Phylogenetic Analysis of cfr Mediated Linezolid Resistance in Clinical Isolates of MRSA isolated from Eastern India

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ABSTRACT
The emergence of cfr mediated linezolid resistance have narrowed down the therapeutic options in treating gram positive infections. The mechanism of cfr mediated resistance to linezolid and other class of antibiotics involves the methylation in A2503 of 23S rRNA by Cfr methyltransferase. The problem increases as with the minor amino acid differences, cfr gene has now been found in many pathogenic bacteria. The aim of the study was to elucidate the mechanism of linezolid resistance in clinical isolates of Staphylococcus aureus and to find out evolutionary aspects of the distribution of Cfr. For the period of four years from West Bengal we identified cfr mediated linezolid resistance in three Methicillin resistant S. aureus (MRSA). The genotyping of the sequences shows high homology in between them. Collectively, the study outlines the phylogenetic structure of strains carrying the cfr gene and lastly our findings further reinforce the relevance of linezolid resistant strains by acquiring a natural resistance gene on the growing burden of antimicrobial resistance.

Keywords: Linezolid, MRSA, cfr, phylogenetic

INTRODUCTION
After the emergence of methicillin resistant Staphylococcus aureus (MRSA) as a nosocomial pathogen in the early 1960s, there have been an increasing number of outbreaks of MRSA infections in hospitals reported from many countries [1]. Methicillin resistance is mediated by a modified penicillin binding protein (PBP2a) which encodes the mecA gene that confers resistance to organism against methicillin and other beta-lactam antibiotics [2]. For treating MRSA, vancomycin and linezolid are the drugs of choice [1,3]. With the emergence of resistance to glycopeptides in MRSA, the only available antimicrobial with proved high activity against MRSA including strains with reduced susceptibility to glycopeptides is linezolid [3].
Most *S. aureus* including MRSA is found to be linezolid-susceptible with a breakpoint of 4µg/mL. [4,5] Linezolid is preferred by the clinicians because it heals faster, orally effective and reduces the stay of a patient in the hospital by almost three weeks. Linezolid comes under the oxazolidinone group of antibiotics and is approved for the treatment of infections caused by methicillin-susceptible (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) strains [3]. It inhibits protein synthesis by binding to the 50S bacterial ribosomal subunit [5]. But unfortunately a year after its introduction as a drug first report of linezolid-resistant in clinical isolate came in 2001[6]. The major mechanism of linezolid resistance is mediated by mutations in the V domain of the 23S rRNA [7] and another mechanism is RNA methylation by methyltransferase encoded by the *cfr* (chloramphenicol florfenicol resistance) gene [8].

The *Cfr* methyltransferase encoded by *cfr* gene primarily methylates C-8 position in A2503 of 23S rRNA which is the peptidyl transferase binding region of bacterial large ribosomes. [9] It is also evident that the *cfr* gene can be horizontally transferred to other hosts, as it is mostly plasmid or transposon [10] borne and confers cross resistance to other group of antibiotics like phenicols, lincosamides, oxazolidinones, pleromutilines and streptogramin A (PhLOPS phenotype) [11]. Thus the spreading of *cfr* gene in pathogenic bacteria is a health threat because many clinically important antibiotics become useless for treatment[12]. Also with the recent reports of emergence of linezolid resistance from various parts of world therapeutic options are decreasing increasingly. Keeping this knowledge into account the study was aimed at molecular characterization of *cfr* mediated linezolid resistance in MRSA isolates isolated from Eastern India (West Bengal) and to further establish their evolutionary relationships with the help of phylogenetic classification.

**MATERIAL AND METHODS**

The study was carried out at Medical College Hospital, Kolkata from Jan 2011 to Dec 2014. Samples such as pus, sputum, blood, urine and body fluid received in microbiology laboratory from various departments were processed.

**PHENOTYPIC IDENTIFICATION OF *S. aureus* ISOLATES**

Samples were inoculated on blood agar and Mac-Conkey’s agar followed by overnight incubation at 37°C. Gram staining was performed and smears showing gram positive cocci in clusters were considered. The culture was further inoculated into Manitol Salt Agar (MSA) & Dnase plates (Hi-media, India). Further, for confirmation of *S. aureus* tube coagulase test was performed by using standard method. *S. aureus* strain ATCC 25923 was used as positive control.

