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RESEARCH ARTICLE

Emergence of Chloramphenicol-florfenicol resistance (cfr) gene mediated linezolid resistance in methicillin resistant *Staphylococcus aureus*, case reports and review of literature.

Bashir Ahmad Fomda [1],Asiya Khan [1],Danish Zahoor [1],Javid Rasool [2],Haroon Rashid Zargar [3]
Department of Microbiology[1], Clinical Hematology [2] and Plastic Surgery[3],
Sher-I-Kashmir Institute of Medical Sciences, Soura , Srinagar

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Corresponding Author

Dr Bashir Ahmad Fomda
Professor, Department of Microbiology,SKIMS,
Soura, Srinagar 190011

Abstract

Context: Despite the use of linezolid, resistance to it has remained stable and extremely low with only sporadic cases have been reported.

Aim: To find out linezolid resistance in MRSA isolates.

Material and Methods: Specimens were collected aseptically and cultured using standard microbiological protocols. Antibiotic susceptibility was done by Kirby-Bauer disc diffusion method according to CLSI guidelines. Minimum inhibitory concentration (MIC) of these isolates were carried out and confirmed by E-test. PCR was performed for *mec A, nuc A* and *cfr* genes. Sequencing of the linezolid resistant isolates was also carried out.

Results: We here report three cases of *cfr* mediated linezolid resistant MRSA isolated from patients. MIC of linezolid to each *Staphylococcus aureus* was ≥8 µg/ml. The MIC of linezolid was further confirmed by E –test. PCR was done for amplification of *mec A, nuc A* and *cfr* genes confirming MRSA and *cfr* mediated linezolid resistance.

Conclusion: Scarcity of newer antimicrobials and increasing resistance to available drugs demands implementation of infection control measures to prevent the dissemination of *cfr* gene mediated linezolid resistant *Staphylococcus aureus*.

Introduction

Linezolid is an oxazolidinone with antimicrobial activity against resistant gram positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* and *Streptococcus* species (Kloss et al 1999).The drug binds to domain V of the 23S ribosomal RNA (rRNA) of 50S subunit of bacterial ribosome's, thus inhibiting protein

synthesis (Aoki et al 2010). Despite the clinical use of linezolid for very long time resistance to it is limited to case reports only (Ikeda-Dantsuji et al 2011; Jones et al 2010). The most common mechanism of linezolid resistance involves mutations in the central loop of domain V of the 23S rRNA. A new non mutational mechanism of linezolid resistance involving acquisition of a natural resistance *cfr* (chloramphenicol-florfenicol resistance) gene has recently been reported in veterinary staphylococcal isolates (Morales et al 2010). Although we have already reported the first case of linezolid resistance coagulase negative *Staphylococcus cohnii a*nd *Staphylococcus kloosii* in India (Peer et al 2011), linezolid resistant *Staphylococcus aureus* has not been reported previously from this region. We here report three *cfr* mediated linezolid resistant strains isolated from three patients.

Case reports

We isolated *Staphylococcus aureus* from bone marrow, burn wound, and swab from central line site of three patients who were admitted in 700 bedded tertiary care hospital. The clinical characteristics of the cases and bacterial profiles of the isolates are described below:

Case 1:

