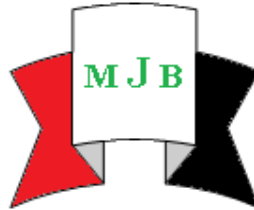


Molecular Detection of CTX-M Genes in *Klebsiella pneumoniae* Isolated from Different Clinical Samples in Baghdad City

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Received 15 December 2014

Accepted 28 January 2015

Abstract

CTX-M extended-spectrum β -lactamase (ESBL) producing *Klebsiella pneumoniae* have been reported to be an important nosocomial infections. A total of 50 *K. pneumoniae* isolates were isolated from different clinical samples in some public hospitals in Baghdad city during the period from October to December 2013. Bacterial identification was done using conventional cultural & chemical methods & VITEK 2 cards (GN) for identification, while the antimicrobial drug susceptibility of *K. pneumoniae* was performed by disk diffusion test and the minimum inhibitory concentration (MIC) testing was performed using VITEK 2 automated system (bioMérieux, France). ESBL production was phenotypically detected by double disk synergy test according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. The presence of *bla*-gene encoded CTX-M was detected by conventional PCR technique.

Out of 50 *K. Pneumonia* isolates, 13 (26%) were ESBL producer by CDT, the minimum inhibitory concentration (MIC) of different antibiotics was performed on these 13 (26%) isolates using VITEK2 AST-GN30 showed that 13 (100%) isolates were Ceftazidime, Ceftriaxone and Cefepime resistant with MIC ≥ 16 - ≥ 64 $\mu\text{g/ml}$, and 8 (61.53%) of ESBL producing isolates were carbapenem sensitive 8 (61.53%) with MIC ≤ 0.25 $\mu\text{g/ml}$. PCR assay revealed that 4 (30.76%) of the ESBL producing isolates harbored *bla*CTX-M gene.

Extended spectrum beta lactamase mediated resistance in *K. pneumonia* is a cause for concern in the therapy of critically ill patients. The ESBL producing *K. pneumoniae* isolates were more resistant to various antimicrobial agents. This suggests that ESBL producing isolates in hospitals may cause serious infections that illustrated when these strains were responsible for a nosocomial outbreak. The findings strongly suggest that there is a need to track the detection of ESBL producers and that judicious use of carbapenems is necessary to prevent the further spread of these organisms. The prevalence of multi-drug resistant *K. pneumoniae* isolates especially ESBL producing bacteria was increased in Baghdad city. Phenotypic and molecular characterization of ESBL provide information about the prevalence of ESBL producing *K. pneumoniae* in Baghdad. The *bla*CTX-M was one of the predominant ESBL genes in *K. pneumoniae* in this study.

Key words: *Klebsiella pneumonia*, ESBL, *bla* genes

الكشف الجزيئي عن جينات CTX-M في بكتريا الكلبسيلا الرئوية والمعزولة من مختلف العينات السريرية في مدينة بغداد

الخلاصة

تعتبر بكتريا الكلبسيلا الرئوية المنتجة لانزيمات CTX-M مثبتة بانها من مسببات المهمة للاصابات والالتهابات المختلفة في المستشفيات. بكتريا الزوائف الزنجارية لها قابلية عالية على مقاومة العديد من المضادات الحيوية المستخدمة حاليا من خلال ميكانيات مختلفة منها داخلية او مكتسبة واهم الميكانيكات هو انتاج انزيمات Extended spectrum B-Lactamase . تم جمع 50 عزلة من بكتريا الكلبسيلا الرئوية من مختلف العينات السريرية من بعض المستشفيات والمختبرات الحكومية في محافظة بغداد للفترة من اب ولغاية كانون الاول لعام ٢٠١٣. تم تشخيص البكتريا باستخدام مختلف الطرق سواء كانت فحوص كيميائية او عن طريق تشخيص البكتريا في اوساط زرعية مختلفة كذلك تم تشخيص العزلات البكتيرية باستخدام جهاز VITEK2 وتم قياس الحساسية الدوائية للعزلات البكتيرية باستخدام نفس الجهاز وكذلك تم التوصل للنتائج التالية:نسبة انتشار العزلات المقاومة لعقارات السيفالوسبورين هي 100% وعدد العزلات البكتيرية المنتجة لانزيماتESBL باستخدام الطرق المظهرية هو 13 ونسبة 26%. تم استخدام تقني ال PCR للعزلات الثلاثة عشر المقاومة المنتجة مظهريا

