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Research Paper

Carbapenem and cefoxitin resistance of *Klebsiella pneumoniae* strains associated with porin OmpK36 loss and DHA-1 β-lactamase production

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Abstract

Clinical isolates of carbapenem-resistant *Klebsiella pneumoniae* (*K. pneumoniae*) strains are being increased worldwide. Five pan-resistant *K. pneumoniae* strains have been isolated from respiratory and ICU wards in a Chinese hospital, and reveal strong resistance to all β-lactams, fluoroquinolones and aminoglycosides. Totally 27 β-lactamase genes and 2 membrane pore protein (porin) genes in 5 *K. pneumoniae* strains were screened by polymerase chain reaction (PCR). The results indicated that all of 5 *K. pneumoniae* strains carried *bla*TEM-1 and *bla*DHA-1 genes, as well as base deletion and mutation of *OmpK*35 or *OmpK*36 genes. Compared with carbapenem-sensitive isolates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the resistant isolates markedly lacked the protein band of 34-40 kDa, which might be the outer membrane proteins of OmpK36 according to the electrophoresis mobility. In addition, the conjugation test was confirmed that *bla*DHA-1 mediated by plasmids could be transferred between resistant and sensitive strains. When reserpine (30 μg/mL) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (50 μg/mL) were added in imipenem and meropenem, the MICs had no change against *K. pneumoniae* strains. These results suggest that both DHA-1 β-lactamase and loss or deficiency of porin OmpK36 may be the main reason for the cefoxitin and carbapenem resistance in *K. pneumoniae* strains in our hospital.

Key words: *Klebsiella pneumoniae*, AmpC β-Lactamase, Porin, molecular biology.

Introduction

K. pneumoniae is one of the most common pathogens in clinical infections, such as pneumonia, urinary tract infections, sepsis, wound infections, meningitis and other diseases. Multidrug-resistant *K. pneumoniae* strains are becoming a severe problem worldwide, and it usually carries one or more extended-spectrum β-lactamases (ESBLs) that confers the resistance to expanded-spectrum cephalosporins (Lee *et al.*, 2007; Huang and Hsueh, 2008). In recent years, *K. pneumoniae* strains have been reported to be resistant to most β-lactamas through mutation genes encoded in chromosome, acquisition of genes from mobile plasmids and integrons (Maltezou *et al.*, 2009; Munoz-Price and Quinn, 2009).

Carbapenem antibiotics are very effective in the treatment of severe bacterial infections caused by ESBL- and AmpC enzymes-producing *K. pneumoniae* strains so that they have been widely used in clinics. Due to carbapenemases, metallo-β-lactamases and porin loss, carbapenem-resistant *K. pneumoniae* strains are increasing (Landman *et al.*, 2009; Pournaras *et al.*, 2010). Up to now, it has been reported that the outer membrane proteins of *K. pneumoniae* strains contain three known porins including OmpK35, OmpK36, and OmpK37 (Hernandez-Alles *et al.*, 1999; Kaczmarek *et al.*, 2006). Furthermore, porins OmpK35 and OmpK36 play critical roles in the penetration of antibiotics into the cells, and the loss of OmpK35 or OmpK36 can resist or reduce susceptibility to cephalosporins and carbapenems, particularly in strains containing

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Ambler group A, B, C, or D β-lactamase (Doumith *et al.*, 2009; Goldfarb *et al.*, 2009; Kontopoulou *et al.*, 2010).

