ORIGINAL ARTICLE

Evaluation of Carba NP Version II Test for Rapid Detection and Categorization of Carbapenemases Produced by *Klebsiella Pneumoniae* Clinical Isolates

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Key words:
Carbapenemases, CarbaNP II test, Antimicrobial resistance

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ABSTRACT

Background: *Carbapenemase producing K. pneumoniae poses severe clinical problems. The accurate phenotypic detection and differentiation of the carbapenemase types in clinical laboratories is now of utmost importance for the determination of appropriate therapeutic schemes and the implementation of infection control measures.* ***Objective:*** *to identify the prevalent carbapenemases among carbapenem non-susceptible K. pneumoniae isolated from AMUH, to evaluate the ability of Carba NP version II test to detect carbapenemase production and identify their Ambler classes compared to PCR as a gold standard, in addition to comparing the Carba NP version II test with MHT and inhibitor-based phenotypic tests regarding sensitivity, specificity, turnaround time and cost effectiveness.* ***Methodology:*** *This study was carried out on 96 non-duplicate carbapenem non-susceptible K. pneumoniae isolates carrying one or more of carbapenemase genes. phenotypic screening methods included: Carba NP II test, MHT, meropenem/PBA CDT and meropenem/EDTA CDT.* ***Results:*** *Carbapenemase class B was the most prevalent (94.8%). Carba NP II showed the highest sensitivity in detecting class B (72.5%) with PPV (95.7%). Increase in the number of class B genes in a given isolate, improved the sensitivity from 55.6% to 80%. The Carba NP II test was found to be 15% sensitive and 91.7% specific in detecting class D and it could not detect class A in any of the 19 isolates that harbored KPC gene.* ***Conclusion:*** *The main advantages of Carba NP II test over CDT and MHT are; the rapidity of the test and its relatively higher sensitivity in detecting class B carbapenemases.*

INTRODUCTION

Klebsiella pneumoniae is a major cause of hospital-acquired infections. Conferring to the World Health Organization (WHO) report released in 2014, resistance to carbapenem antibiotics, the treatment of last resort for life-threatening infections caused by *K. pneumoniae*, has extended all over the globe and in some countries, because of resistance, carbapenem antibiotics would not work in more than half of patients treated for *K. pneumoniae* infections [1].

Resistance to carbapenems is typically caused by several mechanisms: First and most important is acquisition of carbapenemase genes that encode for enzymes capable of degrading carbapenem [2]. The second mechanism is active transport of carbapenem drugs out of the cell, augmented drug efflux. Thirdly, mutation or loss of outer membrane porins (omp), preventing antibiotics from entering the cells. Combinations of these mechanisms can cause high levels of resistance to carbapenems in certain bacterial species, such as *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* [3].

Acquired carbapenemases are the most serious risk and are spreading in *Enterobacteriaceae*. Carbapenemases are classified by their molecular structures into 3 classes: class A, B and D of the Ambler classification system. Class A (e.g. KPC-type enzymes), class B metallo-β-lactamases (e.g.VIM, IMP, NDM.. etc), and class D Oxacillinase (e.g OXA-48) [2].

Proper selection of suspicious isolates with reduced susceptibility to carbapenems is crucial [4]. Additional phenotypic tests can help to identify carbapenemase producer (CP), these include the modified Hodge test (MHT), inhibitor based tests, the Carba NP test and its variants, and the carbapenem inactivation method [5-7].

The Modified Hodge test (MHT) was recommended for years by CLSI as a detector for CP till it was omitted in 2018 [8]. Combined disc tests utilizing boronic acid and EDTA as specific carbapenemase inhibitors are available to assign the carbapenemase class (Ambler class A, B). In its 2013 guidelines for carbapenemases detection, EUCAST recommended combined disk tests (CDT) for carbapenemase confirmation [9]. Nordmann et al [10], described an inhibitor-based biochemical assay for detection of carbapenemase, the Carba NP test, which was published in two versions: The Carba NP assay

provides a positive or negative result ("carbapenemase detected/not detected"), whereas the Carba NP II test was designed to discriminate between carbapenemase classes A,B, and D [11,12]. Carba NP is a biochemical phenotypic assay for speedy detection (≤2h) of carbapenemase production on gram-negative bacilli that detects carbapenemases by measuring the in-vitro hydrolysis of the antibiotic imipenem by a bacterial extract. Imipenem hydrolysis changes the pH and produces a resultant colour change of a pH indicator phenol red (red to orange/yellow) (Figure 1) [11].

