Detection of Genes Encoding of Extended-Spectrum and AmpC β-Lactamases in Klebsiella pneumoniae Isolates from Clinical Specimens

Ghusoon A. Abdulhasan*, Hula Y. Fadhil* and Kifah A. Jasem**

*Department of Biology, College of Science, University of Baghdad, Baghdad-Iraq.
**Ministry of Health, Central Health Laboratory, Iraq.

*E-mail: ghusoon@yahoo.com.

Abstract

Klebsiella pneumoniae is an important pathogen of nosocomial infections and has rapidly become the most common producing beta lactamases that resistance for many antimicrobial agents. Thus, our study aimed to identify K. pneumoniae isolates harboring SHV, TEM, CTX-M and AmpC β-lactamase genes and the relation between them and with some antimicrobial resistance to avoid treatment failure. Sensitivity disc test and PCR technique were done on 24 clinical isolates of K. pneumoniae. The PCR results showed that blashv, blatem, blactx-m and blampa genes were present in 91.67% of the isolates. Significance appearance of resistance genes was 75% for each blashv and blactx-m, 62.5% for blatem, while blampa in 16.7%. Finding pointed out that blampa gene present with highly significant in bacterial isolates which lacking the blashv and blactx-m. Moreover, blashv and blatem occurred on significant correlation with blactx-m. Antimicrobial discs (CTX, CDZ, CRO and CL) correlating with resistance genes (blactx-m, blashv and blatem). Remarkably, 41.67% of bacterial isolates have three of cephalosporine β-lactamase genes due to the common used of cephalosporine third generation for treatment.

Keywords: Klebsiella pneumoniae, β-lactamase, antimicrobial resistance and PCR.

Introduction

Extended spectrum beta lactamases (ESBLs) are enzymes can hydrolyze oxyimino-cephalosporins and monobactams but not cephamycins and carbapenems, inhabited by clavulanate [1].

There are more than 30 different ESBL variants that classified into nine families based on their amino acid sequences [2], [3]. They are frequently encoded by plasmid produced by gram negative bacteria and exchanged between their species [4].

One of the most important nosocomial pathogen produced ESBLs is Klebsiella pneumoniae, frequently causing pneumonia, urinary tract, wound and blood infection resulting in significant morbidity and mortality due to had plasmid encoded ESBLs lead to failure treatment or treatment option limited [5]. The predominant types of ESBLs enzymes in K. pneumoniae are SHV followed TEM and CTX-M that belonged to class A B- lactamases [4].

SHV and TEM are the classical B-lactamase had resistance to pencillin and narrow spectrum cephalosporins, most ESBLs derived from them due to mutation in one or more amino acid around the active side. There are more than 70 SHV and 150 TEM types are known. blashv and blatem are genes responsible for encoded these enzymes, blashv genes originated from chromosome in K. pneumonia and mobilized to the plasmid through insertion element [6].

CTX-M beta lactamase are more active against cefotaxime and ceftriaxone than ceftazidime, but point mutation can increase their activity against it [7]. They are divided into five subgroups having more than 80 enzymes which became dominant in many countries [8], [9] due to blactx-m can transfer horizontally to other bacteria via conjugation and two types of insertion elements [6].

AmpC beta lactamase has cephalosporinase activity, in K. pneumoniae the blampa frequently carried on plasmid with other beta lactamase genes such as blatem-1, blashv, blapes-1 and blactx-m3 [10], [11],[12].

Usually the detection of beta lactamase occur by using antibiogram, but the appearance of new forms of beta lactamases make routine susceptibility test unreliable thus need other test that may be fastidious and too time consuming such as determination isoelectric point of enzymes [13]. However molecular detection and typing of ESBLs can confer
rapid and reliable diagnosis for genes responsible for these enzymes [14].

The aim of this study is the detection of common genes of ESBLs (TEM, SHV, CTX-M and Amp C) found in K. pneumoniae by PCR and study the relationship between them to provide useful information can aid in the treatment of this pathogen and to avoid treatment failure.

**Materials and Methods**

**Bacterial Isolates**

Twenty four isolates of K. pneumoniae were isolated from different specimens (urine, blood, wound and sputum) on MacConkey agar. All isolates were obtained and diagnosed in central health laboratory using API-20E kit (Bio-Mereix, France) according to the manufacture instructions.

**Antimicrobial Sensitivity Disc Test**

The detection of phenotypic resistance mediated by ESBLs in K. pneumoniae isolates was performed by disc diffusion method on Mueller-Hinton agar using ceftriaxone (30mg), cefotaxime (10 mg), cefodizime (30 mg), cephalaxin (30 mg) and ampicillin (10 mg).