In 2010, we isolated 5 *K. pneumoniae* strains resistant to almost all antibiotics from clinical specimens. The minimal inhibitory concentrations (MICs) of piperacillin, piperacillin/tazobactam, amoxicillin/clavulanic acid, cefoperazone/sulbactam, cefoxitin, cefotaxime and aztreonam against 5 *K. pneumoniae* strains were larger than 128 μg/mL, and the MICs of imipenem and meropenem against 5 *K. pneumoniae* strains were larger than 32 μg/mL. In addition, these *K. pneumoniae* strains also revealed the resistance to ciprofloxacin, levofloxacin, gentamicin and amikacin (MIC ≥ 64 μg/mL). The objective of this study was to investigate the cause of high-level carbapenem resistance in *K. pneumoniae* strains. Except for *bla*TEM-1 and *bla*DHA-1 genes, carbapenem-resistant genes were not observed in 5 *K. pneumoniae* strains; however, the base deletion or mutation of *OmpK*35 and *OmpK*36 genes could lead to the alterations of amino acid sequences, protein configuration and pore size of porins, thus impeding the access of antimicrobials. Therefore, high-level resistance to cefoxitin and carbapenem in 5 *K. pneumoniae* clinical isolates is due to the combinatorial action from the production of blaDHA-1 beta-lactamase and the insertional inactivation or loss of porin OmpK36.

Methods

Strains and antimicrobial susceptibility testing

In 2010, 5 carbapenem-resistant *K. pneumoniae* (*Kp*01, *Kp*02, *Kp*03, *Kp*04 and *Kp*05) strains were isolated from sputum, urine and deep venous catheter specimens in a Chinese hospital. Among 5 patients, 1 case was from respiratory ward and 4 cases were from ICU ward. The MICs of piperacillin, peracillin/sulbactam, amoxicillin/clavulanic acid, cefoperazone/sulbactam, cefoxitin, cefotaxime, cefepime, aztreonam, imipenem, meropenem, ciprofloxacin, levofloxacin, gentamicin and amikacin were performed by E-test (Oxiod), and *E. coli ATCC25922* was used as the control.

Extraction of β-lactamase and three-dimensional test

Briefly, a colony was inoculated into 5 mL of tryptic soy broth overnight and the culture was grown at 35 °C for 4 h. The cells were concentrated by centrifugation, and crude enzyme extract was prepared by repeated freeze-thawing cycle for five times. The surface of a Mueller-Hinton agar plate was inoculated with *E.coli* strains as described by the standard disk diffusion method. A 30 μg cefoxitin disk (Oxoid) was placed on the inoculated agar. With a sterile scalpel blade, 4 slit beginning 5 mm from the edge of the disk was cut in the agar in outward radial direction. By using a pipette, 40 μL of crude enzyme extract was dispensed into each slit, beginning near the disk and moving outward. Slit overfill was avoided. The inoculated media were incubated overnight at 35 °C. Enhanced growth of the surface organism at the point where the slit intersected the zone of inhibition was considered a positive three-dimensional testing result and was interpreted as the evidence in the presence of AmpC beta-lactamase or ESBL. *K. pneumoniae* ATCC700603 and *Enterobacter cloacae* (*E. cloacae*) 029 M were used as the control strains.

Transconjugation experiments

In order to determine if the resistance was transferable, transconjugation experiments were performed. Five isolates were used as the donors and *E*C600 (Rif^r) was used as the recipient in transconjugation experiments. The organisms were inoculated into 5 mL of Luria-Bertani (LB) broth (Difco) and incubated at 35 °C for 20 h with shaking. Two organisms were mixed together at a proportion of 1 : 2, and then incubated overnight at 35 °C with shaking. After centrifugation for 5 min at 4,000 *g*, the precipitate was mixed with 1 mL of distilled water and inoculated onto MacConkey agar plates containing 600 μg/mL rifampin and 0.25 μg/mL cefotaxime. Transparent colonies were selected from the agar plates and inoculated into MacConkey agar again for activation. The drug-resistant profile and plasmid electrophoresis were further analyzed.

