

# Mechanisms of linezolid-resistant *Staphylococcus capitis* with the novel mutation C2128T in the 23S rRNA in China

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## Research Article

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# Abstract

**Purpose:** Linezolid is the first oral oxazolidinone antibiotic approved for clinical application. With the widespread use of the linezolid, there are increasing reports of the linezolid-resistant (LZR). The objective of this study was to investigate the molecular characteristics and the potential resistance mechanisms of the LZR *Staphylococcus capitis* isolates from a tertiary hospital in China.

**Methods:** The agar dilution and E-test methods were used to detect antibiotic resistance. The carrying status of the chloramphenicol-florfenicol resistance (*cfr*) gene in the strains was detected by PCR. Whole-genome sequencing (WGS) was used to detect point mutations, L3, L4, and L22 mutations, and also to study the genetic environment of the *cfr* gene and the relationship between strains.

**Results:** A similar pattern in susceptibility profiles were observed in all four *S. capitis* strains, each exhibiting a multidrug-resistant phenotype. A potential novel mutation, C2128T, was identified, and the *cfr* genes of *S. capitis* strains were all positive. Additionally, the same mutation (C2128T and G2600T) was identified in all 23S rRNA sequences of isolates, while the mutation was lacking in the L3, L4, and L22 ribosomal proteins. The genetic environments surrounding *cfr* were identical in all four isolates. A schematic diagram of the phylogenetic tree exhibited that they were closely related to AYP1020, CR01, and TW2795, and a total of seven drug-resistant genes were detected in these strains.

**Conclusions:** The study indicated that the resistance of the *Staphylococcus capitis* strains to linezolid was caused by multiple mechanisms and a potential novel mutation C2128T was identified which may have an impact on bacterial resistance.

## 1. Introduction

*Staphylococcus capitis*, a coagulase-negative staphylococcus (CoNS), is a normal flora of human skin tissue that can cause diseases of the body under certain conditions, causing skin infections, bloodstream infections, and even sepsis<sup>[1, 2]</sup>. In recent years, a wide range of broad-spectrum antibacterial drugs, immunosuppressants, and chemotherapy drugs have been widely used in clinical practice, and various invasive operations have been widely implemented, resulting in an increase in the harvesting rate of bacteria year by year and the continuous improvement of drug resistance. At present, the phenomenon of bacterial drug resistance is increasing at an alarming rate<sup>[3]</sup>. Linezolid is the first oral oxazolidinone antibiotic approved by the U.S. Food and Drug Administration (FDA) for clinical applications in 2000<sup>[4]</sup>. This antibiotic is active against a broad spectrum of gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pneumoniae*, and vancomycin-resistant *Enterococci* (VRE)<sup>[5]</sup>. Linezolid inhibits bacterial protein production by binding to rRNA on the 50S ribosomal subunit<sup>[6]</sup>. Linezolid is primarily used to block the development of the initiation complex during protein synthesis; it can inhibit the formation of the initiation complex but does not affect the elongation or termination of the peptide chain during protein synthesis<sup>[7]</sup>.

The incidence of linezolid resistance is gradually increasing following the widespread use of linezolid. There have been many previous reports of linezolid-resistant (LZR) *S. capitis* infections. The study found that the more well-defined mechanism of change in LZR is the mutation of the 23S rRNA gene target and the acquisition of the chloramphenicol-florfenicol resistance (*cfr*) gene<sup>[8-10]</sup>. 23S rRNA is involved in constituting the bacterial ribosome 50S subunit, which mutates during the action, causing the drug to act on the bacterial ribosome target to change and eventually leads to drug resistance. Gene mutation sites also vary for different strains<sup>[11, 12]</sup>. Mutations in the genes G2447U, and G2576U are currently found to be the most common in *Staphylococcus*<sup>[10, 13]</sup>. The *cfr* gene produces methyltransferase encoding 23S rRNA, which was first discovered in a German cow, in the *Staphylococcal* plasmid, and is considered to be related to the transfer of plasmids between *Staphylococcus*<sup>[14]</sup>. The *cfr* gene can cause methylation at large subunit sites of ribosomes, causing LZR<sup>[15, 16]</sup>.

