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LC-MS/MS: a rapid and simple new method for the determination of carbapenem β-lactamases

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ABSTRACT. We investigated the application of liquid chromatography-tandem mass spectrometry (LC-MS/MS) for pan-drug-resistant *Acinetobacter baumannii* (PDR-AB) with carbapenem resistance. Eight strains were randomly selected from 84 clinical isolates of PDR-AB strains obtained by the Kirby-Bauer and agar dilution methods. An efflux pump inhibition test was used to screen for the efflux pump phenotype. An ethylenediaminetetraacetic acid (EDTA) synergy test was used to screen for the β-lactamase phenotype, and a three-dimensional test was used to detect extended spectrum β-lactamase (ESBL) and ampicillin C, KPC, and carbapenemase. ESBL genes were amplified by polymerase chain reaction and sequenced. Outer membrane proteins were extracted from a sensitive strain and the PDR-AB strains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subjected to LC-MS/MS. Peptide mass fingerprinting data were retrieved, and proteins with differential expression were identified. Results of the efflux pump inhibition tests showed that the minimum inhibitory concentrations for meropenem were decreased in 4 of the 8 strains by at least 25% of the

original value. The results of the EDTA synergy test were negative, and the modified Hodge's tests were positive for all strains. PCR and sequencing confirmed that seven, five, and all eight of the PDR-AB strains contained *blaOXA-23*, *blaTEM-1*, and *KPC-2*, respectively. OXA-23 and CsuC proteins were differentially expressed between the drug-resistant and -sensitive strains. Production of blaOXA-23 enzyme and pilus molecular chaperone to guide synthesis of CsuC protein may be involved in the resistance of *A. baumannii* to carbapenems. LC-MS/MS provides a quick and easy method for carbapenemase detection.

Key words: LC-MS/MS; Carbapenemase; Resistance mechanism

INTRODUCTION

Acinetobacter baumannii is an important pathogen that causes nosocomial infection (Karageorgopoulos and Falagas, 2008), often affecting immunosuppressed patients and those who have undergone tracheostomy or are being treated with mechanical ventilation for pulmonary infection. In addition, the number of pan-resistant *A. baumannii* strains remains high. Carbapenems are a class of antibiotics used to treat Gram-negative bacilli infections. The increased clinical use of antimicrobial drugs has resulted in the development of drug resistance of non-fermenting bacteria (represented by *Pseudomonas aeruginosa* and species of the *Acinetobacter* genus) resistant to carbapenems. In 2012, data from the CHINET antimicrobial resistance survey revealed that the drug resistance rates of *Acinetobacter* bacteria (89.6% of which are *A. baumannii*) to imipenem and meropenem were 57.0 and 61.0%, respectively (Wang et al., 2013). The generation of carbapenemase is the main cause of carbapenem antibiotic resistance (Nemec et al., 2004). These drug-resistant strains can produce carbapenemase and other enzymes to result in nosocomial infection outbreaks, for which no drug-based treatment methods are currently available. Thus, new methods of carbapenemase detection that are fast, easy, and highly sensitive and specific are urgently needed. In this study, we collected 84 pan-drug-resistant *A. baumannii* (PDR-AB) strains, tested them for imipenem and meropenem resistance, and studied the mechanism of resistance in eight randomly selected strains that were sensitive only to colistin. We established a fast and easy method for identifying carbapenem β-lactamase using liquid chromatography-tandem mass spectrometry (LC-MS/MS), which should help to reveal the mechanism of *A. baumannii* resistance to carbapenems.

MATERIAL AND METHODS

Materials

Strain source and screening

We collected clinical isolates of 84 PDR-AB strains (resistant to polymyxin and other antimicrobial drugs, including β-lactamase inhibitor compound, fluoroquinolones, aminoglycosides, and carbapenems) from the First Hospital Affiliated to Anhui Medical University, from January 2010 to December 2010. Duplicated isolates from the same site of the same patient were excluded. We randomly selected 8 strains to study the mechanisms of resistance

to meropenem and imipenem. We selected one strain that was sensitive to 7 antibiotics and showed anti-*Pseudomonas* effects (including to penicillins, cephalosporins, monocyclic antibiotics, fluoroquinolones, aminoglycosides, carbapenems, and colistin) as a control strain.

