Occurrence of *Klebsiella pneumoniae* carbapenemase *KPC* gene in *Klebsiella pneumoniae* isolated from patients in Anbar city of Iraq.

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Abstract:

Carbapenems are the last drugs of choice except colistin against serious infections caused by Gram-negative bacteria. However, there are increasing number of reports indicating prevailing emergence of *KPC*-producing clinical isolates worldwide, which is of harmful genes because many plasmids that carry *KPC* resistance elements concurrently carry other plasmid-mediated resistance elements, such as quinolone (QnrA and QnrB) and aminoglycoside (rmtB) resistance. This study reports *KPC* for the first time among *Klebsiella pneumoniae* from hospitalized patients in Anbar, Iraq. Six clinical isolates of *K. pneumoniae* resistant to carbapenem from 50 isolates were investigated from burned wounds, sputum, C.S.F, and blood samples. The susceptibility to different antibiotics was tested by VITEK-2 system. Where the percentage of resistance to Carbapenem was 22%. The phenotypic detection of carbapenemases by Modified Carba NP, Blue-CARBA Test that all isolates are carbapenem gene-producing, 5/6 (83.33%) gave positive result with the imipenem-EDTA test, and modified Hodg test. The bla*KPC* and other genes were detected by multiplex PCR and the result showed 1/6 (16.67%) strains positive for bla*KPC* gene and 5/6 (83.33%) strains harbored bla*VIM* and bla*OXA-48* genes. Our results showed the coexistence of both bla*VIM* and bla*OXA-48* genes in four strains of *K. pneumoniae*, while indicated widespread *KPC*, *VIM*, and *OXA-48* in Anbar, Iraq. Hence, it is necessary to follow proper infection control practices and physicians should be aware of the patients with such risk factors.

Key words: *K. pneumoniae*, bla*–KPC*, carbapenem-resistance, and bla–*VIM*.


Introduction:

*Klebsiellapneumoniae* is Enterobacteriaceae family member causing serious infections⁴, such as respiratory tract infections, blood stream infections, and urinary tract infections⁵. *K. pneumoniae* is viewed to be the second most prevalent cause of nosocomial Gram-negative pathogen after *Escherichiacoli*. Problems related to multidrug-resistant (MDR) organisms or superbugs are worrisome these days as they are becoming increasingly serious⁶. Carbapenem-resistant Enterobacteriaceae (CRE) is emerging, and carbapenem-resistant *Klebsiellapneumoniae* (CRKP) is the most common CRE detected. Since its first detection in 1996 in North Carolina, CRKP has been reported worldwide, including in Asia⁴. Broad-spectrum carbapenems are often considered as last therapeutic choices for treatment of infections due to multidrug-resistant Gram-negative bacteria⁵. Risk factors for infections caused by carbapenemases-producing strains of *K. pneumoniae* include long hospital stay, and prior administration of antibiotics, especially broad-spectrum cephalosporins and β-lactamase inhibitor combinations⁶. The emergence of carbapenem-resistant Enterobacteriaceae is increasingly notified worldwide and is becoming an important topic in health-care systems⁷. In *K.
pneumoniae, resistance to carbapenems is mainly related to the production of carbapenem-hydrolyzing β-lactamase\(^8\). Recent studies in prevalence of KPC, OXA-48, and metallo-β-lactamase (VIM) producing strains among K. pneumoniae in different countries have been carried out. Transmission of these genes to other gram-negative bacteria should be considered because they are located on mobile genetic elements like transposons and plasmids\(^9\).

Nevertheless, high population density in Al-Ramadi city and its high percentage of pollution as a result of the destruction caused by wars and military operations, the knowledge of the carbapenemases is still limited at presence. In Iraq, little attention has been paid to β-lactamases producing isolates. However, in Ramadi city, there is no information regarding the molecular studies of the occurrence of carbapenemases-producing K. pneumoniae recovered from clinical cases. The aim of this study was to determine the presence of serine and metallo-β-lactamase (MBL) genes including KPC, OXA-48, and VIM genes among carbapenem-resistant K. pneumoniae isolated from hospitalized patients in two hospitals in Anbar, Iraq.

**Material and Methods:**

**Bacterial strains and susceptibility testing**

Fifty K. pneumoniae clinical isolates were isolated from burned wounds, sputum, C.S.F, and blood samples of hospitalized patients in hospitals in Al-Anbar city. These strains were isolated through a period extended from July 2018 to December 2018. Identification of K. pneumoniae was performed by conventional and automated (VITEK-2 system, bioMérieux, France) methods using ID-GNB cards according to the manufacturer’s instructions.

