



Molecular detection of chloramphenicol-florfenicol resistance (*cf*r) genes among linezolid resistant MRSA isolates in Sokoto State, Nigeria

Detecção molecular de genes de resistência ao cloranfenicol-florfenicol (*cf*r) entre isolados de MRSA resistentes a linezolida no estado de Sokoto, Nigéria

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Abstract

Objective: we investigated previous literatures for documentation of the trend in Sokoto, Nigeria and found none. We deemed it fit to determine the frequency of linezolid resistance mediated by *cf*r gene among MRSA isolates from Sokoto State-owned hospitals. **Methods:** Bacterial species identification was carried out with Microgen™ Staph-ID System kit (Microgen, Surrey, UK). Disc agar diffusion method (Modified Kirby-Bauer's) following Clinical and Laboratory Standards Institute (CLSI 2018) guidelines was used in antimicrobial susceptibility testing. The results were interpreted and managed using WHONET 5.6 software (WHO, Switzerland). Oxacillin resistant screening agar base (ORSAB) culture was used to determine phenotypic methicillin resistance. Polymerase chain reaction (PCR) was carried out to determine the presence of *cf*r-gene. **Results:** A total of 81 *S. aureus* isolates were phenotypically identified. Of this number, 46.91% (38/81) were MRSA; Healthcare workers (39.5%), Outpatient (28.9%), In patient (21%), Security men and Cleaners (5.3% each). Importantly linezolid resistance rate among the MRSA isolates was 44.7%. Analysis of antimicrobial susceptibility profile also showed a multiple antibiotics resistance burden of MDR (5.9%), possible XDR (47.1%), XDR (41.1%) and PDR (5.9%) amongst LR-MRSA. About 52.9% (9/17) of LR-MRSA harbored the *cf*r gene. **Conclusions:** This is the first report to document *cf*r gene in LR-MRSA strains in Sokoto. The *cf*r gene was found among the studied LR-MRSA strains and if *cf*r-mediated linezolid resistance is not properly checked, its phenotypic expression may result in an outbreak of multiple antibiotic resistant strains.

Keywords Antibiotics resistance. *Cfr* gene. Linezolid. PCR. MRSA.

Resumo

Objetivo: avaliar a incidência de resistência linezolida *cf*r-mediada entre os isolados de MRSA dos hospitais do Estado de Sokoto. **Métodos:** A identificação das espécies bacterianas foi realizada com Microgen™ Staph-ID System kit (Microgen, Surrey, UK). Método de difusão em ágar de disco (Kirby-Bauer modificado) seguindo as diretrizes do Clinical and Laboratory Standards Institute (CLSI 2018). O resultado foi interpretado e gerido com WHONET 5.6 (OMS, Suíça) software. A cultura ORSAB (Oxacillin resistant screening agar) foi utilizada para determinar a resistência fenotípica à metilina. A PCR foi realizada para determinar a presença de *cf*r-gene. **Resultados:** um total de 81 isolados de *S. aureus* foi identificada fenotipicamente. Desse número, 46,91% (38/81) eram de MRSA; Profissionais de saúde (39,5%), Ambulatoriais (28,9%), Em paciente (21%), Homens de segurança e Limpadores (5,3% cada). A taxa de resistência linezolida entre os isolados de MRSA foi de 44,7%. A análise do perfil de sensibilidade antimicrobiana também mostrou uma carga de resistência a antibióticos múltiplos de MDR (5,9%), possível XDR (47,1%), XDR (41,1%) e PDR (5,9%) entre LR-MRSA. Um total de, 52,9% (9/17) da LR-MRSA abrigava o gene *cf*r. **Conclusões:** Este é o primeiro relatório a documentar o *cf*r-gen nas estirpes LR-MRSA em Sokoto. O gene *cf*r está presente entre as cepas estudadas de LR-MRSA, e se a resistência *cf*r-mediada linezolida não for adequadamente verificada, sua expressão fenotípica pode resultar em um surto de múltiplas cepas resistentes a antibióticos.

Palavras-chave: Resistência a Antibióticos. Gene *Cfr*. linezolida. PCR. MRSA.

