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Genotype and Phenotype Investigation of *CTX-M* Gene among Multidrug Resistant *Klebsiella pneumoniae* Isolates

Esraa A. Ajeel, Rana K. Mohammed

Department of Biotechnology, College of Science, University of Bagdad,

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Abstract: Beta-lactam resistance is a major clinical problem in treating of *Klebsiella pneumoniae* infections. *Klebsiella pneumoniae* (*K. pneumoniae*) is an opportunistic bacterium that can resist Beta-lactams principally by producing the CTX-M enzymes. The objective of this study was to identify the prevalence of the *CTX-M* gene in clinical isolates of multidrug resistant (MDR) *K. pneumoniae* among hospitalized patients in Baghdad metropolis. From 200 various clinical samples (urine, wounds, blood, vaginal, stool, burns and sputum) that collected from hospitals in Baghdad during November 2021 to the end of April 2022, 87 positive *K. pneumoniae* cultures in total were analyzed. *K. pneumoniae* was identified from specimens using the CHROM agar orientation medium, biochemical test, VITEK2 system, diagnosis was verified using molecular identification. The antibiotic susceptibility test results showed high resistant to piperacillin, ceftazidime, cefepime, ceftriaxone, ampicillin, cefotaxime while imipenem and meropenem were more effective against the isolates. All of the *K. pneumoniae* isolate showed a MDR phenotype and Extended Spectrum Beta Lactamase (ESBLs) producers. The results of the molecular diagnosis of the *CTX-M* resistance genes showed the presence of this gene in 87 (100%) bacterial isolates.

Keywords: *Klebsiella pneumoniae*, *Multidrug resistance*, *CTX-M*, beta-lactamase.

Corresponding author: (Email: esraaaltimime@gmail.com).

Introduction

The most prevalent bacterial resistance to beta lactam antibiotics is the synthesis of lactamase enzymes. Klebsiella spp. are particularly prone to developing resistance to third-generation cephalosporins. Recently, all throughout the world, these bacteria have increased their production of (ESBLs). The three primary ESBL kinds are *TEM, CTX, and SHV* variations, while there are many other groups into which they might be split. Most Enterobacteriaceae, particularly *E. coli* and *K. pneumoniae*, have the *CTX-M* type according to the most recent review and new information in Gen-Bank. Penicillin and expanded-spectrum cephalosporin resistance is conferred by these Beta-lactamases, and the majority of the variations hydrolyze cefotaxime more quickly than ceftazidime. (1) *K. pneumoniae* is an *Enterobacteriaceae* family member that colonizes the digestive tract and is a part of the body's natural flora. In hospitals, this bacterium affects newborns and preterm infants, causing bacteremia, septicemia, urinary tract infections, surgical site infections, pneumonia, and meningitis. Illnesses that develop in patients while they are receiving treatment at a medical facility are known as healthcare-associated infections (HAIs) are linked to increased mortality rates and morbidity (2)

Although *K. pneumoniae* possesses mechanisms for naturally resisting specific antibiotic classes, it is becoming more and more common for acquired multidrug resistant *K. pneumoniae* to have ESBL enzyme activity. Multidrug resistant (MDR) Gram-negative bacteria that produce ESBL are a growing source of concern, as MDR has dramatically increased among bacteria of nosocomial infections. (3) ESBL are frequently found on plasmids that can be exchanged between bacterial species and strains. Numerous outbreaks of the infection have been caused by ESBL-producing Enterobacteriaceae, and controlling these epidemics appears to be difficult. (4) For the purposes of epidemiology, it is critical to characterize the genetic makeup of ESBL-producing species. The abundance of ESBL-producing genes in bacteria may point to typical variations in the expression of antibiotic resistance.

Materials and methods

Isolation and identification of isolates

Two hundred specimens were collected from The Baghdad Teaching Hospital and Teaching Laboratories Institute, Burns hospital / Medical city, Al-Kindy Teaching Hospital of patients from November 2021 to the end of April 2022. These specimens included urine, wounds, blood, vaginal, stool, burns and sputum. All specimens have been cultured immediately on the CHROM agar Orientation and MacConkey agar media (Himedia, India), then incubated for 24h at 37°C, bacterial isolated had been identified according to culture charcterization, biochemical test, VITEK 2 system (bioMerieux, France), and validated with molecular detection using 16S rRNA by polymerase chain reaction (PCR).

