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Original article

Evaluation of direct Carba-NP (CNPt) for screening of carbaenemases production in Gram negative bacilli

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ABSTRACT

Background: Carbapenem resistance among gram negative bacilli (GNB) is a major threat to human health. Rapid and accurate detection of carbapenemase production is very important for clinical management, epidemiological purposes and infection prevention and control issues. Direct Carba NP test (CNPt) is a modified CNPt that allows rapid detection of carbapenemases at a lower cost and improved sensitivity. This work aimed to evaluate direct-CNPt for detection of cabapenemases production as a screening and confirmatory test in GNB versus multiplex PCR as the gold standard test. **Methods:** This study was conducted on 50 clinical isolates of GNB derived from different clinical samples according to their resistance to meropenem (zone of inhibition ≤ 23 mm). Direct CNPt was performed on all 50 clinical isolates using bacterial colonies directly. A change in colour was observed after (≥ 2 h). A multiplex PCR was performed on all isolates to detect bla KPC, bla IMP, bla VIM and bla NDM, and bla OXA-48 genes. **Results:** The overall sensitivity and specificity of direct CNPt as compared to multiplex PCR were 95.2% and 100% respectively. The sensitivity to OXA-48 and IMP genes were relatively lower (91.6 % and 85.7 % respectively) than other genes which had 100% sensitivity for each. **Conclusion:** Direct CNPt is a reliable and rapid method that allows detection of different carbapenemases at a reduced cost. It could be used in combination with other phenotypic or genotypic assays in settings where OXA-48 and IMP are of high prevalence.

Introduction

Carbapenems are one of the most important classes of antibiotics that are considered the last tools in the arsenal used in the combat against multi drug resistant Gram negative bacilli (MDR GNB) infections [1, 2].

However, due to the widespread use of carbapenems in clinical practise, there has been a worrying increase of carbapenem resistance in recent years. This could be attributed to carbapenemase production, a decrease in bacterial outer membrane permeability, overexpression Ambler class C β-lactamases (AmpC)/ extended spectrum β-lactamases (ESBL)and/ or efflux pump genes [3, 4].

Among GNB; the main mechanism of carbapenem resistance is the ability to produce different typesof carbaenemases. Carbapenemases are β-lactamases that can breakdown carbapenems in addition to all other β-lactam antibiotics. There are three different classes of carbapenemases; Ambler class A as KPC, B (metallo- β -lactamases as NDM, VIM and IMP, and D –lactamases as OXA-48. They are encoded by bla_{KPC}, $bla_{IMP,}$ $bla_{VIM,}$ bla_{OXA-48} and bla_{NDM} genes that can be horizontally

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transferred to different bacterial species adding challenges to the spread of this type of resistance [5].

Many phenotypic tests have been developed for the detection of carbapenemases; yet, molecular methods are considered the gold standard tests. There are certain drawbacks to the use of molecular methods which include their high cost, lack of required expertise for their detection by many laboratories. In addition timely availability of those tests and the number of genes detected add to the limitations to their use [6].

The Carba NP test (CNPt) is a phenotypic confirmatory test for carbapenemase production that has been approved by the Clinical and Laboratory Standards Institute (CLSI), 2015 which recommends its use for *Enterobacteriaceae, Pseudomonas aeruginosa*, and *Acinetobacter* species. With increased minimal inhibitory concentrations or decreased inhibition zones by disk diffusion for carbapenem antibiotics [7].

Carba NP test allows detection of carbapenemase production on GNB in a short time (around 2 hours). The test is based on the fact that a bacterial lysate producing carbapenmase enzyme can hydrolyze imipenem causing changes in pH values which could then be detected by a colour change using phenol red indicator. However; one of the drawbacks that limits the use of this test especially for economically developing countries is the high cost of the commercial extraction buffer used to generate bacterial extracts [3].

Pasteran et al. 2015 has modified the CNPt protocol allowing the direct use of isolated colonies instead of the extraction buffer which was originally used to produce general extract, which has the benefit of simplifying procedures, lowering costs, and improving carbapenmase detection [8].

