Full Length Research Paper

Evaluation of a real-time PCR assay for the detection of the *Klebsiella pneumoniae* carbapenemase gene (*bla*KPC) in enterobacteriaceae isolates from clinical samples in Menoufia University hospitals, Egypt

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The aim of our work is to study and evaluate a rapid method for detection of *Klebsiella pneumoniae* carbapenemases genes (*bla*KPC) in enterobacteriaceae isolates from clinical samples by using real time PCR and comparison of this method with ordinary phenotypic methods. Outbreaks of carbapenem-resistant enterobacteriaceae (CRE), primarily *K. pneumoniae*, have been reported recently in several regions worldwide. The production of carbapenemases especially *K. pneumoniae* carbapenemase (KPC) is the most important mechanism of enzymatic resistance in enterobacteriaceae. One hundred and fifty clinical isolates from different departments of Menoufia university hospitals were tested by both disc diffusion method (Imipenem 10 μg, Meropenem 10 μg and Ertapenem 10 μg), and imipenem E-test for minimum inhibitory concentration (MIC) then analyzed according to cut off-points of CLSI 2014 guideline. Then all the one hundred fifty clinical isolates were tested for the presence of a *bla*KPC gene by real time PCR. We found Eighty three (83) isolates (55.3%) from 150 were resistant to one or more carbapenems by disk diffusion method, and 88 isolates (58.7%) were resistant by E test while 91 isolates (60.6%) were positive for the presence of *KPC* gene by real time PCR. There was significant difference between disk diffusion method and real-time PCR (*P* < 0.001) and E test and real-time PCR (*P* < 0.001) regarding carbapenem resistance. The highest percent of enterobacteriaceae isolates having *KPC* gene were among *K. pneumoniae* (46.1%). *KPC* positive cases were mainly (74.1%) from urology department. About (97.8%) *bla*KPC PCR positive cases had been exposed to invasive procedures such as mechanical ventilation (*P* < 0.001), and (95.6%) *bla*KPC PCR positive cases had been from hospital acquired infections (*P* < 0.001). There was a history of antimicrobial intake in 70.3% of cases infected with *KPC* PCR positive isolates. *bla*KPC PCR has sensitivity, specificity, negative predictive value, and diagnostic accuracy (99, 87, 98 and 93%), respectively.  

Key words: carbapenem-resistant enterobacteriaceae (CRE), real time PCR, *bla*KPC.

INTRODUCTION

Carbapenems are highly efficacious drugs for treating infections with extended-spectrum β-lactamase–producing gram-negative bacteria. Previously, resistance to carbapenems has been rare; however, the emergence of transmissible carbapenem resistance is now a growing concern. (Raghunathan et al., 2011).
An increasingly common mechanism of carbapenem resistance is the class-A, 
*Klebsiella pneumoniae* carbapenemase (KPC). KPCs have been reported in *K
pneumoniae* and in *Klebsiella oxytoca, Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis,
Citrobacter freundii, Enterobacter spp, Serratia spp, and
Salmonella spp.* (Villegas et al., 2006).
The *blaKPC* genes that encode KPCs are present on 
transferable plasmids and are flanked by transposable 
elements, thus allowing for the gene to move from 
plasmid to the bacterial chromosome and back (Bratu et 
al., 2005).
This potential to disseminate resistance has been 
demonstrated in several reported outbreaks with high
mortality rates (Raghunathan et al., 2011).
Given the limited therapeutic options available, the 
accurate and timely detection of KPC-producing 
enterobacteriaceae is vital in order to control their spread (Nordmann et al., 2009).
The mechanisms of resistance to carbapenems may be 
related to the combination of decrease in bacterial outer 
membrane permeability, increasing production of 
Extended-Spectrum Beta-Lactamases (ESBLs), AmpC 
beta-lactamases and expression of beta-lactamases like  
Carbapenemases. The production of carbapenemases 
especially KPC is the most important mechanism of 
enzymatic resistance in isolated Enterobacteriaceae such as 
*K. pneumoniae* (Nordmann et al., 2012).
The detection of KPC-producing bacteria can be 
challenging because of heterogeneous expression of 
β-lactam resistance. Automated and agar diffusion 
methods of susceptibility testing show some inconsistencies in 
reliably detecting KPC-mediated resistance, and this is 
influenced by the carbapenem that is used for testing 
(Francis et al., 2012; Fallah et al., 2013).
To address these issues, confirmatory tests such as 
several polymerase chain reaction (PCR)-based assays 
have been developed to detect KPC-mediated 
carbapenem resistance. Real-time PCR has been 
employed in the rapid detection of colonization/infection 
with KPC-producing Enterobacteriaceae in various types 
of samples and clinical isolates.
These assays have demonstrated good sensitivity and 
specificity with favorable positive and negative predictive 
values (Schechner et al., 2009).

