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

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## 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 $bla_{SHV}$, $bla_{TEM}$, $bla_{CTX-M}$ and $bla_{AmpC}$ genes were present in 91.67% of the isolates. Significance appearance of resistance genes was 75% for each $bla_{SHV}$ and $bla_{CTX-M}$, 62.5% for $bla_{TEM}$, while $bla_{AmpC}$ in 16.7%. Finding pointed out that $bla_{AmpC}$ gene present with highly significant in bacterial isolates which lacking the $bla_{SHV}$ and $bla_{CTX-M}$. Moreover, $bla_{SHV}$ and $bla_{TEM}$ occurred on significant correlation with $bla_{CTX-M}$. Antimicrobial discs (CTX, CDZ, CRO and CL) correlating with resistance genes ($bla_{CTX-M}$, $bla_{SHV}$ and $bla_{TEM}$). 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 300 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. $bla_{SHV}$ and $bla_{TEM}$ are genes responsible for encoded these enzymes, $bla_{SHV}$ genes originated from chromosome in *K. pneumonia* and mobilized to the plasmid through insertion element [6].

CTX-M beta lactamase are more active against cefotoxime 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 $bla_{CTX-M}$ can transfer horizontally to other bacteria via conjugation and two types of insertion elements [6].

AmpC beta lactamase has cephalosporenase activity, in *K. pneumoniae* the $bla_{AmpC}$ frequently carried on plasmid with other beta lactamase genes such as $bla_{TEM-1}$,$bla_{SHV}$, $bla_{PES-1}$ and $bla_{CTX-M3}$ [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), cefodiziime (30 mg), cephalexin (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 $bla_{\text{SHV}}$, $bla_{\text{AmpC}}$ and $bla_{\text{CTX-M}}$ 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 $bla_{\text{TEM}}$. 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.***

| *primer* | *Gene* | *Sequence 5´- 3´* | *Product size (bp)* | *references* |
|---|---|---|---|---|
| SHV-F | $bla_{SHV}$ | TGGTTATGCGTTATATTCGCC | 800 | [4] |
| SHV-R | | GGTTAGCGTTGCCAGTGC | | |
| AmpC-F | $bla_{AmpC}$ | ATTCGTATGCTGGATCTCGCCACC | 395 | [16] |
| AmpC-R | | CATGACCCAGTTCGCCATATCCTG | | |
| CTX-M F | $bla_{CTX-M}$ | TTTGCGATGTGCAGTACCAGTAA | 544 | [17] |
| CTX-M R | | CGATATCGTTGGTGGTGCCATA | | |
| TEM-F | $bla_{TEM}$ | ATAAAATTCTTGAAGACGAA | 850 | [4] |
| TEM-R | | GACAGTTACCAATGCTTAATC | | |

**Results and Discussion**

Regarding PCR results showed that $bla_{SHV}$, $bla_{TEM}$ and $bla_{CTX-M}$ 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 ($P \leq 0.01$) 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 $bla_{SHV}$ and $bla_{CTX-M}$ genes was observed as single or/and with other tested resistance genes ($P \leq 0.05$). Meanwhile, the appearance of $bla_{TEM}$ 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 $bla_{SHV}$ and $bla_{CTX-M}$ only 75.2% of ESBL isolates for one or more genes [2], whereas the $bla_{CTX-M}$ 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 trimethoprime/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].

