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Detection of methyltransferase *CFR* multidrug resistant gene in bacterial isolates from intensive care unit and ocular infections

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Abstract

We screened a total of 308 bacterial isolates (25 ICU and 283 ocular samples) for the presence of *cfr* gene and a mutation in the V-domain of 23S rRNA, as they are known to confer resistance to various antibiotics, including linezolid. We report, multi drug resistant (MDR) methyltransferase *cfr* gene positive *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from a patient with septicemia admitted to ICU and ocular infection respectively. V-domain mutation has been noticed in five bacterial isolates derived from the ocular infections (G2576T) in the 23S rRNA. In conclusion, the detection of the MDR conferring *cfr* gene in the bacterial isolate implies a need for screening the infectious clinical samples for the presence of *cfr* gene to effectively treat infections, especially which are resistant to linezolid. The ocular samples may also be screened for the presence of *cfr* gene as linezolid is also being used to treat vancomycin resistant ocular infections.

Introduction

Emergence of newer strains of multi drug resistant (MDR) bacteria has been posing a formidable challenge to mankind, worldwide. Newer resistant mechanisms of drug resistance lead to the decrease in efficacy of the treatment leading to prolonged illness, and higher expenditure of healthcare. According to WHO, there are about 4,50,000 newer cases of multidrug-resistant tuberculosis (MDR-TB).[1] The emergence of artemisinin-resistant strains of malaria,[2] high percentage of nosocomial infections including methicillin-resistant *Staphylococcus aureus* (MRSA),[3] or multidrug-resistant gram-negative bacteria,[4] is increasingly being reported.

Newer classes of antibiotics were introduced to treat the MDR microorganisms, including the drug linezolid, the first oxazolidinone a potent drug against MRSA as well as vancomycin resistant *Streptococcus Sp* and *Enterococcus Sp*.[5] Linezolid drug is used to treat ICU patients with MDR bacterial infections.[6] The increased use of linezolid has been reported to treat ocular infections, such as bacterial keratitis and endophthalmitis caused by MRSA and vancomycin resistant gram positive organisms.[7,8,9,10] In addition, the linezolid drug has been successfully used to treat infection caused by *Mycobacterium chelonae*.[11] However, bacteria with chloramphenicol/florfenicol resistance (*cfr*) gene are found to be resistant to linezolid, and other four other classes of antibiotics such as phenicols, lincosamides, pleuromutilins and streptogramin A. Resistance to these antibiotics such as oxazolininones, phenicols, lincosamides, pleuromutilins and streptogramin A has been reported in many countries including USA,[13] Spain,[14] Italy[15] and China.

A recent multi-center study from Japan identified *Staphylococcus aureus* isolates of adult and pediatric clinical samples, resistant to clindamycin, belonging to the lincosamide class of antibiotics.[12] The bacteria may acquire drug resistance due to presence of *cfr* gene which encodes a methyltransferase that catalyzes and initiates the post transcriptional methylation of nucleotide A2503 in 23S rRNA,[13] or because of mutations, mainly at the V-domain of 23S rRNA.[17] Although, hospital based infections due to *cfr* mediated drug resistance has been reported in countries such as USA, Spain, Argentina and China, there is paucity of information on the presence of the bacteria with the *cfr* gene and/or MDR conferring mutations in most other countries, including India. A recent study from Southern India reported the presence of the *Staphylococcus aureus* with *cfr* gene in one Indian patient admitted to ICU.[16] To the best of our knowledge there are no other investigations. In view of the above, the present study was designed to screen the bacterial isolates derived from ocular and ICU infections obtained from health care facilities in and around Hyderabad, Telangana, India.

Materials and Method

Bacterial isolates and antibiotic susceptibility testing: A total of 308 bacterial isolates obtained from ICU (25) and ocular (283) cases. The bacterial isolates (gram positive and gram negative) were obtained from the microbiology laboratories (GHR Micro Diagnostics, Hyderabad, Sarojini Devi Eye Hospital, Hyderabad, India, Jhaveri Microbiology Centre, L.V. Prasad Eye Institute, Hyderabad) during 2013-15. The bacterial isolates were subjected to antibiotic susceptibility testing using Kirby-Bauer method for amikacin,

vancomycin, tigecycline, streptomycin, ciprofloxacin, ofloxacin, cefazolin, cefotaxime, amoxicillin, azithromycin, gentamycin, clindamycin, chloramphenicol, teicoplanin. The Minimum inhibitory concentration (MIC) was performed for linezolid, clindamycin, daptomycin and pristinomycin for the *cfr* positive *Staphylococcus aureus and Staphylococcus epidermidis*. The patients included both males and females. The ICU patients included 13 and 12 female and male subjects, respectively. The average age of the ICU subjects was 36.8 years (range 10-57 years), whereas the ocular samples were obtained from a total of 283 subjects including 185 males and 98 females, 46.5 years (Min- 8 days Max-94 years).