**PATIENTS**

This study was conducted on clinical isolates of 
enterobacteriaceae that were isolated from samples sent 
to microbiology laboratory of Menoufia University 
Hospitals, from July 2013 to September 2014.

**Subjects**

One hundred fifty clinical isolates from different 
departments of Menoufia university hospitals were tested. 
During the study period, all strains of enterobacteriaiae isolated were stored on broth glycerol at-70ºC for 
subsequent PCR analysis.

**METHODS**

Enterobacteriaceae isolates were identified by conventional methods such as culture characteristics and biochemical reactions (Colle et al., 1996). Triple sugar iron agar (TSI), lysine iron agar (LIA), motility indole ornithine (MIO), Simmons citrate agar and urea agar base (Oxoid England) plus identification by API 20E (https://apiweb.biomerieux.com).

**Susceptibility testing**

0.5 McFarland turbidity suspension for each isolate was used to inoculate on Mueller-Hinton agar plates (Oxoid England).

**Disk diffusion**

By using imipenem, meropenem and ertapenem disk diffusion. Results were categorized as sensitive, intermediate and resistant 
as according to Clinical Laboratory Standard Institute (CLSI) 

**E-Test for imipenem**

MIC for imipenem was determined using E-test (bioMérieux) and results were categorized as sensitive, intermediate and resistant as per Clinical Laboratory Standard Institute (CLSI) guidelines 2014 (S ≤1, I: 2, R ≥4) (CLSI, 2014)

**Genotypic detection of KPC**

All clinical isolates of enterobacteriaceae were tested for the 
presence of a *blaKPC* gene by real time PCR:

**DNA extraction**

DNA extraction using the GeneJET Genomic DNA Purification Kit 
(Thermo Scientific K0721, Fermentas, UE). using Gram-negative 
bacteria genomic DNA purification protocol.

**Sample preparation**

After an overnight pure growth on MacConkey, 2 to 3 of bacterial 
colonies were inoculated into 1 ml of nutrient broth water then
overnight incubation.

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Table 1. Reaction setup for real-time.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/reaction (μl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x HOT FIREPOL EvaGreen qPCR Mix plus</td>
<td>4</td>
<td>1x</td>
</tr>
<tr>
<td>Primer forward (10 pmol/μl)</td>
<td>0.5</td>
<td>0.25 uM</td>
</tr>
<tr>
<td>Primer reverse (10 pmol/μl)</td>
<td>0.5</td>
<td>0.25 uM</td>
</tr>
<tr>
<td>Template DNA</td>
<td>5</td>
<td>0.01-10 ng/μl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Total reaction volume</strong></td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

Real-time PCR amplification and detection

Real-time PCR was performed on Spartan RX CYP2 C 19 instrument using Syber Green with the following primers (Raghunathan et al., 2011): Forward primer, 5'-ATGTCACGTATCGCCGTC-3' (80-250 nM final concentration), Reverse primer, 5'-CTCAGTCTGCTACAGAAAAC-3' (80-250 nM final concentration) and 5x HOTFIREPol®EvaGreen®qPCR Mix Plus (no ROX) –Solis biodyne– Cat 08-25-00001, a reaction mix was prepared according to Table 1.