**ANTIBIOTIC SUSCEPTIBILITY TESTING**

All the confirmed *Staphylococcus aureus* strains were subsequently tested for methicillin and Linezolid resistance by Kirby-Bauer disc diffusion method using cefoxitin discs (30 µg/disc) and linezolid disc (30 µg) (Hi- Media, India) respectively on Mueller-Hinton agar. The plates were incubated at 37°C for 24 hrs and zone size was recorded. The isolates were considered methicillin resistant if the zone of inhibition was 24mm or less and linezolid resistant if the zone of inhibition was 21 mm or less. [4] Further again minimum inhibitory concentrations (MICs) of linezolid, clindamycin and chloramphenicol was determined by using Ezy MIC strip according to the instructions given by the manufacturer (Hi Media,India) . The Etest strips were placed on MHA agar plate incubated at 37°C for 24 hours. Isolates were categorized as susceptible or resistant to linezolid according to the breakpoints given by CLSI guidelines. An isolate was classified as susceptible to linezolid if the MIC was ≤4 µg/mL and resistant if MIC was ≥8 µg/mL.[4]
DETECTION OF LINEZOLID RESISTANT GENE

For Polymerase Chain Reaction, Staphylococcal genomic DNA was extracted as described by Phenol-Chloroform Method [13] with minor modification by adding lysostaphin (Sigma–Aldrich, USA) to a final concentration of 15 µg/ml. *Staphylococcus aureus* was confirmed by 16S rRNA gene amplification PCR. The genes encoding methicillin resistant (*mecA*) were amplified using PCR. Finally, all the isolates were studied for distribution of cfr genes for linezolid resistance. For the detection of all the genes DNA amplification was performed in total 25 µl of reaction mixture that contained 5 ng of template DNA, 2.5 µl (10x) buffer and 2 µl (25 mM) MgCl₂, 2.5 µl (200mM) dNPT mix (Sigma–Aldrich, USA), 1.2µl each of a pair of primers forward and reverse (10 pmol/µl) (Sigma–Aldrich, USA), 1 U of Taq DNA polymerase (Ampli Taq). The amplification was carried out in Thermal cycler (Biometra, Germany). The annealing condition and oligonucleotide primers sequence used are given in [Table 1]. The amplification product (10µl) was analysed by electrophoresis on 1 % agarose gel and visualized with ultraviolet light after staining with ethidium bromide.

Table 1: Oligonucleotide primers sequence and amplification conditions:

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Nucleotide Sequence (5'-3')</th>
<th>Product Length (BP)</th>
<th>Location Nucleotide No</th>
<th>Annealing Temperature °C</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S-r RNA-F</td>
<td>GTA GGT GGC AAG CGT TAT CC</td>
<td>228</td>
<td>545–564</td>
<td>64°C</td>
<td>28</td>
</tr>
<tr>
<td>16S-r RNA-R</td>
<td>CGC ACA TCA GC GTC AG</td>
<td>228</td>
<td>773–758</td>
<td>64°C</td>
<td>29</td>
</tr>
<tr>
<td>mecA-F</td>
<td>AGTTGTAGTTGTCGGGTTT</td>
<td>604</td>
<td>41596–41614</td>
<td>54°C</td>
<td>29</td>
</tr>
<tr>
<td>mecA-R</td>
<td>AGTGAACGAAGGTATCATC</td>
<td>604</td>
<td>41009–41028</td>
<td>54°C</td>
<td>29</td>
</tr>
<tr>
<td>cfr-F</td>
<td>ATGAATTTTAATAATAAAACAAAAAG</td>
<td>1043</td>
<td>3467–3490</td>
<td>50°C</td>
<td>30</td>
</tr>
<tr>
<td>cfr-R</td>
<td>CTAATTGCTATTTGATAATTACC</td>
<td>1043</td>
<td>4591–4509</td>
<td>50°C</td>
<td>30</td>
</tr>
</tbody>
</table>

Throughout the study *S. aureus* ATCC 25923 was used as control. a Denaturation and extension temperatures were kept at 94 and 72°C for each gene amplification

SEQUENCING OF CFR GENE:

The amplified PCR products (1043 bp of cfr region) was purified with a PCR purification kit (Qiagen.Hilden, Germany). The purified DNA were subjected to sequencing PCR using big dye terminator ready reaction mixture, forward and reverse primer (3.2 pmoles/µL) at thermal cycling conditions: denaturation at 94°C for 10 seconds of 25 cycles, annealing at 50°C for 5 seconds and extension at 60°C for 4 minutes. Cycle sequencing PCR product was purified by adding 0.1 volume of 3M sodium acetate (pH = 4.5) and 2.5 volume of absolute alcohol and dissolved in 25 µL of template suspension reagent (TSR) and heated at 95°C for 5 minutes followed by snap-chilling to denature the DNA. Samples were transferred in fresh tubes. These samples were loaded on sample tray. The sample was run through performance-optimized polymer (POP6) and electrophoreses at 12.1 kv for 3 hours in 1 X genetic analyser buffer on ABI PRISM 310 DNA sequencer (Applied Biosystems).

