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CFR GENE MEDIATED LINEZOLID RESISTANCE IN STAPHYLOCOCCAL ISOLATES

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ABSTRACT

Linezolid is one of the most potent antibacterial agents against gram positive organisms especially used in the treatment of skin and soft tissue infections and hospital acquired pneumonias. Recently resistant isolates to this drug are increasingly observed in many parts of the world but resistance is still rare in India. We have encountered two such resistant isolates from a tissue specimen culture. The isolates were identified as *S.aureus* and *S.epidermidis* by phenotypic tests. Both the isolates were resistant to linezolid by disc diffusion method. When the minimum inhibitory concentration (MIC) was determined by E- strip method, MIC for *S.aureus* was 32µg/ml and that of *S.epidermidis* was >256 µg/ml. *cfr* gene was detected in both the isolates by PCR. Both the isolates were sensitive to Tigecycline, Vancomycin, Cotrimoxazole and Levofloxacin with variable resistance to other antibiotics. This is a first case report of *cfr* gene mediated linezolid resistance in our country.

KEYWORDS: Linezolid resistance, Staphylococci, *cfr* gene, MIC, PhLOPS$_A$ phenotype.

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INTRODUCTION

Linezolid, the first member of the oxazolidinone group of antibiotics, introduced into the clinical practice in the early 2000, is a potent bacteriostatic drug against many gram positive organisms. It is one of the most extensively used drugs for the treatment of drug resistant pathogens including Methicillin resistant Staphylococci, Vancomycin intermediate S.aureus and Vancomycin resistant Enterococci [1]. Being a completely synthetic drug, development of resistance was considered unlikely to this drug as the natural pool of resistant genes won't exist readily in the environment, unlike many other antibiotics which are derived from microbial origin and have the their set of resistant genes in the environment. Linezolid acts by inhibiting the bacterial protein synthesis. It targets the 50S ribosomal subunit by binding to the peptidyltransferase center [2, 3]. Resistance to linezolid can occur by any one of the different mechanisms, the predominant one being mutation in 23S rRNA. This mutation occurs in the central loop of domain V of region of 23S rRNA, the most common location being position 2576 (G2576T tranversion) [4, 5]. Mutations have also been reported less commonly in other positions of rRNA which include C2534T, T2500A, G2215A, G2447T and T2504A [6, 7]. The second mechanism of linezolid resistance is by modification of ribosomal proteins L3, L4 and L22 which are encoded by *rplC*, *rplD* and *rplV* respectively [8, 9]. Resistance develops slowly because of these two types of mechanisms and is not transferable between species. The third mechanism is mediated by *cfr* gene which encodes a ribosomal methyl transferase which causes the post transcriptional methylation at position A2503 in the 23S rRNA gene of the large ribosomal subunit [10]. The *cfr* gene was first described as chloramphenicol resistance gene in veterinary isolates of *Staphylococcus sciuri* in year 2000 followed by reports of many other animal isolates of Staphylococci [11]. Though, linezolid resistant clinical isolate was reported as early as in 2001[4], the first clinical isolate of MRSA with *cfr* gene mediated Linezolid resistance was described only in the year 2007 [12] . This is followed by many case reports from various regions of the world including Italy, Colombia, Spain, Ireland, The United States and Mexico [13, 14, 15, 16, 17, 18]. Apart from staphylococcus aureus, this gene has been detected in coagulase negative staphylococcus and enterococci. This *cfr* gene is usually located on the plasmids in animal isolates, but unlike the animal isolates, in human isolates this gene was identified predominately in the chromosome as a part of integrated plasmid or transposon with the potential ability for excision and mobilization. This *cfr* mediated methylation renders the bacteria resistant to five structurally diverse groups of antibiotics which are all targeting the peptidyltransferase center. This phenotype is commonly known as $PhLOPS_{A,}$ shows resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A.