Method

1. The reaction mix was mixed thoroughly, and appropriate volume was dispensed into PCR tubes or plates.
2. Template DNA was added (0.01 to 10 ng/μl) to the individual PCR tubes or wells containing the reaction mix.
3. Real-time instrument Spartan was programmed according to the following cycling conditions: 95°C for 15 s, 57°C for 20 s, and 72°C for 20 s. And finally dissociation at 95°C for 30 min.

RESULTS

Eighty three (83) isolates (55.3%) from 150 were resistant to one or more carbapenems by disk diffusion method and sixty seven were sensitive (Table 2).

This table shows that 88 isolates (58.7%) from all enterobactericea isolates (150) were resistant by E test and 62 (41.3%) were sensitive (Table 3). This table shows that 91 isolates (60.6%) from all enterobactericea isolates (150) were positive for the presence of KPC gene by real-time PCR and 59 (39.4%) were negative (Table 4). In this study, the melting temperature was 81.9°C (Figure 3), the positive cases for the presence of blaKPC gene show melting peaks that express fluorescence exceeding the threshold line (Figure 1) while negative cases express fluorescence that does not exceed the threshold line (Figure 2).

This table shows that from 83 carbapenem resistant isolates by disk diffusion, 82 were blaKPC PCR positive and from 67 carbapenem sensitive isolates, 9 were blaKPC PCR positive, and there was highly significant difference (p<0.001) between two methods (Table 5).

This table shows that from (88) resistant isolates by E-test, 82 (93.2%) were blaKPC PCR positive cases from 62 carbapenem sensitive isolates, 9 were blaKPC PCR positive, and there was highly significant difference (p<0.001) between two methods (Table 6).

Real time PCR detected 82 (93.2%) of carbapenem resistant isolates by DDT, thus the sensitivity of the PCR was 99%, specificity was 87% and diagnostic accuracy was 93%, all in relation to DDT as gold standard test (Table 7).

Real time PCR detected 82 (93.2%) of E-test resistant isolates, thus the sensitivity of the PCR was 93.2%, specificity was 85.5% and diagnostic accuracy was 90%, all in relation to E-test as gold standard test (Table 8). As regarding evaluation of the performance of the real-time PCR using disk diffusion susceptibility results, there were 2 cases which were negative for blaKPC by PCR and in the same time were resistant by disk diffusion (Table 9).

The highest percent of enterobactericeae isolates having KPC gene were among K.pneumoniae (46.1%)
Figure 1. Real time PCR amplification curve as shown by Step 1 Applied Spartan Real time PCR equipment. Positive KPC: G3, G8 and G11 expressing fluorescence exceeding the threshold line. Negative case: G5 expressing fluorescence that does not exceed the threshold line.

Figure 2. Negative results (G1, G3, G5, G7 and G9).

Figure 3. Melting point.
Table 5. Relation between disk diffusion and real-time PCR results in detection of carbapenem resistance.

<table>
<thead>
<tr>
<th></th>
<th>DDT</th>
<th>Total</th>
<th>Symmetrical measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CR</td>
<td>CS</td>
<td></td>
</tr>
<tr>
<td><strong>PCR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>Count</td>
<td>82 (90.1)</td>
<td>9 (9.9)</td>
</tr>
<tr>
<td></td>
<td>%Within DDT</td>
<td>98.8%</td>
<td>13.4%</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>Count</td>
<td>1 (1.7)</td>
<td>58 (98.3)</td>
</tr>
<tr>
<td></td>
<td>%Within DDT</td>
<td>1.2%</td>
<td>86.7%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>Count</td>
<td>83</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>%Within</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Symmetrical measurement: p< 0.05 = significant ; PCR, polymerase chain reaction; CR, Carbapenem resistant; CS, Carbapenem sensitive.

Table 6. Relation between E-test and PCR results in detection of carbapenem resistance.

