 [1] dated 05/08/2022. The paired blood samples were collected by the trained phlebotomists following standard aseptic precautions as per inclusion and exclusion criteria.

Inclusion criteria: The paired blood culture samples from the patients of all age groups and genders suspected of having blood stream infections and clinically significant isolates of *Staphylococcus epidermidis* recovered from both the blood culture samples were included in the study.

Exclusion criteria: The blood cultures showing the growth of pathogens other than *Staphylococcus epidermidis* and the samples received from the patients with history of antibiotic treatment prior to sample collection were excluded from the study.

Sample processing:

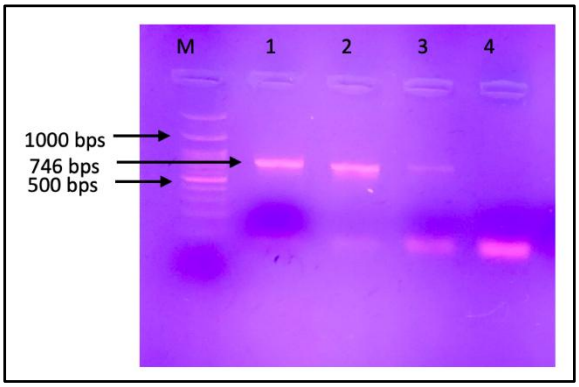
The paired blood culture samples collected in designated blood culture bottles for adult and pediatric age groups as recommended by bioMerieux, France were incubated in BacT/ Alert 3D automated blood culture system (bioMerieux, France). The broths from flagged positive blood culture bottles were processed as per standard bacteriological technique by subculturing the broth on blood agar, chocolate agar and MacConkey agar plates. The agar plates were incubated for 24-48 hours at 37°C aerobically. The growth on agar plates was further processed for preliminary identification by conventional techniques including Gram staining. The species level identification and antibiotic sensitivity testing of presumptively identified isolates of staphylococci was done by VITEK-2 compact automated system (bioMerieux, France) using GP test card and AST P628 card respectively. The staphylococcal isolates identified as *Staphylococcus epidermidis* were further subjected to PCR for molecular characterization of linezolid resistance by targeting *cfr* and *optrA* genes.

Deoxyribonucleic acid (DNA) extraction:

The DNA of *Staphylococcus epidermidis* was extracted using the Trueprep Auto Kit (Molbio Diagnostic Pvt. Ltd. India) following the manufacturer's instructions.

***cfr* and *optrA* gene amplification using polymerase Chain Reaction (PCR):**

Specific primers were used for the final confirmation of the *cfr* and *optrA* genes, detected using 25 µg of template DNA with two specific primer sets. For the *cfr* gene, the forward primer was '5'-TGA AGT ATA AAG CAG GTT GGG AGT CA-3' and the reverse primer was '5'-ACC ATA TAA TTG ACC ACA AGC AGC-3'. Similarly, for the *optrA* gene, the forward primer was '5'-AGG TGG TCA GCG AAC TAA-3' and the reverse primer was '5'-ATC AAC TGT TCC CAT TCA-3' [12,13]. The PCR master mix kit and primers were purchased from Bangalore Genei Private Limited, India. The procedural steps were followed as per manufacturer's instructions. To briefly summarize, the *cfr* gene was amplified by initial denaturation at 94ºC for one minute, annealing at 48ºC for two minutes, and final extension at 72ºC for seven minutes repeated for 30 cycles. The PCR conditions for the *optrA* gene were similar to those for the *cfr* gene, except the annealing temperature which was set at 55ºC for two minutes. The PCR products, corresponding to 746 bp for the *cfr* gene and 1395 bp for the *optrA* gene were detected using one % agarose gel electrophoresis stained with ethidium bromide and visualized under UV trans illumination. The amplification bands on agarose gel electrophoresis for *cfr* gene are shown in Figure 1.