DATA SET ASSEMBLY:

The nucleotide and deduced amino acid sequences of the three isolated linezolid resistant strains were compared to published sequences

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available in GenBank with DNASTAR (DNA Star). PSI- BLAST (Position-specific Iterated-Basic Local Alignment Search Tool) of Cfr region was carried out with the deduced amino acid sequences from isolated strains for the best possible match. The data set for phylogenetic tree was based on the amino acid sequences of isolated cfr region plus additional Cfr-like sequences identified in the NCBI nr database by BLASTP searching with Cfr as query region. Sites were aligned in CLUSTAL W 2.1 (the BLOSUM62 substitution group) selected for phylogenetic analysis.

FORMATION OF PHYLOGENETIC TREE
Evolutionary analyses were conducted in MEGA6 [14] by using the Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model [15]. The bootstrap consensus tree inferred from 100 replicates [16] is taken to represent the evolutionary history of the taxa analyzed [16]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches [16]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. Codon positions included were 1st + 2nd + 3rd + noncoding. The analysis involved 12 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 178 positions in the final dataset.

RESULTS:
Out of total 16388 samples (pus, urine, blood, fluid, swabs, sputum) processed in the past 4 years in the microbiology laboratory, 2008 (12.25%) strains of *Staphylococcus aureus* were isolated. The clinical isolates were identified as *Staphylococcus* based on the characteristic growth on blood agar followed by microscopic observation. The isolate showed positive for mannitol fermentation, DNase and tube coagulase tests. Further the 16S rRNA gene PCR confirmed the isolate as *S. aureus*

ANTIBIOTIC SUSCEPTIBILITY ANALYSIS
Among 2008 *S.aureus*, 384 (19.12%) were MRSA and 1624 (80.87%) were methicillin sensitive *Staphylococcus aureus* (MSSA). Among 384 MRSA isolates, 381 (99.21%) were linezolid sensitive and 3 (0.7%) were linezolid resistant (isolated from pus). The isolate showed high MIC values (>256 µg/ml) of linezolid, clindamycin, chloramphenicol. Brief history of those 3 patients whose post-operative wound infections revealed linezolid resistant isolates & their antibiogram (as shown in figure 1).

**Patient I**: A 50 yrs female patient admitted with right transtrochanteric hip fracture due to road-traffic accident (RTA), underwent internal fixation of the right hip and develop wound infection subsequently.

**Patient II**: A 27 yrs male patient operated in Bangladesh for fracture shaft femur and admitted in our hospital with profuse collection of pus at the site of operation.

**Patient III**: A 35 yrs female patient admitted with Intracapsular fracture neck femur by falling on the road. Dynamic hipscrew was done. After one month, discharge came from the operative site. All the samples of post operative wound discharge & collected from the patients admitted in orthopedic ward all of them had H/O linezolid exposure before sending their samples.
**cfr GENE DETECTION BY PCR ANALYSIS**

The presence of *mec A* in the three isolated strains from pus confirms them as MRSA. Further for the detection of mode of linezolid resistance in these MRSA strains PCR amplification of *cfr* gene was carried out. PCR amplification showed that these three clinical isolates contains the *cfr* gene of 1043bp. (Figure 2)

**SEQUENCING AND NUCLEOTIDE SEQUENCE ACCESSION NUMBERS**

The edited segment of sequence (1043bp) of three Methicillin resistant *S. aureus* *cfr* region have been deposited in the NCBI database under the accession numbers KJ204625-KJ204627.

**ALIGNMENT AND PHYLOGENETIC ANALYSIS OF CFR SEQUENCES**

Phylogenetic tree was derived from three Cfr amino acid sequences of MRSA isolates along with nine previously published strains of Cfr methyltransferase protein (Fig 3), retrieved from the NCBI RefSeq protein database using PSI-BLAST with Cfr as the query. The phylogenetic tree comprises of amino acid sequences of *cfr* gene from various spp of *Staphylococcus, Enterococcus, Bacillales* all showing 99-100% identity with query sequence. The phylogenetic analysis showed that the Cfr of the isolates with accession no KJ204626 & KJ204627 are related to each other by sharing a common ancestor and in the past the Cfr proteins of these strains might have evolved from the same ancestors as of *Staphylococcus aureus* AGO46419 (NCBI GI numbers) chloramphenicol/florfenicol resistance protein & WP 032491462.123S ribosomal RNA
methyltransferase Cfr. On the other hand KJ204625 shares a common ancestor with 23S ribosomal RNA methyltransferase Cfr of *Enterococcus* and it is distantly related with 23S ribosomal RNA methyltransferase Cfr of *Staphylococcus capitis*. The Clustal W (version 2.1) alignment of the deduced amino acid sequences of these three strains KJ204625 - KJ204627 shows around 99% identity within the Cfr regions. But in the strain KJ204625 a point mutation was observed in the 133 position where instead of lysine (K) glutamine (Q) was present. The exact mechanism of this mutation is not known. (Fig 4) All these data shows that *cfr* gene which encodes Cfr methyltransferase may be easily transferred from one strain to another giving rise to resistance.

