Coexistence of multiple antimicrobial-resistance genes in a carbapenem-resistant *Citrobacter freundii* clinical isolate from China

Citrobacter freundii is a nosocomial pathogen associated with diarrhoea, septicaemia, meningitis, and urinary tract and respiratory system infection. Recently, there has been an increase in infections caused by multidrug-resistant *C. freundii* isolates, especially carbapenem-resistant isolates (Lee *et al.*, 2005). *Klebsiella pneumoniae* carbapenemase (KPC)-type enzymes and metallo- β -lactamases are common causes of carbapenem resistance in members of the *Enterobacteriaceae* worldwide (Kitchel *et al.*, 2009; Cendejas *et al.*, 2010). KPC-2 has been found in many *Enterobacteriaceae* species, including *C. freundii*, in China (Shen *et al.*, 2009; Zhang *et al.*, 2008). In this report, we investigated the multiple antimicrobial-resistance genes in a carbapenem-resistant *C. freundii* clinical isolate from a Chinese hospital. *C. freundii* strain NC118 was isolated from the sputum of a 23-year-old male patient hospitalized for acute lymphocytic leukaemia in April 2009 at the second affiliated hospital of Nanchang University, China. A week after admission to hospital, the patient began to cough and developed pneumonia verified by X-ray. Due to haemoptysis and lung infection, the patient died on hospital day 12. Complete biochemical identification of *C. freundii* strain NC118 was performed with the Vitek fully automated microbiology analyser system and an API 20E kit (bioMérieux). Prior to collection of the sample for culture, the patient was treated with intravenous imipenem and etimicin. *C. freundii* strain NC118 was resistant to most clinically used antimicrobial agents, except tetracycline, as determined by disc diffusion testing according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2008), including ampicillin, cefoperazone, cefotaxime, ceftazidime, aztreonam, cefoxitin, imipenem, meropenem, gentamicin, tobramycin, amikacin, ciprofloxacin, levofloxacin and

trimethoprim-sulfamethoxazole. MICs of antimicrobial agents determined by E-test disc diffusion were as follows ($\mu\text{g ml}^{-1}$): cefotaxime, 256; ceftazidime, 256; aztreonam, 256; cefoxitin, >256; imipenem, 16; meropenem, 16; gentamicin, 1024; tobramycin, 1024; amikacin, >256; ciprofloxacin, >256; trimethoprim-sulfamethoxazole, >32; and tetracycline, 4. PCR and DNA sequencing were used for detecting the antimicrobial-resistance genes, including β -lactamase genes, plasmid-borne quinolone-resistance determinants, and 16S rRNA methylase genes. *bla*_{KPC-2} was found in *C. freundii* strain NC118 by PCR and direct DNA sequencing of both strands with an ABI PRISM 3100 genetic analyser (Applied Biosystems). *qnr* genes including *qnrA*, *qnrB* and *qnrS* were determined by multiplex PCR as described previously (Robicsek *et al.*, 2006). *C. freundii* strain NC118 was positive for *qnrA* and *qnrB*. *qnrA*- and *qnrB*-positive PCR products were sequenced and shown to match *qnrA1* and *qnrB1*. *aac(6')-Ib-cr* was detected in the isolate by PCR and direct DNA sequencing (Park *et al.*, 2006a). Extended-spectrum β -lactamase genes were screened by PCR as described previously (Yu *et al.*, 2007). DNA sequencing of both strands was performed by the direct sequencing method. *bla*_{TEM-1}, *bla*_{CTX-M-3} and *bla*_{CTX-M-14} were found. Since *C. freundii* strain NC118 was highly resistant to cefoxitin (MIC >256 $\mu\text{g ml}^{-1}$), plasmid-borne AmpC-lactamase genes were sought by using a multiplex PCR as described previously (Perez-Perez & Hanson, 2002). The isolate was positive for a *bla*_{CMY}-like gene by PCR and DNA sequencing. The entire coding region of the *bla*_{CMY}-like gene was amplified with primers *bla*_{CMY}-F (5'-CAGCAAAAATAA-TCAAAACCGCAAG-3') and *bla*_{CMY}-R (5'-GTATTGCCGGAATTACTCAAGAG-CG-3'). The GenBank accession number of the sequence of the whole 1146 bp *bla*_{CMY}-

