



Occurrence and Detection of Carbapenemase-Producing *Klebsiella pneumoniae* Clinical Isolates in Najaf Hospitals

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Abstract:

Resistance to carbapenems is emerging, and it is a great problem to therapeutics. Carbapenemase producing *Klebsiella pneumoniae* have been reported to be important cause of nosocomial infections. There is no information available on carbapenemase producing *K. pneumoniae* from Najaf. The purpose of this study was undertaken to determine the occurrence of carbapenemase including metallo β -lactamases (MBLS), *Klebsiella pneumoniae* carbapenemase (KPC) and GES- β -lactamase producing *K. pneumoniae*. A total of 770 clinical samples were collected from April to July, 2010. The *K. pneumoniae* isolates were identified according to API 20E system. Phenotypic detection of carbapenemase was performed by using the imipenem-EDTA disk method and confirmed by the modified Hodg test. KPC was detected on KPC CHROMagar medium. The isolates were subjected to polymerase chain reaction (PCR) assays with specific primers for *bla*_{IMP}, *bla*_{VIM}, *bla*_{KPC}, *bla*_{CTX-M} and *bla*_{AmpC}. Only 108 (14.%) *K. pneumoniae* isolates were recovered from clinical infections. only 95 (88%) isolates were β -lactam resistant (resistant to both ampicillin and amoxicillin). Of the β -lactam resistant isolates, 4/95 (4.2%) were found to be carbapenem disk resistant, of which, 2 (50%) gave positive result with the imipenem-EDTA test, 4 (100%) gave positive result with modified Hodg test and all isolates (100%) gave heavy growth on KPC CHROMagar. In PCR experiments using specific primers for *bla*_{IMP}, *bla*_{VIM} and *bla*_{KPC} genes, the results were negative among all isolates. All isolate were harbored *bla*_{CTX-M} and *bla*_{AmpC} genes. The present findings suggest that the spread of carbapenemases in isolates is high in Najaf.

Introduction:

Most notoriously, *K. pneumoniae* is a prominent nosocomial pathogen mainly responsible for bacteraemia, urinary tract, respiratory tract, and wound infections. Most *K. pneumoniae* are hospital associated with a high fatality rate if incorrectly treated. Isolates from hospitals often display antibiotic resistance phenotypes (Woodford *et al.*, 2007), while resistance isolates may also spread into the community settings (Colodner *et al.*, 2004).

The increment of irrational use of antibiotics promotes bacterial resistance to these drugs. Moreover, the resistance mechanisms developed by *K. pneumoniae* is the production of β -lactamase which can destroy the β -lactam antibiotics (Herewana *et al.*, 2008). Extended-spectrum β -lactamases (ESBLs) and AmpC β -lactamases are most prevalent in *K. pneumoniae*, subsequently has resulted in their resistance to third-generation cephalosporins and cephamycin. (Bradford, 2001; Li *et al.*, 2009). Carbapenemases represent the most versatile family of β -lactamases, with a breadth of spectrum unrivaled by other β -lactam-hydrolyzing enzymes, some investigators have preferred the nomenclature "carbapenem-hydrolyzing enzymes" to the term "carbapenemases," suggesting that carbapenems are their substrate spectrum (Queenan and Bush, 2007).



Carbapenemases belong to two major molecular families, distinguished by the hydrolytic mechanism at the active site. The first carbapenemases described were from Gram-positive bacilli. These enzymes were inhibited by EDTA. In the mid to late 1980 s, another set of carbapenem-hydrolyzing enzymes emerged among the *Enterobacteriaceae*, but EDTA did not inhibit their activity (Medeiros and Hare, 1986).

Risk factors for infections caused by carbapenemases-producing strains of *K. pneumoniae* include long hospital stay, and prior administration of antibiotics, especially broad-spectrum cephalosporins and β -lactamase inhibitor combinations (Pai *et al.*, 2004). The molecular biological techniques such as detection of enzyme hydrolysis parameter, isoelectric point, gene detection, could be used for the identification carbapenemases. Recently, molecular analysis like PCR has been developed as the "gold standard" for family-specific plasmid-mediated AmpC β -lactamase detection (Perez-Perez and Hanson, 2002 ; Naas *et al.*, 2004).

