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Rapid detection of carbapenemase-producing *Klebsiella pneumoniae*: A comparative study of CIM and MHT

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ABSTRACT

Objective: This study aimed to compare the performance of the carbapenem inactivation method (CIM) and the modified hodge test (MHT) to screen carbapenemase activity in *Klebsiella pneumoniae* isolates with genotypically confirmed results.

Materials and Methods: A total of 114 carbapenem-resistant K. *pneumoniae* clinical isolates were collected from hospitalized patients. Two methods, the MHT and CIM, were used to investigate carbapenemase production. The CIM test was evaluated at the 6th hour for the preliminary decision and the 24th hour for the final decision. MHT was evaluated at the 24th hour. A polymerase chain reaction (PCR) was performed to detect carbapenemase-encoding genes (bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, *and* bla_{OXA-48}).

Results: Of these isolates in which a carbapenemase enzyme was detected by PCR, bla_{OXA-48} was found in 87.7%, bla_{KPC} in 6.1%, bla_{NDM} in 6.1%, bla_{IMP} in 0.8%, and bla_{VIM} in 0.8%. The most common carbapenemase gene detected was OXA-48. Of the 114 isolates with a genotypically detected carbapenemase enzyme, 98 and 109 were positive by CIM at the 6th and 24th hour, respectively, and 88 by MHT. The isolates producing both bla_{OXA-48} and bla_{NDM} and bla_{IMP} and bla_{VIM} were detected as positive by both phenotypic tests. The sensitivity of CIM at the 6th and 24th hour and MHT was found to be 85.9%, 95.6%, and 77.1%, respectively.

Conclusion: These findings indicate that CIM can be an effective method for accurately and rapidly detecting carbapenemase activity in K. *pneumoniae* infections, particularly in clinical microbiological laboratories with limited resources. To verify the negative tests, molecular methods are recommended to predict OXA-48 activity particularly..

Keywords: Carbapenem-resistant, Carbapenemase, OXA-48, Beta lactamase KPC, PCR

1. INTRODUCTION

Among hospital-acquired (nosocomial) infections, a particular concern is the rise of carbapenem-resistant *Klebsiella pneumoniae* (CR-KP), a type of bacteria belonging to the *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species (ESKAPE) group [1]. CR-KP infections are becoming increasingly common worldwide, posing a significant threat to global public health. This is because CR-KP strains produce enzymes known as carbapenemases that render carbapenem antibiotics ineffective. This leads to increased mortality and morbidity rates, prolonged hospital stays, and higher treatment costs [1].

Carbapenems are often a last-resort treatment for serious infections. The overuse of carbapenems to treat infections caused by extended-spectrum beta-lactamase (ESBL)-producing bacteria contribute to the development of carbapenem resistance in Enterobacterales [2,3]. This resistance is a significant problem because it spreads easily between bacteria through mobile genetic elements such as plasmids, transposons, and integrons [2]. Detecting carbapenem-resistant Enterobacterales is crucial for two main reasons: infection control and epidemiological data. This information is vital for developing strategies to combat this growing threat.

Therefore, to effectively fight the spread of carbapenemase-producing Enterobacterales (CPE) and ensure proper treatment for patients, rapid and accurate susceptibility testing is crucial. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) recommend activity tests based on carbapenem

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hydrolysis. These activity tests are used alongside other methods like PCR or immunochromatographic assays to confirm the specific type of resistance [4-6].

There are various options available for these activity tests. Some popular choices include colorimetric tests like Carba NP and NitroSpeed-Carba NP. Additionally, there are different variations of the carbapenem inactivation method (CIM) [5,7]. The success of these phenotypic methods varies depending on the bacterial species, enzyme type, expression level of the gene encoding the enzyme, and the presence of additional resistance mechanisms [6-8].

It is important to use a high sensitivity and specificity method to prevent the transmission of infections caused by carbapenem-resistant bacteria and to develop treatment models [13]. While the modified Hodge test (MHT) is recommended by the Clinical and Laboratory Standards Institute (CLSI) to screen carbapenemase production in Enterobacterales, recently, the CIM test has been used as a phenotypic test [9-11]. The CIM test can be applied simply and quickly and is suitable for screening [12]. Both tests,the MHT and CIM, are performed on cultured colonies [13].