<table>
<thead>
<tr>
<th></th>
<th>DDT</th>
<th>Total</th>
<th>Symmetrical measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CR</td>
<td>CS</td>
<td></td>
</tr>
<tr>
<td><strong>PCR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>Count</td>
<td>82 (90.1%)</td>
<td>9 (9.9%)</td>
</tr>
<tr>
<td></td>
<td>%Within MIC</td>
<td>93.2</td>
<td>14.5</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>Count</td>
<td>6 (10.2%)</td>
<td>53 (89.8%)</td>
</tr>
<tr>
<td></td>
<td>%Within MIC</td>
<td>6.8</td>
<td>85.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>Count</td>
<td>88</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>%Within MIC</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 7. Clinical performance of PCR in relation to disk diffusion test (DDT) as gold standard test.

<table>
<thead>
<tr>
<th>Item</th>
<th>TP</th>
<th>FN</th>
<th>TN</th>
<th>FP</th>
<th>Sens.</th>
<th>Spec.</th>
<th>PPV</th>
<th>NPV</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>82</td>
<td>1</td>
<td>58</td>
<td>9</td>
<td>99%</td>
<td>87%</td>
<td>90%</td>
<td>98%</td>
<td>93%</td>
</tr>
</tbody>
</table>

TP, True positive; FN, false negative; TN, true negative; FP, false positive; Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value.

Table 8. Clinical performance of PCR in relation to E- test as gold standard test.

<table>
<thead>
<tr>
<th>Item</th>
<th>TP</th>
<th>FN</th>
<th>TN</th>
<th>FP</th>
<th>Sens.</th>
<th>Spec.</th>
<th>PPV</th>
<th>NPV</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>82</td>
<td>6</td>
<td>53</td>
<td>9</td>
<td>93.2%</td>
<td>85.5%</td>
<td>90%</td>
<td>90%</td>
<td>90%</td>
</tr>
</tbody>
</table>

TP, True positive; FN, false negative; TN, true negative; FP, false positive; Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value.

Table 9. Comparison of disk diffusion (imipenem/meropenem or etrapenem) and KPC real-time PCR results.

<table>
<thead>
<tr>
<th>KPC real-time PCR</th>
<th>Susceptibility by disk diffusion (imipenem/meropenem)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>83</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>57</td>
</tr>
</tbody>
</table>

TP, True positive; FN, false negative; TN, true negative; FP, false positive; Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value.
Table 10. Types of KPC PCR positive isolates.

<table>
<thead>
<tr>
<th>Organism</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. pneumoniae</td>
<td>42</td>
<td>46.1</td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td>31</td>
<td>34.1</td>
</tr>
<tr>
<td>E. coli</td>
<td>16</td>
<td>17.6</td>
</tr>
<tr>
<td>Proteus</td>
<td>2</td>
<td>2.2</td>
</tr>
<tr>
<td>Total</td>
<td>91</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 11. Distribution of KPC PCR positive isolates in hospital units.

<table>
<thead>
<tr>
<th>Unit</th>
<th>Total</th>
<th>$\chi^2$ test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICU</td>
<td>34(37.4%)</td>
<td>57.6%</td>
<td>20(21.9%)</td>
</tr>
<tr>
<td>Urology</td>
<td>25(42.4%)</td>
<td>3(3.4%)</td>
<td>7(11.8%)</td>
</tr>
<tr>
<td>Neonatology</td>
<td>59(39.3%)</td>
<td>6(4.0%)</td>
<td>6(4.0%)</td>
</tr>
<tr>
<td>Others</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 12. Distribution of KPC PCR positive isolates in relation to exposure to invasive procedures.