Antibiotic susceptibility test

Antibiotics susceptibility of *K. pneumoniae* isolates were examined towards 19 different antibiotics by using disk diffusion method on Mueller-Hinton agar (Himedia, India) plates. The Mueller-Hinton agar plates were inoculated with a *K. pneumoniae* suspension that was produced to an equivalent turbidity of 0.5 McFarland. According to Clinical and Laboratory Standard Institute (CLSI, 2022) guidelines. These antibiotics included Amikacin (AK 30 μg), Ampicillin (AM 25 μg), Trimethoprim-Sulfamethoxazole (SXT 75 μg), Ceftriaxone (CRO 10 μg), Cefotaxime (CTX 30 μg), Ceftazidime (CAZ 30 μg), Ciprofloxacin (CIP 5 μg), Levofloxacin (LEV 5 μg), Imipenem (IPM 10 μg), Meropenem (MEM 10 μg), Gentamicin (CN10 μg), Piperacillin (PRL100μg), Tobramycin (TOB10 μg), Aztreonam (ATM 30 μg), Cefepime (FEP 30 μg), Tigecycline (TGC15 μg) and Rifampicin (RF 5 μg), (Bioanalyses/Turkey). Colistin (CL10 μg), and Tetracycline (TE 10 μg) (Himedia, India). Then the plates were incubated at 37°C for 24h., and subsequently, the inhibition zone diameters were recorded in millimeters, and interpretation was carried out based on CLSI (2022) (5).

Confirmatory test for detection of extended spectrum β-lactamase:

Double Disc Synergy Test (DDST) was used to identify the ESBL phenotype on Muller Hinton agar. The *K. pneumoniae* suspension's turbidity was adjusted to 0.5 McFarland equivalent, and sterile cotton swabs were used to inoculate the Muller-Hinton agar plates with the suspension.

Forceps under sterilization was used to put the antimicrobial discs on the Muller-Hinton agar plates, Augmentin disc (AMC 30 µg) was placed in the center of plate, around of three side of AMC (30 µg) disc, a disc of Ceftriaxone (CRO 30 µg), Cefotaxime (CTX 30 µg), Ceftazidime (CAZ 30 µg) were placed at distance (15 mm) from center to center of AMC (30 µg) disc. Then the plate was incubated overnight at 37°C. A zone diameter increases of either antibiotic agent tested in conjunction with clavulanic acid of more than 5mm over the agent's original zone diameter was characterized as an ESBL (6).

DNA extraction

The extraction of genomic DNA for all clinical isolates of *K. pneumoniae* was done followed the manufacturing procedure of bacterial DNA extraction kit (ABIOpure/ USA).

Quantitation of DNA

The quantity of extracted DNA was measured using a Quantus Fluorometer to assess the quality of samples for subsequent uses. 199 µl of diluted Quant fluor Dye were combined with 1 µl of DNA. DNA concentration readings were found following a 5-minute incubation period at room temperature.

Molecular identification of *K. pneumoniae* by *16S rRNA* gene:

The PCR reaction was performed for Identification of *K. pneumoniae* by 16SrRNA gene (7). The primer sequence and product size were listed in table (1) and the thermal program listed in table (2).

Table (1): Primer sequences for*16SrRNA* to detection of *K. pneumoniae*

Primer		Sequences 5`-3`	Size bp
16S rRNA	F	GCAAGTCGAGCGGTAGCACAG	260
	R	CAGTGTGGCTGGTCATCCTCTC	

Table (2): Thermal program used to amplify *16SrRNA*

Steps	°C	M:S	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	58	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

Molecular detection of *CTX-M* gene

The *CTX-M* gene was detected by PCR reaction by using specific primer (1). The primer sequence and product size were listed table (3) and the thermal program listed in table (4).

