ORIGINAL ARTICLE

Effectiveness of Modified Hodge Test to detect NDM-1 Carbapenemases: an experience from Pakistan

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Abstract

Objective: To assess the effectiveness of Modified Hodge Test and its clinical utility as a phenotypic method in detecting carbapenemase.

Methods: The prospective study screened all enterobacteriaceae isolated from blood, tracheal aspirate, urine and pus aspirates at the Microbiology Laboratory of Aga Khan University Hospital, Karachi, from June 2009 to July 2010. The isolates were screened according to susceptibility zone size ≤ 23mm of meropenem on Kirby-Bauer disc diffusion followed by confirmation with minimum inhibitory concentrations ?0.5 µg/ml for meropenem by E-test method. The screened isolates were subjected to phenotypic assay (Modified Hodge Test) with subsequent confirmation with the genotypic assay. SPSS 19 was used for statistical analysis.

Results: A total of 7192 enterobacteriaceae were screened. Of these, 100 (1.39%) isolates were prospectively included in the study: Klebsiella pneumoniae 63 (63%); Escherichia coli 32 (32%); others 5 (5%). Out of the 100 isolates, 93 (93%) showed positive polymerase chain reaction results for New Delhi-Metallo-beta-lactamase (NDM-1) gene, and 69 (69%) isolates showed positive Modified Hodge Test. Four (5.8%) polymerase chain reaction negative isolates were found positive by Modified Hodge Test (false positive), which showed sensitivity of 42.8%, and specificity of 69.8% with a positive predictive value of 94.2% and a negative predictive value of 9.6%.

Conclusion: The Modified Hodge Test is a simple cost-effective method for phenotypic of carbapenemases detection in carbapenem-resistant enterobacteriaceae. This phenotypic test can be routinely performed in the clinical laboratories to detect NDM-1 carbapenemases production in the absence of molecular assays in resource-constrained settings.

Keywords: NDM-1, Metallo-beta-lactamase, Modified Hodge Test, Pakistan. (JPMA 63: 955; 2013)

Introduction

Carbapenems are the mainstay in the management of infections by multi-drug resistant (MDR) enterobacteriaceae. However, clinical utility of this group of antibiotic is under threat due to the recent emergence and spread of imipenem/meropenem-resistant enterobacteriaceae throughout the world.[1] According to a report from the SENTRY Antimicrobial Surveillance Programme, enterobacteriaceae collected from 2000 to 2005 in medical centres distributed worldwide, carbapenem resistance was found to be 0.5% and in some centres it was as high as 4%.[2] The mainstay in carbapenem resistance is the production of enzyme carbapenemase that has been classified into four main classes (Ambler Class: A-D) according to their amino acid sequence. Until recently Klebsiella pneumoniae carbapenemase (KPC) first detected in Klebsiella pneumoniae (molecular Class A enzyme), and Verona integron-encoded metallo-β-lactamase (VIM) carbapenemase (molecular Class B enzyme) were considered to be the most frequent types of carbapenemases reported from countries including United States, Israel, Turkey, China, India, the United Kingdom, Greece and Nordic countries.[3,4] However, since 2008, the emergence of a new type of Class B metallo-carbapenemase has been reported New Delhi-Metallo-beta-lactamase (NDM-1) among Klebsiella pneumoniae and E-coli isolates from India and other neighbouring countries, including Pakistan.[5-7]

Owing to plasmid mediated mode of spread of metallo-carbapenemase among enterobacteriaceae, the risk of nosocomial infections, epidemic outbreaks and increased mortality is of great concern.[3] Therefore, an early, reliable and affordable laboratory method for the detection of carbapenemase-producing enterobacteriaceae is of utmost importance in any clinical laboratory. Recent guideline recommends carbapenemase screening and confirmation as standard component of the susceptibility testing on all enterobacteriaceae isolated in routine diagnostic labs.[8]

While genotypic confirmation methods such as polymerase chain reaction (PCR) and sequencing of carbapenemase genes are most sensitive and reliable, they are prohibitively expensive for routine use. A number of relatively cheaper phenotypic methods of confirmation

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of carbapenemase producing enterobacteriaceae have recently been published.[9] Among these, the Modified Hodge Test (MHT) for the detection of diffusible carbapenemase, and Carbapenemase Inhibition Test (CIT) to distinguish between the different classes of carbapenemases, are commonly employed.