Drugs

For susceptibility testing, amikacin, gentamicin, piperacillin, ceftazidime, ciprofloxacin, levofloxacin, cefepime, cefoperazone-sulbactam, piperacillin-triazole Batan, aztreonam, imipenem, meropenem, and polymyxin were purchased from Oxoid (Hampton, UK). The antibiotic powder imipenem was purchased from Hangzhou MSD Pharmaceutical Co., Ltd. (Hangzhou, China). Meropenem, clavulanic acid (CLA), and cloxacillin (CLO) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). L-Phenol propylamino-L-amino acid-beta-naphthylamine (MC-207,110; L-PAβN) was purchased from Sigma (St. Louis, MO, USA).

Medium and reagents

Mueller-Hinton agar culture medium was purchased from Oxoid. The polymerase chain reaction (PCR) detection reagents *Taq* enzyme, buffer, and dNTPs were purchased from Jiuh-Bao Biotechnology (Dalian) Co., Ltd. (Fujian, China).

Primers

Primers were designed as described by Shen et al. (2008) and synthesized by Sangon Co., Ltd. (Shanghai, China).

Instruments

The PROTEAN II Xi Cell vertical slab electrophoresis instrument, Molecular Image Fx gel image scanner, and PDQuest version 8.0 image analysis software were purchased from Bio-Rad (Hercules, CA, USA) for electrophoresis. The LC-MS/MS mass spectrometer was purchased from Thermo Scientific (Waltham, MA, USA).

Methods

Susceptibility testing was performed using the Kirby-Bauer disc diffusion method, recommended by the United States Clinical and Laboratory Standards Institute (2010 edition). The minimum inhibitory concentrations (MICs) of imipenem and meropenem were detected using the agar dilution method. *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as control strains.

Efflux pump phenotypic screening test

The MICs of drug-sensitive and -resistant strains were detected in the presence and absence of 20 mg L-PAβN. After adding L-PAβN, the MIC of the strains resistant to imipenem or meropenem decreased by more than 4-fold. This suggested that the efflux pump plays an important role in the mechanism of drug resistance in these strains (Ribera et al., 2002).

β-lactamase phenotypic screening

Extended-spectrum β-lactamase (ESBL) and ampicillin C (AmpC) β-lactamase were detected by a three-dimensional test (Ni et al., 2005; Xu et al., 2005; Xia and Xu, 2008). β-lactamase in the 8 drug-resistant strains was detected by ultrasonication and a modified three-dimensional test. A sterile cotton swab was used to collect 0.5 McFarland units of *E. coli* ATCC 25922, which was coated uniformly on Mueller-Hinton agar plates. After 10 min, a piece of CRO paper was placed in the center of the plate. A sterile blade was used to radially draw 5 cracks (2 mm wide; 15 mm long) about 5 mm from the edge of the paper inside to outside. The cracks were added to either i) 40 µL crude extraction (ESBLs or AmpC), ii) 32 µL crude extraction + 8 µL CLO (2 mM), iii) 32 µL crude extraction + 8 µL CLA (2 mM), iv) 32 µL crude extraction + 8 µL CLA (2 mM) + 8 µL CLO (2 mM), or v) 32 µL crude extraction + 8 µL ethylenediaminetetraacetic acid (EDTA)-Na_2 (0.1 M). After culturing overnight at 35°C, a sagittal lawn appeared on the plate, indicating that the enzyme could hydrolyze CLO. At the same time, we observed whether the enzyme could be inhibited by CLA, CLO or EDTA-Na_2. *Klebsiella pneumoniae* ATCC 700603 and *Enterobacter cloacae* 029M served as positive control strain of the ESBL and AmpC enzyme assays, respectively. The judgement standards were as follows: positive results for treatments (i), (iii), and (iv) were determined to be ESBL-producing strains; strains with positive results for treatments (i), (ii), and (iv) were determined to be AmpC-producing strains; strain with positive results for treatments (i), (ii), (iii), and (iv) were determined to be ESBL + AmpC-producing strains; strains with positive results for only treatment (v) were determined to be metallo-β-lactamase-producing strains; and strains with positive results for all treatments (i)-(iv) were determined to be metallo-β-lactamase + ESBL + AmpC-producing strains.

