Original Article

Comparison Of Different Phenotypic Carbapenemase Detection Tests With Molecular Detection Of Carbapenemases

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Abstract

Introduction:

Antimicrobial resistance remains a global threat to public health in the 21st century. With limited therapeutic options, early diagnosis of Carbapenem resistance in Enterobacterales remains a keystone to minimizing the spread of carbapenem-resistant Enterobacterales (CRE). Evaluation of several simple techniques was done to ascertain a rapid and reliable test for detection of CRE with molecular methods as comparison.

Materials and Methods:

450 non-duplicate strains of Enterobacterales were collected from various clinical samples and subjected to Kirby-Bauer disk diffusion susceptibility test (KBDDST), Modified Hodge test (MHT), modified carbapenem inactivation method (mCIM) and Carba NP test (CNPt) with gene detection assay for blaVIM, blaNDM, and blaOXA-48 genes also being performed.

Results:

Among the 450 isolates tested, prevalence of carbapenem resistance was 30%. 139 isolates showed carbapenem resistance by KBDDST, 135 by molecular methods, 110 by MHT, 131 by mCIM and 133 by Carba NP. The most common gene detected was blaOXA-48(130/135), followed by blaVIM (127/135) and blaNDM (102/135). All isolates tested showed multiple genetic mechanisms of carbapenem resistance.

Discussion:

In this study, Carba NP test was determined to be reliable and rapid as a screening test compared to other tests. The gene based assays can be used as confirmatory test for detection of specific genetic mechanism of carbapenem resistance which may be useful for tailoring optimal therapy regimen. However, KBDDST remains the best method of detection of novel resistance mechanisms

Keywords: Carbapenemase resistance, Carba NP, mCIM, blaNDM, blaVIM, and blaOXA-48

Introduction:

The predominance of carbapenem-resistant Enterobacterales (CRE) is rising, necessitating the development of rapid detection techniques for clinical diagnostics imperative. [1] The World Health Organization's designation of CRE as a critical priority pathogen emphasizes the increasing role they play as a significant global health problem. [2] are commonly present as a part of the gut microbiome and are a major contributor of both community and healthcare-acquired infections. [3] Compared to carbapenem-susceptible bacterial infections, CRE infections lead to lengthier hospital stays, higher healthcare expenses, and increased mortality. [3]

Carbapenems are the last resort of treatment for severe life threatening infections. Carbapenemases, the enzymes destroying carbapenems, are mostly encoded on plasmids which are highly transmissible. With the very few effective antimicrobial options available for treating CRE, early detection and isolation of CRE infected patients in health care facilities is therefore of paramount importance. [1]

Currently the standard methods for detection of carbapenemase production involve 48-72 hrs incubation. Newer adaptations such as the Carba NP test and gene based assays can reduce the time required to 3-4 hours, however these methods are not easily accessible to all as they require specialized expensive reagents and machines. [4] To handle such highly resistant illnesses in the hospital setting, inexpensive, quick and precise diagnostic tools need to be evaluated for their utility.

This study compared different phenotypic carbapenemase detection tests with molecular methods of detection of carbapenemases to determine the accuracy, cost effective and rapid test for detection of carbapenemases.

Materials and Methods:

This study was conducted in SRM Medical College and Research Centre, Kattankulathur a tertiary care hospital based in Tamil Nadu, South India between March 2021 and October 2021. Institutional ethics approval was obtained prior to initiating the study. In this prospective, cross sectional study, 450 Enterobacterales clinical isolates were subjected to testing for carbapenemase detection by four phenotypic methods: Kirby-Bauer disk diffusion susceptibility test, Modified Hodge test (MHT), modified carbapenem inactivation method (mCIM) and Carba NP test (CNPt). Molecular testing was also performed to determine genotypic resistance mechanisms.

Kirby-Bauer Disk Diffusion Susceptibility Testing (KBDDST):

The isolates were identified as Enterobacterales by routine identification tests. The susceptibility of the isolates to β lactam class of antibiotics was verified by Kirby-Bauer disc diffusion method. This method involves placing an antibiotic infused filter paper disc on a lawn culture of test isolate on Mueller Hinton agar (MHA) plate. After overnight incubation, zones of inhibition of bacterial growth are noted and interpreted as per Clinical & Laboratory Standards Institute (2021) (CLSI-2021) guidelines to determine antimicrobial susceptibility. [5]

Modified Hodge Test (MHT):

