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Short Communication

# The emergence of plasmid-borne *cfr*-mediated linezolid resistant-staphylococci in Vietnam

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ABSTRACT

*Objectives:* Linezolid is one of the last resort antibiotics effectively used in the treatment of infections caused by multidrug-resistant Gram-positive bacteria. Recent outbreaks of Linezolid resistance have been the great concern worldwide, while many countries have not experienced it. In this work, we aimed to evaluate the existence of linezolid resistance and further clarify potential resistance mechanism(s) in staphylococcal isolates obtained from the hospital in Vietnam, a country in which linezolid resistance had not been previously detected.

*Methods:* Seventy staphylococcal clinical isolates including MRSA ($n = 63$) and methicillin-resistant coagulase-negative staphylococci (MRCNS, $n = 7$) were collected and analyzed for linezolid resistance. Linezolid-resistant isolates were submitted for whole genome sequencing to search for the resistance determinants.

*Results:* We identified two coagulase-negative staphylococcal isolates that were resistant to linezolid. Whole genome sequencing revealed several alterations in the 23S rRNA and L3, L17, L22, L24, L30 ribosomal proteins. Importantly, both isolates harbour the chloramphenicol/florfenicol resistance (*cfr*) gene on a plasmid. The plasmid was closely identical to the pLRSA417 plasmid that was originally reported in China.

*Conclusions:* To the best of our knowledge, this is the first report of *cfr*-mediated linezolid resistance in clinically isolated staphylococci in Vietnam. We suggest that adequate surveillance is necessary to monitor the dissemination of linezolid resistance among staphylococcal species and other important pathogens.



## 1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has spread globally with the rate of isolation over 50% in many countries [1]. In Vietnam, MRSA accounts for a significant proportion of *S. aureus* infections, estimated as 74.1% of hospital acquired and 30.1% of community associated *S. aureus* infections [2]. The management of staphylococcal infections frequently represents a challenge for clinicians, given the ability of this bacterium to quickly acquire resistance. As a consequence of the dissemination of MRSA strains and the accumulation of several resistant traits, only a few

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antibiotics such as vancomycin and linezolid are considered for use as a last resort antibiotic.

Vancomycin is indicated for use in the treatment of MRSA and other multidrug-resistant Gram-positive bacteria. Vancomycin resistance has not been described in Vietnam although treatment failure was occasionally reported in vancomycin-susceptible MRSA [3]. Linezolid, an oxazolidinone antibiotic firstly introduced in 2000, is effective against multidrug-resistant Gram-positive pathogens including MRSA, vancomycin-resistant *S. aureus* (VRSA), vancomycin-resistant enterococcus (VRE) and penicillin-resistant pneumococci (PRP).

Linezolid, a member of oxazolidinone class antibiotics, inhibits the initiation of protein synthesis by binding to the 50S ribosomal subunit and prohibiting the 70S ribosome assembly [4]. Linezolid resistance is most often due to target site mutations in the domain V region of the 23S rRNA and/or ribosomal proteins such as L3, L4 and L22. These substitutions are often associated with the prolonged and/or intermittent use of linezolid [5]. Furthermore, the acquisition of a plasmid-borne ribosomal methyltransferase gene, the *cfr* gene, results in transferable linezolid resistance. The methyltransferase encoded by the *cfr* gene methylates adenosine at position 2503 in 23S rRNA within the overlapping binding site for phenicols, lincosamides, oxozolidinones, pleuromutilins and streptogramin A conferring the resistance to all of these antibiotics known as PhLOPSA resistance phenotype [6]. The presence of the *cfr* gene together with the ribosomal mutations results in the high-level resistance [5]. Recently, a new oxazolidinone phenicol transferable resistance (*optrA*) gene was also described to be involved in linezolid resistance [7].

The *cfr* gene has been associated with different plasmid vehicles detected in different countries, and the ability of *cfr*-carrying plasmids to be transmitted between strains and species is of global concern. The identification of vectors such as pSCFS3-like plasmids in the United State [8], the pSCFS7-like plasmids in European countries [9,10] or pSS-01-like plasmids in China [11] indicates a geographically distribution of the *cfr*-vectors. It has been speculated that the coagulase-negative staphylococci (CNS) are a reservoir of the *cfr* gene allowing transmission to *S. aureus* and other pathogenic bacteria [12,13]. Although there is limited information about the transmission of *cfr*-carrying plasmid in a clinical environment, *in vitro* results using pSCFS7-like plasmids showed that horizontal gene transfer mechanisms, such as conjugation and transduction, are involved in the transmission among CNS and *S. aureus* strains [13].

