Original Research Article

Evaluation of Boronic Acid Disk for Detection of *Klebsiella pneumoniae* Carbapenemase in *Klebsiella pneumoniae*

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**ABSTRACT**

The rapid global spread of *K. pneumoniae* producing *Klebsiella pneumoniae* carbapenemase (KPC) with its limited treatment options is of major concern. So its accurate laboratory identification in clinical isolates is critical for limiting the spread of this resistance mechanism. Our aim was to assess boronic acid in detection of KPC production in carbapenem resistant *Klebsiella pneumoniae* isolates using different antibiotic substrates in comparison to Modified Hodge Test (MHT) and bla<sub>KPC</sub> gene by polymerase chain reaction (PCR). 57 clinical isolates of carbapenem resistant *Klebsiella pneumoniae* were collected including 28 extended spectrum beta lactamase (ESBL) producers. All isolates were tested by MHT and by disks containing carbapenems, cefepime and cefoxitin, alone and in combination with boronic acid (BA). 12/57 (21%) isolates are bla<sub>KPC</sub> gene positive by PCR. MHT is positive for all isolates with 100% sensitivity. BA disks show 100% sensitivity for all substrates except cefoxitin, however ertapenem/BA shows the highest specificity (95%). In conclusion, the use of ertapenem/BA disk is a practical, cheap and sensitive screening test for detection of KPC-producing organisms which is important for avoiding spread of infection and implementing infection control measures. Confirmatory molecular investigations for carbapenem resistant genes including bla<sub>KPC</sub> should follow to identify the pattern of resistance.

**Keywords**

*Klebsiella pneumoniae*, Carbapenemase, KPC, bla<sub>KPC</sub> gene, Carbapenem, PCR, Boronic acid

**Introduction**

Carbapenem resistance *Enterobacteriaceae* is one of the major problems for antimicrobial resistance (Tsakris *et al.*, 2009). *Klebsiella pneumonia* cabapenemase (KPC) producing *Enterobacteriaceae* strains are rapidly disseminating in several countries and over large geographic areas (Cuzon *et al.*, 2010). This current wide spread of KPC enzymes makes them a great challenge for infection control and a potential threat to currently available antibiotic-based treatments (Nordmann *et al.*, 2009). KPC-producing bacteria can confer resistance to other antimicrobial classes, including all available β-lactams, fluoroquinolones, and aminoglycosides. Therefore, a limited number of antimicrobials are available for treatment of KPC infections with a high rate of therapeutic failure and at least 50% mortality rates (Lee and Burgess, 2012). It is
also of note that KPCs are mostly plasmid encoded determinants that can easily disseminate to other enterobacterial strains and species. Therefore, the accurate detection of this carbapenem resistance mechanisms is crucial for appropriate treatment of patients and controlling the spread of KPC enzymes among Enterobacteriaceae (Tsakris et al., 2010). Molecular assays are increasingly used for detection of antimicrobial resistance genes, but they are expensive and not easily accessible in all institutions. Thus, several phenotypic methods for identification of carbapenemases producing organisms including KPC are described as reduced susceptibility to ertapenem or meropenem and performance of the modified Hodge test (MHT) (Giske et al., 2010). Utilization of boronic acid disk tests have demonstrated promising results and appear practical for use in clinical microbiology laboratories (Hirsch and Tam, 2010).

Assessment of the use of boronic acid for detection of KPC in Klebsiella pneumonia isolates compared to Modified Hodge Test (MHT) and polymerase chain reaction (PCR) for detection of blaKPC gene.

Materials and methods

Fifty seven clinical isolates of carbapenem resistant Klebsiella pneumonia were collected from different clinical specimens that were referred for routine culture and sensitivity to the Central Microbiology Laboratory, Ain Shams University Hospitals, Cairo, Egypt. Identification of the isolates was confirmed by using the API 20E system (bioMerieux, Marcy l’Etoile, France). The isolates included 29 negative and 28 positive extended spectrum beta lactamase (ESBL) producers (Abou El-Makarem et al. 2012). They were screened for the presence of carbapenemase by Modified Hodge test (MHT). Boronic acid was assessed for the ability of detection of KPC possessing isolates compared to PCR for detection of blaKPC gene as a reference method (Tsakris et al., 2009).

Antimicrobial susceptibility testing and phenotypic screening

Susceptibility testing was carried out by the disk diffusion method according to CLSI (2011) guidelines and interpretative criteria.