In addition, LZR *S. aureus* was reported in the first year of clinical use in China<sup>[17]</sup>. Since then, LZR *Staphylococci* isolates have been documented successively in the United States<sup>[18]</sup>, Mexico<sup>[19]</sup>, Japan<sup>[20]</sup>, Spain<sup>[21]</sup>, and Italy<sup>[22]</sup>. Therefore, it is urgent to elaborate the resistance mechanism of LZR in China. This study was conducted to investigate the clinical conditions of LZR *S. capitis* found in clinical infections, thereby exploring its molecular characteristics and mechanisms of resistance, and providing clinical assistance in the prevention of LZR *S. capitis*.

## 2. Materials And Methods

### Bacterial isolates and patients

From 2018 to 2019, 4 linezolid-resistant bacterial strains isolated from patients and associated data were authorized by the ethics committee of the Fourth Affiliated Hospital of Harbin Medical University, China. Before their inclusion in the current study, all patients signed a written informed permission form. The isolates were recovered from patients with chronic obstructive pulmonary disease (COPD), acute coronary syndrome, rectal cancer, and male pelvic abscess. Clinical data on patients were obtained retrospectively for each isolate, including age, gender, prior exposure to linezolid, and the clinical outcome. The data was kept anonymous.

### Antimicrobial susceptibility testing

The antimicrobial agents tested include oxacillin, penicillin, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, erythromycin, clindamycin, quinupristin/dalfopristin, vancomycin, tetracycline, tigecycline, rifampicin, trimethoprim/sulfamethoxazole, and linezolid, as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines<sup>[23]</sup>. The minimal inhibitory concentration (MIC) (VITEK 2 Compact, Biomerieux, France) of every

antibiotic was calculated by the agar dilution MIC method, and the results were interpreted according to the CLSI. The MIC of linezolid was also calculated by E-test (Biomerieux, France), as per instructions provided by the manufacturer. *S. aureus* ATCC 25923 and ATCC 29213 were tested concurrently for quality control (both with linezolid MIC of  $\leq 1$   $\mu\text{g}/\text{ml}$ , Laboratory Department of the Fourth Affiliated Hospital of Harbin Medical University, China).

### Detection of *cfr* by PCR

PCR was used to determine the existence of *cfr* in *S. capitis*. The colonies harvested from agar plates were incubated for 5 minutes in 500 $\mu\text{l}$  of  $\text{H}_2\text{O}$  at a temperature of 100°C. Following 2 minutes of centrifugation, 1 $\mu\text{l}$  of the resultant supernatant was used as a template in the PCR that was conducted using two *cfr*-specific primers (the forward primer: 5'-GAAGCTCTAGCCAACCGTCA-3' and the reverse primer: 5'-TCTACCTGCCCTTCGTTTGC-3', 458 base-pair (bp) amplicon, GenBank reference sequence AM408573). The amplification conditions were as follows: 5 minutes at a temperature of 94°C; 30 cycles with the same temperature for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, and following a final extension at 72°C for 7 minutes. PCR products were imaged by a Visible light transilluminator (Bioteke, Beijing, China)

### Whole-genome sequencing (WGS)

Products of bacterial genomic DNA were sequenced by Illumina HiSeq (Illumina, America) and PacBio RS (Pacific Biosciences, America) after they were extracted and purified by using the purification kit (TaKaRa, Dalian, China). On the basis of the 16S rRNA nucleotide sequence, a phylogenetic analysis was performed. The four strains' representative 16S rRNA nucleotide sequences were compared against other *Staphylococci* strains stored in GenBank (Supplementary materials). Phylogenetic inferences were made using the Neighbor-joining method within the MEGA software (version 7.0.26).

