ISSN: 0067-2904
GIF: 0.851

Detection of bla_{KPC} Gene in Some Clinical *Klebsiella pneumoniae* Isolates in Baghdad

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Abstract

For the period from February 2014 till May 2014, one hundred and nine lactose fermenter clinical isolates from different samples (urine, stool, wound swab, blood, and sputum) were collected from Alyarmok, Alkadimiya, and Baghdad teaching hospitals at Baghdad governorate. Identification of all *Klebsiella pneumoniae* isolates were carried out depending on macroscopic, microscopic characterizations, conventional biochemical tests, and Api 20E system. Fifty-three (48.62%) isolates represented *K. pneumoniae*; however, 51.73% represented other bacteria. Susceptibility test was achieved to all fifty-three *K. pneumoniae* isolates using five antibiotic disks (Ceftazidime, Ceftriaxone, Cefotaxime, Imipenem, and Meropenem). Most of tested isolates (90.5% and 77.3%) were susceptible to Meropenem and Imipenem, respectively and less susceptible to third generation Cephalosporin. Carbapenemase production was detected by the modified Hodge test, five carbapenem resistant *K. pneumoniae* isolates (K2, K3, K4, K34, and K35) gave positive results. In the other part in this study, detection of bla_{KPC} gene by pcr techinique was carried out on all fifty-three *K. pneumonie* isolates. Even though five isolates gave positive modified Hodge test, only one isolate (K2) gave specific identification for bla_{KPC} gene.

Keywords: *Klebsiella pneumoniae, bla*$_{KPC}$, Carpabenem, Hodge test.

الكشف عن مورث bla_{KPC} في بعض العزلات السريرية للكليبسيلا الرئوية المعزولة من بغداد

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الخلاصة

جمعت 109 عزلة مخمرة لسكر اللاكتوز للمدة من شباط 2014 ولغاية شهر ايار2014 من مختبرات مستشفى اليرموك التعليمي والكاظمية التعليمي ومستشفى بغداد التعليمي في مدينة بغداد حيث شملت مختلف العينات السريرية (ادرار و خروج ومسحات جروح وعينات دم وقشع).عزلت بكتريا *Klebsiella pneumoniae* . تم تشخيص عزلات *K. pneumoniae* اعتمادا على الصفات المظهرية والمجهرية والفحوصات الكيمياحياتية . وقد تم تأكيد التشخيص باستعمال نظام Api 20 E ، مثلت عزلات *K. pneumoniae* مانسبته 48.62% وباقي انواع البكتريا 51.73% . اظهرت نتائج فحص الحساسية الدوائية لثلاث و خمسين عزلة *K. pneumoniae* باستعمال خمسة انواع من مضادات الحياة (سيفتازديم و سفترازون و سيفوتكسايم و اميبينيم و ميروبينيم) ، اظهرت معظم العزلات قيد الدراسة حساسية لمضادي ميروبينيم واميبنيم بنسب مئوية 90.5% و 77.3% على التتابع ، واظهرت العزلات حساسية اقل لمضادات

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سيفالوسبورينات الجيل الثالث. اختبرت العزلات المنتجة لانزيم الكاربابنيميز بوساطة فحص هوج المحدث ، اذ اعطت خمس عزلات مقاومة لمضادات الكاربابنيم (K2 و K3 و K4 و K34 وK35 نتيجة موجبة لهذا الاختبارمن مجموع 53 عزلة لبكتريا *K. pneumoniae*. بينت النتائج في هذه الدراسة على المستوى الجزيئي باستخدام تقنية pcr لتحديد الجين المقاوم لمضادات الكاربابنيم (bla_{KPC}) ل 53عزلة *K. pneumoniae* . على الرغم من وجود خمس عزلات موجبة لفحص هوج المحدث ، فقد اعطت عزلة واحدة (K2) تشخيصا موجبا لجين bla_{KPC}.

