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Original Article

Microbiology Section

Comparative Evaluation of Multiplex PCR and Routine Laboratory Phenotypic Methods for Detection of Carbapenemases among Gram Negative Bacilli

RACHANA SOLANKI[1], LAVANYA VANJARI[3], SREEVIDYA SUBRAMANIAN[3], APARNA B.[4], NAGAPRIYANKA E.[5], VEMU. LAKSHMI[6]

ABSTRACT

Background: Carbapenem resistant pathogens cause infections associated with significant morbidity and mortality.

Objective: This study evaluates the use of Multiplex PCR for rapid detection of carbapenemase genes among carbapenem resistant Gram negative bacteria in comparison with the existing phenotypic methods like modified Hodge test (MHT), combined disc test (CDT) and automated methods.

Material and Methods: A total of 100 Carbapenem resistant clinical isolates, [*Escherichia coli* (25), *Klebsiella pneumoniae* (35) *P. aeruginosa* (18) and *Acinetobacter baumannii* (22)] were screened for the presence of carbapenemases (bla_{NDM-1}, bla_{VIM}, bla_{IMP} and bla_{KPC} genes) by phenotype methods such as the modified Hodge test (MHT) and combined disc test (CDT) and the molecular methods such as Multiplex PCR.

Results: Seventy of the 100 isolates were MHT positive while, 65 isolates were positive by CDT. All the CDT positive isolates with EDTA and APB were Metallo betalactamase (MBL) and *K. pneumoniae* carbapenemase (KPC) producers respectively. bla_{NDM-1} was present as a lone gene in 44 isolates. In 14 isolates bla_{NDM-1} gene was present with bla_{KPC} gene, and in one isolate bla_{NDM-1} gene was present with bla_{VIM}, gene. Only one *E. coli* isolate had a lone bla_{KPC} gene. We didn't find bla_{IMP} gene in any of the isolates. Neither of the genes could be detected in 35 isolates.

Conclusion: Accurate detection of the genes related with carbapenemase production by Molecular methods like Multiplex PCR overcome the limitations of the phenotypic methods and Automated systems.

Keywords: bla_{NDM-1}, bla_{VIM}, bla_{IMP} bla_{KPC} *genes,* Combined disc test, Modified hodge test, Multiplex PCR

INTRODUCTION

Carbapenems are commonly used to treat infections caused by multidrug-resistant *Enterobacteriaceae*. Production of carbapenemases is the most common mechanism of resistance to this class of antibacterial agents in clinically important Gram-negative bacteria. However, detection of the carbapenemase among Gram-negative bacteria is challenging since carbapenemase-producing *Klebsiella pneumoniae* with low carbapenem MICs in the susceptible range according to CLSI or EUCAST have been described [1].

Increased carbapenem MICs in *Enterobacteriaceae* can be a result of two different mechanisms of resistance: (i) hyperproduction of class C-β -lactamases or extended-spectrum -β -lactamases (ESBLs) in combination with porin loss; and/or (ii) carbapenemase production by serine carbapenemase and/or metallo--β b-lactamases [2]. Thus, the new CLSI breakpoints can be useful as an excellent screening test, but they do not identify the mechanism of resistance. Therefore, Molecular based assays are considered the standard tests for the identification of genes related to carbapenemase production [3].

Several phenotypic methods are available for detection of carbapenemases like Modified Hodge test (MHT), Combined disc test (CDT) and inhibitor based E-test [Table/Fig-1]. Phenotypic methods are growth dependent, turnaround time is 18 - 24 h, not clinically useful and results are also subjective. Phenotypic tests like the modified Hodge test are useful for detection of carbapenemases but has low sensitivity [4] and low specificity [1] for NDM. Similarly for Inhibitor based Synergy phenotypic test for detection of *Klebsiella pneumoniae* carbapenemase, false-positive test results occur if AmpC β lactamases are coproduced [5]. Therefore, confirmation by molecular methods is necessary.

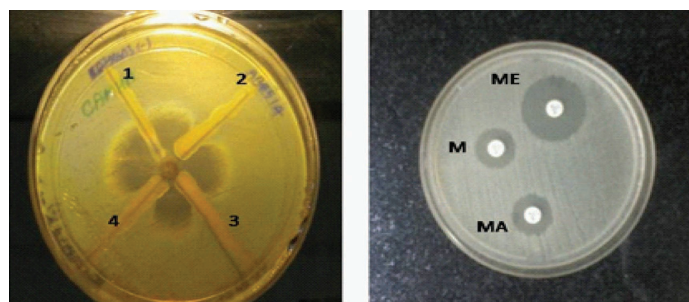
Recently, the molecular diagnostic techniques, like Real time PCR, & its modification such as LAMP have been shown to be sensitive and accurate method for identification of bla_{NDM-1} and bla_{KPC} genes[6-8]. In this prospective study, we would like to evaluate various methods for detection of bla_{NDM-1}, bla_{VIM}, bla_{IMP} and bla_{KPC} genes.