Nevertheless, the knowledge of the carbapenemases is still limited at presence. In Iraq, little attention has been paid to β -lactamases producing isolates. However, in Najaf city, there is no information regarding the molecular studies of the occurrence of carbapenemases-producing *K. pneumoniae* recovered from clinical cases. Therefore, there is an increase demand to investigate the role of these isolates in hospital infections, hence the fundamental aim of this study is to identify the occurrence of carbapenemases in *K. pneumoniae* isolates recovered from Hospital settings in Najaf.

Materials and Methods:

- 1- Materials :** All cultures media, reagents , solutions, antibiotics disk, PCR kits and gene primers (*bla*_{IMP}, *bla*_{VIM}, *bla*_{KPC}, *bla*_{CTX-M} and *bla*_{AmpC}) were manufactured by Oxoid , Difico, Himedia, Promega and Kappa companies.
- 2- Methods:** All cultural, cellular and biochemical tests were used according to MacFaddin (2000) and Collee *et al.* (1996).

2.1 .Screening Test for β -latam Resistance

Ampicillin and amoxicillin were added separately, from stock solution, to the cooled Muller-Hinton agar at final concentration of 100 and 50 μ g/ml, respectively. The medium poured into sterilized Petri dishes, then stored at 4°C. Preliminary screening of *K. pneumoniae* isolates resistance to both antibiotics was carried out using pick and patch method on above plates (NCCLS, 2003 b). Results were compared with *E.coli* ATCC 25922 as negative control.

2.2 . Phenotypic Detection of Carbapenemases

2.2.1 . Imipenem-EDTA Double Disk Synergy Test

Metallo- β -lactamase detection was performed by double disk synergy method according to Lee *et al.* (2003). A 10 μ g imipenem disk was placed in the center of a Muller-Hinton agar plate inoculated with a 0.5 McFarland dilution of the test isolate. An EDTA disk (1900 μ g) was placed at a distance of 15 mm center to center from the imipenem disk. The plate was incubated at 37°C overnight. The zone around the imipenem disk would be extended on the side nearest the EDTA disk for a metallo- β -lactamase producer.

2.2.2 . Modified Hodge Test (MHT)

It was done as described by Lee *et al.* (2001). A 0.5 McFarland dilution of *E. coli* ATCC 25922 was prepared in 5 ml of tryptic soy broth, a lawn was streaked to a Muller-Hinton agar plate and allow to dry (3-5) min, imipenem disk (10 μ g) was



placed in the center of the test area. In a straight line the test organism streaked from the edge of the disk to the edge of the plate, the plates were incubated overnight at 37°C. Four organisms were tested on the same plate with one disk. MHT positive test has a clover leaf-like indentation of *E. coli* ATCC 25922 growing along the test organism growth streak within the disk inhibition zone. MHT negative test has no growth of *E. coli* ATCC 25922 along the test organism (CLSI, 2010).

2.2.3 . Detection of KPC by CHROMagar Technique

Klebsiella pneumoniae carbapenemase CHROMagar plates were streaked in the same day of preparation by overnight growth of *K. pneumoniae*, and incubated at 37 °C for 24 hr according to manufacturer procedure. Growth of blue colonies indicated to suspected KPC producer. The reference strain of *E. coli* ATCC 25922 was inhibited and used as negative control in this test.

2.3 . Detection of *bla* Genes by Polymerase Chain Reaction

2.3.1 . DNA extraction

Extraction of DNA from bacterial cells was performed by salting out method (Pospiech and Neumann, 1995) with some modification to prepare tamplet DNA.

