African Journal of Microbiology Research Vol. 6(23), pp. 5043-5047, 21 June, 2012
Available online at http://www.academicjournals.org/ajmr
DOI: 10.5897/AJMR12.752
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

First report of clinical linezolid-resistant *Staphylococcus cohnii* mediated by the *cfr* gene

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Accepted 28 May, 2012

***cfr* (chloramphenicol-florfenicol resistance) gene, initially associated with chloramphenicol resistance, represents an additionally natural linezolid resistance mechanism, which could be transmitted between species. Coagulase-negative *staphylococci* may be a reservoir of *cfr* gene and *cfr*-carrying isolates may become a serious threat to several potent Gram-positive-active agents. We isolated two linezolid-resistant *Staphylococcus cohnii* from clinical patients in China for the first time. Both isolates were carrying *cfr* gene and no G2576T mutation. They are resistant to quinolones, tetracycline, erythromycin, clindamycin, trimethoprim/sulfamethoxazole and chloramphenicol, and susceptible to vacomycin, teicoplanin, rifampicin, gentamicin, tigecycline, quinupristin/dalfopristin. The minimum inhibitory concentrations (MICs) of linezolid to each *S. cohnii* were more than 256 mg/L; the MICs of vancomycin were 2 mg/L. Our results raise concerns about the future clinical efficacy of several antimicrobial classes; effective measures, such as hygiene, separate and prospective resistance surveillance, should be taken to prevent *cfr* gene transmission.**

Key words: *cfr* (chloramphenicol-florfenicol resistance), resistance, *Staphylococcus cohnii*, linezolid.

INTRODUCTION

Linezolid (LNZ), the first oxazolidinone to be used clinically (Wong et al., 2010), is still effective in the treatment of infections caused by multidrug resistant Gram-positive pathogens, such as vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Kloss et al., 1999; Jones et al., 2007). It is often used for treatrment of complicated infections when other therapies have failed (Ford et al., 1997). However, there comes LNZ resistance not long time after clinical application of LNZ (Tsiodras et al., 2001). In 2008, overall LNZ resistance rate was 0.4% in USA, while rates as low as 0.1% among Gram-positive pathogens from non-USA (Farrell et al., 2009; Jones et al., 2009b). LNZ inhibits protein synthesis by binding to large ribosomal subunit (Lin et al., 1997). LNZ resistance was first associated with mutations in the domain V region of 23 S rRNA genes (Gonzales et al., 2001; Tsiodras et al., 2001; Farrell et al., 2009; Jones et al., 2009a; 2009b). In rare cases, mutations in ribosomal protein L3, L4 have also been associated with LNZ resistance (Locke et al., 2009a, b; Holzel et al., 2010). This type of resistance appears rare, develops slowly because of the redundancy of rRNA genes in bacteria, and is not transferable between pathogenic species. As LNZ is a synthetic compound which does not have natural prototypes, it was initially expected that there is no natural pool of resistance gene which could facilitate the development of clinical resistance. However, *cfr* (chloramphenicol-florfenicol resistnace) gene was first discovered in 2000 during a surveillance study among *staphylococci* from animals (Schwarz et al., 2000) and has recently been reported as nonmutation and acquisition of a natural resistance gene. The product of the *cfr* gene is a methyltransferase that catalyzes methylation of A2503 in the 23S rRNA gene of the large ribosomal subunit, conferring resistant to chloramphenicol, florfenicol, clidamycin, and LNZ (Kehrenberg et al., 2005; Toh et al., 2007). Cfr is

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generally plasmid borne and transpose associated and therefore likely to be horizontally transmitted (Kehrenberg et al., 2007). To date, some cases of LNZ resistant MRSA has been reported (Locke et al., 2009b, 2010a, b; Morales et al., 2010; Nannini et al., 2010; Sanchez Garcia et al., 2010; Farrell et al., 2011), little attention has been put to LNZ resistant coagulase-negative *Staphylococcus* (CoNS) (Petinaki et al., 2009; Bongiorno et al., 2010; Mendes et al., 2010). The presence of CoNS maybe becomes a reservoir of *cfr*-mediated resistance. Horizontal transmission of resistance is a serious threat, because the *cfr* gene can also be transmitted between species, such as *Staphylococcus cohnii* and *Staphylococcus epidermidis*, which although not pathogenic, could become a reservoir for resistance genes. This mode of transmission is difficult to prevent and stop. We reported here the first example of LNZ resistant *S. cohnii* (due to or carrying) by *cfr* gene which is different from the report from Greece (Petinaki et al., 2009) which LNZ resistant *S. cohnii* were caused by G2576T mutation.

MATERIALS AND METHODS

Bacterial isolates

We studied two LNZ-resistant clinical isolates from blood cultures of hospitalized patients at affiliated First Hospital of China Medical University, a 2500-bed hospital in northeastern China. The isolates were identified by the VITEK 2 system (Bio-Merieux, France) and confirmed by the sequencing of the *tuf* gene, as previously described (Kontos et al., 2003), one of which was obtained from patients in the ICU ward, the other was from patients in the respiratory ward.