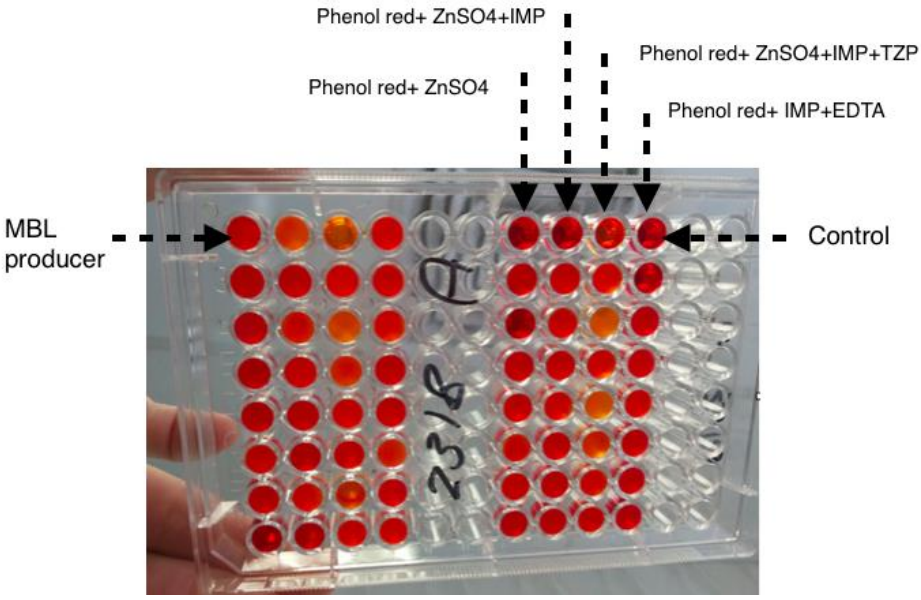

Fig. 1: Carba NP II test.

The aim of the present study is to identify the prevalent carbapenemases among carbapenem non-susceptible *K. pneumoniae* isolated from Alexandria Main University Hospital (AMUH), to evaluate the ability of Carba NP version II test to detect carbapenemase production and identify their Ambler classes compared to PCR as a gold standard and to compare the Carba NP version II test with MHT and inhibitor-based phenotypic tests regarding sensitivity, specificity, turnaround time and cost effectiveness.

METHODOLOGY

The present study was performed on 96 non-duplicate carbapenem non-susceptible *K. pneumoniae* isolates received at AMUH microbiology laboratory. The university ethical committee approved the study design in January 2017 (IRB:00007555 - FWA:00015712), Serial number:0104849.

Cultures of all clinical specimens from the Microbiology lab of AMUH were screened for growth of *Klebsiella pneumoniae* using the standard microbiological methods [13]. Antimicrobial susceptibility was done by modified Bauer-Kirby disc diffusion technique, and interpreted in accordance with the guidelines established by the CLSI [14]. *Klebsiella pneumoniae* isolates resistant or intermediate susceptible to at least one of the three carbapenems (Imipenem, meropenem and ertapenem) were included in the current study.

Conventional PCR was carried out on 110 randomly selected carbapenem non-susceptible *K. pneumoniae* isolates for carbapenemase genes detection. The following genes were amplified; IMP, VIM, NDM, KPC and OXA-48 [15].