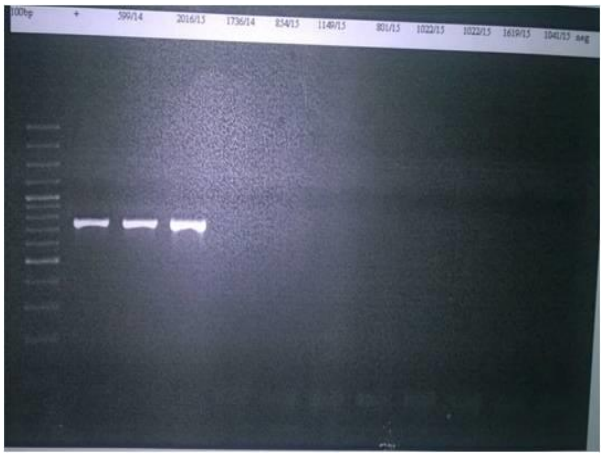
***cfr* gene screening:** In brief, bacterial DNA was isolated using a commercially available DNA isolation kit (Hi PurA™, Bacterial genomic DNA purification kit, Hi Media, India) as per the instructions of the manufacturer. Quality of the DNA was assessed using 1.0% agarose gel electrophoresis (AGE) and the isolated DNA was stored at -20^0 C, until use. All the samples were subjected to PCR within 2-3 days of isolating the DNA. PCR was carried out in a total volume of 50 µl PCR reaction mixture, using specific primers (18). The previously described primers were; Cfr F 5`TGAAGTATAAAGCAGGTTGGGAGTCA-3` Cfr R 5`- ACCATATAATTGACCACAAGCAGC-3 readymade PCR reaction mix, 50-100 ng of bacterial DNA and the total volume of 50 µl was made up with molecular biology grade water. PCR conditions were standardized. The conditions used for the PCR were; Initial denaturation was carried out for 5 minutes at 95^0 C, followed by 35 cycles of denaturation at 95^0 C for 30 seconds, annealing at 49^0 C for 30 seconds, extension at 72^0 C for 30 seconds and a final extension at 72^0 C for 10 minutes. Along with the bacterial isolates, plasmid containing *cfr* gene (a gift from Dr. Gopegui ER, Hospital Universitari Son Espasses, Palma de Mallorca, Spain) was also subjected to PCR amplification. The amplicons obtained were of the expected size 746 as judged by the 1.5% AGE, using 100 bp DNA ladder.

Identification of V region of 23S rRNA mutation in the bacterial isolates: The transitional mutation (G2576T) in bacterial isolates were screened using PCR-RFLP. PCR reaction mixture was same as above excepting the primers. The primers used were; F-5' GCTTTACTGTAGCCTGATATTGA3', R-5'GACAACTGGTACACCAGAGGTA3'. The conditions used for the PCR were; Initial denaturation was carried out for 5 minutes at 95^0 C, followed by 35 cycles of denaturation at 95^0 C for 30 seconds, annealing at 55^0 C for 30 seconds, extension at 72^0 C for 30 seconds and a final extension at 72^0 C for 10 minutes. The PCR generated expected amplicon (628 bp) as judged by the 2% AGE electrophoresis. The amplicons were subjected to RFLP using *Nhe1* restriction enzyme, (Fermentas, USA) as per the instructions of the manufacturer. The RFLP products were resolved on a 3% AGE. The size of the amplicon was determined using 50 and 100 bp DNA ladder. The wild type was found to be digested by the restriction enzyme while the mutated form was not cleaved by the enzyme. The expected size of the products were, wild (G2576); 555 bp and 73 bp, and mutant (T2576) ; 628 bp.