### Genetic environment of the *cfr* gene

The genetic context of the *cfr* gene was determined by WGS. The reference sequence of *S. capitis* pXWZ (GenBank reference sequence MT096435), *S. aureus* pSR01 (GenBank reference sequence CP048644) *S. capitis* p*cfr*-XZ03 (GenBank reference sequence CP077712), *S. xylosus* pSX01 (GenBank reference sequence KP890694) were downloaded from National Center of Biotechnology Information (NCBI). The resistance genes were obtained through the Comprehensive Antibiotic Research Database (CARD) (<https://card.mcmaster.ca/>).

## 3. Results

### 3.1. Characteristics of linezolid-resistant *S. capitis*

A total of 4 linezolid-resistant *S. capitis* isolates were collected. Three strains of linezolid-resistant *S. capitis* were recovered from blood samples, and one strain was recovered from pleural effusion. Table 1 summarized the demographic and clinical characteristics of the patients. All patients had received linezolid for an average of 11.5 days leading up to the isolation of the linezolid-resistant *S. capitis*. A similar pattern in susceptibility profiles was observed in the four *S. capitis* strains. All of the isolates exhibited a multidrug-resistant phenotype (resistant to three or more different antibiotics). Linezolid (MIC 256 mg/L), penicillin, oxacillin, ciprofloxacin, gentamicin, erythromycin, moxifloxacin, and levofloxacin were all resistant to all isolates. The resistance to clindamycin and quinupristin/dalfopristin was variable (Table 2).

### 3.2. Resistance genes and mutations

The PCR screening results were positive for the *cfr* gene in all isolates, which was further confirmed by sequencing. Additionally, an investigation of the 23S rRNA sequences found the identical mutation (C2128T and G2600T) in all isolates, but no alterations in the ribosomal proteins L3, L4, and L22. A potential novel mutation, C2128T was identified in all four linezolid-resistant *S. capitis* isolates (GenBank accession number SUB11152217).

### 3.3. Genetic environment of the *cfr* gene in the plasmids

The genetic environment surrounding *cfr* was similar in all four isolates (Fig. 1). A complete Tn4001-like transposon was identified upstream of *cfr* in the plasmid. Tn4001's aminoglycoside resistance gene pair *aacA-aphD* was flanked by two IS256 elements containing transposase genes. Another copy of the IS256-associated transposase gene was discovered downstream of *cfr*, orientated toward the *cfr* gene and identical in sequence to the right (*cfr*-proximal) transposase gene of the Tn4001-like transposon. This transposase gene is followed by a short open reading frame 1 (ORF1) encoding a protein with similarity to a transcriptional regulator of the helix-turn-helix (HTH) type. The genetic environment surrounding the *cfr* gene exhibited a striking similarity to that of previously reported plasmids, including p*cfr*-XZ03 (100% coverage, 100% identity, CP077712) and pXWZ (100% coverage, 100% identity, MT096435) from *S. capitis* strains, pSR01 (100% coverage, 99.98% identity, CP048646) from *S. aureus* strains, and pSX01 (99% coverage, 99.97% identity, KP890694) from an *S. xylosus* strain. These findings are consistent with the spread of *cfr*-positive pieces between species and genera via various healthcare facilities.

### 3.4. Genetic relationship to other strains and drug-resistant genes

The 16S rRNA genes of the four linezolid-resistant strains were identical to the corresponding region (GenBank accession number SUB11152030), and they were more closely related to AYP1020, CR01, and TW2795 in contrast to other strains (Fig. 2). The comparison with the CARD revealed that the resistance genes carried by four linezolid-resistant strains of *S. capitis* were identical to those shown in Table 3. There were a total of seven drug resistance genes, among which *cfr*, *blaZ*, *qacA*, and *aac(6')-aph(2'')* were located on the plasmids.

Table 1  
Clinical information of patients with linezolid-resistant *Staphylococcus capitis* isolates

Isolate	Age (years)/Gender	Underlying diseases	Date of isolation (dd/mm/yy)	Source	Department	Prior linezolid therapy	Time to isolation of LZR <i>S. capitis</i> (days)	Outcome
701	68/M	COPD	04/08/2018	Blood	ICU	Yes	13	Survived
703	53/M	Acute coronary syndrome	03/09/2018	Blood	Cardiology	Yes	12	Died
708	52/M	Rectal cancer	11/10/2018	Blood	ICU	Yes	8	Survived
709	60/M	Male pelvic abscess	10/07/2019	Hydrothorax	ICU	Yes	13	Survived

Note: ICU, intensive care unit; M, male; COPD, chronic obstructive pulmonary disease; LZR *S. capitis*, linezolid-resistant *Staphylococcus capitis*.