**DNA Extraction**

Boiling method was used for extraction of genomic DNA from K. pneumoniae isolates as the method described by Ahmed et al. [15], with modifications as follow: bacterial cells were harvested in one ml of TE buffer and centrifuged at 12000 g for 2min. Pellet was re-suspended in 100 µl of sterile D.W and boiling at 100ºC for 10 min, cooled on ice then centrifuged at 10000 g for 10 min. The supernatant was stored at - 20 ºC until use.

**PCR amplification**

Multiplex PCR was carried out to detect three genes included blaSHV, blaAmpC and blCTX-M using specific primers (Table 1). The PCR mixture set up in 20 µl total volume consisting of 5 µl of premix Accupouer (Bionear, Korea), 10 pico/ µl of each primer and 5µl of DNA template. The thermal programme was optimized and performed in master cycler (Eppendorf) as follows: 4min at 94 ºC, then 32 cycles of 1min at 94 ºC, 1min at 55 ºC and 1min at 72 ºC then final elongation step at 72 ºC for 10min.

Another primer set used for detection blaTEM. Touchdown PCR was performed, PCR premix prepared as mentioned above. After optimization, the amplification was performed at 95 ºC for 2min then 14 cycles of annealing temperature was initially set at 53 ºC and then decreased 0.5ºC each cycle until it reached to 46 ºC. Nineteen additional cycles were run at 46 ºC. Denaturation was carried out at 95 ºC for 30 sec and extension step was at 72 ºC for 90sec. The final extension step was done at 72ºC for 5min. the negative control was performed with each run. The product of PCR were detected by 1.5% agarose gel electrophoresis and visualized under U.V after ethidium bromide staining.

**Statistical Analysis**

All data were tabulated and analyzed using the SPSS IBM version 20. The Chi-Square test was done to investigate probable correlation between β-lactamase genes present, their frequency and isolates resistance phenotypes, while the Kruskal-Wallis test used to evaluate the genes number for each isolate. The frequencies were checked by applying Person test to estimate the relative correlation between lactamase genes and with phenotype expression. Values were considered statistically significant $P \leq 0.05$. 

**Table (1)**

The primers and their sequences used in PCR for detection of ESBL and AmpC genes in *K. pneumoniae*.

<table>
<thead>
<tr>
<th><strong>primer</strong></th>
<th><strong>Gene</strong></th>
<th><strong>Sequence</strong></th>
<th><strong>Product size (bp)</strong></th>
<th><strong>references</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>SHV-F</td>
<td><em>bla</em>&lt;sub&gt;SHV&lt;/sub&gt;</td>
<td>TGGTTATGC\text{CGTTATATTCGCC}</td>
<td>800</td>
<td>[4]</td>
</tr>
<tr>
<td>SHV-R</td>
<td><em>bla</em>&lt;sub&gt;SHV&lt;/sub&gt;</td>
<td>GGT\text{TACCGTTGCCAGTGC}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AmpC-F</td>
<td><em>bla</em>AmpC</td>
<td>ATTCGTATGCTGGATCTCGCCACC</td>
<td>395</td>
<td>[16]</td>
</tr>
<tr>
<td>AmpC-R</td>
<td><em>bla</em>AmpC</td>
<td>CATGACCCCAGTTCGCCATATCCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX-M F</td>
<td><em>bla</em>CTX-M</td>
<td>TTTCGCCATGTCGACAGTAAT</td>
<td>544</td>
<td>[17]</td>
</tr>
<tr>
<td>CTX-M R</td>
<td><em>bla</em>CTX-M</td>
<td>CGATATCGT\text{CGTTGCTCCATA}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM-F</td>
<td><em>bla</em>TEM</td>
<td>ATAAATATCTTGAAGACGAA</td>
<td>850</td>
<td>[4]</td>
</tr>
<tr>
<td>TEM-R</td>
<td><em>bla</em>TEM</td>
<td>GACAGTTACCAATCTTAAATC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Results and Discussion

Regarding PCR results showed that *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> genes were the most present in studying clinical bacterial isolates from *bla*AmpC gene (Fig. (1) and (2)). However, all these genes manifestation was signed (*P*≤0.01) in 91.67% whether as single or mixed genes among clinical bacterial isolates, while 79.17% of these isolates have phenotype resistance for one or more of antimicrobial used. Similar values were present in Najaf province that 88% of isolates were β-lactam resistance gene and of which 59.1% were able to produce β-lactamases [18]. Likely, the phenotypes that appear without the presence of these genes in genomic DNA suggest the presence of other genes of ESBL may be responsible for resistance phenotypes, or contrary these genes correlated with other antimicrobial resistance. Incidence in equal percentage and significance of resistance genes was 75% of bacterial strains have *bla*<sub>SHV</sub> and *bla*<sub>CTX-M</sub> genes was observed as single or/and with other tested resistance genes (*P*≤0.05). Meanwhile, the appearance of *bla*<sub>TEM</sub> and *bla*AmpC genes were reported in 62.5% and 16.7%, respectively of bacterial strains at single and multiple β-lactam genes (Table (2)). Similar percentages were obtained in previous study in India using specific primers for *bla*<sub>SHV</sub> and *bla*<sub>CTX-M</sub> only 75.2% of ESBL isolates for one or more genes [2], whereas the *bla*<sub>CTX-M</sub> gene constituted about 58% of ESBL isolates in our population [19], [20]. By Ding et al. [21], reported a 10% prevalence of *K. pneumoniae* strains producing Amp C beta-lactamase while it represented as 20% that poses *bla*AmpC gene by local study [22]. The negative amplification in the remaining isolates may be due to the presence of other ESBL genes.

