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ORIGINAL ARTICLE

Rapid and sensitive detection of carbapenemase activity in *Acinetobacter baumannii* using superficially porous liquid chromatography-tandem mass spectrometry

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Abstract *Background:* The emergence and spread of carbapenem-resistant *Acinetobacter baumannii* poses a challenge for optimizing antibiotic therapies and preventing outbreaks. Traditional phenotypic assays such as the modified Hodge test (MHT) or polymerase chain reaction (PCR)-based detection of the carbapenemase genes are time-consuming and complicated. Therefore, new approaches for the efficient detection of carbapenemase-producing *A. baumannii* are urgently required.
Methods: In this study, we used the superficially porous liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay to measure carbapenem hydrolysis in a solution spiked with test strains of *A. baumannii*. The rate of carbapenem hydrolysis during incubation was expressed as the ratio of the carbapenem peak area of the test *A. baumannii* strains to the noncarbapenemase-producing *A. baumannii* ATCC 17978. This method can accurately measure the carbapenem hydrolysis rate and, therefore, can effectively identify carbapenemase-producing strains within 75 minutes.
Results: A total of 112 *A. baumannii* strains were used in this study, including 103 clinical isolates with 68 carbapenem-resistant strains and 35 carbapenem-susceptible strains, seven ATCC strains and two selected mutants. The results of the superficially porous LC-MS/MS assay showed higher detection sensitivity compared to the results of the MHT.

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Conclusion: Our results demonstrate the ability of the former method to routinely detect carbapenemase-producing *A. baumannii*.

Introduction

Over the past few decades, multidrug-resistant (MDR) *Acinetobacter baumannii* has gradually emerged as an important nosocomial pathogen worldwide.[1] Carbapenems, primarily imipenem and meropenem, have been used to treat MDR *A. baumannii* infections.[2] However, there are increasing reports of carbapenem-resistant *A. baumannii* being isolated.[3,4] Previous studies showed that most carbapenem-resistant *A. baumannii* isolates are able to produce OXA-type (oxacillinase) carbapenemase.[3,5] Therefore, developing rapid and effective techniques for the identification of carbapenemase-producing *A. baumannii* will ensure appropriate treatment to patients, along with effective control of nosocomial infections.

Conventional methods such as the modified Hodge test (MHT) have successfully identified the majority of the carbapenemase-producing bacteria,[6] and commercial phenotypic methods [e.g., the VITEK 2 system (bioMerieux, Marcy L'Etoile, France)] are commonly used in microbiology laboratories to identify carbapenem-resistant bacteria. Previously, Pasteran et al[7] proposed a procedure for carbapenem screening. They assumed that the clinical isolates positively identified as carbapenemase producers would be confirmed by additional, more specific methods such as the MHT, together with the minimal inhibition concentration (MIC) test. However, these additional methods are time-consuming (typically >18 hours for the MHT and >4 hours for the VITEK 2 system), relatively complicated to operate, and may require specific media (e.g., the VITEK 2 system).

The Carba NP test is a colorimetric- and pH-based detection of imipenem hydrolysis. Although it is a rapid method to detect carbapenemase producers, it fails to detect OXA-type carbapenemase, which is the most frequently identified carbapenemase among the *Acinetobacter* species.[8] Recently, carbapenemase-producing bacteria have been identified using a number of polymerase chain reaction (PCR)-based methods.[9,10] However, several different enzyme families exhibit carbapenemase activity, and the diversity of the genes encoding these enzymes has limited the development of a single rapid test to identify all carbapenemase-producing organisms.