Table 2  
Antibiotic susceptibilities and linezolid resistance mechanisms of linezolid-resistant *Staphylococcus capitis* isolates

Isolate	MIC (mg/L)																Genetic resistance markers				
	LZD	P	OXA	CN	CIP	LEV	MXF	E	DA	QD	VA	TE	TGC	RA	SXT	cfr	23S rRNA	L3	L4	L22	
701	≥ 256	≥ 0.5	≥ 4	≥ 16	≥ 8	4	2	≥ 8	2	1	≤ 0.5	1	0.25	≤ 0.5	20	+	C2128T, G2600T	-	-	-	
703	≥ 256	≥ 0.5	≥ 4	≥ 16	≥ 8	≥ 8	2	≥ 8	≥ 8	2	≤ 0.5	2	0.5	≤ 0.5	20	+	C2128T, G2600T	-	-	-	
708	≥ 256	≥ 0.5	≥ 4	≥ 16	≥ 8	4	2	≥ 8	≥ 8	2	≤ 0.5	2	0.5	≤ 0.5	20	+	C2128T, G2600T	-	-	-	
709	≥ 256	≥ 0.5	≥ 4	≥ 16	≥ 8	4	2	≥ 8	≥ 8	2	≤ 0.5	2	0.5	≤ 0.5	20	+	C2128T, G2600T	-	-	-	

Note: MIC, minimum inhibitory concentration; LZD, linezolid; P, penicillin; OXA, oxacillin; CN, gentamicin; CIP, ciprofloxacin; LEV, levofloxacin; MXF, moxifloxacin; E, erythromycin; DA, clindamycin; QD, quinupristin/dalfopristin; VA, vancomycin; TE, tetracycline; TGC, tigecycline; RA, rifampicin; SXT, trimethoprim/sulfamethoxazole.

Table 3  
Drug resistance genes from WGS

Isolates	Aminoglycoside	Beta-lactam	Macrolide	Oxazolidinone	Phenicol	Disinfectant
701	<i>AadD;aac(6')-aph(2''); aadD; ant(9)-Ia</i>	<i>mecA;blaZ</i>	<i>ermA;cfr</i>	<i>cfr</i>	<i>cfr</i>	<i>qacA</i>
703	<i>AadD;aac(6')-aph(2''); aadD; ant(9)-Ia</i>	<i>mecA;blaZ</i>	<i>ermA;cfr</i>	<i>cfr</i>	<i>cfr</i>	<i>qacA</i>
708	<i>AadD;aac(6')-aph(2''); aadD; ant(9)-Ia</i>	<i>mecA;blaZ</i>	<i>ermA;cfr</i>	<i>cfr</i>	<i>cfr</i>	<i>qacA</i>
709	<i>AadD;aac(6')-aph(2''); aadD; ant(9)-Ia</i>	<i>mecA;blaZ</i>	<i>ermA;cfr</i>	<i>cfr</i>	<i>cfr</i>	<i>qacA</i>

Note: WGS, Whole-genome sequencing.

## 4. Discussion

Linezolid is a synthetic oxazolidone agent that is commonly used to treat gram-positive infections. It exerts its action via inhibiting the synthesis of bacterial protein through binding to ribosomes. Linezolid binds to the cleft composed of 23S rRNA domain V nucleotides of bacteria, and the binding site is almost entirely composed of RNA, so the main mechanism for drug resistance is the mutation of nearby nucleotides, and the target of the *cfr* gene is also the G2503 site of the bacterial ribosomal 23S rRNA domain V<sup>[11]</sup>. Additionally, linezolid resistance has been linked to amino acid changes in the ribosomal proteins L3, L4, and L22<sup>[10]</sup>.