Modified Hodge test

Screening for the presence of carbapenemase by Modified Hodge test (MHT) was done by inoculating the surface of a Muller-Hinton agar (Oxoid, UK) plate with a culture suspension of E. coli (ATCC25922) adjusted to a one tenth turbidity of a 0.5 McFarland. A meropenem 10µg disc (Oxoid, UK) was placed at the center of the plate and the isolates to be tested were streaked from the edge of the disc to the periphery of the plate. The plates were incubated at 35°C overnight. A clover leaf-like indentation of the E.coli growing along the test organism growth streak within the disk diffusion zone indicates the presence of a carbapenemase producing organism. While no growth of the E.coli along the test organism growth streak within the disc diffusion indicates that this isolate is a non carbapenemase producing organism (Figure1) (CLSI, 2011).

Detection of KPC with boronic acid disk

Detection of KPC possessing isolates was done using boronic acid disk (Tsakaris et al., 2009).

a) Preparation of boronic acid (BA) stock solution: 120 mg of phenylboronic acid (benzeneboronic acid; Sigma-Aldrich, Germany) was dissolved in 3 ml of dimethyl sulfoxide (DMSO). Then 3ml of
sterile distilled water was added to this solution to reach final concentration of 20 g/L (Coudron, 2005).

b) Preparation of boronic acid disks: 20µL boronic acid stock solution was dispensed onto each antibiotic disk (Oxoid, UK) containing 10µg imipenem (IPM), 10µg meropenem (MEM), 10µg ertapenem (ETP), 30µg cefpime (FEP) and 30µg cefoxitin (FOX). The final concentration of BA on each disk was 400µg. Disks were allowed to dry for 60 min and used immediately or stored in airtight vials at 2-8°C for up to 1 month without loss of activity.

c) Interpretation: A ≥5 mm increase in the inhibition zone around a β-lactam disk with boronic acid compared to disk containing the β-lactam substrate alone was considered positive for KPC production (Figure 2).

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed for detection of blaKPC genes (blaKPC-1 through blaKPC-7) in the 57 isolates. Bacterial DNA was extracted using DNA extraction kit (Prep Man Ultra, Germany). The reaction mixture included Qiagen Taq PCR master mix (Qiagen, Germany), Oligonucleotide primers; KPC forward (5'-ATGTCCTGTATCGCCGTCT-3') and KPC reverse 5'- (TTTTCAGAG CTTACTGCC-3') (Midland Certified Reagent Company, Texas, USA) (table 1) in a final volume of 25µL (Table 2). Escherichia coli ATCC 25922 and blaKPC-carrying K. pneumoniae ATCC BAA-1705 were used as negative and positive controls, respectively. The amplification was done as described by (Schechner et al., 2009). The PCR conditions were with initial denaturation step at 95°C for 15 min and 38 cycles of amplification consisting of: denaturation at 94°C for 1 min., annealing at 62°C for 1 min., extension at 72°C for 1 min. and final extension at 72°C for 10 min. Detection of the PCR amplified product was done by capillary gel electrophoresis QIAxcel analyzer (Qiagen, Germany). The blaKPC gene gave band at 893bp (Figure 3).

Statistical analysis

Categorical variables were expressed as number (%). Chi-square test was used to study the association between each 2 variables or comparison between 2 independent groups as regards the categorized data. The probability of error at 0.05 was considered significant, while at 0.01 and 0.001 were highly significant. The diagnostic test evaluation; sensitivity, specificity and the positive and negative predictive values and Cohen’s Kappa coefficient (k) for agreement between two tests were calculated for determining the diagnostic validity of the test. All the analyses were performed with commercially available software (SPSS version 20, SPSS, Inc., Chicago, IL, USA).

Results and Discussion

Clinical isolates

Out of 57 carbapenem resistant Klebsiella pneumonia isolates: 19 (33.3%) are from wound swabs, 15 (26.3%) from sputum, 10 (17.5%) from urine, 5 (8.8%) from pus, 4 (7%) from blood culture and 4 (7%) from miscellaneous specimens (2 central line, 1 pleural fluid, 1 nasal specimens). The twelve (12) K. pneumoniae positive blaKPC isolates are isolated from sputum specimens (7), wound swabs (4) and urine specimen (1).
Phenotypic and molecular testing

Out of the 57 *Klebsiella pneumonia* isolates positive by Modified Hodge (MHT), 28 (49%) are also ESBL producers among which 12 (21%) are positive for (*bla*KPC) gene by PCR. In reference to *bla*KPC PCR, MHT has 100% sensitivity. All the 12 *bla*KPC positive isolates are ESBL producers and harboring the following genes; 6 TEM, 4 TEM and SHV, 1 TEM and AmpC and 1 CTX-M genes. The remaining 45 negative KPC isolates are 16 (28%) positive ESBL and 29 (51%) negative ESBL isolates. The 16 *bla*KPC negative but ESBL producer isolates revealed 11 have TEM and SHV, 3 TEM, SHV and AmpC, 1 TEM and 1 CTX (Figure 4).

