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Detection of *bla*$_{KPC1}$ gene in *Klebsiella pneumoniae* isolated from Iraqi patients

Saade Abdalkareem Jasim[a], Najwa Shihab Ahmed [b], Mohammed Nadhir Maaroof [c]

[a]Al-maarif University College - Department of Medical Laboratory Techniques, Al-anbar-Iraq
saadee1988@auc-edu.org.
[b] Biotechnology Research Center, Al-Nahrain University, Baghdad, Iraq
wahj_dna@yahoo.com
[c]college of Education Pure Sciences, Tikrit University, Salah Al-dain, Iraq

Abstract: Some bacterial species are highly effective in destroying the carbapenem group through their production of Carbapenemases, which are associated with increased bacterial infections through their development of antibiotic resistance mechanisms. To achieve this goal, we collected 180 samples from different sources included (92 blood, 33 smears of wounds, 13 smears of burns and 42 sputum). The samples were taken from patients attended Ibn-Baladi Hospital as well as Yarmouk Teaching Hospital and some external laboratories Baghdad Governorate. Samples were diagnosed based on cultured characteristics on the MacConkey agar media as well as the EMB medium, microscopic examination by observing the diplococci-negative bacteria and biochemical tests. Then used the VITEK2 System to increase accuracy in the diagnostic process before DNA extraction. DNA was isolated from 84 samples, the purity and concentration of samples extracted was measured with Nanodrop.

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Using the PCR technique, the *bla*$_{KPC1}$ gene was detected of the size of 61 base pairs using specialized gene primers.

Keyword: *K. pneumoniae*, Carbapenemases, Nanodrop, VITEK2, PCR, *bla*$_{KPC1}$.

Introduction

Klebsiella pneumoniae is considered the second cause pathogenesis in Enterobacteriaceae family after *E.coli* , these bacteria are gram-negative bacilli and considered from opportunistic pathogen bacteria as endemic to the intestinal tract and nasopharynx and infect the person especially the infant and elderly. The infection occurs in any position in body when transition him. As it causes 17% from UTI and cause respiratory tract infections [Brisse, *et al.*,2009 and Chang, *et al.*,1992]. Also *K. pneumoniae* is considered one of important cause pathogen from nosocomial infection especially for patient who suffers from Immunocompromised or who are taking immunosuppressed drugs and who suffer from increase iron concentration in blood because they possess Siderophore [Arlet, *et al.*,2001]. *K. pneumoniae* is possess number of virulence factors which share a pathogen and such as capsule antigens, adhesion factors, enterotoxin produce like lipopolysaccharide as well as resistance killer effect for serum and system the obtain on iron (Siderophore) and multi resistance for antibiotics which considered the main reason in spread acquired infections in hospitals, as the percentage infections 80% which led to go for find alternative treatments [Chhibber and Kumari, 2008]. *Klebsiella pneumoniae* Carbapenemases (KPCs) constitute a new variant of class A β-lactamase enzymes capable of hydrolyzing all known β-lactam antibiotics and displaying resistance to β-lactamase inhibitors [Bratu, *et al.*,2007]. As with other class A enzymes, they are carried on a variety of plasmids, thereby facilitating horizontal transmission of *bla*$_{KPC}$ genes [Smith Moland, 2003]. Since their initial description in 2001, KPC-producing strains of *K. pneumoniae* and other Enterobacteriaceae have spread rapidly in the New York metropolitan region, with increasing numbers of cases reported across the United States [Woodford, *et al.*,2004]. *K. pneumoniae* resistance to Carbapenem group produced a wide range from β- lactamase (ESBLS), it was differentiated isolates which produce β-lactamase by *bla*$_{KPC}$ genes, which are these genes diagnosis using specific primers via PCR technique. A screening investigation during 2004 of *E.coli* and *K. pneumoniae* collected from four hospitals in Brooklyn, New York, showed that none of the *E.coli* isolates carried a *bla*$_{kpc}$ gene, whereas 24% of the *K. pneumoniae* isolates possessed a *bla*$_{KPC-1}$ or *bla*$_{KPC-2}$ gene. The majority

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(88%) of the bla_{KPC} – carrying isolates belonged to the same ribotype [Bratu, *et al.*,2005]. Aim of the study is to detect of produce β-lactamase by bla_{KPC} genes of local *K. pneumoniae* isolate from Iraqi patients.