Introduction

Klebsiella pneumoniae is a Gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic, rod-shaped bacterium, establish in the normal flora of the mouth, skin, and intestine [1]. This bacteria present in the respiratory tract and feces of about 5% of normal individuals. It causes a small proportion of bacterial pneumonias. It can produce extensive hemorrhagic necrotizing consolidation of the lung. It occasionally produces urinary tract infection and bacteremia with focal lesions in weakened patients [2].*K. pneummiae* is frequently related with hospital-acquired infection. Certain underlying diseases such as malignancy, cirrhosis, biliary tract disorders, urinary and biliary tract infections, osteomyelitis and bacteremia diabetes mellitus (DM), and alcoholism may impair an individual's defenses and increase the risk of *K. pneumoniae* infection. This species is a second most common cause of Gram-negative bacteremia after *Escherichia coli*. *K. pneumoniae* bacteremia causes significant morbidity and mortality in general populations [3].

Although *K. pneumoniae* owns only moderate amounts of chromosomal penicillinases, it is a well-known "collector" of multidrug resistance plasmids that commonly encoded resistance to aminoglycosides, till the end of 1980s, while, later, encoding extended-spectrum β-lactamases (ESBLs), mostly Temoniera (TEMs) and Sulfhydryl variable (SHVs) active against last generation Cephalosporins, as well as a diversity of genes conferring resistance to drugs other than β-lactams [4]. The acquisition of these plasmids and the happening of chromosomal mutations that confer resistance to Fluoroquinolones often makes the treatment of *K. pneumoniae* healthcare-associated infections possible only by using Carbapenems as "last-resort of defense" antibiotics [5].

Carbapenemases denote the most versatile family of Beta-lactamases, with a breadth of spectrum unrivaled by other β-lactam-hydrolyzing enzymes. Although known as "carbapenemases," many of these enzymes distinguish almost all hydrolysable β-lactams, and most are resilient against inhibition by all commercially viable β -lactamase inhibitors [6].

K. pneumoniae carbapenemase-1 (KPC-1), a class A carbapenemase in *K. pneumoniae*, has altered the patterns of carbapenemase dissemination [7]. What was once considered to be a problem of clonal spread has now become a global problem of interspecies dispersion. Because of the proliferation of new members of established carbapenemase families, it is even more important to attempt to understand the properties of these enzymes, with all their strengths and limitations [8]. In.The infection with these highly resistant isolates are increasing and spreading in hospitals and community. This may be due to antibiotic misuse and low hospital finances for controlling the spread of these isolates [9].

This work aimed to investigate the distribution of bla_{KPC} in clinical isolates of *K. pneumoniae* isolated from Iraqi patients in Baghdad medical facilities.

Materials and Methods

One hundred and nine of lactose fermenter clinical isolates were collected from patients (nineteen to sixty years old) visiting Baghdad hospitals include Alyarmok laboratories, Alkadimiya laboratories and Baghdad teaching laboratories. The clinical specimen comprised urine, stool, sputum, blood, and wound swabs. Api 20E system (BioMerieux, France) was employed to confirm the identification results.

Isolation and identification

Identification of isolates was achieved according to Bergey's Manual of Systematic Bacteriology, 2nd edition [10] , included morphological characteristics and biochemical tests. All clinical isolates were collected and directly cultured on blood agar plates and incubated at 37ºC for 24 hours. Opaque grayish or white colonies on blood agar were recultured on MacConkey agar (Salucea, Netherland)

plates for primary identification incubated at 37ºC for 24 hours, the large, pink, and mucoid colonies were selected and subcultured on another MacConky agar to obtain discrete colonies.

Antibiotic susceptibility test

Five antibiotic disks were used in this study summarized in table-1. Antibiotic susceptibility test for *K. pneumoiae* isolates was done according to Baur *et al.* method [11], through inoculated agar plates with bacteria isolates and incubate at 37°C for 24 hr, then 4-5 pure and identical colonies transfer brain heart broth (Salucea, Netherland) and incubate at 37°C for 3-5 hr to obtain turbidity compared with the standard turbidity solution, this approximately equals to $1.5x10^{8}$ CFU/ml. after that by using sterile cotton swab the Mueller-Hinton agar (Salucea, Netherland) plate was inoculated by bacterial suspension in all direction, let dry for 15 min, after that antibiotic disks were placed on the surface of Mueller-Hinton agar using sterile forceps and about 15 mm away from edge of plate, the plates were incubated at 37°C for 24 hr. Afterwards, the inhibition zone was measured and comparison with was accomplished. Results were interpreted according to CLSI [12].