Escherichia coli ATCC 25922 was streaked as lawn culture on a MHA plate. Meropenem disc (10 ug) was positioned in the center and the test isolate was streaked out from disk's edge to the plate's edge in a straight line. After overnight incubation at 37^0C, any enhanced growth of E. coli and formation of clover leaf-like indentation at the juncture of the test isolate touching the E. coli lawn culture was interpreted as Positive result indicating presence of carbapenemase production. [6]

Modified Carbapenem Inactivation Method (mCIM):

In the mCIM method, a 1 µl loopful of the test isolate colonies was mixed thoroughly in 2 mL Tryptic Soy Broth (TSB). After immersing a 10 µg meropenem disc, the tube was incubated for 4 hours in ambient air. At the end of 4

hours of incubation, the disc was squeezed and positioned on a MHA plate freshly inoculated with Escherichia coli ATCC 25922 lawn culture. After overnight growth, the zone of inhibition around the meropenem disk was measured and interpreted according to CLSI-2021guidelines with zone diameter 6-15mm or presence of pinpoint colonies within a 16-18mm zone indicative of carbapenemase production. [5]

Carba NP Test:

After overnight incubation on MHA, bacterial growth was collected with a loop and suspended in 100 µl of 20mM Tris-HCl lysis buffer and emulsified using vortex device for 5 seconds. This bacterial lysate was mixed with a tube (Tube A) containing concentrated solution of phenol red 0.5% w/v with $ZnSO_4$ 0.1mmol/litre, pH adjusted to 7.8. (Solution A) and to a second tube Tube B containing imipenem 6 mg/mL and solution A. The colour change was observed in both tubes after incubation at 37°C for a maximum of 2 hours and results were interpreted accordingly CLSI M100-2021 guidelines. The synthesis of carbapenemase enzymes was detected as the tube B's colour changing from red to yellow or light orange.

Genotypic Testing for Carbapenemase Encoding Genes:

Molecular screening was performed on all carbapenem-resistant isolates to find blaVIM, blaNDM, and blaOXA-48 genes that encode for carbapenemase production by Polymerase Chain Reaction (PCR) amplification.

On nutrient agar culture plates, pure cultures of isolated bacteria were cultivated overnight and further subjected to heat extraction method to obtain genomic DNA (95°C for 15 minutes and subsequently centrifugation at 15000 rpm for 10 minutes). Plasmid DNA was extracted by using Truescreen magnetic bead extraction kit. The forward and reverse primers were selected for blaVIM (F – GATGGTGTTTGGTCGCATA; R – CGAATGCGCAGCACCAG), blaNDM (F – CCGTATGAGTGATTGCGGCG, R –CAATATTATGCACCCGG) and blaOXA-48 (F – GCTTGATCGCCCTCGATT, R – GATTTGCTCCGTGGCCGAAA). The final reaction volume was 25 µl, with 12.5 µl of SYBRTM Green PCR Master Mix, 0.3 M (0.3 µl) of forward and reverse primer, 3 µl of template DNA, and nuclease-free molecular grade water. Cycle conditions included a 15-minute hold period at 95°C and 30 cycles of amplification lasting 30 seconds at 94°C, 90 seconds at 59°C, and 90 seconds at 72°C, with the final extension lasting 10 minutes at 72°C. blaNDM, blaOXA-48 and blaVIM genes were detected by amplification. [7]

Results:

In the current study, 450 clinical isolates of the Enterobacterales family were subjected to a selection of tests to look for the synthesis of carbapenemase enzymes. The species distribution was found to be Escherichia coli 252(56 %), Klebsiella pneumonia 153 (34%), Enterobacter spp. 20(4%), Proteus spp. 18(4%). and Citrobacter spp. 7 (2%)

The results of the antimicrobial susceptibility of the Enterobacterales isolates showed the overall prevalence of CRE by disc diffusion was 31% (139 isolates). The isolates were highly sensitive to Carbapenems (Meropenem Resistance (R)= 28%, Imipenem R=28.3% and Ertapenem R=29%) in contrast to other β lactam class of antibiotics, with resistance to Penicillins being the highest at 73%. Cephalosporins showed decreasing resistance with higher 3rd and 4th generation (Cefotaxime R= 55%) and Cefipime (R=42%) as compared to 1st and 2nd generations Cefazolin (R=64%), Cefuroxime (R=60%). Piperacillin-Tazobactum (R=35%) was found to be more effective as compared to Ceftazidine-Clavulanic acid (R=55%) for therapy in the β-lactam & β lactam inhibitor combinations.

