Detection of OXA-23 among Carbapenem Resistant Clinical Isolates of *Klebsiella pneumoniae* in Hilla.

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Abstract

This study aimed to assess the presence of bla_{OXA-23} in clinical isolates of *Klebsiella pneumoniae*. During the period from April to August 2011, a total of 801 various clinical samples were collected from different hospitals in Hilla city. One hundred and seventy seven (22%) isolates were diagnosed as *Klebsiella* spp.,117 isolates belonged to *K.pneumoniae* . High prevalence of *K.pneumoniae* was detected in Babylon Teaching Hospital for Maternity and Pediatric with 65 isolates. Regarding clinical samples, highest rate were observed for stool samples 38 (27%) followed by sputum 19 (15%). All 117 *K.pneumoniae* isolates were primarily screened for β - lactams resistance, 91 (78%) showed positive results for beta lactams . β- lactam resistance isolates were underwent antimicrobial susceptibility to 26 antibiotics by Kirby-Bauer disk diffusion methods. High resistance rate was recorded for penicillins (carbenicillin and ampicillin) 99% and 94.5%,respectively .Carbapenem resistance was reported in 17 (18.7%) of *K. pneumoniae* isolates. The presence of bla_{OXA-23} gene was checked by Polymerase Chain reaction (PCR) and confirmed in 15 (88.2%) of isolates.

Keywords: *Klebsiella pneumoniae*, carbapenem resistance ,OXA-23 β-lactamase, Polymerase Chain Reaction.

الخلاصة

هدفت الدراسة الحالية الى التحري عن وجود موروث البيتالاكتام نوع OXA-23 بين عزلات بكتيريا الكليبسيلا الرئوية. تم الحصول على 177عزلة بكتيرية عائدة الى انواع مختلفة من جنس *Klebsiella* 117 عزلة تابعة للنوع *K. pneumoniae* من مجموع 801 عينة سريريه ومن مختلف مستشفيات مدينة الحلة وللفترة من بداية نيسان إلى نهاية آب 2011.أظهرت النتائج إن أكثر عزلات *K. pneumoniae* تم عزلها من مستشفى بابل التعليمي للنسائية والاطفال بواقع 65 عزلة .اما حسب مصدر العينة احتلت عينات البراز 38 (27%) اعلى نسبة تلتها عينات القشع19 (15%) . وقد اظهر المسح الأولي لحساسية العزلات لمضادات البيتالاكتام ان 91 عزلة (78%) كانت مقاومة لمضادي الامبسيلين والاموكسيسلين.

اختبرت حساسية العزلات المقاومة لمضادات البيتالاكتام للمضادات الحيوية بطريقة انتشار القرص (كيربي– باور) وأظهرت النتائج إن أعلى نسبة للمقاومة كانت لمضادات البنسلين،99% لمضاد الكاربنسلين و94.5% لمضاد الامبسلين. فيما أبدت 17(18.7%) عزلة من بكتيريا *K. pneumoniae* مقاومة لمضادات الكاربابنيم وخضعت هذه العزلات للكشف عن موروث bla_{OXA-23} بتقنية سلسلة تفاعلات البلمرة (Polymerase Chain Reaction) وكشفت النتائج إن 15 (82.2 %) من العزلات كانت حاملة للمورث bla_{OXA-23}.

الكلمات المفتاحية: بكتيريا الكليبسيلا الرئوية، لمضادات الكاربابنيم، موروث البيتالاكتام نوع OXA-23 ، سلسلة تفاعلات البلمرة.

Introduction

Klebsiella pneumoniae is an opportunistic and major hospital –acquired pathogen that has the potential to cause sever morbidity and mortality, particularly in intensive care units and amongst pediatrics patients but also in medical and surgical wards (Branger *et al*.,1998; Decre *et al*.,1998; Podschun and Ullmann,1998). *Klebsiella* is the cause of 5-7.5 % of all nosocomial infections and the third most–common bacterial cause of hospital -acquired pneumonia (Jones,2010; Khorshidi *et al*.,2011).