Results

Detection of *cfr* gene in bacterial isolates obtained from ICU and ocular infection subjects: We report presence of *cfr* gene in *Staphylococcus aureus* (599) isolated from blood culture derived from patient with septicemia admitted in ICU (Fig. 1). The organism was sensitive to amikacin, vancomycin, tigecycline, streptomycin and resistant to ciprofloxacin, ofloxacin, cefazolin, cefotaxime, amoxicillin, azithromycin, gentamycin, clindamycin, chloramphenicol, intermediate to teicoplanin. MIC confirmed the organism to be resistant to pristinomycin, clindamycin, linezolid and sensitive to daptomycin.

We also report the presence of *cfr* gene in *Staphylococcus epidermidis* (2016) isolated from the corneal scraping of a 55 years male patient suffering with the ocular infection corneal ulcer, conjunctival swab collected and subjected to the culture. The organism was resistant to chloromphenicol, clindamycin, Intermediate to erythromycin, teicoplanin and sensitive to gentamicin, ciprofloxacin, vancomycin, tigicyclin, rifampicin, oxacillin (Fig. 1). MIC showed no zone of clearance for linezolid indicating the resistance, sensitive to daptomycin (Fig. 3, 4).



Identification of *cfr* gene in the S*taphylococcus aureus and Staphylococcus epidermidis* isolated from patient with septicemia admitted in ICU and ocular infection patient.

Lane 1-100bp ladder, Lane 2-*cfr* positive control (746bp), Lane 3-599/14, Lane -4 2016/15 (746 bp),

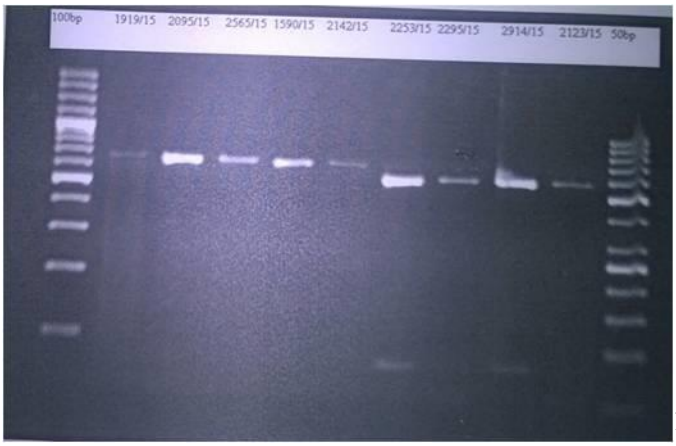


Fig. 2: Detection of V domain mutation: Mutation noticed in 1919/15, 2095/15, 2565/15, 1590/15, 2142/15 (628 bp)

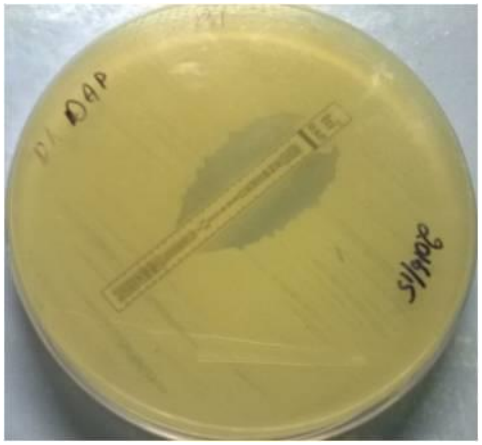


Fig. 3: MIC of *Staphylococcus epidermidis* shows sensitive to daptomycin

The drug resistance pattern in ICU has shown high drug resistance for gentamicin (82.5%), ciprofloxacin (63.6%), amikacin (48%), and piperacillin/tazobactum (53%), and sensitive to imipenem (65%). The drug resistance pattern in ocular cases includes sensitive to gentamycin (70%), ciprofloxacin (58%), gatifloxacin (70%), moxifloxacin (80%) chloromphenicol (84%), vancomycin (74%), ofloxacin (53%), erythromycin (61.5%), clindamycin (71%), Tigicycline (95%), oxacillin (88%), rifampicin (90%) and teicoplanin (74%) (Fig. 5).

