Original Research Article

Detection of \( \text{bla}_{NDM} \) -Metallo-\( \beta \)-Lactamase Genes in \textit{Klebsiella pneumoniae} Strains Isolated From Burn Patients in Baghdad Hospitals

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Accepted 12 February, 2017

Abstract

From the period from March to August 2016, 210 swabs were collected from the burn patients hospitalized in different hospitals in Baghdad City: Al-Karama Teaching Hospital, Special Burn Hospital, Central Teaching Laboratories, Child protection Teaching Hospital, Imam Ali Hospital. Out of 210 clinical isolates, 42 (37.5%) had been shown a single isolated of pathogenic bacteria \textit{K. pneumoniae} and the others were belonged to other bacteria and mixed growth isolates. Identification of all isolates were carried out depending on macroscopic, microscopic characterizations, conventional biochemical tests and Api 20E system. Metallo-\( \beta \)-lactamase (MBL) enzymes were screen by two phenotypic methods (Meropenem-EDTA double disks method and Modified Hodg test). Susceptibility testing were used with The following antibiotic disks: Imipenem, Meropenem, Ceftazidime, Cefotaxime, Pipracillin, Gentamicin, Amikacin and Ciprofloxacin. The percentage of resistance isolates were as followed: Imipenem (21.42%), Meropenem (19.04%), Ceftazidime (69.04%), Cefotaxime (85.71%), Pipracillin (85.71%), Gentamicin (26.19%), Amikacin (19.04 %) and Ciprofloxacin (59.52%). The percentage of the prevalence of \( \text{bla}_{NDM} \)-1 and \( \text{bla}_{NDM} \)-2 genes in \textit{K. pneumoniae} isolates from burn patients in Baghdad hospitals were as followed: 20(100 %) for \( \text{bla}_{NDM} \)-1 genes and 6 (30 %) for \( \text{bla}_{NDM} \)-2 genes.

Key Words: Burn Patients, \( \text{bla}_{NDM} \) -Metallo-\( \beta \)-Lactamase Genes, \textit{Klebsiella pneumoniae}.

الخلاصة

جُمعت 210 مسحوقات قطنية للمرة الأولى من أذار لأغسطس 2016 من مرضى الحروق المرضى في مستشفيات مختلفة في مدينة بغداد: مستشفى الكرامية التعليمي، مستشفى الحروق التعليمي، مختبرات التعليم المركزي. تم تشخيص 42 عزلة (37.5%) من البكتيريا الرئوية، وتم استخدام طريقة كيميائية متعددة للتشخيص، وتستند على استخدام نظام Api 20E للكشف عن إنزيمات البیتالکتامیز المعذنة-1. عالنت نسبة مقاومة العمالات للأدوية، وتم استخدام طريقة من التوصيف الفيزيائي للحصول على النتائج. ظهرت تعاملات البكتيريا الرئوية مع العوامل المقصودة. وعلى التوالي: أميکاسین، میتروفلوكساسین، سیفوکاسین، دیپرسین، سیفوکاسین، سیفوکاسین، و سیفوکاسین. ظهرت نسبتيات انتشار إنزيمات البیتالکتامیز المعذنة-1 بلغت 20 (100% ) ونسبة انتشار إنزيمات البیتالکتامیز المعذنة-2 بلغت 6 (30%).

الكلمات المفتاحية: مرضى الحروق، جينات إنزيمات البیتالکتامیز المعذنة، الكليسیلا الرئوية.
**Introduction**