Isolates carrying a carbapenemase gene (one or more) were subjected to various phenotypic methods:

- Carba NP II test [10,12]:

Two loopful (10µl) of the examined strain grown on a Mueller-Hinton agar were re-suspended in an eppendorf containing 200 µl Tris-HCl 20mM lysis buffer (Lobachemie), vortexed for 1min, and incubated for 30 min at room temperature. The eppendorf was centrifuged for 5 min at 10,000 x g in room temperature. The supernatant formed represent the enzymatic bacterial suspension. Using a microtiter plate, 30 µl of this supernatant was mixed with100µl of (i) Well 1: A diluted red phenol solution with 0.1 mM $ZnSO_4$ (Lobachemie), (ii) Well 2: A diluted red phenol solution with 0.1 mM $ZnSO_4$ and 3 mg/ml imipenem monohydrate, (iii) Well 3: A diluted red phenol solution with 0.1 mM $ZnSO_4$, 3 mg/ml imipenem monohydrate,

and 4 mg/ml tazobactam sodium salt, and (iv) Well 4: A diluted red phenol solution with 3 mg/ml of imipenem monohydrate and 0.003 M of EDTA (Sigma-Aldrich, Steinheim, Germany)[12].

The microtiter plate was incubated at 37°C for 2h maximally [12]. The assay was tested in triplicate for each isolate. *K. pneumoniae* isolates fully susceptible to carbapenems were randomly selected and enrolled as negative control.

The interpretation of the assay (Figure 1) includes:

If the first two wells are read alone (Carba NP), so the isolate was considered CP when the first well remain red and the second well (imipenem + $ZnSO_4$) turn yellow/orange.

If the four wells are read (Carba NP version II): (i) If the color of the wells containing imipenem + $ZnSO_4$, and imipenem + EDTA changed from red to yellow/orange, but the well containing imipenem + $ZnSO_4$ + tazobactam remained red, the strain produced an Ambler class A carbapenemase. (ii)If the color of the wells containing imipenem + $ZnSO_4$ and imipenem + $ZnSO_4$ + tazobactam turned from red to yellow/orange, but the well containing imipenem + EDTA remained red, the strain was an MBL producer.(iii)If the color of the wells containing imipenem + $ZnSO_4$, imipenem + $ZnSO_4$ + tazobactam, and imipenem + EDTA turned from red to yellow/orange, the strain produced a carbapenemase belonging neither to Ambler class A nor class B, and may likely be a class D carbapenemase. (iv) If the color of all wells remained red, the strain was not a CP. (v) If the color of all wells turned from red to yellow/orange, or if the color of the first two wells (well with phenol red solution, well with imipenem + $ZnSO_4$) remained red, while the third and fourth wells turned yellow/orange, the test was considered as not interpretable (invalid) [12].

- Modified Hodge test (MHT)[13]:

According to the CLSI recommendations[8].

- Screening for production of class A and class B carbapenemases using meropenem/phenyl boronic acid and meropenem/EDTA combined disc test [16]:

Phenyl boronic acid (PBA) (Himedia) / EDTA (Sigma-Aldrich, Steinheim, Germany) along with meropenem disc were used for identifying KPC and MBL respectively. The stock solution of PBA was prepared by dissolving PBA in dimethyl sulfoxide [DMSO] (Lobachemie) at a concentration of 20 mg/ml, from which 20 µL (containing 400 µg of PBA) was distributed on discs of meropenem. EDTA 0.1M was prepared as stock solution, from which 10 µL (containing 292 µg of EDTA) was added to meropenem discs. Discs with added inhibitor were left to dry, and used within 60 minutes [16].

The tested isolate was inoculated on the surface of Muller Hinton agar, three discs of meropenem were placed. One disc of the antibiotic with no inhibitor, the second disc containing 400 µg of PBA, and third disc containing 292 µg of EDTA. The plates were incubated overnight at 37°C. Then the inhibitory zones around each disc was measured [17].

Interpretation:

If the inhibitory zone around the disc with PBA was ≥5mm in comparison to inhibitory zone around the disc with no inhibitors, the isolate was considered KPC producer. If the inhibitory zone around the disc with EDTA was ≥5mm in comparison to inhibitory zone around the disc with no inhibitors, the isolate was considered MBL producer. The isolate was considered negative for KPC and MBL production, when none of the two combined-disc tests were positive[18,19].

