Phenotypic and Genotypic Detection of Klebsiella Pneumoniae Carbapenemase

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Abstract

Aim: This study aimed to detect the presence of Klebsiella Pneumoniae Carbapenemase (KPC) enzyme among Enterobacteriaceae by phenotypic tests; the Modified Hodge Test (MHT) and boronic acid inhibition test and to compare both methods to genotypic detection of blaKPC gene by Polymerase Chain Reaction (PCR).

Methodology: This study involved detection of KPC among 72 carbapenem non-susceptible isolates of enterobacteriaceae by phenotypic methods as MHT and boronic acid inhibition test followed by genotypic methods as PCR.

Results: The results of this study showed that the prevalence of carbapenemase producing isolates by MHT was 51% and prevalence of KPC by carbapenem-boronic acid combined disk test was 35%, while it was 49% by genotypic method.

Conclusions: The presence of Klebsiella Pneumoniae Carbapenemase among enterobacteriaceae isolates causing healthcare-associated infections in the current study emphasizes the necessity for early detection of these isolates by phenotypic tests and genotypic tests.

Key Words: Klebsiella pneumoniae carbapenemase – Modified Hodge test – Boronic acid – blaKPC gene.

Introduction

The carbapenems; Imipenem (IMP), Meropenem (MEM), and Ertapenem (ETP) have the broadest antimicrobial spectrum of activity of any β-lactam antibiotics available. Like all β-lactam antibiotics, carbapenems inhibit bacterial cell wall synthesis [1,2]. Carbapenem-resistant enterobacteriaceae have been reported worldwide, primarily as a consequence of widespread acquisition of carbapenemase genes. Carbapenemase-producing strains are characterized by their resistance to virtually all β-lactam antibiotics, including cephalosporins and carbapenems, as well as to fluoroquinolones, aminoglycosides and co-trimoxazole. Invasive infections with these strains are associated with high rates of morbidity and mortality [3].

A large variety of carbapenemases which belong to two major molecular families serine carbapenemases are derivatives of molecular class A and D β-lactamase enzymes, while metallo carbapenemases are molecular class B β-lactamaseses [4-6]. The most common carbapenemases in Enterobacteriaceae isolated are the KPC enzymes, which are Ambler class A, serine β-lactamases, the gene encoding the KPC enzyme is usually flanked by transposon-related sequences and has been identified on conjugative plasmids; therefore, the potential for dissemination is significant [2,7]. The recommended phenotypic tests for detection of carbapenemases in enterobacteriaceae are the MHT as well as carbapenemase inhibition tests with boronic acid for ambler class A carbapenemases [8]. PCR is the most reliable, sensitive and specific test for detecting carbapenemases. PCR is considered to be the reference standard method for detecting carbapenemase production [9,10].

Material and Methods

Clinical isolates:

This study was carried out at Department of Medical Microbiology and Immunology, Faculty of Medicine, Cairo University, Cairo, Egypt between Dec. 2012 and Feb. 2014. The study included 72 different isolates of enterobacteriaceae; identified by the conventional microbiological standard tests (Gram’s stain, glucose fermentation test and oxidase test) and API 20E identification system (BioMérieux, France). The Isolates fulfilled the

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CLSI criterion for performing carbapenemase detection by the MHT; they were non-susceptible to either ETP and/or MEM and non-susceptible to either CFP and/or CTX, by Kirby Bauer disk diffusion method [11].

Table (1): Inhibition zone diameters interpretive standards for Enterobacteriaceae.

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Disk content (µg)</th>
<th>Zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>I</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>10</td>
<td>≥22</td>
</tr>
<tr>
<td>Meropenem</td>
<td>10</td>
<td>≥23</td>
</tr>
<tr>
<td>Cefoprazone</td>
<td>75</td>
<td>≥21</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>30</td>
<td>≥26</td>
</tr>
</tbody>
</table>

The modified hodge test:
A 0.5 McFarland turbidity standard suspension of E.coli ATCC 25922 was diluted 1:10 in sterile physiological saline solution (NaCl 0.9%). A Muller-Hinton agar plate (150mm) was inoculated with this dilution as for a standard E-test or disk diffusion test. Four 10 µg MEM and ETP disks (Oxoid, UK) were placed on the plate. An inoculation loop or swab was used to pick up three to five colonies from an overnight pure culture of a test isolate suspected of being carbapenemase-producer and streaked from the edge of the carbapenem disk to the periphery of the plate. The streak was 20-25 mm long and eight isolates were examined on the same plate. The plate was incubated overnight at 35±2°C in an aerobic atmosphere. Following incubation for 18-24 hours, the intersection of the edge of the inhibition zone and the streak of the test isolate was examined [11].

