Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 876 - 887
Received 05 March 2021; Accepted 01 April 2021.

Detection of CTX-M and Carbapeneme Hydrolyzing Beta – Lactamase KPC in Clinical Isolates of *Klebsiella Pneumoniae*

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ABSTRACT

The aim of this study was to detection *Ctx-M* and *Kpc* genes in clinical isolates of *Klebsiella pneumoniae* . it was isolated 54 isolates of *Klebsiella* spp. from different clinical specimens (urine, stool, sputum , wounds , ENT and burns) that collected from different hospitals in Al-Nasiriyah city , Thi-qar province , Iraq. During period from September 2019 to February 2020. The isolates were identified and diagnosed by microscopic examination , biochemical tests and confirmed by API 20E and Vitek 2 system .*Klebsiella* isolates were tested against 18 antibiotic disks using the disk diffusion method. The results showed a high sensitivity of imipenem and amikacin, at a rate of 83%, for both antagonists,most isolates showed high rates of resistance to cephalosporins especially ceftriaxone (100%) and showed high resistance for the penicillins group (Ampicillin, Amoxicillin-Clavulanic acid), it was recorded 98% and 96% respectively. Two methods were used to detect ESBL, 32(59%) isolates were producers of ESBLs with the screening test. Whereas 35(65%) of isolates were ESBLs producers by modified double –disk synergy test (DDST) . The results of the molecular diagnosis of the *bla*-CTX-M and *bla*-KPC resistance genes showed the presence of the *bla*-CTX-M gene in 31 isolates (58.4%) while the *bla*-KPC gene was not present in any isolate of *Klebsiella pneumoniae*.

Keywords:*Klebsiella pneumoniae* , CTX-M , KPC , beta-lactamase

INTRODUCTION

*Klebsiella*spp. are gram-negative straight rods bacteria, arranged individually, in pairs or short chains, and it's slightly shorter than other Enterobacteriaceae members (Brisse *et al.*, 2005) .The majority of *Klebsiella* infections are related to hospitalization as an opportunistic pathogen , It primarily attacks people who are immune-compromised, and have a severe underlying disease such as diabetes , *Klebsiella* infections are mostly caused by *K. pneumoniae*, which is the most medically important type of the genus and to a much lesser extent, *K. oxytoca* has been isolated from human clinical specimens(Podschun and Ullmann, 1998).

Beta-lactam antibiotics are one of the most frequently antibiotics used in treatment of bacterial infections and the production of β-lactamase enzymes are the most common bacterial resistance

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mechanisms to beta-lactam antibiotics. *Klebsiella* spp. have the ability to acquire resistance to numerous antibiotics, particularly third-generation cephalosporins (Vasaikar *et al.*, 2017). In recent years, Extended-Spectrum Beta-Lactamase (ESBL) production by these bacteria has increased over the world ,the ESBLs are split into numerous groups; the main groups are TEM, CTX, and SHV derivatives (Manoharan *et al.*, 2011). CTX-M type ESBL are plasmid-encoded enzymes that have been detected in at least 26 bacteria. CTX-M is the most common type found in Enterobacteriaceae especially in *E. coli* and *K. pneumoniae* (Zhao and Hu, 2013). These β-lactamases confer resistance to penicillin's and expanded-spectrum cephalosporin's and the majority of the variants present higher rates of hydrolysis to cefotaxime than to ceftazidime , according to the latest review and new data within Gen-Bank, CTX-M-lactamases can be separated into five groups based on their amino acid sequence identities(Mohammed, 2015 ; Qasim and Al-Mayali, 2019).