A 19 years old male, admitted with history of fever and chills for one and half month. The patient also had mild abdominal pain, sore throat, cough, dysuria, and low appetite of the same duration. The patient was suffering from a seizure disorder and was on valproic acid from last 13years. On examination chest, CVS, CNS were normal and abdominal examination showed splenomegaly. With this clinical presentation possibility of brucellosis, typhoid, malaria, tuberculosis and viral hepatitis were kept in mind as the cause of pyrexia of unknown origin. Various investigations were done which revealed Hb 8.6, TLC 1200, DLC (N 47.7, L 46.6, M 3.4, and E 2.3), PLT 186, MCV 88.1, MCH 26.2, MCHC 28.3, ESR 14mm/hr, Creatinin 0.52, Bil 0.24, ALT 20.5, ALP 92.9, AST 8.56, Pro 6.25, Alb 4.44, CK 24, TG 280, HDL 27, LDH 929, UA 4.95, Ca 9.27, urine examination revealed 12-15 pus cells and 1-3 RBC. Urine and blood culture were sterile, urine for AFB was negative. mantoux test, widal test and serology for brucellosis were negative. Blood film for malarial parasite was also negative. The patient's bone marrow was sent for the biopsy that revealed acute myeloid leukaemia FAB-M2. USG abdomen was done that reveals mild hepatosplenomegaly. The patient was treated with cefepime 2gm 8hrly and amikacin 750mg OD for 2 weeks. The patient continued to remain febrile despite treatment and his bone marrow was sent for culture and sensitivity that grew *Staphylococcus aureus.* Antibiotic susceptibility was performed by Kirby-Baur Disc Diffusion method according to CLSI guidelines. VITEK (Phenotype Oxazolidinone resistant, Macrolides/ Lincosamides/ Streptogramins MLSB+SA constitutive).

Case 2:

A 55 years old female patient of multiple myeloma Stage III A (IgA-Type) was admitted for autologous stem cell transplant (ATSC).Various investigations were done which revealed IgA 473,IgG 657,IgM 40,HIV negative, CMV negative, HBSAg negative ,HBV DNA negative. The patient received ATSC and engrafted (PMN engraftment D9 and platelet engraftment on D11). After transplantation patient developed post engraftment non neutropenic fever and was treated empirically (linezolid 600mg BD and voriconazole 50 mg OD for 5 days. The patient continued to remain febrile despite treatment and also developed thrombophlebitis in central and peripheral

catheter site. The catheter was removed and swab from catheter site was sent for culture and sensitivity that grew *Staphylococcus aureus* (Phenotype Oxazolidinone resistant).

Case 3:

A 42 years old male patient was admitted with 40% flame burn of face, chest, both arms, abdomen and back. Various investigations were done which revealed Hb 11, TLC 9.0, PLT 1.27, Urea 30, Creatinin 1.4, Bcl 1.1, ALT 33, ACP 35.The patient was on ceftriaxone 1 gm and linezolid 600mg twice daily for 6 days and was not responding to antibiotics. Burn wound swab was sent for culture sensitivity that grew *Staphylococcus aureus* (Phenotype Oxazolidinone resistant). The patient was managed conservatively with daily antiseptic dressing.

Microbiological investigations:

Specimens were inoculated on blood agar and MacConkey agar and incubated overnight at 37^0C. After the organism was identified as gram positive cocci in bunches by gram staining, various biochemical tests such as catalase, coagulase (slide and tube), phosphatase and DNase tests were performed for confirmation of *Staphylococcus aureus*. Susceptibility testing and interpretation for various antimicrobial agents was performed by Kirby-Bauer disk diffusion method following CLSI guidelines (2012). Various antibiotics like penicillin 10units, cefoxitin 30µg, linezolid 30µg, vancomycin 30µg, erythromycin 15µg, ciprofloxacin 5µg, cotrimoxazole (1.23/ 23.75µg), amikacin 30µg, gentamicin 10µg, teicoplanin 30µg and clindamycin 2µg were used. All antibiotic discs used were purchased from (HiMedia Labs. Mumbai, India). *Staphylococcus aureus* ATCC 25923 was used as a control. Minimum inhibitory concentrations of cefoxitin, oxacillin, vancomycin, clindamycin, erythromycin, cotrimoxazole, gentamicin and linezolid were determined by the broth microdilution method according to 2012 CLSI guidelines . Antibiotic powders of cefoxitin, oxacillin, erythromycin, vancomycin, clindamycin and gentamicin were obtained from Sigma, St. Louis, Mo, USA while as linezolid was eluted by disk elution method (Wilson et al 1990).The minimum inhibitory concentrations were also determined by VITEK 2 compact using GP67 panel (Bio-Merieux, France). The MIC of linezolid was further confirmed by E –test (AB Biodisk, Solna, Sweden).