In addition, the β-lactamase phenotype was detected by an EDTA synergy test, according to the methods described by Shen et al. (2008), using 8 drug-resistant strains and one wild-type strain.

Detection of carbapenemase

Carbapenemase was detected using a modified Hodge test as described in the CLSI M100-S19 guidelines (2009).

Detection of β-lactamase genes

The carbapenemase genes *blaKPC* and *blaOXA*, and the ESBL genes *blaTEM*, *blaCTX-M*, *blaSHV*, and *blaOXA* were detected by PCR amplification. The DNA template was prepared using the boiling method. Primer synthesis and amplification conditions were as described previously (Shen et al., 2008). Positive PCR amplification products were sequenced by Shanghai Biological Engineering Company (Shanghai, China). Sequencing results were analyzed using the BLAST in National Center for Biotechnology Information (NCBI).

Membrane protein analysis

Outer membrane protein (Omp) extraction and separation was conducted according to the methods described by Luo et al. (2009) from 8 PDR-AB strains and a fully sensi-

tive strain. Membrane protein analysis was performed by vertical-slab sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 10 μL processed protein samples. The gels were stained with Coomassie brilliant blue overnight. After destaining (methanol:deionized water:glacial acetic acid; 45:45:10), photographs were acquired using a Bio-Rad GelDoc XR gel imaging system and the Quantity One image software was used to analyze the images.

LC-MS/MS analysis

To construct the protein spectrum system, extensively drug-resistant *A. baumannii* were screened based on increased or decreased expression levels of bands in SDS-PAGE. The protein bands were cut out directly from the gel and placed into a sample tube, washed with 50 mM ammonium bicarbonate, and the buffer was removed. At least 200 mL 30% acetonitrile/50 mM ammonium bicarbonate was added, vortexed for 30 s, maintained at room temperature for 5-10 min, and then the buffer was discarded; this step was repeated until the Coomasie brilliant blue stain was removed. A further 200 mL 50% acetonitrile/50 mM ammonium bicarbonate was added, the buffer was removed and discarded, and the gel was vacuum-pumped for 10-30 min until the gel was completely dried. Fifty microliters of 50 mM bicarbonate containing 0.1 μg trypsin was added to each tube, and digested overnight at 37°C. The enzyme digestion solution was collected, 200 mL 60% acetonitrile/5% trifluoroacetic acid was added, the mixture was sonicated for 1 min, and the above steps were repeated. The sample was frozen, and vacuum-drained for about 4 h, after which 25 μL 50 mM bicarbonate/0.1% formic acid was added. One microliter of the suspension was collected for LC-MS/MS peptide fingerprint analysis. After bleaching and digestion, the mixed peptide segments were subjected to a fully automated, multi-level, one-dimensional LTQ linear ion trap LC-MS instrument workstation for analysis.

To construct the peptide mass fingerprint database, the primary data collected by MS analysis were searched using the Bioworks version 3.2 software (SEQUEST, Thermo Scientific), using a local search server for matching polypeptides of *A. baumannii* non-redundant protein databases, downloaded from NCBI in July 2011.

RESULTS

Drug-sensitivity tests

Eight strains of *A. baumannii* were selected that were resistant to meropenem and imipenem, with MIC values reaching 8-128 mg/L and 16-128 mg/L, respectively. All strains also showed drug resistance for the 12 other antibacterial agents tested; all eight strains were sensitive to polymyxin.

Efflux pump phenotypic screening

The results of the synergistic efflux pump inhibitor test of the 8 PDR-AB strains showed that the MIC values for meropenem decreased to 25% or more of the original value in 4 strains, and the MIC values of imipenem did not decrease to 25% or more than the original values in 8 strains (Table 1).

Table 1. Result of 8 strains of *Acinetobacter baumannii* resistant to carbapenems inhibited by efflux pump inhibitor L-PAβN. (MIC unit: μg/mL).