*Klebsiella pneumoniae* was an opportunistic gram-negative pathogenic bacterium associated with a range of nosocomial infections (e.g. sepsis, pneumonia, bacteremia, meningitis, urinary tract, burn and wound infections) [1]. Furthermore, it was the most medically important species of the genus *Klebsiella*. In recent years, *Klebsiella* have become important pathogens in nosocomial infections [2]. It was also a potential community-acquired pathogen [3]. Antibiotic therapies are widely used for treating infectious diseases. Nowadays, antibiotic-resistant bacteria are a great concern of worldwide public health [4]. The problem of antimicrobial resistance is highlighted by a recent increase of carbapenem-resistant *K. pneumoniae*, which has largely been driven by the emergence and spread of mobile genetic elements carrying carbapenemase resistance genes including the metallo-beta-lactamase [5, 6]. Meropenem and imipenem are carbapenems that remain active against organisms carrying most Ambler classes of beta-lactamases which include many Gram-negative bacilli, including *Klebsiella* spp. One of the major mechanisms of carbapenem resistance in this pathogen is the production of carbapenem-hydrolyzing beta-lactamases. These specific groups of beta-lactamases are categorized into class B metallo beta-lactamases (MBLs) including Imipenemase (*IMP*) and Verona integrin encoded metallob-lactamase (*VIM*), New Delhi metallo-beta-lactamase (*NDMs*) and class D (Oxacillinases) including OXA-23-like, OXA-24/40-like and OXA-58 [7, 8]. The new MBL, New Delhi metallo-beta-lactamase (*NDM-1*), initially reported in *K. pneumoniae* and *E. coli* recovered from a Swedish patient who was previously hospitalized in India in 2008 [9]. The rapid emergence spread of *NDM*-positive bacteria has a complex epidemiology involving a variety of harboring species (principally *Klebsiella pneumoniae* and *E. coli*), inter-strain, inter-species, and inter-genus transmission, which has been related to a diverse moveable plasmid that can be transferred from one bacteria to another, from man to man and even from country to country in more than 40 countries worldwide [10,11]. The bacteria with *NDM-1* gene are known as superbugs and public health must pay more attention to them [12].

Many phenotypic, genotypic, phylogenetic and molecular methods used to detect the production of enzymes by bacteria that responsible about drug resistant which causes increased morbidity and mortality among patients with infections caused by these bacteria and increased healthcare costs due to the extended hospital stay [13]. In recent years, many Iraqi patients were travelled to India and to other countries for medical care purpose which may helped in acquiring *NDM* gene. In Iraq there were no information about the occurrence of *NDM*. *K. pneumoniae* producing clinical isolates. So the proposed aim of this study was to detect MBL genes *blaNDM*-1 among resistant isolates of *K. pneumoniae* obtained from burn patients in Baghdad Hospitals by polymerase chain reaction (PCR).

**Material and Methods**

**Isolation and Identification**

During the period from March to August 2016, 42 *K. pneumoniae* strains were isolated from 210 swabs of burn patients hospitalized in different hospitals in Baghdad City: Al-Karama Teaching Hospital, Special Burn Hospital, Central Teaching Laboratories, Child protection Teaching Hospital, Imam Ali Hospital. Specimens were collected by sterile swabs after the removal of dressing and cleaning the wound surface by 70% alcohol. The isolation and identification of *K. pneumoniae* from wound specimens were streaked on blood agar, MacConkey agar and Eosin methylene blue(EMB) agar (Biomark Lab. Pune. India) and incubated at 37°C for 24hrs. The isolates were identified as *K. pneumoniae* by manual biochemical tests that were used in accordance with the manufacturer’s instructions; based on Gram staining,
catalase test, oxidase test, triple sugar iron (TSI) fermentation, Indole test, Voges-Proskauer (VP) test, Methyl red (MR) test, Simmons Citrate test, Urease test, motility test, and string test [14]. For final confirmation, biochemical tests embedded in the API-20E biochemical kit system (Bio-Merieux, France).

**Antimicrobial Susceptibility Testing**

The susceptibility pattern of isolates to different antibiotics were examined using disk diffusion method (Kirby-Bauer) on Muller-Hinton agar plates (Biomark Lab., Pune, India) according to guidelines of CLSI [15]. The antimicrobial disks were included: Imipenem (10µg), Meropenem (10µg), Ceftazidime (30µg), Cefotaxime (30µg), Pipracillin (100µg), Gentamicin (10µg), Amikacin (30µg) and Ciprofloxacin (5µg) (MAST Co. UK). *Pseudomonas aeruginosa* ATCC27853 were used as a control strain [16].