Boronic acid disk testing

By comparing the different antibiotic disks with and without boronic acid, the number of positive tests among the 57 isolates were higher 21(36.8%) with (FEP/BA), 20 (35%) with (IPM /BA), 19(33%) with (MEM /BA) and 14/57 (25%) with ETP/BA. All carbapenem disks (IPM, MEM, ETP) and FEP showed 100% sensitivity. The least sensitivity was detected with for FOX (42%). However ETP/BA showed the highest specificity for detection of KPC (95%) followed by MEM/BA (84%) then IPM /BA (82%) and the least was for FEP/BA (80%) then FOX /BA (77%) (Table 1).

There is almost perfect agreement between (ETP/BA) test and PCR (Kappa 0.9) for detection of KPC, followed by substantial agreement for MEM/BA (Kappa 0.7), IPM/BA (Kappa 0.66) and FEP/BA (0.63) while FOX/BA has the least agreement (Kappa 0.18) (Table 2).

In the current study, out of 57 isolates 19 (33.3%) were from wound specimens followed by sputum specimens 15(26.3%), urine 10(17.5%), pus and blood culture 5(8.8%) and miscellaneous specimens 4% (7%). The twelve (12) *K. pneumonia* positive *bla*KPC isolates were 7 (58%) from sputum specimens, 4 (30%) from wound swabs and 1 (8%) from urine specimen. While Tsakaris and colleagues (2009) found that out of the 57 KPC-possessing *K. pneumoniae* isolates, 19 were mainly from blood (33.3%), similarly 18 from wound swabs (31.6%), 11 from urine (19.3%), to a lesser extent 6 from respiratory tract specimens (10.5%), and 3 from vascular catheter tips (5.3%).

In the present study, the modified Hodge test detected the 57 *Klebsiella pneumonia* isolates as carbapenemase producers including the 12 *bla*KPC gene positive isolates. Although the sensitivity of MHT method was (100%) for detection of KPC, however, the false positive results may be due to the presence of carbapenemases other than KPC or due to ESBL production as 49% of the isolates harbored ESBL genes (TEM, SHV, CTX-M and AmpC).

In the current study, MHT using an MEM disk showed 100% sensitivity in comparison
to PCR for detection of \textit{bla}_{KPC} gene. Similarly, Seah et al. (2011) reported that MHT detected all carbapenemase producers with 100% sensitivity. Also, in a study conducted by Endimiani et al. (2010), the sensitivity of MHT, performed with MEM and IPM, were 100% for both disks and the specificity were 91\% and 88\%, respectively. Similarly, Pasteran et al. (2010) reported that, the specificity of MHT showed slight differences according the substrate used, being as low as 73\%, 75\%, and 77\% for IPM, MEM and ETP respectively. Similar results were obtained by Thomson (2010) who observed the occurrence of false positive results with high-level AmpC producers, more likely with an imipenem disk than with other carbapenems. The CLSI (2011) recommended the use of ETP or MEM as having better performance than IPM as a screen for carbapenemases.

In the present study, the inhibitory activity of BA with a final concentration of 400 µg/disk was tested using several carbapenems substrates (imipenem, meropenem and ertapenem), cefepime and cefoxitin for the detection of KPC producing isolates. The number of positive tests among the 57 \textit{Klebsiella pneumoniae} isolates were 36.8\% with FEP/BA, 35\% with IPM /BA, 33\% with MEM /BA, 29\% with FOX/BA and 25\% with ETP/BA. All carbapenem disks (IPM, MEM, ETP) and FEP showed 100% sensitivity and the least sensitivity was detected with FOX (42\%). While the highest specificity was with ETP (95\%) followed by MEM (84\%), IPM (82\%), FEP (80\%) and the least was for FOX (77\%). There was almost perfect agreement between ETP/BA test and PCR (Kappa 0.9) for detection of KPC, followed by substantial agreement for MEM/BA (Kappa 0.7) and IPM/BA (Kappa 0.66) while FOX/BA has the least agreement (Kappa 0.18). Similarly, Tsakris et al. (2009) found that, the inhibitory activity of phenylboronic acid using the same antibiotic substrates showed 100% sensitivity when tested against a large collection of KPC-PCR-positive and -negative clinical isolates.