While the sensitivity of MHT has been reported to be 95-100%, its specificity varies based on the presence of other mechanisms of resistance prevailing among the strains such as CTX-M extended spectrum beta lactamase (ESBL), AmpC enzymes or expression of porins etc. Moreover, the value of MHT for the detection of blaNDM-1 containing isolates is still unclear.[9]

Pakistan is witnessing a gradual increase in the rate of carbapenem-resistant enterobacteriaceae. Laboratory-based data of over 3 years (2006-2009) of a tertiary care center has shown increase in resistance rate from zero to 2%.[10] In addition, this group has also reported expression of CTX-M ESBL among the enterobacteriaceae isolates from Pakistan.[11] Despite these reports of alarming increase in the resistance isolates, many diagnostic laboratories in Pakistan do not perform screening and confirmatory tests to detect resistant isolates preemptively, mainly due to resource limitation and also due to lack of screening guidelines based on local isolates.

The current study tested for molecular gene detection of blaNDM-1 by PCR among enterobacteriaceae isolates yielded from clinical samples, and correlated it with MHT to assess its effectiveness and clinical utility as a phenotypic method for the detection of carbapenemases in a diagnostic laboratory in Pakistan.

Materials and Methods

The prospective descriptive cross-sectional study was conducted at the clinical Microbiology Laboratory of the Aga Khan University Hospital (AKUH), Karachi. A total of 7192 enterobacteriaceae were available from different samples during the study period of 13 months from June 2009 to July 2010.

The study was approved by the Ethical Review Committee. Informed consent was personally obtained from in-patients and telephonically sought from out-patients.

The study comprised all enterobacteriaceae: KPC, E.coli, Enterobacter species, Proteus species, Serratia species and Citrobacter species. These were isolated from blood, pus aspirates, urine, tracheal aspirate and sterile body tissues.

Repeat samples from the same patients were excluded, and so were all non-fermenters and bacteria other than enterobacteriaceae.

Based on standard guidelines;[8] the detection strategy included initial screening using disc diffusion followed by confirmation with minimum inhibitory concentrations (MIC) by E-test method for meropenem.

The screened isolates were then tested by phenotypic method in the shape of MHT and genotypic method in the shape of PCR. Since carbapenemases-producing isolates are considered to be multi-resistant, therefore we also sought to test anti-microbial susceptibility of these isolates for other group of antibiotics, including aminoglycosides (amikacin), cephalosporins (ceftriaxone, cefotaxime, and cefixime), monobactams (aztreonam), fluoroquinolones (ofloxacin) and polymyxin B and results were interpreted according to CLSI 2011.[12] Fosfomycin was additionally tested for urinary isolates.

Initial screening for carbapenemase production was performed by disc diffusion and by conducting meropenem MIC using E-test strips (ABbiodisk, Solna, Sweden).[13] The zone diameter for screening breakpoint for meropenem was set at $\leq$23mm, while that for MIC was set at $\geq$0.5µg/ml. All enterobacteriaceae isolates falling in this category were considered potential carbapenemase-producers and were subjected to phenotypic and genotypic confirmation.[8]

The MHT was performed by inoculating Mueller-Hinton agar uniformly with a 1:10 dilution of a 0.5 McFarland equivalent suspension of Escherichia coli ATCC 25922. A carbapenem disk (meropenem 10µg) was applied in the centre of the lawn. The test organism and positive/negative control isolates were streaked, starting from the edge of the carbapenem disk working outwards towards the edge of the plate. The plates were examined after 16-24 hours of incubation for a clover-leaf type indentation at the intersection of the test organism and the control strain E. coli ATCC 25922 within the zone of inhibition of the carbapenem susceptibility disk (Figure-1).