Remarkably, 41.67% of bacterial isolates have three of cephalosporine β-lactamase genes (SHV, CTX, and TEM) together in this study, while other study that reported these three β-lactamase genes in 21% [9]. Significant association with prior antibiotic therapy supports the hypothesis that selection pressure related to overuse of broad spectrum antibiotics, especially third generation cephalosporin play important role for emergence of high level resistance in the family *Enterobacteriaceae*. Graffunder et al. [23], found that use of third generation cephalosporins, aminoglycosides and trimethoprim/sulphamethoxazole were independently associated with infections by ESBL producing strains and patients on prolonged use of ventilator were at the greatest risk of having an ESBL organism. Several other studies had also shown strong association between infection with ESBL-producing *E. coli* or *K. pneumoniae* and antibiotic use [24].
**Fig. (1)** PCR amplification of bla$_{SHV}$ (800bp), bla$_{CTX-M}$ (544bp) and bla$_{AmpC}$ (395bp) in K. pneumoniae isolates, Agarose (1.5%), 5 V/cm for 45min and visualized under U.V after staining with ethidium bromide. Lanes 1,2,7 bla$_{SHV}$. Lanes 3,4,6,9,13,14 bla$_{SHV}$ and bla$_{CTX-M}$. Lanes 5,10,12 bla$_{AmpC}$ and bla$_{CTX-M}$. Lane 11 bla$_{SHV}$, bla$_{AmpC}$ and bla$_{CTX-M}$. Lane 8 K. pneumoniae isolate don’t have these resistance genes. C: negative control. M: 100bp DNA marker.

**Fig. (2)** PCR amplification of bla$_{TEM}$ (850bp) in K. pneumoniae isolates, Agarose (1.5%), 5 V/cm for 45min and visualized under U.V after staining with ethidium bromide. Lanes 1,2,7, 8-13 bla$_{TEM}$ positive. Lanes 2 bla$_{TEM}$ negative. C: negative control. M: 100bp DNA marker.
Reduction in use of ceftazidime or all cephalosporins decreased the occurrence of infection by ESBL producing strains [23], [24].

**Table (2)**

<table>
<thead>
<tr>
<th>Frequency of resistance genes among bacterial isolates according to PCR analysis.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ESBL gene</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>SHV</td>
</tr>
<tr>
<td>CFX</td>
</tr>
<tr>
<td>TEM</td>
</tr>
<tr>
<td>Amp</td>
</tr>
</tbody>
</table>

On the other hand, the PCR results pointed out that AmpC gene present with highly significant ($r=-0.6, P≤0.01$) in bacterial isolates which lacking the SHV gene as shown in table 2. Hence, Appearance of AmpC gene was highly significant correlation with the CTX gene presence ($r=1, P≤0.01$), while there is a non-significant correlation with the TEM gene presence ($r=0.32$), and weak correlation with SHV gene ($r=0.2$). The genetic description by Husickova et al. [25], that revealed mutation in the promotor region of the Amp C chromosomal gene that are associated with it’s over production.

In spite of the TEM gene occurred along with CTX gene in 12 bacterial isolates, there is non-significant correlation between them. As, the SHV gene with CTX gene have no significant correlation ($r=0.48, P=0.04$) within bacterial strains. The origin of the CTX-M enzyme is different from that of the TEM and SHV ESBL, while SHV and TEM-ESBLs were generated by amino acid substitutions of their parent enzyme, CTX-M ESBLs were acquired by the horizontal gene transfer from other bacteria using genetic apparatuses such as a conjugative plasmid or transposon [6].