Reading and interpretation:
- Indentation of the inhibition zone (s) indicated that the test strain attacks carbapenems.
- The test result is non-determinable if the growth of E.coli ATCC 25922 was inhibited by the test isolate (inhibition zone parallel to the streak of the test isolate).
- The test is considered negative for carbapenemase production if there was no enhanced growth.

The boronic acid inhibition test:
The stock solution was prepared by dissolving phenylboronic acid (benzeneboronic acid; Sigma-Aldrich, Germany) in dimethyl sulfoxide at a concentration of 20mg/ml; disks containing boronic acid were prepared as follows: 120mg of phenylboronic acid was dissolved in 3ml of dimethyl sulfoxide and 3ml of sterile distilled water was added to this solution. From this solution, 20 µl of boronic acid were placed onto the agar. The agar plates were incubated at 37°C overnight (18-24 hours) [11].

Interpretation:
- The test was considered positive for the detection of KPC enzyme production when the diameter of the growth-inhibitory zone around a carbapenem disk with boronic acid was 5mm than that around a disk containing the carbapenem alone.

It should be noted that the concentration of boronic acid employed in the present study did not show any detectable effect on bacterial growth, since the boronic acid MIC exceeded 2,500 µg/ml [12].

Genotypic detection of KPC genes:
DNA extraction from the bacterial isolates was done and PCR amplification of the blaKPC gene was performed using the following primers: Sense, 5/-CTT GTC ATC CTT GTT AGG CG-3/ and antisense, 5/-CGT CTA GTT CTG CTG TCT TG-3/ according to the protocol described by [13]. The PCR amplification was performed under the following cycling conditions; an initial denaturation step (94°C for 1 0mins) followed by 36 cycles (94°C for 30sec, 52°C for 40sec and 72°C for 50sec) and a final extension step (72°C for 5mins). A positive and a negative control were included in each PCR run [6]. The amplification was performed in Biometra T Gradient personal thermal cycler. PCR products were analyzed on 2% agarose gel using Biometra T11 Gel Documentation System. Visual detection of DNA bands at 798bp was indicative of a positive specimen for blaKPC gene.

Statistical analysis:
Data were statistically described in terms of frequencies (number of cases) and percentages. Comparison between the study groups was done using McNemar test. Accuracy was represented using the terms sensitivity, specificity, +ve predictive value, –ve predictive value and overall accuracy, p-values less than 0.05 was considered statistically significant. All statistical calculations were done using SPSS version 15.
Results

I- The Modified Hodge Test (MHT):

Out of the 72 suspected carbapenemase-producing enterobacteriaceae isolates, 25 isolates (35%) were positive by MHT as evidenced by the indentation of the inhibition zones of MEM and ETP indicating that the test strain attacked carbapenem(s), while 35 isolates (48%) were negative as there was no indentation of the inhibition zones, whereas 12 isolates (17%) showed mild indentation (flattening) of inhibition zones by MEM and ETP disks Fig. (1).

Fig. (1): Modified Hodge.

Isolates no. 19, 41 and 44, showed positive results, isolates no. 13, 14, 42 and 89 showed mild indentation and isolate 47 was negative.

Thirty seven isolates (the 25 positive isolates and the 12 isolates that produced mild indentation by MHT) were subjected to the second phenotypic confirmatory test for carbapenemase production (the boronic acid inhibition test) and genotypic confirmation of the $\text{blaKPC}$ gene by PCR.

II- The boronic acid inhibition test: Results are shown in [(Table 2) & Fig. (2A,B)].

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boronic-MEM</td>
<td>2</td>
<td>35</td>
<td>37</td>
<td>0.001</td>
</tr>
<tr>
<td>Boronic-ETP</td>
<td>13</td>
<td>24</td>
<td>37</td>
<td></td>
</tr>
</tbody>
</table>

$p$-value <0.05 is considered statistically significant.

The 2 isolates those were positive when using MEM gave also positive results with ETP.

Fig. (2A): Positive potentiation of boronic acid inhibition test.

Results using the boronic acid-based method without (disks on Rt side) or with boronic acid (disks on Lt side); potentiation of carbapenems; MEM & ETP by boronic acid $>5$mm difference (positive test).

Fig. (2B): Negative potentiation of boronic acid inhibition test.