DNA extraction

Strains were grown overnight on MacConkey agar plates at 37 °C, and growth from approximately one-quarter of a plate was resuspended in 180 μL of distilled water. A total of 200 μL of buffer solution (0.01 mM Tris-HCl, pH 7.8; 0.005 M EDTA; 0.5% sodium dodecyl sulfate) and 20 μL of proteinase K (1 mg/mL) were added. The mixture was incubated at 55 °C for 2 h, and then 400 μL of phenol-chloroform solution was added, mixed with gentle agitation, and centrifuged at 12,000 *g* for 5 min. The supernatant was collected and DNA was precipitated after the addition of 0.5 volume of 7.5 M ammonium acetate and 2 volumes of ethanol. DNA was washed with 70% ethanol, dried, and resuspended with 100 μL of Tris-EDTA buffer.

Analysis of β-lactamase and porin genes

The oligonucleotide primers were designed on the basis of the nucleotide sequence in GenBank (Table 1). The 27 β-lactamase and 2 porin genes were screened by PCR. The primer pairs were shown in Table 1. The assay was carried out in 20 μL of reaction mixture containing 0.5 μM each primer, 10 mM KCl, 2 mM $MgCl_2$, 8 mM $(NH4)_2SO_4$, 10 mM Tris-HCl (pH 9.0), 200 μM dNTP and 1 U Taq DNA polymerase. All amplification reactions were performed with an initial denaturation at 93 °C for 3 min followed by 35 cycles of denaturation at 93 °C for 1 min, an-

Table 1 - Primers used in this study.

Target genes	Primer sequence (3' → 5')	Size (bp)	GenBank accession no. of reference
Class A β-lactamases			
TEM	P1: AGGAAGAGTATGATTCAACA		
	P2: CTCGTCGTTTGGTATGGC	535 bp	X54604
SHV	P1: TGCGCAAGCTGCTGACCAGC		
	P2:TTAGCGTTGCCAGTGCTCGA	305 bp	AY326946
CTX-M-1 group	P1: ATGGTTAAAAAATCACTGCGC		
	P2: TCCCGACGGCTTTCCGCCTT	833 bp	GU125714
CTX-M-2 group	P1: ATGATGACTCAGAGCATTCG		
	P2: TCCCGACGGCTTTCCGCCTT	833 bp	AY750915
CTX-M-8 group	P1: ATGATGAGACATCGCGTTAAGCGG		
	P2: TTAATAACCGTCGGTGACGATTTTCGCG	876 bp	AF189721
CTX-M-9 group	P1: CGGCCTGTATTTCGCTGTTG		
	P2: TCCCGACGGCTTTCCGCCTT	793 bp	AB205197
CTX-M-25 group	P1: ATGATGAGAAAAAGCGTAAGGCGGGCG		
	P2: TCCCGACGGCTTTCCGCCTT	876 bp	AY157676
PER	P1: AGTCAGCGGCTTAGATA		
	P2: CGTATGAAAAGGACAATC	978 bp	AJ621265
GES	P1: ATGCGCTTCATTCACGCAC		
	P2: CTATTTGTCCGTGCTCAGG	846 bp	AY219651
VEB	P1: GCGGTAATTTAACCAGA		
	P2: GCCTATGAGCCAGTGTT	961 bp	AY536743
CARB	P1: AAAGCAGATCTTGTGACCTATTC		
	P2: TCAGCGCGACTGTGATGTATAAAC	588 bp	S46063
KPC	P1: ATGTCACTGTATCGCCGTCTA		
	P2: TTACTGCCCGTTGACGCCCAA	882 bp	GU086225
LAP	P1: ATGAAAAAGATCCGCCTTATTATAA		
	P2: TTACCAGTTCTTAATTACTGAATC	858 bp	EF026092
Class B β-lactamases			
IMP	P1: CGGCCGCAGGAGAGGCTTT		
	P2: AACCAGTTTTGCCTTACCAT	587 bp	AJ223604
VIM	P1: ATTCCGGTCGGAGAGGTCCG		
	P2: GAGCAAGTCTAGACCGCCCG	633 bp	AY509609
SPM	P1: CTGCTTGGATTCATGGGCGCG	784 bp	AY341249
	P2: CCTTTTCCGCGACCTTGCTCG		
NDM	P1: TCAGCGCAGCTTGTCGGCCA	813 bp	HQ738352
	P2: ATGGAATTGCCCAATATTATGCA		
Class C β-lactamases			
LEN	P1: ATGCGTTATATTCGCCTGTG	591 bp	AM850914
	P2: GGCGCTCAGATGCTGCGC		
OKP	P1: AAGCGCTTCCCGGCGACGTG	362 bp	AM051141
	P2: TTAGCGTTGCCAGTGCTCGA		
DHA	P1: AACTTTCACAGGTGTGCTGGGT	405 bp	AY585202
	P2: CCGTACGCATACTGGCTTTGC		
ACT	P1: TCGGTAAAGCCGATGTTGCGG	303 bp	EF508682
	P2: CTTCCACTGCGGCTGCCAGTT		

