Original Article
Clinical Microbiology

Ann Lab Med 2017;37:305-312
https://doi.org/10.3343/alm.2017.37.4.305
ISSN 2234-3806 • eISSN 2234-3814

ANNALS OF LABORATORY MEDICINE

Evaluation of Six Phenotypic Methods for the Detection of Carbapenemases in Gram-Negative Bacteria With Characterized Resistance Mechanisms

Kunling Sun, M.D., Xiuyu Xu, M.D., Jinrong Yan, M.D., and Liping Zhang, M.D.

Department of Laboratory Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China

Background: We compared the performance of the modified Hodge test (MHT), Triton Hodge test (THT), Carba NP test (CNPt), simplified Carba NP test (CNPt-direct), blue-Carba NP test (BCT), and carbapenem inactivation method (CIM) for rapid and accurate carbapenemase detection.

Methods: The methods were evaluated by using 256 gram-negative isolates, including 197 *Enterobacteriaceae* (79 *Enterobacter* spp., 74 *Klebsiella* spp., 33 *Escherichia coli*, 10 *Citrobacter* spp., and 1 *Serratia marcescens*), 51 *Acinetobacter baumannii*, and 8 *Pseudomonas aeruginosa* strains. The collection included 117 non-carbapenemase, 18 *Klebsiella pneumoniae* carbapenemases (KPC) producers, 46 New Delhi metallo-β-lactamases (NDM) producers, 11 imipenemases (IMP) producers, and 51 oxacillinases (OXA) producers, and 13 strains harboring two different carbapenemase genes.

Results: The specificity of the THT (91.5%) was significantly lower than other methods, each of which had 100% specificity ($P<0.003$). This can be attributed to the false detection of Ampler class C β-lactamases (AmpC) carriers. The CNPt-direct and CIM yielded the highest sensitivities ($P<0.003$), which were comparable (92.8% vs 93.5%, $P>0.999$). Because of improved detection of NDM carriers, THT showed significantly higher sensitivity than the MHT (84.9% vs 75.5%, $P<0.001$). However, poor performances in detecting OXA still influenced the sensitivities of the CNPt (66.2%) and BCT (82.0%), as well as the MHT and THT.

Conclusions: CNPt-direct and CIM demonstrated the best performance for the efficient detection of carbapenemase among the six evaluated methods. Except the MHT and THT, the detection of carbapenemase-producing *Enterobacteriaceae* by all the other methods was acceptable, when the OXA-type carbapenemase was not prevalent.

Key Words: Evaluation, Phenotypic methods, Carbapenemase, Gram-negative bacteria

Received: May 31, 2016
Revision received: December 16, 2016
Accepted: February 22, 2017

Corresponding author: Liping Zhang
Department of Clinical Laboratory, The First Affiliated Hospital of Chongqing Medical University, No.1 Youyi Road, Yuzhong District, Chongqing 400016, China
Tel: +86-023-89012756
Fax: +86-023-89012513
E-mail: 1309898173@qq.com

INTRODUCTION

The emergence and spread of carbapenem-resistant gram-negative bacteria is a worldwide public health threat [1]. The reduced susceptibility to carbapenems can be assessed by automated microbiology systems in clinical microbiology laboratories; however, the high or low minimal inhibitory concentrations (MICs) do not necessarily reflect the production of carbapenemases, as other mechanisms, such as overexpression of Ampler class C β-lactamases (AmpC) and/or extended spectrum β-lactamases (ESBLs), coupled with loss of porin or increased efflux pump activity, can also cause resistance [2]. Since carbapenemase-encoding genes are often located on plasmids, this type of resistance is more likely to disseminate [3]. Therefore, rapid, simple,

and reliable detection of these bacteria has become imperative to prevent the spread of carbapenemase producers. Accordingly, phenotypic tests such as the modified Hodge test (MHT) and Carba NP test (CNPt) have been recommended by CLSI to monitor the carbapenemases for epidemiological or infection control purposes [4]. Furthermore, a simple improvement of the MHT, called the Triton Hodge test (THT) was shown to improve the poor performance of the MHT in detecting New Delhi metallo-β-lactamases (NDM) carriers in 2015 [5].