Carbapenems are a group of β-lactam antibiotics which considered as the last line of therapy for multidrug-resistant isolates that are prevalent in many Gram-negative bacterial species ,especially those producing ESBLs or/ derepressed AmpC β-lactamase (Oueenan and Bush,2007; Peirano *et al*.,2009)**.** However , more recently carbapenem –resistant *K.pneumoniae* have emerged in United States and various parts of the world (;Woodford *et a*l.,2004; Peirano *et al*.,2009 ;Aktas *et al* .,2012;Sacha *et al*.,2012). This species is resistant to almost all available antimicrobial agents, and infections with this organism have been associated with high rates of morbidity and mortality, particularly among persons with prolonged hospitalization and those who are critically ill and exposed to invasive devices (e.g., ventilators or central venous catheters) (Wachino *et al*.,2004; Schwaber and Carmeli.,2008). The main mechanism of resistance to carbapenems in *K.pneumoniae* is through the production of a carbapenemase , those that hudrolyse imipenem and /or meropenem are classified in either Ambler classes A, B or D (genetic differences) or in Bush –Jacoby- Medieros groups 2f,3a or 3b (substrate preference and molecular structure). Although resistance is not limited to this mechanism solely , another method of resistance includes ESBLs and or / AmpC production coupled with outer membrane porin (OMP) alterations (Thomson, 2001) .

Class D enzymes are OXA (for oxacillin hydrolyzing) enzymes, which are penicillinases capable of hydrolyzing oxacillin and cloxacillin. These serine beta-lactamases are plasmid encoded and are found primarily in *Pseudomonas aeruginosa*, *Acinetobacter baumannii* ,and rarely in isolates of *Enterobacteriaceae* from the United States. The major concern with OXA carbapenemases is their ability to rapidly mutate and expand their spectrum of activity (Marsik and Nambiar.,2011).

The first OXA enzyme with carbapenemase activity was found in an isolate of *A. baumannii* in 1985 from a patient in Scotland. This enzyme was called ARI-1(for *Acinetobacter* resistant to imipenem) which was later renamed OXA-23 (Paton *et al*.,1993) .Among the carbapenem hydrolyzing OXA enzymes, there is 40-70% amino acid homology within groups and within the group the homology is 92.5% or higher. These enzymes have measurable activity against penicillins, early cephalosporins and imipenem (Walther-Rasmussen and Hoiby.,2006 ; Oueenan and Bush,2007).

The present study was conducted to evaluate the prevalence of *K.pneumoniae* isolated from various clinical specimen in Hilla city, determine carbapenem resistant isolates and to detect $bla_{\text{OXA-23}}$ gene by Polymerase Chain Reaction (PCR) method.

Materials and Methods

Bacterial isolates

In the present study, a total of 801 clinical samples were collected during the period of five months from April to the end of August 2011 ,from patients hospitalized / or attended to different hospitals in Hilla city / Babylon Province, included: Babylon Teaching Hospital for Maternity and Pediatric, AL- Hilla Teaching Hospital, Merjan Teaching Hospital and Chest Diseases Center. All samples were cultured on MacConkey's agar (Himedia) and incubated at 37 C° for 24 hrs. Bacterial isolates of *K. pneumoniae* were identified to the level of species by using the standard biochemical tests according to methods described by Collee *et al.,* (2006) and MacFaddin (2000), confirmatory identification was carried out by VITEK 2 system following manufacturers instructions.

Screening Test for β -lactam Resistance

Preliminary screening of *K.pnenmoniae* isolates being resistant to β-lactam antibiotics was carried out using pick and patch method (NCCLS,2003) .Results were

compared with *E.coli* ATCC 25922(College of Medicine ,University of Kufa) as a negative control.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of β-lactam resistant *K.pneumoniae* isolates was performed on Mueller-Hinton agar (Oxoid) plates by using Kirby-Bauer disk diffusion method (Bauer *et al*.,1966). The isolates were tested against the following antibiotics: Ampicillin (10µg), Carbenicillin (100 µg), Piperacillin (100 µg), Amoxicillin-clavulanic acid (30 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), Ceftriaxone (30 µg), Cefepime (30 µg), Cefoxitin (30 µg), Aztreonam (30 µg), Cefaclor (10µg), Cefprozil (30µg), Imipenem (10 µg), Meropenem (10 µg), Ertapenam (10µg), Gentamicin (10 µg), Amikacin (30µg); Kanamycin (30µg) , Nalidixic acid (30 µg) , Ciprofloxacin (5µg) ; Levofloxacin (5µg) ; Trimethoprim-Sulfamethoxazole (25µg) Nitrofurantion (30µg), Chloramphenicol (30µg) ,Tetracycline (30µg) and Doxycycline (30µg). The cultures were incubated at 37 C^{o} for 18 hrs under aerobic conditions and bacterial growth inhibition zones diameter were measured and interpreted in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI,2010).*E. coli* ATCC 25922 was used as the standard strain for antibiotic susceptibility testing.