This study aimed to compare the MHT and CIM 6-hour and 24-hour results in carbapenem-resistant *Klebsiella pneumoniae* isolates to evaluate their use in routine laboratory practice.

2. MATERIALS and METHODS

A total of 139 clinical isolates (114 carbapenem-resistant *K. pneumoniae* and 25 non-carbapenemase-producing) was collected from various hospitalized patients at the Marmara University, Pendik Training and Research Hospital between June 1, 2022, and December 31, 2022. In the case of repeated growths from the same patient, the first growth in the sample was included. The isolates were identified using MALDITOF-MS (bioMérieux, MarcyL'Etoile, France) and susceptibility was performed using Vitek 2 (bioMérieux, MarcyL'Etoile, France). The double disc synergy test was employed to confirm the positive ESBL isolates. Isolates that demonstrated meropenem, with a minimum inhibitory concentration (MIC) of greater than or equal to 0.25 mg/L, were accepted as resistant. The two methods, MHT and CIM, were used to detect carbapenemase production. The CIM test was evaluated at the 6th hour for the preliminary decision and at the 24th hour for the final decision. MHT was evaluated at the 24th hour. All the isolates were also analyzed using the CIM and MHT methods. The carbapenemase genes of these isolates were studied by an in-house PCR. It was performed to detect carbapenemase-encoding genes (bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and bla_{OXA-48}).

This study was approved by Marmara University Faculty of Health Sciences Ethics Committee. (Approval no. 09-2023-193) and was conducted in accordance with the Declaration of Helsinki and ethical standards of our country.

Carbapenem Inactivation Method (CIM)

A loopful of bacteria from the colonies being investigated for carbapenemase production was suspended in sterile distilled water. Then, 10 µL of a meropenem (MEM) disc, a type of antibiotic, was added to the bacterial suspension. This mixture was incubated for two hours, potentially allowing the bacteria to inactivate the meropenem. After incubation, the MEM disc was removed from the bacterial suspension. The disc was then placed onto a Mueller Hinton Agar (MHA) plate containing a strain of *E. coli* known to be susceptible to carbapenems (*E. coli* ATCC 29522). This plate was incubated for six hours at 35°C±2°C. If the bacteria in the original sample inactivated the meropenem during step 2, the disc will not inhibit the growth of the *E. coli* on the plate. This will be seen as an absence of a clear zone (no growth) around the disc. If no inhibition zone was formed around the carbapenem disc at the end of incubation, the test was considered positive.

Modified Hodge Test (MHT)

Carbapenem-susceptible *E. coli* (ATCC 25922) was swabbed onto an MHA plate. Meropenem disks (10 µg) were placed at the center of the MHA plates swabbed with *E. coli*. The suspected carbapenemase-producing organism was streaked in a straight line from the edge of the disc to the edge of the plate. The plate was incubated overnight at 35°C±2°C. A positive result was indicated by a distinct *E. coli* cloverleaf pattern growth surrounding the test *K. pneumoniae* colony within the antibiotic disc diffusion zone. Conversely, a negative result was characterized by the absence of *E. coli* growth in this area.

Molecular detection of carbapenemase resistance genes

After incubating overnight, the bacterial colonies were placed in sterile tubes containing 250 µL of distilled water and mixed thoroughly. The tubes were heated in a heat block at 95°C for 15 minutes to lyse the bacteria. Following centrifugation at 1000 rpm for five minutes, the supernatant was collected for PCR analysis. The PCR was performed using a T100 thermal cycler (Bio-Rad) for 35 cycles. Each cycle 95°C for 5 minutes, 95°C for 30 seconds,58°C for 30 seconds (primer binding), and 72°C for 30 seconds (DNA synthesis). The PCR products were analyzed on a 1.5% agarose gel prepared with 1X TBE buffer (pH 8.0). The primers used in our study are given in Table I.