Carbapenems are a group of β-lactam" antibiotics that are considered the last line of treatment for MDR isolates that are prevalent in many gram-negative bacterial species, especially those that produce ESBLs or AmpCβ lactamase ,the production of carbapenimases, especially the KPC enzyme, is the most important mechanism of enzymatic resistance in Enterobacteriaceae such such as *K. pneumoniae*(Bina *et al.*, 2015). According to the Ambler's classification method, carbapenemases can be divided into classes A, B and D. class A and class D are carbapenemases of serine β-lactamases, and class B carbapenemases are metallo-β-lactamases (MBLs) (Cui *et al.*, 2019). In 2001, the first KPC-producing *K pneumoniae* isolate was reported in North Carolina.The enzyme KPC, an Ambler class A beta-lactamase, KPCs are encoded by the gene *bla*-KPC and can be transferred between different species(Arnold *et al*., 2011). Most bacteria carrying the *bla*-KPCgene typically contain other resistance genes, resulting in resistance to multiple classes of antimicrobials, such as aminoglycosides and fluoroquinolones (Tzouvelekis *et al*., 2012), the several diverse KPC family variants (KPC-1 to KPC-22), the most well-characterized variants are KPC-2 and KPC-3(Lee *et al.*, 2016). Therefore, the main goal of thisstudy was to investigate of antimicrobial resistance genes (*bla*-Kpc and *bla*-Ctx-m) in *K. pneumoniae* isolated from different patients in different hospitals in Al-Nasiriyah city Thi-Qar province in Iraq.

MATERIALS AND METHODS

A-Isolation and identification of isolates

The specimens were collected from patients of (Al-Hussein Teaching , Al Haboubi ,Al-Musawi Children's andBint Al-hudahospitals). These specimens included urine , stool , sputum and swap specimens taken from ENT(ear , nose and throat) , burns and wounds. All specimens have been cultured immediately on the different media (blood agar , MacConkey agar and Eosin-methylene blue (EMB) agar), and incubated for 24 h at 37^0C.

Identification of isolates was based on Morphological examination, Gram stain , Biochemical tests and confirm by API 20E and VITEK 2 system(Macfaddin, 2000)

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B-Antibiotic susceptibility test

Antibioticssusceptibility of *Klebsiella*isolates were examined towards 18 different antibiotics byusing disk diffusion method.using the standard Kirby-Bauer disk diffusion method on Mueller- Hinton agar plates (Oxiod, England) (Bauer *et al,* 1966). These antibiotics includedAmikacin(10µg),Amoxicillin(30µg), Ampicillin (10µg) ,Aztreonam (30µg), Cefepime (30µg),Cefotaxime (30µg), Ceftazidim (30µg), Ceftriaxone (30µg), Chloramphenicol (30µg), Ciprofloxacin (5µg), Doxycycline (10µg), Gentamicin (10µg), Imipenem (10µg), Meropenem (10µg), Nalidixic acid(30µg), Tetracycline (10µg), Tobramycin (10µg) and Trimethoprim(10µg) (Bioanalys ,Turkey).

C-Phenotypic Methods for Detection of ESβLs

1-Primary (screening)test for production of ESBL

This test was done to three kinds of 3rd generation cephalosporins antibiotics: ceftazidime, cefotaxime, and ceftriaxone. If inhibition zone for bacterial isolates were ≤27 mm for cefotaxime, ≤22 mm for ceftazidime and ≤24 mm for ceftriaxone, these results considered as positive result for production of ESBL (CLSI, 2019).

2-Confirmatory test of ESBLs

The presence of ESBLs was detected in *Klebsiella* isolates using the double disk test.A disk containing 30µg amoxicillin–clavulanate was placed at the center of the Mueller–Hinton agar plate. A ceftazidime disk(30µg) andcefotaxime(30µg) were placed at distance 20mm (center to center) from the amoxicillin–clavulanate disk. The plates were incubated at 37^{o}C overnight , ESBLs production was inferred as positive if there was an expansion of the zone of inhibition for cefotaximeor ceftazidime toward amoxicillin – clavulanate disk or both(Livermore and Brown, 2001).

D-Molecular detection of *bla*-KPC and *bla*-CTX-M

1-DNA Extraction

The bacterial DNA was extracted withEasy Pure® Bacteria Genomic DNA Kit provided byTrans Gen Biotech (China).

2-Polymerase Chain Reaction (PCR) .

PCRwas used to detect *bla*-KPC and *bla*-CTX-M. All primers used in this study and all PCR thermo cycling conditions are listed in Table 1 and 2, respectively. All PCR products were loaded on a 1.5% agarose gel with 1µl of loading dye (Bromophenol blue) and were analyzed by gel electrophoresis and the product was visualized under UV-Transilluminator(Abbas, 2019).