**Table 1:** Antibiotic disks used in this study

<table>
<thead>
<tr>
<th>Antibiotic disks</th>
<th>Symbol</th>
<th>Disks potency (µg/ disk)</th>
<th>Company (origin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td>IMP</td>
<td>10 µg</td>
<td></td>
</tr>
<tr>
<td>Meropenem</td>
<td>MEM</td>
<td>10 µg</td>
<td></td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>CAZ</td>
<td>30µg</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>CTX</td>
<td>30µg</td>
<td></td>
</tr>
<tr>
<td>Pipracillin</td>
<td>Pip</td>
<td>100µg</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>GM</td>
<td>10 µg</td>
<td></td>
</tr>
<tr>
<td>Amikacin</td>
<td>AK</td>
<td>30µg</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>CIP</td>
<td>5µg</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAST Co. UK.</td>
<td></td>
</tr>
</tbody>
</table>

**Screening for metallo β-lactamases (MBL)**

Meropenem-EDTA double disks method was performed using disks containing 1900 µg of EDTA plus 10 µg of Meropenem disks were placed on inoculated Muller Hinton agar plates. After 24 hr. incubation, an increase of ≥ 17 mm in zone diameter in the presence of 1900 µg of EDTA compared to Meropenem disk alone were considered as MBL producing *K. pneumoniae* strains (positive results) [17].

**Modified Hodge test (MHT)**

Tested isolates were exposed to MHT test as recommended by [18]; Inoculating an overnight culture suspension of *E. coli* ATCC 25922 was streaked across the entire plate of Mueller-Hinton agar (MHA) plate. After drying 10 µg of Meropenem disk was placed at the center of the plate and up to 4 different isolates of tested organisms were streaked linearly from the periphery of the plate into the direction of Meropenem disk at the center then the test plate was incubated at 37°C for 18 hours. The presence of a clover leaf-like shaped zone of inhibition around each tested strain is interpreted as Carbapenemases producing strain.

**PCR amplification:**

DNA was extracted from the isolates by using genomic extraction mini kit according to the manufacture instructions (Promega company, USA). To amplify the genes encoding carbapenemases, a PCR was run using the primers of NDM-1 and NDM-2 gene (Table-2) as described by [19]. Amplification was performed in a 20µl volume as recommended by Promega Master mix instruction. PCR amplifications were carried out on a thermal cycler (ESCO/USA). The cycling conditions for amplification were as follows: for *blaNDM*-1 and 2 genes, initial denaturation at 95°C for 5 min., 1 cycle, Denaturation at 95°C, 30 sec., Annealing at 55°C, 30 sec., Extension at 72°C for 30 sec., 30 cycles and Final Extension at 72°C for 5 min., 1 cycle.
Table 2: The sequences of primers used in PCR to detect bla NDM-1&2 [19].

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>blaNDM-1 Forward</td>
<td>'5-ATG GAA TTG CCC AAT ATT ATG C-3'</td>
<td>500bp</td>
</tr>
<tr>
<td>Reverse</td>
<td>'5-CGA AAG TCA GGC TGT GTT G-3'</td>
<td></td>
</tr>
<tr>
<td>blaNDM-2 Forward</td>
<td>'5-CAC CTC ATG TTT GAA TTC GCC-3'</td>
<td>1000bp</td>
</tr>
<tr>
<td>Reverse</td>
<td>'5-CTC TGT CAC ATC GAA ATC GC-3'</td>
<td></td>
</tr>
</tbody>
</table>

Agarose Gel Electrophoresis
Amplified products were detected by agarose gel electrophoresis in 1% Tris-borate-EDTA (TBE) agarose (Promega USA), and staining with ethidium bromide, electric current was allowed at 70 volts for 2 hrs. DNA bands were observed using UV-Transilluminator and photographed with Gel documentation system. 100 bp DNA Ladder (Promega) was used to assess PCR product size [20].