Antibiotic susceptibility testing was performed by automated VITEK-2 system (bioMérieux, France) for the following antibiotics: Ticarcillin, Ticarcillin/clavulanic acid, Piperacillin, Piperacillin/Tazobactam, Ceftriaxone, Ceftazidine, Cefipime, Aztreonam, imipenem, meropenem, Amikacin, ciprofloxacin, gentamicin, tobramycin, and trimethoprim/sulfamethoxazole using AST cards according to the manufacturer’s instructions.

**Phenotyping Methods:**

**Modified Carba NP test:**

Bacterial strains were cultivated onto Trypticase soy agar (TSA) at 37 °C for 18–24h. A 10\(\mu l\) calibrated loop full of the test strain was inoculated into 500 \(\mu l\) Tris–HCl (20mM–pH 7.5) The suspension was subjected to vortex homogenization. Then, 30\(\mu l\) of the cell extract was mixed with 100\(\mu l\) of phenol red (Isofar) containing 0.1mM ZnSO4 (meropenem −) and 100 \(\mu l\) phenol red containing 0.1mM ZnSO4 and 6mg/\(\mu l\) meropenem (Meropenem +). The mixtures were incubated at 37 °C for 2h. Tests were performed in duplicate for all isolates. The color change of the meropenem containing vial from red to yellow or orange indicated a positive result. Three independent observers recorded the results with no discordant readings\(^{10}\).

**Blue-CARBA Test**
In the Blue-Carba test variant, bromothymol blue was selected as the indicator, since it includes the optimal pH range (6.0 to 7.6) for most β-lactamases (pH 6.8), which was a key factor for a direct colony approach. A commercially and widely available imipenem (Meropenem : 500; Arwan, France) was used as the substrate for carbapenemases. The test solution consisted of an aqueous solution of bromothymol blue at 0.04% (Merck Millipore, Germany) adjusted to pH 6.0, 0.1 mmol/liter ZnSO₄, and 3 mg/ml of meropenem, with a final pH of 7.0. A negative-control solution (0.04% bromothymol blue solution, pH 7.0) was prepared to control the influence of bacterial components or products in the pH of the solution. A loop of a pure bacterial culture recovered from Mueller-Hinton agar (bioMérieux, France) was directly suspended in 100 µl of both test and negative-control solutions in a 96-well microtiter plate and incubated at 37°C with agitation (150 rpm) for 2 h. Carbapenemase activity was revealed when the test and negative-control solutions, respectively, were (i) yellow versus blue, (ii) yellow versus green, or (iii) green versus blue. Noncarbapenemase producers remained blue or green on both solutions.

**Modified Hodge Test :-**

For identification of carbapenemase-producing bacteria, all carbapenem-resistant strains and some imipenem and meropenem susceptible isolates were diagnosed and then examined with phenotypic MHT. Briefly, a 0.5 McFarland standard suspension of *Escherichia coli* ATCC 25922 was prepared and diluted 1:10 in broth, then inoculate on Mueller-Hinton agar plates for the routine disc diffusion procedure. Appropriate number of meropenem discs was placed on the plates, which followed by overnight growth of *Klebsiella pneumonia*, on a blood agar and inoculation in a straight line from the edge of the disc to edge of plate. Finally all plates incubated at 37°C for 18-22 hrs. Positive Strains with cloverleaf shape around (10) *E. coli ATCC 25922* was used as the carbapenem-susceptible strain and it was obtained from the Ministry of Science and Technology, Baghdad. *ATCC* (American Type Culture Collection)

**Modified Carbapenem Inactivation Methods for Suspected Carbapenemase Production :**

The CIM was performed as previously described the original protocol (10). Briefly, a suspension was made by suspending a full of 10 µl loop, cultured colony of tested isolate, in 2mlTSA. Subsequently, 10 µg meropenem disk was immersed in the suspension and incubated for a two hours at 35°C. After incubation the disk was removed from the suspension and placed on a Mueller-Hinton agar (MHA) plate inoculated with *Escherichia coli ATCC 29522* and subsequently incubated for overnight at 35°C. After this step, the absence of an inhibition zone indicates enzymatic hydrolysis of carbapenem (carbapenemase-positive), whereas a clear inhibition zone appears when the tested isolate does not express carbapenemase activity (carbapenemase-negative).

EDTAmCIM for each isolate, label a second 2ml TSB tube for the eCIM test. Add 20 µl of the 0.5 M EDTA to the 2ml TSB tube to obtain a final concentration of 5 mM EDTA. Follow steps and incubated for a two hours at 35°C. After incubation the disk was removed from the suspension and placed on a Mueller-Hinton agar (MHA) plate inoculated with *Escherichia coli ATCC 29522* and subsequently incubated for overnight at 35°C. After this step, the absence of an inhibition zone indicates serine beta-lactamase whereas a clear inhibition zone appears when the tested isolate metalo-beta lactamase.
Detection of Carbapenemase enzymes by double disk synergy tests (DDST)

Imipenem resistant *K. pneumoniae* isolates were tested by double disk synergy test (DDST) using Imipenem and Imipenem EDTA, as described by Lee et al. A lawn culture of the organism was inoculated onto MHA plate as per CLSI guidelines. An Imipenem disc(10ug) was placed at a distance of 10mm from a blank disc which contains EDTA (750ug). The plate was then incubated at 37 °C overnight\(^{(11)}\).