Statistical analysis

All analyses were performed using SPSS, version 16.0 (SPSS Inc, Chicago, IL, USA). The statistical analysis was done using pearson's chi square test; $P \leq 0.05$ was considered statistically significant.

RESULTS

One hundred and ten carbapenem non-susceptible, biochemically confirmed *K. pneumoniae* isolates were arbitrarily chosen for PCR testing. Fourteen isolates didn't harbour any of the genes tested for in the study, so they were excluded from further testing. The remaining 96 PCR positive isolates were included in the study.

Antimicrobial susceptibility testing of carbapenems revealed 97.9% (94/96) resistance and 2.1% (2/96) intermediate susceptibility to ertapenem. As for meropenem, 85.4% (82/96) were resistant and 9.4% (9/96) showed intermediate susceptibility. Imipenem demonstrated the lowest resistance rate among the tested carbapenems, where 78.1% (75/96) of the tested isolates were imipenem resistant and 19.8% (19/96) showed intermediate susceptibility.

Results of PCR for carbapenemase genes:

Regarding the distribution of different carbapenemase classes, the most predominant class was class B (represented in 94.8% [91/96] of the tested isolates; either alone in 30.2%, or combined with one or more other classes in 64.6% of isolates, the least one was class A (19.8% prevalence), whereas class D existed in 62.5% (60/96).

Regarding gene distribution, the most frequently detected gene was VIM (83.3% of the tested isolates harbored this gene either alone or in association with other genes), followed by NDM (69.8%), OXA- 48 (62.5%), whereas the least numerous was KPC (19.8%) and never detected alone (Table 1).

Table 1: Distribution of carbapenemase genes among the 96 studied isolates

	Genotypic (PCR)	No.	%
I- Class B only (n = 29, 30.2%)	IMP	4	4.2
	VIM	4	4.2
	NDM	1	1.0
	IMP + VIM	5	5.2
	VIM + NDM	6	6.3
	IMP + VIM + NDM	9	9.4
II- Class D only (n = 5, 5.2%)	OXA-48	5	5.2
III- Class B+D (n = 43, 44.8%)	IMP + OXA-48	3	3.1
	VIM + OXA-48	6	6.3
	NDM + OXA-48	1	1.0
	IMP + VIM + OXA-48	1	1.0
	IMP + NDM + OXA-48	1	1.0
	VIM + NDM + OXA-48	16	16.7
	IMP + VIM + NDM + OXA-48	15	15.6
IV- Class B+A (n = 7, 7.3%)	IMP + KPC	1	1.0
	VIM + NDM + KPC	4	4.2
	IMP + VIM + NDM + KPC	2	2.1
V- Class A+B+D (n = 12, 12.5%)	VIM + NDM + KPC + OXA-48	5	5.2
	IMP + VIM + NDM + KPC + OXA-48	7	7.3

Results of Carba NP test:

The Carba NP test demonstrated 84.38% sensitivity and 100.0% PPV (Table 2) compared to PCR as a gold standard. Carba NP test detected production of carbapenemase in 100% of isolates harboring all of the 5 tested genes (100% sensitivity) (Table 3). Lowest sensitivity (71.4%) on the contrary, was demonstrated among isolates harboring only one of the studied genes, with the highest frequency of negative results (28.6%).