MATERIALS AND METHODS

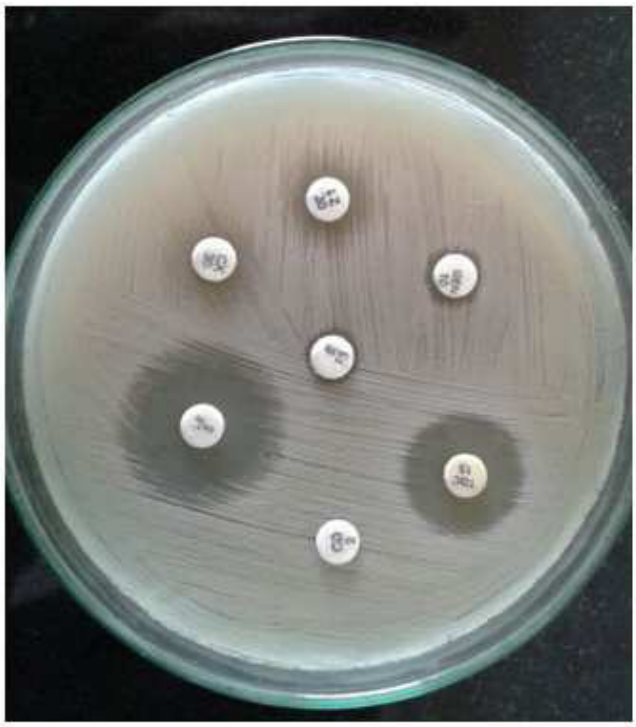
The source of the current study includes two bacterial isolates obtained from the culture of a tissue specimen from orthopedics ward. To brief the history, a 26 year old male patient was admitted in the orthopedic ward with "Infected Non Union of Right Tibia with Discharging Sinus". The patient was put-on linezolid injection for two weeks considering the prior pus culture report of *Staphylococcus aureus* showing sensitivity towards linezolid. Later the patient was operated, the infected tissue was removed and the freshening of the edges was done followed by long bone fixation by LRS (limb reconstructive system) along with bone grafting. The removed tissue was sent to the Department of Microbiology for culture. The tissue specimen was processed according to the standard protocol and the culture was done on blood agar and MacConkey agar plates. Next day, two different morphological types of colonies were isolated from culture plates.

Phenotypic identification of these two organisms was done by performing the standard biochemical tests. Susceptibility testing for both the isolates was done by Kirby Bauer disc diffusion method. As both the isolates were resistant to linezolid by disc diffusion method, minimum inhibitory concentration of these isolates was determined by using linezolid E-strips (Hi-media). *S.aureus* ATCC 25923 and *S.aureus* ATCC 29213 were used as controls throughout the study. The organism was considered resistant when the MIC >8µg/ml. Detection of *cfr* gene was done by polymerase chain reaction (PCR). PCR amplification of *cfr* gene: After isolating the DNA from these bacterial isolates by standard protocol, PCR was done by using the forward and reverse primers described elsewhere[19].PCR conditions were , Predenaturation-94^{o}C (5 min), Denaturation-94^{o}C(30 sec) , Annealing-52^{o}C(30 sec) ,Extention-72 ^{o}C (30 sec) for 35 cycles with a Final extention-72 ^{o}C (07 min).

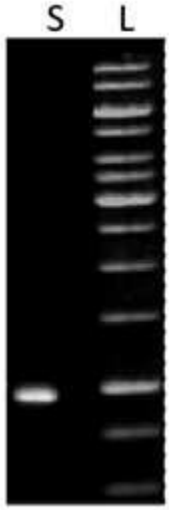
RESULTS

Among the two isolates, one was identified as *S. aureus* and the other as *S.epidermidis*. Both the isolates were resistant to cefoxitin and were therefore considered as methicillin resistant staphylococcal isolates (MRSA and MRCoNS). Both of them were resistant to clindamycin, chloramphenicol, linezolid and gentamicin by disc diffusion method. *S.aureus* was also resistant to erythromycin but susceptible to rifampin. The *S.epidermidis* was susceptible to erythromycin and resistant to rifampin. Both the isolates were sensitive to fluoroquinolones (levofloxacin and moxifloxacin), co-trimoxazole, tigecycline and vancomycin. Minimum inhibitory concentration of linezolid for *S.aureus* was 32µg/ml and for the *S.epidermidis* it was considerably high with MIC >256µg/ml by E-test method. *Cfr* gene was detected in both the isolates.