The healthcare settings have seen a notable increase in isolation of drug-resistant strains following extensive use of linezolid. In the current study, we determined the incidence of the *cfr* gene and mutations of C2128T and G2600T in 23S rRNA that existed in linezolid-resistant *S. capitis* isolates recovered from 4 patients from a tertiary hospital in China. In general, *cfr* methyltransferase mediates resistance to the antibiotics targeting the 50S ribosome subunit, including benzoic acid, lincosamide, oxazolidinone, pleural polysaccharide, and streptavidin<sup>[10]</sup>. Thus, *cfr*-carrying *Staphylococci* exhibited a multidrug-resistant phenotype that was consistent with the resistance profile of the isolates in this study. Notably, the plasmids contain the *cfr* gene, which is amenable to horizontal transmission across *Staphylococci*. Previously in a study, it was shown that the *cfr* gene plays a critical role in mediating linezolid resistance. The isolates expressing the *cfr* gene demonstrated significantly higher levels of linezolid resistance than the isolates without the *cfr* gene<sup>[9]</sup>. The observation of a high prevalence of *cfr* in our drug-resistant strains raises the possibility of horizontal gene transfer, which is a reminder of the need for a proactive approach to infection control.

In addition, previous studies have reported that His 146 and Gly155Arg in L3 from a research lab *S. aureus* and a mutation in the conserved region (63KPWRQKGTGRAR74) of the L4 protein are all related to the cross-resistance of *S. pneumoniae*, *S. aureus*, and *C. perfringens* to linezolid [24–26]. However, the L3, L4, and L22 ribosomal proteins in the four linezolid resistant isolates indicated in our investigation did not exhibit a similar resistance mechanism. Furthermore, a unique mutation C2128T in the 23S rRNA gene was observed that is distinct from the typical changes in the central loop of 23S rRNA domain V in most linezolid-resistant strains such as *Enterococcus* and *Staphylococcus*. To the best of our knowledge, resistance to linezolid has been attributed to G2528U, G2576U, and G2505A (*Enterococcus* strains), G2447U and G2576U (*Staphylococcus* strains), and C2534T, G2447T, G2576T, T2504A, C2109T and G2474T (Coagulase-negative Staphylococci strains)<sup>[13]</sup>. Notably, there was an absence of the most prevalent mutation G2576T, in the V region of the 23S rRNA gene in all of our four linezolid-resistant isolates, whereas G2600T mutation was detected in all isolates. Additionally, investigations have discovered a correlation between the prevalence of linezolid resistance and the number of mutations in the copy of the 23S rRNA-encoding gene [27]. The close genetic relatedness of the clones in this study to the CR01 and AYP1020 genomes is surprising, as both are fully susceptible strains. So far, no explanation has been suggested for this unexpected clustering. Both may represent pre-resistance lineages of linezolid clones, but there is presently no epidemiological evidence to substantiate this hypothesis [28].

Since 3 strains of LZR *S. capitis* in this study were recovered from blood samples, it is important for clinicians to perform blood cultures to confirm the presence of bloodstream infections. Timely detection of linezolid-resistant *Staphylococci* in the early stage is of great significance for ensuring optimal antibiotic treatment and limiting the emergence of multidrug-resistant bacteria. A previous report showed that the mean isolation time of linezolid resistant coagulase negative staphylococci (LRCoNS) strains was  $11.0 \pm 8.0$  days in patients following the linezolid treatment, but resistance strains were obtained in a few cases by cross-infection [13]. This may provide more substantial proof for the theory that consumption of linezolid is a potential independent risk factor for the development of LRCoNS strains<sup>[29]</sup>. This is proven in view of the fact that all patients carrying linezolid-resistant *S. capitis* had linezolid medication for an average of 11.5 days prior to the harvesting of the linezolid-resistant *S. capitis* in our investigation. Taking into consideration that the same drug-resistant clones were recovered from these four patients and the time periods during which the strains were harvested, we hypothesize that this could be due to clonal propagation between strains [30]. Whilst the incidence of LZR *Staphylococci* remains low, factors like prolonged hospitalization, numerous interventions, and irrational antibiotic use may hasten the emergence and spread of LZR *Staphylococci*. The discreet use of linezolid and monitoring of staphylococcal resistance is necessary for therapeutic efficacy.

