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EVALUATION OF CNPt-DIRECT ASSAY AND DEVELOPMENT OF A MODIFIED CNPt-EDTA ASSAY TO DIFFERENTIATE NDM FROM OXA-48 LIKE CARBAPENEMASES

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ABSTRACT

Infections caused by carbapenemase producing bacteria are a worldwide threat, due to their association with high mortality. Early diagnosis is the key to treatment and can help in preventing the spread of infection. In this study, we have evaluated the phenotypic assay, carbaNP test (CNPt)-direct, that is used to identify carbapenemase producers. We have introducedCNPt-EDTA direct, a modification of the (CNPt)-direct test. The CNPt-EDTA direct can be used to distinguish strong metallo carbapenemases producers such as the NDM enzyme from weak hydrolyzers such as the OXA-48 like enzymes.Thirty-one bacterial isolates, including twenty eight clinical isolates, two positive control strains and one negative control strain, were subjected to the CNPt- direct, and the CNPt-EDTA-direct assays. Based on the color produced(from red to yellow or orange), twenty seven clinical isolates tested carbapenemase positive by CNPt-direct, of which eighteen were strong carbapenemase hydrolyzers and nine weak hydrolyzers. Each isolate was then tested by CNPt-EDTA direct and confirmed by gene specific PCR. When analysed by the CNPt-EDTA-direct, the strong carbapenem hydrolysers showed that fourteen isolates harboured the NDM enzyme, while four harboured a strong hydrolyser in association with or without class B enzyme. Out of the nine weak hydrolysers, eight were EDTA insensitive and harboured the OXA-48 like enzyme and one harboured the VIM enzyme. Thus the use of CNPt-direct in conjunction with CNPt-EDTA direct is a cost-effective and sensitive method to identify and classify carbapenemases, especially the NDM and OXA-48 like enzymes.

KEYWORDS :Carbapenemase, Enterobacteraceae, CNPt-Direct, *bla*$_{NDM}$, *bla*$_{OXA-48}$ *like*, NDM.

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INTRODUCTION

Carbapenemases are versatile beta lactamases that degrade most of beta lactam antibiotics, including carbapenems.[1] Early diagnosis of carbapenemase production is vital since it is associated with high mortality rate.[2] Carbapenemases are classified into three major classes, class A (*Klebsiella pneumoniae* carbapenemases-KPC) enzymes inhibited by boronic acid, Class B (New Delhi Metallo-beta-lactamases - NDM) metallo-enzymes inhibited by Ethylenediaminetetraacetic acid (EDTA) and class D (Oxacillinases-OXA 48 like) enzymes, not inhibited by both. [3] Asian and European countries predominantly contain NDM,veronica Integron Metallo-beta-lactamases (VIM) and OXA-48 like enzyme producing clinical isolate.[4-7] Detection of carbapenemase production is done by a phenotypic assay followed by molecular assay -Polymerase chain reaction(PCR) for confirmation. As PCR is a golden standard in detecting carbapenemase producing strains,[8] phenotypic assay are compared to the PCR results for their accuracy. Though phenotypic assays like modified Hodge test, Antimicrobials and Inhibitors Microbiological(AIM) assay and Carba NP test have been developed, their sensitivity and specificity, have been debated in recent past.[9,10] Clinical and Laboratory Standards Institute (CLSI) has recommended carbaNP test that was developed by Nordmann et al., in 2012[11], which works on the principle of enzyme (carbapenemase) substrate (mostly imipenem) hydrolysis followed by change in pH of the medium indicated by appropriate indicator. Though this assay is rapid, sensitive and has become a default assay to detect carbapenemase producers in most of the laboratories, it is cost-ineffective and researchers have reported problems in identifying OXA producers. Since then, several modifications have been done to this assay to make it simpler, sensitive and cheaper.[12,10] One such modified assay is the carbaNP test (CNPt)- direct, in which the expensive extraction buffer was replaced by triton X. This method has proved be more sensitive since it can identify OXA producers and its simplified protocol, makes it easier to perform.[13] This study is focused on evaluating the CNPt-direct against gram negative clinical isolates. We also introduce the CNPt-EDTA direct test, which is a modification of the CNPt-direct that includes an additional step, to differentiate metallo carbapenemases from other classes.