لانتزيمات ESBL للكشف عن وجود الجينات الخاصة بمقاومة المضادات الحيوية *bla*genes والمشفرة لانتزيمات CTX-M وظهرت النتائج ان ٤ عزلات (٣٠.٧٦%) من العزلات المنتجة لانتزيمات ESBL كانت تحوي جينات *bla*CTX-M.

Introduction

Klebsiella pneumoniae is gram negative bacilli, possessing non-motile, facultative anaerobic bacteria. The organisms are usually 3-6µm in length and up to 1.0 µm in width. The encapsulated strains of *Klebsiella* are known to produce mucoid colonies (1).

Klebsiella pneumoniae is an important pathogen that causes urinary tract infections (UTIs), pneumonia, and intra-abdominal infections in hospitalized immunocompromised patients with severe underlying diseases (2). It has been estimated that *Klebsiella* spp. cause 5-7% of the total bacterial nosocomial infections in the world (3). Beta-Lactams are the most widely used antibiotics in clinical medicine and resistance to beta-lactams may become a severe threat because they have low toxicity and are used to treat a broad range of infections (4). Cephalosporins, fluoroquinolones, aminoglycosides and carbapenems are effective for treating infections caused by *Klebsiella pneumoniae* (5, 6).

Resistance to extended spectrum cephalosporins can occur in *K. pneumoniae* via the production of extended spectrum Beta-lactamases (ESBLs) that are capable of hydrolyzing the oxyiminocephalosporins and monobactams (7, 8). Although, today hundreds variant of ESBLs have been described but the most common of them are derivatives of TEM or SHV enzymes also, In recent years a new family of plasmid mediated CTX-M extended spectrum β-lactamases (ESBLs) called CTX-M has arisen with increasing frequency from Europe, Africa, Asia, South America and North America. These ESBLs were named CTX-M type Beta-lactamases, owing to their high activity against cefotaxime (9).

According to a recent review and new data within GenBank, CTX-M-β-lactamases can be divided into five groups

based on their amino acid sequence identities. Group I includes CTX-M-1, -3, -10 to -12, -15, -22, -23, -28, -29, and -30. Group II includes CTX-M-2, -4 to -7, and-20 and Toho-1. Group III includes CTX-M-8. Group IV includes CTX-M-9, -13, -14, -16 to -19, -21, and -27 and Toho-2. Finally group V includes CTX-M-25 and -26. The members of these groups exhibit greater than 94% amino acid identity within the group and about 90% amino acid identity between groups (10).

The CTX-M enzymes have been detected in a many *Enterobacteriaceae* species, from different geographical regions. However, the CTX-M variants are mostly detected in *E. coli*, *S. typhimurium*, *K. pneumoniae* and *Proteus mirabilis* (11).

Infection with the ESBL producing organisms is associated with higher rates of mortality, morbidity, and health care costs, which is at least partly due to the lack of proper screening recommendations. Nosocomial infection involving multi-drug resistant *K. pneumoniae* is a growing problem worldwide (12).

The present study was designed for phenotypic detection of CTX-M type ESBLs and molecular detection of genes encoding CTX-M-β-lactamases in ESBL-producing strains of *K. pneumoniae* isolated from clinical specimens collected in this study.

Materials and Methods

Bacterial Isolates

Fifty isolates of *Klebsiella pneumoniae* were isolated from different clinical samples in Baghdad/Iraq during the period from October to December 2013. The *Klebsiella* isolates (50 isolates) were as follows: burn (17), ear (3), sputum (4), wound (2), urine (19), vagina (2), blood (3). Clinical samples were collected from teaching laboratories of medical city, Al-Yarmouk Hospital, in addition to some private laboratories. Bacteria were

cultured on MacConkey and Nutrient agar in aerobic condition at 42 °C for 24-48 h, Then identified by conventional biochemical tests and by using of VITEK 2 Automated system using (GN) cards.

