Galore International Journal of Health Sciences and Research
Vol.4; Issue: 3; July-Sept. 2019
Website: www.gijhsr.com
Original Research Article *P-ISSN: 2456-9321*

Comparison of Molecular and Conventional Methods in the Detection of Carbapenem Resistance in Gram Negative Bacilli

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ABSTRACT

Aim: To assess the role of Carba NP as a standalone test in the detection of carbapenem resistance in Gram negative bacilli (GNB) compared to other phenotypic and molecular methods.

Materials and Methods: A total of 170 Gram Negative Bacilli (GNB**)** isolated from the 340 clinical samples like urine, pus, tissue, blood, sputum and tracheal aspirate were used for the study**.** We did antibiotic susceptibility testing by Kirby bauer disk diffusion test to detect carbapenem resistance and compared Epsilometer strip test (E test), Modified Hodge Test (MHT) and Multiplex Polymerase Chain Reaction (PCR).

Results: Of the 170 Gram negative bacilli isolated 18.2% (31/170) showed resistance to one or more of the carbapenem drugs tested i.e. Ertapenem, Meropenem, Doripenem and Imipenem. *Escherichia coli (E. coli*) showed maximum presence of carbapenemase with bla OXA 48 being the predominant gene. CarbaNP test had a sensitivity of 87% in detecting carbapenemase activity when compared with other conventional techniques such as Modified Hodge test and Kirby Bauer Disk Diffusion. Among the carbapenemase producing Gram negative Bacteria bla NDM, bla OXA 48 and bla IMP were demonstrated by molecular diagnostic methods using Multiplex PCR.

Conclusion: CarbaNp is a cost effective and rapid screening test in detection of Carbapenem resistant enterobacteriaceae (CRE)

Key words: CarbaNP test, Gram negative bacteria, Kirby bauer test, Modified Hodge test, PCR.

INTRODUCTION

One major public health threat is the prevalence of multidrug- resistant organisms (MDROs) which continues to increase on a global level and is associated with significant morbidity and mortality. [1] Antibiotic resistance has the potential to affect people at any stage of life, as well as the healthcare, veterinary, and agriculture industries, making it one of the world's most urgent public health problems. [2]

β-lactams such as Carbapenems differs from the penicillins because it is unsaturated and contains a carbon atom instead of the sulfur atom, it also contains a fused β-lactam ring and a five-member ring system. This class of antibiotics has a much broader spectrum of activity than most other β-lactam antibiotics and is effective for a wide variety of infections including urinary tract and respiratory tract infections; intra-abdominal and gynecological infections; and skin, soft tissue, bone, and joint infections. [3]

As carbapenem-resistant Enterobacteriaceae (CRE) have become increasingly prevalent worldwide, carbapenems, a last line of defense, are challenged by mobile genetic elements (MGEs) harbouring carbapenemases and other drug resistance genes." [4] The prevalence of CRE, according to some institutions in epidemic area, varies between 24.7% and 29.8%. [5] Indian single center studies overall report a prevalence of carbapenem resistance in up to 12-15% of

of Enterobacteriaceae. Resistance may be associated with hydrolysis β lactam ring, an over expression of β lactamase with no carbapenemase activity or an expression of carbapenemase. [6]

Various carbapenemases have been reported in Enterobacteriaceae such as the following types: Klebsiella pneumonia carbapenemase (KPC; Ambler class); Verona integron encoded metallo β lactamase (VIM), imipenemase (IMI), New Delhi metallo β lactamase (NMD) (all Ambler class B), and oxacillinase-48 (OXA-48; Ambler class D). [7]

Potential carbapenemase producers are detected by phenotypic methods like conventional antibiotic susceptibility testing and Modified Hodge test but these tests are not highly sensitive and specific, hence a newer and rapid method of detecting resistance is needed. A simple rapid test (Carba NP) was developed by Nordmann Poirel et al to identify presence of carbapenemase based on the hydrolysis of the β lactam ring of a carbapenem. This test is rapid, sensitive and specific, and adaptable to any laboratory in the world. [7,8]

