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Detection of plasmid-mediated IMP-1 metallo-β-lactamase and quinolone resistance determinants in an ertapenem-resistant *Enterobacter cloacae* isolate

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Abstract: Objective: To investigate the mechanism of carbapenem resistance and the occurrence of plasmid-mediated quinolone resistance determinants *qnr* and *aac(6')-Ib-cr* in a clinical isolate of *Enterobacter cloacae*. Methods: An ertapenem-resistant *E. cloacae* ZY106, which was isolated from liquor puris of a female gastric cancer patient in a Chinese hospital, was investigated. Antibiotic susceptibilities were determined by agar dilution method. Conjugation experiments, isoelectric focusing, polymerase chain reaction (PCR), and DNA sequence analyses of plasmid-mediated carbapenemases and quinolone resistance determinants were preformed to confirm the genotype. Outer membrane proteins (OMPs) were examined by urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Urea-SDS-PAGE). Results: Minimum inhibitory concentrations (MICs) of imipenem, meropenem, and ertapenem for ZY106 were 2, 4, and 16 μg/ml, respectively. Conjugation studies with *Escherichia coli* resulted in the transfer of significantly reduced carbapenem susceptibility. ZY106 produced IMP-1 metallo-β-lactamase and CTX-M-3 extended-spectrum β-lactamase, and *E. coli* transconjugant produced IMP-1. Plasmid-mediated quinolone resistance determinant *qnrS1* was detected in ZY106. Transfer of the *qnrS1*-encoding-plasmid into *E. coli* by conjugation resulted in intermediate resistance to ciprofloxacin in *E. coli* transconjugant. Urea-SDS-PAGE analysis of OMPs showed that ZY106 lacked an OMP of approximately 38 kDa. Conclusion: It is the first IMP-1-producing Enterobacteriaceae in China and the first report of a clinical isolate that harbors both *blaIMP* and *qnrS* genes as well. The *blaIMP-1*, *blaCTX-M-3*, and *qnrS1* are encoded at three different plasmids. IMP-1 combined with the loss of an OMP possibly resulted in ertapenem resistance and reduced imipenem and meropenem susceptibility in *E. cloacae*.

Key words: Antibiotic resistance, Carbapenem, Enterobacteriaceae, Outer membrane proteins (OMPs)
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INTRODUCTION

Carbapenems are commonly used to treat serious infections caused by multi-resistant gram-negative bacilli, especially strains producing high-level of AmpC cephalosporinases or extended-spectrum β-lactamases (ESBLs) (Baldwin *et al*., 2008). However, the emergence of carbapenemase is becoming a therapeutic challenge. Carbapenemases involved in acquired resistance are of Ambler molecular classes A, B, and D (Walsh, 2008). The class B enzymes, metallo-β-lactamases (MBLs), are the most clinically threatening carbapenemases, as these enzymes are capable of hydrolyzing all β-lactams except monobactams, and are not susceptible to therapeutic β-lactamase inhibitors such as clavulanate, sulbactam, and tazobactam.

The most commonly acquired MBL families include the IMP, Verona integron-encoded metallo-β-lactamase (VIM), Sao Paulo metallo-β-lactamase (SPM), German imipenemase (GIM), and Seoul imipenemase (SIM). SPM, GIM, and SIM MBLs

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were detected in some specific regions of origins, while IMP and VIM, which are becoming common in *Pseudomonas aeruginosa* and other nonfermenting gram-negative bacteria, spread worldwide. IMP-1 was first detected in a *P. aeruginosa* isolate in Japan in 1990 (Watanabe *et al*., 1991). Soon afterward, IMP-1 was prevalent in *Serratia marcescens* and spread into several members of the family Enterobacteriaceae in Japan (Walsh *et al*., 2005; Queenan and Bush, 2007). There has been an increase in the detection and spread of the acquired MBLs in Enterobacteriaceae from Australia and Taiwan region of China, and IMP-4 and IMP-8 were the predominant MBLs (Walsh *et al*., 2005; Queenan and Bush, 2007; Peleg *et al*., 2005; Herbert *et al*., 2007; Wu *et al*., 2007; 2008; Liu *et al*., 2008). MBLs in Enterobacteriaceae have occurred sporadically in other countries. IMP-1 has been reported in *Klebsiella pneumoniae* from Singapore (Koh *et al*., 1999), Brazil (Lincopan *et al*., 2005; 2006), Turkey (Aktas *et al*., 2006), and the UK (Woodford *et al*., 2007), in *Enterobacter cloacae* from Turkey (Deshpande *et al*., 2006), and in *Enterobacter aerogenes* from France (Biendo *et al*., 2008). In China, there were only two reports on the production of IMP in Enterobacteriaceae. An IMP-4-producing *Citrobacter youngae* was isolated from Guangzhou in 2001 (Hawkey *et al*., 2001) and an IMP-4-producing *K. pneumoniae* from Wuhan in 2008 (Mendes *et al*., 2008).