Material and Method

Sample collection and Identification:

One hundred eighty samples from different clinical sources were collected included 92 urine, 33 smears of wounds, 13 smears of burns, 42 sputum samples and information of the like patients (sex and age) have been included. The samples taken from Al-Yarmouk Teaching, Ibin Baladi hospitals and external labs for the period between February 2015 to May 2015. Diagnosed bacterial isolates depending on the microscopic, culture characteristics, biochemical tests, also used the API 20E to diagnosis the Enterobacteriaceae family and used the modern methods of diagnosis such as VITEK2 System according to previous reports [Goldman, and Lorrence, 2009, Stock and Wiedemenn, 2001].

Use of API 20E system

Analytical profile index (API) test strips, produced by (BioMerieux SA, Lyon, France) used to identify members of the Enterobacteriaceae. These consisted of a series of miniature capsules on a moulded plastic strip, each of which contains a sterile dehydrated medium in powder form [Ball, 1997]. The results of the API system yield a seven-digit biotype number from which identification can be made [Atlas *et al.*, 1995]. Here we used API 20E system for confirmed identification and typing of 82 isolates *Klebsiella* spp. which we previously identified by biochemical tests as mentioned before.

Antibiotics susceptibility

The inoculum bacterial was prepare through transferring a single colony to test tube containing on nutrient broth then incubate in 37°C for 24 hours. After preparing incubation and inoculum we took sterile swab and put it in test tube containing inoculum then swabbed evenly across the surface of a Muller-Hinton agar plate, after inoculation the antibiotics [tabe1] puts by forceps with hard pressure, where developed three discs in each dish then plate inverting and incubate 37°C for 18 hours [Mahon and Manuselis, 2000].

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Table1: The Antibiotic discs, which were used throughout the study

Antibiotics	Antibiotic symbol	Concentration µg / disc	Diameters of inhibition zone by (mm)		
			Susceptible	Intermediate	Resistant
Ertapenem	ETM	10	≥ 23	20-22	≤ 19
Doripenem	MEM	10	≥ 22	20-21	≤ 19
Imipenem	IPM	10	≥23	20-22	≤19
Meropenem	DRM	10	≥23	20-22	≤19
Tetracycline	TC	10	≥19	15-18	≤14
Gentamycin	G	10	≥16	14-15	≤13
Ampicillin	Am	10	≥17	14-16	≤13

DNA Extraction:

DNA was extract from 84 samples by DNA extraction kit Genomic DNA Mini Kit, USA, Catalog #: GB 100 [table 2] according to the manufacturer's protocol [Vogelstein., *et al* 1979]. Detection of bla_{KPC1} gene was conduct by using primers for amplification of bla_{KPC1} gene. A fragment 61bp of bla_{KPC1} was amplified using a forward primer were supplied by IDT (Integrated DNA Technologies) company, Canada [table3].

Table 2: Kit contents (Vogelstein et al., 1979)

Name	GB100
RBC Lysis Buffer	135 ml
GT Buffer	30ml
GB Buffer	40ml
W1 Buffer	45ml
Wash Buffer (Add Ethanol)	25 ml (100 ml)
Elution Buffer	30ml

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GD Colum	100pcs
2ml Collection Tube	200pcs

Primer	Sequence	Tm(°C)	GC (%)	Product size	Reference
Forward	5'-GGC CGC CGT GCA ATA C-3'	62.3	55.0	61bp	Design Primer
Reverse	5'-GCC GCC CAA CTC CTT CA-3'	58.3	45.0		