***Table 1**. Primers used in the carbapenemase detection*

Resistance Gene	Primer Sequence	Amplicon size
blaOXA-48	F: 5' TTG GTG GCA TCG ATT ATC GG 3' R: 5' GAG CAC TTC TTT TGT GAT GGC 3'	743
blaNDM	F: 5' GGG CAG TCG CTT CCA ACG GT 3' R: 5' GTA GTG CTC AGT GTC GGC AT 3'	475
blaVIM	F: 5' GTT TGG TCG CAT ATC GCA AC 3' R: 5' AAT GCG CAG CAC CAG GAT AG 3'	389
blaIMP	F: 5' GAA GGY GTT TAT GTT CAT AC 3' R: 5' GTA MGT TTC AAG AGT GAT GC 3'	587
blaKPC	F: 5' TGT CAC TGT ATC GCC GTC 3' R: 5' CTC AGT GCT CTA CAG AAA ACC 3'	880

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Statistical Analysis

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using the genotype as the reference gold standard.

3. RESULTS

Among the clinical samples (n = 139), blood and urine were the most common sample types, each yielding 38 isolates. Deep Tracheal Aspirate (DTA) samples followed with 22 isolates, followed by sputum (n = 12), wound swabs (n = 14), and Bronchoalveolar Lavage (BAL) (n = 4). Endotracheal aspirates contributed 2 isolates, while abscess, CSF, and pleural fluid samples provided 5, 3, and 1 isolate, respectively.

All the isolates were resistant to ampicillin, amoxicillin/clavulanic acid, trimethoprim/sulfamethoxazole, and ciprofloxacin. The isolates also had significant concurrent resistance to the aminoglycosides gentamicin (74%), amikacin (79%), cefepime (84%), and ceftriaxone (97%), as well as piperacillin tazobactam (99%).

A total of 139 isolates were identified as carbapenem-resistant by the Vitek 2 system, with a MIC for meropenem greater than 0.25 mg/L, by the EUCAST recommendation. Of the 139 isolates, 114 (82%) had a carbapenemase enzyme detected by PCR. Out of the carbapenemase genes, bla_{OXA-48} was found in 100 samples (87.7%), bla_{KPC} in 7(6.1%), bla_{NDM} in 7(6.1%), bla_{IMP} in 1(0.8%), and bla_{VIM} in 1(0.8%). The most common carbapenemase gene detected was bla_{OXA-48} (Table II).

Table II. *MHT and CIM results of positive carbapenemase K. pneumoniae isolates*

Carbapenemases	CIM (6thhour)		CIM (24thhour)		MHT	
	Positive n (%)	Negative n (%)	Positive n (%)	Negative n (%)	Positive n (%)	Negative n (%)
OXA-48 (n = 100)	85 (85%)	15 (93.7%)	95 (95%)	0 (0%)	77 (77%)	24 (92.3%)
NDM (n = 7)	7 (100%)	16 (100%)	7 (100%)	5 (100%)	4 (57.1%)	3 (88.4%)
KPC (n = 7)	6 (85.7%)	15 (93.7%)	7 (100%)	5 (100%)	7 (100%)	26 (100%)
IMP (n = 1)	1 (100%)	16 (100%)	1 (100%)	5 (100%)	1 (100%)	26 (100%)
VIM (n = 1)	1 (100%)	16 (100%)	1 (100%)	5 (100%)	1 (100%)	26 (100%)
Total (n = 114)	98 (85.9%)		109 (95.6%)		88 (77.1%)	

CIM:Carbapenem Inactivation Method, MHT: Modified Hodge Test

Of the 114 isolates with a genotypically detected carbapenemase enzyme, 98 were found to be positive by CIM at hour 6, 109 at hour 24, and 88 by MHT. The sensitivity of CIM at the 6th and 24th hour and MHT was found to be 85.9%, 95.6%, and 77.1%, respectively. Out of the 25 negative isolates detected by PCR, 8 (32%) were found to be positive by MHT, 14 (56%) at CIM hour 6 and 8 (32%) at CIM hour 24. The isolates producing both bla_{OXA-48} and bla_{NDM} were found to be positive by both phenotypic tests. Bla_{IMP} and bla_{VIM} were co-positivity detected in one isolate by all three phenotypic methods (Table III).

