INTRODUCTION

*Klebsiella ozaenae*, a Gram-negative bacillus, is described as a respiratory tract and oral colonizer. It has been reported that *K. ozaenae* can cause invasive infections, including otitis media and mastoiditis, urinary tract infections, soft tissue infections and bacteremia associated with neutropenia, pneumonia and meningitis (Goldstein *et al.*, 1978), and even mortality. *K. ozaenae* is naturally sensitive or intermediately sensitive to most antibiotics, such as cephalosporins, aminoglycosides, quinolones, tetracyclines and cotrimoxazole (Stock & Wiedemann, 2001). However, one highly multi-drug-resistant strain of *K. ozaenae* was recently isolated from a 48-year-old male patient in the ICU of a Chinese tertiary hospital. In this study, we report the antibiotic-resistance phenotype and genetic characteristics of this strain.

METHODS

**Bacterial strain identification.** The *K. ozaenae* strain was isolated from the sputum specimen of a 48-year-old male patient in an ICU. The strain was identified by using the Vitek-2 Compact System with GN13 cards and resistance genes were evaluated by PCR and gene sequencing. The strain was resistant to most of the β-lactams and quinolones tested and carried several antibiotic resistance genes, including *bla*<sub>KPC-2</sub>, *bla*<sub>TEM-98</sub>, *bla*<sub>CTX-M-3</sub>, *bla*<sub>SHV-26</sub> and *qnr*S. To our knowledge, this is the first report of β-lactam and quinolone resistance genes co-existing in a *K. ozaenae* strain in China.

**β-lactamase determination.** *Klebsiella pneumoniae* carbapenemase (KPC) production was determined by a Modified Hodge Test (MHT) with an ertapenem disk (10 μg, Oxoid) according to the guidelines of the CLSI. A KPC inhibition test was performed with an additional 10 μl phenylboronic acid (PBA, 300 mg l<sup>−1</sup>) added to the ertapenem disk (Pasteran *et al.*, 2010). Metallo-β-lactamase (MBL) production was evaluated by an imipenem/imipenem–EDTA combined-disk diffusion test (DDT) and an imipenem/EDTA double-disk synergy test (DDST) as described previously (Franklin *et al.*, 2006; Kim *et al.*, 2007). Extended spectrum β-lactamase (ESBL) detection was performed according to CLSI guidelines. A zone of inhibition for ceftaxime/clavulanic-acid discs (Oxoid) 5 mm greater than ceftaxime discs (Oxoid) was interpreted as a positive result for ESBLs. Strains of *K. pneumoniae*, producing KPC-2, and *Acinetobacter baumannii*, expressing VIM-2, were used as positive controls and *E. coli* ATCC 25922 was used as a negative control.

**PCR and amplified fragment sequencing.** Genomic DNA for PCRs was prepared by using the boiling method. The presence of β-lactamase and quinolone-resistance-related genes, including *bla*<sub>KPC</sub>, *bla*<sub>TEM</sub>, *bla*<sub>MDR</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-3</sub>, *bla*<sub>SHV-26</sub>, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-3</sub>, *qnr*A, *qnr*B, *qnr*C, *qnr*D, *qnr*S, *qepA*, *aac(6’)-Ib-cr*, *ggy*A, *par*C, and transferable element *intI* were determined by PCR. The PCR parameters used for all amplifications were as follows: an initial step at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s, and a final elongation step at 72 °C for 5 min. The primers used (SBS Gentech) were described previously (Brisse *et al.*, 1999; Ellington *et al.*, 2007; Hata *et al.*, 2005; Huang *et al.*, 2009; Jeon *et al.*, 2005; Koelman *et al.*, 2001; Labbate *et al.*, 2009; Park *et al.*, 2006; Tran *et al.*, 2005; Walther-Rasmussen & Høiby, 2006; Wu *et al.*, 2007; Zhou *et al.*, 2007). Other PCR components, including *Taq* polymerase, dNTPs and PCR buffer, were also provided by SBS Gentech. Amplified fragments were sequenced by Invitrogen (Shanghai) and Sangon Biotech and analysis was carried out using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Positive controls were generously provided by Professor Yunsong Yu (First Affiliated Hospital of Zhejiang University), and Professor Minggui Wang (Fudan University).
RESULTS

Strain identification

The strain, which was mucus-producing and foul-smelling, was identified as *K. ozaenae* by the Vitek-2 Compact System. About 1.4 kb of the 16S rRNA gene of this isolate was amplified and sequenced; it shared 99% sequence similarity with *K. ozaenae* ATCC 11296. Based on its biological and biochemical characteristics and the results of 16S rRNA gene sequencing, the isolate was confirmed as *K. ozaenae*.