Table 1- Antibiotic disks used in this study

Antibiotic discs	Symbol	Disk potency (µg/disk)	Company (Origin)
Ceftriaxon	CRO	30 µg	Bioanalyse (Turkey)
Ceftazidime	CAZ	30 µg	
Cefotaxime	CTX	30 µg	
Imipenem	IPM	10 µg	
Meropenem	MEM	10 µg	

Modified Hodge test (MHT)

This test was used to detect *K. pneumoniae* carbapenemase (KPC) according to CLSI [12]. In brief; Muller-Hinton agar (MHA) was seeded with *E. coli* ATCC 25922 suspensions compared with 0.5 McFarland standard no. 0.5, plates were allowed to dry for 3-10 min. Thereafter, Meropenem antibiotic disk (10 µg/ml) was placed on the center of MHA plate. By an aid of a loop, 3-5 colonies grown overnight on agar plate were inoculated in straight line out from the edge of the disk. Eventually, Mueller-Hinton plates were incubated at 37°C for 16-20 hr. MHT Positive test has a clover leaf-like indentation of the *E. coli* growing along the test organism growth streak within the disk diffusion zone. MHT Negative test has no growth of the *E. coli* along the test *K. pneumoniae* growth streak within the disk diffusion. A positive MHT indicates that this isolate is producing a carbapenemase. Nonetheless, a negative MHT indicates that this isolate unable to produce carbapenemase.

Polymerase chain reaction (PCR) technique

PCR was done to amplify different fragments of bla_{KPC} in a premix tube (Bioneer, Korea) for the detection of *Klebsiella pneumoniae* carbapenemase encoding gene (carbapenem resistance gene).

Primers selection and preparation

Forward and Reverse primers (Prmega, USA) for the detection of bla_{KPC} were chosen according to Doyle *et al*. [13]. These primers were provided in lyophilized form, dissolved in sterile deionized distilled water to give a final concentration of 100 µM/µl as recommended by provider and stored in a freezer until use. The primers sequences was used in this study are listed in table-2.

Table 2- The sequences of primers used in PCR to detect bla_{KPC} [13].

Primer	**Sequence**	**Amplicon size (bp)**
Forward	5'-TGTCACTGTATCGCCGTC-3'	900
Reverse	5'-CTCAGTGCTCTACAGAAAACC-3'	

Amplification reaction

The extracted DNA, primers and PCR premix were vortexed and centrifuged briefly to bring the contents to the bottom of the tubes. Optimization of polymerase chain reaction was accomplished after several trials, PCR mixture was set up in a total volume of 20 µl included 2 µl of each primer (10 micromole/ µl) and 6 µl of template DNA (50 ng/µl) have been used. The rest volume was completed

with free nuclease water. Negative control contained all material except DNA, in which D.W. was added instead of template DNA. PCR reaction tubes were vortexed and finally placed into thermocycler PCR instrument, Program of DNA amplification as indicating in table-3 [13].

Table 3- PCR amplification program for KPC primer [13].

Stage	Temperature (time)	Number of cycle
Initial denaturation	95°C for (5 min)	1
Denaturation	95°C for (40 sec)	35
Annealing	57°C for (45 sec)	
Extension	72°C for (1 min)	
Final extension	72°C for (8 min)	1

Agarose gel electrophoresis

Agarose gel electrophoresis was used for the separation of DNA fragments and PCR products which visualized with the aid of ethidium bromide and UV transilluminator documentation system [14].