Most phenotypic tests are compared to the results of molecular approaches, which provide an accurate, rapid, and highly sensitive assessment of carbapenemase genes. Several PCRs and real-time PCRs have been developed [9].

The direct CNPt can be used for screening and confirmation of carbapenemases production as it is cost effective and requires simpler protocols which would be more suitable in low income countries which have limited resources and technical expertise [8].

The aim of this study was to evaluate direct-CNPt for detection of cabapenemases production as a screening and confirmatory test in GNB versus multiplex PCR as the gold standard test.

Materials and Methods

This was a cross sectional study that was conducted in the period from January to June 2018 at Ain-shams University Hospitals. A total of 50 non-repetitive clinical isolates of GNB were included in the study. All isolates included in the study were selected according to their resistance to meropenem (zone of inhibition≤ 23 mm) using disk diffusion method as defined by CLSI, 2017 [10].

Direct CNPt:

The direct CNPt was performed following the protocol described by **Pasteran et al., 2015** [8]. Bacteria were grown overnight on Mueller-Hinton agar. An Eppendorf tube (0.1 ml) was used to prepare an indicator solution containing 0.05% phenol red with 0.1 mmol/liter $ZnSO_4$. An 0.1% (vol/vol) of Triton X-100 (Sigma, Aldrich) was added to allow the use of isolated bacterial colonies directly; and so there was no need for using the extraction buffer to generate bacterial lysate; and then the indicator solution was adjusted to pH 7.8.One µl loop was used to scrape off the bacterial colony and suspend it in the indicator solution which was supplemented with 12 mg/ml imipenem-cilastatin (injectable form) that was added to the reaction tube. A bacterial colony was also added to another indicator tube without antibiotic which was used as a control tube using the same method. Before incubation; a vortex device was used to strongly mix the tubes for 5 to 10 second. Tubes were finally incubated at 35°C and monitored throughout 2 hours for colour change. A change in colour from red to orange/yellow in the antibiotic-containing tube was interpreted as a positive result.

Real-time multiplex PCR for carbapenemase genes detection:

The presence of bla KPC, bla NDM, bla IMP, bla VIM, and bla OXA-48 carbapenemase genes in all bacterial isolates was detected using multiplex PCR following the method described by **Monterio et al.,** 2012 [11]:

I) DNA extraction:

DNA was extracted from the bacterial isolates using the spin column method (QIAGEN; GmbH, Hilden, Germany) as per manufacturer's instructions. The ratio of 260 nm to 280 nm absorbance was used to determine the purity of DNA; a ratio of approximately 1.8 was considered "pure" using a nanodrop Spectrophotometer. The extracted DNA

was recovered in 60 µL of the elution buffer and preserved by freezing at -20˚C.

II) DNA amplification:

Five sets of primers (Bioline, UK) were used to amplify DNA according to the manufacturer's recommendations **(Table 1)** [11-13].

A 50 µl reaction mixwas containing 10 mM Tris-HCL (pH 8.3), 50 mM KCL, 1.5 mM MgCl2, 0.1% Triton X100, 200 mM of each of the deoxynucleoside triphosphate, 1U of Thermus aquaticus DNA polymerase, 5 µl of template DNA, and 0.4 mM of each primer was used for the multiplex PCR technique.

PCR conditions:

Initial denaturation at 94°C for 5 min.; 30 cycles of 94°C for 25 s, 55°C for 45 s and 72°C for 30 s.Final elongation step at 72°C for 5 min; and a melt curve step (from 65 ˚C gradually increasing by 0.18C/s to 95 ˚C, with fluorescence data acquisition every1s).

Statistical analysis

The performances of the phenotypic test (direct-CNPt) for detection of different carbapenemases were compared to gene-specific PCR test which was considered as the reference gold standard test. Sensitivity was calculated as the number of true-positive direct-CNPt whereas specificity was calculated from the true negative direct-CNPt. Positive predictive values (PPV) and negative predictive values (NPV) were calculated.