<table>
<thead>
<tr>
<th>Invasive procedures</th>
<th>PCR results</th>
<th>Total</th>
<th>$\chi^2$ test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>P</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%Within MIC</td>
<td>82(90.1%)</td>
<td>9(9.9%)</td>
<td>91(100%)</td>
<td>60.6%</td>
</tr>
<tr>
<td>No</td>
<td>%Within MIC</td>
<td>6.8</td>
<td>85.5</td>
<td>39.4</td>
</tr>
<tr>
<td>Total</td>
<td>Count</td>
<td>88</td>
<td>62</td>
<td>150</td>
</tr>
<tr>
<td>%Within MIC</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

while the lowest percent were among proteus (2.2%) (Table 10) KPC positive cases were mainly (74.1%) from urology department which represented (21.9%) of their isolates, followed by ICU (57.6%) which represented (37.4%) of their isolates (Table 11). About (97.8%) blaKPC PCR positive cases had been exposed to invasive procedures such as mechanical ventilation, urinary catheterization, CVL and cannula insertion (Table 12). About (95.6%) blaKPC PCR positive cases had been isolated from cases with hospital acquired infections (Table 13).There was a history of antimicrobial intake in 70.3% of cases infected with KPC PCR positive isolates (Table 14).

DISCUSSION

The emergence and rapid dissemination of carbapenem-resistant enterobacteriaceae (CRE) worldwide is a cause for concern. Treatment options for infections due to these organisms are extremely limited and effective therapy may be delayed whilst microbiology laboratory confirmatory results are awaited (Burns and Schaffer, 2011).

The rapid detection of KPC-producing enterobacteriaceae is of great importance since these organisms have the potential to spread rapidly in hospital environments and cause nosocomial infections with high mortality rates (Samra et al., 2007; Tibbetts et al., 2008; Burns and Schaffer, 2011). The aim of our study is to study a rapid method for detection of K. pneumoniae carbapenemase genes (blaKPC) in enterobacteriaceae isolates in clinical samples by using real time PCR and comparing phenotypic with genotypic results.

In our study 83 (55.3%) of 150 samples had reduced susceptibility to one or more carbapenems. This is similar to a study conducted by Landman et al. (2005) in New York, where (61.5%) of lactose fermenting gram-negative bacilli were imipenem resistant by disc diffusion. In a study conducted by Patel et al. (2008) in Europe and Hindiyeh et al. (2008) in Israel, the carbapenem-resistant K. pneumoniae by disc diffusion was 26 and 25.1%, respectively.
On the other hand, resistance reported by Marschall et al. (2009) was 2.9%, also in the Faculty of Medicine Vajira Hospital in University of Bangkok, Metropolis, the incidence of CRE was 0.13% and the presence of the resistance was an important public health problem (Phumisantiphong, 2011). For certain reasons there is a wide variability in prevalence of CRE. The possible factors could be different geographical locations, variable proficiency levels of microbiology trained technical staff, different antibiotic cut offs being used, different guidelines being followed and different techniques being used for CRE detection.

In several western studies, prevalence of CRE was less than that found in our study. The higher prevalence compared to western countries can be explained by the fact that western countries had strict infection control policies and practices, efficient and effective antibiotic audit systems, shorter average hospital stay, better nursing barriers and other important health care measures that are known to substantially decrease the chances of acquisition and spread of CRE.

There are several factors that make detection of CRE by susceptibility testing is challenging and make carbapenem-resistant bacteria incorrectly identified as carbapenem susceptible, resulting in inappropriate selection of therapy. One of these factors may be the heterogeneous expression of β-lactam resistance (Chen et al., 2011) or the low level of resistance that cannot be detected by ordinary susceptibility tests Thomson (2010).

In addition, Meropenem and imipenem susceptibility demonstrated poor sensitivity for methods other than BMD (broth microdilution). However, the specificity of meropenem and imipenem susceptibility testing was higher than that for ertapenem susceptibility testing regardless of test method (Benenson et al., 2011). The ertapenem disk-diffusion test has been shown to be a reliable screening method for KPC-mediated resistance (Bratu et al., 2005). Therefore, results for culture-based susceptibility to ertapenem are often used for determining carbapenem resistance in routine clinical microbiology laboratories. However, resistance to ertapenem alone is not a marker for KPC expression; it has been shown that most ertapenem resistance is related to factors such as an extended-spectrum β-lactamase (ESBL) or AmpC production in association with outer membrane porin mutations (Francis et al., 2012).