While there are treatment alternatives for Carbapenem resistant Gram-positive bacteria (e.g. glycopeptides, daptomycin), carbapenem resistant Gram negative bacteria have very limited treatment options. Gram- negative bacteria that are Carbapenemase producers in particular are resistant to all or almost all beta-lactams. These bacteria at the same time bear genes encoding for resistance mechanisms against fluoroquinolones and/or aminoglycosides, therefore, older antimicrobial agents, such as polymyxins and fosfomycin, which were rarely implemented in the past because of efficacy and/ or toxicity concerns, together with the newer tigecycline, have become last- resort choices. [9]

In a large cohort study of ESBL-producing E. coli and Klebsiella spp. isolated from surveillance cultures of perianal swab specimens from critical care patients from 2001 to 2009, it was observed that approximately 10% of isolates were resistant to at least one carbapenem antibiotic and the median MICs of all carbapenems except meropenem appeared to increase among Klebsiella spp. and E. coli during the study period thus highlighting the increase in the incidence of CR-ESBL strains. [10]

What is essential at this time and age is the active participation in surveillance systems to measure and compare the frequency of bacterial resistance to understand the extent of emerging and established multidrug- resistant organisms (MDRO) infections. [11]

Aim and objectives:

To compare the molecular and conventional methods of detecting carbapenem resistance in Gram negative bacteria (GNB) isolated at a tertiary care hospital from January 2017 – January 2018

Specific objective:

1. To evaluate Carba NP test to be used as a rapid screening test for a faster turnaround time (TAT)
2. To find the minimum inhibitory concentration of carbapenem group of drugs by Epsilometer test as a gold standard test
3. To compare the sensitivity and specificity of Carba NP test and MIC test with Polymerase Chain reaction (PCR)

MATERIALS AND METHODS

Ethics:

The ethical committee approval (111/IEC/2017) has been obtained before starting the study. Written consent was obtained from the patients before enrolling in the study.

Study population:

Sample processing:

All the 340 samples were cultured aerobically in blood agar and MacConkey agar plate. A total of 170 Gram negative bacteria were isolated. The isolates were confirmed by biochemical tests using standard protocols. A total of 170 Gram negative bacteria isolated from the 340 samples were used for the study.

Methodology:

The antibiotic susceptibility of the gram negative bacteria to the carbapenem group of drugs was done by Kirby bauer disk diffusion method and Modified Hodge test and compared with CarbaNP test. The MIC for the carbapenem drugs was done by E test as a Gold standard test. The test results were compared with PCR along with the evaluation on the resistant genes.

Kirby bauer disk diffusion test for Antibiotic susceptibility test:

The isolates were inoculated in peptone water and incubated at 37 C for 30 minutes. After adjusting to the turbidity to 0.5 McFarland the isolates were streaked on to Muller Hinton agar plate by lawn culture. The following antibiotics were chosen Imipenem (10 g), Meropenem (10 g), Ertapenem (10 g) from HI- Media Laboratories, BD Diadgnostics Pvt Ltd, India were placed and the plates were incubated for 16-24 hours at 37 C. Results were interpreted based on CLSI guidelines 2017.

Modified Hodge Test For Suspected Carbapenemase Production:

An overnight suspension of E.coli ATCC 25922 adjusted to 0.5 McFarland standard was lawn cultured on to Muller Hinton agar plate (HI-MEDIA, Mumbai, India).After drying, 10 g of Meropenemdisc was placed on the centre of the plate and the test strain was streaked from the edge of the disc to the periphery of the plate in four different directions .Presence of cloverleaf shaped indendation shows presence of carbapenemase activity.