PCR assay for *mecA, nucA* and *cfr* gene

Amplification of targeted genes was carried out by a PCR assay using template DNA. Bacterial DNA was extracted by using 10 mg/ml Lysostaphin (William et al 2001).PCR was carried out for *mecA, nucA* and *cfr* by using following primers *mec-A1* (5'-AAA ATC GAT GGT AAA GGT TGC C-3') ,*mec-A2* (5'- AGT TCT GCA GTA CCG GAT TTG C- 3'),*nuc-A1(5*'-GCG ATT GAT GGT GAT ACG GTT-3'), *nuc-A2(5*'- AGC CAA GCC TTG ACG AAC TAA AGC-3'), *cfr*-fw (5'-TGA AGT ATA AAG CAG GTT GGG AGT CA-3'), *cfr*-rv (5'-ACC ATA TAA TTG ACC ACA AGC AGC-3'),(Kehrenberg et al 2005).Multiplex PCR was performed for, *mec A* and *nuc A* gene in a 25µl reaction volume (200 µl PCR vial) with 1X-PCR buffer containing 10mM Tris-Hcl pH-8.3, 50mM Kcl, 1.5mM $MgCl_2$, 200µM concentration of each deoxynucleoside- triphosphate (dNTPs),2.5Uof taq polymerase, 0.2µM concentration of each primer and 2.5µl template DNA.Thermo cycling was carried out in a (Bio-metra) thermo cycler and the conditions were as follows: Denaturation at 94^0 C for 10minutes , followed by 30 cycles of 94^0 C for 30 seconds, annealing at 59^0 C for 30 seconds and extension at 72^0 C for 30 seconds

with a final extension of 10min at 72^0 C. PCR amplification of *cfr* gene were carried out in a thermal cycler using 200 µl of PCR tubes ,with a reaction mixture volume of 25 µl, each of the reaction mixtures contained 1X-PCR buffer containing 10mM Tris-Hcl pH-8.3, 50mM KCl, 1.5mM $MgCl_2$, 200µM concentration of each deoxynucleoside- triphosphate (dNTPs),2.5U of Taq polymerase, 0.2µM concentration of each primer and 2.5µl template DNA.The cycling conditions were as follows: initial denaturation at 94° C for 2 minutes was followed by denaturation at 94° C for 10 seconds, annealing at 55° C for 30 seconds, and extension at 72° C for 30 seconds for 30 cycles. Final extension of 7 minutes at 72° C. Electrophoresis at 100v for 40minutes was performed to separate the products on 1.5% 1xTBE (8.9M boric acid) and (0.2M EDTA) agarose gel. The gel was stained with 5µg/ml ethidium bromide and the photograph was taken under gel documentation system (Alpha Innotech Corporation, USA).

Sequencing:
PCR products were purified and sequenced.

Sequence analysis:
Previously published sequences of *cfr* mediated linezolid resistant isolates retrieved from the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov) were used as the reference sequence. Nucleotide sequence analysis was performed with BLAST sequence algorithms and sequences were aligned using Clustal W. (Thompson et al 2004)

Ethical clearance:
The study was approved by the Institute ethical clearance committee.

Results

All three isolates were resistant to penicillin, oxacillin, cefoxitin and all other β-lactams. The isolates were also resistant to erythromycin, gentamicin, ciprofloxacin, clindamycin and linezolid [Figure 1], while the isolates were sensitive to vancomycin, amikacin,and teicoplanin.