Strain No.	Meropenem MIC	After adding efflux pump inhibitor MIC	Imipenem MIC	After adding efflux pump inhibitor MIC	Positive phenotype	Efflux pump inhibitor
AB1	32	16	32	16	-	IMP-MEM-
AB8	>128	>128	>128	>128	-	IMP-MEM-
AB18	32	16	32	16	-	IMP-MEM-
AB26	128	16	128	64	+	IMP-MEM+
AB29	64	8	64	32	+	IMP-MEM+
AB43	64	16	64	64	+	IMP-MEM+
AB51	128	8	64	64	+	IMP-MEM+
AB59	64	32	64	64	-	IMP-MEM-

β-lactamase phenotype

The eight PDR-AB strains and a wild-type strain were subjected to an EDTA synergy test, and all of the results were negative.

Detection of ESBLs and AmpC by three-dimensional tests

All eight of the PDR-AB strains were found to produce β-lactamase, which can hydrolyze carbapenems, and its activity was inhibited by CLA, but not by CLO or EDTA. The screening test for β-lactamase with EDTA was negative, which is consistent with the results of the EDTA enhancement test indicated above. These results suggested that the drug-resistant strains may be able to produce ESBLs or blaKPC, blaOXA, and other Class A and Class D enzymes (Figure 1).

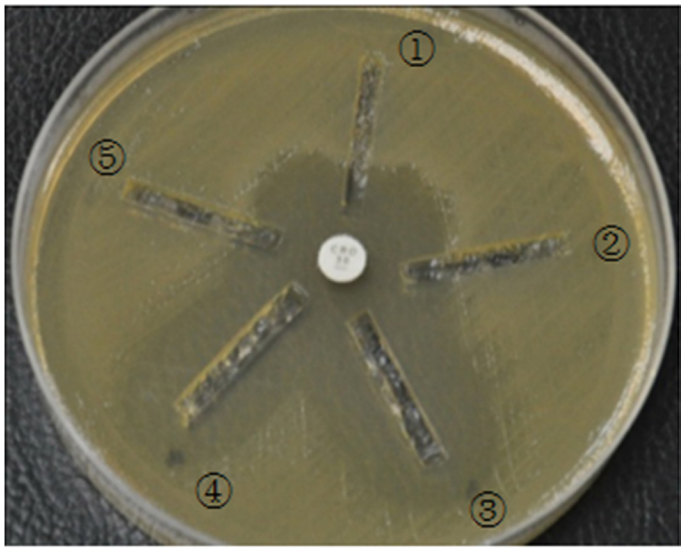

Figure 1. Three-dimensional tests of *Acinetobacter baumannii.* ① crude extraction 40 μL; ② crude extraction 32 μL + 8 μL CLO (2 mM); ③ crude extraction 32 μL + 8 μL CLA (2 mM); ④ crude extraction 32 μL + 8 μL CLA (2 mM) + 8 μL CLO (2 mM); ⑤ crude extraction 32 μL + 8 μL EDTA-2Na_2 (0.1 M) ①, ③, ④ were positive, suggesting that these strains produce super-spectrum β-ESBL.

Carbapenemase phenotype test

The eight PDR-AB strains were subjected to a modified Hodge test, and the results were positive for all strains, whereas the result was negative for the wild-type strain (Figure 2).

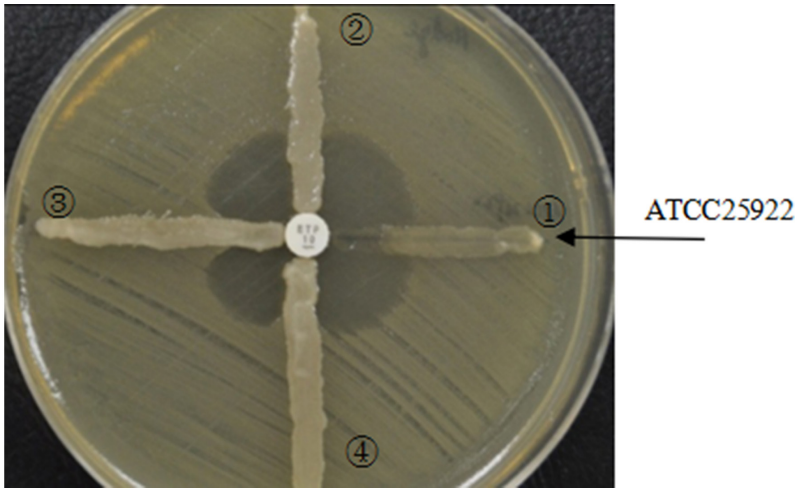

Figure 2. Modified Hodge test of *Acinetobacter baumannii.* ① ATCC25922; ②, ③, ④ PDR-AB26, AB29, AB43.