Minimum Inhibitory Concentration (Epsilometer Strip Test) (HiMedia, Mumbai)

The test isolate is inoculated in peptone water for 30 minutes and adjusted to 0.5 McFarland turbidity. The inoculum is streaked on to Muller Hinton agar plate with a sterile cotton swab by lawn culture.MIC E strips of Imipenem, Meropenem and Ertapenem are placed separately and incubate at 37 C for 16-24 hours. Table 1 shows the MIC range for carbapenem drugs

CarbaNP test for suspected carbapenemase production in Enterobacteriaceae and Pseudomonas aeruginosa [12]

Carba NP test was performed on those isolates that showed resistance in Kirby bauer disk diffusion test to imipenem, meropenem, ertapenem (or) all 3 drugs. One loopful (10 µL) of the tested strain directly recovered from the antibiogram plate was resuspended in a Tris- HCl 20 mmol/L lysis buffer, vortexed for 1 minute and further incubated at room temperature for 30 minutes. The bacterial suspension was then centrifuged at 10,000 × g at room temperature for 5 minutes. 30µL of the supernatant bacterial suspension was mixed in a 96-well tray with 100 µL of a 1-mL solution made of 3 mg of Imipenem pH 7.8, phenol red solution, and 0.1 mm of a phenol red solution 0.5% (wt/vol) with 16.6 ml of distilled water. The pH value was then adjusted to 7.8 by adding drops of 1 N NaOH.

A mixture of the phenol red solution and the enzymatic suspension being tested was placed in one well of the 96 well microtitre plate, to the next well an uninoculated solution was added and to the next a positive quality control of Klebsiella pneumonia ATCC BAA-1705 was used. The microtitre plate was then incubated at 37°C for 2 hours and results read. The interpretation of results are shown in **table 2**

DNA Extraction and purification method:

1.5ml of an incubated overnight broth is transferred to a 2ml eppendorf tube and centrifuged at 6000 rpm for 5 minutes. After discarding the supernatant 100µl of Buffer BE was added to the pellet and vortexed .Transfer the whole microbial suspension to a bead tube and add 40µl Buffer MG and add 10µl Proteinase K. Vortex the tube. Centrifuge the bead tube at 11,000 rpm for 30 seconds to remove the sample attached to the lid and erase the

bubbles. Add 600µl of Buffer MG to the bead tube and mix with vortex for 3 seconds.

Centrifuge the bead tube at 11,000 rpm for 30 seconds. Add 500 to 600µl of the supernatant from the bead tube to the column and centrifuge at 11,000 rpm for 30 seconds. After discarding the filtrate set the column in a new 2ml collection tube. First wash: o Add 500µl Buffer BW to the column and centrifuge at 11,000 rpm for 30 seconds .After discarding the filtrate, set the column on the same collection tube. Second wash o Add 500µl Buffer B5 to the column and centrifuge at 11,000rpm for 30 seconds o After discarding the filtrate set the column on the same collection tube. The column is further centrifuged at 11,000 rpm for 30 seconds. Remove the spin column and place the spin column in a microtube (2ml eppendorf). Add 50µl Buffer BE, close the lid and leave at room temperature for 1 minute .Centrifuge at 11,000 rpm for 30 seconds to elute the DNA and store at -70°C till further use

RESISTANT GENES IDENTIFICATION BY POLYMERASE CHAIN REACTION

Materials Used: (orange ultrapure genomic DNA extraction kit based; cat no. op-3003) orange red dye PCR master –mix components (1ml)

2U of Taq DNA polymerase, 5X Taq reaction buffer, 2mM $MgCl^{2+}$, 1µl of 10mM dNTPs mix and Red Dye PCR additive

Primers used for amplification of blaKPC, blaNDM, blaGES, blaOXA-48, blaIMP, BlaVIM genes in a master cycler (Obtained from Eurofin, Bangalore) are shown in table3

POLYMERASE CHAIN REACTION:

The reaction volume and the reagents used for PCR are shown in **table 4**

PCR Amplification protocol:

"Cycling conditions were: Initial denaturation at 95°C for 5 minutes, followed by 35 cycles of Denaturation at 95°C for 35 seconds, Annealing at 55°C for 30 seconds and Extension at 72°C for 1 minute and final extension at 72°C for 10 minutes respectively."