2.3.2. Preparing the Primers Suspension

The DNA primers were resuspended by dissolving the lyophilized product after spinning down briefly with TE buffer molecular grad depending on manufacturer instruction as stock suspension. Working primer tube was prepared by diluted with TE buffer molecular grad. The final picomoles depended on the procedure of each primer.

2.2.3 . Polymerase Chain Reaction Protocols

a) PCR Mixture and thermocycling conditions

The DNA extract of *K. pneumoniae* isolates were subjected to *bla* genes by PCR, the protocol was used depending on manufacturer's instruction. All PCR components were assembled in PCR tube and mixed on ice bag under sterile condition as in Table (1). The PCR tubes were placed on the PCR machine and the right PCR cycling program parameters conditions were installed as in Table (2).

b) Agarose Gel Electrophoresis

All requirements, technical and preparations of agarose gel electrophoresis for DNA detection and analysis were performed by Bartlett and Stirling (1998). Finally, the gel was photographed using Biometra gel documentation system.

Table (1): Programs of PCR thermocycling conditions

gene	Temperature(°C) / Time					Cycle number
	Initial denaturation	Cycling condition			Final extension	
		denaturation	Annealing	extension		
<i>bla</i> _{IMP}	93/3 min	93/1 min	55/1 min	72/1 min	72/7	40
<i>bla</i> _{VIM}	93/3 min	93/1 min	55/1 min	72/1 min	72/7	40
<i>bla</i> _{KPC}	95/15 min	94/1 min	62/1 min	72/1 min	72/10 min	38
<i>bla</i> _{AmpC}	94/30 sec	94/30 sec	60/1 min	72/1 min	72/10 min	35
<i>bla</i> _{CTX-M}	94/30 sec	94/30 sec	60/1 min	72/1 min	72/10 min	35

Results:

Among the 95 β-lactam resistant *K. pneumoniae* isolates (Table 2), four had previously been identified as resistant to both imipenem and meropenem (4.2 %) by standard disk diffusion method (Table 3). All these isolates were recovered from



clinical samples. Production of carbapenemase was confirmed by three different methods, imipenem-EDTA disk, modified Hodge methods and KPC CHROMagar.

Phenotypic detection of metallo- β -lactamases was performed by using the imipenem-EDTA disk method. Only tow (50 %) isolates demonstrated enhancement of inhibition zone, suggesting production of metallo- β -lactamases, while no remarkable distinct change was noticed in the others isolates. In PCR experiments using specific primers for *bla_{IMP}* and *bla_{VIM}*, the results were negative among all carbapenems resistant *K. pneumoniae* isolates (Table 4).

In this study, the modified Hodge test was used as phenotypic confirmatory method for both KPC and metallo- β -lactamases-production, but did not differentiate between them. The presence of a distorted inhibition zone was interpreted as a positive result for carbapenem hydrolysis screening. However, this test was positive for all (n=4, 100 %) carbapenems resistant *K. pneumoniae* isolates, indicating carbapenemase production (Table 3 and Figure 1). However, all carbapenemase positive isolate harbored both *bla_{CTX-M}* and *bla_{AmpC}* genes for ESBL and AmpC β -lactamase respectively (Figure3 and 4) .

Table (2) : β -lactam resistant *K. pneumoniae* isolates collected from clinical samples

Clinical sample	No. of samples	No. (%) of <i>K. pneumoniae</i> isolates	No. (%) of resistant isolates to both ampicillin and amoxicillin		
Sputum	150	72 (16.0%)	67 (93.0%)		
Type of sample	No. of Carbapen-em disk resistance isolates	No. (%) of phenotypic confirmed carbapenemase producer isolates			
		Imipenem-EDTA disk test	Modefied Hodge test	KPC* chromagar techinque	

Table (3): Phenotypic detection of carbapenemase production in *K. pneumoniae* isolates



Sputum	2	2 (100%)	2 (100%)	2 (100%)
Urine	0	0 (0%)	0 (0%)	0 (0%)
Burn wound	2	0 (0%)	2 (100%)	2 (100%)
Total	4	2 (50%)	4 (100%)	4 (100%)