Antimicrobial susceptibility testing

Susceptibility testing and interpretation for various antimicrobial agents was performed by disk diffusion following the 2012 guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012). The minimum inhibitory concentrations (MICs) of tigecycline, vancomycin, gentamicin, erythromycin, ciprofloxacin, clindamycin, LNZ were determined using VITEK2 panels GP67 (Bio-Merieux, France). The MICs of LNZ and vancomycin were further confirmed using the E-test (AB BIODISK). Resistance to LNZ was defined as an MIC≥8 mg/L. Sensitivity to vancomycin was defined as an MIC ≤4 mg/L. ATCC29213 was the reference strain for quality control in determinations of the MIC. ATCC25923 was the reference strain for quality control in the disk diffusion.

Molecular typing

The two strains were genotyped by PFGE following the protocol (Murchan et al., 2003) with minor modifications. Briefly, the agarose plugs of strains were treated with proteinaseK/lysostaphin and digested with 30U of *Sma*I restriction enzyme (New England Biolabs). The plugs were loaded into 1% agarose gels and electrophoresed using a CHEF-DRIII apparatus (Bio-Rad Laboratories) in 0.5 × TBE buffer. Run time was 20 h with an initial switch time of 5 s and a final switch time of 40 s. Voltage was set at 6 V/cm and the angle at 120°. The dendrogram was constructed using the Dice correlation coefficient and the unweighted pair group method with arithmetic mean with a 3% band tolerance. Isolates showing a similarity coefficient of ≥80% were considered to be genetically related.

Amplification of the cfr gene using polymerase chain reaction (PCR)

The presence of *cfr* was assessed by PCR with use of oligonucleotide primers as previously described by Kehrenberg and Schwarz (2006), forward, 5' - TGAAGTATAAAGCAGGTTGGGAGTCA -3', and reverse, 5'- ACCATATAATTGACCACAAGCAGC -3'. We extracted genomic DNA from the tested strains as templates according to the manufacture (Biospin bacteria Genomic DNA Extraction Kit). PCR conditions were as follows: Initial denaturation at 94°C for 2 min was followed by denaturation at 94°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s for 30 cycles. There was a final extension of 7 min at 72°C.

Amplification of domain V of 23S rRNA gene by PCR

Domain V of the 23S rRNA gene was amplified by PCR using the primers forward, 5' - GCGGTCGCCTCCTAAAAG -3', and reverse, 5'- ATCCCGGTCCTC TCGTACTA -3'. The 420 bp amplicons were treated with restriction enzyme *Nhe*I (MBI Fermentas) and analysed by agarose gel electrophoresis to confirm the absence/presence of the most frequent mutation G2576T, which generates a new *Nhe*I site (Pillai et al., 2002).

Sequences

Fragments were also sequenced for confirmation. Amplified DNA was purified using the Ultra clean PCR Clean-up kit (Biospin). Sequencing was performed in a thermal cycler with use of the dRhodamine Dye Terminator Cycle sequencing Ready Reaction Kit (Applied Biosystems), the same primers as for the PCR, and 2~4 µL of the purified DNA.

RESULTS

Patients and antibiotic susceptibility

The two LNZ-resistant isolates from two hospitalized patients were identified as *S. cohnii* subsp. ureolyticus. Of the two patients, one was male, 34 years old, admitted on 17/11/2009 in respiratory ward, infection occurred at 20/11/2009, and he was dead at 22/11/2009 via serial application of LNZ, imipenem, third cephalosporin, quinolones, enzyme inhibitor. The other patients was male, 60 years old, admitted on 30/11/2009 in ICU ward, infection occurred at 20/12/2009, and he was improved under treatment with glycopeptide, imipenem, LNZ, enzyme inhibitor. These two isolates had the same resistance profile, were susceptible to vacomycin, teicoplanin, rifampicin, gentamicin, tigecycline, quinupristin/dalfopristin, and were resistant to quinolones, tetracycline, macrolides, trimethoprim/sulfamethoxazole and chloramphenicol (Table 1). It is not listed that the MICs of gentamicin, tigecycline, quinupristin/dalfopristin,

Table 1. Antibiotic susceptibility to the linezolid resistant *S. cohnii.*

Antibiotic(μg)	060905190-1		060905673-1	
	mm	I	mm	I
Ciprofloxacin	6	R	6	R
Levofloxacin	6	R	6	R
Linezolid	6	R	6	R
Vancomycin	19	S	18	S
Teicoplanin	17	S	16	S
Rifampicin	38	S	36	S
Tetracycline	11	R	11	R
Erythromycin	6	R	6	R
Clindamycin	6	R	6	R
Chloramphenicol	6	R	6	R

I, Interpretation; mm, bacteria restriction zone diameter; S, Susceptible; R, Resistant.

trimethoprim/sulfamethoxazole tested by VITEK2. The MICs of LNZ to each *S. cohnii* was more than 256 mg/L; the MICs of vancomycin to each *S. cohnii* was 2 mg/L.

