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ORIGINAL ARTICLE

The predominance of *Klebsiella pneumoniae* carbapenemase (KPC-type) gene among high-level carbapenem-resistant *Klebsiella pneumoniae* isolates in Baghdad, Iraq

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Abstract

Background The serine carbapenemase enzymes (KPC) which produce from bacteria *klebsiella pneumoniae* today have been emerged as one of the β-lactamase enzymes that is capable to inactivating the last line of carbapenems. The gene encoding the *K. pneumonia* (bla_{KPC}) belongs to gene carried on plasmid among *Enterobacteriaceae* family, which has modulation for the infections control so this study is aimed to spot the presence and evaluate bla_{KPC} gene expression by real-time PCR in local isolates of *K. pneumonia.*

Methods Forty-seven of *K. pneumonia* isolates were isolated from different clinical samples (blood, sputum, urine, wounds and burns) from patients in separate hospitals in Baghdad., Antimicrobial sensitivity test was carried out by vitik-2 system and Kirby- Bauer method. The PCR was employed to detect carbapenemase gene.

Results The results of this study showed that all explored isolates were resistant to Ertapenem, Meropenem and imipenem 47(100%). Phenotypically, all the isolates had carbapenemase which hydrolyzed the carbapenem antibiotics. Furthermore, the isolates showed (100%) resistance to Cefazolin, Ampicillin and Amoxicillin/ Clavulic acid. However, the most effective antibiotic was Levofloxacin (91.5%). The results of conventional PCR technique for the detection of bla_{KPC} gene showed that 38 (80.9%) isolates of carbapenem-resistant *K. pneumoniae* harboured bla_{KPC} gene (1010 bp), while none carried other carbapenemase genes including bla_{NDM1}, bla_{VIM} and bla_{IMP} genes. High levels of carbapenem resistance was clarified by the imipenem and meropenem MICs determination. All 38 isolates were positive in CNPT. Furthermore, the 38 isolates showed over expression of bla_{KPC} gene compared with housekeeping *rpo* gene in Real-Time PCR.

Conclusions According to these results, the resistant isolates to carbapenem were belong to the present and high level expression of bla_{KPC} gene in our local isolates.

Keywords Carbapenem · *Klebsiella pneumonia* · Carbapenem resistance · KPC-Gene and Real-Time PCR

Introduction

The Carbapenem antibiotics like ertapenem, meropenem and Imipenem considered as the most therapeutic agent importance in health care units. Due to broad spectrum of activity of these antibiotics, antibiotics are recommended as the first line of therapy in cases of severe infections that are caused by bacteria producing Extended Spectrum β-lactamases (ESBL) enzymes, ESBL bacteria related to gram negative multi-drug resistant bacteria and Enterobacteriaceae like *Pseudomonas aeruginosa* as well as *Acinetobacter spp*. [1, 2, 3]. Resistance to carbapenem antibiotics had been rare previously, but the emergence of transmissible carbapenem resistance is now increasing concern [4]. Class-A *K. pneumoniae* carbapenemase (KPC) has the increasingly popular mechanism of carbapenem resistance among bacteria [5].

In the family *Enterobacteriaceae*, resistance to carbapenems is specially among *Escherichia coli* and *K. pneumoniae*, and it is an emerging problem throughout the world [5, 6].

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KPC-type enzymes also can confer the resistance to whole carpenems such as ertapenem, meropenem, imipenem and doripenem [4]. These enzymes were called KPC since they had been mainly identified in *K. pneumoniae* [7], and the KPC enzymes in KPC- *K. pneumoniae* isolates were firstly recorded in North Carolina in the year 2001 [8].