In this study, an ertapenem-resistant *E. cloacae* strain was collected from our hospital and its mechanisms of carbapenem resistance were analyzed. It is the first detection of IMP-1 in Enterobacteriaceae from China. A further study revealed that this *E. cloacae* strain lacked an outer membrane protein (OMP) and harbored a plasmid-mediated quinolone resistance determinant *qnrS1*.

MATERIALS AND METHODS

Bacterial strains

In August 2007, an *E. cloacae* strain (ZY106) was isolated from a purulent exudate sample from abdominal cavity of a patient in oncology ward in the Second Affiliated Hospital of Zhejiang University, China. The patient was diagnosed as gastric cancer and received surgery. During hospitalization, the patient was treated with cefoperazone/sulbactam and ciprofloxacin before the isolation of the ertapenem-resistant *E. cloacae* strain.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined using the agar dilution method according to Clinical and Laboratory Standards Institute (2007) recommendations.

Conjugation experiments

Conjugal transfer experiments were carried out in mixed broth cultures (Zhang *et al*., 2008). Rifampin-resistant *Escherichia coli* EC600 and streptomycin-resistant *E. coli* C600 were used as recipients. IMP-producing *E. coli* transconjugants were selected on Muller-Hinton agar plates containing 400 μg/ml rifampin (or 400 μg/ml streptomycin) and 8 μg/ml ceftazidime, and *qnr* positive *E. coli* transconjugants were selected on plates containing 400 μg/ml rifampin (or 400 μg/ml streptomycin) and 0.25 μg/ml ciprofloxacin. The selected colonies were picked up and identified by Vitek system (bioMérieux, Hazelwood, MO, USA). Plasmids from *E. cloacae* ZY106 and *E. coli* transconjugants were extracted using AxyPrep Plasmid Miniprep Kit (Axygen Scientific, Union City, CA, USA) and examined by electrophoresis.

Isoelectric focusing (IEF) of β-lactamase

Crude β-lactamase preparations were obtained by ultrasonic method. IEF was carried out on Phast-Gel polyacrylamide gel (pH 3 to 9, Amersham Biosciences, Uppsala, Sweden) using the PhastSystem (Pharmacia Biotech, Uppsala, Sweden) by the method of Mathew *et al*.(1975). β-lactamase activity was visualized by staining the gel with Nitrocefin (Oxoid, Basingstoke, Hampshire, England). The isoelectric points (pIs) were determined as described previously (Zhang *et al*., 2008).

Polymerase chain reaction (PCR) amplification and DNA sequence analysis of *bla* and plasmid-mediated quinolone resistance genes

Plasmids from *E. cloacae* ZY106 and *E. coli* transconjugants were used as template. The primers used to amplify bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$ (Yu *et al*., 2007), $bla_{\text{IMP-1}}$, $bla_{\text{IMP-2}}$ (Shibata *et al*., 2003), *qnrA*,

qnrB, *qnrS* (Robicsek *et al*., 2006), and *aac(6')-Ib* (Jiang *et al*., 2008) were described previously. Prepared bacterial DNA of the ZY106 was used as the template for PCR amplifications of *gyrA*, *parC*, and gene of chromosomal cephalosporinase (Deguchi *et al*., 1997; Conceição *et al*., 2004). The reaction was conducted in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR amplification products were purified and sequenced using an ABI3730 Sequencer (Applied Biosystems). We determined partial sequences of the *gyrA* and *parC* genes of the ZY106, including the regions analogous to the quinolone resistance-determining region of the *E. cloacae* ATCC13047 *gyrA* and *parC* genes and the deduced amino acid sequences of the ZY106 *gyrA* and *parC* genes were compared with the corresponding regions of the *E. cloacae* ATCC13047 GyrA and ParC proteins. Detection of KPC-2 of the ZY106 was conducted according to our previous report (Zhang *et al*., 2008).