Molecular detection of *bla*$_{\text{OXA-23}}$ gene

DNA preparation

DNA preparation from bacterial cells was performed by salting out method as described by Pospiech and Neuman (1995) with some modification and used as a template for PCR reaction .

PCR amplification of *bla*$_{\text{OXA-23}}$ gene.

Polymerase chain reaction was used to amplify the entire sequence of *bla*$_{\text{OXA-23}}$ gene. The primer (Bioneer) used for the amplification of this gene was : OXA-23/F (5-TCTGGTTGTACGGTTTCAGC-3) and OXA-23/R (5—AGT CTTT CCA AAA ATTTTG -3).Amplification reaction mixture was carried out in a 25 µl reaction volume using 12.5 µl Go Taq Green Master Mix 2X (Promega), 5 µl DNA template, 2.5 µl of 10 pmol/ µl of specific up stream primers and, 2.5 µl of 10 pmol/ µl of specific down stream primers, 2.5 µl nuclease-free water. Cycling parameters of *bla*$_{\text{OXA-23}}$ were as follows: an initial denaturation at 95 C˚ for 30sec, followed by 30 cycles of denaturation at 95 C˚ for 30 sec, anneling at 51 C˚ for1 min, extension at 72 C˚ for1 min. and a final extension step of 72 at 10 min.(Hujer *et a*l.,2006;Srinvasan *et al*.,2009).The resulting PCR product was run in 1.5 % agarose gels and electric current was allowed at 70 volts for 2 hr. DNA bands were observed using UV-Transilluminator and photographed with Gel documentation system. 100 bp DNA Ladder (Bioneer) was used to assess PCR product size.

Results and Discussion

Results of the present study revealed the presence of 177 (22%) isolates belonged to *Klebsiella* spp. (Table -1) .In a previous study in Hilla by Al- Charrakh (2005), 29(13.8%) *Klebsiella* spp. isolates were obtained from 209 clinical samples. Another study by Al-Muhannak (2010) reported that *Klebsiella* spp. was the second frequently 62 (30.5%) isolated organisms in Najaf hospitals. Podchun and Ullmann (1998) ,documented that the principle pathogenic reservoirs for transmission of *Klebsiella* are the gastrointestinal tract and the hands of hospital personnel which increase the likelihood of person – to -person transmission and contaminated equipments are also important factors promoting the spread of *Klebsiella* spp . Also table (1) showed that 117 (14.6%) isolates were identified as *K. pneumoniae*. This

result is similar to findings of local study in Hilla by Al-Saedi (2000) who stated that *K. pneumoniae* isolates comprised (15.3%) from 725 clinical samples .

However, prevalence and distribution of *K.pneumoniae* varied among Hilla hospitals , Table (1) shows distribution of *Klebsiella pneumoniae* in Hilla hospitals: Babylon Teaching (23%) , Chest diseases centers (14.8%), Al-Hilla Teaching (11.1%) and Merjan Teaching (3%).This variation among hospitals related to specification of each hospital for receiving patients suffering from specific diseases related to those hospitals .High prevalence rate (23%) was detected in Babylon Teaching Hospital, this range may be related to that most patients received were infants and premature babies suffering from diarrhea, vomiting ,meningitis and premature neonates in ICU for long period ,followed by Chest Diseases Center (14.8%), which specified for examining outpatients sputum with respiratory problem most of them for diagnosis of tuberculosis. High prevalence rate may be related to that most patients were debilitated by other diseases like diabetes mellitus and bronchoplumonary diseases .It was mentioned that the oragansim is carried on respiratory tracts of about 10% of normal people ,who are borne to pneumonia if host defense are impaired (Levinson and Jawetz,2000).

Results of the present study revealed that 569/801(71%) of other bacterial spp. isolates were recovered from clinical samples, (Table -1).There is no doubt that hospitals are typical environments for the presence of pathogens such as *S.aureus E. coli, Klebsiella, Proteus, Morganella, Enterobacter, Citrobacter, Serratia, Acinetobacter* and *Pseudomonas* spp. (Kucukates and Kocazeybek, 2002; Azimi *et al*., 2011). However, the dissemination of bacterial isolates in clinical samples may be due to their ability to cause different nosocomial infections and resistance to a wide range of antibiotics.