Antibiotic Susceptibility Testing

All *K. pneumoniae* isolates were cultures on MacConkey agar (selective and differential media). The antimicrobial susceptibility of these isolates was achieved by disk diffusion and VITEK 2 system according to CLSI (13). The MIC for phenotypically ESBL producing isolates was obtained.

Identification of ESBL Producing Isolates

Combined disk test (CDT)

A disk of ceftazidime (30µg) alone and a disk of ceftazidime + clavulanic acid (30µg/10µg) were placed independently, 30mm apart, on a lawn culture of 0.5 McFarland opacity of the test isolate on Mueller Hinton Agar (MHA) plate and incubated for 18-24 hours at 35°C. An increase of ≥ 5 mm zone of inhibition diameter around the ceftazidime/clavulanic acid in comparison to ceftazidime confirmed ESBL production (13).

Molecular detection of *bla*CTX-M genes

Plasmid DNA Extraction

DNA preparation from bacterial cells was performed by salting out method with some modification as following:

1- Bacterial cell of 50 ml culture were precipitated by centrifugation (1000 rpm for 10 minutes). Rewashed 3 times in TE buffer, Then the pellet was resuspended in 5 ml TE buffer.

2- A volume of 600 µl of 25% SDS was added, mixed by inversion to the cell

suspension and incubated for 5 minutes at 55 °C.

3- About 2 ml of 5M NaCl solution was added to the lysate, mixed thoroughly by inversion and let to be cooled to 37°C. Then 5 ml of (phenol: chloroform: isoamylalcohol) (25: 24: 1 v/v) was added to the lysate and mixed by inversion for 30 minutes at 25° C and the spun by centrifuge 4500 rpm for 10 minutes.

4- The aqueous phase was transferred to a fresh tube, which contain the nucleic acid then isopropanol (0.6 volume) was added to the extract and mixed by inversion, after 3 minutes DNA spooled on to a sealed pasture pipette.

5- The DNA rinsed in 5 ml of 70% ethanol, air dried, and dissolved in 300 µl TE buffer, and then DNA extract was kept at -20 °C until use.

Preparation of primers suspension

The DNA primers were resuspended by dissolving the lyophilized primers after spinning down with TE buffer depending on IDT/USA instructions as stock suspension. Working primer tube was prepared by diluted with TE buffer. The final picomoles depended on the procedure of each primer.

Detection of CTX-M genes by PCR

The CTX-M genes were detected for the phenotypically resistant isolates by using primers targeting *bla*CTX-M gene. The PCR amplification mixture has been prepared according to the manufacturer's instructions (Intron, Korea).

A- Primers:

The primers used to amplify the genes encoding the CTX-M enzymes are listed in Table -1 below:

Table 1 : primers sequences for detection of *blaCTX-M* genes

Gene	Primer sequence (5'-3')	Size of product	Ref.
<i>blaCTX-M</i> F	CGCTTTGCGATGTGCAG	550 bp	(14)
<i>blaCTX-M</i> R	ACCGCGATATCGTTGGT		

B-The reaction mixture:

Amplification of DNA was carried out in a final volume of 20µl containing the

following (Table-2). and The PCR product was detected using agarose gel electrophoresis

Table 2: Contents of the reaction mixture

No.	Contents of reaction mixture	Volume
1.	2X PCR ImaxII master mix	4µl
2.	Upstream primer	1µl
3.	Downstream primer	1µl
4.	DNA template	5µl
5.	Nuclease free water	9µl
Total volume		20 µl

Results

Out of the 50 *K. pneumoniae* isolates studied, only 13(26%) appear to be phenotypically ESBL producer by using Combined Disk Test (CDT). (Table-3, Figure-1).

The antibiotic susceptibility test was done for all isolates *K. pneumoniae* isolates by disk diffusion method. In present study, the MIC of 10 antibiotics listed in Table (4) was done by VITEK2-Compact using AST-GN30 for testing the antibiotic susceptibility for the ESBL producing *K. pneumoniae* isolates (no.13) and the MIC values were interpreted according to the CLSI 2012 (11).