Table 3: Sequence of bla_{KPC1} gene

PCR Procedure

The PCR amplification was perform in a total volume of 25µl containing 1.5µl DNA, 12.5 µl Taq Master Mix PCR (Promega, USA), 1µl of each primer 10 pmol then distilled water was add into tube to a total volume of 25µl [table 4]. PCR amplification was conduct under the following conditions: 5 minutes at 94°C, followed by 40 cycles of 94 °C for 1 minute, 60°C for 1:45 minute, 72 °C for 1 minute and a final extension of 72°C for 10 minutes[table 5] using a thermal Cycler made by Labnet (Labnet international, Inc, MultiGene OptiMax, Catalog #: TC9610-230, USA). The PCR products were separate on a 1.5% agarose gel electrophoresis and visualized by exposure to ultra violate light 302 nm after ethidium bromide staining [Yong *et al.*, 2009].

Table 4: Mixture of the specific interaction for diagnosis bla_{KPC1} gene

bla_{KPC}	
Components	**Concentration**
Green Master Mix	12.5µl
Forward primer bla_{KPC1}	10 picomols/µl
Reverse primer bla_{KPC1}	10 picomols/µl
DNA	1-1.5µl

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Distal water	9 - 9.5µl
Final volume	25µl

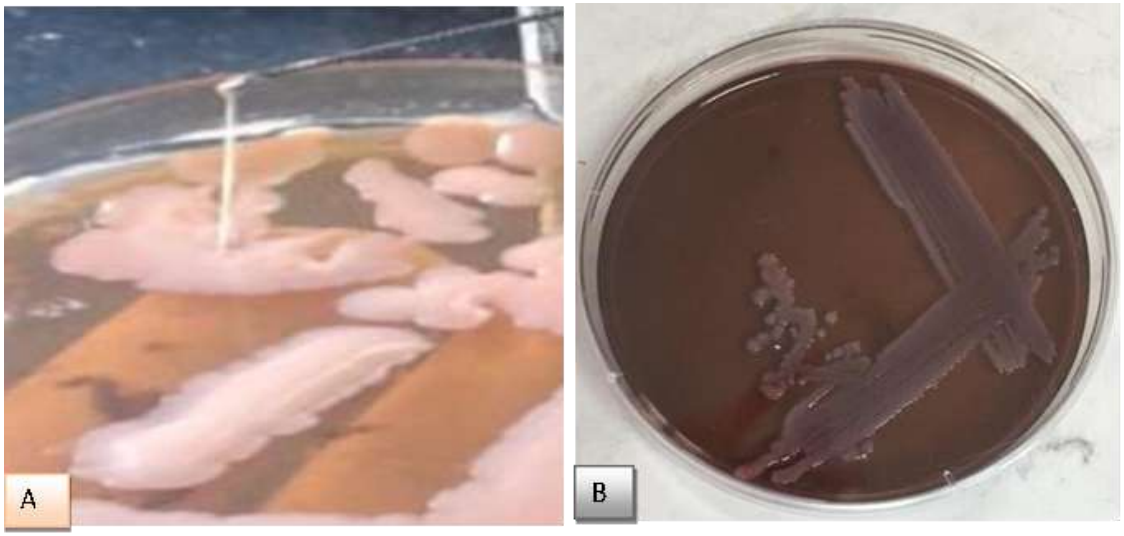
Table 5: The optimum condition of detection bla_{KPC1}

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	94°C	5 min.	40cycle
2-	Denaturation -2	94°C	1 min.	
3-	Annealing	60°C	1:45min.	
4-	Extension-1	72°C	1 min.	
5-	Extension -2	72°C	10 min.	

Results and Discussion

Isolation, Identification and susceptibility test

Initial isolation results were shown that there are 82 isolates given the qualities of colonies *Klebsiella* spp. from the total aggregate samples 180. Those samples have the ability to



lactose fermentation when it is cultured on MacConkey agar medium [figure 1A] and gave the pink glamorous colonies with mucus texture of which it is one of the important

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qualities of *Klebsiella* spp. microscopic examination, culture characteristic and biochemical tests [table 6] have shown that 82 isolates of the total 180 sample they have given a positive result to *Klebsiella* spp. in 45.6%, this percentage clarifies the importance of this genus in between other pathogens [Green and Sambrook, 2012, Malik *et al.*, 2003].