Table (3): **Primer sequences for PCR detection of *CTX-M* gene in *K. pneumoniae***

Primer		Sequences 5`-3`	Size bp
CTX-M	F	CGATATCGTTGGTGGTGCCATA	544
	R	TTTGCGATGTGCAGTACCAGTAA	

Table (4): Thermal program used to amplify *CTX-M* gene

Steps	°C	M:S	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	62	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

The mixture of PCR amplification consisted (2 µl) DNA templet, (12.5 µl) of Green Master Mix that contains (Tag DNA polymerase, $MgCl_2$, deoxy nucleosides (dNTP), reaction buffer) (Promega/USA), (1 µl) of primer forward, (1 µl) of primer revers each primer for each specific gene, up to the final volume (25 µl) with nucleases free water (8.5 µl). all tubes were vortexed and centrifuged briefly in micro centrifuge (My Fugene/China) for 10 s to bring the contents to the bottom of the tubes.

PCR Program and identification of *K. pneumoniae* and *CTX-M* gene

Thermal cycler (Thermos Fisher Scientific/USA) was used to transfer the PCR tubes and initiate the amplification reaction using a customized protocol for each primer). By using 1.5% agarose gel electrophoresis with ethidium bromide (Promega/USA) in 1X TAE buffer, amplified products were analyzed. As a molecular weight marker, a 100 bp DNA ladder from Promega (USA) was employed. The amplified DNAs underwent a 60-min electrophoresis at 100 V. The Gel imaging equipment was used to view the Ethidium bromide-stained gel bands (Taiwan's major science).

Statistical methods

The statistical Packaged for Social Sciences (IBM SPSS) version 28.0 was used to enter data into Excel systems and perform precise test. Pearson's chi-square test used to calculate the probability (8).

Results

From 200 bacterial cultures that were obtained from various clinical specimen, 87(43.5%) of isolated bacteria were diagnosed as *K. pneumoniae.* The percentage of patients who were male was 47/87 (54.22%), while 40/87 (45.97%) of the patients were female. *K. pneumoniae* colonies looked mucoid and metallic blue when cultivated on CHROM agar media (Figure1). On MacConkey agar media, it had mucoid pink to crimson colonies. (Figure2). The biochemical characters could identify *K. pneumonia* simply. They were positive for Voges Proskauer, Citrate utilization, and Urease tests. However, they produce negative reactions with Indole and Methyl red tests (Figure3). VITEK2 system was used to verified the identification of *K. pneumoniae* isolates. Molecular identification was performed by PCR using specific primer *16SrRNA*. The number and percentage of *K. pneumoniae* isolates were: 26(29.88%) from urinary tract infection, 25(28.73%) from blood, 11(12.64%) from burn patients, 8(9.19) from stool, 9(10.34%) from vaginal,

4(4.59%) from sputum and 4(4.59%) from wound infections.

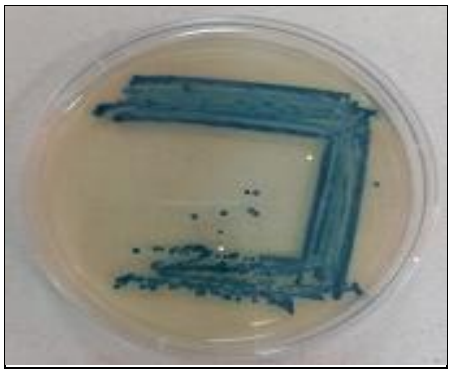

Figure (1): *K. pneumoniae* on CHROM agar.

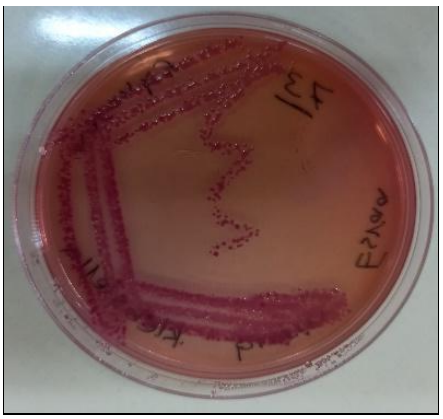

Figure (2)*: K. pneumoniae* on MacConkey agar.

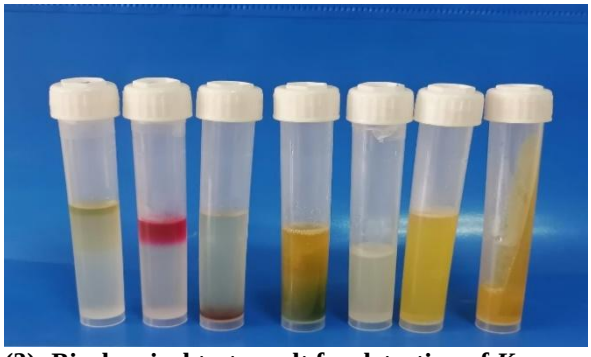

Figure (3): Biochemical test result for detection of *K. pneumoniae*.

