EVALUATION OF PHENOTYPIC METHODS USED FOR THE DETECTION OF CARBAPENEMASE: MODIFIED HODGE TEST, CARBAPENEM INACTIVATION METHOD, AND CARBANP TEST

Prasanth Manohar, Nazia Hassan, Abha Kumari, Vaikhari Kale, Nachimuthu Ramesh*

Antibiotic Resistance and Phage Therapy Laboratory, Department of Biomedical Sciences, School of Bioscience and Technology, Vellore Institute of Technology, Vellore-632014, Tamil Nadu, India

ABSTRACT

Introduction: Infections caused by carbapenem-resistant Enterobacteriaceae are one of the serious clinical problems, considering carbapenems are the last resort of antibiotics for treating multi-drug resistant Enterobacteriaceae.

Objective: In this study, we compared different phenotypic screening methods used for the identification of carbapenemase-producing Enterobacteriaceae.

Methods: The clinical isolates were initially studied for their minimal inhibitory concentration and PCR based carbapenem-resistance gene analysis. The Modified Hodge Test (MHT), carbapenem inactivation method (CIM) and carbaNP test were performed.

Results: The results showed that the MHT and carba NP test can produce false-negative results but carbapenem inactivation method (CIM) can produce relatively positive results for all the isolates. In the CIM method, the use of ertapenem disks produced accurate results. The carbaNP test was found to be rapid in analyzing the results but CIM was suitable for general clinical laboratories with its high sensitivity and specificity.

Conclusion: This study highlighted the use of different phenotypic methods to evaluate the carbapenemase producers in the clinical laboratories.

Keywords: Carbapenem-resistant *Enterobacteriaceae*, Carbapenemase, Modified Hodge Test, Carbapenem Inactivation Method, CarbaNP test.

REZUMAT

Introducere: Infecțiile cauzate de enterobacteriile rezistente la carbapenem sunt una dintre problemele clinice grave, deoarece carbapenemele reprezintă ultima soluție de antibiotice pentru tratarea acestor infecții multirezistente.

Obiectiv: În acest studiu, am comparat diferite metode de screening fenotipic, utilizate pentru identificarea enterobacteriilor producătoare de carbapenemază.

Metode: Izolatele clinice au fost inițial studiate pentru a se determina concentrația lor minimă inhibitorie și pentru analiza prin PCR a genelor pentru rezistența la carbapeneme. Au fost efectuate testul Hodge modificat (MHT), metoda de inactivare a carbapenemelor (CIM) și testul carbaNP.

Rezultate: Analiza a arătat că testele MHT și carbaNP pot produce rezultate fals negative, dar metoda de inactivare cu carbapenem (CIM) poate produce rezultate fals pozitive pentru toate izolatele. În metoda CIM, utilizarea discurilor de ertapenem a produs rezultate corecte. Testul carbaNP s-a dovedit a fi rapid în analiza rezultatelor, CIM fiind potrivit pentru laboratoarele clinice generale, datorită sensibilității și specificității sale ridicate.

Concluzie: Acest studiu a evidențiat necesitatea utilizării diferitelor teste fenotipice în laboratoarele clinice, în vederea evaluării microorganismelor producătoare de carbapenemază.

Cuvinte-cheie: enterobacterii rezistente la carbapenem, carbapenemază, test Hodge modificat, metoda de inactivare a carbapenemului, test CarbaNP.

INTRODUCTION

Carbapenems are active members of the β-lactam class of antibiotics and can resist hydrolysis by β-lactamases (also called carbapenemases).

As carbapenems are considered to have an extensive antibacterial spectrum, these antibiotics are one of the last recourse for controlling bacterial infections [1]. Resistance to carbapenems in Gram-negative bacteria

**Corresponding author:* Dr. N. Ramesh, Assistant Professor, Vellore Institute of Technology, Vellore, India. E-mail:drpnramesh@gmail.com

has become a major public health issue which has emerged globally; notably, it is mediated by transferable genes. As clinicians often encounter patients with multidrug resistant infections, there is a possibility of the emergence of untreatable infections [2].

The medical community depends on clinical expertise for deciding empirical therapy. Therefore, the concern lies in the early identification of carbapenemase producers to prevent the development of nosocomial and community outbreaks [3, 4].

