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Efficacy of Modified Carbapenem Inactivation Method for Carbapenemase Detection and Comparative Evaluation with Polymerase Chain Reaction for the Identification of Carbapenemase-Producing *Klebsiella pneumonia* Isolates

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ABSTRACT

Introduction: Rapid and reliable detection of carbapenemase-producing microorganisms is an important element of antimicrobial stewardship. Various phenotypic methods for the detection of these organisms have their limitations.

Aims: This study was conducted to assess the performance of the Modified Carbapenem Inactivation Method (mCIM) which is a recent specific phenotypic method described by CLSI 2018 guidelines for the detection of carbapenemase production.

Methodology: In the study, mCIM was performed in 80 Gram-negative isolates that were resistant to meropenem by the Kirby-Bauer disc diffusion method. Separately, another 20 *bla*KPC(*Klebsiella pneumoniae* carbapenemase) polymerase chain reaction (PCR) positive and 15 *bla*KPC PCR negative *Klebsiella pneumoniae* isolates were tested by mCIM and their results were compared.

Results: The positivity of mCIM was 92.7% and 80% in the case of Enterobacteriaceae and *Pseudomonas* spp. respectively. The test also showed 100% sensitivity for detecting carbapenemase production in PCR positive *Klebsiella pneumoniae* isolates harbouring the *bla*KPC gene.

Conclusion: mCIM is a user-specific phenotypic test that is simple and easy to carry out for the detection of carbapenemase-producing microorganisms in microbiology laboratories.

Key Words: Carbapenem resistance, Carbapenemases, mCIM, Phenotypic method, Polymerase chain reaction

INTRODUCTION

Carbapenemases have become a frequent cause of drug resistance in Enterobacteriaceae , *Pseudomonas aeruginosa and Acinetobacter baumannii* during the last decade.[1] Multiple carbapenemase genes have been described till now. The common determinants contributing to carbapenem resistance include *bla*KPC (Ambler class A), *bla*NDM(Ambler classB), and *bla*OXA-48-like(Ambler class D) genes. These enzymes are routinely isolated from *Klebsiella pneumoniae and Escherichia coli* along with other Gram-negative non-fermenters like *Pseudomonas aeruginosa* and *Acinetobacter* spp.[2] Infections due to carbapenemase-producing Gram-negative bacteria can cause serious illness leading to a prolonged period of hospitalization and increased mortality ratio. Therefore, monitoring the development of resistance against carbapenems is of utmost importance.[3]

Various phenotypic, as well as genotypic methods, have been used to detect carbapenemases. Modified Hodge test was a useful phenotypic test previously included in the CLSI guidelines but it had the disadvantage of giving false-positive results mostly with *Enterobacter* spp. harbouring AmpC enzymes and alterations in porin channels. It also suffered false-negative results in New Delhi Metallo-beta-lactamase(NDM) producing isolates.[4] Other phenotypic tests like the Carba NP test requires the use of specific and exclusive reagents, also the interpretation of the Carba NP test is

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subjective and it has shown poor sensitivity for the detection of OXA-48-type carbapenemases.[5,6] Genotypic assays can principally detect only known targets and thus the performance of such test could be seriously affected by the occurrence of mutations within the targets.[4]

Modified carbapenem inactivation method (mCIM) is included as a screening test for carbapenemase-producing Enterobacteriaceae in CLSI 2018.[7] Compared to Carba NP, which has a rapid turnaround time of 2 hours, mCIM is time-consuming, requiring an overnight incubation for the detection of carbapenemases. But it is relatively simple and has shown sensitivity and specificity of more than 99%.[8]

The emergence and spread of carbapenem-resistant bacteria is an issue of great clinical and public health concern.[4] Thus, the study was carried out to evaluate the usefulness of mCIM for the identification of carbapenem-resistant isolates in our hospital because the accurate diagnosis of infections due to carbapenemase-producing Gram-negative bacteria is important for epidemiological purposes, infection-prevention measures and expediting appropriate therapy in such infected patients.

MATERIAL AND METHODS

This prospective cross-sectional study was conducted in the Department of Microbiology, Sher-i- Kashmir Institute of Medical Sciences (SKIMS) India, a tertiary care institute from October 2019 to March 2020.