In order to overcome such shortcomings when treating infections caused by enterobacteriaceae, the Clinical and Laboratory Standards Institute (CLSI) has recently lowered the susceptibility breakpoints for meropenem, imipenem, ertapenem and doripenem (Chen et al., 2011). Also, the presence of scattered inner colonies along the inhibition zone can lead to perceived increased resistance. The presence of scattered colonies may be

<p>| Table 13. Distribution of PCR results in relation to hospital acquired infection. |
|-----------------------------------------------|---------------|-------------|-------------|-------------|</p>
<table>
<thead>
<tr>
<th>PCR results</th>
<th>P</th>
<th>N</th>
<th>Total</th>
<th>χ² test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>Count</td>
<td>87(85.3%)</td>
<td>15(14.7%)</td>
<td>102(100%)</td>
<td>81.02</td>
</tr>
<tr>
<td>%Within PCR results</td>
<td>95.6%</td>
<td>25.4%</td>
<td>68.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>Count</td>
<td>4(8.3%)</td>
<td>44(91.7%)</td>
<td>48(100%)</td>
<td>3.12</td>
</tr>
<tr>
<td>%Within PCR results</td>
<td>4.4%</td>
<td>74.6%</td>
<td>32.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>Count</td>
<td>91(60.6%)</td>
<td>59(39.4)</td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td>%Within PCR results</td>
<td>100</td>
<td>100</td>
<td>68.0%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<p>| Table 14. Distribution of KPC PCR positive isolates in relation to antimicrobial intake. |
|-----------------------------------------------|---------------|-------------|-------------|-------------|</p>
<table>
<thead>
<tr>
<th>PCR results</th>
<th>P</th>
<th>N</th>
<th>Total</th>
<th>χ² test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>Count</td>
<td>64(56.6%)</td>
<td>49(43.4%)</td>
<td>113(100%)</td>
<td>3.12</td>
</tr>
<tr>
<td>%Within PCR results</td>
<td>70.3%</td>
<td>83.1%</td>
<td>75.3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>Count</td>
<td>27(73.0%)</td>
<td>10(27.0%)</td>
<td>37(100%)</td>
<td>29.7%</td>
</tr>
<tr>
<td>%Within PCR results</td>
<td>29.7%</td>
<td>16.9%</td>
<td>24.7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>Count</td>
<td>91(60.6%)</td>
<td>59(39.4)</td>
<td>150</td>
<td>100</td>
</tr>
</tbody>
</table>
due to decreased expression of the porin channel OmpK36, which has been found in isolates expressing blaKPC (Bulik et al., 2010).

Our study showed that the resistant isolates to imipenem by E-test were 88 out of 150 (58.7%). While in a study conducted by Gupta et al. (2011) in New York City, carbapenem resistance by E-Test was reported in (10.8%) of isolates that were associated with certain device-related infections.

Girlich et al. (2013) found on 133 well-characterized enterobacterial isolates, KPC and meropenem-containing MP/ MPI Etest had high sensitivity (>92 %) and specificity (>97 %).

Although we included the E test method in our study, but determining resistance and susceptibility for imipenem with E test was difficult because of the colonies that were present within the zones of inhibition and make lack of consensus on reading E test method.

In Rapp and Urban (2012), most KPC-producing isolates had a carbapenem MIC ≥ 2 µg/ml, but some have been reported to be susceptible to carbapenems. The reason for this discrepancy is that full resistance to carbapenems usually requires the presence of a second mechanism of resistance such as a defect in the permeability of outer membrane. Detection of blaKPC genes by PCR has been proposed as the gold standard for detection of KPC-bearing organisms. To date, several PCR based detection methods have been described, including two real-time PCR assays, as well as a method that uses PCR in conjunction with electrospray ionization mass spectrometry (PCR/ESI-MS) (Chen et al., 2011).