Currently used detection techniques involve prior screening of resistant strains followed by phenotypic and/or genotypic confirmatory assessment [5]. Several techniques are built and routinely used in clinical laboratories for rapid detection of carbapenemases. It includes the disk diffusion test that involves the first careful analysis of susceptibility testing followed by the minimum inhibitory concentration (MIC) [6]. Unfortunately, the battle towards carbapenems is difficult to expose by conventional disk diffusion method used by several laboratories and microbiologists [7, 8].

MIC is considered as a conventional method for identification of antibiotic resistance in many laboratories. The Modified Hodge test (MHT) is widely used as a phenotypic method for the detection of carbapenemase producer [9]. However, this technique may provide positive results for the strains that are producing other types of β -lactamases [10]. The demonstration of enzyme hydrolysis of carbapenem in resistant Gram-negative rods using susceptibility testing disks has been developed as the Carbapenem Inactivation Method (CIM) [11]. The CIM is a rapid test that will simplify the carbapenemase test [12]. The CarbaNP test is a recently developed method and it is a rapid, sensitive, and accurate detection method, as it helps in differentiating carbapenemase producers from the other strains [13]. Perhaps it is appropriate for the brisk prevention and regulation of infections in clinics and hospitals.

The newly developed, CarbaNP test has the capacity to overcome the problems on other phenotypic tests because the results are observed as a change in pH [14]. All these phenotypic tests readily give results based on the detection of diffusible carbapenemases within a discrete period of time.

However, considering efficiency and appropriate workflow of each test will allow us to distinguish them on the basis of specificity and reliability in clinical laboratories, their cost-effectiveness, and time consumption. Therefore, in this study, we evaluated the different phenotypic methods used to identify the carbapenemase producer and compared each method for their specificity and sensitivity.

MATERIALS AND METHODS

Clinical isolates

A total of 43 non-repetitive, Gram-negative bacterial isolates were used in this study to analyze the different carbapenemase detection techniques. All the isolates were recovered from the clinical samples that include blood, urine, pus, sputum and wound swab. All the isolates were collected from clinical laboratories in Chennai and further study was carried out in Antibiotic Resistance and Phage Therapy Laboratory, VIT, Vellore.

Minimal Inhibitory Concentration (MIC)

Agar dilution method was followed to determine the MIC values of meropenem and imipenem (CLSI guidelines). Briefly, Muller-Hinton Agar (MHA) plates were prepared using different concentrations of meropenem/imipenem ranging from 0.06 to 128 mg/L. The bacterial inoculum was prepared (0.5 McFarland Turbidity Standard) and one μL of the bacterial inoculum was placed on the agar plates. The plates were dried and incubated at 37°C for 20h. The results were interpreted as per the CLSI guidelines [15].

Modified Hodge Test (MHT)

In the Mueller Hinton Agar (MHA) plates, the bacterial lawn was prepared using *E. coli* DH5α (susceptible strain). Meropenem/imipenem/ertapenem disk was placed at the center of the agar plate and bacterial isolates were streaked in a single straight line from the rim of the disk to the boundary of the plate. The MHA plates were incubated at 37°C for 20h. Any enhanced growth around the test organism and the zone of inhibition indicated the test organism producing carbapenemase.

All the results were interpreted as per the CLSI guidelines [15].

Carbapenemase inactivation method (CIM)

A loop full of bacterial culture was taken and a suspension was prepared using one mL of Muller-Hinton broth (MHB). To the suspension, meropenem/imipenem/ertapenem disk (10 μg) was immersed and incubated for two hours at 37°C. The susceptible-testing disks were removed by using sterile forceps and placed on agar plate where the bacterial lawn was prepared using *E. coli* DH5α and the plates were incubated at 37°C to analyze the zone of inhibition. In the case of test bacterium producing carbapenemase, no zone of inhibition was observed and the bacterium that does not produce carbapenemase showed the clear zone of inhibition. When the bacteria are producing carbapenemase enzyme, the antibiotic in the disk is inactivated that allows the uninhibited growth of *E. coli* DH5α but when the bacteria is a non-carbapenemase producer, the antibiotic remains in the disk that forms the clear inhibition zones of *E. coli* DH5α [11].