Since its first report in 2012, the CNPt has been extensively shown to possess relatively high sensitivity and specificity, as well as a fast turn-around time [2, 6]. However, the use of the CNPt and related commercialized kits are limited by the high cost of the commercial bacterial protein extraction buffer and the imipenem reference standard powder, accompanied by invalid results, which occurred with certain oxacillinases (OXA)-type carbapenemase-producing isolates. As a result, several improved CNPts have been proposed recently. A simplified CNPt, the CNPt-direct, was optimized in 2015 by using intravenous imipenem-cilastatin instead of the imipenem reference standard powder to lower the cost. Furthermore, the direct addition of bacterial colonies to the CNPt reaction solution containing 0.1% Triton X-100 instead of the commercial extraction buffer to extract bacterial proteins yielded 98% sensitivity and 100% specificity in the detection of carbapenemases in *Enterobacteriaceae*, *Pseudomonas* spp., and *Acinetobacter* spp. [7]. Additionally, a CNPt-based variant called the blue-Carba NP test (BCT) was formulated by directly using the bacterial colonies and bromothymol blue as an indicator, which spans an optimal pH range (6.0 to 7.6) for most β-lactamases (pH=6.8) [8]. The BCT was reported to significantly improve the detection of the OXA-type carbapenemases commonly identified in *Acinetobacter* spp. [8, 9]. Additionally, a new bioassay called the carbapenem inactivation method (CIM), which showed excellent specificity, sensitivity, and low operation costs, was developed in 2015 to detect various carbapenemase activities in gram-negative rods [10].

The aim of this study was to comprehensively evaluate the performances of the MHT, THT, CNPT, CNPt-direct, BCT, and CIM in the detection of carbapenemase. There have been some reports evaluating the abilities of few of the phenotypic methods mentioned above [11-13]; however, considering that the existing evaluations were performed in separate laboratories with different isolates under varying experimental conditions, the assessment of the individual performances of these assays in one evaluation panel was lacking. To the best of our knowledge, this is the first large-scale single model evaluation of the performances of all the available phenotypic methods on certain gram-negative bacteria with characterized resistance mechanisms.

METHODS

1. Bacterial isolates, testing of antimicrobial susceptibility, and molecular detection of β-lactamase genes

Two hundred and fifty-six gram-negative bacterial isolates, including 139 carbapenemase producers and 117 carbapenemase non-producers, were collected in this study. The strains were recovered between 2011 and 2015 from various clinical samples from the first affiliated hospital of the Chongqing Medical University, China, a 3,500-bed tertiary university hospital with an annual admission of more than 100,000 in-patients. All the isolates were identified to the species level by the VITEK2 compact or VITEK MS (bioMerieux, Hazelwood, MO, USA). The MIC values of Ertapenem, imipenem, and meropenem were determined by the VITEK2 compact (bioMerieux) and verified by the reference broth microdilution method according to the CLSI methods [4]. The results of susceptibility testing were interpreted by using the CLSI guideline [4]. All the β-lactamase genes, including those encoding carbapenemases, ESBLs, and AmpC, were determined by PCR [14-16]. Molecular detection by PCR and sequencing was considered as the gold standard for β-lactamase characterization. In our study, we verified these strains by the broth microdilution method, PCR, and the six phenotypic tests at the same time. For the different phenotypic tests described below, strain ATCC BAA-1705 of *Klebsiella pneumoniae* was used as the positive control, whereas *K. pneumoniae* ATCC BAA-1706 was used as the negative control.

2. Modified Hodge test and Triton Hodge test

The MHT was performed according to the CLSI Document M100 [4]. The THT was performed as described previously [5]. Briefly, a Mueller-Hinton agar (MHA) plate was flooded with 50 μL Triton X-100 (0.2% v/v in the MHA plate). The detergent was dripped in the center of the plate and quickly distributed by streaking 4 to 6 times with a swab over the entire sterile agar surface until complete absorption. Before inoculation with the indicator organism, excess surface moisture was removed by evaporation at 35°C. The subsequent procedures were identical to the MHT. For both the MHT and THT, the cut-off value of "growth of the indicator strain no less than 3 mm" for the interpretation of positive result was utilized [5, 17]. The strains were tested in triplicates, and three independent technicians interpreted the results with both protocols.