## 5. Conclusion

In conclusion, we analyzed mutations in the 23S rRNA of 4 LZR *S. capitis* strains, combined with sensitive strains isolated at the same time and quality control strains, to identify a potential novel mutation C2128T that may have an impact on linezolid resistance. This also provides new treatment ideas for strengthening the rational use of antibacterial drugs and the prevention and control of hospital infections in the future and minimize the emergence of multi-drug resistance.

## Declarations

### Ethical Approval and consent to participate

All methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols were approved by the ethics committee of the Fourth Affiliated Hospital of Harbin Medical University. The informed consent was obtained from all subjects and/or their legal guardian(s).

### Consent to Publish

Not applicable.

### Acknowledgement

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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### Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

### Author Contributions

Xiao Han: Data analysis and writing original draft preparation; Guiling Zou: Conceptualization and Data curation; Jiaren Liu, Chun Yang, and Xuefei Du: Project administration and Supervision; Guoyu Chen, Zhe Sun, Xinyu Zhang, Yu Sun, and Wanying Zhang: Investigation, Resources, Validation; Xiaofeng Jiang: Writing – review and editing. All authors contributed to the study conception and design and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

### Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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## Figures

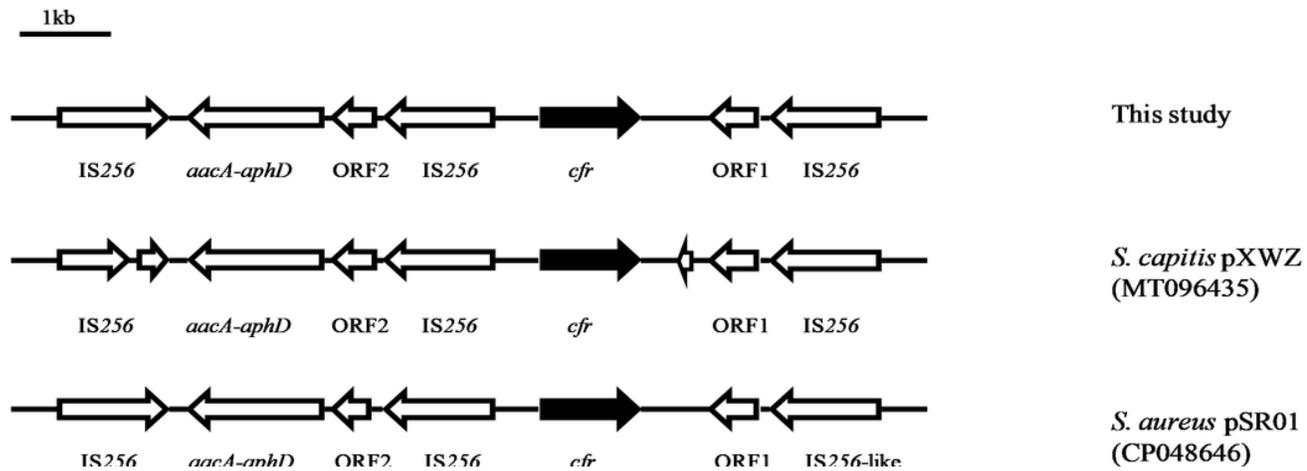


Figure 1

**Schematic diagram of the genetic environment of the *cfr* gene in this study.** The arrows represent the positions and direction of the elements. The *cfr* gene is shown in black.

Figure 2

**Schematic diagram of the phylogenetic tree of the four strains.** The strains of the research group have been marked with emphasis. The strains and their corresponding GenBank accession numbers for 16S rRNA genes are shown following the organism names. Numbers at the branching nodes are percentages of bootstrap values based on 500 replications. Bootstrap values greater than 50% are shown at the branch points. The scale bar represents 0.002 substitutions per nucleotide position.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterials.docx](#)