CarbaNP Test

CarbaNP test was performed to scrutinize the phenotypic carbapenemase-producing bacteria. Briefly, 0.05% phenol red solution and 0.1 mmol/L $ZnSO_4$ (pH-7.8±0.1) was mixed to prepare CNP solution A. CNP solution B was prepared using 12 mg/mL imipenem monohydrate-cilastatin and CNP solution was prepared by mixing solution A and B on the experiment day. Each bacterial isolate was inoculated into the microcentrifuge tube containing one mL of MH broth followed by centrifugation at 10,000 x g for 10 min. Then, 100 μL of lysis buffer (Tris HCl-20mM/L and 0.1% Triton-X 100) was added to the retrieved pellet, vortexed for 2 min and stored at room temperature for 10 minutes. To the 100 μL of CNP solution in the microtiter plate, 10 μL of lysate was added, and incubation for 2 h at 37°C. The change in color of the solution from red or light-orange color to yellow or yellowish-orange color was interpreted as the presence of carbapenemase enzyme which is further considered to be a positive test [13].

Genotypic detection of carbapenem resistance genes

The presence of β-lactamase/carbapenem-resistance genes $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}8}$, $bla_{CTX\text{-}M\text{-}9}$, $bla_{CTX\text{-}M\text{-}25}$, bla_{NDM}, $bla_{OXA\text{-}48\text{-}like}$, bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{DIM}, bla_{BIC}, bla_{GIM}, bla_{SIM} and bla_{AIM} were studied using multiplex polymerase chain reaction (PCR) as explained elsewhere. The primers and reaction conditions used for bla_{NDM}, $bla_{OXA\text{-}48\text{-}like}$, bla_{KPC}, bla_{IMP}, and bla_{VIM} were as detailed by Doyle *et al*., [16] and for bla_{DIM}, bla_{BIC}, bla_{GIM}, bla_{SIM} and bla_{AIM} as explained by Gheorghe *et al*., [17]. All the PCR amplified gene products were sequenced and analyzed.

RESULTS

A total of 43 isolates (including *E. coli* DH5α) belonging to Enterobacteriaceae were used in this study. The isolates included 21 *E. coli* (3 susceptible, 3 CTX-M, and 15 carbapenem-resistant), 10 *K. pneumoniae* (2 susceptible, 2 CTX-M, 6 carbapenem-resistant), *K. oxytoca* (2 carbapenem-resistant), *E. cloacae* (3 carbapenem-resistant), *E. hormaechei* (3 carbapenem-resistant), *P. rettgeri* (2 carbapenem-resistant) and *S. marcescens* (2 carbapenem-resistant). The isolates harbouring carbapenem-resistance genes (PCR based detection) were considered as carbapenemase producer throughout this study. The isolates included 33 carbapenemase-producers (NDM, OXA-48, IMP) and 10 non-carbapenemase producers (carbapenem sensitive, ESBL). The MIC values for imipenem were ranging between 8 and >128 μg/mL, and for meropenem, the ranges were between 4 and >128 μg/mL (Table 1). The MHT showed that the false-positive results were observed for *E. coli* and *K. pneumoniae* producing ESBLs and false-negative results were observed for *E. coli* producing NDM-1 and *P. rettgeri* producing OXA-48-like. In the MHT, sensitivity (%) and specificity (%) for ertapenem were 90.9 and 90.0; for imipenem 90.0 and 80.0 and for meropenem 90.9 and 80.0 respectively. The CIM was performed using three antibiotics; ertapenem, imipenem and meropenem. For CIM, false-positive results were observed for *E. coli* and *K. pneumoniae* producing ESBLs and false-negative results for *E. coli*, *K. oxytoca* and *P. rettgeri*. In the case of CIM, the

Table 1. Comparison of different methods used in the phenotypic detection of carbapenemase producing Gram-negative bacteria