In Vietnam, linezolid resistance has not been detected. In this work, we aimed to evaluate the extent of linezolid resistance among the clinical staphylococcal isolates and identify potential resistance mechanism(s).

## 2. Materials and methods

### 2.1. Bacterial isolates and identification

Methicillin-resistant *S. aureus* and other CNS (MRCNS) isolates were collected from Microbiology department, University Medical Center, Ho Chi Minh City, from November 2017 to August 2018. Bacterial identification was performed using the BD Phoenix™ automated identification system and API Staph Identification kit (bioMérieux).

### 2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested by disc diffusion method according to the CLSI 2018 guideline. MIC of vancomycin was determined by broth microdilution method. MICs of linezolid and various antibiotics of the two linezolid-resistant isolates were measured using the MIC Dry Plate (Eiken Chemical Co., Japan).

### 2.3. Whole genome sequencing (WGS)

Two linezolid-resistant coagulase-negative staphylococci (LRCNS) isolated in this study were submitted for WGS. The bacteria were cultured on a tryptic soy agar (TSA) plate supplemented with chloramphenicol 25 mg/L and incubated at 37 °C. Colonies on the plate were suspended in 10 mL of TSB supplemented with chloramphenicol 25 mg/L and incubated for 24 h with shaking. Whole DNA was extracted using GeneJET genomic DNA purification kit (Thermo Scientific) according to the manufacture's instruction, except that cell lysis was facilitated by the addition of lysostaphin (Sigma–Aldrich) at the final concentration of 1 mg/L. WGS was conducted using illumina MiSeq with Nextera XT libraries prepared according to manufacturer's guideline and paired-end 300 bp reads generated with the MiSeq run kit (v3). Short read data were assembled *de novo* using SPAdes (version 3.8.0). Putative gene function annotation was assigned to genes using PROKKA (version 1.12-beta). Assembled genomes of the two LRCNS were deposited in the GenBank database under the accession numbers: SRR9047052 (*Staphylococcus haemolyticus*) and SRR9047051 (*Staphylococcus cohnii*). Resistance genes were

**Table 1**
Antimicrobial resistance genes identified in the two clinical LRCNS.

| Antimicrobial resistance category | Resistance genes | |
|---|---|---|
| | *S. haemolyticus* | *S. cohnii* |
| Aminocoumarin | *alaS* | *alaS* |
| Aminoglycoside | *aacA-aphD*; *aph(3′)-IIIa* | *aacA-aphD*; *ANT(4′)-Ib*; *ANT(9)-Ia* |
| Beta-lactam | *blaZ*, *mecA* | *mecA* |
| Diaminopyrimidine | *dfrC*; *dfrG* | *dfrC*; *dfrG* |
| Fluoroquinolone | *mfd* | *mfd* |
| Isoniazid | *fabI* | *fabI* |
| Lincosamide | *ermC*; *cfrA* | *ermA*; *ermC*; *cfrA* |
| Linezolid | *cfrA* | *cfrA* |
| Macrolide | *ermC*; *cfrA*; *mphC* | *ermA*; *ermC*; *cfrA* |
| Mupirocin | *ileS* | *ileS* |
| Nucleoside | *SAT-4* | – |
| Phenicol | *cfrA* | *cfrA* |
| Streptogramin | *ermC*; *cfrA* | *ermA*; *ermC*; *cfrA* |
| Triclosan | *fabI* | *fabI* |
| Multidrug resistance efflux pump complex | *taeA*, *arlR*, *arlS*, *lmrB*, *mepA*, *mgrA*, *msrC*, *norA*, *norB*, *sav1866*, *vgaALC* | *taeA*, *arlR*, *arlS*, *lmrB*, *mgrA*, *norA*, *norB*, *qacA*, *salA*, *sav1866* |

identified using ARIBA (Antimicrobial Resistance Identification By Assembly; version 2.12.1) [14]. Mutations in the 23S rRNA and 50S ribosomal proteins were identified by sequence alignment using the reference sequences (*S. haemolyticus* JCSC1435, accession no. AP006716 and *S. cohnii* strain FDAARGOS_334, accession no. CP027422).

### 2.4. Detection of cfr and molecular analysis of the cfr-carrying plasmid

The presence of *cfr* in the two LRCNS isolates was revealed by WGS and confirmed by PCR [13,15]. Contigs of the two isolates were aligned with sequences deposited in NCBI to search for the possible *cfr*-carrying plasmid using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The regions flanking the *cfr* gene-carrying contig were confirmed by mapping PCRs using the primers designed based on the available sequence of *cfr*-carrying plasmids. List of primers used in this study are described in Table S2.