Table 1 (cont.)

Target genes	Primer sequence (3' → 5')	Size (bp)	GenBank accession no. of reference
MOX	P1: GCTGCTCAAGGAGCACAGGAT	520 bp	EU515248
	P2: CACATTGACATAGGTGTGGTG		
ACC	P1: ACAGCCTCAGCAGCCGGTTA	345 bp	AJ133121
	P2: TTCGCCGCAATCATCCCTAG		
FOX	P1: AACATGGGGTATCAGGGAGAT	190 bp	AY034848
	P2: CAAAGCGCGTAACCGGATTGG		
Class D β-lactamases			
OXA-1 group	P1: CTGTTGTTTGGGTTTCGCAAG	440 bp	GQ438248
	P2: CTTGGCTTTTATGCTTGATG		
OXA-2 group	P1: CAGGCGCTGTTCGTGATGAGTT	233 bp	FJ855130
	P2: GCCTTCTATCCAGTAATCGCC		
OXA-10 group	P1: GTCTTTCAAGTACGGCATTA	822 bp	AY509609
	P2: GATTTTCTTAGCGGCAACTTA		
Porin			
ompK35	P1: ATGATGAAGCGCAATATTCTGGCAGTGG	684 bp	GU945384
	P2: TCGGCTTTGTCGCCATTGCCGTCA		
ompK36	P1: ATGAAAGTTAAAGTACTGTCCCTC	1076 bp	HM000057
	P2: GCCGGTATCTCTACCGACGAC		

nealing at 55 °C for 1 min and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 7 min. Aliquot (20 μL) of each sample was subjected to electrophoresis by 2% agarose gels. The amplicons were purified with PCR Cleanup kits (Roche Molecular Biochemicals) and sequenced on an ABI PRISM 377 analyzer (Applied Biosystems).

Outer membrane protein analysis

K. pneumoniae was inoculated overnight in 5 mL of LB medium at 35 °C with shaking, the culture was added in 200 mL of LB liquid medium at 35 °C with shaking for 5 h. After centrifugation (4 °C) for 5 min at 4,000 *g*, the supernatant was discarded. The precipitate was washed three times with 50 mM (pH 7.0) phosphate buffer solution (PBS), and suspended in 10 mM Tris-HCl (pH 7.4). The ultrasonic fragmentation of bacteria was performed in ice bath conditions. In order to remove unbroken bacteria, centrifugation was performed at 4,000 *g* for 30 min, and then the supernatant was centrifuged at 25,000 *g* for another 30 min. The precipitation was resuspended with buffer, and phenylmethylsulfonyl was added. The outer membrane protein was denatured at 100 °C for 5 min before electrophoresis and then separated by SDS-PAGE with Mini-sub cell horizontal electrophoresis tank (Biorad Co.). The gel concentration was 12.0%, and stained with Coomassie brilliant blue R250 for 30 min.