Antibiotic susceptibility testing of S.aureus and S.epidermidis



Amplified Cfr Gene



DISCUSSION

Emergence of antibiotic resistance in staphylococcal isolates is an age old phenomena with isolates developing resistance to antimicrobials appeared as soon as the drugs were introduced into market. Though, linezolid is introduced into clinical practice in the year 2000, Till now development of linezolid resistance in staphylococcal isolates is considerably low with only sporadic case reports from different countries [13, 14, 15, 16, 17]. Observance of Linezolid resistance was relatively more in coagulase negative staphylococci compared to *S.aureus* with only less than 0.05% of *S.aureus* are resistant and the coagulase negative staphylococci being 1.4% [20,21]. In an extensive literature search and review done by Bing Gu et al[22] in 2012 has shown that linezolid resistance was more frequently seen in Coagulase Negative Staphylococci than in *S.aureus*. Majority of the linezolid resistant *S.aureus* (LRSA) were reported from North America and Europe (77%) and only 20% were from the Asia. And the linezolid resistant coagulase negative staphylococci (LRCoNS) were reported to an account of only 1.1% from Asian countries and almost all others from America and Europe. Among the nine different species of LRCoNS described, majority were *S.epidermidis* (76.4%) followed by *S.hominis* and *S.hemolyticus* (9.1% and 8.8% respectively) Reports of Linezolid resistance in India have appeared in the literature in the year 2011 and majority of them being coagulase negative staphylococci which include *S.cohnii, S.kloosii, S.hominis, S.lugdunensis, S.hemolyticus*[23,24,25]. Resistance in *S. aureus* is extremely low in India with only single published report till date[26]. None of these reports from India have investigated the genomics of resistance in these isolates and many of them were sensitive to clindamycin which gives a clue that *cfr* gene might not have been responsible for resistance and probably mutations might have played a role in exhibiting such resistance. In the current study, we report a first case report of *cfr* gene mediated linezolid resistance in two staphylococcal isolates (LRSA and LRCoNS) isolated from a single patient. Though, inadvertent use of florfenicol in animals might have caused the origin of this type of resistance, in human isolates this has been explained by the over-prescription of the linezolid, non adherent to the therapy and the selective pressure created on the Staphylococci especially CoNS for the emergence and transfer of this kind of resistance[26]. This may be the same reason in

our case too as the patient was on linezolid treatment for two weeks before the appearance of such isolates in culture.

Cfr gene, though responsible for increase in the MIC for linezolid the level of MIC rarely exceeds 32µg/ml. This may be true for our *S.aureus* isolate which had MIC of 32µg/ml but the very high MIC (>256 µg/ml) observed in *S.epidermidis* needs to be investigated for other associated factors. Bonilla et al(2010)[27] and LaMarre et al (2013)[28]have observed in their studies that linezolid resistant *S.epidermidis* with very high MIC values were associated with both cfr gene and also with rRNA and/or mutations in the genes of ribosomal proteins L3andL4 which might have acted synergistically for developing high MIC values [27,28]. In our study, both the isolates were uniformly sensitive to tigecycline and vancomycin levofloxacin and cotrimoxazole. Majority of the studies, revealed similar findings where the isolates were sensititve to tigecycline, vancomycin and daptomycin but the susceptibility to fluroquinolones and cotrimoxazole showed variability[22]. In our study *S.aureus* was resistant to erythromycin but *S.epidermidis* was sensitive. This can be explained by the fact that in *S.aureus* the *cfr* gene is clustered in the chromosome along with the *erm* genes in the form of a transcriptional unit named *mlr* operon as described by Arias et al[29]. This may be true in our case also but this needs further workup to know the associated genes which was not performed in our study. With the wide prevalence of MRSA in our country only few alternative drugs are available, linezolid being the important one among them[30]. To preserve the potency of this highly potent drug great care should be taken in understanding the underlying mechanisms. Irrespective of genomics, whenever a clinical microbiologist observes such a typical phenotype ($PhLOPS_A$) with the organism showing resistance to chloramphenicol, clindamycin and linezolid (streptogramins are not routinely tested and pleuromutilins are not available yet), the possibility of *cfr* mediated resistance should be considered and great care should be taken microbiologically, epidemiologically as well as therapeutically in controlling the infection so that further transmission of this resistance can be minimized, rendering the potency of linezolid intact in treating multidrug resistant staphylococcal isolates.

CONCLUSION

To our knowledge, this is the first case report of *cfr* mediated linezolid resistance in staphylococcal isolates from India. Though this is just an initial report, observing such a potentially transferable type of resistance mechanism is really worrisome which if spreads, can take back the linezolid into pre-antibiotic era. In addition, observing this gene in coagulase negative staphylococci is of major concern as they act as reservoir for these resistance determinants, especially in a hospital environment and have the potential ability to horizontal spread of this resistance to other Gram positive organisms.

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