Table (1): Distribution of bacterial isolates recovered from clinical samples among different hospitals in Hilla city.

Hospital's name	No. of sample	No. (%) of *Klebsiella* spp. isolates	No.(%) of *K. pneumoniae* isolates	No. (%) of other bacterial spp. isolates	No. (%) of no growth cultures
Babylon Teaching Hospital for Maternity and Pediatric	282	103 (36.5%)	65(23%)	179 (63.5%)	0 (0%)
Al- Hilla Teaching Hospital	261	51 (19 %)	29 (11.1%)	195 (75%)	15 (6%)
Merjan Teaching Hospital	130	4 (3%)	4(3%)	86 (66%)	40 (31%)
Chest Diseases Center	128	19 (14.8%)	19(14.8%)	109 (85.2%)	0 (0%)
Total	801	177(22%)	117(14.6%)	569(71%)	55(7%)

The results of Table (2) showed that the majority of *K. pneumoniae* isolates 38/141(27%) were obtained from stool samples. High prevalence of *K. pneumoniae* in stool samples was demonstrated by other researchers , Al-Saedi (2000) in Hilla , (14%) , Ali *et al*.(2010) in Jordon, (20%) .In sputum , *K.pneumoniae* was detected in

19/128 (15%) of samples .Increasing prevalence of *K.pneumoniae* in sputum was observed by other researchers ,Al- Muhannak (2010) ((15.7%) and Al-Sehlawi (2012) ,(16%) .

In this study, VITEK 2 system was used to confirm identification of *Klebsiella* to species and subspecies levels and to avoid variability in findings of biochemical tests. Results of the present study indicated that *K.pneumoniae* subsp. *pneumoniae* was the most frequent subspecies 77/117(9.6%), followed by *K.pneumoniae* subsp.*ozaenae* 34/117(4%) and *K.pneumoniae* subsp. *rhinoscleromatis* 6/117 (1%) (Table -2). Dominance of *K.pneumoniae* subsp. *pneumoniae* among all other subspecies was supported with a report documented by Al-Sehlawi (2012) ,who stated that *K.pneumoniae* subsp. *pneumoniae* was the most frequent occurring subspecies., accounting for 88.9% .

Results showed that 26/141 (18%) of *K.pneumoniae* subsp. *pneumoniae* ,8/141 (6%) of *K.pneumoniae* subsp.*ozaenae* and 4/141(3%) of *K.pneumoniae* subsp. *rhinoscleromatis* were obtained from stool samples .Similar findings were recorded by Al-Charrakh (2005) who found that most *Klebsiella* spp. were recovered from stool samples and *K.pneumoniae* subsp *pneumoniae* was the most frequently 87% occurring subspecies followed by *K.pneumoniae* subsp.*ozaenea* (9.5%) and *K.pneumoniae* subsp. *rhinoscleromatis* (3.5%) .

In sputum samples, the prevalence rate was 8/128 (6%) for *K.pneumoniae* subsp.*pneumoniae* , 9/128 (7%) for *K.pneumoniae* subsp. *ozaenae* and 2/128 (2%) for *K.pneumoniae* subsp. *rhinoscleromatis*. One study pointed out that the detection rates of *K.pneumoniae* subsp. *pneumoniae*, *K.pneumoniae* subsp. *ozaenae* and *K.pneumoniae* subsp. *rhinoscleromatis* in patients with lower respiratory tract infections in Najaf were (4.3%),(8.6%),and (8.6%), respectively (Al-Muhannak,2010).

Table (2): Numbers and percentages of *K. pneumoniae* subspecies among different clinical samples.

