Performance of modified carbapenem inactivation method and inhibitor-based combined disk test in the detection and distinguishing of carbapenemase producing Enterobacteriaceae

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Background: This study aimed to evaluate the performance of modified carbapenem inactivation method (mCIM) combined EDTA-carbapenem inactivation method (eCIM), and inhibitor-based combined disk test (CDT) in the detection and distinguishing of carbapenemase production in Enterobacteriaceae.

Methods: A total of 101 nonrepetitive carbapenem insensitive Enterobacteriaceae [minimal inhibitory concentration (MIC) ≥2 μg/mL] were tested by mCIM, eCIM and CDT respectively, and the major carbapenemase genes including blaKPC, blaNDM, blaIMP, blaVIM and blaOXA-48-like genes were detected by polymerase chain reaction (PCR) as control.

Results: Seventy-nine (78.2%) of isolates were found to harbour one or more carbapenemase genes by PCR, with blaKPC and blaNDM being the most common genes. OXA-48-like genes were undetectable. The coincidence rate of mCIM combined eCIM and CDT was 97.5% (77/79) and 96.2% (76/79) respectively, compared with gene detection. Both assays had a misclassification in two $bla_{KPC+NDM}$-producing isolates of *Klebsiella oxytoca*. The sensitivity and specificity of two assays above were 100.0% *vs.* 95.0% and 98.4% *vs.* 98.4%, respectively in distinguishing serine-carbapenemase, while they were 95.1% *vs.* 97.6% and 100% *vs.* 100.0%, respectively in distinguishing metallo-carbapenemase.

Conclusions: mCIM combined eCIM and the CDT are both useful tools for the reliable detection and distinguishing single serine-carbapenemase or metallo-carbapenemase, but not for mixed types.

Keywords: Modified carbapenem inactivation method (mCIM); EDTA-carbapenem inactivation method (eCIM); combined disk test (CDT); carbapenemase-producing Enterobacteriaceae (CPE)

Introduction

Carbapenems, a class of broad spectrum β-lactam antibiotic, have been widely used in clinical practices and play an important role in treatment of multiple drug-resistant bacterial infections, mixed aerobic and anaerobic infections, and infections in immunodeficient patients (1). SENTRY monitoring in United States failed to find Enterobacteriaceae insensitive to carbapenems between 1998 and 2004 (2), carbapenem-resistant Enterobacteriaceae (CRE) has gradually increased around the world, including China, since 2005 (3-6).

The main mechanism underlying the resistance of CRE is the production of carbapenemase (7). Many carbapenems coding genes are located in plasmids, transposon or other

removable genetic elements, which promotes the horizontal transfer of resistance genes between gram-negative organisms, and thus it is imperative to develop rapid and accurate methods for the identification of these organisms. The new antimicrobial drug ceftazidime/avibatan can be used to treat carbapenemase-producing Enterobacteriaceae (CPE) which has no metalloenzyme (8), and therefore the detection of genotype is also necessary.

Several approaches have been developed for the detection of carbapenemases in clinical laboratories, such as chromogenic agars, MALDI-TOF MS, Carba NP, CIM, and polymerase chain reaction (PCR) based method (9-11). In these approaches, phenotypic assay is the simplest and easiest to be popularized in primary settings.

The antimicrobial activity may decrease when the carbapenem disk is incubated with CPE, and then the activity of disk is detected to determine whether carbapenemases are produced, which is also known as the modified carbapenem inactivation method (mCIM) (12). The Clinical and Laboratory Standards Institute (CLSI) has recommended combined use of mCIM and EDTA-carbapenem inactivation method (eCIM) to distinguish metallo-carbapenemase from serine-carbapenemase in 20–22 h (13).

Combined disk test (CDT) is another phenotypic assay which was firstly reported for the direct identification of CPE with surveillance rectal swabs in 2013 (14). It is an inhibitor-based test and relies on the fact that metallo-carbapenemase activity can be inhibited by EDTA and serine-carbapenemase be inhibited by phenyl boronic acid (PBA) (15,16). In the present study, the performance of both methods was compared in the detection and distinguishing of carbapenemase production, using PCR as a reference.