Mass spectrometry (MS) technologies, such as matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF MS) and ultra-performance liquid chromatography-tandem MS (UPLC-MS/MS) are also able to characterize carbapenemase-producing bacteria.[11,12] MALDI-TOF MS can be used to detect molecules with a broad range of masses. However, the small molecular size of antibiotics (<1000 Da) complicates the analysis in two ways; one is the possible interactions of the antibiotics with the matrix, and the other is the interference from a high background signal.[13] For example, meropenem can be observed at a mass-to-charge ratio (m/z) of 383.4. The dimer of alpha-cyano-4-hydroxycinnamic acid, a common matrix, is observed at m/z 380. The peak of meropenem is not clearly visible in a spectrum with a high matrix signal. In contrast, LC-MS/MS, especially triple quadrupole MS/MS, provides better spectral resolution and good quantification performance in clinical chemistry laboratories.[14] It is the gold standard for determining small molecule compounds because of its high sensitivity and specificity. In general, a reduction in the particle size of the LC column leads to a decrease in mass transfer and an increase in column efficiency. Totally porous sub-2-μm particle LC columns provide high-resolution chromatography, but must be operated at high back pressure.[15] In recent years, sub-3-μm superficially porous columns, a solid core surrounded by a porous layer, have been developed. The particle size distribution of the superficially porous columns is significantly narrower than that of the totally porous columns.[16] The narrow particle size distribution allows the formation of optimally packed beds, consequently leading to high efficiency and stability. Additionally, the superficially porous columns exhibit column back pressure which is one-half to one-third of that of the columns with totally porous sub-2-μm particles at the same flow rate, and they are potentially compatible with the conventional high-performance LC instruments (back pressure limit, 400 bar).[16,17] Moreover, the re-equilibrium volume of a superficially porous column requires less than three column volumes of the initiating solvent, which reduces the analysis time.[18] Therefore, in this study, we chose to use a superficially porous column to improve the separation efficiency.

In this work, we used superficially porous LC-MS/MS assays to measure carbapenem hydrolysis in a solution spiked with the test strains of *A. baumannii*. Our results show that this method can accurately measure the rates of carbapenem hydrolysis, and thus can effectively identify carbapenemase-producing *A. baumannii* within 75 minutes.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are summarized in Table 1. Among the 112 strains of *A. baumannii*, seven carbapenem-susceptible reference strains were obtained from the American Type Culture Collection (ATCC), and two were selected mutants generated from the parental strain ATCC 17978. Sixty-eight carbapenem-resistant and 35 carbapenem-susceptible clinical isolates were non-repetitively collected from three branches (Hualien,

Table 1 Result of genotypic and phenotypic tests of *Acinetobacter baumannii*.

A. baumannii reference strains ($n = 9$)	Description	MIC (μg/mL) of IPM	MHT result of IPM		Hydrolysis rate of IPM (%)
ATCC17978	ATCC strain	<2	−		0
ATCC15884	ATCC strain	<2	−		0
ATCC15886	ATCC strain	<2	−		0
ATCC19606	ATCC strain	<2	−		0
ATCC15308	ATCC strain	<2	−		0
ATCC15151	ATCC strain	<2	−		0
ATCC19003	ATCC strain	<2	−		0
ATCC17978 IPM-2m	Imipenem-selected mutant (carbapenem-borderline-susceptible)	2	−		100
ATCC17978 IPM-8m	Imipenem-selected mutant (carbapenem-resistant)	>8	−		100
No. of *A. baumannii* susceptible isolates ($n = 35$)	Resistance mechanism(s) determined by multiplex PCR	MIC (μg/mL) of IPM	MHT result of IPM		Hydrolysis rate of IPM (%)
9	OXA-51	<2	−		0–40
1	OXA-51, TEM-1	<2	−		56
13	OXA-24, OXA-51	<2	−		0–37
1	OXA-24	<2	−		0
1	OXA-23, OXA-51	<2	−		0–21
10	All negative	<2	−		0–14
No. of *A. baumannii* resistance isolates ($n = 68$)	Resistance mechanism(s) determined by multiplex PCR	MIC (μg/mL) of IPM	MHT result of IPM		Hydrolysis rate of IPM (%)
			Positive	Negative	
3	OXA-23, OXA-51, TEM-1	>8	2	1	100
1	OXA-24, OXA-51	>8	1	0	100
9	OXA-24, OXA-51, TEM-1	>8	9	0	100
34	OXA-51, TEM-1	>8	29	5	84–100
1	OXA-51, TEM-1	>8	0	1	19
16	OXA-51	>8	15	1	86–100
1	OXA-23, OXA-58, OXA-51, TEM-1	>8	1	0	100
2	OXA-23, OXA-51	>8	2	0	100
1	All negative	>8	0	1	100

ATCC = American Type Culture Collection; IPM = imipenem; MHT = modified Hodge test; MIC = minimal inhibition concentration; PCR = polymerase chain reaction.