Agarose Gel Electrophoresis

The products of PCR were added to the wells of prepared 2% agarose gel and subjected to eleptrophoresis at 100V. The bands were observed under UV transilluminator and Gel documentation was done and shown in fig 1

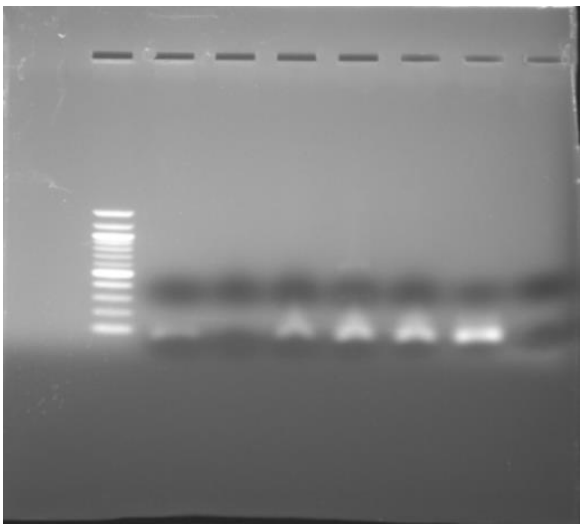

Figure 1: Agarose gel electrophoresis pattern

Statistical analysis:

The sample size for the study was derived using the formula $n=4pq/L^2$. Processing of data (variable) was done in the statistical package for social science (SPSS) software for windows, version 22.

RESULTS

Of the 340 samples processed 170 were Gram negative bacteria. E.coli was isolated from 78 samples (48.8%), Pseudomonas aeruginosa from 32 samples (18.8%), Klebsiella pneumoniae from 26 samples (15.3%).The remaining were Acinetobacter baumannii, Citrobacter freundii and Proteus mirabilis as shown in fig 2.

Among the 170 Gram negative bacilli that were analysed from carbapenem

sensitivity 31 (18%) were resistance to one or more of the carbapenem drugs tested namely Imipenem, Meropenem, Ertapenem, Doripenem and the remaining 139(82%) were carbapenem sensitive as shown in fig 3. Carbapenem resistant isolates were E. coli 15(48.4%), Pseudomonas aeruginosa and Acinetobacter baumannii 5 each (16.1%), Klebsiella pneumonia 3 (10%), Citrobacter freundii 2(7%), Proteus mirabilis 1 (3%) as shown in fig 4

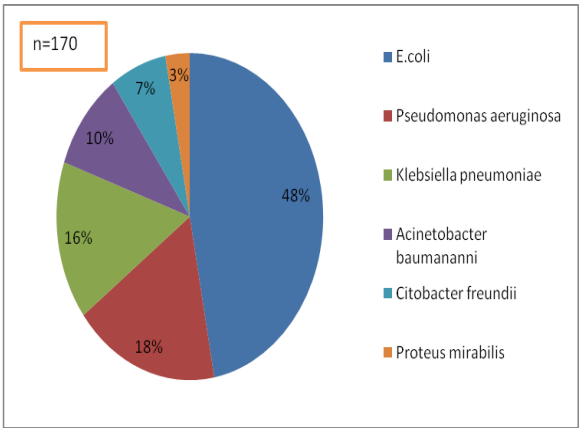

Figure 2: Distribution of the Gram negative bacteria

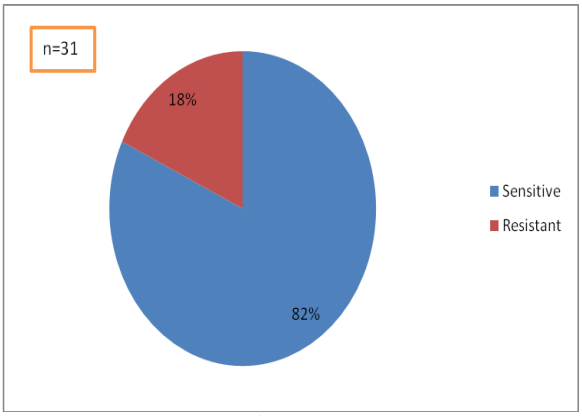

Figure 3: Distribution of sensitive and resistant isolates

Conventional Kirby bauer test detected 31/31 isolates (100%) whereas Carba NP test and Modified Hodge test detected 27/31 resistant isolates (87%) isolates(with a sensitivity of 87%. The comparision between the three tests are shown in fig 5.

