Detection of antibiotics resistance genes in clinical isolates of *Klebsiella pneumonia*

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Abstract:
Total of 46 isolates of *Klebsiella pneumoniae* were collected from patients attending (Al-Yarmook Hospital and Education Labs / medical city), and isolates were re-identified, depending on morphology and biochemical tests . Disk diffusion method was employed to determine antibiotic susceptibility of forty six isolates by using eleven antibiotics .The results revealed the sensitivity of six isolates (9.3%) to Imipenem and Meropenem . On the other hand the isolates were showed 23.9% resistant against Ciprofloxacin, while some isolates shown higher resistant against several antimicrobial agents such as 65.2%, 69.0% for Amikacin and Cefepime consequently , 71.1%, 71.7 % for Amoxicillin -Clauvulanic acid and Gentamicin and 82.6% against Piperacillin , Nitrofurantoin and Ceftazidime. The isolates also appeared high level of resistance against Cefotaxime at a percentage 91.3% . Depending on the obtained results 6 isolates were selected assigned (K21, K32, K33, K37, K38, K43) for detection of *blaTEM*, bla*SHV*, bla*KPC* and *AmpC* because of its resistance of almost chosen antibiotics. The selected isolates were PCR-positive for *blaTEM* which showed bands in 209 pb., on the other hand the result revealed that the isolates K33,K32 posses encoding to *blaSHV* of 509pb in size,. In regard to *blaKPC* gene only K37 (16%)gave 811 pb . All selected isolate gave negative results to *AmpC*. The selected isolates were detected of beta-lactamase production by using acidimetric tests (tube method) , all isolates gave positive result.

Keywords: *K. Pneumoniae,* antibiotics resistance, genes.

التحري عن المورثات المشفره لصفه المقاومه للمضادات الحيويه في عزلات سريرية لبكتريا *Klebsiella pneumonia*

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الخلاصه:
جمعت (46) عزله ل *Klebsiella pneumoniae* من مستشفى اليروموك والمختبرات التعليميه في مدينه الطب . اعيد تشخيصها اعتمادا على الاختبارات الكيموحيوية .اظهرت نتائج اختبارحساسيه العزلات تجاه احدى عشر مضاد حيوي وتضمنت مقاومه 9.3% لكل من Imipenem و Meropenem و 3.9% مقاومه لمضاد Ciprofloxacin فضلا عن امتلاك العزلات مقاومه عاليه للعديد من المضادات شملت 65.2% لمضاد

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Amikacin و 69.0% لمضاد Cefepime و71.1% لمضادAmoxicillin–Clavulanic acid و% 71.7 لمضاد Gentamicin و82.6% لكل من Piperacillin ,itrofurantoin و Ceftazidime في حين اظهرت العزلات 91.3% مقاومه للمضاد Cefotaxime . وبناءا على نتائج اختبار الحساسيه اختيرت 6 عزلات (K21, K32, K33, K37, K38, K43) والتي تمثل الاكثر مقاومه لمعظم المضادات البكتيريه المستعمله . تم استخدام تقنيه PCR للتحري عن امتلاك العزلات 6 المختاره على المورثات المشفره *bla TEM blaSHV, blaKPC, AmpC*. اظهرت العزلات جميعها امتلاكها للمورث المشفر *bla TEM* ذو حجم 209 زوج قاعدي وامتلاك العزلتين 33,32 للمورث المشفر *blaSHV* ذو حجم 509 زوج قاعدي واظهرت عزله واحده امتلاكها للمورث المشفر *bla KPC* ذو حجم 811 زوج قاعدي ولكن جميع العزلا ت لم تمتلك المورث المشفر *AmpC* . اختبرت قابليه العزلات على انتاجها لانزيم البتالاكتميز باستخدام طريقه acidimetric tests (tube method) اظهرت النتائج ان جميع العزلات منتجه لانزيم البيتالاكتميز.

Introduction:

Antimicrobial resistance is a serious problem in many bacterial pathogens concern for hospital-acquired problem nosocomial infections. *K. pneumoniae* is important cause of the pneumonia ,urinary tract infection sepsis and meningitis and other infections [1].