Table 2: Sensitivity & PPV of Carba NP test compared to PCR

Phenotypic [Carba NP]	Genotypic (PCR) (n = 96)	
	No.	%
Positive	81	84.4
Negative	15	15.6
Sensitivity	84.38	
PPV	100.0	

PPV: Positive predictive value

Table 3: Impact of number of genes on the performance of Carba NP test

Phenotypic [Carba NP]	Number of genes										χ^2	^{MC}p
	1 gene (n = 14)		2 genes (n = 22)		3 genes (n = 31)		4 genes (n = 22)		5 genes (n = 7)			
	No.	%	No.	%	No.	%	No.	%	No.	%		
Positive	10	71.4	20	90.9	25	80.6	19	86.4	7	100.0	3.542	0.457
Negative	4	28.6	2	9.1	6	19.4	3	13.6	0	0.0		

χ^2: Chi square test

p: p value for comparing between the studied categories

Regarding the utility of Carba NP version II test to identify different carbapenemase classes, it was found that; out of 96 isolates, 69 (71.9 %) were identified as class B, 12 (12.5%) belong to class D and no isolate was proved to belong to class A. The invalid test results represented 15.6% (15/96).

On comparing Carba NP II results to PCR results, the highest sensitivity of Carba NP II was in detection of class B carbapenemases (72.5%) with PPV 95.65%. For class D detection, Carba NP II showed only 15% sensitivity with 75% PPV. Carba NP II test could not detect class A in any isolate of the 19 harboring the KPC gene.

It was noticed that, an increase in the number of class B genes in a given isolate, improved the sensitivity of Carba NP II test in detecting class B carbapenemases from 55.6% to 80%, while the rate of invalid /negative results dropped from 44.4% to 20% (Table 4).

Table 4: Impact of number of detected genes of class B alone (n=29) on Carba NP II results

Phenotypic [Carba NP II]	Number of genes for class B only				χ^2	^{FE}p
	1 gene (n = 9)		More than one gene (n = 20)			
	No.	%	No.	%		
Class B	5	55.6	16	80.0	1.857	0.209
Invalid/ Negative	4	44.4	4	20.0		

χ^2: **Chi square test** **FE: Fisher Exact**
p: p value for comparing between the studied categories

Results of Modified Hodge test:

The test identified 59 isolates out of the 96 PCR positive (61.5%) as CP with 61.46% sensitivity and 100% PPV. A statistically significant association (P=0.039) was detected between the increase in the quantity of carbapenemase genes and positivity of MHT (Table5).

Table 5: Impact of number of genes on the results of MHT

Number of genes (PCR)	Phenotypic [MHT]				χ^2	p
	Negative (n = 37)		Positive (n = 59)			
	No.	%	No.	%		
1 gene	9	64.3	5	35.7	10.100*	0.039*
2 genes	10	45.5	12	54.5		
3 genes	13	42	18	58		
4 genes	4	18	18	82		
5 genes	1	14.3	6	85.7		

χ^2: Chi square test **p:** p value for comparing between the studied categories ***:** Statistically significant at $p \leq 0.05$

Results of combined disc test:

a) Meropenem/ EDTA CDT:

It identified 61(67.0%) of 91 class B PCR positive isolates, with 60% sensitivity and 96.83% PPV.

Similar to Carba NP II test, the higher the number of class B genes within a given isolate the greater the sensitivity of meropenem/ EDTA CDT in detecting class B carbapenemases from 33.3% to 90%, while the rate of negative results dropped from 66.7% to 10% (Table 6). These results are statistically significant (p= 0.004).

Table 6: Impact of number of detected genes of class B alone (n=29) on Meropenem/ EDTA CDT results

CDT (EDTA)	Number of genes (ClassB)				χ^2	^{FE}p
	1 gene (n = 9)		More than one gene (n = 20)			
	No.	%	No.	%		
Negative	6	66.7	2	10.0	9.977*	0.004*
Positive	3	33.3	18	90.0		

χ^2: **Chi square test** **FE: Fisher Exact**
p: p value for comparing between the studied categories *: Statistically significant at $p \leq 0.05$

b) Meropenem/ PBA-CDT:

It identified 11(57.9%) of 19 class A PCR positive isolates with 57.89% sensitivity and 21.57% PPV

DISCUSSION

K. pneumoniae is one of the major bacterial pathogens responsible for infections in hospital. The ESBLs producing *K. pneumoniae* has been reported worldwide, leaving carbapenems as the only group of β-lactams available for therapy. Recently, carbapenem resistant *K. pneumoniae* has arose as a grave challenge in health-care settings. Carbapenemase producing bacterial isolates pose severe clinical problems as non-susceptibility to β-lactams is frequently accompanied by co-resistance to additional drug classes. Subsequently, alarmingly limited treatment options are available for CP, and may include antimicrobials with substantial side effects as a last choice [20-22].