Inhibitory effects of reserpine and CCCP against efflux

Susceptibility test was carried out using agar dilution method. MIC changes were observed in the absence or presence of reserpine (J & K Chemical) and CCCP (Sigma) at concentrations of 30 μg/mL and 50 μg/mL, respectively. An inoculum of each isolate at the amount of 5 x 10^4 cfu/mLwas inoculated onto MH medium containing serial dilutions of imipenem and meropenem. A phenotype for positive efflux was detectable after at least 4-fold dilutions of MIC for imipenem or meropenem in the absence or presence of reserpine and CCCP.

Results

Antimicrobial susceptibility test

In vitro tests showed that the MICs of piperacillin, piperacillin/tazobactam, amoxicillin/clavulanic acid, cefoperazone/sulbactam, cefoxitin, cefotaxime and aztreonam against 5 *K. pneumoniae* strains were larger than 128 μg/mL, and the MICs of imipenem or meropenem against 5 *K. pneumoniae* strains were larger than 32 μg/mL. In addition, 5 *K. pneumoniae* strains also revealed strong resistance to ciprofloxacin, levofloxacin, gentamicin and amikacin (Table 2).

Table 2 - MICs of β-lactams, fluoroquinolones and aminoglycosides against *K. pneumoniae* strains.

Antimicrobial agents	MIC (μg/mL)					
	*Kp*01	*Kp*02	*Kp*03	*Kp*04	*Kp*05	*EC600*
Piperacillin	> 256	> 256	> 256	> 256	> 256	1
Piperacillin/sulbactam	> 256	> 256	> 256	> 256	> 256	1
Amoxicillin/clavulanic acid	> 256	> 256	> 256	> 256	> 256	0.5
Cefoxitin	> 256	> 256	> 256	> 256	> 256	4
Cefotaxime	> 256	> 256	> 256	> 256	> 256	0.25
Cefoperazone/sulbactam	> 256	> 256	> 256	> 256	> 256	≤ 0.125
Cefepime	128	128	> 256	> 256	128	≤ 0.125
Aztreonam	> 256	> 256	> 256	> 256	> 256	≤ 0.125
Imipenem	> 32	> 32	> 32	> 32	> 32	≤ 0.125
Meropenem	> 32	> 32	> 32	32	32	≤ 0.125
Ciprofloxacin	> 128	> 128	> 128	64	128	≤ 0.125
Levofloxacin	> 128	> 128	> 128	64	64	≤ 0.125
Gentamicin	> 128	> 128	> 128	> 128	128	≤ 0.125
Amikacin	> 128	> 128	> 128	128	128	≤ 0.125

*Kp*01, *Kp*02, *Kp*03, *Kp*04 and *Kp*05: carbapenem-resistant *K. pneumoniae* strains.

Three dimension test

All 5 isolates exhibited positive three-dimensional test results, and their extracts promoted the growth of one surface organism of *E.coli* ATCC25922 on MH agar. *E. cloacae* 029 M could produce AmpC enzyme to exhibit an interference with the growth of ATCC25922. ATCC700603 was used as the negative control (Figure 1).

Sequence analysis of β-lactamase, *OmpK35* and *OmpK36* genes

The PCR and sequence analysis showed that *Kp*01, *Kp*02, *Kp*03, *Kp*04 and *Kp*05 contained TEM and DHA β-lactamase genes. Compared with *bla*TEM-1 (Accession No. FJ668746) and *bla*DHA-1 (Accession No. HM193083) in GenBank, these strains shared 100% identity. Similarly, *OmpK*35 and *OmpK*36 genes in 5 multidrug-resistant *K. pneumoniae* strains were detected by PCR amplification and sequence analysis. Among 5 *K. pneumoniae* strains, the base deletions of *OmpK35* and *OmpK36* genes were observed in *Kp*01 and *Kp*03 strains, and the base deletion of *OmpK35* gene was also observed in *Kp*02 and *Kp*05. In *OmpK*36 gene, insertion and deletion as well as the mutations of short DNA fragment (1-5 bp) could result in the alteration of open reading frame and early termination of translation. In addition, mutations of *OmpK35* gene occurred in *Kp*04 (Table 3). Compared with GU945384 in GenBank, *OmpK35* gene mutations of G → C at base 465