Clinical Isolates: 80 Gram-negative isolates recovered from various clinical samples (blood, pus, urine, sputum and other body fluids) were included in the study. The isolates comprised of *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

Antibiotic susceptibility was performed by Kirby-Bauer disc diffusion method according to CLSI guidelines and the isolates resistant to meropenem(10 µg) were selected.[7] These isolates were subjected to a modified carbapenem inactivation method(mCIM). In addition, 20 *bla*KPC PCR positive and 15 *bla*KPC PCR negative *Klebsiella pneumonia* isolates were tested by mCIM separately.

mCIM testing: It was done according to CLSI 2018 guidelines.[7] Using a sterile inoculating loop, 1µl of the test organism(*Escherichia coli / Klebsiella pneumoniae*) or 10µl of *Pseudomonas aeruginosa* was added into a tube containing 2 ml of tryptic soy broth(HIMEDIA); the bacterial suspension was vortexed for 10 to 15s. Aseptically, a 10µg meropenem disc (HIMEDIA) was added to this bacterial suspension and was incubated for 4 h ±15 min at 35°C ± 2°C in ambient air. Preceding the completion of the 4-hour carbapenem inactivation step, a 0.5 McFarland standard suspension of the mCIM indicator organism (*Escherichia coli* ATCC 25922, a carbapenem-susceptible strain) was prepared and inoculated on the Mueller-Hinton agar plate(HIMEDIA) using the method for standard disc diffusion susceptibility testing. The meropenem disc was then removed from the tryptic soy broth bacterial suspension using a 10µl inoculating loop which was dragged along the edge of the tube to remove excess liquid, and the disc was placed on the inoculated MHA plate. It was then incubated for 18 to 24 h at 35°C ± 2°C in ambient air.[7]

mCIM result interpretation: The test was interpreted according to CLSI 2018 guidelines.[7] The zone of inhibition around each meropenem disc was measured. A zone diameter of 6 to 15 mm or presence of colonies within 16-18mm zone was considered as a positive result (i.e. carbapenemase production), a zone diameter of more than or equal to 19 mm was considered a negative result (i.e. no carbapenemase production) and a zone diameter of 16-18 mm was considered an indeterminate result.[7] A small ring of a growth adjacent to the meropenem disc, representing carryover of the test organism from the Tryptic soy broth was ignored (Fig.1). For intermediate results, mCIM for the test isolate was repeated after checking for the purity of *Escherichia coli* ATCC 25922 indicator strain and the meropenem disc integrity by subjecting it to repeat disc diffusion test.

Figure 1: mCIM interpretation showing isolates 1 and 3(mCIM positive), isolates 2 and 4(mCIM negative).

Polymerase chain reaction (PCR): Molecular identification of KPC-producing *Klebsiella pneumoniae* was done by *bla* KPC PCR using bacterial lysates prepared by removal of 200µl of overnight broth culture. The lysates were centrifuged at 12,000 × *g* for 2 min, and then re-suspended in 200µl of molecular-grade water followed by boiling at 95°C for 10 min, and discarding the cellular debris by centrifugation at 12,000

× *g* for 2 min at 4°C. 1µl of cell lysates were then subjected to PCR analysis using the following primers designed to identify all *bla*KPC genes (bla_{KPC-1} through bla_{KPC-7}):

1. KPC forward (ATGTCACTGTATCGCCGTCT),
2. KPC reverse (TTTTCAGAGCCTTACTGCCC).

The reaction was set up in a PCR vial, after the addition of master mix, primers and the extracted DNA. 25µl of Master Mix contained 10X Taq buffer, 0.4mM dNTPs mix, 2mM Mgcl2, and 2U *Proofreading* Taq DNA polymerase.(Thermo scientific, USA). Lysates derived from *bla*KPC carrying *Klebsiella pneumoniae* strain 1705 and *Escherichia coli* ATCC 25922 were used as positive and negative controls respectively. The PCR was set up at the following conditions: 15 min at 95°C and 38 cycles of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C, which was followed by an extension step of 10 min at 72°C.[9] The PCR products were analysed by electrophoresis on 2% agarose gel stained with ethidium bromide and visualized with UV light. The *bla*KPC gene gave a band at 893bp.

RESULTS

Out of the total 80 isolates tested for carbapenemase production by mCIM, 71 (88.7%) were carbapenemase producers. Initially, an indeterminate result was present in 12 isolates(8 *Klebsiella pneumoniae* and 4 *Pseudomonas aeruginosa* isolates). After repeating mCIM of these isolates 6 among 8 *Klebsiella pneumoniae* isolates were mCIM positive and 2 were mCIM negative. Among the 4 *Pseudomonas aeruginosa* isolates, 2 were positive and 2 were negative after repeating mCIM. The positivity of mCIM in different isolates is shown in Table-1.

Table 1: Detection of carbapenemase production by mCIM in different isolates.