Result and Discussion

Out of 109 clinical lactose fermenter isolates, 53 (48.62%) isolates were assigned as *K. pneumoniae*, and the remaining isolates belonged to other bacteria figure-1. The fifty-three *K. pneumoniae* isolates obtained from urine, stool, swabs, blood, and sputum specimens at percentages of 45.3%, 37.7%, 9.4%, 3.7%, and 3.7%, respectively.

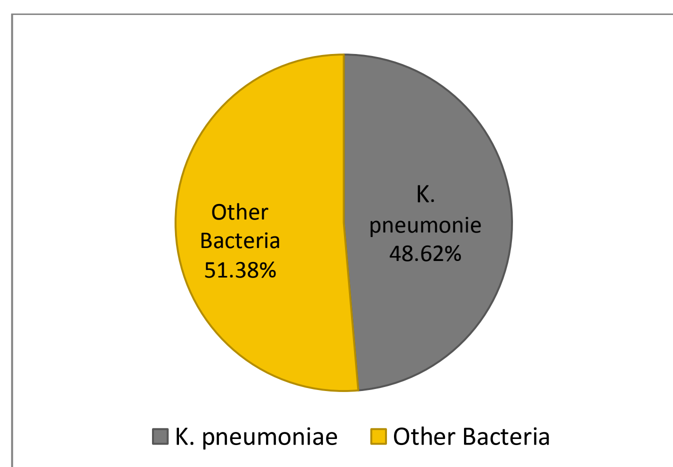

Figure 1- percentage of *K. pneumonia* isolates in all clinical specimens.

In a local study done by Hasson (2012), *K. pneumoniae* was isolated more frequently from urine 21.4 % and less from sputum 12.5%; while Assal (2010) isolated *K. pneumoniae* from urine (42.16%) more than wound (31.25%) then sputum (28.57%).*Klebsiella pneumoniae* associated with hospital-acquired infection accounting for 34–36% of cases of *K. pneumoniae* bacteremia [15].

Susceptibility test

Five antibiotic disks were used in this study included three types of third generation Cephalosporins included; Ceftazidime (CAZ) , Ceftriaxone (CRO), Cefotaxime (CTX) and two types of Carbapenems antibiotics; Imipenem (IPM), Meropenem (MEM). Figure-2 summarizes the results of antibiotic susceptibility test and reflects fifty-three isolates were resistance to all antibiotics; Imipenem (5.6%), Meropenem (9.4%), Ceftazidime (58.5%), Ceftraixone (50.9%), and Cefotaxime (43.3%). Furthermore, some isolates exhibited intermediate susceptibility to Imipenem (16.9%), Ceftazidime (28.3%), Ceftriaxone (30.1%), and Cefotaxime (28.3%). While 7.5% isolates displayed susceptibility to all antibiotics and 5.6% of isolates were non susceptible to all antibiotics.

In local study, at Baghdad city, Al-Qafaji [16] referred that 100% and 94.5% of *K. pneumoniae* isolates were resistance to Cefotaxime and Ceftazidime, respectively. Al-Obadi [17] reported that 90.5%, 87.5%, and 97.5% of isolates were resistant to Cefotaxime, Ceftriaxone, and Imipenem, respectively. The present study gave about 50% resistance to third generation Cephalosporin in compaired to local studies but not agree with Imipenem result in local study.

While Kevin *et al.*[18] pointed out to the resistance of *K. pneumoniae* isolates to Cefotaxime (69.5%), Ceftazidime (68.5%). Sanchez *et al.*[29] reported *K. pneumoniae* clinical isolates were resistant to Cefotaxime (93%), Ceftazidime (97%), and Cetraixone (87%), and these results in agreement to the present study.

In present work, only 3 (5.6%) isolates were sensitive to all antibiotics. However, most of isolates (90.5%) exhibited sensitivity to Meropenem. Nevertheless, 13.2%, 20.7%, 28.3%, and 77.3% of isolates were sensitive to Ceftazidime, Ceftraixone, Cefotaxime, and Imipenem respectively.