Among the 139 CRE isolates, the most common bacteria isolated were Escherichia coli (n=62; 44.6%), Klebsiella pneumoniae (n=75; 53.6%), and Enterobacter spp. (n=2; 1.44%). Of the 450 Enterobacterales isolates, 110(24.4%) were positive by MHT, 131(29.1%) were positive by mCIM and 133 (29.56%) isolates were positive by Carba NP tube test. (Table 1)

Table 1: Analysis of results of Modified Hodge Test (MHT), Modified Carbapenem Inactivation Method (mCIM), Carba NP test, and gene based assay of Carbapenem resistance.

METHOD					
N=450	**Gene based assay**	**KBDDST**	**MHT**	**mCIM**	**Carba NP**
Carbapenem Resistant	135	139	110	131	133
Carbapenem Sensitive	315	311	323	319	317

Real-time quantitative PCR was used to identify the genes blaNDM, blaVIM, and blaOXA-48 that code for carbapenem resistance in all isolates. Many CRE isolates were discovered to include more than one Carbapenem resistance expressing genes (blaNDM, blaVIM, and blaOXA-48) inside their plasmid DNA.

blaOXA-48 gene was discovered to be present in the plasmid DNA of 130 of the 135 CRE isolates which showed presence of genes encoding carbapenem resistance. The second most common gene, blaVIM, was found in 127 isolates, followed by blaNDM, which was found in 102 isolates.

Almost half of the tested isolates (45%) contained all three genes. blaVIM and blaOXA-48 genes were present in the genomes of 36% of isolates. The least frequent gene combination in the tested isolates was found to be blaNDM and blaOXA-48 (16%). No isolate had a single genetic mechanism of resistance. (Table 2)4 isolates which were CRE by KBDDST did not show presence of the genes blaNDM, blaVIM, and blaOXA-48.

Table 2: Presence of gene combinations observed among CRE isolates

GENE	**NUMBER**
OXA-48 + NDM	22
VIM + OXA 48	50
VIM + OXA 48+ NDM	63
TOTAL	135

Considering the phenotypic tests, the highest sensitivity for detection of CRE was shown by disc diffusion (100%) and least by MHT (87.60%). Carba NP test and mCIM test showed the highest specificity (99.68%) and the highest PPV (99.25%).The highest NPV was for disc diffusion (100%) closely followed by Carba NP (99.37%). (Table 3)

Table 3: Performance characteristics of phenotypic tests

METHOD				
N=450	**KBDDST**	**MHT**	**mCIM**	**Carba NP**
Sensitivity	100.00	87.60	97.01	98.51
Specificity	98.73	98.78	99.68	99.68
Positive predictive value (PPV)	97.12	96.36	99.24	99.25
Negative predictive value (NPV)	100.00	95.59	98.75	99.37

Discussion:

The rapid worsening spread of CRE has concerning implications for treatment of bacterial infections, patient mortality and hospital infection control practices. [8] Carbapenem resistance may be due to carbapenemase production, hyperproduction of AmpC or ESBL enzymes or upregulated efflux pump or decreased influx due to major outer membrane proteins (OMPs) porin loss.[9] It is important to differentiate between the different mechanisms as carbapenemases are typically encoded on plasmids, can spread quickly in medical settings.[8] The universally used disc diffusion method requires two days for results and is also inappropriate for the higher end antibiotics, such as colistin, tigecycline which do not have KBDST breakpoints as per CLSI. [10] There is a need to evaluate a rapid precise method for identification of CRE to make informed decisions about antibiotic therapy and to curtail the spread.

The modified Hodge test (MHT), the first CRE confirmatory technique suggested by CLSI, has been discontinued from the recent guidelines as it showed unsatisfactory effectiveness. [5] Nonetheless, it is still employed in underdeveloped nations to identify CP-CRE. (10). In this study, MHT was found to have relatively low overall sensitivity (90.7%) and specificity (92.1%), and low PPV (56.0%). Similar findings were reflected by Tamma PD, et al.,[11] Girlich D, et al., [12] and Kumudunie WG , et al.,[10]. MHT has been previously reported to have better sensitivity for detecting Ambler classes A and D carbapenemases [10, 11, 12] but lower sensitivity in detecting NDM. Even though it's inexpensive and simple to perform, the major drawbacks of MHT are that the interpretation is subjective, the long turnaround time and the low specificity. [10, 12].