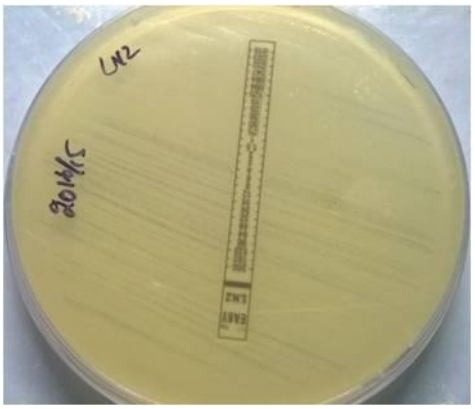


Fig. 4: MIC of *Staphylococcus epidermidis* shows resistant to linezolid

Fig. 5: General representation of antibiotic disc diffusion test

V region mutations: Mutations in the V region has been found in the samples the *haemolytic streptococcus* (1919*),* *Streptoccocus* Sp.(2095), *Staphylococcus aureus* (2565), *Enterococcus Sp.* (1590), *Streptococcus Sp.* (2142) (Fig. 2), *Streptococcus* (1919) is sensitive to all except gentamicin, *Streptococcus* Sp (2095) is sensitive to all found to be resistant to ciprofloxacin and ofloxacin, *Staphylococcus aureus* (2565) is sensitive to all except intermediate to ciprofloxacin, *Enterococcus* Sp (1590) is sensitive to all except ofloxacin and *Streptococcous* Sp (2142) is resistant to ofloxacin and clinamycin and sensitive to all.

Table 1: Specimens and bacterial isolated from the ocular and ICU infections

Samples	Specimens	Bacteria Isolated
	Anterior Chamber tap-8	*Acinetobacter Sp-3*
Ocular infections	Anterior chamber aspirate-1	*Bacillus Sp-1*
	Anterior chamber wash-1	*Enterococcus Sp-4*
	Blood centrline-1	*Klebsiella Sp-1*
	Conjunctival swab-45	*Micococcus Sp.-1*

	contact lens-1	*Pesudomonas Sp-28*
	half corneal button-3	*Pseudomonas auroginosa-31*
	Sclera -2	*Staphylococcus epidermidis-106*
	Pus-5	*Staphylococus aureus-89*
	Endothelial graft-1	*Staphylococcus Sp-3*
	Exenterated tissue-1	*Streptococcus Sp-16*
	Lens fluid-1	
	Lid abcess-1	
	Lacrimal sac-2	
	Corneal scrapping -173	
	Vitreous-36	
	Tear film-1	
ICU infections	Tracheostomy sputum-2	*E.coli -5*
	Blood -3	*Klebsiella Sp. -3*
	Ear swab-2	*Pseudomonas Sp. -9*
	Pus-4	*Staphylococcus aureus-8*
	Sputum-3	
	Urine-6	
	Tissue-1	
	Eustachian tube-4	

Discussion

Our finding of *cfr* gene in *Staphylococcus aureus* is of clinical significance as the linezolid is the only approved drug to treat MRSA cases as well as vancomycin resistant enterococci and penicillin resistant pneumococci. We evaluated the samples obtained from both ocular and ICU specimens accounting of total 308 cases, we detected the presence of *cfr* gene in *Staphylococcus aureus* isolated from the blood culture from patient suffering with septicemia admitted in ICU. We also report first time the isolation of the *cfr* in the *Stapylococcus epidermidis* isolated from the ocular infection. None of the other bacterial strains were positive for *cfr* gene, though the gene confers drug resistance not only to *Staphylococcus aureus* but also to other Gram positive organisms such as *Bacillus Sp, Enterococcus faecalis, Macrococcus Sp.* and Gram negative includes *Proteus Sp. and E.coli.*[17]

Since the presence of *cfr* gene has been documented in the *Staphylococcus sciuri* isolated from the animals,[18] the outbreak of linezolid resistant organisms has been reported in several countries including USA, China, European countries.[19,20] A recent study also reported the presence of the *cfr* gene from the bacterial isolates in India, i.e., coagulase negative *Staphylococcus hemolyticus* isolated from a 60 yrs male patient admitted in hospital for oedema and cellulitis in the left lower limb and he is on the prolonged linezolid therapy.[16] In our study, *cfr* gene positive *Staphylococcus aureus* isolated from a male, 57 years, septicemic patient admitted in ICU and ocular infection 55 years male patient suffering with the ocular infection was treated with the linezolid. We also report the presence of the mutation in the V domain region, in the gram positive organisms isolated from the ocular infections

In conclusion our finding of the *cfr* gene positive bacteria in India implies for a better surveillance of emerging MDR bacteria and screening of MDR patients for presence of the *cfr* gene positive bacteria, especially patients resistant to linezolid.

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Conflict of Interest

The authors declare no conflict of Interest.

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