β-lactam resistance phenotypes of *Klebsiella* include Carbapenem-hydrolyzing enzymes (including one produced by a variant of the chromosomally-encoded bla_{SHV1} gene and plasmid-mediated *ampC* β-lactamases, which are generally resistant to β - lactamases inhibitors [19] . However, Carbapenems are very active in vitro against the vast common of *Klebsiella* strains [20].

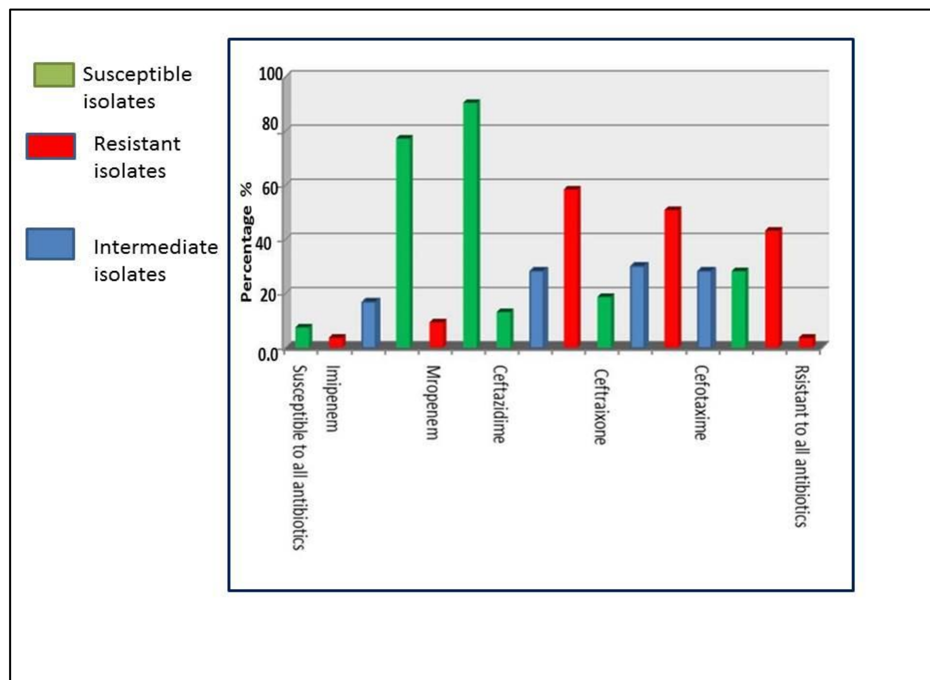

Figure 2- Antibiotic susceptibility test of fifty three of *K. pneumoniae* isolates.

Modified Hodge test (MHT)

Carbapenemase production was detected by the MHT when the test isolate produces the enzyme and allows growth of a carbapenem susceptible strain (*E. coli* ATCC 25922) towards a Carbapenem disk figure-3. Five carbapenem resistant *K. pneumoniae* isolates (K2, K3, K4, K34, and K35), out of fifty-three, gave positive Hodge test.The result is a characteristic cloverleaf-like indentation. Result depicted in Figure-3 for K2 and K3 isolates that gave positive Hodge test while K42 isolate gave negative result.

Musafer [21] stated that among fifty-eight *Pseudomonas aeruginosa* strains evaluated with E test, 2 (3.4°%) strains were intermediate, and 9 (15.5%) strains were resistant to Imipenem. All these 9 strains were previously isolated from patients with cystic fibrosis, no carbapenemase activity was found among the intermediate and resistant strains, as it is confirmed by the Hodge test. A positive strain develop a 'cloverleaf shaped' zone of inhibition due to carbapenemase production. The strains displayed negative results (undistorted zone of inhibition) these results suggested that the Imipenem resistance is due to *oprD* malfunction rather than carbapenemase. Metwally *et al.*[22] pointed that out of 45 *K. pneumoniae*, 20 isolates (44.4%) were reported as non-susceptible (intermediate and resistant) to Ertapenem . Isolates that were non-susceptible to Ertapenem (i.e. resistant and intermediate isolates) were also tested by the MHT previously described. 17(85%) out of 20 *K. pneumoniae* non-susceptible (intermediate and resistant) to Ertapenem showed MHT positive.