## 3. Results

### 3.1. Bacterial identification and antimicrobial resistance profiles of clinical isolates

A total of 70 clinically isolated MRSA ($n = 63$) and MRCNS ($n = 7$) were collected during the research period. Bacterial identification and antibiotic susceptibility of all the isolates are shown in Table S1. All the isolates are resistant to beta-lactam antibiotics (penicillin and cefoxitin) (Table S1) and harbour *mecA* gene (data not shown). None of them were vancomycin resistant. Of note, two CNS (one *S. haemolyticus* and one *S. cohnii*) showed resistance to linezolid (MIC = 128 mg/L and 64 mg/L, respectively) (Tables S1 and S3). Both LRCNS were obtained from blood culture of patients admitted to the intensive care unit (ICU) in the University Medical Center, HCMC.

### 3.2. Sequence analysis of the two LRCNS isolates

Analysis of assembled sequences identified the genes related to various antimicrobial resistances in the two strains as shown in Table 1.

A series of single nucleotide polymorphism (SNP) in the 23S rRNA were found in the two LRCNS. Among the SNPs, alterations G2604T and G2215A were previously described to be responsible for linezolid resistance [16]. We also found mutations in several 50S ribosomal proteins including L3 (Arg-138 → Val, Met-156 → Thr), L17 (Ala-57 → Asp), L24 (Val-94 → Ala), L30 (Lys-49 → Arg) in *S. haemolyticus* and L3 (Ser-158 → Phe), L22 (Cys-99 → Arg) in *S. cohnii*.

### 3.3. Two clinical LRCNS carry the cfr gene on a pLRSA41-like vector

PCR detection of the *cfr* gene in two LRCNS showed positive amplification in both strains (Fig. 1). WGS and aligning contigs with reported sequences in NCBI showed that both LRCNS harbour a similar plasmid having 98.73% (in *S. haemolyticus*) and 99.99% (in *S. cohnii*) identity compared with the pLRSA417 vector (accession no. KJ922127) [17] with the coverage of 99% and 100%, respectively. The plasmid pLRSA417 was firstly detected in the clinical isolated CNS and MRSA in China [18,19]. The *cfr* gene on this plasmid is flanked by the ISEnfa4 segment and a Tn4001-like transposon that is composed of an ISenfa4, the aminoglycoside-resistance gene *aacA-aphD*, and the IS256-like transposon fragment (Fig. 1A). PCR