Table 1. PCR primers used in carbapenemase genes identification.

Primer	Primer sequence 5'-3'	Amplicon size (bp)	Reference
bla KPC type	KPC-F TCGCTAAACTCGAACAGG KPC-R TTACTGCCCGTTGACGCCCAATCC	785	[12]
bla NDM-1type	NDM-F TTGGCCTTGCTGTCCTTG NDM-R ACACCAGTGACAATATCACCG	82	[11]
bla IMP type	IMP-F GAGTGGCTTAATTCTCRATC IMP-R AACTAYCCAATAYRTAAC	120	[13]
bla VIM type	VIM-F GTTTGGTCGCATATCGCAAC VIM-R AATGCGCAGCACCAGGATAG	382	[13]
bla OXA-48	OXA-48 F TGTTTTTGGTGGCATCGAT OXA-48 R GTAAMRATGCTTGGTTCGC	177	[11]

Results

During the study period, 50 meropenem-resistant GNB were chosen (*Klebsiella spp* (21/50) 42 %; *E.coli* 12/50 (24 %); *Acinetobacter spp* 6/50 (12 %); and *Proteus spp* 2/50 (4 %).The details of PCR tests of all carbapenem-resistant isolates are displayed in **table (2)**. Based on PCR assay results; 42 (84%) of all tested 50 isolates were positive for one or more carbapenemase Genes. The most prevalent carbapenemase genes were KPC and NDM (19/50 isolates; 38% for each of them). Multiple carbapenemase genes were found in 19 (38%) isolates. Carbapenem-resistant isolates without carbapenemase gene represented 8/50 (16%) isolates.

In the case of direct CNPt, 40 out of 50 isolates were positive, accounting for 80% of the total. **Table 3** shows the details of the direct CNPt results for each species. Direct CNPt was negative for all 8/50 (16%) isolates that tested negative for carbapenemase genes by PCR. Two carbapenemase-positive isolates from *Klebsiella* species that were positive for the OXA-48 gene and one Pseudomonas species that were positive for the IMP gene provided false negative results when tested with direct CNPt.

Multiplex PCR was used as gold standard to determine sensitivity and specificity of the directCNPt.The overall sensitivity and specificity of the direct CNPt was 95.2% and 100% respectively. Positive predictive value and NPV were 100% and 80% respectively. The performance parameters of direct CNPt are described for each carbapenemase gene in **table (4)**.

Table 2. Pattern of carbapenem resistance among Gram-negative bacilli.

No and species of isolates	Carbapenem resistance genes							Direct CNPt
	KPC	**OXA-48**	**IMP**	**VIM**	**NDM**	**Carbapenem resistance without genes**	**Multiple carbapenemase genes**	
***Klebsiella spp* (21/50) 42%**	9 (42.8%)	6 (28.5%)	0 (0%)	2 (9.5%)	7 (33.3%)	2 (9.5%)	5 (23.8%)	18/21 (85.7%)
***E.coli* 12/50 (24%)**	7 (29.1%)	5 (20.8%)	0 (0%)	1 (4.1%)	5 (20.8%)	1 (4.1%)	6 (50%)	11/12 (91.6%)
***Pseudomonas aeruginosa* 9/50 (18%)**	1 (11.1%)	1 (11.1%)	5 (55.5%)	2 (22.2%)	3 (33.3%)	2 (22.2%)	5 (55.5%)	6/9 (66.6%)
***Acinetobacter*s spp 6/50 (12%)**	1 (16.6%)	0 (0%)	0 (0%)	0 (0%)	3 (50%)	3 (50%)	1 (16.6%)	3/6 (50%)
***Proteus* spp 2/50 (4%)**	1 (50%)	0 (0%)	2 (100%)	1 (50%)	1 (50%)	0 (0%)	2 (100%)	2/2 (100%)
Total 50 (100%)	19 (38%)	12 (24%)	7 (14%)	6 (12%)	19 (38%)	8 (16%)	19 (38%)	40 (80%)

Table 3. Results of direct CNPt for detection of carbapenemases in tested isolates.