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Table 1:Sequence of primers that used in this study

No.	Primer	Product size (bp)	Primer Sequence(5'-3')		Reference
1	*bla*-KPC	196	F	CAGCTCATTCAAGGGCTTTC	Findlay *et al*., (2012)
			R	GGCGGCGTTATCACTGTATT	
2	*bla*-CTX-M	544	R	TTTGCGATGTGCAGTACCAGTAA	Edelstein *et al*., (2003)
			F	CGATATCGTTGGTGGTGCCATA	

Table 2:Thermo cycling conditions of genes that used in the present study

1	**Amplification conditions of *Kpc* gene**		
	PCR Steps	Time	Temperature
	Initial denaturation	5min	94°C
	Number of cycles: 40 cycles		
	Denaturation	1 min	94°C
	Annealing	1:45 min	52°C
	Extension	1min	72°C
	Final extension	10min	72°C
2	**Amplification conditions of *Ctx-M* gene**		
	PCR Steps	Time	Temperature
	Initial denaturation	30 sec.	94°C
	Number of cycles: 30 cycles		
	Denaturation	15-30 sec.	94°C
	Annealing	15-60 sec	61°C
	Extension	1 min	68°C
	Final extension	5 min	68°C

RESULTS

Bacterial isolation

It was identified 54 isolates of *Klebsiella* spp. That distributed to :18 isolates from urine ,14 isolates from stool ,10 isolates from sputum ,8 isolates from wound and 4 isolates from ENT , while the burn smear did not show any isolate from *Klebsiella.* Fifty four of *Klebsiella* spp. was distributed into two species : *K. pneumoniae*in first order that recorded 53 (98%) isolates and *K. oxytoca* 1 (2%) isolate, Table (3).

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Table 3: Distribution of *Klebsiella* isolates according to source of specimens

Species / Source of specimens	No. (%)of *K. pneumoniae*	No. (%) of *K. oxytoca*	Total of *Klebsiella* spp.
Urine	18 (100%)	0(0%)	18 (33%)
Stool	13 (93%)	1 (7%)	14 (26%)
Sputum	10 (100%)	0 (0%)	10 (19%)
Wound	8 (100%)	0 (0%)	8 (15%)
ENT	4 (100%)	0 (0%)	4 (7%)
Burn	0 (0%)	0 (0%)	0 (0%)
Total	53 (98%)	1 (2%)	54 (100%)

Antimicrobial susceptibility tests

The results demonstrated that 98% of isolates were resistant to ampicillin and 96% to amoxicillin-clavulanic acid . *Klebsiella* spp.isolates showed high resistance tocephalosporins , all isolates (100%) were appeared resistant to ceftriaxone and 81% were resistant to cefotaxime,the resistant to other cephalosporins antibiotic that used in this study were variant , it was registered 54% for cefepime and 44% for ceftazidime. Monobactam antibiotic (Aztreonam) resistant was found 50%. The bacterial resistant to carbapenems were low , the isolates were registered high sensitive to this β-lactam antibiotic , it was recorded 83% for imipenem and 81% for meropenem .The other non β-lactam antibiotics that used in this study was aminoglycosides , the result showed low resistant of isolates against this group , it was appeared 17%, 22% and 24% for amikacin , tobramycin and gentamycin respectively .*Klebsiella* spp. were appeared low resistance to quinolones group (ciprofloxacin and nalidixic acid) ,which reached to 35% and 37% respectively . The result showed high sensitivity (80%) to chloramphenicol . it was found relatively higher levels of resistance (96% and 70%) for tetracycline and doxycycline respectively . Resistance of isolates to trimethoprim registered 56% figure (1) .