Detection of β-lactamase genes

Carbapenamase genes were detected in the eight PDR-AB strains by PCR, using the primers for *KPC*, including those of the *OXA23*, *OXA24*, *OXA48*, *OXA50*, *OXA55*, *OXA58*, *OXA60*, and *OXA64* groups. PCR amplification was also conducted for the ESBL genes *TEM*, *SHV*, *CTX-M*, and *OXA*. Of the 8 PDR-AB strains, five were positive for the *TEM* gene, all eight strains were positive for *KPC* genes, including seven PDR-AB *OXA23* gene-positive strains. The rest were negative (Figures 3 and 4).

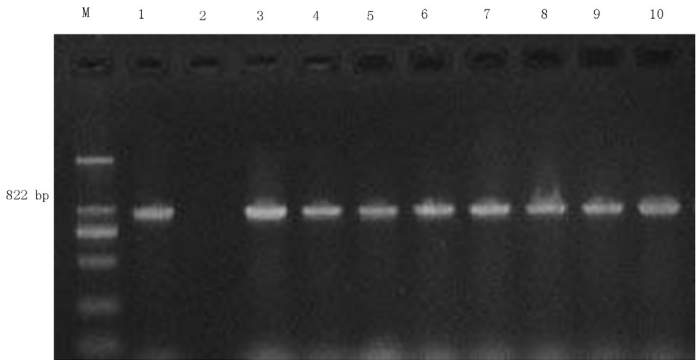

Figure 3. Amplification electrophoretogram of the primer of *Acinetobacter baumannii* OXA23 entire coding sequence. *Lane M* represents DL2000 marker; *lane 1* represents positive control; *lane 2* represents negative control; *lanes 3-5* and *7-10* represent PDR-AB-positive strains; *lane* 6 represents PDR-AB strain.

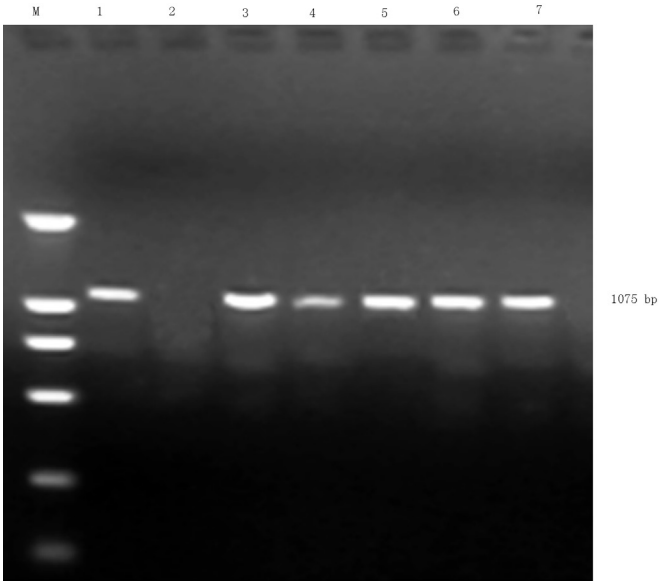

Figure 4. Amplification electrophoretogram of the primer of *Acinetobacter baumannii* TEM-1 entire coding sequence. *Lane M* represents DL2000 marker; *lane 1* represents positive control; *lane 2* represents negative control; *lanes 1-5* represent PDR-AB-positive strains.