Organism	Charactersitics	Direct CNPt	
		positive	Negative
Klebsiella spp	KPC (9)	9	0
	Oxa-48 (6)	5	1
	IMP (0)	21	0
	VIM (2)	2	0
	NDM (7)	7	0
	Carbapenemase negative (2)	0	2
	Multiple genes (5)	5	0
E.coli	KPC (7)	7	0
	Oxa-48 (5)	5	0
	IMP (0)	12	0
	VIM (1)	1	0
	NDM (5)	5	0
	Carbapenemase negative (1)	0	1
	Multiple genes (6)	6	0
Pseudomonas aeruginosa	KPC (1)	1	0
	Oxa-48 (1)	1	0
	IMP (5)	4	1
	VIM (2)	2	0
	NDM (3)	3	0
	Carbapenemase negative (2)	0	2
	Multiple genes (5)	5	0
Acinetobacter	KPC (1)	1	0
	Oxa-48 (0)	6	0
	IMP (0)	6	0
	VIM (0)	6	0
	NDM (3)	3	0
	Carbapenemase negative (3)	0	3
	Multiple genes (1)	1	0
Proteus	KPC (1)	1	0
	Oxa-48 (0)	2	0
	IMP (2)	2	0
	VIM (1)	1	0
	NDM (1)	1	0
	Carbapenemase negative (0)	0	0
	Multiple genes (2)	2	0

Table 4. Performance parameters of direct CNPt as a screening test.

Carbapenemase	TP*	FP*	TN*	FN*	Sensitivity (%)	Specificity (%)	PPV* (%)	NPV*(%)
KPC (19)	19	0	10	0	100	100	100	100
Oxa-48(12)	11	0	9	1	91.6	100	100	90
IMP	6	0	9	1	85.7	100	100	90
VIM	6	0	10	0	100	100	100	100
NDM	19	0	10	0	100	100	100	100
Total	40	0	8	2	95.2	100	100	80

*TP: true positive, FP: false positive, TN: true negative, FN: false negative, PPV: positive predictive value, NPV: negative predictive value

Discussion

The ability to diagnose carbapenemase production by Gram negative bacteria quickly and accurately is critical for implementing prompt and suitable therapy as well as proper infection control protocols. In clinical laboratories, phenotypic assays such as the modified Hodge test (MHT) and CNPt are commonly employed for first-line detection of carbapenemase-producing isolates. Given the limitations of these methods, numerous new phenotypic techniques for detecting carbapenemases as the direct CNPt were developed to reduce both costs and incubation time. [8, 14, 15].

Multiplex PCR was employed to detect carbapenemase genes in meropenem-resistant GNB in this study. A total of 42 (84%) of the 50 isolates tested positive for one or more carbapenemase genes. The common detected genes were KPC and NDM, which were found in 19 (38 %) of the isolates. Several studies investigated the prevalence of carbapenemase genes using a variety of genotypic techniques. Similar findings were reported by **Rudresh et al.** who detected carbapenemase genes in 103 carbapenem resistant organisms (71.53 %) using PCR. The distribution of carbapenemase genes was as follows: NDM 52%, OXA-48 28%, multiple genes 20%,and VIM 3%, no KPC or IPM genes were detected [16]. In a study conducted by **Sahin et al.**; OXA-48 was determined to be the most dominant carbapenemase, followed by NDM, while KPC, IMP, and VIM were not detected in any isolates [17]. According to **Mushi et al.** the most common carbapenemase gene was IMP, which was found in 49 (21.6%) of isolates, followed by VIM in 28 (12.3%), OXA-48 in 11 (4.9%), KPC in 8 (3.5%), and NDM in 7 (3.1 %). Of 80 bacterial isolates with carbapenemase genes, 15 (6.6%) had multiple carbapenemase gene [18]. In Egypt, **El Naggar** found that 28 (75.7%) of the 37 bacterial isolates which were resistant to carbapenems and were proven to produce carbapenemases by phenotypic methods were also positive for carbapenemase genes using multiplex PCR; KPC 6 (21.4 %), NDM 9 (31.2 %), OXA 7 (25 %), IMP 5 (17.9%), and VIM 1 (3.6 %) [19].