https://doi.org/10.3343/alm.2017.37.4.305

3. Carba NP test and simplified Carba NP test

The CNPt was performed following the protocol recommended by the CLSI [4]. The simplified CNPt (CNPt-direct) was modified to use colonies directly instead of bacterial extracts [7]. Specifically, 0.1% v/v of TritonX-100 was added before the pH adjustment. A full loop of colonies (1 μL) from the MHA was directly suspended in 100 μL aqueous indicator mix, supplemented with 12 mg/mL imipenem-cilastatin (Tienam 500, Merck Sharp & Dohme, Elkton, VA, USA; equivalent to 6 mg of imipenem standard powder) (reaction tube) or without the antibiotic (control tube). For both the protocols, the tubes were incubated at 35°C and monitored for 2 hr. A color change from red to light orange, dark yellow, or yellow in the reaction tube was interpreted as a positive result. The strains were tested in triplicates, and three independent technicians interpreted the results with both protocols.

4. Blue-Carba NP test

Bromothymol blue was used as an indicator in the BCT [8]. Additionally, the test solution consisted of 6 mg/mL imipenem-cilastatin (Tienam 500, Merck Sharp & Dohme; equivalent to 3 mg of imipenem standard powder). Approximately 5 μL colonies were recovered from the MHA plate by a microbial loop, directly suspended in 100 μL of both the test and negative control solutions in a 96-well micro titer plate, and incubated at 37°C with agitation (150 rpm) for 2 hr. Carbapenemase activity was confirmed when the colors of the test versus negative control solution were (i) yellow versus blue, (ii) yellow versus green, or (iii) green versus blue, respectively; the non-carbapenemase producers remained blue or green in both solutions. The test was performed in triplicate for all the isolates.

5. Carbapenem inactivation method

To perform the CIM [10], a 10 μL loop full of test culture from the MHA plate was suspended in 400 μL water. Subsequently, a 10 μg meropenem disk (Oxoid Ltd, Basingstoke, United Kingdom) was immersed in the suspension and incubated for a minimum of two hours at 35°C. After the incubation, the disk was removed and placed on a MHA plate inoculated with *Escherichia coli* ATCC 29522. Subsequently, the plate was incubated overnight at 35°C (at least 6 hr). Disks incubated in suspensions that did not contain carbapenemases yielded a clear inhibition zone, whereas no inhibition zone was observed if the test strain was a producer of carbapenemase. The strains were tested in triplicates, and three independent technicians interpreted the results.

6. Statistical analysis

The 95% confidence intervals (CI) of sensitivities and specificities of the six tested methods were calculated. To compare the test sensitivity and specificity, the McNemar's tests were performed by using the 139 positive and 117 negative isolates, respectively [17-19]. If sensitivity or specificity was 100%, CI was determined by the exact McNemar's test; otherwise, CI for sensitivity or specificity was determined by the asymptotic McNemar's test. Additionally, according to the corrected significant level $\alpha' = 2\alpha/k(k-1) = 0.0033$ (k: the number of multiple comparisons), the statistical significance was defined at $P < 0.0033$. To evaluate the appropriateness of the sample size, we first calculated the respective accuracies of the six methods by using the formula: Accuracy=(TPR+TNP)/N*100% (TPR: true positive rate; TNP: ture negative rate; N: the sample size), and further calculated the sample size by using the formula mentioned in statistical literature [20]. All statistical analyses were performed with the SPSS software version 21.0 (Chicago, IL, USA) and SAS version 9.4 (Cary, NC, USA).

RESULTS

To compare the carbapenemase detection efficiency of the MHT, THT, CNPt, CNPt-direct, BCT, and CIM, 139 previously characterized strains (88 *Enterobacteriaceae* and 51 *Acinetobacter baumanii*) producing *Klebsiella pneumoniae* carbapenemases (KPC), imipenemases (IMP), NDM, and OXA were tested. All the six phenotypic tests successfully detected the presence of 18 KPC-2 producing isolates (Table 1). The 57 metallo-β-lactamase (MBLs) positive strains tested positive with the CNPt, CNPt-direct, BCT, and CIM, including three *Klebsiella pneumoniae* NDM-1 producers with an imipenem MIC of 0.25 μg/mL. In contrast, the MHT detected 71.9% (41/57) MBL producers but missed detecting 16 NDM-1 producers, which included 7 *K. pneumoniae* strains, 6 *Enterobacter cloacae* isolates, 1 *Escherichia coli* isolate, and 2 *Enterobacter aerogenes* isolates, respectively. The detection rate for the positive MBL producers improved to 93.0% (53/57) by THT, with only 4 NDM-1 producers being missed.