The MIC for cefoxitin was >32 µg/ml,oxacillin>256µg/ml, vancomycin 1µg/ml, clindamycin >8 µg/ml, erythromycin 64 µg/ml, gentamicin >128 µg/ml, and linezolid>32µg/ml by broth microdilution method.MICs were also determined by VITEK 2 compact using GP 67 panel (Bio-Merieux, France). Out of the three isolates the two isolates had the same resistance profile and were susceptible to vancomycin1µg/ml, moxifloxacin1µg/ml, quinupristin/ dalfopristin 1µg/ml and were resistantto benzylpencillin ≥0.5 µg/ml, oxacillin ≥4 µg/ml, gentamicin ≥16 µg/ml, ciprofloxacin ≥8 µg/ml, erythromycin ≥8 µg/ml, clindamycin ≥8 µg/ml, cotrimoxazole ≥320 µg/ml, rifampicin ≥32 µg/ml, tigecycline 1µg/ml. The isolates were intermediate sensitive to tetracycline 8µg/ml and

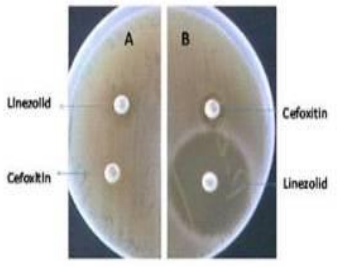

Figure 1: (a). Linezolid resistant MRSA strain, (b). Linezolid sensitive MRSA strain.

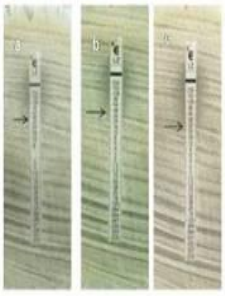

Figure 2: Methicillin resistant *S.aureus* isolates showing an MIC of (a) 32µg/ml, (b) 48µg/ml, and (c) 32µg/ml by E test.

levofloxacin 4µg/ml.The third isolate had a different susceptibility pattern.This isolate was susceptible to moxifloxacin 2µg/ml, quinupristin/dalfopristin 1µg/ml, and was resistant to benzylpencillin ≥0.5 µg/ml, oxacillin ≥4 µg/ml, gentamicin ≥16 µg/ml, ciprofloxacin ≥8 µg/ml, erythromycin ≥8 µg/ml, clindamycin ≥8 µg/ml, rifampicin ≥32 µg/ml, cotrimoxazole ≥320 µg/ml , levofloxacin ≥8 µg/ml and intermediate sensitive to vancomycin with an MIC of 4µg/ml. The MIC of linezolid to each *Staphylococcus aureus* was ≥8 µg/ml. The MIC of linezolid was further confirmed by E–test (AB Biodisk, Solna, Sweden) [Figure 2]. Resistance to linezolid was defined as an MIC≥8 µg/ml and for oxacillin MIC≥4 µg/ml.

Molecular analysis

All the resistant strains showed an amplification band of the expected size (*mec A* 533bp, *nuc A* 270 bp), confirming MRSA and (*cfr* 746 bp) mediated linezolid resistance [Figure 3, 4]. Sequencing of amplified gene was also carried out,.Nucleotide sequence of all the 3 isolates were aligned with reference sequence. All the isolates showed 100% homology with the reference strain.

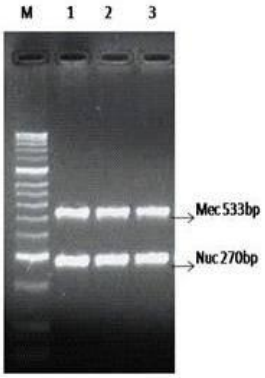

Figure 3: Agarose gel electrophoresis of PCR amplified products with *mecA* and *nucA* specific primers. Lane M. 100bp ladder, lane 1, 2, 3 showing *mec-A* gene (533bp) and *nuc-A* gene (270bp).

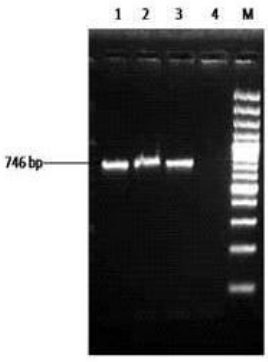

Figure 4: Agarose gel electrophoresis of PCR amplified products with *cfr* specific primers Lane 1, 2, 3 showing amplification of *cfr* gene (746bp), Lane 4 negative control and Lane M. 100bp ladder.