Conventional PCR detected all of the 31 isolates to have resistant genes. Carbapenem resistant organism along with their resistant gene is shown in table 5.

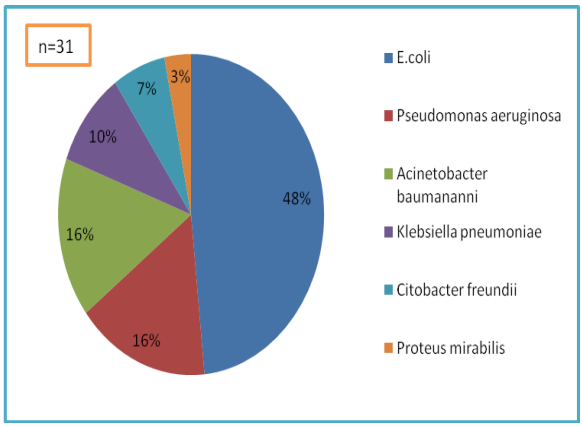

Figure 4: Distribution of the resistant isolates

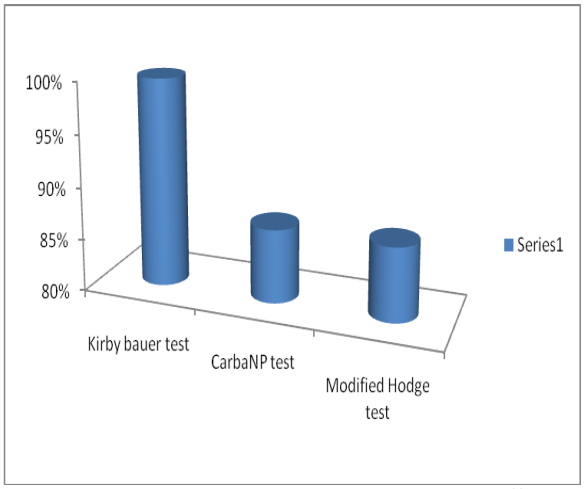

Figure 5: Comparison between Kirby Bauer Disk Diffusion, CarbaNP test and MHT

Table 1 shows the MIC range for carbapenem drugs

DRUG	MIC RANGE	LOT NO.
Meropenem	0.002 – 32mcg/ml	0000315068
Ertapenem	0.002 – 32mcg/ml	0000301322
Imipenem	0.002 – 32mcg/ml	0000311387

Table 6 shows the comparision between Kirby bauer test and E test for the different carbapenem drugs among the resistant bacterial isolates.

Table 2: Interpretation of CarbaNP test

Results for Patient and QC Tubes		
Tube "a": Solution A (serves as internal control)	Tube "b": Solution B	Interpretation
Red or red-orange	Red or red- orange	Negative, no carbapenemase detected
Red or red-orange	Light orange, dark yellow, or yellow	Positive, carbapenemase producer
Red or red-orange	Orange	Invalid
Orange, light orange, dark yellow, or yellow	Any color	Invalid

Table 3: Primers used for amplification of carbapenemase genes

Gene detected	Primer	Amplicon (bp)
bla KPC	F – TCGCTAAACTCGAACAGG R – TTACTGCCCGTTGACGCCCAATCC	785
bla NDM	F – TTGGCCTTGCTGTCCTTG R - ACACCAGTGACAATATCACCG	82
bla GES	F – CTATTACTGGCAGGGATCG R - CCTCTCAATGGTGTGGGT	594
bla OXA 48	F – TGTTTTTGGTGGCATCGAT R - GTAAMRATGCTTGGTTCGC	177
bla IMP	F – GAGTGGCTTAATTCTCRATC R - AACTAYCCAATAYRTAAC	120