MATERIALS AND METHODS

Bacterial isolates and Antimicrobial Sensitivity testing (AST)[14]:

Thirty-one non redundant bacterial isolates, including 28 clinical isolates and two positive control strains, whose genetic content was pre-determined (*bla*$_{NDM}$ n=1, *bla*$_{KPC}$ n=1) and one negative control strain -without any antibiotics resistance, were used in this study. Bacterial isolates were subjected to the disc diffusion test to identify their sensitivity to imipenem (Hi-media, India). Test was performed as per the CLSI guide on a Muller-Hinton agar (Hi-media, India), but the zone diameter screening breakpoint for imipenem disk (10µg) was set as ≤21mm.

CNPt- direct[13]

CNPt–direct was performed as described earlier. Briefly one loop full (1 µl) of each test isolates were suspended in 100 µl of CNPt-direct mix (0.05% phenol red, 0.1 mmol/liter ZnSO4, 0.1% (vol/vol) of Triton X-100, adjusted to pH 7.8 (contains 12 mg/ml imipenem-cilastatin injection)) in a microfuge tube and cultures were suspended evenly by vortex mixer and incubated at 37° C for maximum two hours. Change in colour from red to yellow or orange was considered positive for carbapenemase producers.

CNPt-EDTA direct test

CNPt-EDTA direct test was performed similar to CNPt-direct test, with additional ingredient 0.5M EDTA. Briefly to 100 µl of CNPt-direct mix, 2 µl of 0.5M EDTA (0.01M) and one loop full of bacterial culture was suspended evenly by vortex mixer and incubated at 37 ° c for maximum of two and a half hours. This test is to be performed after CNPt- direct test and results are to be compared. Isolates that turned yellow in the absence of EDTA and the same isolate that did not turn yellow or orange in the presence of EDTA, were supposed to harbour metallo carbapenemase gene. Change in colour from red to yellow or orange was considered positive and insensitive.

PCR Detection of carbapenemases in clinical isolates [15-16]

To determine the genetic content of bacterial isolates, total DNA was extracted as described and presence of carbapenemase gene was determined by multiplex Polymerase Chain Reaction (PCR) assay for the following four genes: *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{OXA-48}$ like, *bla*$_{KPC}$. For Gene specific primers, PCR mixture and conditions from previous references were followed. Taq DNA polymerase from M/S Bangalore Genie, Merck Millipore, Bangalore, India, dNTP stock from M/S Cinnagen, Tehran, Iran, were used.

RESULTS

Identification of Carbapenemase producers

Thirty-one bacterial isolates were tested for imipenem susceptibility. Imipenem resistance cut off was fixed as ≤21mm, where all the strains except for the negative control were resistant to imipenem. Out of the 28 clinical isolates tested by CNPt-direct assay, 27 were identified as carbapenemase producers. Out of these, 18 isolates produced yellow colour, 9 isolates orange colour and 1 isolate was a non carbapenemase producers since it remained red. The presence of yellow colour indicates a strong carbapenem hydrolyser while orange indicates a weak carbapenem hydrolysing enzyme [Table 1]. We hypothesized that NDM is present in all the strong carbapenem hydrolysing isolates while OXA-48 like enzyme is present in the weak hydrolysing isolates[17] [Table1].

Table 1
Results of AST, CNPt direct, CNPt -EDTA direct and gene specific PCR in Strong Carbapenemase producers.