The emergence of the carbapenemase is becoming an increasing therapeutic concern as these enzymes are not only the carbapenems but also cephalosporins, penicillins, and monobactams [4, 8]. Resistance to carbapenems antibiotics usually correlated with resistance to many antibiotics belong to β-lactam and non β-lactam antibiotics. Hence, the treatment of those infections were caused by carbapenemase producing *K. pneumoniae* organisms that are hardly due to their high resistance, resulting in the increase in the mortality rates [3, 9]. For these reasons, this study aimed to investigate the *K. pneumoniae* isolates resistant to antimicrobial agents including the carbapenem antibiotics and detection of carbapenemase gene by phenotypic and molecular techniques.

Methods

Bacterial isolates

Forty-seven carbepenem- resistant *K. pneumonia* isolates were collected from clinical samples during (2018 to 2020). the clinical samples including (sputum, blood, wounds, urine and burns) of patients which hospitalised in separate hospitals in Baghdad. All the bacterial isolates under the study were identified depending on biochemical tests and the morphological characteristics [4, 9]. The identification was confirmed by using Vitek-2 system (BioMeriuex, France) and ID-GNB cards depending on manufacturing instructions.

Antimicrobial susceptibility test

All (47) *K. pneumonia*e clinical isolates were subjected to antimicrobial susceptibility test, Each *K. pneumonia*e isolate was determined to be sensitive or resistant to ertapenem, meropenem, imipenem and many other antimicrobials using Vitek-2 system with AST cards and Kirby-Bauer methods depending on CLSI [10].

Carbapenems and aminoglycosides MICs

The MICs of imipenem, meropenem, gentamicin and tobramycin were carried out by using agar dilution methods depending on CLSI guidelines.

Carba-NP-test

The Carba NP-test was performed for the detection of phenotypic of carbapenemase production as recommended in CLSI [10], that was done by suspended 1 µl of scraped overnight mass colonies on (MHA) in eppendrof have (100 µl) of 20mM Tris-Hcl as lysis buffer, mixed well by vortex for 5s. (100 µl) of an aqueous indicator solution containing (0.1 mmol/litter $ZnSo_4$) with 0.05% phenol red (pH 7.8, 6 mg/ml imipenem). The test phenol red was an indicator and imipenem, which was used as a substrate (reaction tube), control tube contained phenol red solution without imipemem.

Molecular detection of carbapenemase genes

A conventional PCR was done for all 47 studied carbapenem-resistant *K. pneumoniae* isolates to detect bla_{kpc} gene by using specific primers as listed in Table 1. The reaction contained (12.5 µl) of 2X master mix (Kapa, India), (2 µl) of each primers, DNA samples (5 µl) and nuclease- free water was completed final volume to (25 µl). The temperature of the cyclic conditions included first step with initial denaturation at 95 °C/5 min. This was followed by 35 cycles as denaturation step at 95 °C/ 1 min, and then it was annealed at 58 °C/ 30 s, and it was extended at 72 °C/ I min. The final extension step was at 72 °C/ 10 min. [11] The gel electrophoresis with ethiduim bromide and UV transilluminator documentation system were used, and the products of conventional PCR were visualized [12].

RNA extraction

RNA was extracted from bacterial isolates by utilizing RNA extraction kit from (Qiagen-USA) according the manufacture instructions. Total RNA was mixed with DNase enzyme to prevent the contamination and rapidly stored at -70 °C. The purity and concentration of RNA was checked by nanodrop.

Real-time PCR reaction

The reaction of RT-PCR was achieved by using GoTaq®qPCR master mix (promega/ USA). The mixture of reaction was contained 10 µl of master mix, 1 µl of each primers as list in Table 1, template DNA 3 µl and completed volume to 20 µl with nuclease free water. The thermal profile of RT-PCR conditions contained 95 °C/ 5 min. followed by 40 cycles of 95 °C/ 15 s as initial denaturation, and annealing at 58° C/ 20s finally 72 °C/ 20s for the extension step. After that, the differences between the (CT) mean threshold cycle of the reference gene from the main (CT) of the gene for both test samples and control were calculated. The ΔCT