MATERIALS AND METHODS

This prospective study was done over a period of 9 months in department of Microbiology of Nizam's Institute of Medical Sciences. A total of 100 carbapenem resistant, clinically significant, non duplicated Gram negative isolates were included in this study (25 *E. coli*, 35 *K. pneumoniae*, 18 *P. aeruginosa* and 22 *A. baumanii*). Identification and antimicrobial susceptibility was done by Vitek 2 system, using the ID GN and the N90 AST panels.

Phenotypic tests (MHT and CDT) [9,10] were performed with all the 100 study isolates [Table/Fig-1].

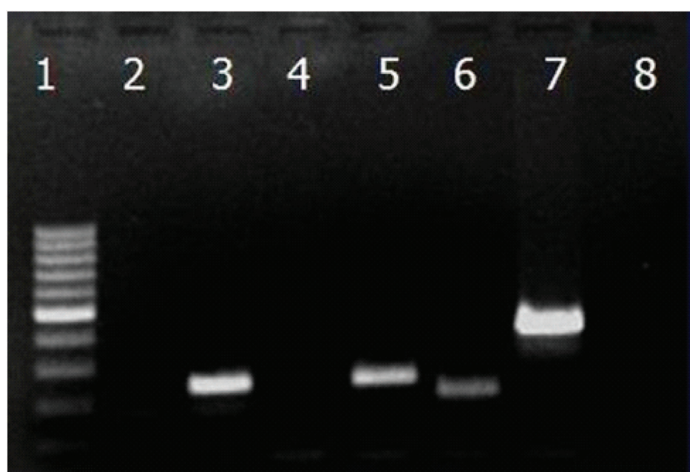
CDT was done using Mueller Hinton agar (Merck) with 10 µg of meropenem (BD, USA) plain disc and with 10 µl 600µg of 3'



[Table/Fig-1]: Modified Hodge Test (MHT) and Combined Disc test (CDT)
Left figure: MHT (1: K. pneumoniae ATCC BAA 1706 - Negative control, 2: K. pneumoniae ATCC BAA 1705 -Positive control, 3 and 4: Positive samples)
Right figure: CDT (M: Meropenm, ME: Meropenm + EDTA, MA: Meropenem +APB) showing MBL production

Gene	Primer Sequence (5'-3')	Product size
NDM-1 FP	GCATAAGTCGCAATCCCCG	237
NDM-1 RP	CTTCCTATCTCGACATGCCG	
VIM FP	GTTTGGTCGCATATCGCAAC	382
VIMRP	AATGCGCAGCACCAGGATAG	
IMP FP	GAAGGCGTTTATGTTCATAC	587
IMP RP	GTAAGTTTCAAGAGTGATGC	
KPC FP	TCGAACAGGACTTTGGCG	201
KPC RP	GGAACCAGCGCATTTTTGC	

[Table/Fig-2]: Primer sequences of 3 target genes (*bla*$_{NDM-1}$, bla$_{VIM}$, *bla*$_{IMP}$ and *bla*$_{KPC}$ genes) for Multiplex PCR



[Table/Fig-3]: Agarose gel results of KPC, NDM-1, VIM and IMP
(Well 1: 100bp ladder (fermentas), Well 2: Negative control, well 3: NDM-1 positive sample, Well 4: Negative sample, Well 5: NDM-1 positive control, Well 6: KPC positive control, Well 7: VIM positive control, Well 8: Negative sample)

aminophenylboronic acid (APB) (Sigma, St.Louis, MO, USA) & 0.5 M EDTA (Himedia, India) per disk. An increase in the zone of inhibition of ≥4 mm with APB indicates presence of the KPC carbapenemase and ≥7 mm with EDTA indicates presence of an MBL.

Molecular detection of *bla*$_{NDM-1}$, *bla*$_{VIM}$, *bla*$_{IMP}$ and *bla*$_{KPC}$ genes

DNA extraction was done according to CDC protocol by the boiling method [11] from all the 100 isolates and the ATCC standard strains. (Commercially procured from Sterisure, Mumbai)

K. pneumoniae ATCC strain BAA1705 (positive control for *bla*$_{KPC}$) [11] and *K. pneumoniae* ATCC BAA 1706 (negative control) were used.

A clinical isolate of *K. pneumoniae*, harboring *bla*$_{NDM-1}$ gene, identified by PCR and gene sequencing, was included as positive control for $_{NDM-1}$, because of inaccessibility of NDM-1 positive standard strain. Similarly *bla*$_{VIM}$ positive isolate was confirmed by sequencing & used as positive control.