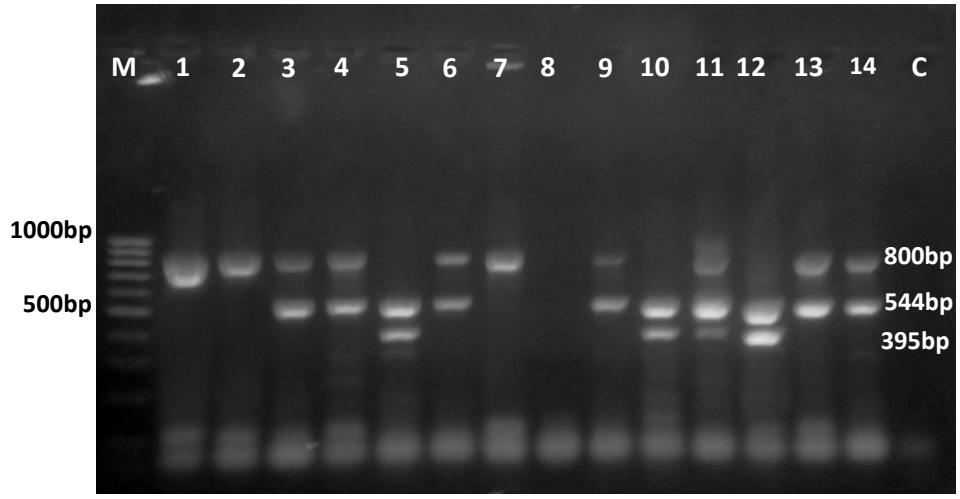


***Fig.(1) PCR amplification of $bla_{SHV}$ (800bp), $bla_{CTX-M}$ (544bp) and $bla_{AmpC}$ (395bp) in K. pneumoniae isolates, Agarose (1.5%), 5 V/cm for 45min and visualized under U.V after staining with ethidium bromide. Lanes 1,2,7 $bla_{SHV}$. Lanes 3,4,6,9,13,14 $bla_{SHV}$ and $bla_{CTX-M}$. Lanes 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.***

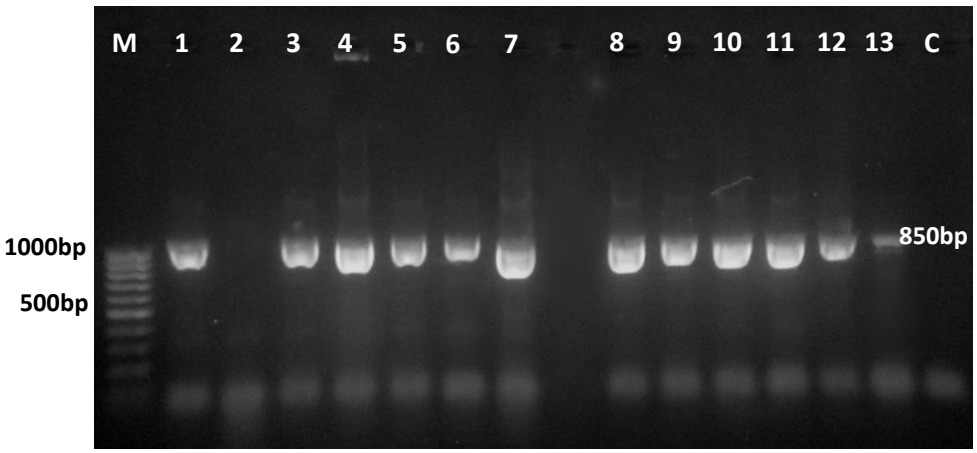


***Fig. (2) PCR amplification of $bla_{TEM}$ (850bp) in K. pneumoniae isolates, Agarose (1.5%), 5 V/cm for 45min and visualized under U.V after staining with ethidium bromide. Lanes 1,2-7, 8-13 $bla_{TEM}$ posative. Lanes 2 $bla_{TEM}$ negative. C: negative control. M: 100bp DNA marker.***

Reduction in use of ceftazidime or all cephalosporins decreased the occurrence of infection by ESBL producing strains [23], [24].

***Table (2)***
***Frequency of resistance genes among bacterial isolates according to PCR analysis.***

| *ESBL gene* | *Genotype appearance (no.) in bacterial isolate* | | *No. (%) isolate have gene* |
|---|---|---|---|
| | *Multiple genes* | *Single gene* | |
| SHV | TEM+CTX(10), CTX(4), TEM(2), TEM+CTX+Amp(1) | SHV (1) | 18 (75) |
| CTX | TEM+SHV(10), TEM+Amp(1), Amp(2), SHV(4), TEM+SHV+Amp(1) | --- | 18 (75) |
| TEM | CTX+SHV(10), CTX+Amp (1), SHV(2), CTX+SHV+Amp (1) | TEM (1) | 15 (62.5) |
| Amp | TEM+CTX (1), CTX (2), TEM+SHV+CTX (1) | --- | 4 (16.7) |