L.S.D. (0.05) Samples = 9.143 , Methods = 8.94*KPC: *K. pneumoniae* carbapenemase,

Table (4): Molecular detection of *bla* genes in carbapenemase producing-*K.*

Type of sample	No. of Carbapen-emase positive isolates	Molecular detection of <i>bla</i> genes				
		<i>bla</i> _{IMP}	<i>bla</i> _{VIM}	<i>bla</i> _{KPC}	<i>bla</i> _{CTX-M}	<i>bla</i> _{AmpC}
Sputum	2	-	-	-	+	+
Burn wound	2	-	-	-	+	+
Total	4	0 (0%)	0 (0%)	0 (0%)	4 (100%)	4 (100%)

pneumoniae isolates

The KPC supplemented CHROMagar test (Figure 2) was also evaluated for detection of KPC-mediated resistance in all *K. pneumoniae* isolates positive in the modified Hodge test. All isolates able to gave overnight heavy growth onto this medium. The isolates that yielded a positive result with the KPC supplemented CHROMagar test underwent KPC polymerase chain reaction (PCR) testing to confirm the presence of a KPC β -lactamase. Table (4) shows no isolate of *K. pneumoniae* was found to harbor the *bla*_{KPC} gene.

Results of present study, indicated that using of modified Hodge test and KPC CHROMagar technique were more significant ($P < 0.05$) than imipenem-EDTA disk test on phenotypic carbapenemase detection.

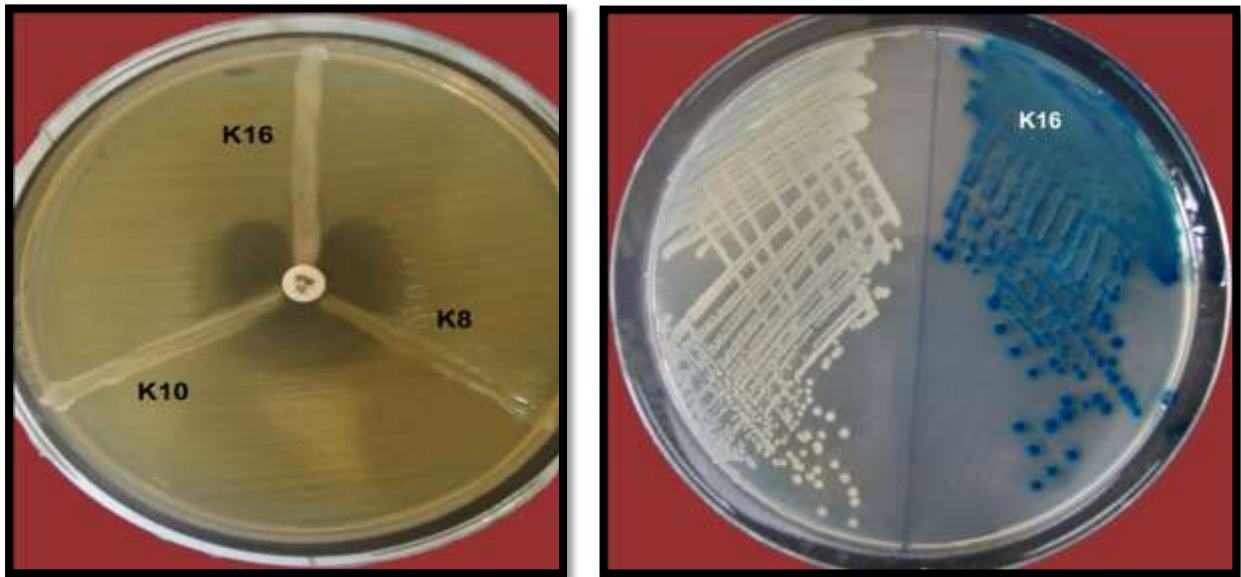


Figure (1): The modified Hodge test of carbapenemase detection in *K. pneumoniae*. Growth of *E. coli* ATCC 25922 strain around straight line of test isolates, K16 isolate, exhibit clear distortion of the inhibition zone of imipenem disk. K8 and K10 isolates exhibit negative result, (IPM: imipenem).