The design of the primers for detection of *bla*$_{NDM-1}$, *bla*$_{VIM}$, *bla*$_{IMP}$ and *bla*$_{KPC}$ genes

For detection of *bla*$_{VIM}$, *bla*$_{IMP}$ genes previously published primers were used, while for detection of *bla*$_{NDM-1}$ & *bla*$_{KPC}$ genes primers were designed in house [12]. The sequences of the primers are shown in [Table/Fig-2].

Procedure of the Multiplex PCR assay: A 237 bp region of *blaNDM* -1, 382 bp region of *bla*$_{VIM}$, 587 bp region of *bla*$_{IMP}$ & 201 bp region of *bla*$_{KPC}$ gene were amplified through the Multiplex PCR using NDM-1, VIM, IMP & KPC specific primers (synthesized at Active oligos, ILS, Gurgaon, India).

The Quick-load Taq 2X PCR Master Mix (New England BioLabs, Inc) was used, 1x PCR contains 10mM Tris-HCL(pH 8.6, @25° C), 50 mM KCL,1.5 mMMgCl2, 0.2 mM of each dNTP, 5% glycerol, 0.08% NP-40, 0.05% Tween-20, 0.024% Orange G, 0.0025% Xyelene Cyanol FF, 50 units/ml Taq DNA polymerase and nuclease-free water to make up the final volume (25 µl). Thermal cycling (Perkin Elmer, USA) for 30 cycles was done at 94°C for 1 min, 54°C for 1 min and 72°C for one and half min. And the final extension step was performed for 5 min at 72°C. The PCR product containing amplicons was analysed in a 2% agarose gel in 1x TAE buffer at 80 V for 1.5 hr and was visualized with ethidium bromide using a gel documentation system (Syngene, UK) [Table/Fig-3].

Organism & carbapenemase	Imipenem MIC (µgm/ml)					Meropenem MIC (µgm/ml)				
	2	4	8	16	64	2	4	8	16	64
VIM (6)										
E. coli (2)	-	-	1	1	-			-	1	1
A. baumannii (1)	-	-	-	1	-			-	-	1
P. aeruginosa (3)	1	2	-	-	-		1	1	1	-
KPC (15)										
E. coli (1)	1	-	-	-	-	-	-	1	-	-
K. pneumoniae (5)	-	-	1	4	-	-	-	-	2	3
A. baumannii (9)	-	-	-	8	1	-	-	-	-	9
NDM-1 (59)										
E. coli (14)	2	4	6	2	-	-	3	4	5	2
K. pneumoniae (30)	-	3	8	19	-	-	-	3	8	19
A. baumannii (15)	-	-	-	14	1	-	-	-	-	15

[Table/Fig-4]: Correlation of MIC with carbapenemase production among GNB

Organism	VIM	IMP	NDM-1	KPC	VIM & NDM-1	KPC & NDM-1	Total
E. coli	2	-	12	1	-	2	17
K. pneumoniae	-	-	25	-	-	5	30
A. baumannii	-	-	7	-	1	7	15
P. aeruginosa	3	-	-	-	-	-	3
Total	5	-	44	1	1	14	65

[Table/Fig-5]: Results of Genotypic test (Multiplex PCR)

RESULTS

Results of Phenotypic methods

Out of the 100 carbapenem resistant isolates, 70 isolates were MHT positive, while 65 isolates were CDT positive. Five isolates which were MHT positive but CDT was negative, none of the 4 genes were detected. Correlation of MIC and carbapenemase production among *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* is shown in [Table/Fig-4].

Results of Genotypic methods

The results of the Multiplex PCR for four target genes are shown in [Table/Fig-5]. Out of 100 carbapenem resistant isolates, 65 isolates harboring one or more than one genes, while in 35 isolates none of the gene was detected. The most common resistance gene was *bla*$_{NDM-1}$ (59/100) followed by *bla*$_{KPC}$ (15/100) while the *bla*$_{VIM}$ gene was least frequent (6/100). We didn't find *bla*$_{IMP}$ in any of the isolates. Correlation of Multiplex PCR with MHT and CDT among carbapenemase producing isolates is mentioned in [Table/Fig-6].

DISCUSSION

Resistance of Carbapenem agents is due to carbapenemase and presence of other resistance mechanisms, such as ESBLs, porin mutations and/or presence of efflux pumps [13]. In our study 65 isolates were carbapenemase producers while 35 isolates were negative suggesting resistance mechanism other than carbapenemase production.