Bacterial code	Bacterial species	Zone diameter in mm Imipenem 10 µg disk	CNPt- direct	CNPt-EDTA- direct	Carbapenemase Gene present-PCR
Strong Carbapenemase producer – EDTA sensitive (n=14)					
B19032-8	*Klebsiella SP*	11	YELLOW-P	Sensitive	bla_{NDM}
B36771-17	*Klebsiella SP*	11	YELLOW-P	Sensitive	bla_{NDM}
B18783-18	*Klebsiella SP*	12	YELLOW-P	Sensitive	bla_{NDM}
B14650-19	*Klebsiella SP*	13	YELLOW-P	Sensitive	bla_{NDM}
B37305-20	*Escherichia coli*	12	YELLOW-P	Sensitive	bla_{NDM}
B20143-22	*Klebsiella SP*	<10	YELLOW-P	Sensitive	bla_{NDM}
B15042-25	*Klebsiella SP*	14	YELLOW-P	Sensitive	bla_{NDM}
B15773-28	*Klebsiella SP*	12	YELLOW-P	Sensitive	bla_{NDM}
B15055-34	*Escherichia coli*	13	YELLOW-P	Sensitive	bla_{NDM}
B1385-40	*Escherichia coli*	10	YELLOW-P	Sensitive	bla_{NDM}
B5488-5	*Klebsiella SP*	NZ	YELLOW-P	Sensitive	bla_{NDM}, bla_{VIM}
B22081-32	*Escherichia coli*	12	YELLOW-P	Sensitive	bla_{NDM}, bla_{VIM}
B18956-37	*Escherichia coli*	10	YELLOW-P	Sensitive	bla_{NDM}, bla_{VIM}
B31273-39	*Escherichia coli*	13	YELLOW-P	Sensitive	bla_{NDM}, bla_{VIM}
Possess a strong carbapenemase producer and likely to have additional non-metallo-carbapenemase enzyme (n=4)					
B21443-29	*Klebsiella SP*	NZ	YELLOW-P	Insensitive	bla_{NDM}, bla_{OXA-48} like
B18461-30	*Klebsiella SP*	10	YELLOW-P	Insensitive	bla_{NDM}, bla_{OXA-48} like
B25203-9	*Escherichia coli*	11	YELLOW-P	Insensitive	bla_{NDM}
B35735-38	*Klebsiella SP*	<10	YELLOW-P	Insensitive	bla_{OXA-48} like
P = POSITIVE					

Distinguishing metallo-carbapenemases from other classes

To identify the metallo-carbapenemase producers out of these, the 28 carbapenemase producers were tested by CNPt—EDTA direct test. The metallo-carbapenemases are inhibited by EDTA and these cultures will not result in a change of color from red to yellow. When the 28 carbapenemase producers were subjected to CNPt-EDTA, out of the 18 isolates that produced yellow colour, 14 isolates were EDTA sensitive, suggesting the presence of a metallo-carbapenemase. In the remaining four strong carbapenem hydrolyzers, the carbapenemase was insensitive to EDTA. It is likely that these isolates might harbour a class A or Class D carbapenemase in addition to a metallo-carbapenemase. Out of the nine isolates that produced orange colour in the CNPT – direct assay, in 8 isolates the carbapenemase was insensitive to EDTA. They produced the orange color indicating weak carbapenem hydrolysis. These eight isolates do not have a metallo carbapenemase and are likely to harbour the OXA-48 like enzyme [Table2].

Table 2
Results of AST, CNPt direct, CNPt -EDTA direct and gene specific PCR in weak Carbapenemase producers and controls.