Lane 1, 2, and 3 showing amplification bands for *cfr* gene (746 bp)

Figure 1: Agarose gel electrophoresis showing *cfr* gene amplification ladder

Statistical Analysis:

The data analysis was done using Stata/MP version 17 (StataCorp LLC, Texas, USA) and the Statistical Package for Social Sciences (SPSS) software version 27 (IBM Corp. Armonk, New York). The proportion of linezolid resistance was calculated and presented as frequencies (%). The association between two categorical variables was analysed using the Chi-square test. The p-value of less than 0.05 was considered statistically significant at 95% of the confidence level.

3. Result & analysis

Out of a total of 522 CoNS isolates, 120 were confirmed as *Staphylococcus epidermidis*. The clinical isolates of *Staphylococcus epidermidis* were predominantly methicillin resistant comprising 97% showing 100% resistance to penicillin. All of the *Staphylococcus epidermidis* isolates were sensitive to vancomycin. The antibiotic susceptibility pattern of all *Staphylococcus epidermidis* isolates is shown in Table 1.

On phenotypic testing only 4 (3.3%) were linezolid resistant *Staphylococcus epidermidis* (LRSE) isolates showing ≥8µg/ml of minimum inhibitory concentration (MIC) for linezolid. All of the LRSE isolates were methicillin resistant *Staphylococcus epidermidis* (MRSE). The comparative analysis of linezolid resistant against methicillin resistant *Staphylococcus epidermidis* isolates is shown in Table 2. On molecular characterization of linezolid resistance, only 3% of *Staphylococcus epidermidis* isolates were positive for *cfr* gene while *OptrA* gene was not detected in any of the *Staphylococcus epidermidis* isolates. The comparative analysis of phenotypic and genotypic detection of linezolid resistant *Staphylococcus epidermidis* isolates is shown in Table 3.

Table1: Antibiotic susceptibility pattern of *Staphylococcus epidermidis* (n=120)

Antibiotics	Sensitive		Resistant	
	Number	%	Number	%
Penicillin	0	0.0	120	100
Erythromycin	21	17.5	99	82.5
Clindamycin	40	33.33	80	66.66
Ciprofloxacin	42	35	78	65
Co-trimoxazole	62	51	58	49
Tetracycline	79	65.8	41	34.1
Gentamicin	88	73.33	32	26.66
Vancomycin	120	100	0	0.0
Linezolid	116	96	4	3.33

Table 2: Correlation between LRSE and MRSE (n=120)

	LRSE	LSSE
MRSE (n=117)	4	113
MSSE (n=3)	00	3

LRSE-Linezolid Resistant *Staphylococcus epidermidis*; LSSE Linezolid Sensitive *Staphylococcus epidermidis;* MRSE-Methicillin Resistant *Staphylococcus epidermidis;* MSSE-Methicillin Sensitive *Staphylococcus epidermidis*

Table 3: Comparative analysis of phenotypic and genotypic detection of linezolid resistance in *Staphylococcus epidermidis* (n=120)

LRSE	Phenotypic method		Genotypic method		Chi- Squire	p-value
	Number	%	Number	%		
Positive	4	3.33	3	2.5	0.147	0.7012
Negative	116	96.66	117	97.5		

4. Discussion

Among Gram positive cocci, CoNS are emerging as important and significant nosocomial pathogens, with *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* being the most commonly reported species. The increasing number of immunocompromised hospitalized patients in association with the use of invasive devices and implants, the importance of these nosocomial pathogens has increased in healthcare settings. Although CoNS possess fewer number of virulence factors as compared to *Staphylococcus aureus*, they are more difficult to treat due to their higher degree of antimicrobial resistance (AMR). India is facing a unique challenge to address the threat of AMR due to vast geography and population dynamics, healthcare spending, and misuse, inappropriate use or overuse of antimicrobials. Linezolid as the first oxazolidinone antimicrobial agent, was approved by the food and drug administration (FDA) in 2000 [8]. Linezolid is considered among one of the last resort antimicrobial drugs against MDR Gram positive bacteria due to its unique action and relatively low resistance around the world [14]. Additionally, its safety profile as well as pharmacokinetic properties are excellent, with nearly 100% bioavailability after oral administration. Due to all these factors, the extensive use and overuse of linezolid have been widely observed, contributing to the emergence of linezolid resistant strains [15]. The first cases of linezolid resistant *Staphylococcus epidermidis* strains were identified and reported from an outbreak investigation in intensive care unit (ICU) patients from Ireland [16]. Furthermore, two outbreaks of LRSE confined to ICU patients have also been reported from the Republic of Ireland, emphasizing the necessity for surveillance of linezolid resistance [17,18].