Analysis of outer membrane proteins (OMPs)

OMPs of *E. cloacae* ZY106 and *E. cloacae* ATCC13047 were isolated and separated on 11.6% (w/v) acrylamide-0.4% (w/v) bisacrylamide-0.1% (w/v) sodium dodecyl sulfate gel containing 20% (w/v) urea (urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Urea-SDS-PAGE) as described previously (Zhang *et al*., 2008).

RESULTS

Antibiotics susceptibility

E. cloacae ZY106 showed reduced susceptibility to imipenem and meropenem with MICs of 2 and 4 μg/ml, and resistance to ertapenem with MIC of 16 μg/ml. The isolate was highly resistant to β-lactams except piperacillin/tazobactam and aztreonam. MICs of carbapenems significantly decreased (≥4-fold) in the presence of ethylenediaminetetraacetic acid (EDTA) (a constant concentration of 320 μg/ml) (Table 1). These results suggest the production of MBL. Similarly, MIC of ceftazidime was reduced from >256 μg/ml to 1 μg/ml, and that of cefotaxime was reduced from 256 μg/ml to 64 μg/ml, suggesting the production of CTX-M ESBL. *E. cloacae* ZY106 was also resistant to quinolones and aminoglycosides.

Table 1 Antimicrobial susceptibility results of *E. cloacae* ZY106 and *E. coli* transconjugants

Antimicrobial agents	*MIC* (μg/ml)					
	E. cloacae ZY106	*E. coli* C600	Transconjugant A	*E. coli* EC600	Transconjugant B_1	Transconjugant B_2
Imipenem	2	≤0.0625	0.5	0.125	0.125	0.125
Imipenem+EDTA*	0.5	≤0.0625	≤0.0625	0.125		
Meropenem	4	≤0.0625	0.25	≤0.0625	≤0.0625	≤0.0625
Meropenem+EDTA	0.25	≤0.0625	≤0.0625	≤0.0625		
Ertapenem	16	≤0.0625	0.5	≤0.0625	≤0.0625	≤0.0625
Ertapenem+EDTA	0.5	≤0.0625	≤0.0625	≤0.0625		
Ceftazidime	>256	≤0.125	256	≤0.125	≤0.125	≤0.125
Ceftazidime+EDTA	1	≤0.125	≤0.125	≤0.125		
Cefotaxime	256	≤0.125	32	≤0.125	≤0.125	≤0.125
Cefotaxime+EDTA	64	≤0.125	0.25	≤0.125		
Cefepime	128	≤0.125	8	≤0.125	≤0.125	≤0.125
Cefoperazone/sulbactam	>256	≤0.125	128	≤0.125	≤0.125	≤0.125
Ampicillin	>256	1	128	8	8	8
Piperacillin	256	0.25	4	1	1	1
Piperacillin/tazobactam	16	0.25	2	1	1	1
Cefoxitin	>256		>256	4	4	4
Aztreonam	32	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125
Ciprofloxacin	>32	≤0.125	≤0.125	≤0.125	2	2
Gentamicin	16	0.5	0.5	0.5	1	16
Amikacin	16	0.5	0.5	0.5	1	16

*The final concentration of EDTA was fixed at 320 μg/ml

Transfer of antibiotics resistance and plasmid analysis

E. cloacae ZY106 failed to transfer carbapenem resistance to *E. coli* EC600 by conjugation, but it was successful for *E. coli* C600. The bacteria grew on plates containing streptomycin and ceftazidime were named as *E. coli* transconjugant A. The plasmid profiles indicated that ZY106 harbored several plasmids and *E. coli* transconjugant A acquired a plasmid with a size of approximately 40 kb (Fig.1). Transfer of ciprofloxacin resistance from *E. cloacae* ZY106 to *E. coli* EC600 was successful. Two different *E. coli* transconjugants were obtained. The one with a 60-kb plasmid was named *E. coli* transconjugant B_1, and the other with an extra 6-kb plasmid was named *E. coli* transconjugant B_2.