| **Table (3)** |
| Observation of K. pneumoniae resistance for some antimicrobial discs that harbor tested resistance genes among 24 clinical isolates. |
| **Antimicrobial disc** | **No. (%) resistance isolate** | **No. of isolate (harbor genes)** |
| | | |
| Cefotaxime (CTX) | 14 (58.3) | 8(CTX+SHV+TEM), 3(CTX+SHV), 1(CTX+TEM+Amp), 1(CTX+Amp), 1(CTX+SHV+TEM+Amp) |
| Cefodizime (CDZ) | 10 (41.7) | 3(CTX+SHV+TEM), 1(CTX+Amp), 2(SHV+TEM), 2(CTX+SHV), 1(TEM), 1(CTX+SHV+TEM+Amp) |
| Ceftriaxone (CRO) | 9 (37.5) | 5(CTX+SHV+TEM), 1(CTX+Amp), 2(SHV+TEM), 1(CTX+SHV) |
| Cephalexin (CL) | 5 (20.8) | 4(CTX+SHV+TEM), 1(CTX+TEM) |
| Ampicillin (Amp) | 2 (8.3) | 1(Amp), 1(non) |

Our finding displayed revealed that antimicrobial discs (CTX, CDZ, CRO and CL) correlating with resistance genes (CTX, SHV and TEM) present as shown in Table (3), as mentioned by other investigators [26]. Meanwhile, in a study carried out in Iran, showed no relation between presence of TEM, SHV and CTX-M β-lactamase genes with β-lactamse production or ESBL phenotype [4]. Basically, all bacterial isolates that cefotaxime resistance (CTX) have carried CTX gene with highly significant ($r=0.7, P≤0.01$), along with other genes as noticed in Colum 3, Table (3). Many investigators listed that emphasized the cefotaxime-resistant strains producing only CTX-M type β-lactamase [6], [8]. In addition, the occurrence of cephalexin with cefotaxime and ceftriaxone with cefodizime resistance were significant ($P≤0.05$) in the same strains. However, there are four strains harbor resistance genes without expression any resistance for antimicrobial discs.
Fig. (3) demonstrates that 45.8% of clinical bacterial strains had three of tested resistance genes followed by 33.4% had two of these genes. Thus, significant existence of resistance genes was found when these strains carrying three genes as compared with others ($P \leq 0.05$). In contrast, other study of Goyal et al. [2], reported single ESBL gene was present in 42.7% of typeable isolates, bla CTX-M, bla SHV and bla TEM being the most common followed two ESBL genes (41.5%), while the combination of three ESBL genes represented 15.9%.

Moreover, our finding showed two of these strains (8.3%) were negative in PCR for the experimented resistance genes, but the AmpC phenotype was positive there may be lack the Amp gene in sub-culturing process prior the amplification. Presence of ESBLs can be masked by the expression of chromosomal or plasmid mediated AmpC β-lactamases. Also, ESBL producing strains with AmpC β-lactamases can cause a false negative in ESBL detection [27]. More importantly, expression of β-lactamases genes depend upon the environmental conditions such as the presence of antibiotics and gene presence shown by PCR does not necessarily indicate its expression [4].

**Conclusion**

In this study, the high occurrence of extended spectrum beta lactamases production of *K. pneumoniae* is necessary to avoid treatment failure condition and need to adopt appropriate control measures to reduce the ESBL. Also, this study showed the usefulness of PCR technique for detection of the ESBLs and AmpC genes and their relation with each other and effect on antimicrobial resistance. Remarkably, 41.67% of bacterial isolates have three of cephalosporine β-lactamase genes and this could be due to the common used of cephalosporine third generation for treatment.

**References**


Ghusoon A. Abdulhasan


الخلاصة

تعتبر بكتريا Klebsiella pneumoniae من الممرضات السريرية المهمة لتسببها بالعديد من الالتهابات وسرعان ما أصبحت الأكثر شيوعا في انتاج إنزيمات البيتا لاكتاماز المقاومة للعديد من المضادات الحيوية. لذلك هدفت هذه الدراسة إلى الكشف عن عزلات K. pneumoniae الحاملة لجينات مقاومة بيتا لاكتاماز TEM و SHV و العلاقة بين هذه الجينات مع بعضها ومع AmpC و CTX-M المقاومة لبعض المضادات الحيوية لتجنب فشل العلاج.

اختبار الحساسية بواسطة أقران المضادات الحيوية وتقنية تفاعل سلسلة البلمرة (PCR) قد استخدمت على 24 عزلة من K. pneumoniae. أظهرت نتائج PCR جينات المقاومة مع جينات blaCTX-M و blaTEM و blaSHV موجودة بنسبة 91.67% من العزلات. اظهرت النتائج ان% 75 من جينات المقاومة عائدة لجينات blaAmpC و blaSHV بينما blaTEM و blaCTX-M موجد في العزلات التي لوحظ ان جين blaAmpC موجود في 16.7% اضافة الى وجود ارتباط blaCTX-M و blaSHV كبير بين جينات CTX, CDZ, CRO كما ان اقران المضادات الحيوية (blaCTX-M, blaSHV, blaTEM) من اللافت للنظر ان% 41.67 من العزلات كانت حاملة لإنتاج من جينات البيتا لاكتاماز المقاومة للسيفالوسبورين نتيجة للاستخدام الشائع للجيل الثالث من السيفالوسبورين.