Using the primers of *TEM*, *OXA23*, and *KPC-2*, the full-coding sequences were PCR-amplified and sequenced for strains that were positive for these genes. The *OXA23*-amplified products from the 7 *OXA23*-positive strains were purified, sequenced, and subject to a BLAST. Homology with the *OXA-23* gene in GenBank was 99%; a G was deleted at bp 818, which resulted lack of the corresponding leucine residue. The sequencing results confirmed that five of the drug-resistance strains were positive for the *TEM-1* gene, and all eight strains were positive for the *KPC-2* and *TEM-1* gene; homology with the *KPC2* and *TEM-1* gene in GenBank was 100% (Figures 5 and 6).

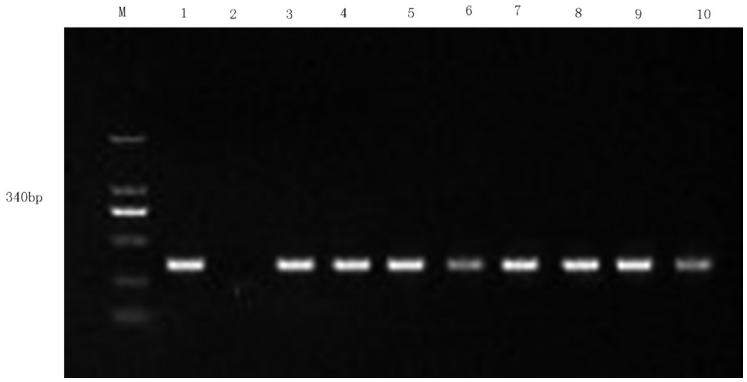

Figure 5. Amplification electrophoretogram of the primer of *Acinetobacter baumannii* KPC universal sequence. *Lane M* represents DL2000 marker; *lane 1* represents positive control; *lane 2* represents negative control; *lanes 3-10* represent PDR-AB-positive strains.

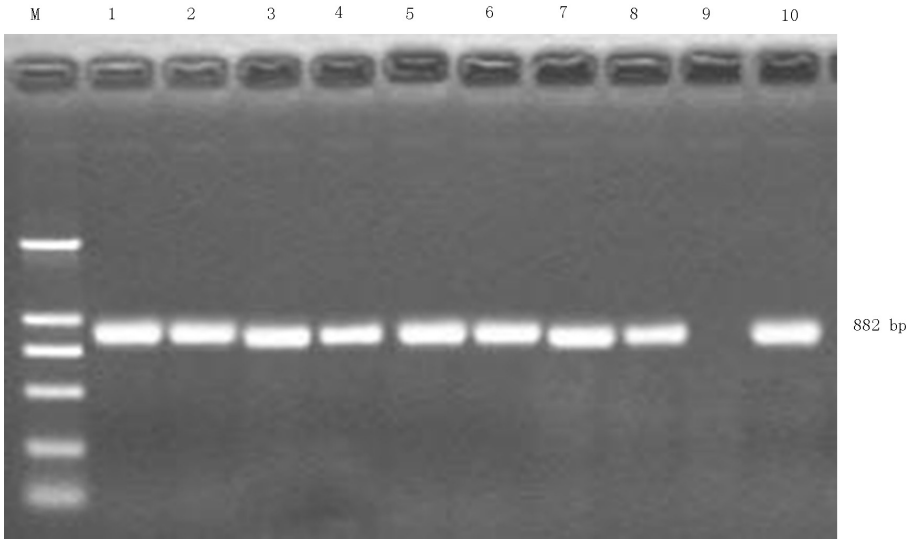

Figure 6. Amplification electrophoretogram of the primer of *Acinetobacter baumannii* KPC-2 entire coding sequence. *Lane M* represents DL2000 marker; *lanes 1-8* represent positive control; *lane 9* represents negative control; *lane 10* represents positive control.

SDS-PAGE of Omp

SDS-PAGE of the Omp of a wild-type strain and the eight PDR-AB strains showed the absence of a band at 25 kDa in the resistant strains, which was present in the drug- sensitive strain. Instead, the drug-resistant strains all had a 28-kDa protein band, which was not detected in the drug-sensitive strain (Figure 7).