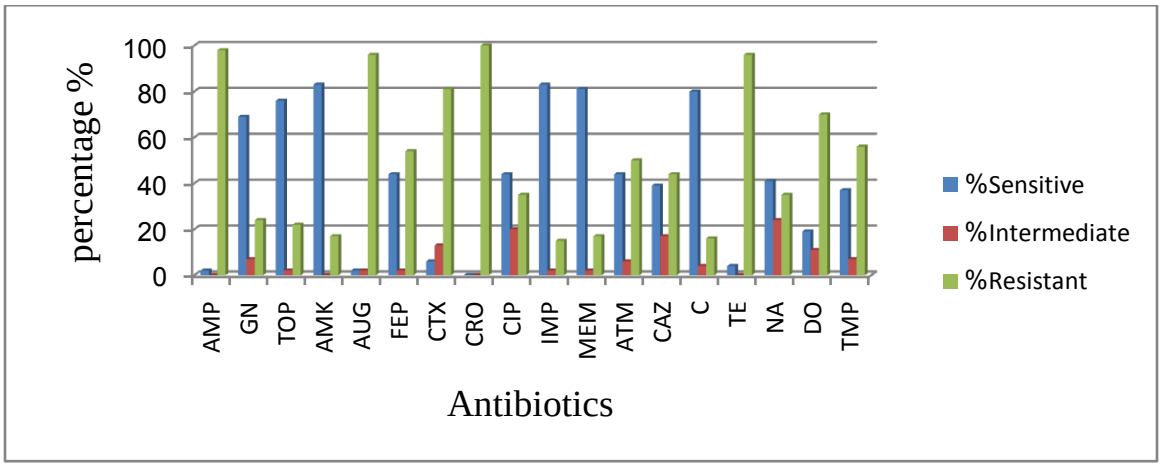

Figure 1 : Antibiotic susceptibility of *Klebsiella* spp.

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AMP: Ampicillin, GN: Gentamicin,TOP: Tobramycin,AMK: Amikacin, AUG: Augmentin, FEP: Cefepime , CTX: Cefotaxime, CRO: Ceftriaxone, CIP : Ciprofloxacin, IPM : imipenem, MEM : Meropenem, CAZ: Ceftazidime, C: Chloramphenicol, TE: Tetracycline, NA: Nalidixic acid, DO: Doxycycline, TMP: Trimethoprim

Phenotypic Method for Detection of ESβLs

The results of both ESβLs detection tests are shown in the table(4).

Table-4: production of ESBLs by phenotypic tests

TEST	No. & (%) Of ESBLS Producers Isolates	No. & (%) Of ESBLS Non Producers Isolates
Screening test	32 (59%)	22(41%)
Confirmatory test	35(65%)	19(35%)

Molecular detection of *bla*-Kpc and *bla*-Ctx-M in *Klebsiella pneumoniae*

DNA was extracted from 53 *K .pneumoniae* for amplification, to provide a template for PCR technique . Nanodrop had been used to estimate concentration and purity of DNA, the results showed a concentration between 53- 293(ng/µl) and purity (1.68 - 2.01nm) .The results of agarose gel electrophoresis were showed all isolates (53) of *K. pneumoniae* were negative for *bla*-KPCgene , they are not appear any bands of this gene in expected size (196bp).Results appeared that , out of 53 isolates , 31 (58.4%) isolates of *K. pneumoniae* were reveled band of *bla*-CTX-M in size 544 bp after electrophoresis in agarose gelfigure (2).

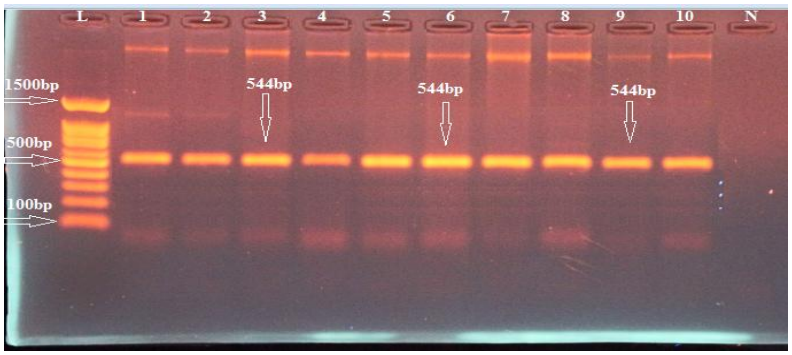

Figure 2 : Gel electrophoresis of *bla*-CTX-M gene (544 bp) (L: DNA Ladder100-1500bp, lane N :negative result, Lanes 1-10represent bands of *K. pneumoniae* isolates). Agarose: 1%, voltage: 100.

DISCUSSION

The dominance of *K. pneumoniae* among all *Klebsiella* spp. It was supported by the report documented by Hansen (1997) who found this species to be the most common among others .The results of the study showed , the frequency of *K. oxytoca* 1 (2%) isolates were less common than *K. pneumonia*

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.these finding were agreement with Al-Obadi (2014) who found only 2 isolates belong to *K. oxytoca.* Rubaye *et al* ., 2016 who found the number of *K. oxytoca* was 4 (6.9%).