like gene obtained is GQ402541. This encoded a new CMY variant designated CMY-49 by the β -lactamase database (<http://www.lahey.org/Studies/other.asp#table1>). At the protein sequence level, CMY-49 had the highest similarity of 98% (376/381) with CMY-45 (ACU00152.1). Compared with CMY-2 (PABU97164), CMY-49 has 14 amino acid substitutions. 16S rRNA methylase genes, including *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA*, were determined by PCR and DNA sequencing as described previously (Yu *et al.*, 2009). Only *armA* was found in the isolate. In order to determine whether the plasmid-bearing resistance was transferable, a conjugation experiment was carried out in Luria-Bertani broth with *Escherichia coli* J53 as the recipient. Transconjugants were selected on tryptic soy agar plates containing sodium azide (100 $\mu\text{g ml}^{-1}$) for counterselection and ampicillin (10 $\mu\text{g ml}^{-1}$), ciprofloxacin (0.5 $\mu\text{g ml}^{-1}$) and amikacin (10 $\mu\text{g ml}^{-1}$) to select for plasmid-encoded resistance. Repeated attempts to acquire transconjugants containing plasmid-encoded resistance were unsuccessful. Plasmid DNA of *C. freundii* strain NC118 was extracted with the Qiagen Plasmid Midi kit according to the manufacturer's instructions. A transformation experiment was used to further investigate plasmid-encoded resistance. Cefotaxime (10 $\mu\text{g ml}^{-1}$), cefoxitin (10 $\mu\text{g ml}^{-1}$), ciprofloxacin (0.5 $\mu\text{g ml}^{-1}$) and amikacin (10 $\mu\text{g ml}^{-1}$) were used for selecting transformants with plasmid-encoded resistance determinants. Although the transformants harbouring quinolone-resistance determinants *bla*_{CTX-M}, *bla*_{KPC-2} and *armA* were obtained, acquiring the transformant harbouring *bla*_{CMY-49} was unsuccessful after repeated attempts. *bla*_{CMY-49} was probably located on the chromosome of *C. freundii* strain NC118. The presence of class 1 integrons was determined by PCR with primers 5'CS (5'-GGCATCCAAGCAGCAAGC-3') and

3'CS (5'-AAGCAGACTTGACCTGAT-3') located in the 5' and 3' conserved segments. Only an approximate 2 kb PCR fragment was obtained from the isolate. The class 1 integron contained a 5' conserved region, *dfrA12* associated with resistance to trimethoprim, *orfF*, *aadA2* conferring resistance to streptomycin and spectinomycin antibiotics and a 3' conserved region.

bla_{KPC-2} has been found coexisting with extended-spectrum β -lactamase genes and *ampC* in *C. freundii* isolates from several countries including China (Rasheed *et al.*, 2008; Zhang *et al.*, 2008). 16S rRNA methylase genes and plasmid-borne quinolone-resistance determinants have also been found in *C. freundii* isolates (Park *et al.*, 2006b; Tamang *et al.*, 2008). However, *bla_{KPC-2}* coexisting with 16S rRNA methylase genes and plasmid-borne quinolone-resistance determinants in the same *C. freundii* strain has not been reported before to our knowledge.

In conclusion, a total of 11 antimicrobial-resistance genes, *bla_{KPC-2}*, *qnrA1*, *qnrB1*, *aac(6')-Ib-cr*, *bla_{TEM-1}*, *bla_{CTX-M-3}*, *bla_{CTX-M-14}*, *armA*, *aadA2*, *dfrA12* and *bla_{CMY-49}*, were simultaneously detected in *C. freundii* strain NC118, which resulted in *C. freundii* strain NC118 being resistant to most of the clinically used antimicrobial agents.

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Jingye Pan,^{1†} Longhua Hu,^{2†}
Fangyou Yu,³ Chong Chen¹
and Xueqing Zhang³

¹Department of Intensive Care Unit, The First Affiliated Hospital of Wenzhou

Medical College, Wenzhou 325000, PR China

²Department of Laboratory Medicine, The Second Affiliated Hospital of Nanchang University, Nanchang 336000, PR China

³Department of Laboratory Medicine, The First Affiliated Hospital of Wenzhou Medical College, Wenzhou 325000, PR China

Correspondence: Fangyou Yu
(wjxyfy@163.com)

†These authors contributed equally to this work.

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