Currently DNA sequencing is the definitive method for identification of blaKPC gene. However, sequencing is impractical for studies involving large sample sizes, as well as for rapid identification in clinical settings. In contrast, methods such as real-time PCR offer rapid, robust, and cost-efficient alternatives to DNA sequencing for blaKPC gene (Chen et al., 2011).

Our study showed that 91 out of 150 (60.6%) had KPC gene by real-time PCR. This result is higher than that from a United State (0.5%) (Deshpande et al., 2006). However, prevalence rates of (KPC-Kp) isolates of >30% have been recorded in the eastern United States (Nordmann et al., 2011). Another study of Brooklyn hospitals reported 38% prevalence of blaKPC (Landman et al., 2007). In this study, there were 2 cases which were negative for blaKPC by PCR and in the same time were resistant by disk diffusion. These results were not due to inhibition of the PCR reactions because the internal control target was successfully amplified.

The negative PCR result with the presence of disk diffusion resistance can be due to presence of eleven types or alleles of KPC gene (KPC1-11) while we used only single primers for detection of blaKPC (Arnold et al., 2012), or it is possible that the carbapenem resistance was due to one of several other mechanisms, including changes in outer membrane permeability, increased activity of antibiotic efflux systems, or the production of AmpC β-lactamases, ESBLs, or non-KPC carbapenemases (Queenan and Bush, 2007).

In addition, it is also possible that the amount of template DNA for these samples was inadequate or that sequence alterations in the bla KPC gene affected the binding of the primers or probes used in the assay (Francis et al., 2012).

One of the disadvantages of PCR is specificity for the particular target sequence, so they cannot be used to monitor the emergence of novel variants (Raghunathan et al., 2011).

In this study, out of the 91 blaKPC positive isolates 46.1% were K. pneumonia. These results are in accordance with those from a study of Landman et al. (2007), where (95%) of blaKPC positive isolates were K. pneumoniae. Another study conducted by Qi et al. (2011) shows that all clinical isolates of K. pneumoniae (100%) with carbapenem resistance were confirmed as KPC producers by PCR.

This is different from study done by (Francis et al., 2012), who showed that the most common KPC positive enterobacteriaceae was Escherichia coli (44.9%), and a study done by Marschall et al. (2009), where (37.0%) of isolates were E. coli.

K. pneumoniae remains the most prevalent bacterial species carrying KPCs. The rapid global spread of KPC-producing K. pneumoniae is now understood, it is a largely clonal phenomenon. A specific clone of KPC-producing K. pneumoniae, called ST258, is globally distributed. ST stands for sequence type, and is assigned by multilocus sequence typing, which is a nucleotide sequence-based bacterial typing method where seven genes on the chromosome are sequenced. ST258 predominates among KPC-producing K. pneumoniae in the United States. ST258 as well as ST512, which is closely related to ST258, has been found commonly in Israel and Italy, whereas ST11 and ST437 appear to predominate in China and Brazil, respectively (Doi and Paterson, 2015).

These STs are all closely related to ST258 suggesting the presence of a common origin, most likely in the mid-Atlantic United States. On the other hand, plasmids carrying the KPC gene are diverse in structure and often capable of self-transmission to other strains by conjugation (Doi and Paterson, 2015).

In addition, the production of carbapenemases especially KPC is the most important mechanism of enzymatic resistance in isolated enterobacteriaceae such as K. pneumoniae. KPCs are encoded by the gene blaKPC, whose potential for different species and universal spreading is mainly elucidated by its location within a Tn3-type transposon, Tn4401. This transposon is able to inserting into varied plasmids of Gram-negative bacteria. Plasmids carrying bla-KPC are related to resistance factors for other antibiotics, the enzyme has been identified in several other Gram-negative bacilli (Bina et
Hospital acquired infections (HAI) are defined as infections not present at the time of admission to hospital. Most infections that become clinically evident after 48 h of hospitalization are considered hospital-acquired (Edwards et al., 2008).

