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

Citrobacter freundii is a nosocomial pathogen associated with diarrhea, sepsicaemia, 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 (μg 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. blaKPC-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. acc(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. blaTEM-1, blaCTX-M-3 and blaCTX-M-14 were found. Since C. freundii strain NC118 was highly resistant to cefoxitin (MIC >256 μg 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 blaCMY-like gene by PCR and DNA sequencing. The entire coding region of the blaCMY-like gene was amplified with primers blaCMY-F (5’-CAGCAGAAAATATTCAAACCGCAAAG-3’) and blaCMY-R (5’-GATTGCGGAATTCGAGCGC-3’). The GenBank accession number of the whole 1146 bp blaCMY-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 previously (Yu et al., 2009). Only armA was found in the isolate. In order to determine whether the plasmid-borne 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 μg ml⁻¹) for counterselection and ampicillin (10 μg ml⁻¹), ciprofloxacin (0.5 μg ml⁻¹) and amikacin (10 μg 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 μg ml⁻¹), cefoxitin (10 μg ml⁻¹), ciprofloxacin (0.5 μg ml⁻¹) and amikacin (10 μg ml⁻¹) were used for selecting transformants with plasmid-encoded resistance determinants. Although the transformants harbouring quinolone-resistance determinants blaCTX-M, blaKPC-2 and armA were obtained, acquiring the transformant harbouring blaCMY-49 was unsuccessful after repeated attempts. blaCMY-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′-GGCATCAAGCAGCAAGCG-3′ and...
3’CS (5’-AAGCAGACTTGACCTGAT-3’) located in the 3’ and 3’ conserved segments. Only an approximate 2 kb PCR fragment was obtained from the isolate. The class 1 integron contained a 3’ conserved region, dfrA12 associated with resistance to trimethoprim, orfB, aadA2 conferring resistance to streptomycin and spectinomycin antibiotics and a 3’ conserved region.

blaKPC-2 has been found coexisting with extended-spectrum β-lactamases 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, blaKPC-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-resistant genes, blaKPC-2, qnrA1, qnrB1, aac(6’)-Ib-cr, blaTEM-1, blaCTX-M-3, blaCTX-M-14, armA, aadA2, dfrA12 and blaCMY-49, were simultaneously detected in C. freundii strain NC118, which resulted in C. freundii strain NC118 being resistant to multiple β-lactam-hydrolysing enzymes and decreased porin expression. None of the β-lactamase genes were detected in C. freundii strain NC118.

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