Molecular identification of *K. pneumoniae* by *16S rRNA* gene:

The identification of *K. pneumoniae* isolates was verified by using PCR at the 260 bp domain of the *16SrRNA* gene.

The results showed that all 87 isolates (100%) were correctly identification by VITEK2 system and biochemical test.

Antibiotic susceptibility test

Clinical *K. pneumoniae* isolates showed high levels of resistance to the most of the tested antibiotics, according to the antibiogram for the isolates under study.

The results explained that all *K. pneumoniae* clinical isolates test resistance to: Rifampicin (100%) followed by Piperacillin (98.9%),

Ceftriaxone (98.9%), Cefotaxime (96.6%), Ceftazidime (95.4%), Ampicillin (94.3%), Cefepime (92%), Trimethoprim (92%), Aztreonam (82.8%), Ciprofloxacin (71.3%), Tetracycline (69%), Tigecycline (62.1%), Tobramycin (62.1%), Levofloxacin (58.6%), Gentamicin (58.6%), Amikacin (50.6%), Colistin (44.8%), Meropenem (46%) and Imipenem (46%).

In the current study, High beta-lactam antibiotic resistance was evident such as Piperacillin, ceftriaxone, cefotaxime, Ceftazidime, Ampicillin, cefepime, aztreonam. All of the *K. pneumoniae* isolate showed a MDR phenotype which resisted to more than three classes of chosen antibiotics as shown Figure (4), and Extended Spectrum Beta Lactamase (ESBLs) producers as shown Figure (5).

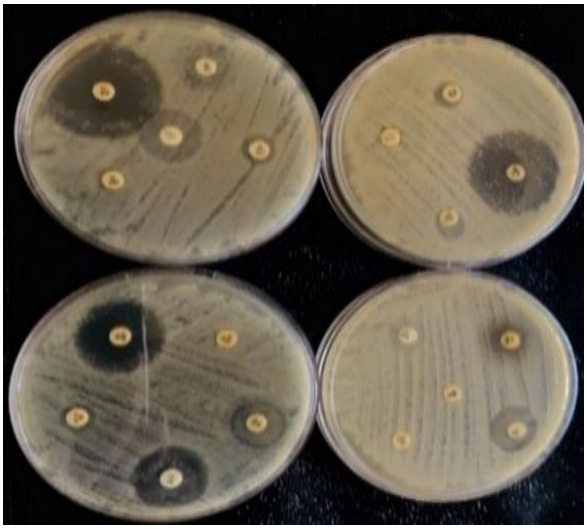

Figure (4): Antibiotic susceptibility test.

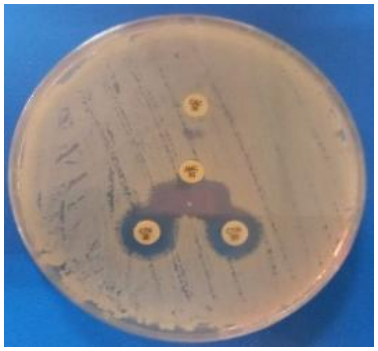

Figure (5): Positive result for ESBL produce.

Molecular detection of *CTX-M gene* in *K. pneumoniae*

DNA was extracted from 87 *K. pneumoniae* for amplification, to provide a template for PCR technique. Quantus Fluorometer had been used to estimate concentration and purity of DNA, the results showed a concentration between DNA concentration was ranged (13-55) (ng/µl). The results of agarose gel electrophoresis were showed all isolates (87) of *K. pneumoniae* were positive for *CTX-M* in size 544 bp after electrophoresis in agarose gel as shown in Figure (6).