Taipei, and Dalin) of the Buddhist Tzu Chi General Hospital in Taiwan. All carbapenem-resistant clinical isolates collected during 2007 were stored at −80°C in trypticase soy broth (Difco Laboratories, Detroit, MI, USA), supplemented with 20% glycerol, before testing. The isolates were then transported to the clinical microbiology laboratory at Tzu Chi University for further experiments. These bacteria were cultured at 37°C in brain heart infusion broth, according to a standard protocol.

A. baumannii ATCC 17978 was used as a carbapenem-susceptible parental strain to develop an LC-MS/MS assay for carbapenemase detection that would be suitable for all clinically relevant *A. baumannii*. The carbapenem-borderline-susceptible and carbapenem-resistant selected mutants were generated from the parental strain using a method reported earlier.[19] The selected strains exposed to 2 mg/L and 8 mg/L imipenem were collected during the induction period and were referred to as 17978 IPM-2m and 17978 IPM-8m, respectively. The carbapenem susceptibilities of the selected mutants were tested using the broth dilution method, according to the 2014 Clinical and Laboratory Standards Institute (CLSI) guidelines.

Identification of strains and evaluation of antimicrobial susceptibilities

A. baumannii strains used in this study were identified using the Vitek system (bioMerieux). Bacterial resistance to carbapenem was double-checked using the broth dilution method, in accordance with the 2014 CLSI guidelines. The breakpoints for imipenem and meropenem as per these guidelines were susceptible (S) ≤2 mg/L and resistant (R) ≥8 mg/L.

To detect the carbapenemase activity in *A. baumannii* strains, MHT was performed as recommended by the CLSI. Briefly, a 0.5 McFarland dilution of *Escherichia coli* 25922 in 5 mL of saline was prepared. A 1:10 dilution was inoculated on the surface of a Mueller-Hinton agar plate and disks with

imipenem (10 μg) were placed onto the middle of the agar plate. Test organisms were inoculated onto the plate in a straight line from the disk to the edge of the agar plate. After overnight incubation, the presence of *E. coli* was interpreted as a positive result for carbapenem hydrolysis screening.

Inhibition base test

According to Tsakris et al,[20] both class A *Klebsiella pneumonia* carbapenemase (KPC) and class B metallo-β-lactamases (MBLs) can be differentiated by a simple method which can also detect the possible coproduction of both carbapenemases. Briefly, the test was performed by inoculating Mueller-Hinton agar for the standard diffusion method, then preparing one disc of imipenem without any inhibitor and three discs of imipenem containing 400 μg of phenylboronic acid (PBA), 292 μg of EDTA, or both 400 μg of PBA and 292 μg of EDTA. The agar plates were incubated at 37°C overnight. The diameters of the growth inhibitory zone around the imipenem discs with inhibitors were compared with the plain imipenem disc.

The hydrolysis rates of imipenem by clinical isolates were detected with inhibitor added; methods are the same as the LC-MS/MS sample preparation and instrumentation described below.

Genotypic characterization of *A. baumannii* isolates

There are many different enzyme families that exhibit carbapenemase activity; therefore, it is difficult to screen all potential targets in one single method. In this study, all clinical isolates were analyzed for the presence of the common oxacillinase genes (bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51}, bla_{OXA-58}) and class A β-lactamase gene (bla_{TEM-1}) using a multiplex PCR method described by Ben et al.[21]

Chemicals and materials

Meropenem was purchased from the United States Pharmacopeia. Imipenem and meropenem-d_6 were obtained from Toronto Research Chemicals (Molsheim, France). Meropenem and imipenem were prepared in water, and meropenem-d_6 was prepared in methanol. All meropenem and imipenem solutions were stored at −80°C, without multiple refreezing, for 1 month. Methanol was purchased from Mallinckrodt (Phillipsburg, NJ, USA). Formic acid and sodium hydroxide were purchased from Riedel-de-Haën (Seelze, Germany). All organic solvents and chemicals were reagent grade.