MHT positive test with a clover-leaf indentation of the E. coli 25922 growing along the test organism growth streak within the disk diffusion zone indicated that the isolate was producing a carbapenemase. MHT negative test with no growth of the E. coli 25922 along the test organism growth streak and no growth within the disc diffusion indicated that the isolate was not producing a carbapenemase.

Quality Control (QC) testing was done with each set of tests done by using following controls:

Positive control: Klebsiella pneumoniae ATCC BAA 1705

Negative control: Klebsiella pneumoniae ATCC BAA 1706

The control strains were maintained in a carbapenem-

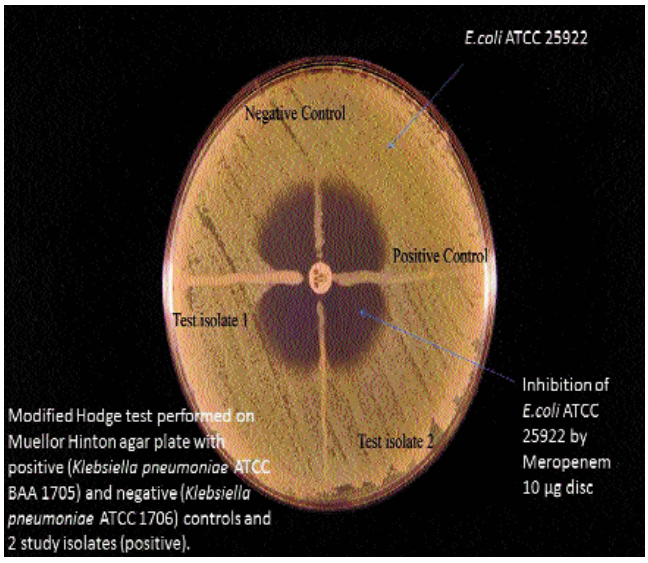


Figure-1: Modified Hodge test performed on Mullor Hinton Agar plate on two isolates (positive) with positive (Klebsiella pneumoniae BAA 1705) and negative control (Klebsiella pneumoniae BAA 1706).

containing medium prior to inoculating them onto the media used with each batch of testing as it has been documented that Klebsiella pneumoniae ATCC® BAA-1705 may undergo a spontaneous loss of the plasmid encoding the carbapenemase, leading to false-negative QC results.[14]

blaNDM-1 genes were detected through conventional PCR, using primer sequence:

F GGG CAG TCG CTT CCA ACG GT; R GTA GTG CTC AGT GTC GGC AT (4)

Dioxyribonucteic acid (DNA) was extracted by Qiagen DNA extraction kit (Qiagen, CA, USA) according to the manufacturer's instructions. Optical density of the extracted DNA was calculated by Nanodrop to check for DNA content and purity. PCR for the detection of the blaNDM-1 genes was performed and a 475bp product was amplified by the NDM primers and visualised on 3% agarose gel. Positive and negative controls were used along with molecular weight markers with each batch of PCR.

Statistical analysis of the data was done using SPSS version 19.0. Results were reported as frequencies (percentages) for categorical variables i.e. type of organism and source of specimen. The sensitivity and specificity of the MHT for the detection of carbapenemase-producing enterobacteriaceae were calculated and compared with PCR results for the detection of NDM-1 as the gold standard.