The method showed lower sensitivity when cefoxitin was used as substrate (96.5\%). Also, the specificity for ETP disk was (95.3\%) as five isolates were AmpC producers that gave false-positive results. The specificity for the other substrates was 100\% for IPM, MEM and FEP disks. When using FOX as antibiotic substrate, the specificity was 60.4\% which was lower than ours (77\%). These findings were supported by Pournaras et al. (2010) where cefepime, imipenem and meropenem with and without BA exhibited the highest sensitivities and specificities for the detection of KPC enzyme. While combination of boronic acid with ertapenem tends to exhibit some false-positive results in AmpC producing strains which were not observed with imipenem or meropenem, which was in contrary to our results. In contrast to our results, Pasteran et al. (2009) found that IPM-BA was the best combination with 100% sensitivity and specificity while ETP-BA and MEM-BA did not achieve the performance of IPM-BA (sensitivities 86\%, 89\%, specificities 95\%, 97\% respectively). Another study carried out by Giske et al. (2011) and Seah et al. (2011) evaluated the ability of meropenem discs supplemented with β-lactamase inhibitors (dipicolinic acid, EDTA, aminophenyl boronic acid (APBA) and cloxacillin) to discriminate between various carbapenemase-producing \textit{Enterobacteriaceae}, and carbapenem non-susceptible isolates with ESBL/ AmpC hyper production in combination with porin changes. They found that the sensitivity of the boronic acid test for detection of KPC was 100\%, and the specificity was 98\% if the additional criterion of a negative cloxacillin result was included.
Table.1 Diagnostic performance of modified Hodge test and boronic acid test compared to PCR for detection of KPC production in carbapenem resistance Enterobacteriaceae isolates

<table>
<thead>
<tr>
<th>Antibiotic used in boronic acid disk test</th>
<th>Total MHT positive isolates (n=57)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Possessing <em>bla</em>&lt;sub&gt;KPC&lt;/sub&gt; (n=12)</td>
<td>Not possessing <em>bla</em>&lt;sub&gt;KPC&lt;/sub&gt; (n=45)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPM/BA</td>
<td>12</td>
<td>8</td>
<td>100</td>
<td>82</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>MEM/BA</td>
<td>12</td>
<td>7</td>
<td>100</td>
<td>84</td>
<td>63</td>
<td>100</td>
</tr>
<tr>
<td>ETP/BA</td>
<td>12</td>
<td>2</td>
<td>100</td>
<td>95</td>
<td>86</td>
<td>100</td>
</tr>
<tr>
<td>FEP/BA</td>
<td>12</td>
<td>9</td>
<td>100</td>
<td>80</td>
<td>57</td>
<td>100</td>
</tr>
<tr>
<td>FOX/BA</td>
<td>5</td>
<td>10</td>
<td>42</td>
<td>77</td>
<td>33</td>
<td>83</td>
</tr>
</tbody>
</table>


Table.2 Agreement between antibiotics with and without boronic acid test compared to PCR for detection of KPC production in carbapenem resistance Enterobacteriaceae isolates

<table>
<thead>
<tr>
<th>Antibiotic used in boronic acid disk test</th>
<th>Positive agreement (%)</th>
<th>Negative agreement (%)</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
<th>Kappa</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPM/BA</td>
<td>75</td>
<td>90</td>
<td>46</td>
<td>87</td>
<td>0.66</td>
<td>Substantial</td>
</tr>
<tr>
<td>MEM/BA</td>
<td>77</td>
<td>92</td>
<td>49</td>
<td>90</td>
<td>0.70</td>
<td>Substantial</td>
</tr>
<tr>
<td>ETP/BA</td>
<td>92</td>
<td>98</td>
<td>77</td>
<td>100</td>
<td>0.90</td>
<td>Almost perfect</td>
</tr>
<tr>
<td>FEP/BA</td>
<td>73</td>
<td>89</td>
<td>42</td>
<td>83</td>
<td>0.63</td>
<td>Substantial</td>
</tr>
<tr>
<td>FOX/BA</td>
<td>37</td>
<td>80</td>
<td>-10</td>
<td>46</td>
<td>0.18</td>
<td>Slight</td>
</tr>
</tbody>
</table>

Kappa test *values for agreement: 0.81-1.0= almost perfect, 0.61-0.80= substantial, 0.41-0.60=moderate, 0.21-0.40 fair, 0-0.2=slight, <0=no agreement
**Figure 1** Modified Hodge test (MHT) using MEM disk

Isolate A: KPC positive MHT, Isolate B and C: KPC negative MHT

**Figure 2** Antibiotic substrate disks without (A) and with (B) boronic acid

A: No inhibitory zones around carbapenem antibiotics without boronic acid, B: ≥5mm increase in carbapenems zone diameter by boronic acid.
Figure 3: PCR gel electrophoresis

Lane 1: positive control, Lane 2: negative control, Lane 5:bblKPC positive samples, Lanes 3, 4 and 6-12 are bblKPC negative samples.