In our study, we found that 95.6% of KPC PCR positive isolates were from hospital acquired infection cases and this was also statistically highly significant (P < 0.001). This was in accordance with Aggeliki et al. (2012) who reported the recent emergence of carbapenemase-producing enterobacteriaceae strains which represented a major threat for hospitalized patients in Greek hospitals, and also show that duration of hospitalization before bacteremia was the only risk factor for multidrug bloodstream infections.

The description of outbreaks indicates that producer strains seem to benefit from selective advantages in hospitals where antimicrobial use is much higher and opportunities for transmission more frequent than in the community (Grundmann et al., 2010). According to Papadimitriou-Oliveris et al. (2012) there was no patient positive for blaKPC without prior hospitalization or antibiotic use before ICU admission.

In our study we found that the high percentage of KPC-producing enterobacteriaceae spp. by PCR was found in patients with history of antibiotic administration (70.3%). This also coincides with Gasink et al. (2009) who found a correlation between the selective pressure of antimicrobial agents and the presence of KPC resistance genes residing on the plasmid.

According to Woodford et al. (2010) and Gasink et al. (2009) it was postulated that prior use of an extended-spectrum cephalosporin and ciprofloxacin may be selective for KPC enzymes and also it was reported by Kwak et al. (2005) that the previous use of carbapenems and cephaplorins were identified as independent risk factors for acquisition of carbapenem resistant K. pneumonia. In rectal and tracheal KPC- K. pneumoniae colonized patients, prolonged antibiotic therapy administered for non KPC-Kp infection predisposes patients to subsequent KPC-Kp ventilator associated pneumonia (VAP). Short prophylaxis of early pneumonia with amoxicillin/clavulanic acid, reducing the need for subsequent antibiotic use, may be associated with reduced risk for KPC-Kp VAP (Sbrana et al., 2016).

According to the study of Tuon et al. (2012), Fluoroquinolones were an independent risk factor for KPC production, which might be explained by the fact that plasmid-encoded qnr genes, which determine low-level fluoroquinolone resistance, have been identified in the same conjugative K. pneumonia plasmid as CP genes (specifically blaKPC-2 and qnrB4).

In our study, we found that (97.8%) of KPC PCR positive isolates had been exposed to invasive procedures such as mechanical ventilation, urinary catheterization, central venous line (CVL) and cannula insertion, and this was statistically highly significant (P < 0.001).

This is in accordance with Lee (2012) who reported that mechanical ventilation is a risk factor for infection with KPC producing organisms, and Kwak et al. (2005) who reported that catheterization is a risk factor for carbapenem-resistant acquisition.

KPC positive cases were mainly from urology department (74.1%) followed by ICU (57.6%). This is similar to that reported by Lee (2012) who showed that KPC positive cases were mainly isolated from patients who had high ICU admission status (72%) this may be due to that most of ICU patients were immunocompromised and may be due to the selective pressure imposed by extensive use of antimicrobials and the potential for patient-to-patient transmission of organisms was greatest.

Our study showed that 82 (98.8%) of carbapenem resistant isolates by using disk diffusion method, were blaKPC PCR positive cases. We found that the sensitivity of the PCR was 99%, specificity was 87%, PPV of 90%, NPP of 98% and diagnostic accuracy was 93%, all in relation to DDT as gold standard test.

This is similar to Hindiyeh et al. (2011) who reported that real-time PCR assay is sensitive and specific compared with culture-based methods of detecting carbapenem resistance attributable to KPC.

Also, Cole et al. (2009) reported that direct detection of blaKPC by PCR shorten the time to identify patients colonized or infected with carbapenem resistant organisms and is more sensitive than culture.

Conclusion

Real time PCR for detection CRE through detection of blaKPC gene in enterobacteriaceae, is a sensitive, accurate, and rapid method with a shorter turnaround time than those with culture based protocols. Beside it has high negative predictive value to rule out the resistance to cabapenems.

Conflict of Interests

The authors have not declared any conflict of interests.

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