Results using the boronic acid-based method without (disks on Rt side) or with boronic acid (disks on Lt side); no potentiation of carbapenems by boronic acid; <5mm difference (negative test).

Performance of MEM in MHT and boronic acid inhibition test:

Out of the 25 MEM-MHT positive isolates, 2 isolates (8%) gave positive results with boronic acid inhibition test, while all the isolates that produced weak indentation gave negative result with boronic acid inhibition test (Table 3).

Performance of ETP in MHT and boronic acid inhibition test:

Out of the 25 ETP-MHT positive isolates, 11 isolates (44%) gave positive results with boronic acid inhibition test, while out of the 12 isolates
that produced weak indentation, 2 isolates (16.7%) gave positive results with boronic acid inhibition test (Table 4).

Table (3): Comparison between MEM in MHT and boronic acid inhibition test.

<table>
<thead>
<tr>
<th>Total (37 isolates)</th>
<th>Boronic-MEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>MHT-MEM positive (25 isolates)</td>
<td>23 (92%)</td>
</tr>
<tr>
<td>MHT-MEM weak indentation (12 isolates)</td>
<td>12 (100%)</td>
</tr>
</tbody>
</table>

Table (4): Comparison between ETP in MHT and boronic acid inhibition test.

<table>
<thead>
<tr>
<th>Total (37 isolates)</th>
<th>Boronic-ETP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>MHT- ETP Positive (25 isolates)</td>
<td>14 (56%)</td>
</tr>
<tr>
<td>MHT- ETP weak indentation (12 isolates)</td>
<td>10 (83.3%)</td>
</tr>
</tbody>
</table>

III- Genotypic detection of \textit{blaKPC} genes:

The prevalence of \textit{blaKPC} gene-harbouring Enterobacteriaceae isolates by PCR was as follows: Out of the 37 isolates, 18 were found positive for the presence of \textit{blaKPC} gene as evidenced by PCR. The remaining 19 isolates were found negative Fig. (3).

![Fig. (3): Gel electrophoresis of PCR detection of \textit{blaKPC} gene.](image)

Lane 1: Negative control.
Lane 2, 7: Positive isolates harboring \textit{blaKPC} gene as denoted by a positive band at 798bp.
Lanes 3, 5, 6 & 8: Negative isolates.

\textit{Performance of MHT as compared to PCR as the gold standard test:}

Out of 18 positive isolates by PCR, 11 isolates (61%) were positive by MHT and 7 isolates (39%) produced weak indentation (flattening) by MHT. While out of the 19 isolates negative by PCR, 14 isolates (74%) were positive by MHT and 5 isolates (26%) produced weak indentation by MHT (Table 5).

Table (5): Correlation between results of MHT method and PCR (the gold standard test).

<table>
<thead>
<tr>
<th>PCR for \textit{blaKPC} gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>MHT weak indentation (12 isolates)</td>
</tr>
<tr>
<td>MHT positive (25 isolates)</td>
</tr>
<tr>
<td>Total (37 isolates)</td>
</tr>
</tbody>
</table>

The MHT gave a sensitivity of 61% and a specificity of 26%.

\textit{Performance of the boronic acid inhibition test as compared to PCR as the gold standard test:}

A- By MEM disk:

Two (11%) out of the 18 isolates that were positive by PCR gave positive results by MEM boronic acid inhibition test whereas the 19 negative isolates by PCR were all negative by the MEM boronic inhibition test (Table 6).

Table (6): Correlation between results of boronic acid inhibition test by MEM and PCR for \textit{blaKPC} gene (the gold standard test).

<table>
<thead>
<tr>
<th>PCR for \textit{blaKPC} gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>Boronic-MEM positive (2 isolates)</td>
</tr>
<tr>
<td>Boronic-MEM negative (35 isolates)</td>
</tr>
<tr>
<td>Total (37 isolates)</td>
</tr>
</tbody>
</table>

The MEM boronic acid inhibition test gave a sensitivity of 11% and a specificity of 100%.

B- By ETP disk:

Out of the 18 isolates positive by PCR, 6 (33%) gave positive results by ETP boronic acid inhibition test, while out of the 19 negative isolates by PCR, 12 (63%) were negative by ETP boronic acid inhibition test (Table 7).

Table (7): Correlation between results of boronic acid inhibition test by ETP and PCR (the gold standard test) for \textit{blaKPC} gene.