Table 1 Specific primers for the bla$_{KPC}$*K. pneumoniae*

Gene	Oligonucleotide		References
bla$_{kpc}$ forwarded primer	5` -TGTCACTG-TATCGCCGTC- 3`	1010 bp	11
bla$_{kpc}$ reversed primer	5` -CTCAGTGCTC-TACAGAAAACC-3`		
Rpo forwarded primer	5`-GGCGAAATG-GCWGAGAAC- 3'	1056 bp	4
Rpo reversed primer	5`-GAGTCTTC-GAAGTTGTAA- 3'		

index was calculated in two control samples and test, ΔΔCT was calculated by the differences between two ΔCT.

Results

Bacterial isolates and antimicrobial susceptibility test

In the current study, the forty-seven clinical isolates of K. pneumoneai resist to carbapenem were isolated and identified from different clinical samples including; sputum 19(40.4%), blood 10(21.3%), wounds 9(19.1%), burns 7(14.9%) and urine 2(4.3%) of patients which were hospitalised in separate hospitals in Baghdad during a period from 2018 to 2020.

As shown in Table 2, in the antimicrobial susceptibility test, all isolates of *K. pneumoniae* 47(100%) were resist to the carbapenem antibiotics including; Ertapenem, Imipenem and Meropenem (the isolates that showing intermediate levels of susceptibility were considered as resistant isolates) [10]. Also, all isolates had carbapenemase which hydrolyzed the carbapenem antibiotics phenotypically.

Also all *K. pneumonia*e were resistant to Ampicillin, Amoxicillin/Clavulin acid and Cefazolin (100%). All isolates appeared resistant to many antibiotic under this study except the Levofloxacin and Ciprofloxacin which showed the highest sensitivity rates (91.5%) and (85.1%), respectively.

Carbapenems and aminoglycosides MICs

In the MICs of imipenem, meropenem, gentamicin and tobramycin using agar dilution method, 39/47, 40/37, 40/47 and 41/47 of isolates were resistant exhibiting a high level of resistance. Noticeably, 39 isolates exhibited imipenem and meropenem MICs > 16 µg/ml.

Table 2 Results of antimicrobial susceptibility test of 47 *Klebsiella pneumoniae* isolates

Antimicrobials	N(R%)	N(I%)	N(S%)
Ampicillin	**47(100%)**	**0(0%)**	**0(0%)**
Amoxicillin/Clavulanic acid	**46(97.9%)**	**1(2.1%)**	**0(0%)**
Piperacillin/ Tazobactam	**44(93.6%)**	**2(4.3%)**	**1(2.1%)**
Cefazolin	**47(100%)**	**0(0%)**	**0(0%)**
Ceftazidime	**40(85.1%)**	**3(6.4%)**	**4(8.5%)**
Ceftriaxone	**41(87.2%)**	**1(2.1%)**	**5(10.6%)**
Cefepime	**40(85.1%)**	**2(4.3%)**	**5(10.6%)**
Meropenem	**43(91.5%)**	**4(8.5%)**	**0(0%)**
Imipenem	**45(95.7%)**	**2(4.3%)**	**0(0%)**
Ertapenem	**47(100%)**	**0(0%)**	**0(0%)**
Gentamicin	**44(93.6%)**	**0(0%)**	**3(6.4%)**
Tobramycin	**42(89.4%)**	**1(2.1%)**	**4(8.5%)**
Ciprofloxacin	**5(10.5%)**	**2(4.3%)**	**40(85.1%)**
Levofloxacin	**3(6.4%)**	**1(2.1%)**	**43(91.5%)**
Nitrofurantoin	**37(78.7%)**	**4(8.5%)**	**6(12.8%)**
Trimethoprim/ Sulfamethoxazole	**31(66%)**	**0(0%)**	**16(34%)**

Carba NP-test

In Carba NP-test to detect phenotypic carbapenemase production (38/47) of isolates could produce carbapenemase enzymes.