Results and Discussion
Bacterial strains, antibiotic susceptibility and MBL phenotypic test.
In this study a total of 210 sample swabs of clinical isolates of burn wound infections were cultured, examined and identified. Out of 210 clinical isolates, 42 (37.5%) had been shown a single isolated of pathogenic bacteria *K. pneumonia* and the others were belonged to other bacteria: 36 (32.14%) *Pseudomonas* spp., 20 (17.86%) *E. coli*, 10 (8.93%) *S. aureus* and 4 (3.57%) *Proteus* spp., while mixed growth isolates frequency as the following: *K. pneumoniae* and *Pseudomonas* spp.64 (65.31), *Pseudomonas* spp. and *E. coli* 18 (18.37%), *K. pneumoniae* and *E. coli* 7 (7.14%), *Pseudomonas* spp. and *Proteus* spp. 4 (4.08%), *K. pneumoniae* and *S. aureus* 3 (3.06%) and *Proteus* spp. and *E. coli* 2 (2.04%).
In a local study done by Mohammed(2007), who isolated *K. pneumoniae* from burn wound infection (36.7%) 20[21]; while Assal, (2010) isolated *K. pneumoniae* from wound (31.25%). These results were agreement with this study. Kehinde.et.al(2004) also found that *Klebsiella* spp. (34.4%) was the most common isolate from infected burn wounds [22]. *K. pneumoniae* associated with hospital-acquired infection accounting for 34–36% of cases of *K. pneumoniae* bacteremia [23].

Antibiotic Susceptibility Testing
Antimicrobial resistance to the carbapenems (e.g. imipenem and meropenem) mediated by metallo-β-lactamase (MBL) enzymes has remarkable clinical implications since the carbapenems are usually the last options of treatment for bacterial infections caused by multidrug resistant organisms (e.g. producers of extended spectrum B-lactamasases) [24]. Eight antibiotic disks were used in this study included two types of Carbapenems antibiotics; Imipenem (IPM), Meropenem (MEM) and two types of third generation Cephalosporins included; Ceftazidime (CAZ), Cefotaxime (CTX). Table(1) summarizes the results of antibiotic susceptibility test and reflects forty-two isolates were resistance to the following antibiotics; Imipenem (21.42%), Meropenem (19.04%), Ceftazidime (69.04%), Cefotaxime (85.71%), Pipracillin (85.71%), gentamicin (26.19%), Amikacin (19.04%) and Ciprofloxacin (59.52%). Furthermore, some isolates exhibited intermediate susceptibility to Imipenem (9.52%), Meropenem (4.76%), Ceftazidime (11.90%), Pipracillin (4.76%), Amikacin (4.76%) and Ciprofloxacin (2.38%). While some isolates showed susceptibility to the antibiotics as the following: Gentamycin and Amikacin (78.57%), Meropenem (76.19%), Imipenem (69.04%), Ciprofloxacin (38.09%), Ceftazidime (19.04%) and both Cefotaxime, Pipracillin (9.52%).
A high degree of resistance to the tested antibiotics was noted among the bacteria isolates especially to the third-generation cephalosporins; Cefotaxime(85.71%), Ceftazidime (69.04%), this results of the study agreement with Ejikeugwu et al., 24[25] who reported that *K. pneumoniae* was not agree. Results from table (1) revealed that higher resistant rate was found for Piperacillin (85.71%); this result in agreement with a previous studies; Al-Asady (2009) and Al–Hilli (2010) who found that Enterobacteriaceae isolates were resistant to piperacillin (100%) and (81%) respectively. High resistance to this class of antibiotics may be due to widespread use of antibiotics in hospitals [31,32].

### Screening for metallo β-lactamases (MBL)

Detection of metallo β-lactamases (MBL) were performed by Meropenem-EDTA double disks method and modified Hodge test. Some carbapenem resistance *K. pneumoniae* isolates were MBL producers. 20 from 30 of isolates(66.6%) showed overnight growth an increase of ≥ 17 mm in zone diameter in the presence of 1900 μg of EDTA compared to Meropenem disk alone (Figure-1). Also all these isolates showed the presence of a clover leaf-like shaped zone of inhibition around each tested strain, which was interpreted as a phenotypic evidence of MBL production (Figure-2).
Genotypic detection of $bla_{NDM-1,2}$ genes:
PCR was carried out on the DNA of 20 carbapenem resistance $K.\ pneumoniae$ isolates for $bla_{NDM-1,2}$, using specific primer for $bla_{NDM-1,2}$ forward and $bla_{NDM-1,2}$ reserve (Table-2). Amplification was performed in a 25μl volume as recommended by Promega Master mix instruction. DNA molecular size marker (500-bp ladder for $NDM-1$gene and 1000-bp for $NDM-2$gene). PCR revealed Lanes (K1 to 20) of $K.\ pneumoniae$ isolates showed positive results with $bla_{NDM-1}$gene (100 %) (table 5) (Fig.3a,b). Lanes (K 2, 10,16,17,18, and 20) show negative results with $bla_{NDM-2}$ genes 6 (30 % ). (Fig.4a,b.).