The performances of the different phenotypic tests varied for the 51 *A. baumanii* isolates carrying the class D carbapenemase genes. Generally, CIM yielded the highest positive detection (42/51, 82.3%), missing 2 OXA-23- and 6 OXA-51-like-producing isolates as well as one strain harboring both the OXA-23- and OXA-51-like genes. Meanwhile, CNPt-direct was as efficient as CIM in testing the class D carbapenemase, which captured 80.4% of the involved OXA gene carriers (Table 1). Both MHT and THT

Table 1. Results of the modified Hodge test, Triton Hodge test, Carba NP test, simplified Carba NP test, Blue-Carba NP test, and carbapenem inactivation method with 139 carbapenemase-positive isolates

Group (N)	Species (N)	MIC (μg/mL)			N (%) of isolates with positive results of					
		ETP	IPM	MEM	MHT	THT	CNPt	CNPt-direct	BCT	CIM
Class A										
KPC-2 (18)	*K. pneumoniae* (5)	128–512	8–64	8–64	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)
	E. cloacae (2)	128	8	16	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)
	E. coli (7)	4–16	4–16	1	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)
	C. koseri (1)	16	8	8	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
	C. braakii (1)	32	8	4	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
	C. freundii (2)	4–16	4–16	2–8	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)
All (18)					18 (100)	18 (100)	18 (100)	18 (100)	18 (100)	18 (100)
Class B										
NDM-1 (46)	*K. pneumoniae* (20)	1–512	0.25–64	0.25–32	13 (65)	18 (90)	20 (100)	20 (100)	20 (100)	20 (100)
	E. cloacae (17)	4–256	2–64	1–32	11 (64.7)	15 (88.2)	17 (100)	17 (100)	17 (100)	17 (100)
	E. coli (3)	8–16	4–16	2–8	2 (66.6)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
	E. aerogenes (5)	8–16	2–16	4–8	3 (60)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)
	K. oxytoca (1)	8	4	2	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
IMP-4 (8)	*K. pneumoniae* (1)	64	16	8	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
	E. cloacae (4)	4	0.5–2	0.5–1	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)
	K. oxytoca (3)	4–8	2–16	2	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
IMP-8 (3)	*E. cloacae* (3)	4–64	1–8	1–8	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
All (57)					41 (71.9)	53 (93)	57 (100)	57 (100)	57 (100)	57 (100)
Class D										
OXA-23 (6)	*A. baumannii* (6)	-	≥16	≥16	3 (50)	3 (50)	1 (16.7)	5 (83.3)	4 (66.7)	4 (66.7)
OXA-51-like (13)	*A. baumannii* (13)	-	≥16	≥16	8 (61.5)	8 (61.5)	1 (7.7)	9 (69.2)	2 (15.4)	7 (53.8)
OXA-23 + OXA-51-like (32)	*A. baumannii* (32)	-	≥16	≥16	23 (71.8)	23 (71.8)	2 (6.3)	27 (84.4)	20 (62.5)	31 (96.9)
All (51)					34 (66.7)	34 (66.7)	4 (7.8)	41 (80.4)	26 (51.1)	42 (82.3)
KPC-2 + NDM-1 (10)	*K. pneumoniae* (5)	8–64	1–32	1–8	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)
	C. freundii (4)	64–128	16–32	8–16	3 (75)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)
	E. aerogenes (1)	64	32	16	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
IMP-8 + NDM-1 (1)	*E. cloacae* (1)	128	128	32	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
KPC-2 + IMP-4 (2)	*C. freundii* (1)	32	16	4	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
	C. koseri (1)	32	8	8	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)

Abbreviations: ETP, ertapenem; IPM, imipenem; MEM, meropenem; MHT, modified Hodge test; THT, Triton Hodge test; CNPt, Carba NP test; CNPt-direct, simplified Carba NP test; BCT, blue-Carba NP test; CIM, carbapenem inactivation method; KPC, *Klebsiella pneumoniae* carbapenemases; OXA, oxacillinases; NDM, New Delhi metallo-β-lactamases.