Clinical sample	No. of sample	No. (%) of *K. pneumoniae* isolates	No. (%) of *K . pneumoniae* subspecies		
			K. pneumoniae* subsp. *pneumoniae	***K. pneumouiae* subsp. *ozaenae***	***K. pneumoniae* subsp. *rhinoscleromatis***
Stool	141	38 (27%)	26(18%)	8(6%)	4(3%)
Sputum	128	19 (15%)	8(6%)	9(7%)	2(2%)
Vagina	116	18 (15.5%)	18(15.5%)	0(0%)	0(0%)
Burn	153	18 (11.7%)	12(7.8%)	6(3.9%)	0(0%)
Urine	97	10 (10%)	3(3%)	7(7%)	0(0%)
Wound	60	8 (13.3%)	5(8.3%)	3(5%)	0(0%)
Blood	58	3 (5%)	2(3%)	1(2%)	0(0%)
Ear	30	2 (6.6%)	2(6.6%)	0 (0%)	0(0%)
Eye	8	1 (12.5%)	1(12.5%)	0(0%)	0(0%)
Throat	10	0(0%)	0(0%)	0(0%)	0(0%)
Total	801	117(14.6%)	77(9.6%)	34(4%)	6(1%)

As shown in table (3) , 91/117 (78%) of *K. pneumoniae* isolates were resistant to ampicillin and amoxicillin. This result is in accordance with a study in Hilla by Al-Charrakh (2005) who stated that 73.8% *Klebsiella* isolates obtained from clinical samples were resistant to both ampicillin and amoxicillin . Higher resistant to these antibiotics could be attributed not only to the production of β - lactamases, but also other resistance mechanisms like decrease the affinity of target PBPs or decrease permeability of the drug into the cell (Jacoby and Munoz-Price,2005).There are three further resistance mechanisms include conformational changes in PBPs, permeability changes in the outer membrane, and active efflux of the antibiotic(Amyes,2003). Other studies reported that *qnr* genes (integron-associated) are associated with resistance to several classes of antibiotics including β-lactam (Paterson,2006)

Table (3): β - lactam resistant *Klebsiella pneumoniae* isolates recovered from different clinical samples.

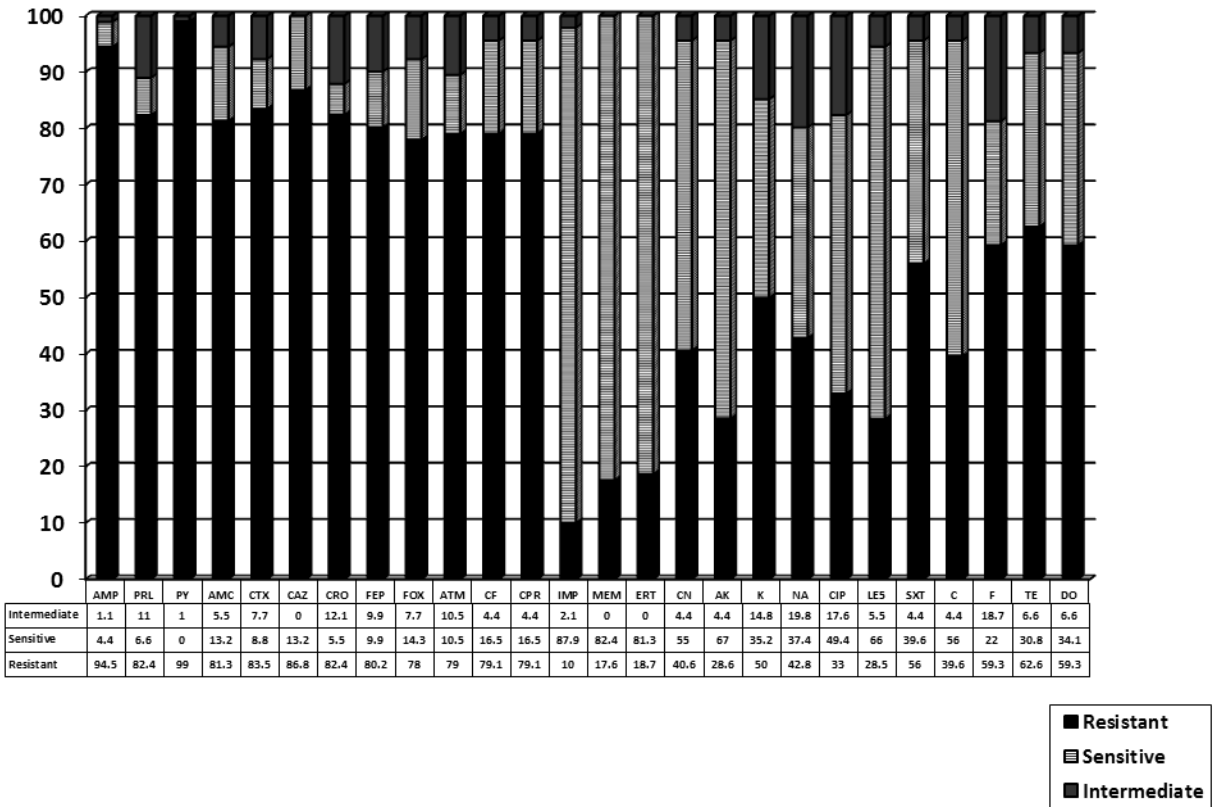
No. of *K. pneumoniae* isolates	Susceptibility to ampicillin and amoxicillin	
	No. (%) of resistant isolates	No. (%) of sensitive isolates
117	91 (78%)	26 (22%)