Antibiotic susceptibility

The strain of *K. ozaenae* isolated in this study was resistant to most of the clinical antimicrobials tested, including imipenem, ertapenem, ampicillin/sulbactam, piperacillin–tazobactam, aztreonam, ceftazidime, cefepime, levofloxacin and ciprofloxacin, but it was sensitive to gentamicin with an MIC < 1 μg ml⁻¹. The MICs obtained using the Vitek-2 compact system with AST-GN13 cards are shown in Table 1.

Phenotype detection of β-lactamase

In the MHT, a strong positive result was obtained. But in KPC inhibition tests, no positive result was observed, although this β-lactamase was inhibited by the addition of PBA. The strain was positive for the ESBL phenotype in screening but no positive result was discovered from the DDT and DDST.

Antibiotic resistance genes

A ~950 bp amplified fragment was obtained by PCR that was 100% identical to the partial sequence of the *K. pneumoniae* plasmid *bla*KPC-2 gene (GenBank accession no. EU594576), according to sequence analysis. Another three β-lactamase-encoding genes were identified, which shared the same deduced amino sequences as TEM-98, SHV-26 and CTX-M-3. For the quinolone-resistance-related genes, the strain was positive for *qnrS*, *aac(6’)-Ib*, *gyrA* and *parC* genes. No nucleotide acid mutations in the *qnrS* and *aac(6’)-Ib* genes were found compared with the published sequences (GenBank nos AB187515 and AF479774). Compared with the corresponding sequence of *E. coli*, no amino acid substitutions were found coded in the *gyrA* and *parC* gene sequences (Brisse *et al.*, 1999). An *intI* gene fragment of expected size was obtained by PCR but PCR results were negative for the following antibiotic-resistance genes: *bla*TEM, *bla*IMP, *bla*SHV, *bla*GIM, *bla*TEM, *bla*OXA-23*, *bla*OXA-24, *bla*OXA-58*, *bla*CTX-M-2*, *bla*CTX-M-9*, *qnrA*, *qnrB*, *qnrC*, *qnrD* and *qepA*.

DISCUSSION

*K. ozaenae* has been described as a respiratory tract and oral colonizer (Goldstein *et al.*, 1978). The strain of *K. ozaenae* that was isolated from the sputum specimen of a 48-year-old male patient was mucus-producing and foul-smelling.

This *K. ozaenae* isolate was resistant to most cephalosporins, including cefotetan, cefazolin, ceftriaxone, ceftazidime and cefepime with MICs of 16, ≥ 64, ≥ 64, ≥ 64 and 16 μg ml⁻¹, respectively. The strain had an ESBL-positive phenotype with TEM-, SHV- and CTX-M-type β-lactamase-encoding genes detected by PCR. According to the sequence analysis, these genes were *bla*TEM-98, *bla*SHV-26, and *bla*CTX-M-3 respectively, which was in agreement with previous reports (Huang *et al.*, 2009). The presence of ESBL genes in such strains may pose a threat to this class of antibiotic.