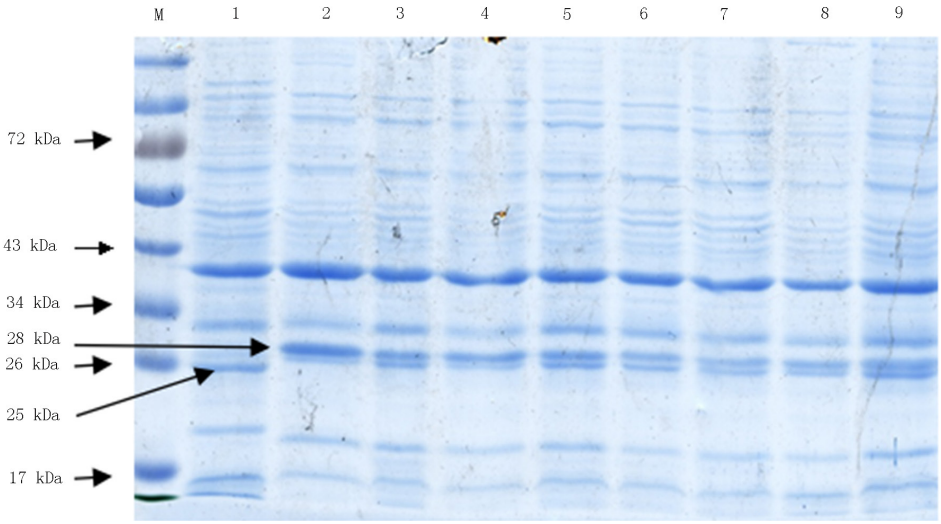

Figure 7. SDS-PAGE protein electrophoretogram of the outer membrane protein of wild strain and PDR-AB strains. *Lane M* represents DL2000 marker; *lane 1* represents wild strain, *lanes 2-9* represent PDR-AB.

Differential expression of Omp between drug-resistant and -sensitive strains based on LC-MS/MS

The major protein bands at 28 kDa in the drug-resistant strains were identified to be OXA-23 protein (molecular weight: 28 x 10^3) and CsuC protein (molecular weight: 30 x 10^3); the major protein band at 25 kDa in the drug-sensitive strain was identified to be OmpW (molecular weight: 23 x 10^3) (Table 2).

Table 2. Identification of *Acinetobacter baumannii* outer membrane proteins shown in SDS-PAGE map by LC-MS/MS.

Spot	Protein name	MW (Da)/pI	Accession No.	Score	Sequense coverage (%)
1	beta-lactamase OXA-23	28665.7/2.82	335346935	210.28	72.73
2	CsuC	30624.0/3.48	37933549	110.24	49.46
3	putative outer membrane protein W	23123.0/1.41	169153379	20.17	18.18

DISCUSSION

The drug resistance mechanism of *A. baumannii* resistant to carbapenemase antibacterial drugs is complex, and involves inactivation of carbapenamase caused by hydrolysis, outer membrane permeability dysfunction, and the presence of a multi-drug resistance efflux pump on the inner membrane (Zarrilli et al., 2009). In this study, we used proteomic analysis to screen for the differential expression of membrane proteins between antibiotic-sensitive and -resistant *A. baumannii* strains. The results of SDS-PAGE and LC-MS/MS were combined with preliminary tests for the hydrolase phenotype, genotype, and functional testing of the efflux pump.

When treated with the efflux pump inhibitor L-PAβN, the imipenem MIC values of the PDR-AB strains were not significantly decreased, whereas the meropenem MIC values dropped by more than 25% in four of the eight strains. This result suggested that the mechanism of drug resistance to meropenem is mediated by an efflux pump mechanism in some strains, whereas the drug resistance mechanism of imipenem may be unrelated to the efflux pump.