INTRODUCTION

Scientists have based a considerable part of the regular prevention and treatment of human diseases in the healthcare settings on the effectiveness of antibiotics. These bacteria-fighting drugs now face immense crisis because of their misuse for both human and livestock¹. The incessant antibiotic selective pressure contributes to the mobilization of horizontally acquired antimicrobial resistance genes². All over the world, methicillin resistant *Staphylococcus aureus* (MRSA) associated infections are ever more difficult to treat³. Linezolid is classified in the Reserve group of the AWaRe (Access, Watch, and Reserve) category of World Health Organisation's (WHO's) Essential Medicines List as last-resource treatments

for multidrug-resistant infections⁴. Decades after its first report, the spread of linezolid resistance (LR) amongst MRSA has been limited. But, some studies have recently reported its swift spread in hospital and community settings^{5,6,7}. Resistance to oxazolidinone group of antibiotics in MRSA is facilitated by two central mechanisms, either through genetic mutation of genes that code for *23S*rRNA which then cause change to the peptidyl transferase centre where conserved residues interact with linezolid or by horizontal acquisition (non-mutational) of the natural *cf*r (chloramphenicol-florfenicol resistance) gene that codes for *23S*rRNA methylase enzyme that targets the adenine residue A2503⁸. The plasmid-carried *cf*r gene when acquired

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can be transferred to other MRSA strain and ultimately spring resistance to drugs with ribosomal target sites (lincosamides, phenicols, oxazolidinones, streptogramin A, some macrolides and pleuromultilins)⁹.

The mortality rate arising from antibiotics-resistant infections is estimated to be around fifty-eight thousand monthly and could reach millions by the year 2050¹⁰. Health-care professionals rely on antibiotics like linezolid as primary treatment for MRSA related infections. Recently, instances of resistance to linezolid has been reported in many studies^{11,12}.

In low and middle-income countries (LMICs) like Nigeria, policies on antibiotics stewardship, sanitation and infection transmission control are inadequately enforced¹³. Consequently, there is a gradual rise of *S. aureus* and MRSA strains with reduced susceptibility to linezolid in Nigeria. Until recently linezolid resistance (LR) amongst *S. aureus* and MRSA strains in the country was largely undocumented^{14,15}. More recent surveys have recognized escalations in the frequency and proliferation of LR-MRSA from 13.3% to 22.4% and 79.1%^{16–18}. The need to uphold the potency of linezolid against strains with LR determinants cannot be overemphasized. We investigated previous literatures for the documentation of the trend in Sokoto, Nigeria and found none. So, we deemed it fit to determine the frequency of linezolid resistance mediated by *cfr* gene among MRSA isolates from Sokoto State-owned hospitals.

METHODS

Bacterial isolates

Approval for the study was provided by the Sokoto State ministry of health's Ethical Review Board (Reference number SMH/1580/V. IV.). Samples were obtained from three Sokoto State-owned Hospitals (Maryam Abacha Women and Children Hospital, Specialist Hospital and Orthopedic Hospital Wamakko) between February 2018 and July 2018. The study participants comprised health care workers, inpatients, outpatients, security men and cleaners. By means of commercially available, swab sticks pre-moistened with sterile normal saline, specimens were collected from the nasal cavity of consenting participants and transported in an ice pack to the Central Research laboratory of the Faculty of Veterinary Medicine, Usmanu Danfodiyo University Sokoto, Nigeria for further processing.

Samples were streaked on mannitol salt agar (Oxoid, UK) and incubated overnight at 37 °C. Bacterial species identification was conducted with Microgen™ Staph-ID System kit (Microgen, Surrey, UK). The isolates were inoculated in Oxacillin resistant screening agar (ORSAB) plate¹⁹ and the resultant isolates after overnight culture were further subjected to a panel of ten (10) antibiotics.

Antimicrobial susceptibility test

The susceptibility of emergent isolates to ten commercially

available antibiotics was determined by Modified Kirby-Bauer's Disc agar diffusion method as suggested by Clinical and Laboratory Standards Institute (CLSI, 2018)²⁰. The results were interpreted and managed using WHONET 5.6 software (WHO, Switzerland) with laboratory algorithm set to match CLSI antibiotics breakpoints²¹. The commercially available 6 mm antibiotic discs (Oxoid, UK) used includes; clindamycin (2µg), erythromycin (15µg), ceftazidime (30µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), chloramphenicol (30µg), linezolid (30µg), tetracycline (30µg), cefoxitin (30µg), levofloxacin (5µg), gentamicin (120µg). The resistance patterns were described according to the definitions of²², multiple antibiotics resistant strains were divided into MDR (Multiple Drug-Resistant), XDR (Extensively Drug-Resistant) and PDR (Pan drug-resistant). MDR bacteria are defined as resistant to one agent in at least three classes of antibiotics. XDR bacteria are characterized by their sensitivity to only three class of antibiotics or less and the PDR bacteria are resistant to all classes of antibiotics used in the study²².