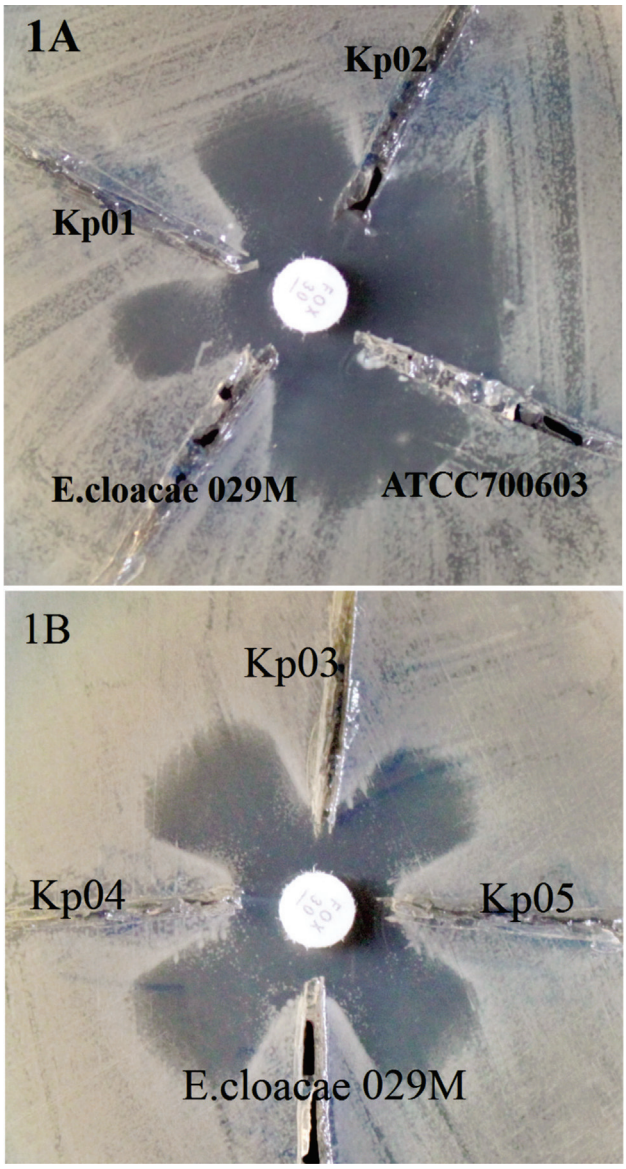

Figure 1 - Three-dimensional test for five strain isolates. (1A) Enhanced growth of *E.coli* ATCC25922 was observed near agar slits containing enzyme extracts of *Kp*01, *Kp*02 and *E. cloacae* 029 M. Except for ATCC700603, all of them were AmpC producers. (1B) *Kp*03, *Kp*04, *Kp*05 and *E. cloacae* 029 M could interfere with the growth of *E. coli* ATCC25922.

and T → C at base 466 in *Kp*04 could also result in the substitution from Gln to His at position 155 and substitution from Tyr to His at position 156. A new subtype has been registered in GenBank (Accession No. HQ259027).

Transconjugation test of *K. pneumoniae*

*EC*600 was used as the recipient in the conjugation studies. In addition, *bla*DHA-1 resistant genes from *Kp*01, *Kp*02, *Kp*03 and *Kp*05 were transferred into the recipient *EC*600 and confirmed by PCR. *EC*600 resulted in drug resistance from *Kp*01, *Kp*02, *Kp*03 and *Kp*05 to a certain degree, and the MICs of piperacillin, piperacillin/sulbactam,

amoxicillin/clavulanic acid and cefotaxime exhibited 2-16 fold increase (Table 4).