Bacterial isolate	Resistance gene	MIC (µg/mL)		MHT			*CIM			Carba NP test
		IMP	MER	ERT	IMP	MER	ERT	IMP	MER	
Time to obtain the result		**18 h**			**20 h**			**12 h**		**2 h**
E. coli DH5α	Susceptible	0.06	0.06	-	-	-	29	27	27	-
E. coli EC1	Susceptible	0.12	0.25	-	-	-	24	24	22	-
E. coli EC2	Susceptible	0.12	0.12	-	-	-	26	24	24	-
E. coli EC3	CTX-M-2	16	4.0	-	-	-	24	26	24	-
E. coli EC4	CTX-M-9	4.0	2.0	-	-	-	26	28	26	-
E. coli EC5	CTX-M-9, CTX-M-25	16	8.0	-	+	+	26	0	0	±
E. coli EC6	NDM-1	>128	64	+	+	+	0	0	0	+
E. coli EC7	NDM-1	32	32	+	+	+	0	0	0	+
E. coli EC8	NDM-1	>128	>128	-	-	-	0	0	0	+
E. coli EC9	NDM-1, CTX-M-9	16	32	+	+	+	0	0	0	+
E. coli EC10	NDM-1, CTX-M-25	32	64	+	+	+	0	0	0	+
E. coli EC11	NDM-1	32	32	-	-	-	0	0	0	+
E. coli EC12	NDM-1, OXA-48-like	64	>128	+	+	+	0	0	0	+
E. coli EC13	NDM-1, IMP-1	>128	64	+	+	+	0	0	0	+
E. coli EC14	OXA-48-like	2.0	8.0	+	+	+	0	0	0	+
E. coli EC15	OXA-48-like, CTX-M-9	2.0	4.0	+	+	+	0	0	0	±
E. coli EC16	OXA-48-like, CTX-M-25	16	16	+	+	+	0	0	0	+
E. coli EC17	OXA-48, NDM-1	8	16	+	+	+	0	0	0	+
E. coli EC18	OXA-48-like	32	16	+	+	+	0	0	22	+
E. coli EC19	IMP-1, CTX-M-9	128	64	+	+	+	0	0	0	+
E. coli EC20	IMP-1	>128	>128	+	+	+	0	0	0	+
K. pneumoniae KP1	Susceptible	1	0.25	-	-	-	22	22	24	-
K. pneumoniae KP2	Susceptible	2	0.5	-	-	-	25	24	24	-
K. pneumoniae KP3	NDM-1	64	64	+	+	+	0	0	0	+
K. pneumoniae KP4	NDM-1, CTX-M-9	32	32	+	+	+	0	0	0	+
K. pneumoniae KP5	NDM-1	>128	32	+	+	+	0	0	0	+
K. pneumoniae KP6	OXA-48-like	16	32	+	+	+	22	25	25	+
K. pneumoniae KP7	OXA-48-like, CTX-M-2	32	>128	+	+	+	0	0	0	+
K. pneumoniae KP8	IMP-1	64	32	+	+	+	0	0	0	+
K. pneumoniae KP9	CTX-M-9	1.0	1.0	+	+	+	25	28	22	-
K. pneumoniae KP10	CTX-M-25	0.5	0.25	-	-	-	26	24	0	-
K. oxytoca KO1	NDM-1	32	64	+	+	+	0	0	0	+
K. oxytoca KO2	OXA-48	128	32	+	+	+	0	0	24	-
E. cloacae EL1	NDM-1	32	32	+	+	+	0	0	0	+
E. cloacae EL2	OXA-48	16	4	+	+	+	0	0	0	+
E. cloacae EL3	IMP-1	16	64	+	+	+	0	0	0	±
E. hormaechei EH1	NDM-1	>128	32	+	+	+	0	0	0	+
E. hormaechei EH2	OXA-48	8	4	+	+	+	0	0	0	+
E. hormaechei EH3	NDM-1	32	>128	+	+	+	0	0	0	+
P. rettgeri PR1	OXA-48	16	128	-	-	-	0	0	0	+
P. rettgeri PR2	OXA-48	64	16	+	+	+	0	22	0	±
S. marcescens SM1	OXA-48	32	32	+	+	+	0	25	0	+
S. marcescens SM2	OXA-48	8	2	+	+	+	0	0	0	+

[a]The grey highlights are false-positive and false-negative results; [b]± represents obscure results

*CIM results are represented as zone of inhibition diameter (mm), diameter of <20 mm was considered as susceptible.

Table 2. Details of the sensitivity and specificity of the phenotypic methods used for the detection of carbapenemase producing *Enterobacteriaceae*

	CIM			MHT			Carba NP test
	ERT	IMP	MER	ERT	IMP	MER	
Sensitivity (%)	100	93.9	93.9	90.9	90.9	90.9	96.9
Specificity (%)	100	90	80.0	90.0	80.0	80.0	100

sensitivity and specificity of imipenem was 93.9% and 80.0% and meropenem was 93.9% and 90.0% respectively. When ertapenem was used for CIM, the sensitivity and specificity was 100%. The carbaNP test results showed that false-negative was observed only for *K. oxytoca* producing OXA-48. The sensitivity and specificity of carbaNP test was 96.9% and 100% respectively (Table 2).