All 117 *K. pneumoniae* isolates were screened for their antibiotic resistance against selected antibiotic agents of different classes (Fig.1).

In the present study a high resistance was observed for penicillins (carbenicillin and ampicillin) with rates of resistance 90(99%) and 86(94.5%),respectively, whereas 75(82.4%) of isolates were resistance to piperacillin. This result is in agreement with a pervious study in Hilla by Al- Asady (2009) who found that all 15 (100%) β-lactam resistant *Enterobacteriaceae* isolates were resistant to ampicillin , piperacillin and carbencillin . High resistance to this class of antibiotics may be due to widespread use of these antibiotics in Hilla hospitals.

The lower resistance rate was observed to carbapenem antibiotics when imipenem displayed (10 %) resistance rate. In spite of the low level of resistance, this result is higher than that reported by other local studies contacted in Iraq which reported that the susceptibility of *K.pneumoniae* isolates collected from clinical and environmental samples to imipenem was (100%) (Hadi,2008; Al-Asady,2009;Al-Muhannak,2010). Pathak *et al*(2012) demonstrated 2% resistance to imipenem by *K.pneumoniae* in a surveillance study in two hospitals in India .Reasons behind resistance may be due to inappropriate duration of antibiotic therapy and subtherapeutic concentrations of the drug (Paquero *et al.*,1997;Philippe *et al*.,1999).

Meropenem antibiotics showed (17.6%) resistant rate. Meropenem is well – tolerated and offers several potential advantages , including greater *in vitro* activity against Gram –negative pathogens and the option of bolus administration (Verwaest *et al*.,2000) Beside these , problem of renal metabolism of imipenem , and risk of seizures (Prakash,2006)and availability of meropenem only in Hilla hospitals might be the reasons behind possible greater use of meropenem over imipenem and hence the high prevalence of resistance.



	AMP	PRL	PY	AMC	CTX	CAZ	CRO	FEP	FOX	ATM	CF	CPR	IMP	MEM	ERT	CN	AK	K	NA	CIP	LE5	SXT	C	F	TE	DO
Intermediate	1.1	11	1	5.5	7.7	0	12.1	9.9	7.7	10.5	4.4	4.4	2.1	0	0	4.4	4.4	14.8	19.8	17.6	5.5	4.4	4.4	18.7	6.6	6.6
Sensitive	4.4	6.6	0	13.2	8.8	13.2	5.5	9.9	14.3	10.5	16.5	16.5	87.9	82.4	81.3	55	67	35.2	37.4	49.4	66	39.6	56	22	30.8	34.1
Resistant	94.5	82.4	99	81.3	83.5	86.8	82.4	80.2	78	79	79.1	79.1	10	17.6	18.7	40.6	28.6	50	42.8	33	28.5	56	39.6	59.3	62.6	59.3

Figure (1): Antibiotics susceptibility profile of *Klebsiella pneumoniae* isolates by disk diffusion method (n=91).

AMP,Ampicillin;PRL,Piperacillin;PY,Carbenicillin;AMC,Amoxi-clav;CTX,Cefotaxime;CAZ,Ceftazidime;CRO,Ceftraiaxone;FEP Cefepime; Fox,Cefoxitin; ATM,Aztreonam; CF,Cefaclor; CPR,Cefprozil; IMP,Imipenem; MEM,Meropenem; ETP,Ertapenem; CN, Gantamicin ; AK, Amikacin ; K, Kanamycin ; NA , Nalidixic acid ; CIP, Ciprofloxacin ; LE[5],Levofloxacin ; SXT, Trimethoprim-Sulfamethoxazole;C,Chloramphenicol;F,Nitrofurantion;TE, Tetracycline;DO,Doxycycline.