detected 34 out of the 51 class D carbapenemase carriers (66.7%), and missed detecting 3 OXA-23- and 5 OXA-51-like-producing isolates and 9 strains harboring both the OXA-23- and OXA-51-like genes. In contrast, BCT showed 48.9% of the false-negative results with 25 strains, most of which tested positive by the CNPt-direct and CIM. In this regard, the CNPt yielded the most unsatisfactory result, detecting only 4 out of the 51 strains producing OXA gene products (7.8%). Among the 139 carbapenemase-positive isolates, there were 13 strains harboring a combination of class A and class B carbapenemases or two different types of class B carbapenemases. They were all successfully detected by the five phenotypic methods, with the exception of one *Citrobacter freundii* isolate carrying KPC-2 plus NDM-1 that was missed by MHT.

https://doi.org/10.3343/alm.2017.37.4.305

Table 2. Results of the modified Hodge test, Triton Hodge test, Carba NP test, simplified Carba NP test, Blue-Carba NP test, and carbapenem inactivation method with 117 carbapenemase-negative isolates

Group (N)	Species (N)	MIC (μg/mL)			N (%) of isolates with positive results of					
		ETP	IPM	MEM	MHT	THT	CNPt	CNPt-direct	BCT	CIM
AmpC (19)	*E. cloacae* (3)	1–64	0.5–4	0.5–2	2 (66.7)	2 (66.7)	0 (0)	0 (0)	0 (0)	0 (0)
	K. pneumoniae (16)	2–64	1–16	0.5–8	1 (6.25)	2 (12.5)	0 (0)	0 (0)	0 (0)	0 (0)
AmpC + porin loss (5)	*E. cloacae* (2)	32	8	8	2 (100)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)
	K. pneumoniae (2)	8–128	0.5–16	0.5–8	1 (50)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)
	S. marcescens (1)	8	1	4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
All (24)					6 (25)	7 (29.2)	0 (0)	0 (0)	0 (0)	0 (0)
ESBLs (40)	*E. aerogenes* (3)	0.25–2	1–4	0.25–2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	E. cloacae (7)	2–8	0.5–1	0.5–1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	E. coli (16)	1–8	0.5–4	0.5–1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	K. pneumoniae (14)	1–256	0.5–16	0.5–64	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
ESBLs + porin loss (11)	*E. aerogenes* (2)	8	2–16	1–2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	E. cloacae (5)	2–64	0.5–4	0.5–8	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	E. coli (3)	2–8	1–4	0.5–1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	K. pneumoniae (1)	16	2	2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
All (51)					0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
AmpC + ESBLs (21)	*E. cloacae* (15)	1–64	0.5–2	0.5–8	1 (6.7)	3 (20)	0 (0)	0 (0)	0 (0)	0 (0)
	E. coli (2)	1–8	0.5–2	0.5–1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	K. pneumoniae (4)	4–16	2	0.5–2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
AmpC + ESBLs + porin loss (7)	*E. cloacae* (5)	2–32	1–8	1–8	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	E. coli (1)	2–32	0.5–1	0.5–2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	K. pneumoniae (1)	2	0.5	0.5	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
No β-lactamases (14)	*E. aerogenes* (3)	2–4	0.5–2	0.25	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	E. cloacae (1)	8	1	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	E. coli (1)	1	1	0.5	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	K. pneumoniae (1)	16	2	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	P. aeruginosa (8)	-	4–16	2–8	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Abbreviations: ETP, ertapenem; IPM, imipenem; MEM, meropenem; MHT, modified Hodge test; THT, Triton Hodge test; CNPt, Carba NP test; CNPt-direct, simplified Carba NP test; BCT, blue-Carba NP test; CIM, carbapenem inactivation method; AmpC, Ampler class C β-lactamases; ESBL, extended spectrum β-lactamases.

To further comprehensively assess the specificities in the detection of carbapenemases by the phenotypic methods, we tested the reaction of 117 non-susceptible carbapenemase-negative isolates to at least one kind of carbapenem. One hundred and three strains carried at least one type of the plasmid-mediated AmpC and/or ESBLs, with or without alterations in outer membrane permeability, while the other 14 isolates showed reduced susceptibilities to carbapenems without harboring any acquired β-lactamases. Overall, except the MHT and THT, the other phenotypic methods yielded 100% negative results, regardless of the bacterial antibiotic resistance characterization (Table 2). In detail, the MHT yielded 7 false-positive results in 5 *E. cloacae* isolates and 2 *K. pneumoniae* isolates, and the THT yielded 10 false-positive results in 7 *E. cloacae* isolates and 3 *K. pneumoniae* isolates; however, interestingly, these strains harbored the plasmid-mediated AmpC genes.