SDS-PAGE of outer membrane protein

The outer membrane proteins of 4 strains were detected by SDS-PAGE. Compared with the sensitive strains, the lack of outer membrane proteins in 3 carbepenem-resistant *K. pneumoniae* strains (*Kp*01, *Kp*02 and *Kp*04) were detected by SDS-PAGE, and molecular weights of these outer membrane proteins were 34-40 kDa, which suggested that only porin loss of OmpK36 in *Kp*01, *Kp*02 and *Kp*04 strains when compared with *K. pneumoniae* ATCC700603 and sensitive strains. In contrast, the porin of OmpK35 and OmpK36 was remained in *Kp*03 (Figure 2).

Inhibitory effects of reserpine and CCCP on the efflux of *K. pneumoniae*

All strains with a concentration of 5 x 10^4 cfu/mL can grow in the presence of 256 μg/mL reserpine and 128 μg/mL CCCP. When reserpine (30 μg/mL) and CCCP (50 μg/mL) were added in imipenem and meropenem, no change of MICs was observed in the presence of reserpine and CCCP.

Table 3 - Expression of two porins in five *K. pneumoniae* strains.

Strains	*bla*TEM-1	*bla*DHA-1	*Omp*K36 gene	*Omp*K35 gene
*Kp*01	+	+	Base deletion	Base deletion
*Kp*02	+	+	Insertion	Base deletion
*Kp*03	+	+	Base deletion	Base deletion
*Kp*04	+	+	Insertion	Mutation
*Kp*05	+	+	Insertion	Base deletion

Table 4 - The antimicrobial susceptibility of transconjugants.

Antimicrobial agents	MIC (μg/mL)			
	Tr01	Tr02	Tr03	Tr05
Piperacillin	8	16	4	8
Piperacillin/sulbactam	4	8	8	4
Amoxicillin/clavulanic acid	4	8	4	4
Cefotaxime	0.5	1	0.5	1
Cefoperazone/sulbactam	0.25	0.125	0.125	0.25
Aztreonam	0.25	0.25	0.25	0.5
Cefepime	≤ 0.125	≤ 0.125	≤ 0.125	≤ 0.125
Imipenem	≤ 0.125	≤ 0.125	≤ 0.125	≤ 0.125
Meropenem	≤ 0.125	≤ 0.125	≤ 0.125	≤ 0.125
Ciprofloxacin	≤ 0.125	≤ 0.125	≤ 0.125	≤ 0.125
Amikacin	0.25	≤ 0.125	0.25	0.25

Transconjugants: Tr.

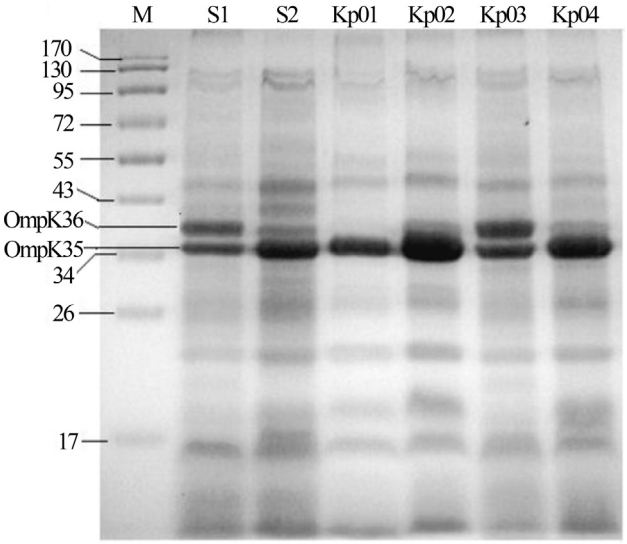

Figure 2 - SDS-PAGE of outer membrane proteins. M: Marker; S1: ATCC700603; S2: sensitive strain.