Table 4 : Reaction volume and reagents used in PCR

Components	bla KPC	bla NDM	bla GES	bla OXA 48	bla IMP	bla VIM
Orange RedDye PCR Master mix	4µl	4µl	4µl	4µl	4µl	4µl
Primer Mix - blaKPC	12 µl	-	-	-	-	-
Primer Mix - blaNDM	-	12 µl	-	-	-	-
Primer Mix - blaGES	-	-	12 µl	-	-	-
Primer Mix - blaOXA 48	-	-	-	12 µl	-	-
Primer Mix - blaIMP	-	-	-	-	12 µl	-
Primer Mix - blaVIM	-	-	-	-	-	12 µl
Purified bacterial DNA	4µl	4µl	4µl	4µl	4µl	4µl
Total volume	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl

Table 5: Conventional PCR Results

Total no. of strains detected with carbapenemase	5/5	2/2	15/15	3/3	1/1	5/5	31/31
No. withblaVIM	0	1	0	0	0	0	1
No. withblaIMP	3	0	3	0	0	2	8
No. with blaOXA48	0	1	8	0	0	0	9
No. withblaGES	0	0	0	0	0	0	0
No. withblaNDM	2	0	4	0	1	3	10
No. withblaKPC	0	0	0	3	0	0	3
No. of isolates	5	2	15	3	1	5	31
Organism	Acinetobacter baumannii	Citrobacter freundii	Escherichia coli	Klebsiella pneumoniae	Proteus mirabilis	Pseudomonas aeruginosa	Total

Table 6: Comparison of resistance pattern between Disk diffusion and Epsilometer strip Test for carbapenem drugs of resistant bacterial isolates

Resistant organisms	Imipenem		Ertapenem		Meropenem	
	Disk Diffusion	Epsilometer strip Test	Disk Diffusion	Epsilometer strip Test	Disk Diffusion	Epsilometer strip Test
Escherichia coli (n=15)	14(93.3%)	12(80%)	15(100%)	13(86.6)	15(100%)	14(93.3%)
Klebsiella pneumonia (n=3)	3(100%)	2(66.7%)	3(100%)	3(100%)	3(100%)	3(100%)
Pseudomonas aeruginosa (n=5)	5(100%)	4(80%)	5(100%)	4(80%)	5(100%)	4(80%)
Acinetobacter baumannii (n=5)	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)	5(100%)
Citrobacter freundii (n=2)	2(100%)	2(100%)	2(100%)	2(100%)	2(100%)	2(100%)
Proteus mirabilis (n=1)	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)
Total (n= 31)	30/31 (Sens-96.7%)	26/31 (Sens-83.8%)	31/31 (Sens-100%)	28/31 (Sens-90.3%)	31/31 (Sens-100%)	29/31 (Sens-93.5%)

Table 7: Percentage of Carbapenem Resistance Worldwide

Year	Country	Carbapenem resistance (%)	Reference
2004 - 2010	China	0.7 - 2.7	[15]
2000 - 2009	Korea	0.1 - 0.5	[16] [17]
2011	Indonesia	5.8	[18]
2012	Chennai, India	20	[19]
2013	New Delhi, India	24	[20]

Table 8: Comparison between MHT and Disk Diffusion method

Author	Modified Hodge Test Sensitivity/ Specificity (%)	Kirby Bauer Disk Diffusion Sensitivity/ Specificity (%)	Reference
Daniel et al	97.5/ 93.3	97.5/ 100	[24]
Shivani Shinde at al	54.72/ 93.88	66.04/ 90.48	[25]
K.F Anderson et al	100/ 100	90/ 90	[26]
Present Study	87/ 100	100/ 100	

Table9: Association of Carba NP Test with Modified Hodge Test

Author	Carba NP Sensitivity/ Specificity (%)	Modified Hodge Test Sensitivity/ Specificity (%)	Reference
Pranita D. et al	97/ 99	86/ 91	[27]
Shawn Vasoo et al	100/ 100	98/80	[28]
Present Study	87	87	