The overall performance of the involved phenotypic methods are listed in Table 3. The CNPt-direct and CIM yielded comparable sensitivities (92.8% vs 93.5%, $P=1$), which were higher than that of the MHT, CNPt, and BCT ($P<0.003$). Owing to the improved detection of the NDM carriers, the sensitivity of the THT was significantly higher than that of the MHT (84.9% vs

Table 3. Sensitivity and specificity of the six different phenotypic tests

Performance characteristics (compared to presence of carbapenemase genes)	MHT	THT	CNPt	CNPt-direct	BCT	CIM
Sensitivity, % (95% CI)	75.5 (68.4–82.7)	84.9 (78.9–90.9)	66.2 (58.3–74.1)	92.8 (88.5–97.1)	82.0 (75.6–88.4)	93.5 (89.4–97.6)
Specificity, % (95% CI)	94.0 (89.7–98.3)	91.5 (86.4–96.5)	100 (96.9–100)*	100 (96.9–100)*	100 (96.9–100)*	100 (96.9–100)*

*These data were determined by the exact McNemar's test. Other data were determined by the asymptotic McNemar's test.
Abbreviations: MHT, modified Hodge test; THT, Triton Hodge test; CNPt, Carba NP test; CNPt-direct, simplified Carba NP test; BCT, blue-Carba NP test; CIM, carbapenem inactivation method; CI, confidence interval.

75.5%, $P<0.001$). Similarly, compared with the CNPt, the sensitivity of the CNPt-direct increased to 92.8%, which was attributed to the improvement in the detection of the OXA carriers (66.2% vs 92.8%, $P<0.001$). Regarding specificity, the CNPt, CNPt-direct, BCT, and CIM showed 100% specificity, whereas the specificities of the MHT and THT were comparable (94.0% vs 91.5%, $P=0.25$). The specificity of the THT was significantly lower than that of the other four methods with 100% specificities ($P<0.003$). Taken together, the CNPt-direct and CIM possessed the best performance for the efficient detection of carbapenemase among the six evaluated methods.

DISCUSSION

The rapid and accurate detection of carbapenemase is crucial for implementing timely and appropriate treatment and correct infection control procedures. Phenotypic tests, like the MHT and CNPt, are widely used in clinical laboratories for a first-line detection of the carbapenemase-producing isolates. Given the respective shortcomings of these methods, several new phenotypic methods were designed to detect carbapenemases with lower cost and shorter incubation time [7, 8, 10]. In this study, a total of 256 gram-negative bacterial isolates with reduced susceptibilities to carbapenem, including 139 carbapenemase producers and 117 non-carbapenemase producers, were used to evaluate the performances of the MHT, THT, CNPt, CNPt-direct, BCT, and CIM for detection of carbapenemase.

The MHT is used as the earliest developed phenotypic method to detect carbapenemase, however, the poor sensitivity in detecting of the NDM carriers has hindered its wide clinical application [19]. In this study, the sensitivity of the MHT was 75.5%, with 34.8% (16/46) of the NDM-1 producers and 33.3% (17/51) of the OXA- producers being missed, respectively. To overcome the unsatisfying performance of the MHT with the NDM carriers, the THT was proposed in 2015 [5]. The performance of the THT with the NDM carriers was also evaluated in our study, with improvement in detection sensitivity from 65.2% (30/46) to 91.3% (42/46), thereby highlighting the THT as an inexpensive and simple method for the detection of clinical NDM carriers.