All carbapenem resistant isolates were screened by PCR for the presence of bla_{OXA-23}. Results confirmed that 15 (88.2%) isolates carrying bla_{OXA-23} (Fig.2). This represents the first report of OXA-23 among clinical isolates of *K.pneumoniae* in Hilla. Recently , bla_{OXA-48} mediated resistance to carbepenem has been reported in *K.pneumoniae* in Turkey [9].The present study found that the prevalence rate of bla_{OXA-23} in carbapenem –resistant *K.pneumoniae* isolates from Hilla hospitals is undoubtedly high.

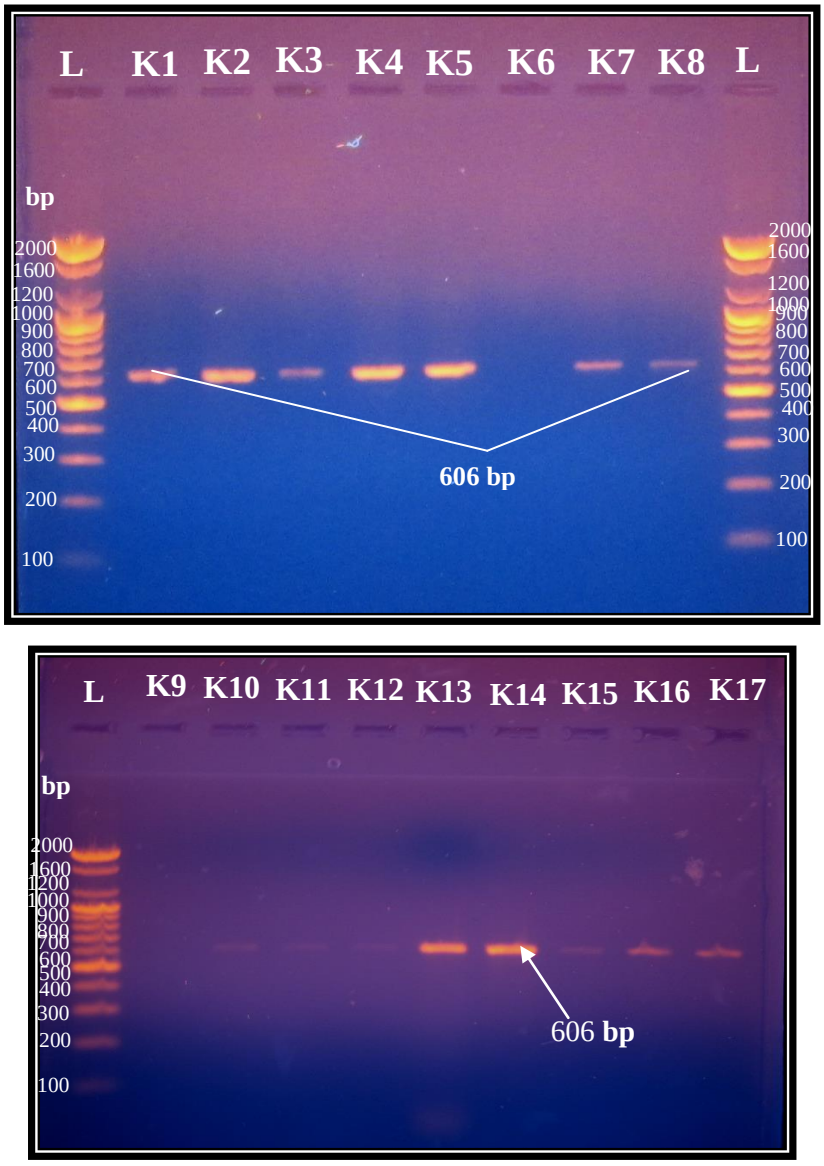


Figure (2): Agarose gel electrophoresis (1.5%agarose,70 % volt for 2-3 hrs) for bla_{OXA-23} gene product (ampilified size 606 bp) using DNA template of carbapenem-resistant *K. pneumoniae* isolates extracted by using salting out method. Lane (L), DNA moleculer size marker (100- bp Ladder).Lanes (K1,2,3, 4, 5, 7, 8, 10,11,12,13,14,15,16 and 17) of *K. pneumoniae* isolates show positive results with bla_{OXA-23} gene , lanes (K 6 and 9) show negative results with bla_{OXA-23} gene.

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