The ESBL producing *K. Pneumoniae* in this study (no. 13) differ in the level of resistance to different antibiotics including the cephalosporins as showed in Table (4). All of the ESBL isolates showed MIC ≥ 16 for ceftazidime and ≥ 64 µg/ml for both

ceftriaxone and cefepime. The resistance profile of ESBL producing isolates against carbapenems was different, Five ESBL producing *K. pneumoniae* isolates were resistant imipenem and meropenem with MIC ≥ 16 µg/ml, while the other eight isolates were sensitive to carbapenems with MIC ≤ 0.25 µg/ml.

The study showed that 10 (76.9%) of ESBL producing isolates were resistant to ciprofloxacin MIC ≥ 4 µg/ml, while only 5 (38.4%) were resistant to levofloxacin. The results showed that 12 (92.3%) of ESBL producing *K. pneumoniae* were resistant to aminoglycosides used in this study (Gentamicin and Tobramycin).

Table 3: Prevalence of ESBL producing *K. pneumoniae* isolates

Total number of isolates	Positive for ESBLs	
	Numbers	%
n=50	13	26%

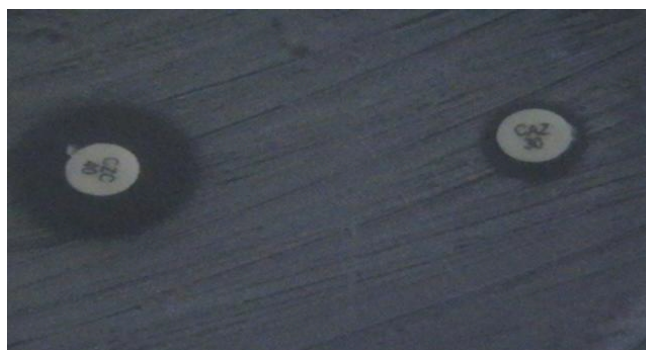


Figure 1: Positive Phenotypic confirmatory test (combination disk method) for detection of ESBL, Muller – Hinton agar plate showing an isolate *Klebsiella pneumoniae* resistant to ceftazidime (CAZ) (30µg) and along with an increase zone of inhibition around ceftazidime clavulanic acid (CZC) (30ug/10).

Most of the ESBL producing *K. pneumoniae* isolates were recovered from urine (Table- 4).
All the isolates are resistant to Imipenem MIC ≥ 16 , while only 4 isolates are

resistant to Meropenem MIC ≥ 16 & these isolates were found to be multi drug resistant (MDR) to the 10 antibiotics (Table- 4).

Table 4: Antibiotic susceptibility of ESBL producing *K. pneumoniae* isolates

Isolate	Specimen	MIC (µg/ml) of selected antibiotics determined by VITEK 2 system									
		IMP	CIP	GM	CAZ	AMC	CRO	TOB	MER	FEP	LEVO
k1	Burn	R ≥ 16	R ≥ 4	R ≥ 16	R ≥ 64	R ≥ 32	R ≥ 64	R ≥ 16	R ≥ 16	R ≥ 64	R ≥ 8
K2	Burn	S ≤ 0.25	R ≥ 4	R ≥ 16	R 32	R ≥ 32	R ≥ 64	R ≥ 16	S ≤ 0.25	R ≥ 64	S 1
K3	Burn	S 0.5	R ≥ 4	R ≥ 16	R ≥ 64	S 1	R ≥ 64	R ≥ 16	S ≤ 0.25	R ≥ 64	S 1
K4	Burn	R ≥ 16	R ≥ 4	R ≥ 16	R ≥ 64	R ≥ 32	R ≥ 64	R ≥ 16	R ≥ 16	R ≥ 64	R ≥ 8