Molecular detection of carbapenemase genes

The results of conventional PCR technique showed that the out of 47 carbapenem-resistant *K. pneumoniae* isolates, 38(80.9%) isolates harboured the *bla*$_{KPC}$ gene with 1010 bp size, but none carried other carbapenemase genes (Fig. 1). From these results, it can be noticedthat our local isolates have carbapenem resistance, that's belongs to produce *bla*$_{KPC}$ gene.

Real-time PCR

The results of this study showed all 38 isolates with positive of bla$_{kpc}$ gene have high expression of of bla$_{kpc}$ gene compared with housekeeping *rpo* gene, the Ct results of *rpo* gene were ranged from (16.72–18.96) within average Ct. (17.32), while the Ct value of *bla$_{kpc}$* gene ranged from (15.21–20.23) within average Ct.(17.86) by fold (0.02–4.23) for *bla$_{kpc}$* gene as shown in Fig. 2.

Discussion

Centers for Disease Control (CDC) reported bacteria *k. pneumonia* have been responsible for 3% of nosocomial outbreaks and 8% of hospital-acquired infections [13]. The most public health and clinical concern are the vast spread of

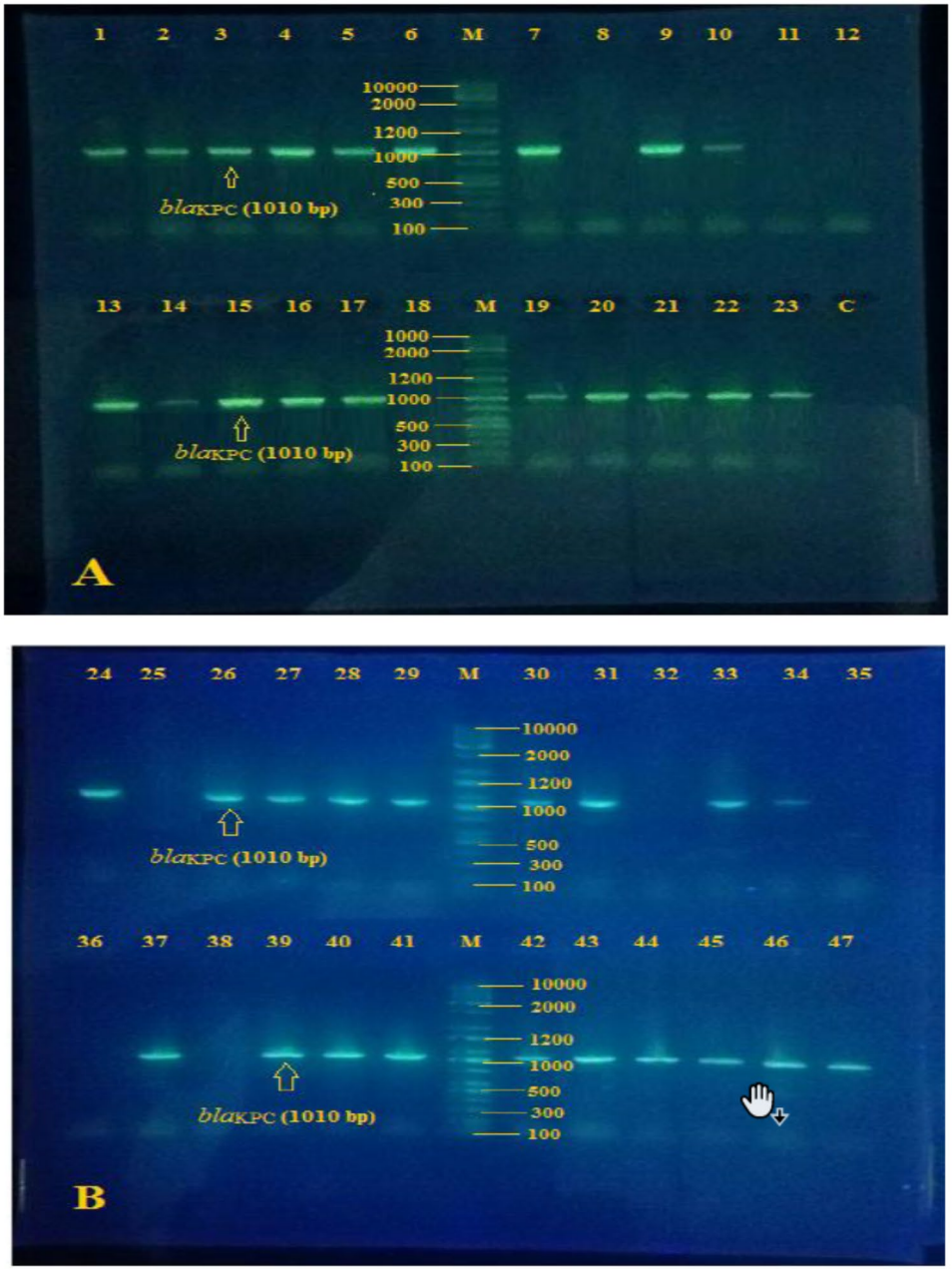

Fig. 1 (A & B): Agarose gel (1%) electrophoresis PCR product of of bla_{KPC} (1010 bp). Lanes 1–47: *K. pneumoniae* isolates; Lane C: control negative; M: Kappa express DNA ladder

KPC-producing *K. pneumoniae*, and they are rising in new locations throughout the world, referring to ongoing process [1] **that** belong**s** to the us**age** of carbapenem antibiotics in the first-line to treat unidentified microoraganisms and last-line antibiotics to treat infections [1]. The dissemination of KPC- producing *k. pneumoniae* cross**es** the worldwide sporadic, epidemic or endemic has been reported, **it becomes** endemic pathogen in some hospitals and their considered responsible for rising the numbers of outbreaks in several healthcare units in Eastern USA [1], Greece [13] and Israel. It increases the rate of antimicrobial resistance [6, 17]. The PCR assay offers an advantage of the rapid detection of this gene [4, 1]. Our result showed the presence of bla_{KPC} gene in 38(80.9%) isolates by conventional PCR assay. The results of using RT-qPCR technique showed over expression of bla_{KPC} gene compared with expression of housekeeping *rpo* gene, that explain the high rate of resistance.

Carbapenems are most effective agents against multiple antibiotic resistant *K. pneumonia* however recent studies have demonstrated high-level resistance to carbapenems [5, 17]. Despite of this type of resistance has been studied excessively in the world, in Iraq the studies are still scare and fail to cover the depth of serious problem till now specially carbapenem antibiotic very restricted in treatment