Besides the THT, the CNPt was recommended for epidemiological or infection control purposes by the CLSI in 2015 [4] because of its relatively high sensitivity and specificity, short turnaround time, and wide application range. However, the CNPt has certain disadvantages, such as the high cost of the commercial bacterial protein extraction buffer, imipenem reference standard powder and inconsistent detection of the OXA-type carbapenemases, especially the OXA-48 positive strains [21, 22]. Despite no OXA-48 carbapenemase was tested in this study, poor sensitivity (7.8%, 4/51) of the CNPt was observed with 51 *A. baumannii* isolates harboring the OXA-23 gene and/or OXA-51-like gene. Only one out of six OXA-23 carriers tested positive, and the false-negative rates were even higher for the strains harboring the OXA-51-like gene or both the OXA-23 and OXA-51-like genes (92.3% and 93.7%, respectively); it was probably due to the variation in enzyme activities caused by different mutations in the OXA-51 gene sequence. It has been demonstrated that the protein extraction buffer used in the CNPt hindered the color change accompanying the reaction, which was the main reason leading to the poor detection of the OXA producers (buffer effect) [7]. This motivated the development of the CNPt, a phenotypic method specific for *Acinetobacter* spp. that utilizes a modified lysis buffer [23].

There are more simplified and improved versions of the CNPt such as the CNPt-direct, which not only lowered the operation cost, but also improved the sensitivity by avoiding the buffer effect and enhancing the release of carbapenemase by Triton X-100. In our study, the overall sensitivity and specificity of the CNPt-direct were 92.8% and 100%, respectively, and the false-negative rate was reduced to 7.2% (CNPt: 33.8%), which was consistent with previous reports [7, 11]. The use of bromothymol blue in the modified BCT instead of phenol red as the indicator, resulted in 100% sensitivity and 100% specificity with all the carbapenemase producers mentioned in the original report [8]. However, in this study, the sensitivity of the BCT was only 82.0%, and nearly a half of the tested OXA carrying *A. baumannii* isolates were missed, albeit achieving 100% specificity. The dis-

https://doi.org/10.3343/alm.2017.37.4.305

crepancy would have been caused partially by the different subtypes and consequential activities of the OXA carbapenemases that were tested in this study and other reports. In addition, we found that the color changes in some test wells versus control wells were not evident enough for determination of positive results according to the positive result interpretation criteria for the OXA carbapenemase producers. It is suggested that increased inoculum and sufficient homogenization could improve the performance of the BCT [12].

Compared with all the CNPt based methods, the carbapenem inactivation method (CIM) has shown very promising results based on its sensitivity, specificity, low cost, and easy interpretation [10]. In this study, the CIM yielded the most satisfying performance among the six methods, with 93.5% sensitivity and 100% specificity. The CIM missed only 9 out of 51 OXA carbapenemase producers, which probably possessed low-level of carbapenemase activities [24]. However, the relative long incubation time for CIM (from 8 hr to overnight) cannot be ignored.

In conclusion, except for the MHT and THT, performances of all the other methods are acceptable for detecting carbapenemase-producing *Enterobacteriaceae*, if the OXA type carbapenemase is not prevalent. The CNPt-direct and the CIM are the most accurate tests among the six phenotypic methods evaluated in this study. In summary, these two methods hold the promise for easy and rapid implementation in routine clinical microbiology laboratories for epidemiological and infection control purposes.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

Acknowledgments

This work was supported in part by the National Natural Science Foundation of China (Grant No. 81471992 and 81272545), and the Science Foundation of Yuzhong district of Chongqing (Grant No. 20140120).