Bacterial code	Bacterial species	Zone diameter in mm Imipenem 10 µg disk	CNPt- direct	CNPt-EDTA- direct	Carbapenemase Gene present-PCR
Weak Carbapenem Hydrolysing enzyme, insensitive to EDTA - Class D carbapenemase producers (n=8)					
B31804-16	*Escherichia coli*	18	ORANGE-P	Insensitive	bla_{OXA-48} like
B16470-21	*Klebsiella SP*	12	ORANGE-P	Insensitive	bla_{OXA-48} like
B15800-23	*Klebsiella SP*	13	ORANGE-P	Insensitive	bla_{OXA-48} like
B16850-24	*Klebsiella SP*	11	ORANGE-P	Insensitive	bla_{OXA-48} like
B36789-35	*Escherichia coli*	18	ORANGE-P	Insensitive	bla_{OXA-48} like
B23994-2	*Escherichia coli*	13	ORANGE-P	Insensitive	bla_{OXA-48} like
B20839-27	*Klebsiella SP*	14	ORANGE-P	Insensitive	bla_{OXA-48} like
B4097-36	*Escherichia coli*	17	ORANGE-P	Insensitive	bla_{OXA-48} like
Weak Carbapenem hydrolysing enzyme, sensitive to EDTA – bla VIM producer (n=1)					
B44535-33	*Escherichia coli*	17	ORANGE-P	Sensitive	bla_{VIM}
POSITIVE CONTROL (n=2)					
KF220657*	*Escherichia coli*	NZ	YELLOW-P	Sensitive	bla_{NDM}
ATCC BAA-1705	*Klebsiella SP*	NZ	YELLOW-P	Insensitive	bla_{KPC}
NEGATIVE CONTROL (n=2)					
MTCC7407	*Klebsiella SP*	30	RED-N	Not done	----------
B26655-31	*Escherichia coli*	20	RED-N	Not done	bla_{OXA-48} like
P: POSITIVE	**N : NEGATIVE**	*** : ACCESION NUMBER**	**NZ: NO ZONE**		

PCR identification of Carbapenemase enzymes and Correlation with phenotypic assay

The accuracy of the phenotypic assay was cross checked with PCR analysis. The 14 isolates that produced yellow colour (strong carbapenem hydrolysers) and were sensitive to EDTA possessed the bla_{NDM} gene with or without bla_{VIM} gene. Of the four isolates that were strong carbapenem hydrolysers but were insensitive to EDTA two had a bla_{NDM} gene and an additional bla_{OXA-48} like gene. In B25203-9, a bla_{NDM} gene was observed. It is likely that there is an additional non-metallo-carbapenemase gene in this isolate

accounting for the EDTA insensitive phenotype. In B35735-38, a *bla*$_{OXA-48}$ like gene was observed, but a *bla*NDM gene was not observed. In our PCR assay, we tested only for NDM and VIM. It is possible there is an unknown metallo-carbapenemase in this isolate. Out of the nine isolates that produced orange colour, eight were insensitive to EDTA and these harboured the *bla*$_{OXA-48}$ like gene in the absence of *bla*$_{NDM}$ gene. One isolate, that produced orange color but was sensitive to EDTA, had the *bla*$_{VIM}$ gene. The *bla*VIM gene on its own is not as strong carbapenemase as *bla*$_{NDM}$ [Figure 1]. [17] Finally, B26655-31, according to the phenotypic test did not show a carbapenemase but the PCR analysis showed the presence of *bla*$_{OXA-48}$ like gene. It is likely that the *bla*$_{OXA-48}$ like gene in this isolate is not expressed. The borderline carbapenem resistance could be due to other factors such as porin loss.[18]