Methods

Bacterial isolates

The study was conducted at Renmin Hospital of Wuhan University between January 2017 and December 2018. A total of 101 nonrepetitive carbapenem insensitive Enterobacteriaceae [minimal inhibitory concentration (MIC) ≥2 μg/mL] were collected, including 47 *Klebsiella pneumoniae*, 26 *Escherichia coli*, 8 *Klebsiella oxytoca*, 8 *Enterobacter aerogenes*,7 *Enterobacter cloacae*, 2 *Citrobacter freundii*, 2 *Morganella morganii*, and 1 *Providencia rettgeri*. 38 were isolated from urine, 33 from sputum, 12 from blood, 6 from secretions, 4 from bile, 2 from ascites,

2 from pus, 1 from peritoneal dialysate, 1 from cerebrospinal fluid, 1 from feces, and 1 from the tip of a catheter. Isolates were re-examined by MALDI-TOF MS (Bruker Biotyper, Germany). Antimicrobial susceptibility was determined by PhoenixTM 100 (Becton, Dickinson and Company, America). Quality control strains included *E. coli* ATCC25922, *E. coli* ATCC35218, *K. pneumoniae* ATCC BAA-1705, *K. pneumoniae* ATCC BAA-1706, *K. pneumoniae* ATCC BAA-2146.

mCIM and eCIM

Detection of CPE was performed with the mCIM and eCIM following the CLSI guideline (13). Two tubes containing 2 mL of Trypticase soy broth (TSB) were used for each strain in parallel: one tube was added with 20 μL of 0.5 M EDTA (Sigma); the other tube had no EDTA. One loop fresh colony of tested strain was taken to each of the tube with 1 μL of inoculating loop. A 10-mg meropenem disk (Oxoid) was incubated in a suspension of tested strain for 4 h at 35 ℃. Then, meropenem disks from two tubes were placed on the same Mueller Hinton agar plates, followed by inoculation with the E. coli ATCC 25922 indicator strain. The mCIM showed positive when the inhibition zone diameter was 6–15 mm, or 16–18 mm with small colonies in the inhibitory zone. The eCIM results should only be interpreted if the mCIM result indicated the presence of carbapenemase. A 5-mm increase in the zone diameter for eCIM as compared to that for mCIM suggests the likelihood of produce metallo-carbapenemase.

CDT

As reported by Pournaras *et al.* (14), 10 μL of 40 mg/mL PBA(Sigma) and 10 μL of 0.5 M EDTA (Sigma) were independently added to 10-mg meropenem disk, which was dried at room temperature. A 10-mg meropenem disk, a meropenem disk plus 10 μL of 40 mg/mL PBA and a meropenem disk plus 0.5 M EDTA were independently pasted on the same Mueller Hinton agar plates which inoculated with 0.5 McFarland of tested isolates, then incubated at 35 ℃ in ambient air for 16–18 h. If the inhibition zone diameter of meropenem was ≤19 mm and difference in the inhibition zone between meropenem disks with or without inhibitors (PBA, EDTA) was ≥5 mm, producing serine-carbapenemase or metallo-carbapenemase could be defined.

Table 1 Primers used for PCR

Gene	Nucleotide sequence (5'–3')	Size (bp)	Annealing temperature (℃)
bla_{KPC}	F: ATCGCCGTCTAGTTCTGCTG	811	60
	R: TCGCTGTGCTTGTCATCCTT		
bla_{NDM}	F: AGGACAAGATGGGCGGTATG	281	60
	R: CTTGGCCTTGCTGTCCTTGA		
bla_{IMP}	F: GGGCGGAATAGAGTGGCTTA	365	60
	R: GTGATGCGTCTCCAGCTTCA		
bla_{VIM}	F: GAGCCGAGTGGTGAGTATCC	370	60
	R: GAATCTCGTTCCCCTCTGCC		
$bla_{OXA-48like}$	F: TTGGTGGCATCGATTATCGG	743	60
	R: GAGCACTTCTTTTGTGATGGC		

PCR, polymerase chain reaction.

Genotyping of carbapenemase genes

Total DNA was extracted using the boiling method. Carbapenemase genes including bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM} and $blaO_{XA-48-like}$ genes were amplified using the primers shown in *Table 1*. Template DNA 1.5 µL, 2× Taq PCR Master Mix (10 µL), and forward and reverse primers (0.5 µL for each) were added to the PCR mixture (final volume: 20 µL), PCR was done as follows: 95 ℃ for 5 min; 32 cycles of 95 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 30 s; and 72 ℃ for 10 min, on the T100™ PCR System (Bio-Rad, America). PCR products were analyzed by agarose gel electrophoresis (SYNGENE, UK).

Statistical analysis

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy, and the consistency analysis were determined to assess the performance of mCIM combined eCIM and CDT in the identification and distinguish carbapenemase using PCR results as a standard. Statistical analyses were performed using SPSS 22.0. Cohen' kappa <0.4, poor consistency; Cohen' kappa=0.4–0.75, a fair degree of consistency; Cohen' kappa >0.75, excellent consistency.