In the present study, Modified carbapenem inactivation method (mCIM) has been evidenced as a reliable and effective method for carbapenemase detection with sensitivity, specificity and positive predictive value. These findings have been corroborated by previous studies as well. [4,8,10]. mCIM shows high specificity and sensitivity for almost all classes of carbapenemases such as class A (KPC), class B (IMP, VIM, NDM), and class D (OXA-48 like) carbapenemases. [4, 10, 13]. It is an inexpensive method for detection and interpretation of mCIM has less observer variability+ than that of MHT. However, the long turn-around time (18–24 h) remains a major drawback of this method. [4, 10]

Carba NP test demonstated better sensitivity and specificity as compared to MHT in the current study. Specificity was comparable to that of mCIM. Review of literature however highlights the low sensitivity of Carba NP test particularly in mucoid strains or OXA-48-like enzymes with low carbapenemase activity. [14,15,16,17] This may be explained by the low prevalence of OXA-48 as the sole carbapenemase resistance gene. The isolates tested predominantly showed multiple co existing mechanisms of carbapenemase production. Carba NP test detects class A and class B carbapenemases with high sensitivity and specificity. [4] The major advantage of this test is the short turnaround time. (5 min-2 h) [4]

However, all of the methods mentioned above have some specificity or sensitivity limitations, require bacterial isolates, and are unable to determine the exact class of carbapenemase, which can be overcome by gene-based assays. These assays can be performed directly on the sample which drastically cuts down the time required for starting targeted antibiotic therapy. [4] Newer antibiotics combinations such as ceftazidime-avibactam have enzyme targeted action specifically against KPC and OXA-48-like (i.e. serine carbapenemase) producing CRE. [10]

In this study, no isolate showed a single genetic mechanism of resistance, with maximum isolates having all three genes present. Only 16% of the isolates had both blaNDM and blaOXA-48 present in their genomes, compared to 48% of isolates with three genes present. 36% of isolates had both blaVIM and blaOXA-48 present in their genomes.Multiple genetic methods are frequently used by bacterial pathogens to render the Carbapenem class of antibiotics inactive. [18] Two isolates which were resistant by disk diffusion but did not show presence of blaNDM, blaVIM, and blaOXA-48 may have other resistance genes such as blaIMP and blaKPC or alternative mechanisms such as porin loss.

These findings are reflected in other studies though the exact prevalence of the genes vary greatly as per geographical area. blaNDM gene showed the highest prevalence (61.7%) followed by blaVIM (30.8%) and blaOXA-48 (5%) in a study from New Delhi. [19]. Another study from Maharashtra highlighted the most widespread mechanism as blaOXA-48 gene with prevalence of 44.1 % compared to the prevalence of other mechanisms blaNDM-1 (8.2 %), blaVIM (2.3%) and blaKPC (1.7 %). The blaNDM gene and the blaOXA-48 coexisted in the genomes of 8.2% of the CRE isolates. [20] Previous studies by Nachimuthu et al., 2016 [21] and Manohar P. et al., 2017 [22] showed that the most common gene in this area is blaNDM, while an emerging carbapenemase gene is blaOXA-48. Similar findings are reflected in the current study.

Gene based testing is the most rapid and reliable method of determining carbapenemase production. There are numerous commercial kits available today that target the five most prevalent genes that code for carbapenemase (KPC, IMP, VIM, NDM, and OXA-48 like). Therefore, resistance due to other mechanisms (presence of an efflux pump and/or porin loss) or novel mutations may be missed. Additionally, depending on how abundantly the resistance gene is expressed, the presence of a gene does not always correlate with the status of carbapenem resistance. [4] It is impossible to define all carbapenemases, their variants, or all types of resistance that lead to carbapenem resistance. As a result, antibiotic susceptibility testing remains the standard for determining appropriate therapy. Drawbacks of PCR based assays remain their non-availability at peripheral centers, requirement for expensive set up and technical skills.

Limitations of this study which may be rectified in future research are inclusion of primers for detection of blaIMP and blaKPC. Presence of other mechanism such as hyperproduction of AmpC or major outer membrane proteins (OMPs) porin loss could not be confirmed. To further measure the expression of these genes, a quantification experiment might be carried out, providing a clearer understanding of the phenotypically predominant mechanism of resistance.

Conclusion:

Rapid and reliable CRE testing is crucial because early detection can improve antibiotic therapy and reduce needless or inappropriate medication prescription. The advancement of rapid molecular diagnostic tests has evidenced the reliability and rapid results of these gene based assays. Among the phenotypic tests, Carba NP test and mCIM were superior to MHT. Optimal detection of carbapenemases may be achieved by using Carba NP test to provide a reliable, rapid and easy screening test, with the gene based assays being put into practice as confirmatory tests to determine the exact genotypic mechanism.

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Data availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Statement

A formal consent from the institutional ethical committee was taken and clearance was obtained from the institute's ethics committee. As only bacterial isolates were utilized, individual patient's consent were not required for the study.

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