Discussion

In the present study, we screened 27 β-lactamase genes including Ambler class A, B, C and D by using PCR technique. The metallo-β-lactamases, OXA-type and KPC-type β-lactamases were not detected by PCR in 5 *K. pneumoniae* strains. However, all of 5 strains carried *bla*TEM-1 and *bla*DHA-1 β-lactamase genes. DHA-1, a plasmid-mediated AmpC type β-lactamase belonging to Ambler class C, can confer the resistance to oxyimino-cephalosporins (cefotaxime and ceftazidime) and cephamycins (cefoxitin and moxalactam), and transfer of the resistance has been confirmed by transconjugation (Lee, 2007; Ding *et al.*, 2008; Yamada *et al.*, 2009). In the present study, *bla*DHA-1 resistant genes from *Kp*01, *Kp*02, *Kp*03 and *Kp*05 may be transferred into the recipient *EC*600, leading to 2-16 fold MIC increase of piperacillin, piperacillin/sulbactam, amoxicillin/clavulanic acid and cefotaxime. These findings indicate that *bla*DHA-1 β-lactamase gene can spread rapidly between the same and different bacteria.

The base deletion of *OmpK*35 and *OmpK*36 genes in *Kp*01 and *Kp*03 was simultaneously observed. Similarly, the base deletion of *Omp*K35 also existed in *Kp*02 and *Kp*05. In addition, there were still genetic insertion of *Omp*K36 in *Kp*02, *Kp*04 and *Kp*05. SDS-PAGE analysis of the OMPs revealed the only loss of *Omp*K36 in *Kp*01, *Kp*02 and *Kp*04 strains when compared with *K. pneumoniae* ATCC700603 and a sensitive strains. However, the loss of porin was not observed in *Kp*03, which suggested that mutations or base deletions of porin-coding genes can lead to the alterations of open reading frame, amino acid sequences and protein configuration. Thus, the pore size of porin OmpK35 or OmpK36 can be affected and the accessibility

of drugs can be impeded. Moreover, the porin OmpK36 plays an important role in the resistance or reduced susceptibility to carbapenems in *K. pneumoniae* strains (Hernandez-Alles, 1999; Wang *et al.*, 2009). In this study, high-level carbapenem resistance in 5 *K. pneumoniae* strains was associated with the deletion or mutation of porin genes. The lack or deficiency of outer membrane protein can result in the change of permeability and reduction of cell accessibility for antibiotics or other drugs, which plays an important role in the main channels (Martinez-Martinez, 2008; Endimiani *et al.*, 2009; Jiang *et al.*, 2009; Landman, 2009). Therefore, *bla*DHA-1 β-lactamases combined with the absence of outer membrane proteins may confer to the carbapenems and cefoxitin resistance in *K. pneumoniae* strains (Su *et al.*, 2008; Jiang *et al.*, 2010).

Efflux systems that contribute to antibiotic resistance have been described for a number of clinically important bacteria (Guglierame *et al.*, 2006; Coyne *et al.*, 2010; Husain and Nikaido, 2010). The overexpression of multidrug efflux pumps can lead to low-level multi-drug resistance (Guglierame, 2006; Piddock, 2006). It has been reported that reserpine and CCCP were used as the pumps inhibitors (Shi *et al.*, 2005). Reserpine is a well-established inhibitor of efflux pumps among Gram-positive microorganisms and non-fermenting Gram-negative microorganisms. CCCP can destroy the proton gradient of bacterial transcytoplasm membrane, causing transport proteins to lose energy supply and eventually leading to the increasing accumulation of drug concentration (Zhang *et al.*, 2010). In this study, when reserpine and CCCP were added, MIC changes of imipenem and meropenem were not observed. The results indicated that the resistance of *K. pneumoniae* strains to carbapenems seems to be irrelevant to efflux.

Taken all together, the expression of porin OmpK36 coupled with *bla*DHA-1 β-lactamase genes plays an important role in conferring resistance of *K. pneumoniae* strains to carbapenems and cefoxitin in our hospital. Our findings highlight the urgent need to develop the strategies for the prevention and control of infections. Limited application of antimicrobials, especially for fluoroquinolones and cephalosporins, may be the effective strategies.

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