Molecular detection of carbapenemases by Multiplex PCR assay :

Genomic bacterial DNA was extracted from all Six carbapenem-resistant *K. pneumoniae* strains using a commercial purification system rapid bacterial genomic DNA isolation kit (bio-basic, Korea). Primers used in this study (Alpha DNA, Canada) were provided in lyophilized form then dissolved in sterile deionized distilled water (Table I).

**Table I. The sequences of primers used in this study**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers' Sequences (5'→3')</th>
<th>TM(°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>KPC</strong></td>
<td>F:TGTTGCTGAAGGAGTGGGC</td>
<td>59.4</td>
<td>340</td>
</tr>
<tr>
<td></td>
<td>R:ACGACGGCATAGTCATTGTC</td>
<td>57.3</td>
<td></td>
</tr>
<tr>
<td><strong>VIM</strong></td>
<td>F:CAGCGGAGATTGARAAGCAGAA</td>
<td>56.3</td>
<td>247</td>
</tr>
<tr>
<td></td>
<td>R:CACGGACCRGGATAGAAARA</td>
<td>59.4</td>
<td></td>
</tr>
<tr>
<td><strong>OXA48</strong></td>
<td>F:AACGGGCGGAACCAAGCATT</td>
<td>57.9</td>
<td>597</td>
</tr>
<tr>
<td></td>
<td>R:TGAGCTTCTTTTTGTGTGGCT</td>
<td>58.9</td>
<td></td>
</tr>
</tbody>
</table>

* F: Forward sequences,  R: Reverse sequences.

For PCR method, the initial denaturation phase for each PCR assay with different primers was established on 94°C for 5 min also denaturation was 94°C for 1 min. The annealing time was 1 min for all primers and temperature was 50 for bla KPC, bla VIM, bla and blaOXA-48, respectively. The extension time was 1 min in 72°C. The final extension for all genes was done at 72°C for 7 min.

The reaction of PCR consisted of 2 × of 25 Master mix (Bioneer, USA), 2 μl of each forward and reverse primers (bla KPC, bla VIM, and OXA-48), 3 μl of template DNA, and 5 μl PCR grade water to a final volume 20 μl. The products of PCR were electrophoresed for 1.5 hr and visualized with the aid of RedSafe staining (iNtRON, Korea) and UV transilluminator documentation system.

**Results**

**Bacterial strains :**

During the period of July 2018 to December 2018, Fifty *K. pneumoniae* were collected from Ramadi city in Al-anbar - Iraq. The collected isolates were from different clinical specimens (urine, wound, swabs, sputum, and blood) of in patients in this hospital. All isolates were identified by using the automated Vitek-2 system (Bio-Merieux)
Antibiotic susceptibility testing

The antibiotic susceptibility test revealed that (22%) carbapenem-resistant *K. pneumonia* from 50 clinical strains were multidrug-resistant and they were resistant to most antibiotics under test and it showed an elevated resistance to numerous classes of β-lactam and non-β-lactam antibiotics.

Table II

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>R (%)</th>
<th>I (%)</th>
<th>S (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ticarcillin</td>
<td>90</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Ticarcillin/clavulanic acid</td>
<td>66</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>98</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Piperacillin/Tazobactam</td>
<td>82</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>80</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>70</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Cefepem</td>
<td>70</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>46</td>
<td>0</td>
<td>54</td>
</tr>
<tr>
<td>Imipenem</td>
<td>22</td>
<td>0</td>
<td>78</td>
</tr>
<tr>
<td>Meropenem</td>
<td>22</td>
<td>0</td>
<td>78</td>
</tr>
<tr>
<td>Amikacin</td>
<td>36</td>
<td>0</td>
<td>64</td>
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<tr>
<td>Gentamicin</td>
<td>34</td>
<td>0</td>
<td>66</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>46</td>
<td>0</td>
<td>54</td>
</tr>
<tr>
<td>CIPFloxacin</td>
<td>38</td>
<td>0</td>
<td>62</td>
</tr>
<tr>
<td>Minocycline</td>
<td>7</td>
<td>0</td>
<td>93</td>
</tr>
<tr>
<td>Trimethoprim/sulfamethazole</td>
<td>64</td>
<td>0</td>
<td>36</td>
</tr>
</tbody>
</table>

Phenotypic detection of carbapenemases:

The phenotypic detection of carbapenemases by using : Modified Carba NP,Blue-CARBA Test*, modified Hodge test, modified carbapenem inactivation methods for Suspected Carbapenemase Production, and double disk synergy tests (DDST).