The accurate phenotypic detection and differentiation of the currently known classes of carbapenemases is of paramount importance for the

execution of infection control measures and the determination of suitable therapeutic plans [6].

The aim of the current study was to identify the prevalent carbapenemases among carbapenem non-susceptible *K. pneumoniae* isolated from AMUH, to evaluate the ability of Carba NP version II test to detect carbapenemase production and identify their Ambler classes compared to PCR as a gold standard. Additionally, we intended to equate the performance, turnaround time, and cost effectiveness of Carba NP II test with MHT and inhibitor-based phenotypic tests.

Ninety six carbapenem non- susceptible *K. pneumoniae* isolates harbouring one or more gene for carbapenemases were studied. With regard to resistance to the three tested carbapenems, it was found that not a single isolate was ertapenem sensitive. On the contrary, five CP (5/96) were meropenem sensitive, and two (2/96) were imipenem sensitive. Accordingly, ertapenem seems the most appropriate carbapenem in screening for the CP, a finding that was also documented by other investigators [11, 23].

According to PCR results, the predominant carbapenemase genes belonged to class B (91/96; 94.8%), either alone (29/91; 32%), or in association with other classes, mainly with class D (43/91; 47.3%). Regarding class D *(bla $_{OXA-48}$)*, it was identified in 62.5% of the tested isolates (60/ 96), where the majority (43 /60; 72%) was accompanied with class B. On the other hand, class A (*bla $_{KPC}$*) was detected only in 19.8% of isolates and no single isolate harboured *bla$_{KPC}$* alone. The most predominant gene was *bla$_{VIM}$* , where it was identified in 83.3% (80/96), followed by *bla$_{NDM}$* which was found in 69.8% (67/96) of the isolates under study. Our results are more or less in agreement with a study from Menoufia, Egypt, where, *bla$_{VIM}$* was the most frequently detected gene, while *bla$_{KPC}$* gene was present in 17.5% of imipenem resistant isolates [24]. In Saudi Arabia, however, *K. pneumoniae* isolates demonstrated a very low prevalence of *bla$_{VIM}$*, and a low prevalence of *bla$_{NDM}$* (1.6% and 20% respectively), while *bla $_{OXA-48}$* was the most prevalent being detected in 78% of the studied isolates [25], and none of the isolates harboured either *bla $_{KPC}$* or *bla $_{IMP}$*. In Turkey, a very low frequency of *bla $_{VIM}$* (5%) was reported, none of the isolates harboured *bla $_{NDM}$*, *bla $_{KPC}$*, or *bla $_{IMP}$*, whereas *bla $_{OXA-48}$* represented the gene with the highest prevalence (43%) [26]. On the other hand, study from China reported *bla $_{KPC}$* as the most prevailing carbapenemase gene among *K. pneumoniae* [21].

Recently, the CLSI guidelines recommended the utility of Carba NP as a supplemental test for suspected CP in *Enterobacteriaceae* and *Pseudomonas* for epidemiologic and infection control purposes[8]. Nordmann et al [10] found that, the Carba NP test perfectly differentiated CP from strains that are carbapenem resistant due to non–carbapenemase-mediated mechanisms. Interpretable positive results were attained in <2 hours, reducing remarkably the time for the first feed- back information from clinical laboratories, and making it possible to implement rapid containment measures to limit the expansion of CPs.

In the current study, the Carba NP test, when compared to PCR as a gold standard, showed an overall sensitivity of 84.4%, and 100% PPV in detecting carbapenemase production. It was also noticed that the test worked with 100% sensitivity for isolates harbouring all the five tested genes, while the sensitivity dropped to 71.4% for isolates harbouring only one of the examined genes. Tijet et al stated 72.5% sensitivity for Carba NP test[27]. Higher sensitivities were documented by other investigators when using different types of protocols[28-30]. The difference in sensitivity may also be ascribed to differences in test format; in house versus commercial kits.