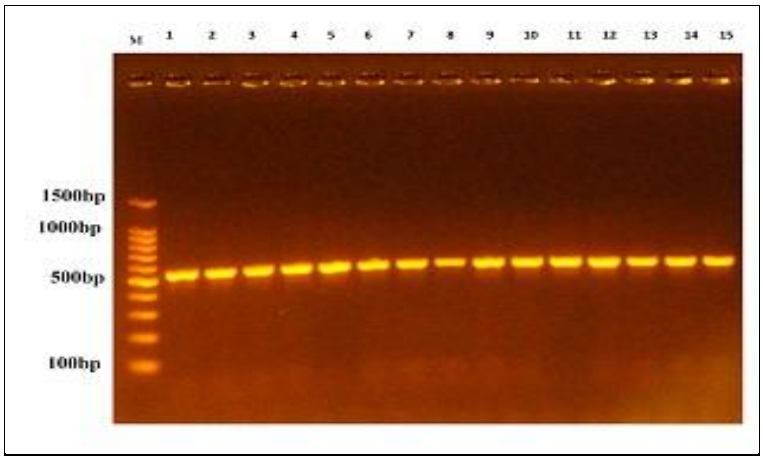

Figure (6): The amplification results of the *bla CTX* primers of *Klebsiella pneumoniae* samples fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker.

Discussion

In the current study, 87(43.5%) of total specimen 200 *K. pneumoniae* has been detected from a number of clinical sample sources. Which was higher than those obtained in the previous study (31.9%) by Raouf *et al*., (8) and lower than the forms study (54%) by Jaaffar *et al*. from Iraq (9).

Due to the simpler identification of various colonies on CHROM agar Orientation medium, these media are dependable tool for the detection of Gram-negative aerobic bacteria. Because of its great accuracy, quick identification, and extremely low false positive rate, CHROM agar Orientation medium is chosen medium (3).

K. pneumoniae infection was the most common pathogen found in the urine samples 26/87(29.88%) of the patient. Urinary tract infections are most frequently caused by *K. pneumoniae*, according to several research conducted in Iraq (11,12) This finding could be attributed to a number of factors, including the fact that women's urethras shorter and closer to the anus than men's, which is a common cause of UTIs in women (12). According to the findings of this investigation, male patients had a higher frequency of *K. pneumoniae* isolates from different clinical samples. 47/87 (54.22%) compared to female patients 40/87 (45.97%). Compared to female patients, male patient had a higher rate of infection, this result was in line with Nirwati *et al.* (12). and different with reported from Raouf *et al* in AL-Najaf City (9) and Naqid *et al.* in Duhok city (12) they found that female patients had a higher rate of infection than male, and no gender related differences were discovered to be significant in the research of Ferreira *et al.* in Brazil (14). Smoking and drinking were poor lifestyle choices that were associated to the sex and *K. pneumoniae* incidence.

All of these isolates were ESBL producers and MDR. The results of earlier research' other findings were consistent with the high incidence of MDR *K. pneumoniae* patterns. (9, 15,16) confirmed the same result, while Adeosuns *et al*., (2019) found that 95.16% of isolates were MDR (17).

Inappropriate use of antibiotics or the transfer of resistant genes between organisms via plasmids capable of taking up resistant genes, integrons, bacteriophages, and transposons are possibilities, as evidenced by the rising prevalence of multidrug resistant strains of *K. pneumoniae* (17,18).

K. pneumoniae showed high susceptibility to Meropenem this result was in line with previous studies which found that susceptibility to Meropenem (97.5%, 85.7%, 27.42%) respectively (2,3,17). The result of PCR showed that 87(100%) of *K. pneumoniae* isolates were found to be positive for *CTX-M* gene. The finding of this study was in line with those of earlier investigations, which identified the *CTX-M* gene as the most prevalent ESBL subtype in *K. pneumoniae* isolates (9).

In contrast to this study findings, Carvaloho *et al*. from Portugal (15) found that *K. pneumoniae* had higher prevalence of *SHV* than *CTX-M*. these variances could be brough on by variation in the study population, sample size, and geographic locations.

K. pneumoniae strains that produce *CTX-M* are presently a concern in Iraq, and they may be linked to the improper use of third generation cephalosporins. The choice of the best antibiotic for treatment and infection control there for depends on the identification of ESBL-producing bacteria in patients through isolation. In order to track, manage, and stop the further spread of these isolates in Iraqi healthcare system, a routine surveillance program must be established in response to the introduction of MDR *K. pneumoniae* strains carrying ESBL resistance gene.

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