K5	Sputum	S ≤0.25	R ≥4	R ≥16	R 32	R ≥32	R ≥64	R ≥16	S ≤0.25	R ≥64	S 1
K6	Sputum	R ≥16	R ≥4	R ≥16	R ≥64	R ≥32	R ≥64	R ≥16	R ≥16	R ≥64	R ≥8
K7	Urine	R ≥16	R ≥4	R ≥16	R ≥64	R ≥32	R ≥64	R ≥16	R ≥16	R ≥64	R ≥8
K8	Urine	S ≤0.25	R ≥4	R ≥16	R 32	R ≥32	R ≥64	R ≥16	S ≤0.25	R ≥64	S 1
K9	Urine	S ≤0.25	S ≤0.25	R ≥16	R 16	R ≥32	R ≥64	R ≥16	S ≤0.25	R ≥64	S 1
K10	Urine	S ≤0.25	R ≥4	R ≥16	R 16	R 16	R ≥64	R ≥16	S ≤0.25	R ≥64	S 1
K11	Urine	S 0.5	S 0.5	S ≤1	R 16	R 16	R ≥64	S ≤1	S ≤0.25	R ≥64	S 1
K12	Urine	R ≥16	R ≥4	R ≥16	R ≥64	R ≥32	R ≥64	R ≥16	R ≥16	R ≥64	R ≥8
K13	Urine	S ≤0.25	S ≤0.25	R ≥16	R 16	S 1	R ≥64	R ≥16	S ≤0.25	R ≥64	S 1

Abbreviation:IPM, imipenem; CIP, ciprofloxacin; GM, gentamicin; CAZ, ceftazidime; AMC, amoxicillin-clavulanic acid; CRO, ceftriaxone ;TOB, tobramycin; MEM, meropenem; FEP, cefepime; LEV, levofloxacin

All *Klebsiella pneumoniae* isolates that were resistant to ceftazidime and phenotypically ESBL producers (n=13) were further investigated for the presence of or plasmid mediated *blaCTX-M* using specific primer. The presence of CTX-M genes was detected by conventional PCR technique.

PCR analysis for CTX genes was accomplished for the 13 ESBL producer

isolates. The distribution of plasmid mediated CTX-M genes within study isolates are shown in Figure (2). The plasmidic *blaCTX-M* was detected in 4 (30.76%) isolates, these are K1, K2, K4 and K6 respectively. These results achieved using specific *blaCTX-M* gene primers.

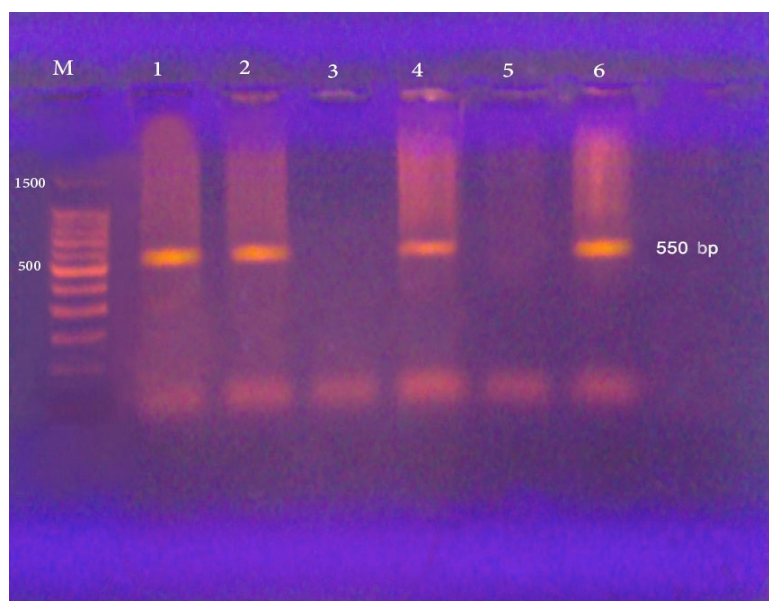


Figure 2: Ethidium bromide stained agarose gel showing PCR amplification products with *bla*CTX-M gene (550 bp) primers for *K. pneumoniae* extracted DNA.