The distribution of the carbapenemase genes vary according to different geographical locations, use of antimicrobial agents, infection control preventive measures and pattern of prevalent microorganisms. This mechanism of resistance is more likely to propagate since carbapenemase-encoding genes are usually located on plasmids. To curb the spread of carbapenemase producers, fast and easy to perform phenotypic identification of carbapenemases has become of ultimate importance [20].

In our study, all 8/50; 16% isolates that were negative for carbapenemase genes by PCR were also negative by CNPt. The presence of carbapenem-resistant isolates that did not harbor any of the carbapenemase genes could be attributed to the presence of other mechanisms of resistance, such as overexpression of AmpC and/or ESBLs, coupled with loss of porin or increased efflux pump activity [21].

All carbapenemase producing GNB (40 isolates) detected by PCR were also positive for CNPtexcept for 2 isolates. The two carbapenemase positive isolates by PCR that gave false negative results by CNPt were one OXA-48 positive *Klebsiella* species and one IMP positive *Pseudomonas* species. The PCR test was used as gold standard to detect sensitivity and specificity of direct CNPt. The overall sensitivity and specificity of the direct CNPt was 95.2% and 100% respectively; PPV and NPV were 100% and 80% respectively.

Kumudunie et al. reported similar results as allisolates that gave negative direct CNPt results were either carbapenem sensitive or carbapenem resistant but negative for carbapenmase production by PCR resulting in 100% specificity and PPVs. The sensitivity and NPVs were 83.3% and 98.2%, respectively. The sensitivity of the test was 100% for all carbapenemase genes except for OXA-48 (79.5%).Therefore, they concluded that the OXA-48 producers were the main cause of false negative results of direct CNPt leading to reducing the overall sensitivity of the test. No IMP genes were found in their study [22]. These results are in accordance with our results where reduced sensitivity was only found with OXA-48 and IMP (91.6% and 85.7% respectively). **Pasteran et al.** found the sensitivity and specificity of direct CNPt to be 98% and 100% respectively which align with our results. Only one IMP producing *P. mirabilis* isolate and two OXA producing isolates were found to be false negative (one *Klebsiella pneumoniae* and one *E. coli* isolates) [8].

An immunochromatographic phenotypic assay conducted by **Hopkins et al.** found that only 12/17 (70.6%) of IMPs were detected by this assay which comes in accordance with our results (6/7; 85.7%) [23].The lower sensitivity of direct CNPtto

detect OXA-48 could be explained by the fact that these enzymes have relatively weaker carbapenemase activity when compared to other carbapenemase enzymes [4].The relatively lower sensitivity of direct CNPt to detect IMP could be attributed to the presence of different IMP variants that could give false negative results. *Pseudomonas aeruginosa* and less frequently *Enterobacteriaceae* are the most common sources of IMP (Imipenemase), an Ambler class B metallo-betalactamase group. It has at least 52 different variations [24, 25].

Conclusion

Direct CNPt could be reliable and rapid method to detect different carbapenmeases in GNB with 100% specificity for all carbapenmases, 100% sensitivity for KPC, NDM and VIM and lower sensitivity for OXA-48 and IMP. It is recommended especially in low income countries particularly in settings where OXA-48 and IMP are of low prevalence. It could be used in combination with other phenotypic assays or genotypic methods which remain the gold standard test. Wider scale studies with large number of isolates at different geographical locations and settings are recommended.

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Limitations: Small number of isolates tested due to high cost of multiplex PCR test.

Authorship

Abd-El-Latif (MD) and M. Erfan (MD) initiated the study idea and contributed to analysis interpretation of data, drafting the article and revising it critically for important intellectual content, and final approval of the version to be submitted.

Each author listed in the manuscript had approved the submission of this version of the manuscript and takes full responsibility for it.

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