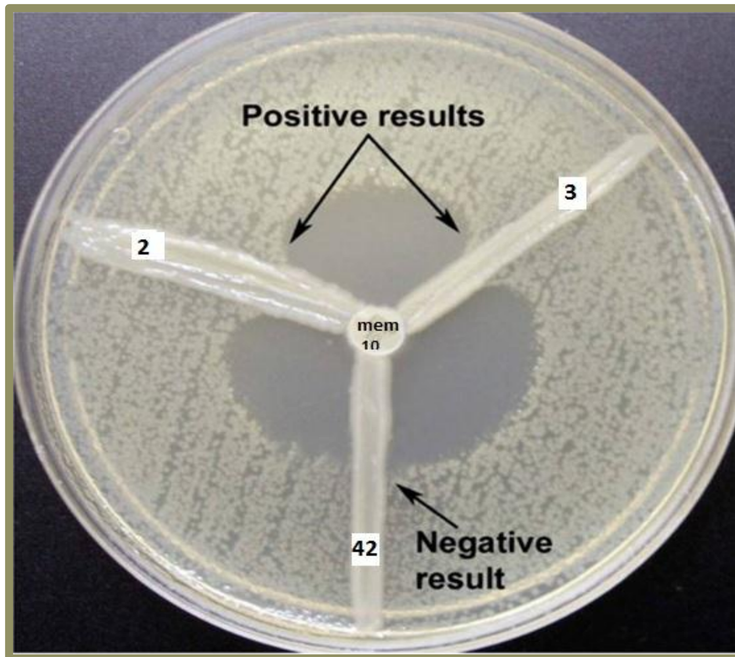

Figure 3- Hodge test of carbapenem resistant *K.pneumoniae*

Detection of *bla*$_{KPC}$ gene in *Klebsiella pneumonie* isolates

PCR were carried out to fifty three *K. pneumonie* isolates which included (sensitive, intermediate, and resistant to tested Carbapenems) In spite of five isolates gave positive MHT, only one isolate (K2) gave specific identification for *bla*$_{KPC}$ gene Figure-4.

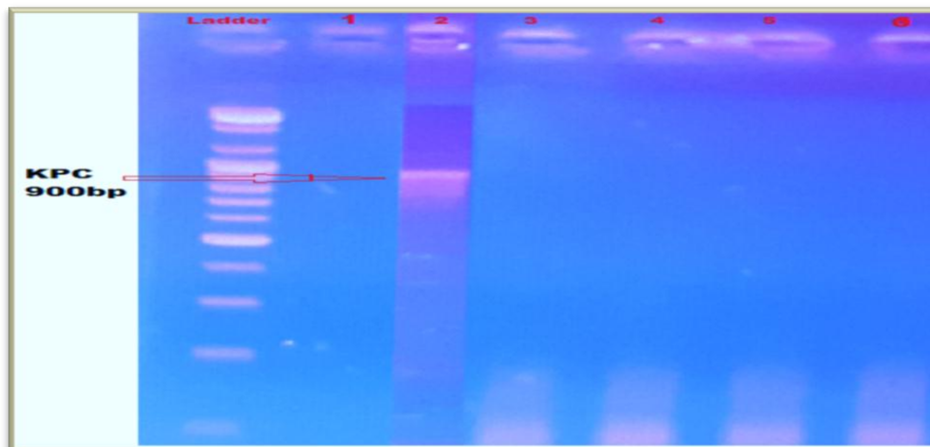

Figure 4- Agarose gel electrophoresis analysis of *bla*$_{KPC}$ (~ 900bp). Lanes 1-6 represent *K. pneumoniae* isolates (K1,K2,K3,K4,K5,K6), respectively. Ladder denotes to 100 pb DNA ladder. Detection was accomplished on agarose gel (1.5%) at 5 V/cm for 1.5 hour, stained with ethidium bromide and visualized on a UV transiluminator documentation system.