<table>
<thead>
<tr>
<th>PCR for \textit{blaKPC} gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Boronic-ETP positive (24 isolates)</td>
</tr>
<tr>
<td>Boronic-ETP negative (13 isolates)</td>
</tr>
<tr>
<td>Total (37 isolates)</td>
</tr>
</tbody>
</table>

The boronic-ETP gave a sensitivity of 33% and a specificity of 63%.
positive when there is an indentation of the inhibition by the MHT and were tested for the presence of carbapenemases by the boronic acid inhibition test using the PCR as the gold standard.

<table>
<thead>
<tr>
<th>Item</th>
<th>MHT BA inhibition</th>
<th>BA inhibition -MEM</th>
<th>BA inhibition -ETP</th>
</tr>
</thead>
<tbody>
<tr>
<td>True positive</td>
<td>11 isolates</td>
<td>2 isolates</td>
<td>6 isolates</td>
</tr>
<tr>
<td>False negative</td>
<td>7 isolates</td>
<td>16 isolates</td>
<td>12 isolates</td>
</tr>
<tr>
<td>True negative</td>
<td>5 isolates</td>
<td>19 isolates</td>
<td>12 isolates</td>
</tr>
<tr>
<td>False positive</td>
<td>14 isolates</td>
<td>0 isolates</td>
<td>7 isolates</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>61%</td>
<td>11%</td>
<td>33%</td>
</tr>
<tr>
<td>Specificity</td>
<td>26%</td>
<td>100%</td>
<td>63%</td>
</tr>
<tr>
<td>Positive predictive values</td>
<td>44%</td>
<td>100%</td>
<td>46%</td>
</tr>
<tr>
<td>Negative predictive values</td>
<td>42%</td>
<td>54%</td>
<td>50%</td>
</tr>
<tr>
<td>Accuracy</td>
<td>43%</td>
<td>57%</td>
<td>49%</td>
</tr>
</tbody>
</table>

### Discussion

Multidrug resistance in enterobacteriaceae is an increasing problem that might lead to dangerous limitations of treatment options. Of special importance is the resistance to carbapenems, which is caused mainly by carbapenemase production [14] or by porin loss combined with the expression of Beta (β)-lactamases like an Extended-spectrum β-Lactamase (ESBL) or Ambler class C (AmpC) [15].

Identification of carbapenemase resistance among Enterobacteriaceae is of primary importance since carbapenemase producers are resistant not only to most (if not all) β-lactams but also to other main classes of antibiotics including quinolones, aminoglycosides and trimethoprim-sulfamethoxazole [16-20]. However, a distinction between porin loss combined with an ESBL or AmpC β-lactamase or a carbapenemase is difficult on the basis of the antibiogram in carbapenem-resistant isolates [21]. Phenotypic detection confirmation of a carbapenemase-producing member of the Enterobacteriaceae is based on an initial screening test for ETP or MEM resistance by the disk diffusion method or MIC determination, followed by the modified Hodge test for confirmation. Ertapenem nonsusceptibility is the most sensitive indicator of carbapenemases production [11].

In the present study, 72 isolates (72%) fulfilled the CLSI criteria (non-susceptibility to either ETP and/or MEM and non-susceptibility to either CFP and/or CTX) for performing carbapenemase detection by the MHT and were tested for the presence of carbapenemases by the MHT.

The MHT, the phenotypic test proposed by CLSI to confirm the presence of carbapenemases is relatively easy to perform and is considered positive when there is an indentation of the inhibition zone around the carbapenem disk due to enhanced growth of E.coli ATCC 25922 caused by the carbapenemase produced by the study isolate, while the test is considered negative if there was no enhanced growth [11]. However, Yusuf et al., [22] has raised the problem of difficulties in the interpretation of weak carbapenemase producers and considered flattening at the intersection of the test organism and E.coli ATCC 25922 within the zone of inhibition to be positive [23]. Uncertain results need to be confirmed by other tests or molecular methods [24].

Out of the 72 isolates, 37 (51%) were suspected of being KPC producers by MHT [25 isolates (35%) were found to be positive and 12 isolates (17%) produced weak indentation] and were subjected to further confirmation by boronic acid combined test and PCR. Similarly, Lari et al., reported that 19 out of 35 (54%) gave positive results with MHT [25]. Also, this result showed partial agreement with a study done by Ambretti et al., who reported that 71/108 (65.7%) enterobacteriaceae isolates were positive by MHT [26]. On the contrary, a higher rate was reported by Mosca et al., who showed that 32/38 (84%) carbapenem resistant enterobacteriaceae were MHT positive [27]. Also, much lower rates than that detected in the current study were reported by Kiratisin and Henprasert and Yusuf et al., who found that 6/35 (17%) and 8/29 (28%) of their enterobacteriaceae isolates suspected of carbapenemase production, yielded positive results by MHT, respectively [22,28].