The strain of *K. ozaenae* investigated in this study expressed resistance to imipenem and ertapenem (MICs ≥ 16 and ≥ 8 μg ml⁻¹, respectively). The MHT resulted in the detection of a KPC phenotype, which could be inhibited by the addition of PBA. MBL screening was negative both in DDTs and DDSTs. These results strongly indicated that this isolate produced KPC-type β-lactamases. The strain was tested for the presence of carbapenemase-encoding genes, including *bla*KPC, *bla*VIM, *bla*IM, *bla*GIM, *bla*SIM, *bla*SPM, *bla*OXA-23*, *bla*OXA-24 and *bla*OXA-58* of all of which gave negative results by PCR except for the *bla*KPC gene, which was positive. According to sequence analysis, this gene shared 100% sequence similarity with the *K. pneumoniae* *bla*KPC-2 gene (GenBank no. EU594576). A previous study reported that the *bla*KPC-2 gene is located within a 10 kb transposon named Tra4401 on a plasmid of *K. pneumoniae* (Naas *et al.*, 2008). Whether the *bla*KPC-2 gene of *K. ozaenae* is plasmid-borne requires further investigation.

The MICs of levofloxacin, lirofloxacin for this strain, obtained using the Vitek-2 compact system with GN13 cards were 32 μg ml⁻¹, 32 μg ml⁻¹, 16 μg ml⁻¹, 16 μg ml⁻¹, 64 μg ml⁻¹, 64 μg ml⁻¹, 1 μg ml⁻¹, 8 μg ml⁻¹, 4 μg ml⁻¹, 8 μg ml⁻¹, 16 μg ml⁻¹, 8 μg ml⁻¹, 128 μg ml⁻¹.

Table 1. MICs of various antimicrobials against a highly multi-drug-resistant strain of *K. ozaenae*

The antimicrobial susceptibility tests were performed with the Vitek 2 compact system, using AST-GN13 cards (bioMérieux). The results were interpreted according to CLSI guidelines. Amp, ampicillin; Sam, ampicillin/sulbactam; Amk, amikacin; Azm, aztreonam; Tms, trimethoprim–sulfamethoxazole; Ctn, cefotetan; Cax, ceftriaxone; Cfx, cefazolin; Cpe, ceferpine; Caz, ceftazidime; Gm, gentamicin; To, tobramycin; Lvx, levofloxacin; Cp, ciprofloxacin; Imp, imipenem; Etp, ertapenem; Ptz, piperacillin/tazobactam; S, susceptible; R, resistant.

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cards, were ≥4 and ≥8 μg ml⁻¹, respectively. Quinolone resistance normally arises by mutations in the chromosomal genes for DNA gyrase and type IV topoisomerases (Brisse et al., 1999; Tran et al., 2005) but no mutations were detected in the quinolone resistance-determining regions of the gyrA and parC genes. Plasmid-mediated quinolone resistance genes, including qnrA, qnrB, qnrC, qnrD, qnrS, qepA and aac(6’)-Ib-cr, were evaluated using PCR and sequence analysis. Amplified fragments of qnrS and aac(6’)-Ib genes were obtained, which shared 100% sequence similarity with those of previous reports (Hata et al., 2005; Park et al., 2006; GenBank nos. AB187515 and AF479774, respectively). QnrS showed 59% deduced amino acid similarity with Qnr and the resistance-encoding activity of the qnrS gene was similar to that of the qnr gene, preventing quinolone inhibition due to DNA gyrase alterations (Hata et al., 2005; Tran et al., 2005). The aac(6’)-Ib-cr gene is reported to encode a ciprofloxacin-modifying enzyme (Park et al., 2006), but no cr variant, responsible for low-level ciprofloxacin resistance, was found in this isolate.

Cephalosporins, carbapenems and quinolones are frequently used in anti-infection therapy in our hospital. This strain of K. ozaenae showed no sensitivity to several antibiotics. Five antimicrobial resistance-associated genes were discovered in this strain, including blakPC-2, blatem-98, blactMX-M-3, blastIV-26 and qnrS, most of which are reported to be plasmid-borne (Huang et al., 2009; Jeon et al., 2005; Naas et al., 2008; Walther-Rasmussen & Høiby, 2006; Wu et al., 2007). As such, it may be critical for us to intensify surveillance to reduce the risk of nosocomial cross infection.

Meanwhile, this strain of K. ozaenae was positive for the intI gene. Class I integrons encoded by this gene contain a site-specific recombination system that is capable of integrating and expressing genes and represents a major reservoir of antibiotic-resistance genes (Labbate et al., 2009). This may play an important role in acquisition of the multi-drug resistance genes.

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