REFERENCES

1. Bush K and Fisher JF. Epidemiological expansion, structural studies, and clinical challenges of new β-lactamases from gram-negative bacteria. Annu Rev Microbiol 2011;65:455-78.
2. Nordmann P, Dortet L, Poirel L. Rapid detection of extended-spectrum-β-lactamase-producing *Enterobacteriaceae*. J Clin Microbiol 2012;50:3016-22.
3. Carattoli A. Plasmids and the spread of resistance. Int J Med Microbiol 2013;303:298-304.
4. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Twenty-fifth Informational supplement M100-S25. Wayne, PA: CLSI, 2015.
5. Pasteran F, Gonzalez LJ, Albornoz E, Bahr G, Vila AJ, Corso A. Triton Hodge test: improved protocol for modified Hodge test for enhanced detection of NDM and other carbapenemase producers. J Clin Microbiol 2016;54:640-9.
6. Dortet L, Agathine A, Naas T, Cuzon G, Poirel L, Nordmann P. Evaluation of the RAPIDEC® CARBA NP, the Rapid CARB Screen® and the Carba NP test for biochemical detection of carbapenemase-producing *Enterobacteriaceae*. J Antimicrob Chemother 2015;70:3014-22.
7. Pasteran F, Tijet N, Melano RG, Corso A. Simplified protocol for Carba NP test for enhanced detection of carbapenemase producers directly from bacterial cultures. J Clin Microbiol 2015;53:3908-11.
8. Pires J, Novais A, Peixe L. Blue-carba, an easy biochemical test for detection of diverse carbapenemase producers directly from bacterial cultures. J Clin Microbiol 2013;51:4281-3.
9. Pasteran F, Veliz O, Ceriana P, Lucero C, Rapoport M, Albornoz E, et al. Evaluation of the Blue-Carba test for rapid detection of carbapenemases in gram-negative bacilli. J Clin Microbiol 2015;53:1996-8.
10. van der Zwaluw K, de Haan A, Pluister GN, Bootsma HJ, de Neeling AJ, Schouls LM. The carbapenem inactivation method (CIM), a simple and low-cost alternative for the Carba NP test to assess phenotypic carbapenemase activity in gram-negative rods. PLoS One 2015;10:e0123690.
11. Yan Q, Liu Q, Li Y, Li H, Liang X, Zou M, et al. Detection of carbapenemase-producing gram-negative bacteria using a simplified Carba NP test. J Microbiol Methods 2016;123:1-3.
12. Pires J, Tinguely R, Thomas B, Luzzaro F, Endimiani A. Comparison of the in-house made Carba-NP and Blue-Carba tests: Considerations for better detection of carbapenemase-producing *Enterobacteriaceae*. J Microbiol Methods 2016;122:33-7.
13. Tijet N, Patel SN, Melano RG. Detection of carbapenemase activity in *Enterobacteriaceae*: comparison of the carbapenem inactivation method versus the Carba NP test. J Antimicrob Chemother 2016;71:274-6.
14. Poirel L, Walsh TR, Cuvillier V, Nordmann P. Multiplex PCR for detection of acquired carbapenemase genes. Diagn Microbiol Infect Dis 2011;70:119-23.
15. Woodford N, Ellington MJ, Coelho JM, Turton JF, Ward ME, Brown S, et al. Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. Int J Antimicrob Agents 2006;27:351-3.
16. Zhang C, Xu X, Pu S, Huang S, Sun J, Yang S, et al. Characterization of carbapenemases, extended spectrum β-lactamases, quinolone resistance and aminoglycoside resistance determinants in carbapenem-non-susceptible *Escherichia coli* from a teaching hospital in Chongqing, Southwest China. Infect Genet Evol 2014;27:271-6.
17. Kim HK, Park JS, Sung H, Kim MN. Further modification of the modified Hodge test for detecting metallo-β-lactamase-producing carbapenem-resistant *Enterobacteriaceae*. Ann Lab Med 2015;35:298-305.
18. Vasoo S, Cunningham SA, Kohner PC, Simner PJ, Mandrekar JN, Lolans K, et al. Comparison of a novel, rapid chromogenic biochemical assay, the Carba NP test, with the modified Hodge test for detection of carbapenemase-producing Gram-negative bacilli. J Clin Microbiol 2013;51:3097-101.
19. Lifshitz Z, Adler A, Carmeli Y. Comparative study of a novel biochemical assay, the Rapidec Carba NP test, for detecting carbapenemase-producing *Enterobacteriaceae*. J Clin Microbiol 2016;54:453-6.

20. Desu MM and Raghavarao D. Sample size methodology. New York: Academic Press, 1990.
21. Heinrichs A, Huang TD, Berhin C, Bogaerts P, Glupczynski Y. Evaluation of several phenotypic methods for the detection of carbapenemase-producing *Pseudomonas aeruginosa*. Eur J Clin Microbiol Infect Dis 2015; 34:1467-74.
22. Genc O, Aksu E, Gulcan A. The identification of carbapenemase types in *Enterobacteriaceae* by using molecular assay and phenotyping confirmation tests. J Microbiol Methods 2016;125:8-11.
23. Dortet L, Poirel L, Errera C, Nordmann P. CarbAcineto NP test for rapid detection of carbapenemase-producing *Acinetobacter* spp. J Clin Microbiol 2014;52:2359-2364.
24. Bush K and Fisher JF. Epidemiological expansion, structural studies, and clinical challenges of new β-lactamases from gram-negative bacteria. Annu Rev Microbiol 2011;65:455-78.

https://doi.org/10.3343/alm.2017.37.4.305