Figure 1: Meropenem-EDTA double disks method.

Figure 2: Modified Hodge test (MHT)

Figure 3a: Agarose gel electrophoresis in 1% for $bla\ NDM-1$gene product show positive results(1-10).Ethidium bromide stain (0.5%), Amplicon size (500bp), DNA Ladder (100bp), the electric current at 100 volt for 1hr.
**Figure 3 b.** Agarose gel electrophoresis in 1% for blaNDM-1 gene product show positive results (11-20). Ethidium bromide stain (0.5%), Amplicon size (500bp), DNA Ladder (100bp), the electric current at 100 volt for 1hr.

**Figure 4 a.** Agarose gel electrophoresis in 1% for blaNDM-2 gene product show negative results (2,10). Ethidium bromide stain (0.5%), Amplicon size (1000bp), DNA Ladder (100bp), the electric current at 100 volt for 1hr.

**Figure 4 b.** Agarose gel electrophoresis in 1% for blaNDM-2 gene product show negative results (16,17,18,20). Ethidium bromide stain (0.5%), Amplicon size (1000bp), DNA Ladder (100bp), the electric current at 100 volt for 1hr.
Table 5: Occurrence of \textit{blaNDM}-1 and 2 genes between \textit{K. pneumonia} isolates

<table>
<thead>
<tr>
<th>Frequency</th>
<th>isolates No.</th>
<th>\textit{NDM}-1 gene</th>
<th>\textit{NDM}-2 gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>+</td>
<td>-</td>
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<tr>
<td>3</td>
<td>3</td>
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<td>17</td>
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<td>18</td>
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<tr>
<td>20</td>
<td>40</td>
<td>+</td>
<td>-</td>
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</table>

This is the first report of \textit{blaNDM}-1 and \textit{blaNDM}-2 genes in Baghdad hospitals among \textit{K. pneumoniae} isolates. There were many types of \textit{blaNDM} gene which was located mostly onto conjugative plasmids belonging to several incompatibility groups [32]. So an important consideration should be taken when designing genetic tools to the target carbapenem resistance genes. The occurrence of isolates contain \textit{blaNDM} in Baghdad hospitals may be resulted from transfer of plasmid among resistant isolates from medical care purpose which may helped in acquiring \textit{NDM} gene when many Iraqi patients were travelled to India and to other countries for medical care purpose. Comparing our results that showed high prevalence of \textit{NDM}-1 and 2 genes, with a study was carried out in the period between April 2009 and February 2011 in Mubarak Al Kabeer Hospital in Kuwait agreed with our results; three isolates were \textit{NDM}-1 positive in \textit{K. pneumoniae} [33]. Multiple reports showed infected cases with \textit{NDM}-1 positive organisms; 44 isolates with \textit{NDM}-1 were identified in south India (Chennai), 26 in north India (Haryana), 37 in the UK, and 73 in other sites in India and Pakistan [34]. While Molecular investigations revealed the first identification of the \textit{NDM}-1 gene in Australia [35] from a man who had been previously hospitalized in Bangladesh and then transferred to Australia. In local study which was carried out in Hillah hospital by AL-Harmoosh and Jarallah [36] revealed the first identification of the \textit{NDM}-1,2 genes in Iraq harbored \textit{Acinetobacter baumannii} isolates among patients with different infections.

**Conclusion**

The study had shown the spreading of \textit{blaNDM}, \textit{pneumoniae} isolates among patients with burn infections. The rapid spread of \textit{blaNDM} genes of \textit{K. pneumoniae} isolates among patients with burn infections in this study poses an increased threat to hospitalized patients in Iraq and more importantly, avoiding misuse, overuse of antibiotics may converse the undesired effects of multidrug resistant and \textit{NDM} producing bacteria. Furthermore we would expect more \textit{NDM} variants to be discovered in the next years in Iraq.
References