PCR for *cfr* (linezolid resistance) gene

Qiagen DNA extraction KIT (Qiagen, Hilden, Germany) was used in the isolation of the total genomic DNA of linezolid resistant MRSA (LR-MRSA) strains as groundwork for Simplex PCR for the detection of *cfr* gene²³. The primers used were designed with Primer3Plus Software²⁴ on a matrix of *cfr* oligonucleotide sequence (GenBank Accession no.MF579752) that eventually (after production) resulted in a primer (Forward: TAGTGAGGAACGCAGCAAAT, Reverse: CTCAGGTGCACTTATTGTAGGA) with an expected amplicon size of 746 bp. A PCR cocktail comprising 5 µl of template DNA, 12.5 µl of Qiagen master mix, 2.5 µL of Q-reagent, 1 µL of each of each primer pair (2 µL totally) and 3 µl molecular grade water. DNA was amplified with a thermocycler (Applied bio systems 9700). PCR conditions were as follows: denaturation for 2 min at 94 °C, 30 cycles of denaturation for 10 s (94 °C, annealing for 30 s at 55 °C), extension (30 s at 72 °C), and a final extension (7 min at 72 °C). The bands indicative of the presence of *cfr* gene was visualized in an electrophoretogram after the PCR products were electrophoresed in a 1.5% ethidium bromide pre-stained agarose gel.

RESULTS

Out of 756 nasal samples obtained, a total of 81 *S. aureus* isolates were phenotypically identified from Maryam Abacha Women and Children Hospital (31.6%), Specialist Hospital (39.5%) and Orthopedic Hospital Wamakko (28.9%). Of this number, 46.91% (38/81) were MRSA; Healthcare workers (39.5%), Outpatient (28.9%), In patient (21%), Security men and Cleaners (5.3% each). Importantly, linezolid resistance rate among the MRSA isolates was 44.7% (17/38). The data gathered from this study submits that all the seventeen-linezolid resistant MRSA were multiple antibiotics resistant strains; the isolates were all resistant to a minimum four (4) agents including linezolid, ceftazidime and cefoxitin. As shown in figure 1, we learnt that 70.6% (12/17) of

linezolid-resistant strains were resistant to levofloxacin, 47.1% (8/17) to chloramphenicol, 58.8% (10/17) to erythromycin, 35.3% (6/17) to clindamycin, 29.4% (5/17) to trimethoprim/sulfamethoxazole, 64.7% (11/17) to tetracycline and 70.6% (12/17) to levofloxacin. Analysis of antimicrobial susceptibility profile also showed a multiple antibiotics resistance burden of MDR (5.9%), possible XDR (47.1%), XDR (41.1%) and PDR (5.9%)

amongst LR-MRSA.

Genetically (Figure 2), the percentage of linezolid resistant MRSA that harbored the *cfr* gene was 52.9% (9/17). Almost all of the *cfr* positive LR-MRSA isolates (66.7%) displayed clindamycin, chloramphenicol and linezolid resistance phenotype.