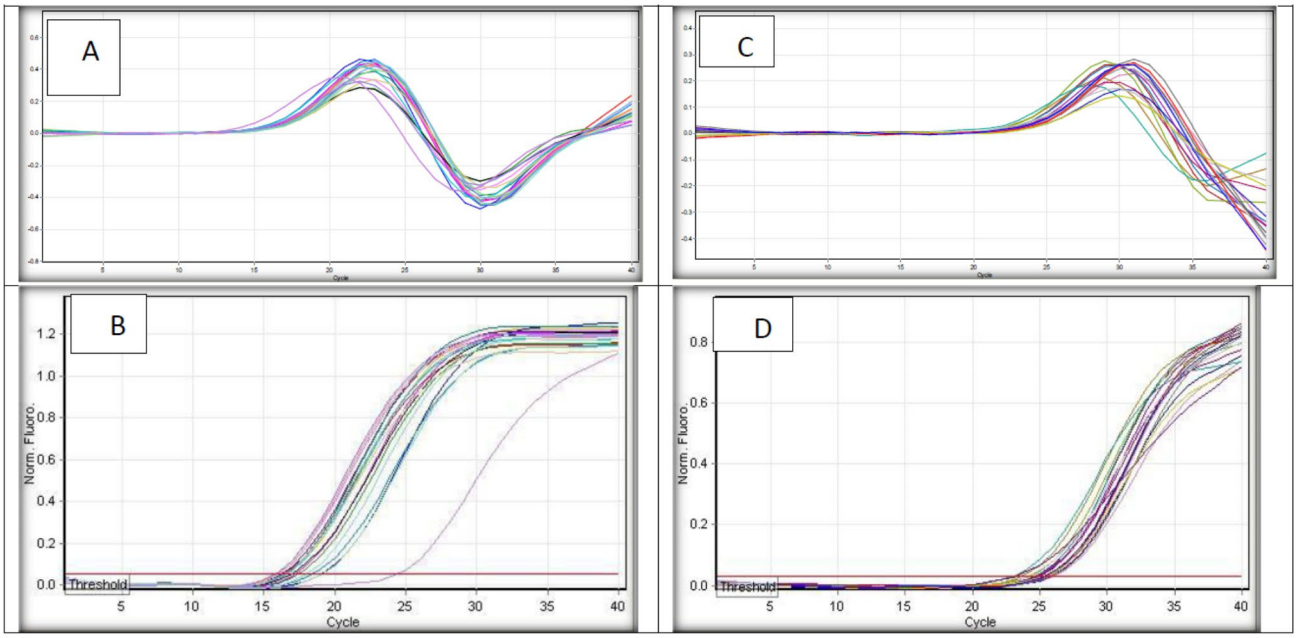

Fig. 2 A-Comparative quantitation analysis of bla_{KPC} gene; B-amplification curve of bla_{KPC} gene; C-comparative quantitation analysis of *rpo* gene; D-amplification curve of *rpo* gene

the bacterial infection. Many outbreaks by KPC-producing *k. pneumoniae* were recorded in Canada, Korea, Greece, Kenya and Italy, many reports showed the mortality rate in Greece is 37% and in Italy 41.6% [1, 6, 18]. The frequency of resistance is consistent with dramatically increase in the recurrence of producers among clinical isolates in several regions and parts of the world [19, 20]. The progressive increasement of Carbapenems resistant strains is still documented via the worldwide, and it is still considered as a major and global threat the worldwide [1, 6]. We observed through the disc diffusion method that all isolates exhibited high antibiotics resistance toward antibiotics which was used in this study specially imipenem, gentamicin and the most effective antibiotic for there is ciprofloxacin, while the MIC appear at < 16 µg/ml for imipenem and meropenem antibiotics. The high rate of carbapenemase production Carba NP-tests. we recommend doing many studied about this gene and study the relatedness with virulence factors of bacteria, study the prevalence of gene in other bacteria, and doing genetic analysis to it by sequencing to know which type are carried in Iraqi bacterial isolates specially now appeared many types of it's like kpc1,kpc2 and others.

Conclusions

High level of carbapenems resistance was clarified by the imipenem and meropenem MICs determination (MIC > 16 µg/ml). The most of carbapenemase-producing isolates harbored the bla_{KPC} gene, but none of them carried other genes. Moreover, in the RT-qPCR the over expression of bla_{KPC} gene was noticed. According to these results, the carbapenemase resistance in baghdad city belongs to production of bla_{KPC} gene.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Consent to Participate (Ethics): the Ethics Committee of the Mustansiriyah University approved and oversaw this study.

Consent to Publish (Ethics): All authors agree to publish this work.

Informed consent All patients gave their written informed consents before inclusion.

Research involving human and animals participants This study involved the forty-seven clinical isolates of K. pneumoneai from patients who are suffering from infections of sputum, blood, wounds, burns and urine at Baghdad hospitals, Baghdad, Iraq.

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