All resistance isolates showed the presence of the carbpenem genes by using Modified Carba NP, Blue-CARBA Test. The modified Hodge test was used as phenotypic confirmatory method for both serine and metallo-β-lactamases production, Therefore 5/6 (83.33) gave a positive result for this examination. Phenotypic detection of metallo-β-lactamases was performed by using the imipenem-EDTA disk method. Only three (50%) isolates demonstrated enhancement of inhibition zone.
In this study, modified carbapenem inactivation methods for Suspected Carbapenemase Production was used as phenotypic confirmatory method for distinguishing between serine (OXA-48 and KPC) and metallo-β-lactamases production. Where the results showed that 5/6 (83.33%) was serine β-lactamase while 1/6 (16.67%) was metallo-β-lactamase.
Figure 1. Phenotypic methods for the detection of carbapenemase-producing carbapenem-resistant organisms. (A) Modified Carba NP test. Tube a, no meropenem added, red, Tube b, meropenem added, orange. (B) Manual Blue Carba positive result. Tube a, meropenem added, yellow, Tube b, no meropenem added, blue. (C) Modified Hodge Test. (D) mCIM and eCIM results.

Molecular detection of bla KPC, VIM, and OXA-48 in Klebsiella pneumoniae by multiplex PCR.

The results showed the presence of a bla-KPC gene (340bp) in 1 (16.67%) carbapenem-resistant K. pneumoniae strains. On the other hand, 5 (83.33%) strains of carbapenem-resistant K. pneumoniae did not harbor bla-KPC. The results showed the presence of bla-VIM (247bp) and OXA-48 (597bp) in 5/6 (83.33%) carbapenem-resistant K. pneumoniae strains, and 1 (16.67%) of the strains not harboring (bla-VIM, bla-OXA-48).

Figure 2. Multiplex PCR amplification fragments for the detection of blaOXA-48 gene (597bp), bla KPC gene (340bp), and bla VIM (247bp) among carbapenem-resistant Klebsiella pneumoniae strains. Lanes K1 – K6: Klebsiella pneumoniae; Lane M: 100-bp DNA ladder; Lane C: negative control. Amplicons were electrophoresed on agarose gel (1%) at 70 V/cm for 1.5 h, stained with RedSafe (iNtRON, Korea), and visualized using an UV transilluminator documentation system.
Discussion:

Carbapenems are the drugs of choice against serious infections caused by Gram-negative bacteria (K. pneumonia, E. coli, P. aeruginosa and A. baumannii), but several studies have reported the prevalence of bla-KPC producing clinical strains worldwide. Among carbapenemase genes, the first KPC-producing K. pneumoniae isolate was reported in North Carolina, U.S. KPCs are now endemic in both Israel and Greece. Enterobacteriaceae-producing KPCs have also been reported in Brazil, China, Colombia, Norway, United Kingdom, India, Sweden, and more recently, Italy, Finland, Iran, Jordan, Egypt. KPCs are increasingly reported in other genera of the Enterobacteriaceae family, such as Escherichia, Proteus, Serratia, Salmonella, and Citrobacter. Worse still, KPC resistance has been reported in inherently-resistant organisms such as Acinetobacter baumannii and Pseudomonas spp.

The results showed that the phenotypic methods were identical to almost molecular methods. Different genes are involved in carbapenem resistance among Enterobacteriaceae, which may vary from country to country. In this study, blaKPC, bla OXA-48 and bla VIM genes were detected by Multiplex PCR and the result showed 1/6 (16.67%) strains harbored blaKPC gene, but only 5/6 (83.33%) strains harbored blaVIM and bla OXA-48 genes. Also, the results showed the coexistence of both blaOXA-48 and blaVIM genes in four strains of K. pneumonia under the study. Our report is in contrary with other reports from Baghdad and Najaf, where bla VIM OXA-48 genes were not reported in Iraq.

In conclusion, Iraq is also facing an alarming threat with the emergence of the imported KPC gene in Enterobacteriaceae which is considered a very dangerous gene because many plasmids that carry KPC resistance elements concurrently carry other plasmid-mediated resistance elements, such as quinolone (Qnr A and Qnr B) and aminoglycoside (rmt B) resistance. Hence, it is necessary to follow proper infection control practices and physicians should be aware of the patients with such risk factors. A multidisciplinary approach to limit the spread of such organisms is essential followed by prevention, detection, proper antimicrobial stewardship, and adequate infection control measures should help in limiting the spread of these organisms.

References:
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