Phenotypic detection of KPC production is based on the susceptibility of KPCs to boronic acid and its derivatives, i.e., phenyl boronic and 3-aminophenylboronic acid; boronates preferentially inhibit KPC-type β-lactamases [29]. This report was soon followed by studies proposing detection techniques using boronic acids combined with a carbapenem, mostly in the combined disk test format. However, specificity problems may arise with isolates producing AmpC-type β-lactamases (cephalosporinases), since boronic acid derivatives are potent inhibitors of these enzymes [10].

In the current study, phenotypic detection for KPC carbapenemase producers by the boronic acid combined-disk test was found to be 35% (13/37) of selected MHT isolates by ETP while only 5% (2/37) gave positive results with MEM. This result disagreed with Hung et al., who stated that only 4 out of 56 positive MHT Enterobacteriaceae (7.1%) appeared to be positive by ETP-boronic acid combined-disk test [30]. On the other hand, Bora et al.,
producing strains which are not observed with IMP. Positive results were obtained only with ETP. The specificity was 100%, whereas ETP displayed sensitivity 11% and specificity 26%. The specificity in this study may be attributed to that MHT is not specific for KPC; positive results could be due to other carbapenemases. Also, false-positive results may be due to non-carbapenemase enzymes, such as AmpC and/or ESBLs, combined with porin loss. This finding was in partial agreement with recent studies by Girlich et al. [15,33]. Moreover, Hatipoglu et al., considered the weakest aspect of the MHT is specificity [34]. However, in contrast to the results of the current study, Priyadarshini et al., reported sensitivity 90% and specificity 60% [23].

In the present study, carbapenemase production by boronic acid inhibition test using MEM and ETP was compared to PCR results of blaKPC gene. When testing MEM, the sensitivity was 11% and the specificity was 100%, whereas ETP displayed a sensitivity of 33% and a specificity of 63%; false-positive results were obtained only with ETP. The discrepancy between the results obtained by both carbapenems could be explained by the possibility that the combinations of boronic acid with ETP tend to exhibit some false-positive results in AmpC-producing strains which are not observed with IMP or MEM [30]. However, Tsakris et al., showed that the IMP and MEM disk with phenyl boronic acid exhibits sensitivity of 100% and specificity of 97.6% in the phenotypic detection of KPC producers [35].

Conclusion:

The use of MHT and boronic acid inhibition test using both MEM and ETP may improve KPC detection. Molecular methods including PCR are still the gold standard techniques to detect different types of antimicrobial resistance including carbapenemase production in Enterobacteriaceae, however, in laboratories where PCR is not available for diagnosis in a suspected carbapenemase-producing enterobacteriaceae, MHT can be done.

References


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الملخص العربي

الدراسة الحالية أجريت للكشف وجود إنزيم كريبيبيتينيز الكليبيسيلا الرئوية ضمن سلالات البكتيريا الأمعائية عن طريق الاختبارات المظهرية مثل اختيار هودج المعدل وباشر اختبار تثبيت الكريبيبيتينيز باستخدام حمض البروريك ومقارنتها بالاختبارات الوراثية مثل اختيار سلسنة تفاعل blakgcp.

شملت الدراسة الكشف عن كريبيبيتينيز الكليبيسيلا الرئوية ضمن اثنان وسبعين بكتيريا أمعائية غير حساسية لعقار الكريبيبيتين عين طريق الاختبارات المظهرية مثل اختيار هودج المعدل وباشر اختبار تثبيت الكريبيبيتينيز باستخدام حمض البروريك والاختبارات الوراثية.

وقد أسفرت النتائج هذا البحث أن نسبة وجود إنزيمات الكريبيبيتينز ضمن سلالات البكتيريا الأمعائية باستخدام اختبار هودج المعدل كانت 61% ونسبة وجود إنزيم كريبيبيتينيز الكليبيسيلا الرئوية باستخدام اختبار تثبيت الكريبيبيتينز باستخدام حمض البروريك 42% والاختبار الوراثي كانت 74%.

إن وجود كريبيبيتينيز الكليبيسيلا الرئوية ضمن سلالات البكتيريا الأمعائية السببية للعووى المرتبطة بالرعاية الصحية في الدراسة الحالية أكد على ضرورة الكشف المبكر على وجود تلك السلالات بالاختبارات المظهرية والاختبارات الوراثية.