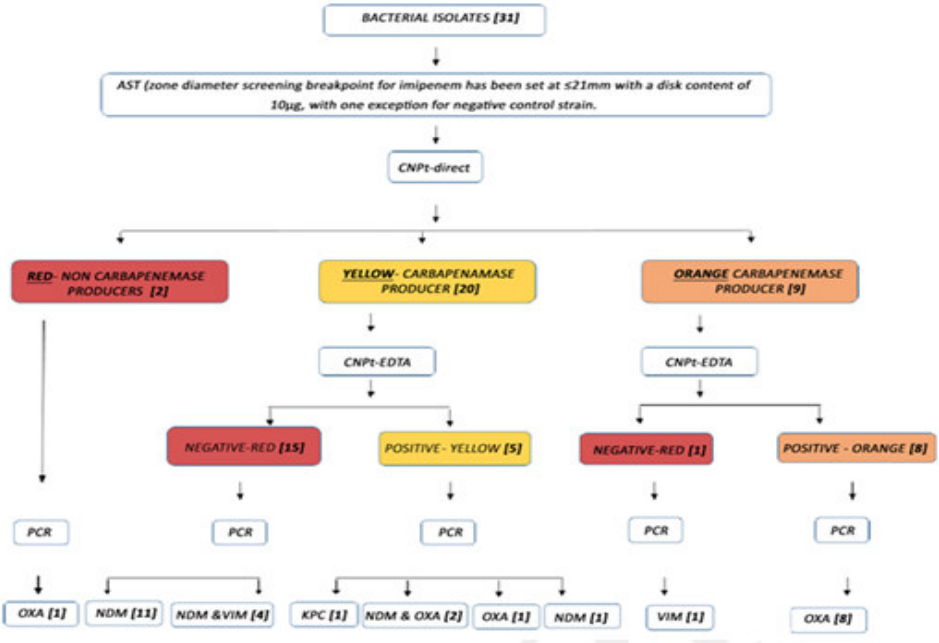

Figure 1
Diagnosis scheme to detect and differentiate carabapenemase classes using CNPt-direct and CNPt-EDTA direct phenotypic assay

DISCUSSIONS

Imipenem resistance cut off was fixed as ≤21mm based on our previous work.[6] Researchers around the globe have disagreed with The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and CLSI cut-off range [14,19, 3] because values based on global sampling may not fit for samples tested in particular locations. The originally developed carbaNP test has high sensitivity and specificity in detecting carbapenemase producing isolates, when compared with modified Hodge test and other phenotypic assays. It also has the advantage of not being influenced by the presence of AmpCs and or ESBLs genes.[11] Despite this, the main drawbacks are the expensive commercial buffer and the inability to identify OXA-48 like carbapenemase.[13] Therefore, in this study we confirmed that the CNPt-direct -modified assay, with a simplified protocol and cost effective ingredients, has a higher sensitivity than the carbaNP test and modified Hodge test. It has no problems in identifying metallo enzymes or OXA-48 like producers. The strong carbapenemase producers like NDM could be differentiated from OXA-48 like producers. Only in one isolate (B26655-31) which tested negative with the phenotypic assay despite carrying the *bla*$_{OXA}$ gene. This can be accounted for by studies that report that the lack of appropriate upstream promoters to *bla*$_{OXA-48}$ like gene might lead to its silence.[20] Their resistance to imipenem could be also be related to the loss of porins.[18] Therefore, from this we suggest that the CNPt direct and CNPt EDTA direct are a simple method to identify and distinguish strong and weak carbapenemase producers. EDTA has proven to be an effective inhibitor of metallo enzymes in other phenotypic assays.[14] This study is the first attempt made to incorporate EDTA with CNPt-direct assay to differentiate metallo carbapenemases from the rest of the other classes. EDTA concentration was selected after performing preliminary assays from 20 µl to 2 µl, where addition of 20 µl and 10 µl had difficulty in differentiating class B from other classes, while 5 µl could differentiate class A (n=1), but not all class D from class B. The addition of 2 µl along with an extended incubation time lead to the differentiation of class B producers from all the other classes. This study proves the efficiency of CNPt-EDTA direct, in distinguishing production of metallo from non metallo carbapenemases. While most of the metallo carbapenemases were identified accurately, one strain (B25203-9) harbouring *bla*$_{NDM}$ alone, was not influenced by the presence of EDTA. This may be due to the production of additional non metallo carbapenemases, which were not focused on in this study. Prevalence of mostly NDM, VIM and OXA producers has been reported in Europe and Asia.[21] As studies have shown that the prevalence of *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{OXA}$ gene in the Asian subcontinent,[16, 6] in this study we used primers to identify *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{OXA-48}$, and *bla*$_{KPC}$ genes alone.

CONCLUSION

Over all, from the experiment conducted, we recommend that the CNPt-direct test could be used to detect carbapenemase producers from clinical origin. It is sensitive enough to identify OXA-48 like enzymes. The difference in color from orange and yellow can be used to differentiate strong carbapenem hydrolyzers such as NDM and weak carbapenem hydrolyzers such as OXA-48 like and VIM enzymes. The CNPt-EDTA-direct test was sensitive enough to differentiate isolates which contained only class B (metallo carbapenemases) from rest of the classes and can be included in routine diagnosis. This method is simple and cost effective for detection and classification of carbapenemase producers in clinical isolates

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CONFLICT OF INTEREST

Conflict of interest declared none.

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