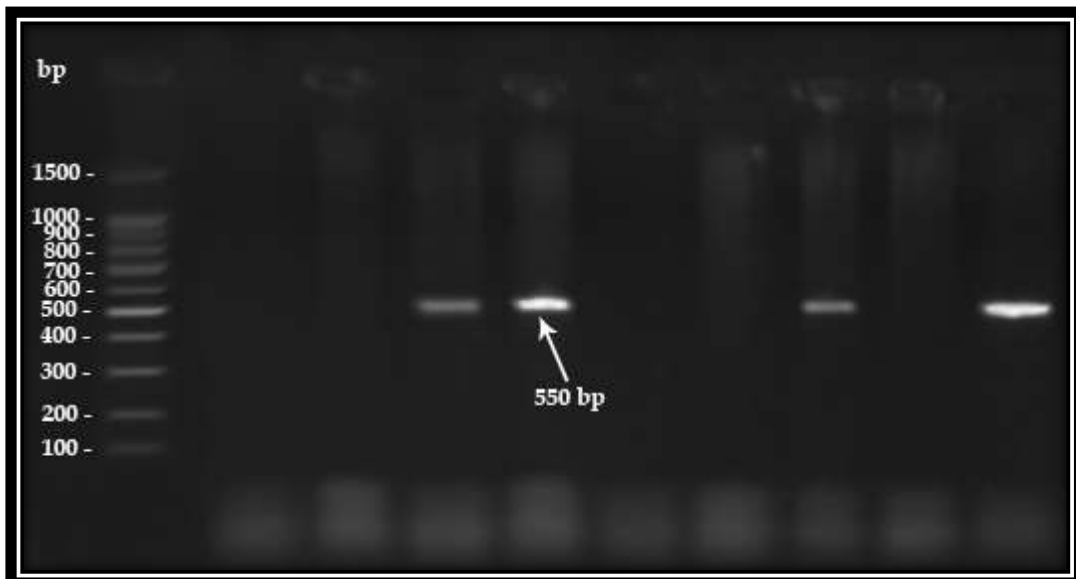


Figure (3): Ethidium bromide-stained agarose gel of PCR amplified products of *K. pneumoniae* isolates and amplified with primers *bla*_{CTX-M} gene (550 bp). The electrophoresis was performed at 70 volt for 1.5 hr. with DNA molecular size marker (1500 bp ladder).

Figure (2): KPC CHROMagar medium to detect carbapenemase producer *K. pneumoniae*, K16 test isolate exhibit blue growth indicate to KPC positive on the right side, and KPC positive of non fermentative Gram negative bacteria exhibit white to creamy growth on the left.