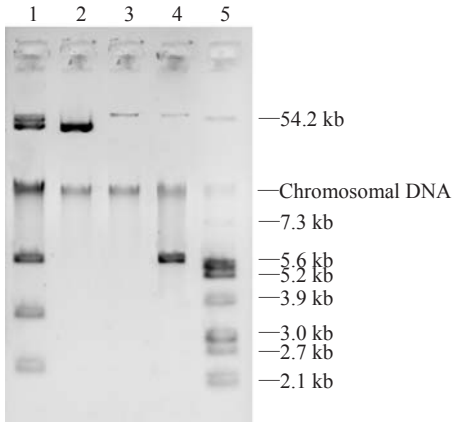

Fig.1 Plasmid profiles of *E. cloacae* ZY106 and *E. coli* transconjugants. Lane 1: *E. cloacae* ZY106; Lanes 2~4: *E. coli* transconjugants A, B_1 and B_2; Lane 5: *E. coli* V517

As shown in Table 1, MICs of imipenem, meropenem, and ertapenem for *E. coli* transconjugant A were 0.5, 0.25, and 0.5 μg/ml, respectively, which increased at least 4-fold compared with those for *E. coli* C600 (MICs of ≤0.0625 μg/ml), although *E. coli* transconjugant A remained susceptible to carbapenems according to the Clinical and Laboratory Standards Institute (CLSI) breakpoints. *E. coli* transconjugant A was resistant to several β-lactams but was susceptible to piperacillin, piperacillin/tazobactam, and aztreonam. *E. coli* transconjugant A had a dramatic decrease in the MICs of carbapenems and cephalosporins in the presence of EDTA. *E. coli* transconjugant B_1 was intermediately resistant to ciprofloxacin (MIC of 2 μg/ml) and was susceptible to other tested antibiotics. Conjugation experiments and antimicrobial susceptibility results of *E. coli* transconjugant B suggest the presence of plasmid-mediated quinolone resistance determinants. The antimicrobial susceptibility patterns of *E. coli* transconjugant B_2 were similar to those of B_1, except its resistance to aminoglycosides.

IEF analysis

IEF analysis demonstrated that both *E. cloacae* ZY106 and *E. coli* transconjugant A had a band with β-lactamase activity with a pI of approximately 9.0 (Fig.2).

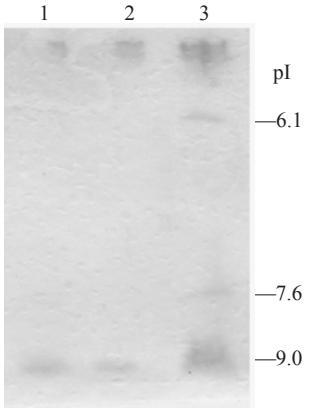

Fig.2 Isoelectric focusing patterns of crude β-lactamase extracts from *E. cloacae* ZY106 and *E. coli* transconjugant A. Lane 1:, *E. cloacae* ZY106; Lane 2: *E. coli* transconjugant A; Lane 3: strains producing TEM-28 (pI of 6.1), SHV-7 (pI of 7.6) and ACT-1 (pI of 9.0)

PCRs and DNA sequence analysis

The above-mentioned antibiotic resistance genes were analyzed by PCR and DNA sequencing. *E. cloacae* ZY106 was positive for $bla_{\text{IMP-1}}$, $bla_{\text{CTX-M}}$, *qnrS*, *aac(6′)-Ib* genes and chromosomal AmpC lactamase gene, while *E. coli* transconjugant A was only positive for the $bla_{\text{IMP-1}}$ gene, B_1 was positive for the *qnrS* gene, and B_2 was positive for *qnrS* and *aac(6′)-Ib* genes. DNA sequencing results identify the genes as $bla_{\text{IMP-1}}$, $bla_{\text{CTX-M-3}}$, *qnrS1*, and *aac(6′)-Ib*. These results indicate that the four genes were carried on separated plasmids. The sizes of plasmids encoding IMP-1, *qnrS1*, and *aac(6′)-Ib* were approximately 40, 60, and 6 kb, respectively, and CTX-M-3 was presumed to be encoded on a 50-kb plasmid (Fig.1). AAC(6′)-Ib-cr, a variant aminoglycoside acetyltransferase capable of modifying ciprofloxacin and

reducing its activity, was not found. The *aac(6')-Ib* conferred the resistance to gentamicin and amikacin but did not elevate the ciprofloxacin MIC for *E. coli* transconjugant B_2 compared with that for B_1. Although *E. cloacae* ZY106 produced two β-lactamases, only one band with β-lactamase activity was observed in IEF. It was because that IMP-1 and CTX-M-3 had an overlapping band at the same pI of approximately 9.0 (Baldwin *et al*., 2008; Jeong *et al*., 2005).

Since the KPC-2 carbapenemase was often detected in carbapenem-resistant Enterobacteriaceae from our hospital (Zhang *et al*., 2007; 2008; Cai *et al*., 2008), we had also analyzed the KPC-2 carbapenemase in ZY106. However, we confirmed that this isolate did not produce KPC-2 carbapenemase.