Multidrug-resistant *K. pneumoniae* isolates are being a growing problem ,it usually carries one or more extended spectrum β-lactamase (ESBLs).Extended -spectrum β-lactamase are usually derived from plasmid mediated *blaTEM* , (classs A- β-lactamases) or *bla*SHV classs A- β-lactamases) β-lactamase through the mutation or mutations that lead to one or more amino acid changes and that result in the alteration of the binding to and hydrolysis of specific substrates at the active site .These enzymes especially TEM and SHV are most commonly found in *K.pneumoniae* and *Escherichia coli* [2] .One study observed that SHV-type gene is ubiquitous in *K.pneumoniae.* The classic *blaSHV*-1gene which is usually encoded by the plasmid of Enterobacteriaceae ,is normally encoded by the chromosome in *K. pneumoniae* .It has been postulated that SHV-may have originated by separation from the chromosome of *K. pneumoniae* and extrachromosome spread to other bacteria [3].

*Ampc*β-lactamase group 1cephalosporinase (class C β-lactamases) that confer resistance to a wide variety of β-lactam antibiotics such as cefoxitin and broad spectrum cephalosporins ,aztreonum and rarely inhibited by β-latamase inhibitors such as clavulinic acid. Genes for *Ampc*β-lactamase are found on the chromosome of the several members of the Enterobacteriaceae. Plasmid mediated *AmpC* β-lactamase has arisen through the transfer of chromosomal genes for the inducible *AmpC*β-lactamase on the plasmids this transfer has resulted in plasmid mediated *Ampc* β -lactamase in *E.coli* and *K. pneumonia* [4].

bla-KPC (K. pneumoniae *carbapenemase*)(classA) enzymes are among the most common *β* -lactamases mediating carbapenem resistance among isolates of Enterobacteriaceae .KPC enzymes are class *A* β -lactamases that mediate resistance to extended -spectrum cephalosporins in addition to carbapenems ,bla KPC are usually plasmids mediated [5] . hence, the present study was carried out to achieve the following aims :determination of multi-drug resist among *K.*pneumoniae isolates by using antibiotic susceptibility test ,molecular detection of *β*– lactamase genes *blaTEM ,bla SHV, blaKPC* and AmpC, *and phenotype detection of β-lactamase* .

Materials and Methods:

Bacterial isolates:

Total of 46 identified isolates of *K. pneumoniae* were collected from patients attending (Al-Yarmook Hospital/ Education Labs / medical city).

Identification of *K. pneumoniae* :

Isolates were Re identified depending on morphology and biochemical tests. The biochemical tests were employed Citrate utilization, Ureae production, Methyle red, Voges- prouskauer, Motility Triple -

sugar Iron and indol as compared with identification scheme described by [6,7,8] and according to VITEK 2 system Kit (Bio-Meriaux,France)as confirmatory test.

Susceptibility of *K. pneumoniae* isolates to different antimicrobial agents:

In present study the isolates of *K. pneumoniae* were adjusted to 0.5 Mcfarland turbidity strands test and subjected to susceptibility test against eleven antibiotics were commonly used .Susceptibility was determined by disk -diffusion technique of kirby-Bauer on Muller Hinton agar plates. Disks containing Imipenem IMP (10µg) Meropenem MEM (10µg), Cefepime FEP (30µg) Cefazidime CAZ (30µg) Ciprofloxacin CIP(5µg) , Nitrofurantion F (100µg) , Cefotaxime CTX (10µg) , Piperacillin PRL (100µg) Amoxicillin-Clavulanic acid AMC(30µg), Amikacin. AK(30 µg) and Gentamicin GEN (10 µg) .All disks were obtained from Bioanalysis (Turkey) After 24 hr .incubation at 37^0C,organisms were classified as Sensitive(S), Intermediately resistant(I) or Resistant (R) on the basis of the size of the zone of the bacteria growth inhibition according to the guidelines of the [9,10,11].