Results

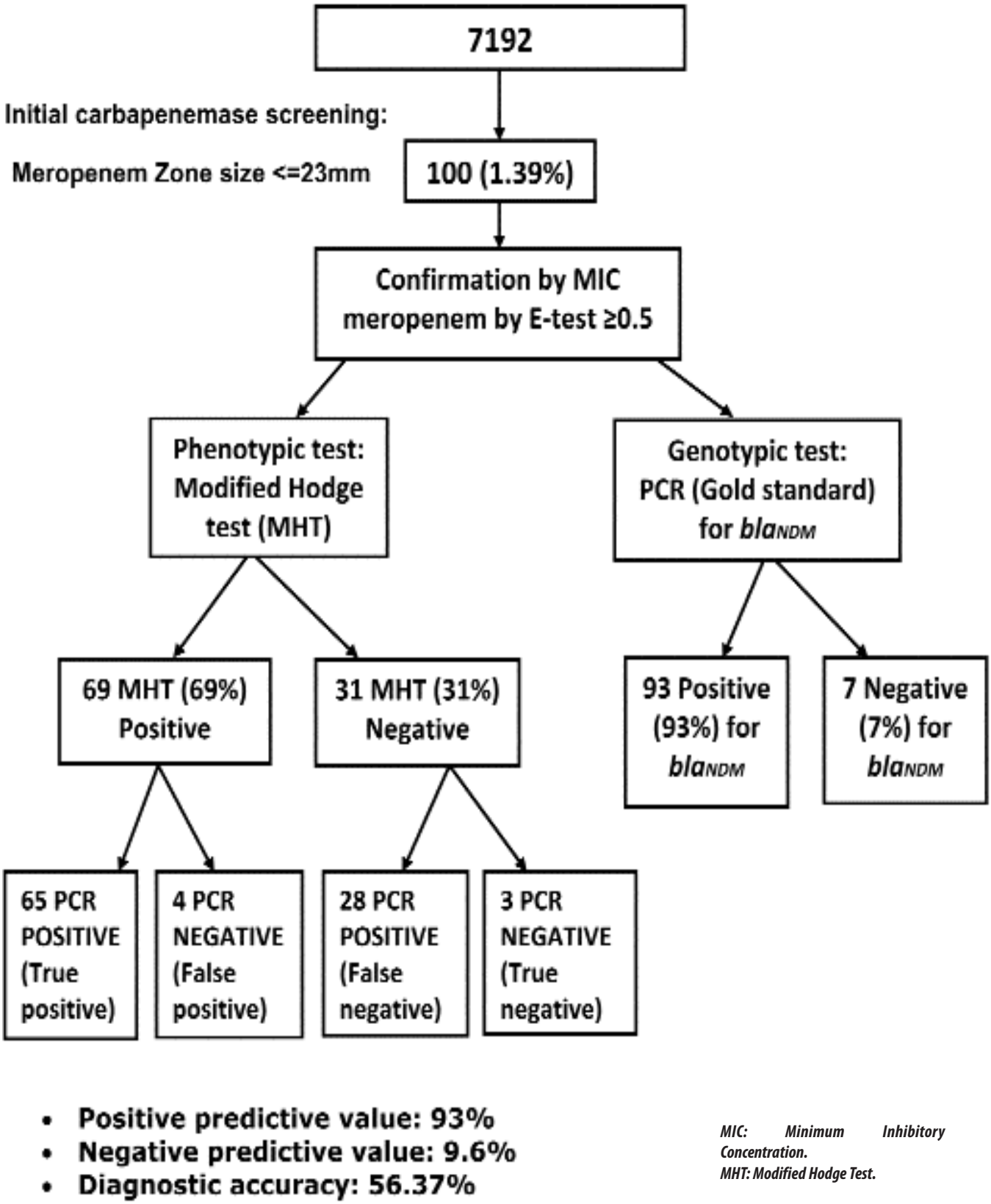
Of the 100 (1.39%) isolates that were screened from the available 7192, there were 63 (63%) Klebsiella pneumoniae, followed by E.coli 32 (32%). Enterobacter species (n=2; 2%), Citrobacter species (n=2; 2%) and Serratia species (n=1; 1%). KPC was isolated mostly from blood cultures (n=46; 73%), followed by urine cultures (n=7; 11%) while E-coli was yielded mostly from urine cultures (n=17; 53%) and 7 (41%) from blood culture. Majority of the isolates (n=82; 82%) belonged to in-patients, while 18 (18%) were from the community. Many of the patients in the community had a history of hospitalisation or healthcare visit in the preceding 3-6 months, hence, the demarcation of community versus hospital acquired infection could not be ascertained.