Figure 4: Flow chart for the distribution of both bblKPC and ESBL genes among the isolates.
In the present study, the inhibitory activity of BA with a final concentration of 400 µg/disk was tested resulting in 100% sensitivity. Tsakris et al. (2011) used disks containing imipenem without and with 300 µg APBA, 600 µg APBA, or 400 µg PBA (sensitivities were 64.3%, 82.1%, and 100%, respectively). Using disks containing meropenem without and with 300 µg APBA, 600 µg APBA, or 400 µg PBA (sensitivities, 77.7%, 96.4%, and 100%, respectively). All combined-disk tests employing imipenem or meropenem correctly identified 124 of the 127 non-KPC producers (specificity, 97.6%). Eventually, the comparative study showed that meropenem with final concentration 400 µg on the disk was a more sensitive substrate than imipenem for the detection of KPCs which was in agreement to our results.

In the current study, 12 out of 57 carbapenem resistant isolates (21%) were positive for blaKPC by PCR and 45 out of 57 isolates (79%) were negative. The remaining 45 negative KPC isolates included 16 (28%) positive ESBL producer isolates revealed 11 have TEM and SHV, 3 TEM, SHV and AmpC, 1 TEM and 1 CTX. The absence of blaKPC in these carbapenem resistant isolates may be explained by: a)The presence of eleven types or alleles of (KPC1/2-12) and the primers used in this study were for detection of blaKPC1-7 (Arnold et al., 2012; Richter et al., 2012); b) Presence of carbapenem resistance mechanisms other than KPC as AmpC hyperproduction or a combination of CTX-M and porin mutations (Francis et al., 2012); c) Presence of other carbapenemases e.g metallo-β lactamase or oxacillinase (Tsakris et al. (2009).

Studies done by Moland et al. (2003) and Villegas et al. (2006) revealed that carbapenem susceptibility testing by the disk diffusion method is unreliable in predicting the presence of blaKPC. Possible explanations for undetected blaKPC carriage are an unexpressed blaKPC gene, the inoculum effect and misinterpretation of the resistance pattern to signify an extended-spectrum beta-lactamase producer. So, PCR-based screening might increase the chance of detecting resistance (Marschall et al., 2009). The direct detection of blaKPC by PCR may shorten the time to identify patients colonized or infected with carbapenem-resistant organisms and may be more sensitive than culture (Cole et al., 2009). But it has several disadvantages: first, it does not allow identification of the bacterial host of the resistance gene with its phenotypic characteristics and its antibiotic susceptibilities. This may lead to delayed identification of epidemiologically important clusters or the emergence of new phenotypes or the spread of blaKPC to previously unaffected species. Second, where mechanisms other than blaKPC are prevalent other methods should be used. Third, a wider PCR-based screening approach may be implemented for identification of various carbapenemase genes. Also PCR-based approach may not be available as a screening tool due to resource limitation (Schechner et al., 2009).

The main limitation of our study was that we could not perform the molecular analysis of several genes that confer resistance to carbapenems.

In conclusion the modified Hodge test is a sensitive method for detection of KPC however not specific. Boronic acid assay using ETP 10µg disk is a simple, cheap, highly sensitive and the most specific disk for the phenotypic confirmation of KPC possessing K. pneumoniae isolates. Molecular tests are rapid but expensive method for detection of blaKPC in K. pneumonia isolates and are used to confirm
the phenotypic results. However, they cannot predict the other causes of carbapenem resistance. So we recommend the use of disc containing ETP/BA as a cheap, accurate screening test for detection of KPC-producing organisms for the rapid therapeutic considerations that may largely impact patient management and for the implementation of infection control measures especially at institutions experiencing increasing rates of carbapenem resistance Enterobacteriaceae. Confirmatory molecular investigations for genes responsible for carbapenem resistance including \( \text{bla}_{\text{KPC}} \) should follow to identify the pattern of resistance.

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