The antimicrobials susceptibility analysis inthe current studyshowed high resistant to cephalosporins group antibiotics, including ceftriaxone, cefotaxime, cefepime, and ceftazidime. Theseresult were inagreement with Shallouf (2018) in Libya who reported high resistant rates to ceftriaxone(89.06%) . Bandari *et al.* (2020) in Iran found that 83.5% of isolates resistant to cefotaxime. Also, in local study it corresponds to the Al-Bassam (2015) showed high level of *Klebsiella* spp. resistance against cefotaxime at a percentage 91.3% , 82.6% to ceftazidime and resistance to the cefepime were 69.0%. The current study results showed high resistance to penicillin group included ampicillin, amoxicillin-clavulanic acid (Augmentin). These results were consistent with results of Alves *et al.* (2006) in Brazil who found that all isolates (100 %) were resistant to ampicillin. Also , these results were in agreement with local study of Al-obadi (2014) who noticed that the percentage of resistance to ampicillin and amoxicillin-clvulanic acid were 97.5 % for both. These high percentage also agreed with the results of Al-Taai (2015)who demonstrated all isolates of *Klebsiella* spp. were resistant to ampicillin and amoxicillin (Nori et al.,2021). .

Results also showed that the low resistance level of *Klebsiella* isolates to the aminoglycosides group included amikacin, gentamicin, and tobramycin . Similarly, Chinedu *et al.,* (2017) in Nigeria , reported that 8 (15.4%) of *Klebsiella* spp. isolates were resistant to gentamicin. Lobo and Moosabba (2020) in India , showed the resistance of amikacin was 24.1%.Al-MathkhuryandAssal (2012) in Baghdad, found that 22.2% of *Klebsiella* spp. isolates were resistant to the gentamicin and 48.1 % were resistant to the tobramycin. Abd Al-Rhman and Al-Aubydi (2015) showed all *Klebsiella* isolates were sensitive to amikacin and gentamicin. The present study showed low resistance of *Klebsiella* isolates to chloramphenicol. Similarly,Mahato *et al.*(2019) in Nepal found 33.3% of *Klebsiella* isolates were resistant to chloramphenicol .In local studies , Kadher and Jarallah(2019) in Hilla city showed that resistant rate for chloramphenicol was 42.6%. Regarded to tetracyclines (doxycycline and tetracycline), the results showed that most isolates of *Klebsiella* spp. were resistant to these antibiotics. Abbas and Jarallah (2017) recorded 62.6% of *K .pneumoniae*were resistant for tetracycline . Omar-Zahid (2009) recorded 71.5% of *Klebsiella* species were resistant for doxycycline(Tahmasebi et al.,2021).

Quinolones such as ciprofloxacin and nalidixic acid also used in this study , the results appeared that resistant rate of *Klebsiella* isolates to these group of antibiotics were low,these results were agreement with results ofAhmed *et al.* (2013) in Egypt and Ghanem *et al*. (2017) in Saudi Arabia , these studies foundthat, the resistant of isolates forciprofloxacin were 6.7% and 3.9% respectively .Albassam (2015) reportedthat 23.9% of *K. pneumoniae* isolates were resistant for ciprofloxacin . Al-Gerir (2012) in Mosul showed 41.4% of *Klebsiella* isolates were resistant to nalidixic acid . Garza-Ramos *et al.* (2018) in México noticed that the percentage of resistance to nalidixic acid was 20.5%. Relatively moderate resistant of *Klebsiella* isolates was recorded to trimethoprim in this study, these results were in agreement with Ali *et al.,* (2019)who noticed that the percentage of resistance to trimethoprim was 61.54%.The current study showed that the *Klebsiella* resistant to monobactams antibiotic (Aztreonam) was low , these results were agreement with results of Pereira and Vanetti (2015) in Brazil recorded 38.1% of *Klebsiella* isolates were resistant to this antibiotic. The results of this study appeared that, the

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isolates were sensitive to imipenem and meropenem ,the result of current study agreement with many studies, Al-Obadi, (2014)showed that *K. pneumoniae* was sensitive to imipenem in rate 97.5 %. Chinedu *et al.* (2017) showed that 100 % of *Klebsiella* isolated from infections in Nigeria were sensitive for both antibiotics. Ghanem *et al.*, (2017) in Saudi Arabia , found 99.5% and 100% of *Klebsiella* isolates were sensitive to imipenem and meropenem, respectively.