Organism	KPC positive (15)			NDM -1 positive (59)			VIM positive (6)		
	PCR positive	MHT positive	CDT positive	PCR positive	MHT positive	CDT positive	PCR Positive	MHT positive	CDT Positive
E.coli (25)	3	3	3	14	12	14	2	2	2
K. pneumoniae (35)	5	5	5	30	30	30	-	-	-
A. baumannii (22)	7	7	7	15	15	15	1	1	1
P. aeruginosa (18)	-	-	-	-	-	-	3	3	3

[Table/Fig-6]: Correlation of Multiplex PCR with MHT and CDT among carbapenemase producing isolates

Accurate susceptibility data is required to provide effective therapy. However, automated susceptibility systems may be unreliable for detection of carbapenem resistance [14,15]. A review of several automated systems showed that they incorrectly labeled up to 87% of carbapenemase-producing *K. pneumoniae* isolates as susceptible to imipenem, as well as reporting varying susceptibilities for the same isolate from day to day [14]. Ertapenem resistance seems to be a marker for carbapenemase production when automated testing methods are used [14,15]. This necessitates the need for further testing by Phenotypic & genotypic methods. If resources are limited, an elevated MIC for ertapenem could be used as a screening method to determine which isolates need further testing [14,15].

Carbapenem MICs for Carbapenemase producing isolates may vary within a broad range of values, from 0.12 to >256 mg/L [16,17]. Although VIM enzymes have strong carbapenem- hydrolytic activity, a proportion of VIM-producing *K. pneumoniae* isolates have low carbapenem MICs. In Our sudy 50% of VIM producing isolates had an MIC ≤ 4 mg/L [17]. In contrast, isolates producing the NDM – 1 have higher carbapenem MICs, 71% of our isolates have MIC ≥ 16 mg/L [18]. Of the total carbapenemase producing isolates, most resistant isolates were *A. baumannii*, all of which had MIC ≥ 16 mg/L.

Phenotypic methods like MHT give variable results. MHT performed well for KPCs and OXA-48-like enzymes but poorly for NDMs, VIMs, and IMPs [19]. Only 66% of MBL producing isolates of *P. aeruginosa* and *Acinetobacter* spp. gave positive results by the MHT (Lee et al.,) in the same study 10 more isolates with equivocal results became positive with incorporation of zinc sulfate [20].

In 5 MHT positive and CDT negative isolates none of the genes included in our study were amplified, which can be explained by presence of bla_{OXA} genes. There is currently no phenotypic test capable of detecting OXA-48. This again necessitates the need of Molecular assay.

Compared to MHT, CDT is a satisfactory and inexpensive method for detection and characterization of the carbapenemase, as results are very well correlated with PCR. Considering PCR as the gold standard test, our data suggest, CDT has 100% sensitivity and specificity.

There are very few available data of KPC from India [21]. To the best of our knowledge, our study is the first report on Multiplex PCR for detection of bla_{NDM-1}, bla_{VIM}, bla_{IMP} and bla_{KPC} genes among *E. coli*, *K. pneumoniae*, *P. aeruginosa* & *A. baumannii* and second report for the combined detection of bla_{NDM-1} and bla_{KPC} genes from India [21].

We found bla_{KPC} genes among 14 isolates with bla_{NDM-1} and in one isolate as a lone gene. Overall, sensitivity and specificity of MHT is 58% and 93%. However The PCR had 100% sensitivity and specificity [19]. We recommend molecular methods like Multiplex PCR for the optimal detection of carbapenemase.

CONCLUSION

Our results suggest that the CDT should be preferred over the MHT for the detection of carbapenemases. The Multiplex PCR was found to be more sensitive than existing phenotypic methods. Multiplex PCR will also help in simultaneous detection of various genes, reducing material, manpower & cost. It helps in determining epidemiology related to these genes & infection control.

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PARTICULARS OF CONTRIBUTORS:
1. Senior Resident, Department of Microbiology, Nizam's Institute of Medical Science, Panjagutta, Hyderabad, Telangana, India.
2. Research Scholar, Department of Microbiology, Nizam's Institute of Medical Science, Panjagutta, Hyderabad, Telangana, India.
3. Junoir Resident, Department of Microbiology, Nizam's Institute of Medical Science, Panjagutta, Hyderabad, Telangana, India.
4. Technician, Department of Microbiology, Nizam's Institute of Medical Science, Panjagutta, Hyderabad, Telangana, India.
5. Junior Research Fellow, Department of Microbiology, Nizam's Institute of Medical Science, Panjagutta, Hyderabad, Telangana, India.
6. Professor & Head, Department of Microbiology, Nizam's Institute of Medical Science, Panjagutta, Hyderabad, Telangana, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Rachana Solanki,
Senior Resident, Department of Microbiology, Nizam's Institute of Medical Sciences,
Panjagutta, Hyderabad – 500082, Telangana, India.
Phone : 0091-040- 66618773, Fax : 0091- 040 - 23310076, E-mail : lakshmi57vemu@gmail.com

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