On the other hand, the PCR results pointed out that AmpC gene present with highly significant (r=-0.6, $P\leq0.01$) 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, $P\leq0.01$), 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 cefataxime resistance (CTX) have carried CTX gene with highly significant (r= 0.7, $P\leq0.01$), 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 cefataxime and ceftriaxone with cefodizime resistance were significant ($P\leq0.05$) 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%0, while the combination of three ESBL genes represented 15.9%.

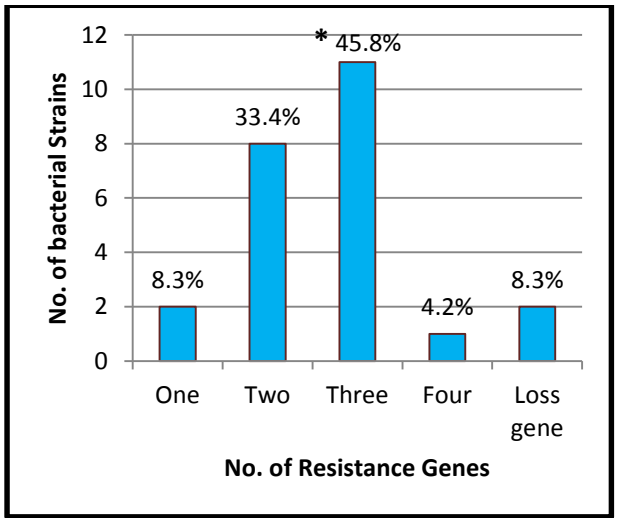


***Fig.(3) Distribution of resistance genes among bacterial isolates according to PCR analysis. Asterisk indicates to significant occurrence for bacterial isolates that carrying three genes compared to other (P≤0.05).***

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.

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كبير بين جينات $bla_{SHV}$ و $bla_{TEM}$ مع جين $bla_{CTX-M}$, كما ان اقراص المضادات الحيوية CTX, CDZ, CRO (and CL) اظهرت ارتباط مع جينات المقاومة ($bla_{CTX-M}$, $bla_{SHV}$, $bla_{TEM}$). من اللافت للنظر ان 41.67% من العزلات كانت حاملة لثلاثة من جينات البيتا لاكتاميز المقاومة للسيفالوسبورين نتيجة للاستخدام الشائع للجيل الثالث من السيفالوسبورين.


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**الخلاصة**


تعتبر بكتريا *Klebsiella pneumoniae* من الممرضات السريرية المهمة لتسببها بالعديد من الالتهابات وسرعان ما اصبحت الاكثر شيوعا في انتاج انزيمات البيتا لاكتاميز المقاومة للعديد من المضادات الحيوية. لذلك هدفت هذه الدراسة الى الكشف عن عزلات *K. pneumoniae* الحاملة لجينات مقاومة بيتا لاكتاميز SHV و TEM و CTX-M و AmpC وعلاقة هذه الجينات مع بعضها ومع المقاومة لبعض المضادات الحيوية لتجنب فشل العلاج.

اختبار الحساسية بواسطة اقراص المضادات الحيوية وتقنية تفاعل سلسلة البلمرة PCR قد استخدمت على 24 عزلة من *K. pneumoniae*. اظهرت نتائج PCR ان جينات المقاومة $bla_{SHV}$ و $bla_{TEM}$ و $bla_{CTX-M}$ و $bla_{AmpC}$ موجودة بنسبة 91.67% من العزلات. اظهرت النتائج ان 75% من جينات المقاومة عائدة لجينات $bla_{SHV}$ و $bla_{CTX-M}$ و 62.5% لجين $bla_{TEM}$ بينما 16.7%. لوحظ ان جين $bla_{AmpC}$ موجود في العزلات التي تفتقر لجينات $bla_{SHV}$ و $bla_{CTX-M}$ اضافة الى وجود ارتباط