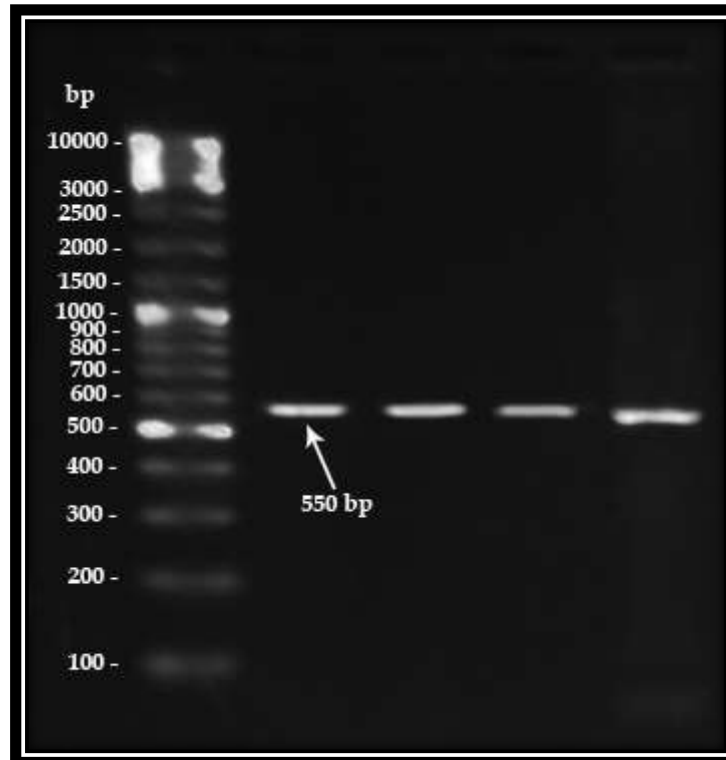


Figure (4): Ethidium bromide-stained agarose gel of PCR amplified products of *K. pneumoniae* isolates and amplified with primers *bla*_{AmpC} gene (550 bp). The electrophoresis was performed at 70 volt for 1.5 hr. with DNA molecular size marker (10000 bp ladder).

Discussion:

Carbapenems are group of β -lactams consists of (imipenem, meropenem, doripenem, ertapenem, panipenem and biapenem). This group has the widest spectrum of antibacterial activity of the antibiotic agents that are currently available. Carbapenems have provided a highly effective treatment option for serious infections with ESBL and/or AmpC-positive *Enterobacteriaceae*. They are active against most of the common β -lactamase enzymes (AmpC and ESBLs). The use of carbapenems in the treatment of infection has been compromised by the emergence of carbapenems-hydrolyzing β -lactamases (Queenan and Bush, 2007).

The present study revealed that most β -lactam resistant *K. pneumoniae* isolates were susceptible to both imipenem and meropenem. Only four (4.2%) of screened isolates were resistant to both imipenem and meropenem by standard disk diffusion method. In the same manner, the study results were in agreement with a Turkish study conducted by Bicmen *et al.* (2004) who pointed that resistance to carbapenems among Gram-negative bacteria recovered from various specimens of hospitalized patients was 24 % for imipenem and 21.3% for meropenem.

In present study, the PCR technique had been confirmed that all carbapenem-resistant isolates were harbored *AmpC* and *CTX-M* genes. Three methods were used to



confirm the carbapenemase production in these isolates. Imipenem-EDTA disk test was less significant effect ($P < 0.05$) in compare with other methods (confirmed only 50% of carbapenems disk resistant isolates as phenotypic carbapenemase producers). There are two isolates were gave negative results with EDTA disk synergy test. That is mean the using of EDTA may not inhibit the activity of all β -lactamases, suggesting the absence of a class B1 enzyme (like IMP and VIM), or these isolates may produced other enzymes (like IMI, GES and KPC) that not inhibited by EDTA. Moreover, the resistance to carbapenems may involve several combined mechanisms other than carbapenemases, include modifications to outer membrane permeability and up-regulation of efflux systems associated with hyperproduction of AmpC β -lactamases and cephalosporinases (ESBLs) (Nordmann *et al.*, 2009).

Recently, modified Hodge test became the most acceptable confirmatory test to detect all type of carbapenemases for infection control and epidemiological purpose. The present study showed that all carbapenems resistant *K. pneumoniae* (4, 100%) gave positive test. In related studies, Al-Hilli (2010) stated that 1(2.4%) of *Klebsiella* spp. isolates recovered from hospital environment in Hilla was confirmed as carbapenemase producer using modified Hodge test, whereas the same isolate gave negative result with imipenem-EDTA synergy test.

Besides, KPC CHROMagar technique was used to select the carbapenemase-producers *K. pneumoniae* on medium that enhanced by KPC supplement, this medium is allow to grow the *K. pneumoniae* carbapenemase-producers only and inhibit the KPC negative bacteria. Because the originality of this method, there is no available studies previously used this technique. However, KPC CHROMagar technique revealed that all carbapenems resistant *K. pneumoniae* isolates (in screening test) were successful gave overnight heavy growth. This result interpreted that possibility of these isolates to carry serine type carbapenemase KPC or OXA carbapenemase.