DISCUSSION

Carbapenems are broad-spectrum antibacterial drugs that are considered as one of the last resort for infections caused by Gram-negative bacteria.

Carbapenemase-producing *Enterobacteriaceae* (CPE) is one of the major concerns worldwide because of the multidrug resistance nature of these bacteria [18]. Because of the increased infections caused by CPE, a more rapid and highly sensitive detection method is necessary for clinical laboratories to prevent and control CPE infections.

At present, there are different methods that can be used for the detection of CPE such as Modified Hodge test (MHT), carbapenem inactivation method (CIM) [11] and carbaNP test (CNPt). According to the Clinical Laboratory Standard Institute (CLSI) guidelines, both MHT and CNPt are recommended for the detection of CPE [15].

In this study, we evaluated the performance of different screening methods that can be used for the detection of CPE. In the MHT, false-positive results were observed for ESBLs (CTX-M) and false-negative results were observed for both NDM and OXA-48 producers.

Though three different carbapenem antibiotics, such as imipenem, meropenem, and ertapenem were used for the MHT, the sensitivity and specificity of the tests were always <100%. The MHT is preferred routinely as a dependable phenotypic method to identify carbapenemase-producing *Enterobacteriaceae* in many clinical and research laboratories.

The earlier studies also reported the drawbacks in using MHT as a confirmatory method to identify carbapenemase-producing *Enterobacteriaceae* [1, 12].

No false-positive results were observed in the case of carbaNP test but false-negative result was observed for OXA-48 producers. The carbaNP test is a rapid method to identify carbapenemase producing *Enterobacteriaceae* [13].

Some earlier studies reported 100% sensitivity and specificity for carbaNP test in identifying carbapenemase producer [13] and few studies showed the false-negative results of carbaNP test [1, 12]. The CIM results were more accurate than the other two phenotypic (MHT, carba NP) methods which yielded 100% sensitivity and specificity when ertapenem disk was used.

The accuracy of the CIM test results was reduced when imipenem and meropenem disks were used. In the case of CIM results for imipenem and meropenem, false-positive results were observed for ESBLs (CTX-M) and false-negative results for OXA-48 producers. For the CIM, the use of ertapenem disk is preferable in order to obtain accurate results.

Similar results were reported by Yamada *et al*., in which the specificity of ertapenem was 100%. Thus, our results indicated that the CIM should be preferred to identify the carbapenemase-producing *Enterobacteriaceae* than the other two methods (MHT or carbaNP test) because the CIM has more sensitivity and specificity.

Previously, Tijet *et al*., (2016) and Yamada *et al*., (2016) have reported the comparison between the different phenotypic methods used for the identification of carbapenemase producers [1, 12, 18]. Tijet *et al*., reported the evaluation report on carbaNP test and

CIM that clearly showed the high sensitivity and specificity of CIM than carbaNP test. Similarly, Yamada *et al*., compared MHT, CIM and carbaNP test to detect carbapenemase-producing *Enterobacteriaceae,* and the results showed that the CIM had more sensitivity and specificity than MHT and carbaNP test. Accordingly, our data also showed the similar results of CIM having 100% sensitivity and specificity compared to MHT and carbaNP test.

Other recent reports also evaluated the use of phenotypic methods for carbapenemase production [19, 20, 21]. It was also observed that the use of ertapenem or meropenem disk provided more accurate results than imipenem disks. From this study, the use of CIM for the routine identification of carbapenemase-producing *Enterobacteriaceae* is preferable than MHT and carbaNP test.

One limitation of this study is the number of carbapenemase producer and non-carbapenemase producers used is minimal because of the availability of the isolates. Further studies using the bacterial isolates harbouring more carbapenem-resistance genes may provide highly accurate results with the large quantity of data. Our results showed that the CIM is a very easy method to perform in any clinical laboratory without any expensive equipment.

The carbaNP test is more rapid to detect the carbapenemase producers but considering the sensitivity and specificity of the method, CIM is preferable. Still, carbaNP test can be used for the rapid detection of carbapenemase producers based on the clinical condition of the patients.

This study sheds light on the screening methods in the evaluation of carbapenemase-producing *Enterobacteriaceae* so that in the future clinical laboratories can follow a rapid and highly accurate detection method for CPE.

Conflict of interests: The authors declare no conflict of interest.

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