Accession numbers

Accession numbers of the isolates obtained from Genbank are, Case1 KF264959, Case2 KF264960, and Case3 KF264961.

Discussion

Methicillin resistant *Staphylococcus aureus* is a major cause of health care associated infection traditionally, because of the universal resistance of MRSA to β-lactams and lack of other alternative, the glycopeptides particularly vancomycin became the mainstay of treatment (Nian et al 2012). However creeping MIC of vancomycin and reports of several frank resistances in *Staphylococcus aureus* has lead to limited therapeutic options for severe MRSA infections. The

recently developed antimicrobial agent linezolid has been probably one of a few choices for treatment of vancomycin resistant MRSA. Linezolid is widely used in critical care because of its antimicrobial spectrum, favorable short-term safety profile, pharmacokinetics/ pharmacodynamics, and effectiveness (Perez et al 2008).Linezolid resistance in staphylococcus was reported only 1 year after the clinical application of this drug (Tsiodras et al 2001). Subsequently, linezolid resistant enterococci and staphylococci have been sporadically isolated all over the world (Aoki et al 2002, Jones et al 2009, Bonora et al 2006 and Sanchez et al 2006) . Linezolid resistance, as described in numerous reports, has been mediated by mutations in 23S rRNA or in L3, L4 ribosomal protein genes, implying the slow dissemination of resistance by these mechanisms. However, the detection of a plasmid-borne *cfr*-mediated linezolid resistance gene in *staphylococci* adds a new dimension to the threat for clinical use of several antimicrobials, including linezolid and clindamycin (Morales et al 2010).This gene is a part of integral plasmid that is capable of excision and mobilization and could be transferred to other pathogen strains and spread rapidly.

We here report three *cfr* mediated linezolid resistant MRSA strains isolated from three patients carrying *cfr* gene, as demonstrated by PCR and sequencing. The MIC of linezolid was determined by the broth micro dilution method, Vitek 2 compact and further confirmed by E-test. The *cfr* gene was initially described in a bovine *Staphylococcus sciuri* isolate (Schwarz et al 2000). It has been found primarily in plasmids and appears to be capable of horizontal transfer between *staphylococci*. The product of the *cfr* gene is a methyltransferase that catalyses methylation of A2503 in the 23S rRNA gene of the large ribosomal subunit, conferring antibiotic resistance (William et al 2001). The methylation confers combined resistance to five different classes of antibiotics resulting in a phenotype called PhLOPS, for resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A (Longa , Vester 2012). In 2007, Toh *et al* reported the first *cfr*-mediated, linezolid-resistant clinical isolate of MRSA.Two new cases of *cfr*-mediated resistance in clinical isolates of *Staphylococcus epidermidis* and *Staphylococcus aureus* were described in 2008 in the United States and subsequently other studies have demonstrated cfr mediated linezolid resistance. (Mendes et al 2008, Morales et al 2010, Kehrenberg et al 2005 and Nian et al 2012) .With the increasing number of immuno-compromised patients, frequent interventions, use of intravascular devices, and prolonged hospital stays, methicillin resistant *S.aureus* are emerging as important pathogens which cause life threatening infections. The increasing rise in MRSA strains is limiting the utility of all beta-lactam agents, thus limits the options of treatment and therefore attention should be paid to the fact that these strains might also be co-selected under use of any of these drug classes, which might increase the risk of development of linezolid resistant strains. In present study three isolates were also resistant to gentamicin, ciprofloxacin, erythromycin, clindamycin, cotrimoxazole, rifampicin, tigecycline. Out of the three strains one strain was intermediate sensitive to vancomycin. It is high time that we recognize this threat and closely monitor and track resistance to linezolid by resistance surveillance studies. Scarcity of newer antimicrobials and increasing resistance to available drugs demands implementation of infection control measures to prevent the dissemination of *cfr* gene mediated linezolid resistant *Staphylococcus aureus.*

Acknowledgment

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