DISCUSSION

In the recent years there has been an alarming rise in resistance particularly among the Enterobacteriaceae family to carbapenem group of drugs. Hence it has become increasingly important to know the prevalence of resistance seen among Gram negative bacteria and their pattern of resistance seen. Knowledge of this can lead to prevention of further rise in resistance to carbapenems and their dissemination among the hospital environment. Carbapenem sensitivity analysis shows a resistant rate of 18% among the 170 isolates tested while 82% of the isolates tested sensitive for the carbapenem drugs. The resistance observed was noted against one or more of the carbapenem drugs tested i.e. Ertapenem, Meropenem, Doripenem and Imipenem. Pravin K. Nair et al 2013 had a prevalence of 12.26% CRE seen among ICU patients in a tertiary care hospital in Mumbai, India. [13] K. Kumarasamy et al, 2010 in a multicentered study showed a resistance range of 4% to 24% of Carbapenem Resistant Enterobacteriaceae from studies done in Chennai, Haryana and other sites in India. [14] There has been a steady rise in CRE over the past few years seen worldwide.

Of the 31 isolates found resistant in our study E. coli was found to have maximum resistance among the other isolates 48.4% (15/31) followed by Acinetobacter 16.1% (5/31) and Pseudomonas 16.1% (5/ 31) which showed equal resistance. This is similar to studies done by Payal Deshpande et al and Muriel Gazin et al [21,22] showed that E. coli was isolated most among the carbapenem resistant enterobacteriaceae. The next commonest carbapenem resistant organisms in their studies were Klebsiella pneumoniae.

In our study it was found that among the 31 isolates found resistant the conventional Kirby Bauer Disk Diffusion method detected all of the 31 isolates to be resistant whereas Modified Hodge Test detected 87% i.e. 27/31 to be resistant with a sensitivity rate of 87% and specificity rate of 100%.

In our study there was a significance between the drug found resistant and Modified Hodge Test. Imipenem, [Chi-Square = 129.69; p value <0.001), Ertapenem [Chi-Square = 117.99; p value <0.001), Meropenem [Chi-Square = 122.37; p value <0.001) and Doripenem [Chi-Square = 16.39; p value <0.001]. Various studies have been done evaluating the effectiveness of MHT which shows a range common sensitivity range between 80% to 100% as shown in table 7.

A study done by A. Amjad et al showed that 69% of isolates which showed intermediate or susceptible zone sizes on disc diffusion were detected positive by MHT indicating the huge importance of this simple test for two reasons, firstly the patient would end up in treatment failure and secondly unnecessary usage of carbapenems would further expose this antimicrobial with potential for more resistance. [23] The comparison between the two tests is shown in table 8.

In our study among the 31 resistant isolates the Carba NP test was positive for 87% of the isolates i.e. 27/31 while the Modified Hodge Test also picked out carbapenemase activity in 87% of the 31

isolates i.e. 27/31. The Carba NP test was tested against the Modified Hodge Test and was found to be equal in sensitivity i.e. 87%. Various other studies have also compared the effectiveness of Carba NP and MHT or the Carba NP as a standalone test as shown in table 9.

The molecular characterization of phenotypically confirmed carbapenemase producing Gram Negative Bacilli in the present study showed a higher prevalence towards blaNDM 32%. blaOXA 48 29% and blaIMP 26%. Statistical analysis showed no significance [Chi square=35.279;p value = 0.563] between the type of carbapenemase and the clinical department isolated from. In our study we compared the effectiveness of Carba NP test with that of Conventional PCR.

Carba NP test managed to pick out 27/31 isolates for carbapenemase and out of the 4 isolates 3 were found to harbour the blaOXA 48 gene and the remaining 1 was found to have the blaIMP gene. Statistical analysis was done for PCR and resistant isolates to carbapenems and was found to be <0.005 which was significant.

CONCLUSION

Resistances to carbapenems are on an alarming rise worldwide and are seen as a global threat to mankind. The threat is even more since carbapenems come under the reserve category of WHO's classification of antibiotics.

This study provides the analytical data of the comparison of various methods of detecting carbapenem resistance with emphasis on rapid detection method CarbaNP test.

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How to cite this article: Mangayarkarasi V, Sneka P, Stephen PM. Comparison of molecular and conventional methods in the detection of carbapenem resistance in gram negative bacilli. Galore International Journal of Health Sciences & Research. 2019; 4(3): 93-102.

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