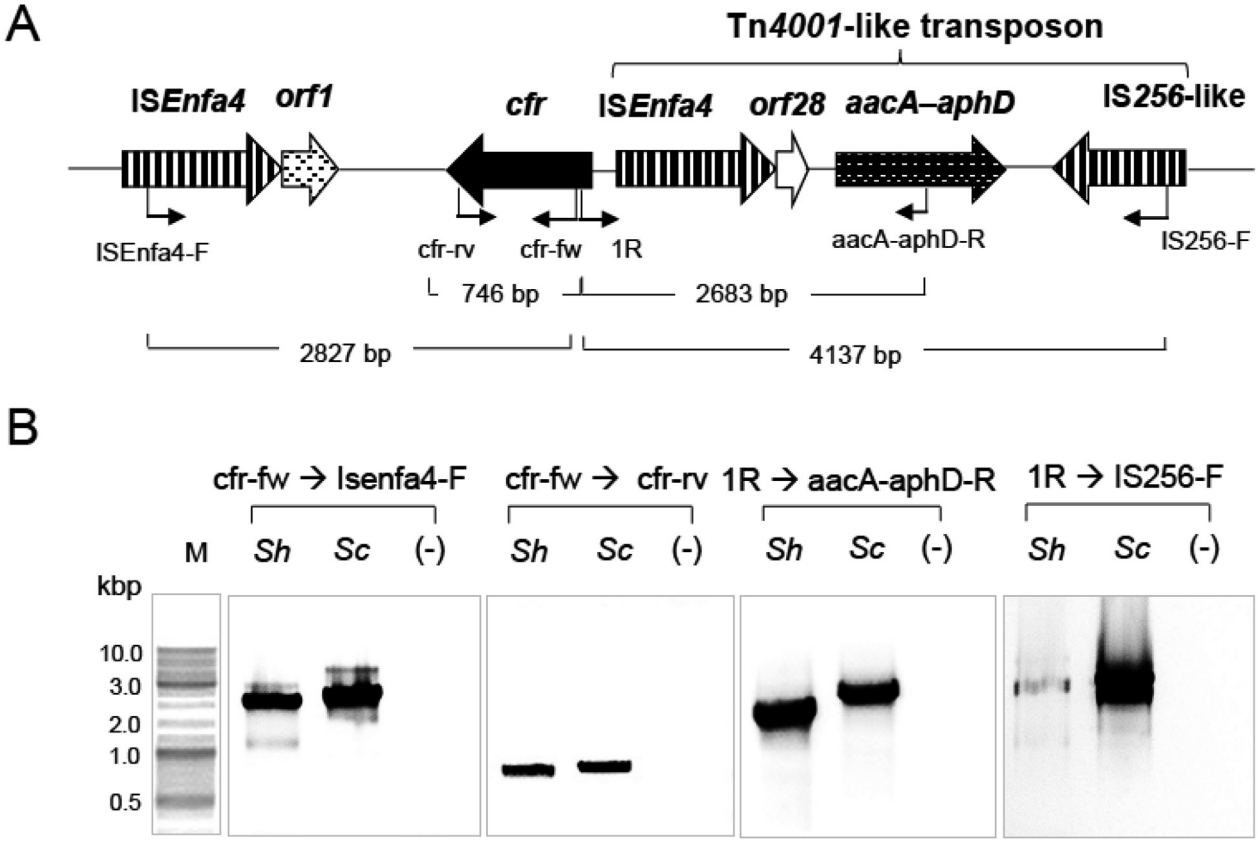


**Fig. 1.** Molecular characterization of genetic structure carrying *cfr* gene in the two linezolid resistant staphylococci. (A) Scheme of mapping PCR determination based on pLRSA417 plasmid sequence; and (B) PCR amplification of genetic environtment surrounding *cfr*. *Sh* – *S. haemolyticus*; *Sc* – *S. cohnii*.

amplification of the genetic environment surrounding the *cfr* gene using the primers designed based on the pLRSA417 sequence showed expected amplicons (Fig. 1). In addition, the presence of conjugative genes (*traA* and *nes*) that locate on the pLRSA417 could also be detected in both isolates as revealed by WGS and confirmed by PCR (data not shown). Thus, the results suggest two LRCNS firstly detected in Vietnam harbour the pLRSA417-like vector which was originally reported in China [17]. The *optrA* gene was not detected in the two isolates (Table 1).

## 4. Discussion

In this study, we identified two *cfr*-positive linezolid-resistant strains among 70 clinical methicillin-resistant *Staphylococcus* sp. Genome sequencing showed a series of alterations in 23S rRNA, and 50S ribosomal proteins L3, L17, L22, L24, L30. Mutations in L3, L4, L22 are frequently found in linezolid resistant clinical isolates [12], but further studies are necessary to clarify the role of substitutions found in L17, L24, L30. In addition to the mutations, both LRCNS harboured the *cfr* gene located in a plasmid, which showed high homology with the previously described pLRSA417-like plasmid [17].

The pLRSA417 plasmid was reported in China, in 2015. It was initially found in five CNS and later was detected in six MRSA from the same hospital [18,19] showing its ability to be transmitted among different staphylococcal strains and species. The *cfr* genetic environment of pLRSA417 comprises a 8,487-bp fragment containing a Tn*4001*-like transposon [17]. This fragment shares an identical sequence to the pSS-01 plasmid, a livestock-associated vector, suggesting the role of this genetic environment in the *cfr* mobilization, which might have reached the clinical staphylococcal population through pLRSA417 plasmid. Once they arrived at the clinical settings, the *cfr* gene would be transferred among the clinical staphylococcal pool, even in the absence of linezolid treatment [17]. Attention should be paid to the fact that linezolid-resistant strains might be selected under the treatment with drugs sharing their target, such as phenicols or macrolides and aminoglycoside.

We must learn from the experience in the countries where *cfr* already became prevalent. In China, *cfr*-mediated linezolid resistance has been described extensively in both Gram-positive and Gram-negative bacteria of animal origin [11]. Subsequently, an emergence of *cfr*-mediated linezolid resistance in clinical setting was reported in 2011 [18]. Linezolid resistance in human origin CNS and the *cfr* gene on different plasmids have frequently been reported among resistant isolates [17,20]. Vietnam seems to be at the early stage of linezolid resistance dissemination. Since many CNS are usually considered as part of the normal skin flora, most clinical laboratories do not test antimicrobial susceptibility of CNS unless isolated from a normally sterile site such as blood. Therefore, the prevalence of linezolid resistance may be under-reported. Improvement of the surveillance of linezolid resistance in CNS not only in the clinical settings but also in the environment is necessary to control the spread of linezolid resistance in the community.

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## Competing interests

None declared.

## Ethical approval

Not required.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.jgar.2020.04.008.

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