M: 100 bp standard size reference marker. Lane 1: *K1* shows positive result with *bla*CTX-M gene. Lane 2: *K2* shows positive result with *bla*CTX-M gene. Lane 3: *K3* shows negative result with *bla*CTX-M gene. Lane 4: *K4* shows positive result with *bla*CTX-M gene. Lane 5: *K5* shows negative result with *bla*CTX-M gene. Lane 6: *K6* shows positive result with *bla*CTX-M gene.

Discussion

Klebsiella pneumoniae is a common pathogen causing nosocomial infection. In the present study, ESBL-producing isolates show a high level of resistance to all β -lactam antibiotics including β -lactamase inhibitor, aminoglycosides, and quinolones. Resistance to aminoglycosides was present in most ESBL-producing isolates (13).

According to various studies, ESBL production ranged from 10% to 65%. In the present study, ESBL productions were 26% in isolates of *Klebsiella pneumoniae* using CDT (confirmative test). ESBL-positive isolates lead to serious therapeutic failure because they carry multidrug-resistant genes, and imipenem is the drug of choice for treatment (14).

Currently, no standardized method for ESBL detection has been proposed, and despite PCR being highly accurate and reliable, its accessibility is often limited to reference laboratories (15).

The results of antibiotic sensitivity

obtained in this study clarified that the *K. pneumoniae* isolates showed high resistance to most common antibiotics of β -lactams, aminoglycosides, fluoroquinolones, were suspected to be highly producers of ESBLs; therefore, all *K. pneumoniae* isolates tested phenotypically for ESBL production. The isolates that produce ESBLs were 13 (26%) and they were more frequently among urinary tract infections. A study done by Al-Muhanna showed that isolates that produce ESBLs were predominant more frequently among burn infections in 11 (42.3%) isolates followed by 7 (26.9%) of UTI. (16).

Ceftazidime is a third-generation cephalosporin used frequently for the treatment of infections caused by *K. pneumoniae*. However, the resistance to ceftazidime is increasing at an alarming rate, complicating the clinical management of patients infected with such isolates. In this study, a high level of resistance to ceftazidime was observed among the *K. pneumoniae* isolates.

recovered from different clinical samples as showed in Table (4) . This result was close to the results obtained by Al-Muhannak (2010) for ceftazidime 89.8% (17).

In this study, ESBL producing isolates were significantly more resistant to all antibiotics tested as compared to non-ESBL producing isolates. other studies have reported on cross resistance to aminoglycosides, fluoroquinolones, and trimethoprim in ESBL producing organisms (18). Mechanisms of co-resistance are not clear, but one possible mechanism is the co-transmission of ESBL and resistance to other antibiotics within the same conjugative plasmids (19). The results achieved by using PCR revealed that only 4 (30.7%) of ESBL producing isolates have *blaCTX-M* genes. The ESBL gene was not detected in the remaining isolate that identified as ESBL producer by phenotypic method. As the PCR is the gold standard technique. So, The phenotypic result may give a false positive result or the isolates had an ESBL variants or other ESBL genes that not detected by the primers used in this study. The *blaCTX-M* determinants have rapidly established a condition of high-level endemicity in Europe. Even the large outbreak reported in Greece was caused by a single clone and was apparently confined to the hospital wards (20).

In a multi-centric study from Russia, *CTX-M* gene was reported in 35.9% of *E. coli* and 34.9% of *K. pneumoniae* ESBLs isolates (21).

Conclusion

The prevalence of multi-drug resistant *K. pneumoniae* isolates especially ESBL producing bacteria was higher than that which has been found in survey of later years. The present study concluded that ESBL producing *K. pneumoniae* isolates were recognized in both phenotypic and molecular methods in local isolates recovered from health centers in Baghdad. The antibiotic resistance was increased against all third generation cephalosporins.

Although the carbapenem was the drug of choice for ESBL producing *K. pneumoniae* isolates, the emerging of ESBL producing bacteria poses a threat to antibiotic treatment program in Baghdad hospitals.

The *blaCTX-M* was the predominant among the ESBL genes of *K. pneumoniae* in this study. The *blaCTX-M* genes are encoded by a plasmid and this resistance mechanism can cause nosocomial outbreaks.

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