Figure 1. Antibiotic resistance profile in Linezolid resistant-MRSA strains

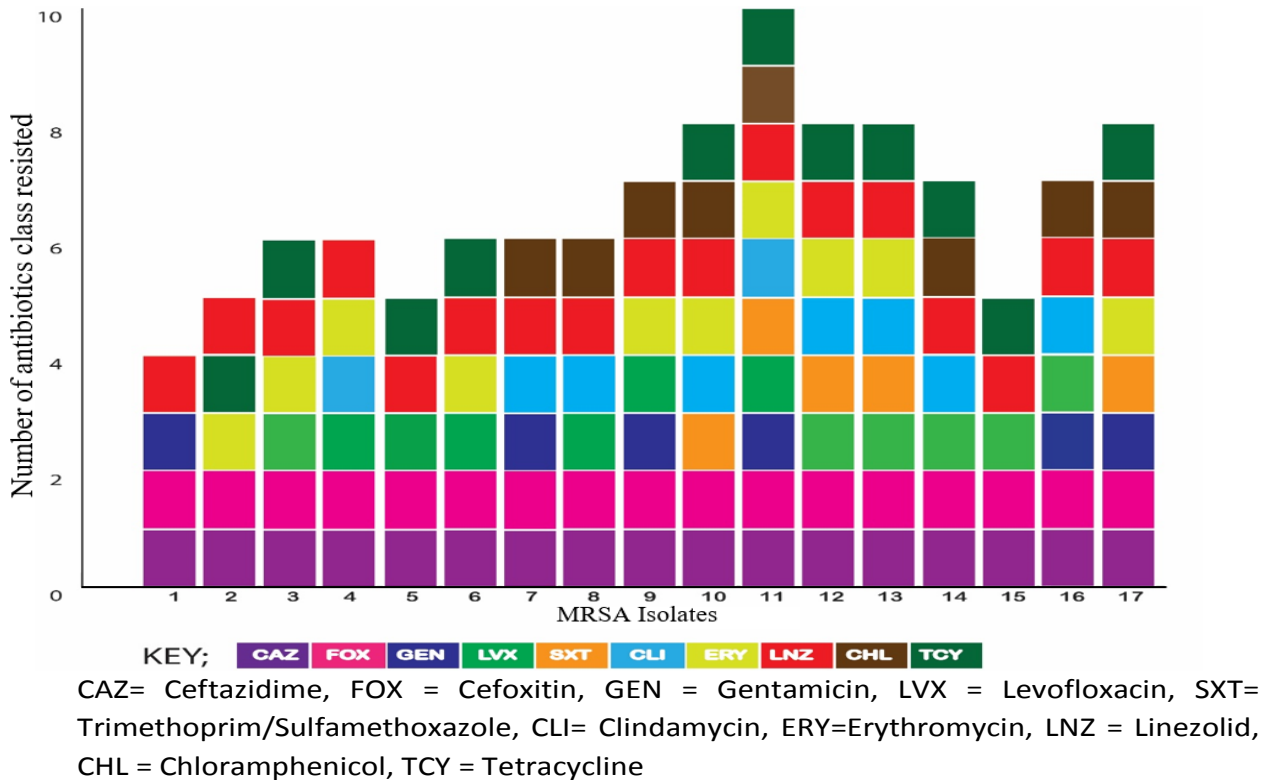
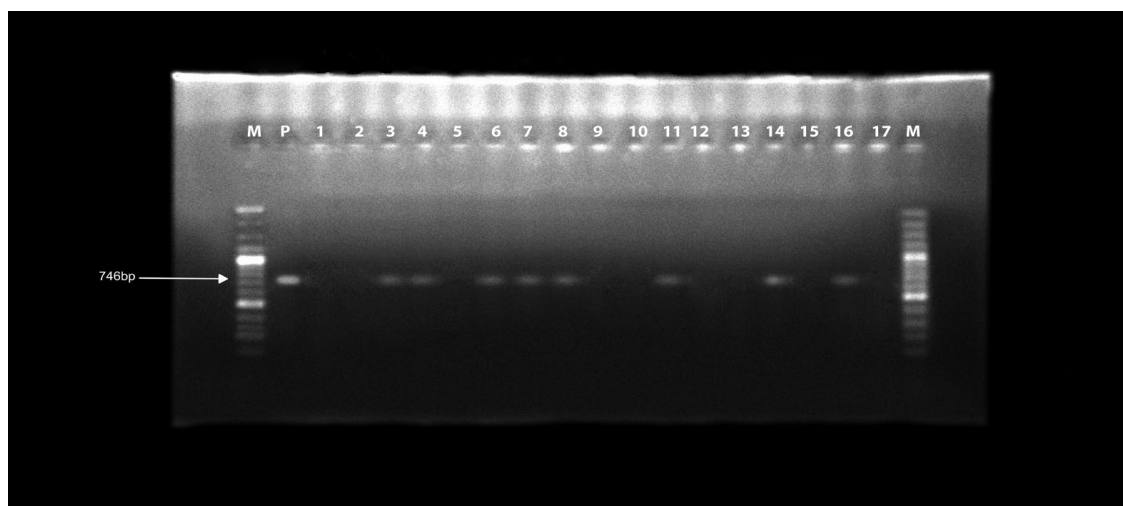


Figure 2. Electrophoretogram for the *cfr* genes of linezolid resistant isolates. In the figure: *cfr* (746 bp), M is 100 bp+ DNA ladder, P = Positive control (*S. aureus* ATCC 25923) Lane 1-17 = Samples