Table III. *Measures of performance of the phenotypic tests for carbapenemase detection*

Test	Sensitivity %	Specificity %	PPV %	NPV %
MHT	77.1%	68%	91.6%	39.5%
CIM (6th hour)	85.9%	56%	89.9%	46.6%
CIM (24th hour)	95.6%	72%	93.9%	78.2%

CIM:Carbapenem Inactivation Method, MHT: Modified Hodge Test, PPV: Positive Predictive Value, NPV:Negative Predictive Value

Some of the isolates with bla_{OXA-48} carbapenemase genes detected by PCR are shown in Figure 1. Figure 2 shows some examples of positive and negative MHT and CIM results.

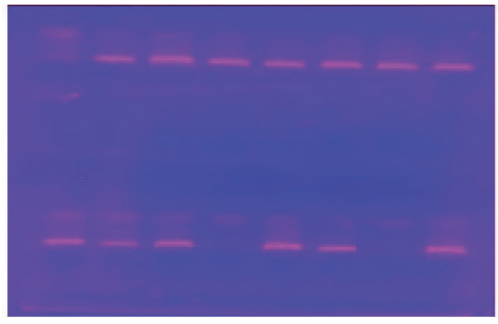

Figure 1. *PRC detection of bla_{OXA-48} in some of the isolates*

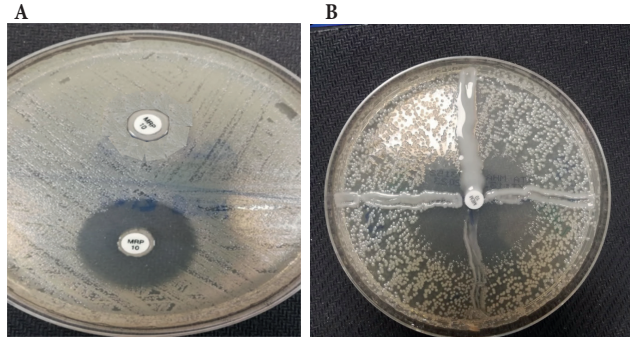

Figure 2. *Images of the CIM test on the left and the MHT method on the right. CIM:Carbapenem Inactivation Method MHT: Modified Hodge Test images of CIM positive isolates (A) and CIM negative isolates (B) image of MHT positive isolate*

4. DISCUSSION

Carbapenem resistance is a rapidly spreading global public health problem among Enterobacterales species [14]. Resistance through genes encoding carbapenemase can spread rapidly and cause epidemics [15]. The accurate identification of carbapenemase-producing isolates in routine microbiology laboratories is essential to prevent transmission, determine

appropriate treatment strategies, and formulate infection control plans [16].

Rapid phenotypic identification of carbapenemases offers a potentially cost-effective strategy to guide antimicrobial therapy. However, phenotypic detection in *K. pneumoniae* has been often challenging due to the absence of standardized methods. This study evaluated three phenotypic techniques. In this study, 114 of 139 isolates tested positive for carbapenemase genes. According to the PCR results, $bla_{\text{OXA-48}}$ was detected in 100 isolates, bla_{NDM} in 7, bla_{KPC} in 7, bla_{IMP} in 1, and bla_{VIM} in 1. Carbapenemases show different geographical distributions [17]. For example, although all carbapenemases have been reported in Turkey, $bla_{\text{oxa-48}}$is the most common [18,19]. However, other carbapenemases may be dominant in different parts of the world.

Of the positive isolates, 98 tested positive by CIM at hour 6, 109 at hour 24, and 88 by MHT. Of the 114 PCR-positive isolates, MHT yielded false negative results for 26 (22.8%) isolates, including a significant proportion (24/26) of $bla_{\text{OXA-48}}$-positive strains and two NDM-positive strains. Conversely, MHT exhibited low specificity, identifying 8 (32%) of the 25 PCR-negative isolates as positive. This study indicated that the positivity of CIM was found to be 85.9% for hour 6 and 95.6% for hour 24. The CIM test at 6 hours missed 16 of the 114 (14.1%) PCR-confirmed resistant isolates and had lower sensitivity than the CIM test at 24 hours, which only missed 5 isolates and had higher sensitivity (95.6%).