ESBLs are more common in *K. pneumoniae* than any other type of enteric bacteria(Al-Mohana *et al*., 2010). In the present study, using the double disk test to detect ESBL ,the current results show that the high percentage of isolates produces ESBLs,the results of the present study were identical with the results of local study, Al-Janaby and Al-Hasani(2016) who reported that rate of ESBL producing *K. pneumoniae* was 62.5% in AL-Najaf province ,and similar to the results Shabaa (2014) who found *Klebsiella* isolates gave positive ESBLs in percentage 59.6%,and the results of international studies, Ullah *et al*. (2009) in Pakistan showed 58.7% isolates were found to be ESBL producers. Gharrah *et al.*, (2017) in Egypt referred to 50% of isolates were ESBL producers .

Polymerase chain reaction(PCR) is considered to be the best efficiency technique for detecting ESBLs because it is faster than the phenotypic methods and too detects the existence of poorly or non-expressed (silent) genes that are difficult to determine by phenotypic method (Diekema *et al* ., 2004). CTX-M type of ESBLs genes was detected in this study,it was found from the results of this study , 58.4% of isolates have been positive for the presence of *bla*-CTX-M genes. The resistance of the third generation cephalosporins that we observed in high proportions among our isolates may be attributed to the spread of β-lactamases CTX-M types .The results of this study were in agreement with other studies, in Kurdistan region , Iraq whenKhalid *et al.* (2013) referred to 58% of *Klebsiella* spp. isolates possess *bla*- CTX-M gene . Abbas and Edi (2015) in Thi-Qar Province found 28 (62.22) of *Klebsiella* isolates have been positive for the presence of genes *bla*-CTX-M. In international studies, Jemima and Verghese (2008) from India , stated 25 of 62 (40%) of *Klebsiella* species had *bla*-CTX-M gene. In South Africa Vasaikar *et al.* (2017) who performed PCR on 157 isolates , 89 (56.7%) isolates gave positive results for*bla*-CTX-M gene . The high prevalence of *bla*-CTX-M among *K. pneumoniae* may represent selective pressure due to the wide prescriptions of cephalosporins, particularly cefotaxime and ceftriaxone in many geographical regions However, the prevalence rates vary not only among different countries but even between different hospitals in the same country (Hamam *et al*., 2019) .

Several genes are involved in carbapenems resistance among *Enterobacteriaceae*, which may differ from country to country. In this study, *bla*-KPC gene was detection by conventional PCR .The results appeared all isolates of *K. pneumoniae* were negative for amplification of the *bla*-KPC gene. KPC producing *K. pneumoniae* has detected in several countries, particularly Greece and Italy and has quickly become endemic in some countries such as China, Colombia, and dissemination of KPC around the world highlights the role of *bla*-KPC gene in the spread of antimicrobial resistance; Thus strains harboring *bla*-KPC gene are a main cause of concern for healthcare systems around the world (Ghasemnejad *et al* ., 2019). In this study, the results of *bla*-KPC gene in *K. pneumoniae* indicated a no prevalence inthe city of Al-Nasiriyah. As an resistance to carbapenems may be attributed to other mechanisms such as porin-mediated resistance, efflux pumps and other enzyme-mediated resistance which is mediated via the acquisition of carbapenemase genes (Elshamy and Aboshanab, 2020). The

results of our current study were consistent with the results obtained by Bina *et al.* (2015) in Iran who found 270 *K. pneumoniae*isolates , gave a negative result when amplifying the *bla*-KPC gene in the PCR method. Chinedu *et al*. (2017) in Nigeria, indicated that of the 52*Klebsiella* isolates tested for PCR ,none of these isolates yielded a positive result for this gene.While the local studies, Al Sehlawi*et al* ., (2013) in Al-Najaf, found all isolates were negative for *bla*-KPC.

In conclusion, the results of our study revealed that CTX-M gene were showed high prevalence in the tested isolates and all *K. pneumoniae* isolates don't carry *bla*-KPC gene. High degrees of antimicrobial resistance indicate monitoring of antimicrobial resistance mechanisms and a focus on the rational use of antimicrobials to reduce the spread of ESBL-producing bacteria and this study recommendsto study another antimicrobial resistance genes within *Klebsiella* spp.

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