Sample preparation for LC-MS/MS analysis

Each strain was injected into the LC-MS/MS system for the detection of the hydrolysis of imipenem or meropenem. The strains were cultured overnight on Mueller-Hinton agar. The bacteria were suspended in normal saline with the optical density at 600 nm (OD_{600}) adjusted to 2 or 4. A 200-μL volume of each suspension was incubated with 8 μg/mL of imipenem or meropenem for 1 hour or 2 hours at 37°C in a 96-well microtiter plate under smooth agitation. The suspensions were then centrifuged at 12,000*g* for 5 minutes, and 100 μL of the supernatant was subsequently mixed with 700 μL of methanol containing meropenem-d_6 (1.5 μg/mL). After the centrifugation at 12,000*g* for 5 minutes, 200 μL of supernatant was mixed with 800 μL of water for further LC-MS/MS analysis.

Instrumentation and LC-MS/MS parameters

The abundance of imipenem and meropenem was measured using an LC-MS/MS system comprising a Thermo Accela LC system (Thermo Fisher Scientific Inc., Waltham, MA, USA) coupled to a TSQ Quantum tandem triple-quadrupole MS. Chromatography separation was performed using a superficially porous column (Agilent poroshell 120 EC-C_{18}; 2.1 mm × 100 mm, 2.7 μm). The flow rate was 0.32 mL/min and the column temperature was set at 25°C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The gradient was as follows: 30% of component B at the time of injection was increased linearly to 55% B over the first 0.3 minutes, and subsequently to 100% B over 1.5 minutes. This composition was maintained for 0.2 minutes. At 1.9 minutes, the gradient returned to the initial condition and was equilibrated for 1.6 minutes, resulting in a total run time of 3.5 minutes. The injection volume was 10 μL. Ionization was achieved using electrospray in positive ionization mode (ESI^+) with a spray voltage of 2800 V. Nitrogen was used as the sheath and auxiliary gas at 28 units and 2 units (arbitrary units). The heated capillary temperature was 270°C and the argon collision gas pressure was 1 mTorr. The multiple-reaction-monitored (MRM) parameters were optimized by post-column infusion of the stock solution (1 μg/mL) using Quantum Tune Master software (Thermo Fisher Scientific Inc.). Optimal MRM parameters include tube lens (102 V for imipenem and 107 V for meropenem) and collision energy (27 eV for imipenem and 16 eV for meropenem). The transitions of $[M+H]^+$ precursor ion products were 383.9 *m/z* to 141.2 *m/z* for meropenem, 300.1 *m/z* to 142.2 *m/z* for imipenem, and 390.1 *m/z* to 147.1 *m/z* for the internal standard (meropenem-d_6). The scan time for each MRM transition was set to 15 milliseconds. The hydrolysis rates of imipenem and meropenem were calculated by dividing the respective postincubation carbapenem concentrations by that of the reference strain.

Results

Susceptibility testing

The antibiotic susceptibility results of all *A. baumannii* strains used in this study are shown in Table 1. All of the *A. baumannii* reference strains and carbapenem-susceptible clinical isolates were susceptible to imipenem and meropenem (MIC < 2 μg/mL), and carbapenem-resistant clinical isolates of *A. baumannii* were resistant to imipenem and meropenem (MIC > 8 μg/mL). The MIC of the imipenem-selected mutant 17978 IPM-2m to imipenem and meropenem was 2 μg/mL. The MIC of the imipenem-selected mutant 17978 IPM-8m to imipenem and meropenem was >8 μg/mL. Thus, 17978 IPM-2m was identified

as a carbapenem-borderline-susceptible strain, and 17978 IPM-8m was identified as a carbapenem-resistant strain, according to the 2014 CLSI guidelines.

Phenotypic and molecular screening

The MHT showed that 59 of the 68 carbapenem-resistant clinical isolates possessed carbapenemase, and nine of the clinical isolates had no sign of being able to hydrolyze imipenem (Table 1). According to the inhibitor base test, among the 68 clinical carbapenem-resistant strains, 10 strains produced both KPC and MBL enzymes, four strains produced KPC only, one strain produced MBL type, and the remaining 53 strains produced neither KPC nor MBL types. After defining to which classes the clinical strains belonged, we chose several strains to examine the hydrolysis rate with the inhibitors added. We discovered that the clinical strains producing KPC could be separated from other clinical strains by adding PBA.