Organism	m CIM Positive N (%)	m CIM Negative N(%)
Klebsiella pneumoniae (N=40)	38 (95%)	2 (5%)
Escherichia coli (N=15)	13(86.6%)	2 (13.3%)
Pseudomonas aeruginosa (N=25)	20 (80%)	5 (20%)
Total =80	71(88.7%)	9 (11.3%)

While comparing mCIM of the *bla*KPC *Klebsiella pneumoniae* isolates, it showed 100% sensitivity in the detection of carbapenemase production in PCR positive *Klebsiella pneumoniae* isolates harbouring the *bla* KPC gene. (Table 2)

Table 2: Comparison of PCR (blaKPC) and mCIM for the known Klebsiella pneumonaie isolates

Isolates	PCR Positive N (%)	PCR Negative N (%)
mCIM positive	20 (100)	4 (26.6)
mCIM negative	0	11(73.4)
TOTAL	20	15

DISCUSSION

Resistance to carbapenems is a worrisome international public health problem.[2] Rapid detection of carbapenemase-producing Enterobacteriaceae is important both for taking effective precautions against the infection and for starting the appropriate treatment procedure.[3] Polymerase chain reaction is considered the gold standard for carbapenemase gene identification, but it has some limitations. False-negative PCR results can occur due to a carbapenemase gene not tested in the PCR reaction, or mutations affecting the annealing of primers. False-positive PCR results can be due to the detection of inactive genes (with no carbapenemase expression). Such results can delay infection-control measures or, oppositely, initiate them when they are not required.[10]

Different phenotypic methods also have their shortcomings. The sensitivity and specificity of these tests vary depending on the bacterial species, enzyme type and expression level of the gene that codes for the enzyme or carbapenem resistance.[3]

In our study, we evaluated the performance of the mCIM (recommended by CLSI 2018) which is a simple test and has been seen to have better sensitivity and specificity than other phenotypic methods for the detection of carbapenemases.

The overall positivity of mCIM for Enterobacteriaceae and non-Enterobacteriaceae (*Pseudomonas* spp.) in our study was 88.7%. 11.3 % of our isolates that were resistant to meropenem by disc diffusion were mCIM negative which could be because of the various other mechanisms of resistance in these isolates rather than carbapenemase production. It was observed that the detection of carbapenemases was high (92.7%) in the case of Enterobacteriaceae (*Klebsiella pneumoniae* and *Escherichia. coli*). The overall sensitivity of mCIM for Enterobacteriaceae is 93% to 100% in a study by Pierce VM et al. [4] In the case of *Pseudomonas* isolates, the positivity of mCIM in our study was 80%. In a study by Luiz F et al., the sensitivity of mCIM was 81% for Pseudomonas isolates.[11] In another multicentric study by Simner PJ et al. the sensitivity of the mCIM for detection of carbapenemase-producing *Pseudomonas* isolates across 10 sites was found to be 98.0%.[12] However in a comparative study by Howard JC et al. the sensitivity of mCIM was found to be only 71.4%.[13]

Since PCR is the gold standard test for the detection of carbapenemases, we compared the results of mCIM and conventional PCR in a separate group of *bla*KPC gene-positive and negative isolates of *Klebsiella pneumonia*. All the 20 *bla*KPC PCR positive isolates were positive by mCIM, thus giving a sensitivity of 100% for this gene. However, in the case of 15 *bla*KPC PCR negative isolates, 4(26.6%) were positive which indicates the presence of a carbapenemase other than KPC carbapenemase. Further 11(73.4%) *bla*KPC PCR negative isolates were negative by this test also because these could have a different resistant mechanism other than carbapenemase production (porin mutations, efflux pumps etc).

CONCLUSION

The mCIM was inexpensive, easy to perform and interpret, supported by the overall excellent reproducibility of the results in Enterobacteriaceae. Comparing with PCR it was found to be an accurate method for the identification of carbapenemase production in *Klebsiella Pneumoniae* isolates. This test also shows good reproducibility of the results in *Pseudomonas* isolates. Thus the test aids in the rapid and reliable identification of carbapenemase-producing carbapenem-resistant Enterobacteriaceae in microbiological laboratories and helps in understanding the local epidemiology of resistance to carbapenems, thus serving as one of the important tools to prevent the spread of these drug-resistant pathogens.

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Author`s contributions: Author Benazir Shazia designed the study, wrote the protocol and performed the laboratory methods. Author Bhat Asifa wrote the first draft of the manuscript, performed the laboratory methods and the literature searches. Author Fomda BA managed the analysis of the study. All the authors read and approved the final manuscript.

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