To the best of our knowledge, Carba NP version II test has not been extensively studied. Data regarding its performance in detecting different carbapenemase classes are very few in the literature. In the present study, the Carba NP II test was evaluated as a method for detecting the carbapenemase class using PCR as a gold standard. The test demonstrated the highest sensitivity (72.5%) in detecting class B, with 95.7% PPV, whereas the specificity was 40%, with 7.4% NPV, and an accuracy of 70.8%. The low specificity could be assigned to the fact that isolates that yielded false positive results may carry other class B genes that were not searched for as we targeted the three most prevalent class B genes; *bla$_{VIM,}$ bla* $_{NDM}$ & *bla* $_{IMP}$. We observed a direct relationship between the capability of the test to detect class B and the increasing in number of class B genes within an isolate (sensitivity increased from 55.6% in isolates carrying one gene to 80% in those carrying more than one gene), however, this observation was not statistically significant.

Carba NP II test was unreliable for accurate identification of OXA-48 producing strains (15% sensitivity, 75% PPV) which have emerged globally. Again, there was an association between lack of detecting class D and the co- existence of *bla* genes related to other classes, suggesting that false negative Carba NP II results in identifying a given class are more likely to occur in isolates harboring genes representing other additional classes. Tijet et al [27] who studied the original version of the test (Carba NP) correlated its results, as positive or negative for carbapenemase production, to PCR results. Such method of assessment demonstrated a low sensitivity of the assay in detecting OXA-48- producing isolates which was in parallel to our results while using the second version of the assay (Carba NP II). Tijet et al stated that increasing the bacterial inoculum increases the sensitivity of the assay particularly in isolates harbouring *bla* OXA-48 gene.

In contrast to our results, Dortet et al [12], the inventors of the test, reported that Carba NP II test

differentiates, with a 100%, specificity, the three principal Ambler classes A, B, and D. Thus, in our hands the Carba NP II test gave suboptimal results compared to the originally described results [12]. However, it should be highlighted that the panel of *Enterobacteriaceae* included in their study (n= 97) harbored only a single carbapenemase gene, except for one isolate (*Citrobacter freundii*) that harboured three genes; *bla* $_{NDM-1}$, *bla* $_{VIM-4}$, and *bla* $_{OXA-181}$, which is not the case in our study, where the majority of the isolates (62/ 96, 65%) carried an array of different carbapenemase genes from different classes in different combinations. Even within the 29 isolates harboured class B only, 20 isolates (69%) harboured two or three genes (IMP+ VIM, VIM+ NDM, and IMP+ VIM+ NDM in five, six, and nine isolates respectively).

Unfortunately, we found the Carba NP II test unreliable for accurate identification of KPC producers. Opposite to the situation in the present study, all Maurer's KPC- producing isolates (n= 7) harboured *bla* $_{KPC}$ gene only. From these data, it appears that comparing our results to Dortet & Maurer [11,12] regarding the performance of Carba NP II in detecting class A is not valid as all *bla* $_{KPC}$ positive isolates in the present study harboured genes of other classes along with *bla* $_{KPC}$. Failure of Carba NP II test to obtain class A pattern in the current study could be ascribed to absence of isolates harbouring class A only. The influence of different genetic backgrounds on the accuracy of Carba NP II test needs clarification.

The MHT, suggested for years by CLSI as a confirmatory phenotypic test for detection of CPs in *Enterobacteriacae*, is cheap and, in principle, simple to perform. However, it displays significant investigator dependence, interpretation is subjective, it cannot distinguish between the carbapenemase classes, furthermore, high false positive rates were noticed, mainly caused by Amp C hyperproduction and production of ESBL coupled with porin loss or porin mutations [16,19].