Of the 100 isolates, 92 (92%) fell in the screening breakpoint cutoff while 8 (8%) isolates showed MIC of <0.5 µg/ml. These 8 isolates were of special interest as these were resistant to meropenem on disc diffusion

Table: Association of MIC of meropenem with MHT.

MIC Meropenem (µg/ml)	No. of MHT Positive isolates	No. of MHT Negative isolates	Total percentage
>32	25	5	30%
24	2	-	2%
16	4	-	4%
12	7	4	11%
8	2	2	4%
6	7	5	13%
4	6	6	11%
3	5	1	6%
2	6	-	6%
1	5	0	5%
<0.5	-	*8	8%
Total	69	31	100%

*These were found resistant by disc diffusion on repeated testing with ATCC controls.
MIC: Minimum Inhibitory Concentration. MHT: Modified Hodge Test.



MIC: Minimum Inhibitory Concentration.
MHT: Modified Hodge Test.

Figure-2: Flowchart of carbapenemase detection in enterobacteriaceae.

method with zone size less than 16mm (confirmed on repeat testing with ATCC controls) but had MIC< 0.5 µg/ml and were, therefore, further subjected to phenotypic and genotypic confirmation.

MHT was found to be positive in 69 (69%) isolates. Among these, 69 MHT-positive isolates, 25 (36.2%) had high MIC of >32µg/ml. All the 31 (31%) MHT-negative, isolates had meropenem zone size of <16mm. Overall, 4 (4%) isolates showed high MIC of >32µg/ml, while 13 (13%) had MIC in the range of 0.75 to 8 µg/ml.

All 8 isolates of special interest which had very low MIC ranging 0.023 to 0.47 µg /ml were resistant to meropenem on disc diffusion (zone size <16mm).

NDM-1 gene was detected in 93 isolates selected after screening. Taking PCR as the gold standard for confirmation of carbapenemase production, 65 (65%) isolates were found to be positive by MHT and PCR (true positives), while 29 (29%) tested positive by PCR were found negative on MHT (false negative). Among the 7(7%) PCR-negative isolates, 4 (4%) were detected positive by MHT (false positive). Therefore, the positive and negative predictive value was found to be 94.2% and 9.6% respectively. Out of the 8 isolates of special interest that were MHT-negative and had low MIC of meropenem, 6 (75%) were positive for NDM-1 gene by PCR (Table and Figure-2).

All the isolates selected on initial screening, were found to be extended spectrum beta- lactamases-producers by phenotypic method (two disc method) and, hence, were resistant to penicillin, cephalosporin and monobactams. All isolates remained uniformly susceptible to polymyxin-B and showed variable susceptibility to fosfomycin that was tested for 25/29 (86%) urinary isolates; 21 (84%) isolates were susceptible to the antibiotic.

Discussion

MDR enterobacteriaceae are emerging at an alarming rate in Pakistan. A study has previously reported the emergence of CTX-M group-1 ESBL in KPC in which meropenem was reported as the only available treatment option.[10] The current study found NDM-1 metallo-β-lactamases as the commonest type of carbapenemase (93% study isolates) circulating among enterobacteriaceae in Pakistan, rendering meropenem ineffective for treatment of infections by such isolates.

NDM-1 has recently been reported as one of the emerging carbapenemase that is circulating in the Indian subcontinent and imported into UK and other European countries as a consequence of active medical tourism.[5] A study conducted in military hospitals in Pakistan in 2011 saw the prevalence of faecal carriage of Enterobacteriaceae with NDM-1 carbapenemases, and recommended the use of chromogenic medium as a screening tool for the isolation of Enterobacteriaceae harbouring the NDM-1 enzyme.[14]

Early detection of NDM-1 carbapenemase is therefore of utmost importance from epidemiological as well as infection control surveillance point of view.