DISCUSSION

As a member of the oxazolidinone group of antibiotics, clinicians prefer linezolid in treatment of MRSA because of its therapeutic effectiveness and corresponding reduction in duration patients' hospitalization. The outcome of linezolid resistance rate among MRSA isolates observed here implicates the rather swift dissemination linezolid resistance of 44% (22/50) reported by¹⁰. Our finding is within the spectrum 13.3% to 79.1% resistance rates reported by some Nigerian studies^{16–18}. This may be associated modifications at the binding sites of linezolid on the ribosome (V loop of 23S rRNA). Furthermore, speculation can be made about the possibility of a secondary resistance like cell wall thickness or biofilm formation being responsible for the observed LR²⁵. Conversely, the development of clinically important LR phenotypes require mutation of more than one allele of the wild type 23SrRNA. In MRSA there are multiple copies of these alleles (4 to 6 copies)²⁶. This may explain relatively high linezolid susceptibility rate documented in this study. This may also explain the fairly uniform activity of linezolid over the years.

The discovery that all seventeen-linezolid resistant MRSA were multiple antibiotic resistant is not surprising owing to the fact that the isolates were obtained from a hospital environment, there is high chance that prior antimicrobial use that may have increased the odds of MRSA isolate to become resistant to multiple antibiotics²⁷. However the burden multiple drug resistance profile documented in this study is similar to the work of²⁸ in which almost all the LR-MRSA were MDR.

Furthermore, the band pattern evidenced by our electrophoretogram reveals that *cfr* gene mediated LR in this study occurred in 52.9% of the isolates. This gives credence to the existing CLSI protocol, which suggest the use of a second method in addition phenotypic method in the confirmation LR in staphylococci²⁰.

The finding that most (66.7%) of the *cfr* positive LR-MRSA strains exhibited the clindamycin, chloramphenicol and linezolid resistance phenotype validates previous suggestions that the phenotype is an indicator for the likely presence of the *cfr* gene²⁹. Our findings identify with several previously reported *cfr* distribution of 100% by^{30–32} amongst LR-MRSA but contradicts the results of lower rates of 6.25% and 7.1% reported by^{29,33}. The observed contrasts among these studies might be due to variations in sampling technique and phenotypic confirmatory methods³⁰.

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All nine (9) of the *cfr*-positive linezolid-resistant MRSA were phenotypically MDR which agrees with a previous study by³⁴. The difference in hospital drug prescription practices is a potential source of pressure that may contribute the evolution of MDR strains.

The predominance of *cfr*-positive LR-MRSA strains in Sokoto where linezolid is not frequently prescribed relates to the hypothesis that any antibiotic within the resistance spectrum of *cfr* gene can trigger its acquisition with relative ease because of the low fitness cost of the multidrug resistant gene²⁷. In other words, the effect of mutation is alleviated by corresponding alterations in group L (Large) ribosomal proteins thus resulting in an increased resistance³⁵.

Presence *cfr* negative strains (47.1%) in this study may have occurred as a result of mutation of the wild type 23S rRNA³⁶. The *cfr* gene is typically situated in mobile genetic elements like on multidrug resistant plasmids which enables fast transfer of the gene into other pathogenic bacteria, susceptible population and back again (cycle)³⁷. Therefore, the *cfr* negative strains may have lost the gene because of its highly mobile nature. Despite the disparity between the results of the phenotypic and genotypic method in terms of LR these MRSA stains clearly did not display wild type susceptibility to linezolid. In essence, the result for this study only confirms the possible mediation of LR by *cfr* gene in PCR positive strains.

Our study suffered several limitations; Firstly, we did not investigate mutations in the 23SrRNA genes and the predisposition LR-MRSA strains to harbour both *cfr* and 23SrRNA genes. Secondly, PCR positive strains were not sequenced to gain insight into the clonality of the circulating strains.

CONCLUSIONS

This is the first report to document *cfr* gene in LR-MRSA strains in Sokoto. The *cfr* gene was found among LR-MRSA isolates of Sokoto state. Because of the ease of its acquisition by *S. aureus*, our study emphasizes the necessity of appropriate surveillance for the bacterial strains harboring *cfr* genes in the hospitals. If *cfr*-mediated linezolid resistance is not properly checked, its phenotypic expression may result in an outbreak of multiple antibiotic resistant strains.

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