Clonal relationship and resistance mechanism to linezolid

PFGE analysis of the two strains, we found that they were clonally related, belonging to the same clone. PCR of the *cfr* gene was performed to ensure the resistance mechanism (data not shown). The two resistant isolates showed an amplification band of the expected size (746 bp), which was compatible with the *cfr* fragment. To confirm the nature of the amplified DNA, the PCR products were chosen for sequencing, and sequences were identical to the *cfr* sequence of *Staphylococcus sciuri* under GenBank accession no. NC_005076.1 (100% identity) and *Enterococcus faecalis* under GenBank accession no. NC_014508.2 (100% identity). To further confirm the mechanism and rule out mutations in domain V of 23S rRNA, the isolates underwent PCR with specific primer sets for domain V of 23S rRNA, PCR products were digested by *Nhe*I, no mutation of G2576T was found (data not shown). Given that the G2576T mutation is the common mutation site in the domain V region described as conferring LNZ-resistance among clinical bacterial isolates and that the 23S rRNA gene is highly conserved among bacteria, this restriction enzyme method may be useful in determining the mutation in other LNZ-resistant bacterial species for which a complete genomic sequence is not available.

DISCUSSION

Clinical LNZ-resistant staphylococcal strain came (Tsiodras et al., 2001) only 1 year after the clinical application of LNZ. Subsequently, LNZ-resistant enterococci and staphylococci have been sporadically isolated all over the world (Bonora et al., 2006; Jones et al., 2009b). LNZ-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 LNZ resistance gene in staphylococci adds a new dimension to the threat to the clinical use of several antimicrobials, including LNZ and clindamycin. This article reports, for the first time to the best of our knowledge, that 2 of *cfr*-mediated LNZ-resistant *S. cohnii* isolates from blood cultures of clinical patients. To date, there are only three studies associated with LNZ-resistant *S. cohnii*, which had either different mechanism or zoonotic source. In Greece (Petinaki et al., 2009), four of LNZ-resistant *S. cohnii subsp*. ureolyticus isolated from blood of variant patients all carried the G2576T mutation and no isolates carried the *cfr* gene. In an Indian study (Peer et al., 2011), there came one LNZ-resistant *S. cohnii subsp.cohnii* with MIC of >256 mg/L from a burn patient, in which the mechanisms of resistance were not studied further. Recently in a Chinese swine farm, *cfr*-carrying *S. cohnii* were found from swine (Wang et al., 2012). Our study, for the first time, shows *cfr*-carrying *S. cohnii subsp. ureolyticus* isolated from clinical patients, which indicated that infection caused by *S. cohnii* carried *cfr* gene is transferring from animals to human and attention should be paid to the fact that *cfr* gene could disseminate to the other staphylococci, which would infect patients and result in outbreak of multidrug-resistant infection in the hospital and may spread to community as well. As we have not isolated LNZ-resistant *Staphylococcus aureus* until now, the dissemination of *cfr* gene should cause our attention to take measurement to prevent its spreading.

The two strains' resistant profile was similar to other

reports of *cfr*-mediated LNZ-resistant staphylococci (Toh et al., 2007; Mendes et al., 2010; Morales et al., 2010), such as resistance to erythromycin, ciprofloxacin, clindamycin, trimethoprim/sulfamethoxazole, chloramphenicol and susceptibility to vancomycin, tigecycline, teicoplanin.

Most of the LNZ resistance reported in staphylococci has emerged after prolonged exposure to LNZ. However, some LNZ resistant isolates have been isolated from patients with no prior LNZ exposure (Gales et al., 2006; Mendes et al., 2008). *cfr* gene transmission may be have not to depend on LNZ application and still lead to acquisition of LNZ resistance. In our study, although the 2 patients have been treated with LNZ before LNZ-resistant *S. cohnii* was isolated, application of LNZ, which did not lead to the mutation in 23S rRNA in our study, would not be the reason of appearance of LNZ-resistant *S. cohnii.* The dissemination of *cfr* gene was an important factor.

The acquisition of a LNZ resistant mechanism based on a modification of A2503 mediated by *cfr* gene localized on a transferable element, indicates a potential to disseminate among Gram-positive pathogenic strains. This gene are also resistant to phenicols, lincosamides, pleuromutilins and streptogramin A class antibiotics (Long et al., 2006), as well as macrolides (Smith and Mankin, 2008), 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 multiply the risk of development of LNZ-resistant strains. Our results raise concerns about the future clinical efficacy of several antimicrobial classes; effective measures, such as hygiene, separate and prospective resistance surveillance, should be taken to prevent *cfr* gene transmission.

ACKNOWLEDGEMENT

This project was financially supported by the National Natural Science Foundation of China (Grant Nos. 81101290).

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