**Figure. 3** Molecular Phylogenetic analysis of 3 Lz resistant MRSA isolates in pus samples from West Bengal. The Maximum Likelihood Phylogenetic tree based on amino acid sequences of Cfr region of MRSA isolates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Vales of Node statistic are given. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. Numbers in taxon names are NCBI GI numbers The isolates sequences with accession number obtained in this study were marked with filled triangle symbols.

**Fig: 4 CLUSTAL O(1.2.1) multiple sequence alignment of protein sequence.**

KJ204625   TDQVLYFHLHGQIDSIFSMGMGEALRNQVFDALSDFTDPNLFAARLSISTIGHIP
KJ204626   TDQVLYFHLHGKIDSIFSMGMGEALRNQVFDALSDFTDPNLFAARLSISTIGHIP
KJ204627   TDQVLYFHLHGKIDSIFSMGMGEALRNQVFDALSDFTDPNLFAARLSISTIGHIP

KJ204625   SIKKITQYEYQVNLTSFLSHPSYSEERSLMPINDRYPIDEVMNILDEHIRTSDKYIAY
KJ204626   SIKKITQYEYQVNLTSFLSHPSYSEERSLMPINDRYPIDEVMNILDEHIRTSDKYIAY
KJ204627   SIKKITQYEYQVNLTSFLSHPSYSEERSLMPINDRYPIDEVMNILDEHIRTSDKYIAY

**DISCUSSION**

*Staphylococcus aureus* is a leading cause of pneumonia, skin-scalded syndrome, post-operative wound infections, bacteraemia and other infections. In 1961 MRSA was first identified [17]. Since that time, MRSA has spread
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worldwide, and the prevalence of MRSA has increased in both healthcare and community settings. However, linezolid-resistant MRSA has been reported recently [18,19]. Some recent outbreaks of cfr-mediated linezolid-resistant strains have been reported, such as in the Spain where 12 patients were affected by the epidemic strain involved in surgical site infections, ventilator-assisted pneumonia and septicemia in an intensive care unit.[19]

The resistant cells were found to have mutations in the G2576T [20, 21] .T2500A [22] encoding the 23S ribosomal RNA (23S rRNA) or in the ribosomal proteins L3 and L4 [23, 24]. Some additional mutations that have been found in vitro which includes G2447T in S. aureus and G2505A, G2512T, G2513T, and C2610G in enterococci [25, 26]. Bacteria possess multiple copies of the 23S rRNA gene, S. aureus strains contain five or six rRNA operons. The number of rRNA genes mutated depends on the dose and duration of linezolid exposure and has been shown to influence the level of linezolid resistance [27]. Non-mutational resistance to oxazolidinones are mediated by the gene encoding an rRNA methyltransferase (designated cfr, for chloramphenicol-florfenicol resistance) that has primarily been found on plasmids with transferable sequences or in a few cases on chromosomes but flanked by transferable sequences and appears to be capable of horizontal transfer between the different strains [8,10]. The mechanism of cfr-mediated resistance to linezolid and chloramphenicol involves the methylation of A2503 in the 23S rRNA of the large ribosomal subunit [9]. It has been found that all cfr genes were very similar in nature, with only one or two amino acid changes which correlates with our study. Despite of the ubiquitous nature of the plasmid borne cfr genes, yet there is no indication of the direct origin of the cfr that has been found in clinical and veterinary samples [12]. The classification of cfr gene can then be verified by phylogenetic analysis, which we carried out in this study. According to our study the isolated strain from this geographical area closely related to each other and they might have evolve from the same family. It can be taken into consideration that this database represent only a small part of bacterial diversity and with future genome sequencing it is much likely that more cfr sequences will be revealed. Also in the last few years, linezolid-resistant staphylococci, mainly in patients undergoing prolonged therapy, have been reported.[18] In our study, all the three patients had been treated with Linezolid empirically 600mg twice daily for atleast 10 days due to post-operative wound infections and MRSA isolated from them showed cfr-mediated resistance to linezolid. So it is a matter of concern that Linezolid is losing its shine against gram positive infections and we have reached a near-end point of a road where there is no new drug in the pipeline to treat gram positive bacteria. Linezolid should not be used empirically. It should be used after confirmation by culture sensitivity.

CONCLUSION:
Phylogenetic analysis is useful in understanding the geographical spread or transmission of gene in the bacteria. The study suggests that in Eastern India, MRSA strains with cfr mediated Linezolid resistance are prevalent with 99 % homology .It is not known from where the cfr evolved but their presence and their action against multiple antibiotics make them a matter of concern for antibiotic resistance.

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CONFLICT OF INTEREST: “The authors declare that there is no conflict of interest regarding the publication of this manuscript.”

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