It has been reported that hydrolysis of carbapenem β-lactamases is the most common mechanism of *A. baumannii* drug resistance (Perez et al., 2007). The most common forms of these carbapenamase are Class D (OXA), Class A (KPC), and Class B (IMP, VIM, SIM, and other metalloenzymes). KPC carbapenemase and TEM ESBLs belong to Class A serine enzymes. Class A and Class D carbapenemases can be inhibited by CLA, but cannot be inhibited by EDTA; the reverse is true for Class B carbapenemases. In this study, the results of modified three-dimensional tests showed positive β-lactamase activity in all eight PDR-AB strains, which could be inhibited by CLA, but not by CLO or EDTA, suggesting that the strains produced KPC, OXA, and ESBLs. The modified Hodge test results were positive for carbapenemase in all eight strains, suggesting that they may produce OXA and KPC carbapenemase; the EDTA synergy test did not detect the production of metalloenzymes. This result is consistent with the results of the modified three-dimensional test, suggesting that resistance of these PDR-ABs to carbapenemase may be unrelated to metal enzymes. The genetic test for KPC-, OXA-, and ESBL-encoding genes showed that seven of the eight (87.5%) strains contained *blaOXA-23* genes. OXA-23 was the first reported OXA carbapenemase in *A. baumannii* (Donald et al., 2000). Five drug-resistant strains were found to produce the broad-spectrum enzyme TEM-1, and all eight strains contained the *blaKPC* gene. KPC-2-producing strains are mostly associated

with *Klebsiella pneumoniae*, although the widespread use of imipenem in recent years has induced *A. baumannii* to produce the KPC enzyme. Ten KPC-positive strains were found among β-lactamase multi-drug resistant *A. baumannii* strains studied in Puerto Rico (Robledo et al., 2010), which supports the results of the present study. Whether or not these are clones remains to be confirmed by further studies.

The genotype results were consistent with the phenotype results in the present study. Since a gene is only the carrier of genetic information, protein analysis is also required to determine the actual biological function. The DNA sequence cannot reveal the real-time expression of certain a gene, expression level, post-translational processing and modification of proteins, or the sub-cellular distribution, etc.; therefore, determining the overall level of protein expression and function is becoming increasingly important in addition to sequence analysis. A few studies (Burckhardt and Zimmermann, 2011; Hrabak et al., 2011) also identified NDM-1, VIM-1, VIM-2, KPC-2, and various types of imipenem enzymes from carbapenem-resistant *P. aeruginosa*, *K. pneumoniae*, and *E. coli* strains by matrix-assisted laser desorption time-of-flight MS within only 1-2.5 h, with a detection sensitivity of 96.67% and specificity of 97.87%. In the present study, the carbapenemase OXA23 was very accurately identified by MS, and was consistent with the results of phenotype and genotype tests.

A high expression level of OmpW membrane protein was detected in the sensitive strain. OmpW can form a hydrophobic channel, and its expression was found to be significantly reduced in drug-resistant strains of *Salmonella typhimurium* (Hu et al., 2005); however, no studies have shown that OmpW participates in the mechanism of resistance to carbapenems.

The most common cause of *A. baumannii* infection is via medical devices in hospitals. In particular, it has the ability to adhere to indwelling catheters and respiratory epithelial cells; in particular, clinical strains related to urinary tract or blood stream infections isolated from catheters show characteristics of forming biofilms and antimicrobial resistance (Sarkisova et al., 2005). The pilus is essential to the process of biofilm formation in the *A. baumannii* ATCC19606 strain. In this study, CsuC protein was detected, which is involved in the secretory process of pilus assembly, namely acting as a chaperone-usher for the pili assembly system. The function of a chaperone protein is to combine with and stabilize the pilus subunit, which prevents its hydrolysis. Such usher proteins can form a porous channel in the bacterial outer membrane, and cooperate with chaperone proteins to assemble and secrete pilus. Cells adhere to the abiotic surface through the pilus and start the microcolony formation stage (Tomaras et al., 2008). Biofilms can induce the expression of bacterial drug-resistance genes and the multidrug-resistance pump gene (Otto, 2006).

In conclusion, the use of LC-MS/MS to detect carbapenemase protein shows high sensitivity, high accuracy, good speed and automation, and enables the simultaneous detection of all types of carbapenemases. In addition, it could detect unknown carbapenemases and overcome omission problems. This study employed advanced MALDI-TOF-MS technology, and established a rapid and simple method for detecting carbapenemase. In the future, we intend to use this method to detect Gram-negative bacillary carbapenemase of clinical isolates showing carbapenem resistance. This method should prove useful for elucidating the drug resistance mechanism of Gram-negative bacteria resistant to carbapenems and to help delay the currently rapid development of drug resistance.

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