Multiplex-PCR results showed that OXA-51 was detected in most of the clinical isolates of *A. baumannii*, and TEM-1 was also detected in most of the carbapenem-resistant *A. baumannii* isolates. Other genes such as OXA-23, OXA-24, and OXA-58 were detected randomly in different isolates. The gene distribution pattern does not match the classes defined by the drug resistance results.

Development of superficially porous LC methods for meropenem and imipenem

A rapid (3.5 minutes) and effective chromatographic separation was achieved using a sub-3-μm superficially porous particle column in this study. The retention times of imipenem and meropenem were 0.81 minutes and 1 minute, respectively (Figure 1). The bacteria were coincubated with imipenem or meropenem in a 96-well microtiter plate for large scale analysis. Protein precipitation was used in the sample preparation in the current study to avoid the degradation of imipenem and meropenem which was reported in earlier studies.[22,23] Protein precipitation minimized the risk of instability of the drugs by quickly removing the matrix. The effect of the matrix during the extraction process was evaluated by comparing the peak areas obtained for the target compounds in the culture medium to those obtained for the target compounds in water, in triplicate. Matrix enhancement or suppression, expressed as a percentage, was measured using the ratio of the mean peak area of the blank medium extract to that of water. The matrix effects of imipenem and meropenem were 73.5% and 89.2%, respectively. The matrix-match calibration curves of imipenem and meropenem were linear over a concentration range of 0.25−10 μg/mL, with a correlation coefficient value > 0.9.

Optimization and validation of the superficially porous LC methods

To develop an efficient method for the detection of carbapenemase activity in *A. baumannii*, we optimized the assay parameters, including drug concentration (0.25−10 μg/mL), incubation time (1 hour or 2 hours), and bacterial inoculum ($OD_{600} = 2$ or 4). The effects of varying these parameters were investigated using a set of three isolates derived from the ATCC 17978-type strain, which were known to be susceptible, borderline-susceptible, and resistant to carbapenem. The incubation conditions are listed in Table 2. The hydrolysis rates of imipenem and meropenem increased with an increase in the inoculums and the incubation time. The hydrolysis rate of meropenem was slower than that of imipenem in the mutant 17978 IPM-2m. In order to enhance the detection efficiency, an inoculum density of $OD_{600} = 4$ and an incubation time of 1 hour were selected. Moreover, the hydrolysis rate thresholds for carbapenemase-positive strains were set to >75% for imipenem and >33% for meropenem. These thresholds were decided according to the hydrolysis rate of carbapenem by 17978 IPM-2m (carbapenem-borderline-susceptible strain) at the lower inoculum ($OD_{600} = 2$) and shorter incubation time (1 hour) (Table 2). Our design was to establish a more stringent threshold to avoid missing any carbapenemase-producing strains.

According to this optimization design, the mean hydrolysis rate of imipenem and meropenem in all carbapenem-susceptible reference strains was <1%, and the mean hydrolysis rate in carbapenem-susceptible clinical isolates was <9% (8.47% for imipenem and 8.04% for meropenem). By contrast, among the 68 resistant strains, there were 67 strains that showed an average hydrolysis rate of 99% (ranging from 84% to 100%) for imipenem and 68% (ranging from 36% to 100%) for meropenem. Interestingly, among the 68 resistant strains, there was one strain which showed only 19% and 14% hydrolysis rates for imipenem and meropenem, respectively. According to this result, we suspect the carbapenem-resistant-mechanism of this clinical isolate has no correlation with carbapenemase, but that another mechanism leads to its resistance to carbapenem. Comparing the results of the MHT and porous LC-MS/MS, we discovered that of the nine clinical isolates which show no sign of MHT, eight were found to produce carbapenemase, suggesting that our LC-MS/MS method is more sensitive than the MHT. The hydrolysis rates with inhibitor added can only differentiate class A carbapenemase (data not shown). Our results showed that using the thresholds determined as described above, high agreement can be achieved with the MIC result for the detection of carbapenem-resistant *A. baumannii* (Table 1).