In the current study, MHT identified 61.5% of PCR verified carbapenemase gene carrying isolates. A higher sensitivity (77.5%) was reported at Menoufia University Hospitals, Egypt [24], whereas, a lower sensitivity (48.4%) was exhibited in Makkah, Saudi Arabia, by Khan et al[31].

In the contemporary study, we recognized a statistically significant association between sensitivity of MHT and the number of carbapenemase genes harboured by a given isolate, which may partly explain the variability in the test sensitivity between different studies with different genetic composition of the examined isolates. When correlating its results with PCR, it was found that MHT detected only 10 out of 29 isolates (34.5%) that harboured class B only. False negative results with isolates producing MBLs have also been reported in other studies [29,32]. Additionally, several authors documented low specificity of the test[29,33,34]. The MHT has slow turnaround. It is worth mentioning that the test has been omitted from the CLSI in 2018 as a recommended phenotypic method for detecting CPs [8].

For differentiating carbapenemase classes, CDTs utilizing boronic acid and EDTA as specific inhibitors for carbapenemase were used for years. However, those two phenotypic methods are time consuming and false negative results often arise, in particular when low level of resistance is observed [35].

In the present study, Meropenem/ EDTA CDT showed 67% sensitivity with 96.8% PPV in uncovering MBL producers. An association between false negative results and the co- presence of other classes within a given isolate was detected (73% of false negative isolates). This association was also reported by other researchers who observed failure of the EDTA CDT to recognize the existence of MBL in isolates harboring both *bla* $_{VIM}$ and *bla* $_{KPC}$, despite the high carbapenems MICs of those isolates. Thus, researchers reported that; in isolates conveying both class A and B the synergy tests utilizing a single inhibitor were inadequet [36,37]. Another important point to be considered is that discs containing EDTA are prepared in house manually, this may raise the statistical error. Contrastingly, the escalation in the number of class B genes within a given isolate was coupled with a statistically significant rise in the sensitivity of meropenem/ EDTA CDT in detecting class B carbapenemases from 33.3% to 90%.

Regarding class A phenotypic detection, meropenem/ PBA-CDT showed 57.9% sensitivity & 48.1% specificity. Such low sensitivity may be caused by the co- existence of *bla* genes from other classes in all isolates harboring *bla*$_{KPC}$ in our study.

On comparing Carba NP assay to MHT, it was found that the Carba NP overall sensitivity for revealing CPs was 84.4% which is higher than that of MHT being 61.5%. The PPV for both tests were 100%.

On comparing Carba NP II to CDT, regarding class B detection, our results revealed the higher sensitivity of Carba NP II test in comparison to EDTA-CDT. Out of 91 class B isolates, Carba NP II test detected 66, while CDT detected 61. Thus, sensitivities were 72.5% versus 67.0%, with 95.7% versus 96.8% PPV. However, regarding specificity, CDT demonstrated to be better with 60 % specificity and 9.1% NPV versus 40% specificity and 7.4% NPV for Carba NP II test.

As for class A, it was better detected by PBA-CDT with sensitivity 57.9% and 21.6% PPV than Carba NP II test which did not detect any of class A isolates. However, Carba NP II test was more specific with 100% specificity and 80.2% NPV, while CDT reported 48.1 % specificity and 82.2 % NPV.

The main advantages of Carba NP II over CDT and MHT are; the rapidity of this test compared to others, as it needs only two hours incubation and then the results are interpreted and reported to the clinician on the same day, and its relatively higher sensitivity in detecting

class B carbapenemases. However, MHT and CDT require an over-night incubation and hence delay the time-to-result by a whole day. Regarding the cost, MHT and CDT are considered cheaper than Carba NP II as this test needs reagents (phenol red, tris-HCL) and pure antibiotic (imipenem) powder, thus raising the cost in comparison to the other phenotypic tests.

CONCLUSION

Carba NP II test demonstrated a relatively higher sensitivity in detecting class B carbapenemases, thus for laboratories concerned with the widely disseminated MBLs producers, it can be a cost effective screening method (compared to molecular techniques) to rapidly identify potential MBLs carrier isolates. Negative and invalid results can be tested by PCR.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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