In a study done by Metwally *et al.*[22] on three types of Carbapenems (Ertapenem , Meropenem, and Imipenem) to detect *bla*$_{KPC}$ gene, mentioned that 20 of *K. pneumoniae* isolates which are non-susceptible (intermediate or resistant), 3 of isolates gave MHT-ve/ PCR-ve , and result of 17 isolates MHT +ve , 3 Of the Ertapenem non susceptible isolates gave MHT+VE/KPC-PCR-negative . Hu *et al.*[23] show The *bla*$_{KPC}$ gene was detected in 58/77 (75.3 %) isolates and in all cases the gene encoded KPC-2-type carbapenemase, Among these 58 strains, this gene was coupled with the *bla*$_{VIM-1}$ gene in two isolates, the *bla*$_{IMP-2}$ gene in two isolates and the *bla*$_{IMP-1}$ gene in one isolate .

In January 2009, the Clinical and Laboratory Standards Institute (CLSI) recommended all *Enterobacteriaceae* with elevated but susceptible Carbapenem . MICs be tested with a modified Hodge test. Another emerging test is a chromogenic medium CHROM agar KPC, which has been shown to have a sensitivity of 100% and specificity of 98.4% relative to PCR [24]. Schechner *et*

al.[25], exhibited for 20 swabs; 9 were bla_{KPC} negative and carbapenem-resistant *Enterobacteriaceae* (CRE) culture positive, and 11 were bla_{KPC} positive and CRE culture negative. After repeat testing, a total of 64 samples were classified as bla_{KPC}-positive CRE. The sensitivity and specificity of the PCR analysis were 92.2% and 99.6%, respectively; a PCR-based method for CRE surveillance has several disadvantages:

First, KPC PCR possibly will be falsely negative due to inhibitory substances in the reaction or to technical inexperience of the laboratory [25].

Seconed, CLSI [26] stated the most probable reason could be the presence of other carbapenemases, such as the metallo-beta-lactamases a member family of carbapenem-hydrolyzing beta-lactamases, SME-1, which can yield a positive result for MHT but negative for bla_{kpc} . So although the new CLSI recommendations lowered the breakpoints of carbapenems and removed the requirement for testing for carbapenemase (e.g. MHT) to determine susceptibility.

Third, the PCR-based approach may not be appropriate as a screening tool in resource-limited centers due to limited availability of both qualified laboratory personnel and the equipment necessary to do molecular testing [25].

Fourth, Bratu *et al*. [27] demonstrated that the isolates are difficult to identify because the majority of them do not obvious high-level resistance to carbapenems (e.g., MICs of 1 to 8 µg/ml for IPM and MEM, based on susceptibility breakpoints currently in use.

Fifth, Endimiani *et al*. [28], stated that approximately 60% of KPC-Kp isolates have IPM or MEM MICs in the susceptible range. This low-level expression of the resistance mechanism is the main reason that automated and non-automated phenotypic tests have difficulty in detecting KPC-Kp isolates. In divergence to the case for IPM and MEM, KPC-Kp strains frequently have ERT MICs in the resistant range. Thus, the use of ERT has been suggested to screen for the KPC production among *Enterobacteriaceae*. These isolates are related with loss or decreased expression of outer membrane porins combined with production of ESBLs [29].

As a conclusion in order to detect bla_{kpc}; the CLSI (20013), modified the Carbapenem interpretations for *Enterobacteriaceae* and recommended performing the MHT, using either MEM (10µg) or ERT (10µg) disks for strains with intermediate or resistant breakpoints and resistant to one or more third generation Cephalosporins (Cefotaxime, Ceftazidime, Ceftraixone..etc). Most of tested clinical *K. pneumoniae* isolates (90.5% and 77.3%) were susceptible to Meropenem and Imipenem, respectively and less susceptible to third generation cephalosporin. Even though, five isolates (K2, K3, K4, K34, and K35) gave positive Hodge test, only one isolate harboured bla_{KPC}.

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