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 *Staphylococcus epidermidis* from a tertiary care hospital. Overall, resistance to linezolid in CoNS ranges from as low as no resistance to as high as 16% from India [19]. In this study, we reported linezolid resistance in 3.3% of our clinical isolates of *Staphylococcus epidermidis.* There are the variations in the reported data of linezolid resistance observed in various studies worldwide, including studies by Karavasilis *et al.*, Baos *et al.*, and Dembicka *et al.*, [20-22]. The overall reported linezolid resistance remained at <1% from 2011 to 2015. The staphylococci, especially *Staphylococcus epidermidis* showed a range of linezolid resistance mechanisms [23]. To the best of our knowledge this is the first study reported from this geographical area of Westen Uttar Pradesh which has looked for the presence of *cfr* and *optrA* gene in phenotypically confirmed LRSE. Our finding revealed the presence of *cfr* gene in 2.5% of *Staphylococcus epidermidis* isolates. A study conducted by Tewhey *et al*. reported 48% of *Staphylococcus epidermidis* isolates harbouring *cfr* gene [24]. Outbreak of LRSE mediated through the horizontally transferable *cfr* gene leads to the adoption of educational programmes aimed at limiting the transmissibility of drug resistant organism and controlling the prescription of linezolid [17]. Mendes and colleagues documented transmission of a mobile *cfr* gene onto two plasmids that were then acquired by *Staphylococcus epidermidis* isolated from the blood of two patients with sepsis [25]. Overall, the presence of *cfr* gene in such a high number of clinical isolates is a matter of therapeutic concern because linezolid is one of the high end reserved antibiotics.

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

In our study, both *cfr* and *optrA* gene were not detected in one of the phenotypically confirmed isolate of LRSE. The reason behind the absence of resistance genes may be the presence of other mechanism or resistance genes other than *cfr* and *optrA* genes associated with linezolid resistance.

The frequency of isolation of LRSE especially in India, is likely underestimated due to limited reporting. The rise in infections due to MDR *Staphylococcus epidermidis* can be a serious issue in near future due to the limited therapeutic options available. Therefore, understanding the epidemiological profile of linezolid resistance is crucial for addressing this critical emerging issue. The ongoing surveillance of antimicrobial susceptibility patterns is of utmost importance to manage the emergence of these MDR strains. The *cfr* gene is the most common genetic mechanism of resistance to linezolid among these strains. The correct and reliable identification of these isolates, judicious use of linezolid, and stringent implementation of infection control measures are important to control the spread of these *cfr* harbouring bacterial strains in the nosocomial environment. It is crucial to closely monitor the linezolid resistance, particularly when frequent and extended linezolid therapy is administered in a clinical setting. The paucity of newer antimicrobials demands the judicious use of linezolid through strict implementation of antimicrobial stewardship programs in healthcare settings. This study highlights the importance of continuous monitoring of linezolid resistance in *Staphylococcus epidermidis.*

There were certain limitations of this study including detection of other genetic elements and genome sequencing was not done due to limited resources. Furthermore, as this study was limited to a small sample size from a particular geographical area, the findings of the study cannot be generalized to the entire population.

5. Conclusion

The emergence of resistance to linezolid is a significant therapeutic concern. The management of such resistant strains is of utmost importance as these strains may spread horizontally to various inpatient units especially in a hospital setting. The plasmid-mediated *cfr* gene is primarily responsible for linezolid resistance in *Staphylococcus epidermidis* which can serve as an important target gene for rapid and reliable identification of these strains leading to early institution of antibiotic treatment to decrease the length of hospital stay, morbidity, and mortality.

Conflict of Interest: None

Funding Information: Nil

Ethical Information:

This study was approved by university ethics committee with reference number SU/2022/175[1] dated 05/08/2022.

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