Results

Seventy-nine CPEs were detected with PCR in 101 non-repetitive isolates of Enterobacteriaceae, which were insensitive to imipenem or meropenem (MIC ≥2 µg/mL). The CPEs included 38 KPC (38 *Klebsiella pneumoniae*), 32 NDM (22 *Escherichia coli*, 3 *Enterobacter cloacae*, 3 *Klebsiella oxytoca*, 2 *Citrobacter freundii*, 1 *Klebsiella pneumoniae*, and 1 *Providencia rettgeri*), 5 IMP (3 *Klebsiella oxytoca*, 2 *Klebsiella pneumoniae*), 1 VIM (one *Enterobacter cloacae*), 2 KPC + NDM (2 *Klebsiella oxytoca*) and 1 NDM + IMP (1 *Klebsiella pneumoniae*).

In the present study, the coincidence rate of mCIM combined eCIM and CDT was 97.5% (77/79) and 96.2% (76/79) respectively, compared with gene detection. Most of carbapenemase producers identified by two methods were KPC- and NDM-producers, mainly in *Klebsiella pneumoniae* and *Escherichia coli*. However, it was worth noting that, one bla_{IMP}-producing isolate of *Klebsiella oxytoca* had misclassified as serine-carbapenemase using CDT. And two $bla_{KPC+NDM}$-producing isolates of *Klebsiella oxytoca* had also misclassified, mCIM combined eCIM showed serine-carbapenemase, while CDT showed metallo-carbapenemase. Of 101 isolates, none of $bla_{OXA-48like}$ was detected, and thus it was unable to show the capacity of both methods in the detection and distinguishing of organisms carrying this gene. The results of mCIM, eCIM and CDT in different isolates are shown in *Table 2*. Results of detection of control isolates with or without carbapenemase genes were consistent with the expectations for phenotypic tests.

There was a high agreement between mCIM and PCR assays in the detection of carbapenemase enzymes, except one false-positive on mCIM, which did not identify the five main carbapenemase genes by PCR. The sensitivity was

Table 2 Carbapenem inactivation method and CDT for CPE isolates (n=79)

Clinical isolates (n=79)	Carbapenemase	No. of isolates with a positive result/no. of isolates tested	
		mCIM + eCIM	CDT
Klebsiella pneumoniae [38]	KPC	38/38	38/38
Klebsiella pneumoniae [1]	NDM	1/1	1/1
Klebsiella pneumoniae [2]	IMP	2/2	2/2
Klebsiella pneumoniae [1]	NDM + IMP	1/1	1/1
Escherichia coli [22]	NDM	22/22	22/22
Enterobacter cloacae [3]	NDM	3/3	3/3
Enterobacter cloacae [1]	VIM	1/1	1/1
Klebsiella oxytoca [3]	NDM	3/3	3/3
Klebsiella oxytoca [2]	KPC + NDM	0/2	0/2
Klebsiella oxytoca [3]	IMP	3/3	2/3
Citrobacter freundii [2]	NDM	2/2	2/2
Providencia rettgeri [1]	NDM	1/1	1/1

CDT, combined disk test; CPE, carbapenemase-producing Enterobacteriaceae; mCIM, modified carbapenem inactivation method; eCIM, EDTA-carbapenem inactivation method.

Table 3 Performance of mCIM and PCR in the identification of carbapenemase-producers

mCIM	PCR positive	PCR negative	Sensitivity	Specificity	PPV	NPV	Accuracy	Cohen's kappa
Positive	79	1	100.0%	95.5%	98.8%	100.0%	99.0%	0.970
Negative	0	21						

mCIM, modified carbapenem inactivation method; PCR, polymerase chain reaction; eCIM, EDTA-carbapenem inactivation method.

Table 4 Performance of two phenotypic methods in distinguishing serine-carbapenemase and metallo-carbapenemase

Carbapenemase classification	Phenotypic method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	Cohen's kappa
Serine-carbapenemase	mCIM + eCIM	100.0	98.4	97.6	100.0	99.0	0.979
	CDT	95.0	98.4	97.4	96.8	97.0	0.938
Metallo-carbapenemase	mCIM + eCIM	95.1	100.0	100.0	96.8	98.0	0.959
	CDT	97.6	100.0	100.0	98.4	99.0	0.979

PPV, positive predictive value; NPV, negative predictive value; mCIM, modified carbapenem inactivation method; eCIM, EDTA-carbapenem inactivation method; CDT, combined disk test.

100%, specificity was 95.5%, κ=0.970 (*Table 3*).

Two phenotypic methods had similar performance in distinguishing serine-carbapenemase and metallo-carbapenemase. The sensitivity, specificity, PPV, NPV, accuracy, and Cohen' kappa value are shown in *Table 4* respectively.