Figure: 1A: Positive string test on a hypervirulent strain of *K. pneumoniae* on MacConkey agar **B**: *K. pneumoniae* colonies on EMB agar.

We tested a collection of 82 isolates of *Klebsiella* spp. by API20E and this test was used to differentiate between the *Klebsiella* species which gave positive result in biochemical tests. When the diagnosis of all isolates was observe that there is diversity among species where the highest percentage appeared to *Klebsiella pneumoniae* 61 isolates 74.4%, the appearance of a higher percentage of *K. pneumoniae* were compatible with other studies [Paterson *et al.*, 2004], and 12 isolate of *K. terrigena* 14.6% and fewer isolates appeared for *K.oxytoca* 9 isolate 11% [figure 2].

Table 6: Biochemical tests used for identification and differentiation between *Klebsiella* spp.

Tests	***K. pneumonia***	***K. oxytoca***	***K. terrigena***
Capsule	+	+	+
Oxidase	-	-	-
Catalase	+	+	+
Indole	-	+	-
Methyl red	-	-	+
Voges proskauer	+	+	+
Citrate utilization	+	+	+
Urease production	+	+	-
Motility	-	-	-

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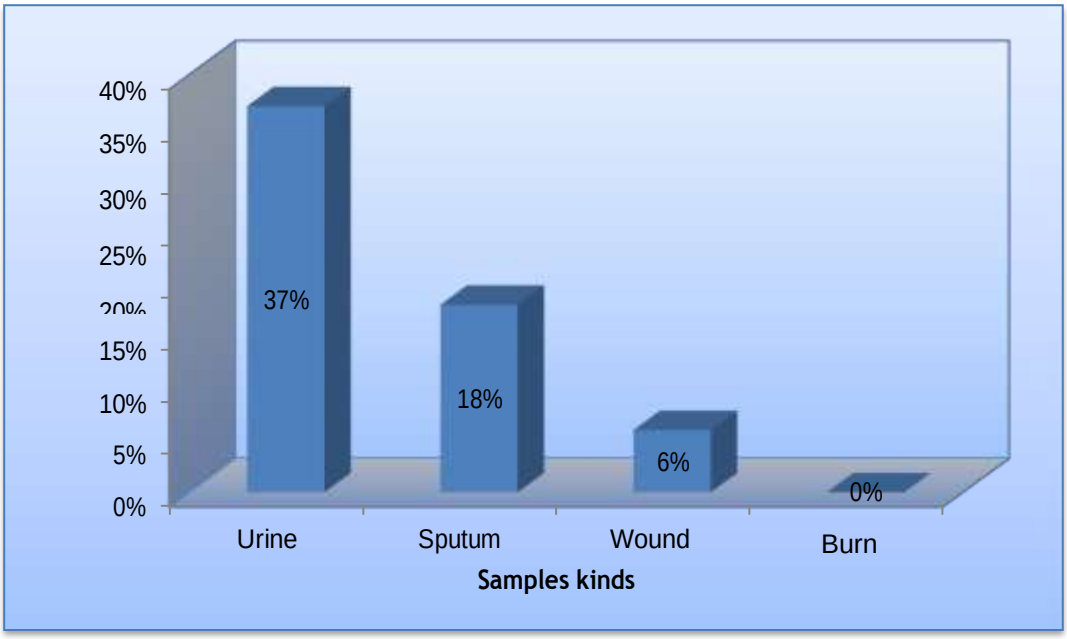

Figure 2: Percentage of *K. pneumoniae* isolates according to source of isolation

Table 7: percentage of diagnosis by API 20E

Species \ Source	Urine	sputum	Wound smear	Burn smear	Total
K. pneumoniae	**37**	**18**	**6**	**0**	**61 (74.4%)**
K. terrigena	**2**	**1**	**2**	**0**	**5 (6.1%)**
K. oxytoca	**8**	**4**	**4**	**0**	**16 (19.5%)**