Discussion

Imipenem-resistant *A. baumannii* bacteremia cause high mortality which is associated with inappropriate initial antimicrobial therapy. Rapid identification of imipenem-resistant *A. baumannii* and early start of appropriate antimicrobial therapy are crucial to reduce mortality in these high-risk patients.[24,25] In recent years, MS technologies such as MALDI-TOF MS and LC-MS/MS have been applied to characterize carbapenemase-producing bacteria,[11,12,26] however, few studies have focused on carbapenemase-producing *A. baumannii*.[12,27] Most of these earlier studies differed in the type of instrument used, the carbapenem used, the incubation conditions, and the interpretive criteria. All of these factors can affect the assay performance.

Carricajo et al[11] reported the detection of carbapenemase-producing bacteria using a UPLC-MS/MS

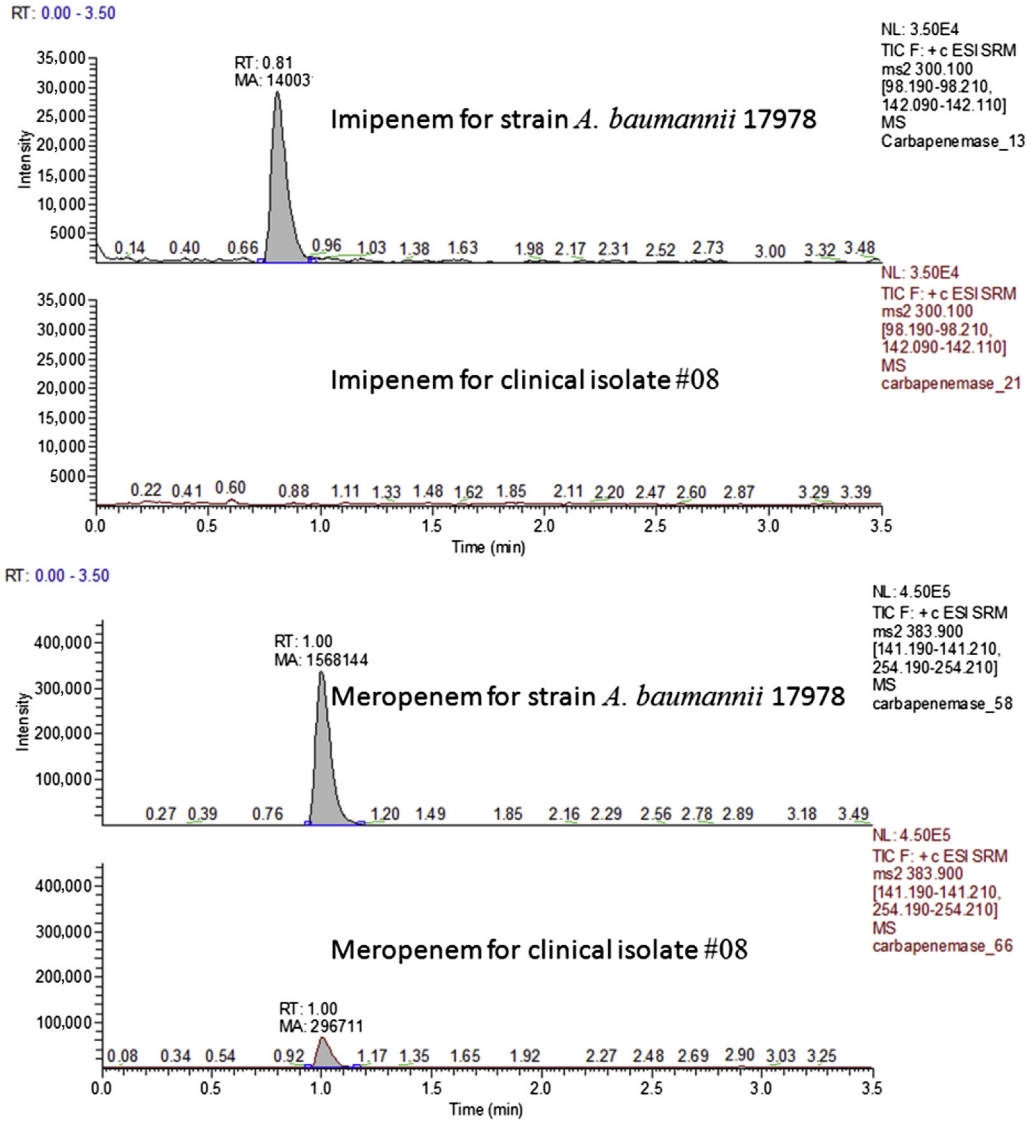

Figure 1. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) chromatograms for imipenem and meropenem for reference strain *A. baumannii* ATCC 17978 and clinical isolate *A. baumannii* #08 (hydrolysis rate of imipenem is 100% and that of meropenem is 87%). ESI = electrospray; SRM = selected reaction motoring; MA = manual integration area; NL = normalized intensity level (counts per second); MS = mass spectrometry, RT = retention time; TIC F = total ion chromatography (full scan).

method. In their study, the tested bacteria required incubation for 3—4 hours, consuming a large amount of culture medium and carbapenem solution. In order to decrease costs while maintaining the detection efficiency, our method reduced the bacterial incubation volume to 200 μL, and decreased the use of antibiotics to 8 μg/mL. Because the incubation volume was reduced to 200 μL/sample, the incubation procedure could be performed in a 96-well

Table 2 Relationship between incubation condition and hydrolysis rates.

A. baumannii strain	MIC (range) (μg/mL)		LC-MS/MS hydrolysis rate (%)							
			OD_{600} = 2, 1 h		OD_{600} = 2, 2 h		OD_{600} = 4, 1 h		OD_{600} = 4, 2 h	
	IPM	MEM	IPM	MEM	IPM	MEM	IPM	MEM	IPM	MEM
ATCC 17978 (carbapenem-sensitive)	<2	<2	<1	<1	<1	<1	<1	<1	<1	<1
ATCC 17978 IPM-2m (carbapenem-borderline-susceptible)	2	2	75	33	97	56	100	66	100	100
ATCC 17978 IPM-8m (carbapenem- resistant)	>8	>8	100	100	100	100	100	100	100	100