Discussion

The emergence of CPE has been a global challenge and a major threat to public health worldwide (17). The prevalence of CPE varies among different countries. A recent prospective multinational study investigated

the epidemiology of carbapenem-producing *Klebsiella pneumoniae* and *Escherichia coli* in Europe (18), and results showed the distribution of four main types of carbapenems (bla_{KPC}, bla_{NDM}, bla_{IMP} and $bla_{OXA-48-like}$ genes) in *Klebsiella pneumoniae* and *Escherichia coli* was significantly different among countries: $bla_{OXA-48-like}$ gene was common, and bla_{VIM} was the most uncommon gene. A report from China CRE Network shows that 89% of clinical isolates (n=155) produces carbapenemases, with a majority of isolates producing KPC (50%) or NDM (33.5%)-type gene among *K. pneumoniae* and *E. coli* (19). In our study, five major gene types were detected and results showed bla_{KPC} and bla_{NDM} were most common, followed by bla_{IMP}, $bla_{KPC + NDM}$, bla_{VIM} and $bla_{NDM + IMP}$. *OXA-48-like* genes were not identified in the present study.

Our study showed, after overnight incubation, mCIM had favorable performance in the phenotypic detection of carbapenemase producers except one *Enterobacter aerogenes* showed "false-positive". It maybe not a false-positive, because of other serine-carbapenemase had not been detected in our study, such as IMI, SME, NMC, GES or other unknown carbapenemase. It just the advantage of phenotypic methods, which was much easier to find unknown resistance genes than target PCR.

The performance of mCIM combined eCIM and CDT were compared with PCR in the classification of carbapenems. Results showed the two phenotypic methods had a high agreement with PCR in the detection of a single gene such as 38 *Klebsiella pneumoniae* carrying bla_{KPC}, 22 *Escherichia coli* carrying bla_{NDM}, and the detection of the same type gene carried by CPEs, such as one *Klebsiella pneumoniae* carrying $bla_{NDM + IMP}$. For different types of carbapenemase, two phenotypic methods were incorrect for two *Klebsiella oxytoca* which carried $bla_{KPC + NDM}$. When metallo-carbapenemase was inhibited by EDTA, the co-existence of serine-carbapenemase could also hydrolyze meropenem, make the inhibition zone diameter decreased and showed serine-carbapenemase positive. So CLSI warned that eCIM was not suitable for the isolate carried both serine and MBL carbapenemase, because it can show false negative (13). And in our study, CDT was also can't distinguish the two types in one isolate, it might be related with the relative amount and effect between the two inhibitors. Nevertheless, it was few that isolate both encoding serine and MBL carbapenemase. Zhou *et al.* evaluated four phenotypic methods in the detection of Class A and B CPE in Peking Union, Medical College Hospital, only two isolates carried $bla_{KPC + IMP}$ were collected, among

342 CRE in half a year (20).

Both of mCIM combined eCIM and CDT had a high sensitivity and specificity in distinguishing serine-carbapenemase and metallo-carbapenemase (>95%), and had excellent consistency with gene detection results (κ>0.9). The results were in accordance with other study evaluating improved methods based on CIM, like CIMplus test (21).

Of note, 21.8% (n=22) of isolates, which were non-carbapenemase-producing carbapenem insensitive Enterobacteriaceae, were negative to the five tested primers. Most of them were *Enterobacter cloacae* and *Enterobacter aerogenes*. Maybe it contained other carbapenemases gene, such as bla_{GES}, bla_{SME}, $bla_{OXA-181}$, $bla_{OXA-244}$, or *ESBL* and *AmpC* gene over-expression, or loss/alteration of *OmpF* and *OmpC* genes (22).

In this study, other carbapenem genes such as bla_{OXA} were not detected simultaneously. Since *OXA-48* and related variants had no specific inhibitors available, the CDT was not suitable for detecting *OXA-like* gene positive isolate (23). At the same time, due to the weak hydrolytic ability to carbapenems, the CIM sometimes fails to detect *OXA-48* in a limited time, while the sensitivity of mCIM was improved without compromising from the specificity (24).

The tests we evaluated in this study have many advantages, like low cost, and easy to interpretable. However, there are also some limitations. The test genes were not enough and we need great varieties gene types. And there was no statistical difference between the two phenotypic methods, maybe we need a large number of CPE and non-CPE. However, for experimental operations, CDT is simpler and easy to be commercialized, but needs to be further verified.

Acknowledgments

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Ethics Committee of Renmin Hospital of Wuhan University (2016K-C025).

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