In this study, the identity between modified Hodge test and KPC CHROMagar in enzyme detection may due to the modified Hodge test has $>90\%$ sensitivity and specificity in detecting of KPC and variable sensitivity and specificity to detect the other metallo- β -lactamases. However, there is no significant difference ($P < 0.05$) between modified Hodge test and KPC CHROMagar. Moreover, this investigation found low efficiency of EDTA synergy test to confirm all type of carbapenemase production in compare with CHROMagar technique and modified Hodge test. This may be, the EDTA synergy test has some problems that interfere with results. Firstly, EDTA may potentiate imipenem with unconnection to inhibited by metallo- β -lactamases, perhaps bacterial cell permeability or zinc affect on the regulation of the OprD porin, which facilitates the entry of imipenem. Secondly, some metallo- β -lactamase producers remain almost fully susceptible to carbapenems in vitro (Livermore and Brown, 2005).

Carbapenemases production was also detected in *bla*_{AmpC} harbouring *K. pneumoniae* isolates by PCR experiments. Using most common carbapenemases specific primers for *bla*_{IMP}, *bla*_{VIM}, and *bla*_{KPC}. The results were negative among all isolates. One limitation of the carbapenemase-specific assay for carbapenems resistant isolates surveillance in this study is that dose not detected all carbapenems resistant genotypes. The results in this investigation indicated that the four isolates were negative in PCR, which could be due to either that absence of *bla*_{IMP}, *bla*_{VIM}, and *bla*_{KPC} genes or the presence of other type of gene variants that could not be targeted by the primers used in this study. In contrary, many reports from Japan, Singapore, and Taiwan (Arakawa *et al.*, 2000 ;Yan *et al.*, 2001) and recently in Lebanon (Daoud



et al., 2010) indicated that IMP, VIM varieties have been detected in most Gram-negative bacilli isolated from nosocomial infections.

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الخلاصة:

تعتبر مقاومة مضادات الكاربابنيم من المشاكل التي تواجه العلاجات الدوائية حالياً. والذي يتوسطه في كثير من الأحيان إنتاج إنزيمات الكاربابنيميم الذي تنتجه بكتريا الكليسيلا الرئوية والمسببة لعدوى المستشفيات المكتسب. ولعدم وجود أي معلومات تفيد عن انتشار البكتريا المنتجة لهذا الإنزيم والمقاومة لمضادات الكاربابنيم في مستشفيات النجف، جاء هدف هذه الدراسة لتحديد انتشار هذه البكتريا في محافظة النجف. جمعت 770 عينة سريرية من مستشفيات النجف للفترة بين نيسان وتموز لعام 2010. استخدمت الاختبارات المظهرية التأكيذية لإنزيمات الكاربابنيميم والتي أكدت باستخدام تقنية تفاعلات البلمرة PCR للتحري عن الجينات المنتجة لهذا الإنزيم. تم تشخيص 108 (14.0%) عزلة تابعة لبكتريا الكليسيلا الرئوية. وجد إن 95 (88%) منها كانت مقاومة لمضادات البيبتالاكتام بشكل عام. أربعة عزلات (4.2%) فقط أبدت مقاومة مظهرية لمضادات الكاربابنيم بطريقة انتشار القرص لكيريبي-باور، منها عزلتان (50%) أعطت فحصاً موجباً لاختبار imipenem-EDTA test. كما إن جميع العزلات المقاومة للكاربابنيم كانت موجبة لاختباري modified Hodg test و KPC CHROMagar. كانت نتيجة PCR سلبية لجميع جينات الكاربابنيميم bla_{IMP} , bla_{VIM} في حين أعطت هذه العزلات نتيجة إيجابية للجينات المنتجة لإنزيمات البيبتالاكتانيز واسعة الطيف CTX-M وجين AmpC.



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