In 2010, the CLSI recommended the use of carbapenemase screening and confirmation as standard component of the susceptibility testing on all enterobacteriaceae isolated in routine diagnostic labs.[11] The zone diameter for the screening cutoff was set at relatively larger size (≤23mm) as opposed to resistant-zone diameter of 16mm for meropenem. This was intended as safety net to screen potential isolates which would otherwise be reported as sensitive. With current revised recommendation (CLSI 2012), the cutoff for carbapenem resistance has been increased to zone diameter of 23mm. This appears to have diminished clinical utility of MHT. However, its role for hospital infection surveillance and epidemiological tool remains unabated as CLSI encourages its use for such purposes.[15] The findings of our study are important because the utility of MHT as phenotypic confirmatory method for local isolates where resistance mechanisms other then carbapenemases such as CTX-M are prevalent, is being reported for the first time from Pakistan. Moreover, our findings have shown its value in the detection of class B carbapenemases (NDM-1) in diagnostic laboratory of a resource-limited country from where limited data has been published so far.

In the current study, 8 isolates were identified that showed meropenem MIC <0.5, but were resistant on Kirby Bauer disc diffusion with zone sizes 16mm of meropenem. These isolates yielded from clinical samples of patients of extreme ages (6 neonates and 2 elderly aged 70 years) were subjected to phenotypic and genotypic assay. Though they all tested negative on MHT, 6 of them were positive by PCR for blaNDM-1. and these 6 patients succumbed to infection perhaps due to delay in treatment with susceptible antibiotics as all of them were being treated with meropenem till the time they were communicated by lab for possible treatment failure. While exact cause of low MIC in an isolate with positive NDM-1 gene needs further exploration, this finding is alarming as such isolates may be missed on routine testing by MIC only. Caution must be applied in cases where MHT is negative in isolates with relatively small zone size on disc diffusion. Such isolates should be subjected to further analysis, using molecular techniques such as PCR to detect NDM-1.

Our findings are in accordance with other published literature as NDM-1 carbapenemase must be suspected among enterobacteriaceae showing MDR to other groups

of antibiotics including cephalosporins, aminoglycosides, fluoroquinolones and monobactams.[16,17] The expression of co-resistance to other non-related groups of antibiotics, including aminoglycosides, macrolides, sulphamethoxazole and fluoroquinolone, has been reported for NDM-1-producing strains. This is noticeable due to co-expression of CTX-M 15 oxacillinases, AmpC production.

The high positive predictive value (PPV) of MHT in our study favours the use of this test as an effective tool for screening of potential carbapenemase producers in enterobacteriaceae in resource-limited settings. Although the sensitivity of this test was found to be low in the study, this can be explained by small sample size. In such cases, a high PPV is a better indicator as even the weak carbapenemase activity enzyme can be detected.[3] Other methods like isoelectric focussing and imipenem hydrolysis can also be used for the same purpose, but these methods are labour intensive and require continuous electrical supply which is questionable in resource-limited settings. Adequate detection of the production of carbapenemase in enterobacteriaceae isolates is crucial for infection control measures and epidemiological analysis. In addition, it is important to make appropriate choice of anti-microbial therapy. Therefore, screening and confirmation of such isolates with simple and cost-effective methods is recommended in the absence of molecular techniques.

Conclusion

MHT is a simple, cost-effective method for phenotypic detection of NDM-1 carbapenemases in carbapenem-resistant enterobacteriaceae. This phenotypic test can be routinely performed in the clinical laboratories to detect NDM-1 carbapenemases production in the absence of molecular assays in resource-constrained settings. However caution must be applied for MHT-negative isolates with reduced zone diametres on Kirby Bauer disc diffusion method. Further exploration of such isolates using molecular methods is warranted.

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