ATCC = American Type Culture Collection; IPM = imipenem; LC-MS/MS = Liquid chromatography-tandem mass spectrometry; MEM = meropenem; MIC = minimal inhibition concentration.

microtiter plate. The incubation conditions, including bacterial concentration and incubation time, were assessed and the results are shown in Table 2. The bacterial incubation density of $OD_{600} = 2$ and an incubation time of 1 hour could sufficiently distinguish between the imipenem-resistant and -susceptible strains. This optimized method is not only cost-effective, but also feasible for large-scale screening of carbapenemase-producing *A. baumannii*.

Using LC-MS/MS, Kulkarni et al[26] determined carbapenemase activity within 75 minutes. Our method spends approximately the same time, but only requires one-tenth of the incubation volume. In addition, Kulkarni et al[26] used three different columns for individually detecting ertapenem, meropenem, and imipenem, which was time-consuming, because of the switching between the columns. Their system lacked sensitivity for detecting ertapenem- and meropenem-resistant bacteria, although it could efficiently identify imipenem-resistant bacteria alone.[26] In our method, only one superficially porous column was used to detect both meropenem and imipenem, which efficiently detected carbapenemase in *A. baumannii*.

Porous LC-MS/MS has a high accuracy to detect the hydrolysis rate of *A. baumannii* against carbapenems. Using this method, the hydrolysis rate of clinical *A. baumannii* with a weak MHT result can be detected. The hydrolysis rate is directly detected and would not be affected by the genotype diversity, unlike molecular detection, thus making it more efficient. We think the main disadvantage of this method is that the initial cost of the equipment is high and the operator needs to be well trained.

In conclusion, the average turnaround time, including the incubation and analysis time for this method, was estimated to be 75 minutes. Our study clearly demonstrates that this superficially porous LC-MS/MS assay could be routinely used in clinical microbiology laboratories to identify carbapenemase-producing *A. baumannii* strains.

Conflicts of interest

None.

Acknowledgments

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