Some studies indicated that *K. pneumoniae* was predominant species 86% among *Klebsiella* species the clinically isolates [Paterson *et al.*,2004] and also note diversity in these bacteria in clinical isolates [Malik *et al.*, 2003], 61 isolates from *K. pneumoniae*, it explains high percentage had obtained from urine 37 isolates 60.5%, sputum 18 isolates 30% and Wound smear 6 isolates 9.5% while the burn smear it didn't appear any isolate

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from *K. pneumoniae* [table 7]. The blood samples were diagnosis 60 samples in Ibin Al-Baladi Hospital and given the diagnosis results by VITEK2 System varying percentage between the bacterial genus, its appeared high percentage to *K. pneumoniae* 23 isolates, while appeared 17 isolates from *Staphylococcus aureus* and appeared other isolates very low percent as *Escherichia coli* 4 isolates, *Enterococcus faceium* 3 isolate, *Salmonella typhi* 1 isolate and *Pseudomonas aeruginosa* 1 isolate [table 8].

Table8: percentage of diagnosis by VITEK2 System

No. of specimens	*K. pneumoniae*	*Staphylococcus aureus*	*E. coli*	*Enterococcus faceium*	*Salmonella typhyi*	*Pseudomonas aeruginosa*
100	**23**	**17**	**4**	**3**	**1**	**1**

Susceptibility tests were performing for 50 isolates *K. pneumoniae*, susceptibilities of all isolates to Carbapenem group such as: Meropenem, Imipenem, Ertapenem and Doripenem were determining by disc diffusion method. From gained results we note all isolates were resistance to carbapenem group but each antibiotic different from the other in the inhibition zone formed around the colony, where it was highest resistance to Imipenem then Doripenem then Ertapenem and Meropenem respectively [Hansen,1997] and each tetracycline, gentamycin and ampicillin don't have any effect with *K. pneumoniae* [table 9].

Table 9: Susceptibility test of *K. pneumoniae* to antibiotics

Antibiotics	Concentration µg / disc	Diameters of inhibition zone by (mm)	No. of Isolates resistance (%)	No. of isolates susceptible (%)
Ertapenem	10	13	50	0
Doripenem	10	11	50	0
Imipenem	10	8	50	0
Meropenem	10	17	50	0
Tetracycline	10	11	50	0
Gentamycin	10	3	50	0
Ampicillin	10	2	50	0

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Optimization of the PCR conditions

Beginning all the isolates 61 from urine, sputum, wound and 23 isolates from blood are grown on Blood agar medium for purpose activate bacteria which it was reserve on the Maintenance medium and then transferred to the Nutrient broth instead of Luria – Berloni and brain-heart infusion medium which used in other studies. After end the DNA extraction [figure 3], should measurement the concentration and purity of DNA by Nanodrop, the results showed a concentration between 54- 294 ng/µl and purity 1.69 - 2.02. Through the use of PCR kit according to the company's instructions promega the interaction was doing in volume 25µl. Taken 12.5µl from Master Mix which consist of $MgCl_2$, dNTPs and Taq polymerase and this was use constant volume for 84 samples. The special compounds for diagnosis the bla_{KPC1} gene 61bp Green Master Mix 12.5µl, Forward primer 10 Pico mole, Reverse primer 10 Pico mole, DNA 1-1.5 µl and D.W. 9 - 9.5µl and change condition for purpose of reaching to optimum condition for each primer through the manipulation of annealing temperature, time and cycle number.

Diagnosis the bla_{KPC1} gene

This gene has identified through determination of the optimal condition of interaction, where used 1 µl from Forward and Reverse primers 10 picomole/ µl concentration and used 1.5 µl from DNA template. Placed the initial temperature for beginning interaction 94°C for 5 minutes for one cycle and also the work of gradient PCR through used varied temperature in annealing stage (57,58,59,60,61 and 62°C) for 1.45 minutes depending on Garcia [2010] which used the temperature 60 °C for 1 minute for 40 cycles, and when the product has deported electrically its appeared the best temperature to annealing primer 60°C, Figure 1 shows the product and marker DNA for the package 61bp[figure 4].