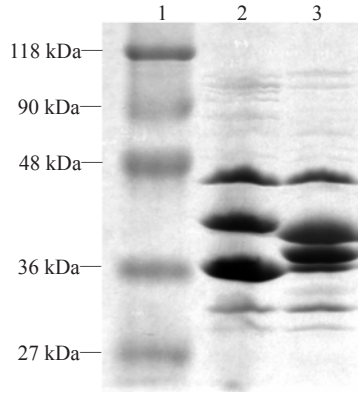

Fig.3 Urea-SDS-PAGE analysis of outer membrane proteins (OMPs) of *E. cloacae* ZY106. Lane 1: protein molecular weight standard (MBI Fermentas); Lane 2: *E. cloacae* ZY106; Lane 3: *E. cloacae* ATCC13047

Detection of mutations in *gyrA* and *parC* genes

As shown in Table 1, transconjugants B_1 and B_2 exhibited rather high ciprofloxacin MICs. Usually transconjugants containing *qnrS* determinants only show ciprofloxacin MICs of less than 0.5 mg/L. To investigate whether other determinants had been contributing to the high ciprofloxacin resistance, we analyzed the partial sequences of the *gyrA* and *parC* genes of ZY106. ZY106 had two mutations generating Ser(S)83→Phe(F) and Asp(D)87→Ala(A) changes in GyrA and a Ser(S)83→Ile(I) mutation in ParC (data not shown). Therefore, the resistance to ciprofloxacin of ZY106 is attributed to the mutations in *gyrA* and *parC* in the quinolone resistance-determining region (Deguchi *et al*., 1997).

Urea-SDS-PAGE analysis of OMPs

Urea-SDS-PAGE analysis showed that *E. cloacae* ATCC13047 expressed four major OMPs, with molecular weights of 47, 41, 38, and 36 kDa. The four OMPs were analogous to *E. coli* LamB, OmpC, OmpF, and OmpA, respectively (Szabó *et al*., 2006). The 41- and 38-kDa proteins were considered to be porins, and the later was absent in *E. cloacae* ZY106 (Fig.3).

DISCUSSION

IMP-type MBLs were most frequently detected among gram-negative nonfermenters. IMP-producing Enterobacteriaceae were mainly reported from Japan, Australia, and Taiwan region of China. Production of IMP in Enterobacteriaceae was rare in mainland China, including IMP-4 in *C. youngae* and *K. pneumoniae* (Hawkey *et al*., 2001; Mendes *et al*., 2008). In this study, we detected bla_{IMP-1} and *qnrS1* genes along with alternations in *gyrA* and *parC* in a clinical isolate of ertapenem-resistant *E. cloacae*. To our knowledge, no other Enterobacteriaceae harboring such genes were recovered so far. In addition, further studies revealed the loss of an OMP, which enhanced the carbapenem resistance in this isolate. However, for *E. cloacae* ZY106, the MIC of ertapenem (16 μg/ml) was much higher than those of imipenem (2 μg/ml) and meropenem (4 μg/ml). *E. coli* transconjugant A that produced IMP-1 exhibited significantly reduced susceptibility to carbapenems (MICs of 0.25 to 0.5 μg/ml). However, it was not sufficient to explain the relatively high carbapenem resistance in *E. cloacae* ZY106 with imipenem MIC of 2 μg/ml, meropenem MIC of 4 μg/ml, even ertapenem MIC of 16 μg/ml. Therefore, other mechanisms such as deficiency in the OMPs (Bush *et al*., 1985; Lee *et al*., 1991; 1992) and existence of efflux pump might also be contributing to the ertapenam resistance (Szabó *et al*., 2006), although the efflux mechanism is not common in the Enterobacteriaceae.

The Urea-SDS-PAGE analysis of OMPs proved that ZY106 lacked the OMP (38 kD), which suggests that at least reduced outer membrane permeability might be involved. However, it requires further investigation to prove whether the efflux mechanism was also involved in the ertapenam resistance in

ZY106. These results suggest that production of IMP combined with the OMP deficiency and possibly efflux mechanism had more effect on susceptibility to ertapenem than on that to imipenem and meropenem in *E. cloacae*, and that ertapenem may be the preferred antibiotic for screening such isolate.

IMP alone does not confer high-level carbapenem resistance in Enterobacteriaceae, and it might spread without attracting attention. Microbiologists should be aware of suspicious gram-negative bacteria with reduced susceptibility to carbapenems, and examine their carbapenemase activity.

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