This study demonstrated a higher concordance rate between the CIM and the PCR in detecting positive carbapenemase than the MHT. A significant advantage of the CIM is its rapid turnaround time of six hours. Rizvi et al., also reported the higher reliability of the CIM for detecting OXA and NDM carbapenemases compared with the MHT [13]. Furthermore, previous studies have highlighted the subjective nature of interpreting the MHT and its lower sensitivity than the CIM [20].

Akhi et al., compared the performance of the MHT and CIM with a molecular method using 245 carbapenem-resistant *Pseudomonas aeruginosa* isolates [21]. Of the 121 isolates confirmed to produce carbapenemases, MHT and CIM yielded 40 and 35 positive results with 10 and 1 false positives, respectively. The study highlighted the high positive predictive value of the CIM and its ability to provide results within six hours, suggesting its potential as a valuable diagnostic tool. Given its low cost and excellent specificity, the authors proposed that CIM could be implemented as a routine method for detecting carbapenem-producing *P. aeruginosa* in clinical laboratories [21].

There are several studies currently underway that investigate the potential of various readily applicable methods for the phenotypic detection of carbapenemases [22,23]. One study compared the performance of MHT, Triton-MHT (THT), CIM, modified CIM (mCIM), and Triton-CIM (TCIM) on 135 genotypically confirmed *A. baumannii* isolates [22]. All the methods demonstrated 100% specificity. Another study tested 256 Gram-negative bacteria using MHT, THT, Carba NP, simplified Carba NP, blue-Carba NP, and CIM [23]. MHT had limited efficacy in detecting OXA-48, while CIM exhibited the highest performance among the tested methods. Recently, CIM-based variants have been developed for detecting rare carbapenemases [24]. MHT and CIM were applied to 25 isolates whose carbepenemase resistance genes were not detected by PCR. Of 25 positive isolates MHT, 6-hour CIM, and 24-hour CIM detected 32% ($n = 8$), 56% ($n = 14$), and 32% ($n = 8$) of them, respectively. This result indicated that at least 11 isolates that were found carbapenem-resistant by Vitek2, were not detected by PCR and MHT or CIM. It is possible that carbapenem resistance is caused by other resistance mechanisms, such as rare carbapenemase, the modification of other membrane proteins, penicillin-binding proteins, or efflux pumps [25]. However, these false negative isolates should not be ignored during PCR or phenotypic methods. This study indicated that a total of 56 (40.2%) isolates was positive for ESBL. According to our country's data, the disadvantage of the MHT is that CTX M-type ESBLs are frequently detected in OXA producers, and this may give a false positive. In addition, the carbapenem MIC has been on the rise [17]. It should be noted that the test may give false negative results, especially for NDM-type metallo-beta-lactamases, which have increased in our country (Turkey) in recent years [18].

Molecular-based technologies cannot be applied in all laboratories providing routine services due to disadvantages, such as high cost, the need for experienced personnel, and the inability to detect new unidentified genes. When detecting carbapenem susceptibility to any isolate in a microbiology laboratory, it is recommended to perform phenotypic tests and biochemical tests based on enzyme hydrolysis [6].

It would be more rational for laboratories to develop phenotypic methods for common carbapenemases. Perhaps a combination of these inexpensive, practical, and easily applicable methods will give much more accurate results.

Conclusion

The escalating prevalence of carbapenem-resistant Enterobacterales (CRE) necessitates rapid and accurate phenotypic detection methods. Given their critical role in infection management and prevention, diagnostic tools must exhibit high sensitivity and specificity. The findings of this study demonstrate that CIM is a valuable option for efficiently detecting carbapenemase activity in *K. pneumoniae* infections, particularly in resource-limited clinical microbiology laboratories. CIM can serve as a confirmatory test for negative molecular results and is recommended for predicting OXA-48 activity.

Compliance with Ethical Standards

Ethical approval: This study was approved by Marmara University Faculty of Health Sciences Ethics Committee. (Approval no. 09-2023-193). The study was conducted in accordance with the Declaration of Helsinki and ethical standards of our country.

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