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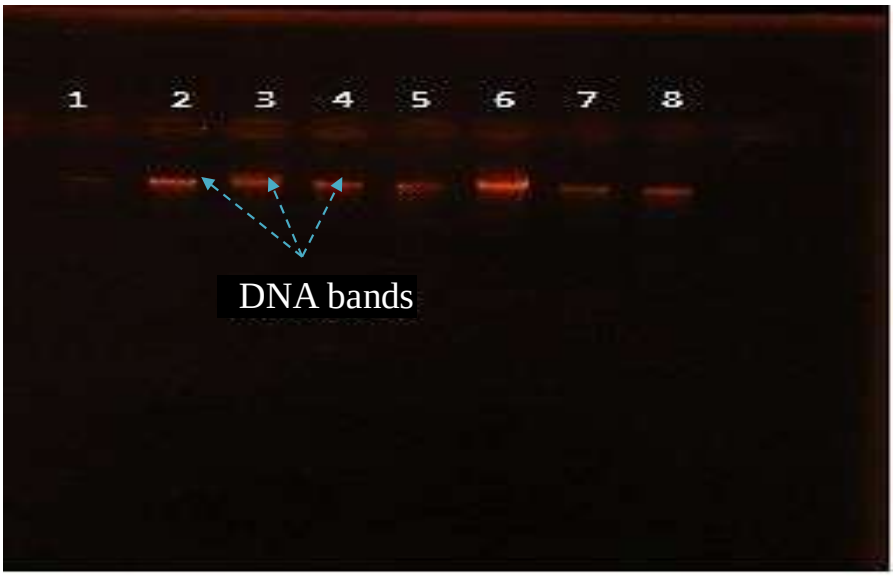

Figure 3: Gel electrophoresis of genomic DNA extraction from *K. pneumoniae* 1% agarose gel at 5 vol /cm for 1:15 hours.

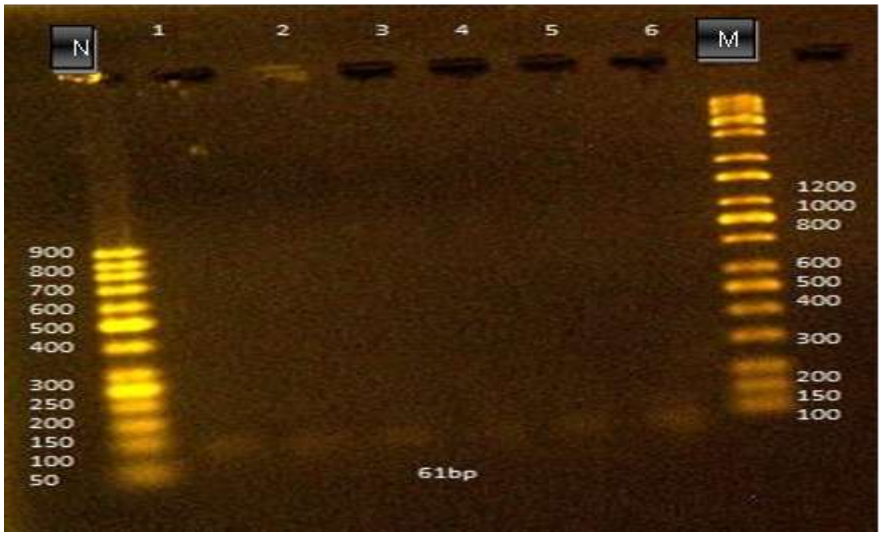

Figure 4: PCR products the band size 61bp. The products were electrophoresis on 2% agarose at 5 volt/cm^2. 1x TBE buffer for 1:45 hours. N: DNA ladder (50- 900), M: DNA ladder (100-10000bp), lane (1-6) PCR product of band size 61bp. visualized under U.V light.

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