Extraction of DNA from the Selected isolates:

Genomic DNA was extracted by using two methods, the ExiPrep ™Plus Genomic DNA kit (Bioneer company ,Korea). The second method is described by [12] with some modification to isolate both plasmid and chromosomal DNA .

Polymerase chain reaction (PCR):

Primers:

All the primers used in this study were manufactured by (Alpha DNA \Canada) in lyophilized form. The oligonucleotide specific primers for *blaTEM*, *blaSHV* , *blaKPC* and *AmpC* genes of *K.pneumoniae* isolates which was used to conform the detection of antibiotics resistance. The primer and their sequences were listed in table -1. Primers were prepared by dissolving lyophilized product in nuclease free water to prepare 100 pmolof stock solution forward and reverse primers then were used to prepare working solution. All four primers were designed locally by university of Baghdad/college of science \Biotechnology Department.

Table 1- Oligonucleotide primer sequences and PCR product for antibiotic resistance genes.

Primer	Sequence5'----------3'	PCR Product
blaTEM -F	TTGATCGTTGGGAACCGGAG	209bp
blaTEM -R	AATAAACCAGCCAGCCGGAA	
blaSHV -F	GCTGGAGCGAAAGATCCACT	509bp
blaSHV -R	CCACAATCCGCTCTGCTTTG	
blaKPC -F	ATCGCCGTCTAGTTCTGCTG	811bp
blaKPC -R	TCGCTGTGCTTGTCATCCTT	
AmpC-F	GGACAGCACCATTAAACCGC	356pb
AmpC-R	GATCCGCACGGCTTTTTACC	

PCR reaction:

Polymerase chain reaction was performed in a total volume of 25 µl containing 1µl of both the forward and reverse of the primer,12.5 µl master mix, 8.5 µl Nuclease free water and 2µl of the DNA , then DNA amplification was carried out with the thermal cycler.

PCR program:

The program of mixtures was adopted in PCR analysis of *blaTEM, blaSHV* , *blaKPC* and *AmpC* primers as shown in table -2.

Table 2- The program of antibiotic resistance primer used in PCR analysis.

Primer name	Initial Denaturation	Denaturation	Annealing	Extension	Final extension
blaSHV* , *AmpC* and *blaTEM	94º C for 5min	95ºC for 30sec	50-60ºC for 30 sec	72 ºC for 30 sec	72 ºC for 5 min
blaKPC	94º C for 5min	94 ºC for 1min	50-60 º C for 1 min	72 ºC for 1 min	72 ºC for 5 min

Determine the Molecular Size of Amplicons:

The PCR products were separated on 2% agarose gel electrophoresis in the presence of 100 bp DNA ladder marker (Promega, USA) ,(Bioneer,Korea)and visualized under the ultraviolet light (302nm) after staining with ethidium bromide [13].

Detection of B-lactamae by using acidimetric tests (tube method):

The six selected isolates were tested its ability to produce β-lactamase , 2ml of 5% (w\v) phenol red solution is diluted with 16.6ml distilled water and 1.2 g of benzylpencillin is added .The pH is adjusted to 8.5with 1M NaOH . Before use ,100µl portion are distributed into tubes and incubated with bacteria from culture plates. A yellow color within 5 min indicate a positive result [14].

Results and discussion:

Isolation and characterization of *K. pneumoniae*:

Forty six local isolate were characterized depending on cultural and microscope characteristic. Genus and species were characterized by using biochemical tests and only selected six isolates (K21, K32,K 33, K37,K 38,K 43) were fully identified by using VITEK2 system.

Cultural characteristics:

The colonies of isolates *K. pneumoniae* were appeared as pink and mucoid colonies on Macconkey agar as described by [6].

Microscopically characteristics:

Microscopical examination of the bacteria appeared as coccobacilli and gram negative when it stained with gram stain as described by [8].

Biochemical characteristics :

Several biochemical tests were done to characterize *K. pneumoniae* . All the 46 isolates of the *K. pneumoniae* showed positive results to the biochemical test Citrate Utilization, Voges-proskaure (VP), Urease production and Triple sugar Iron (TSI) but all isolates were negative to biochemical tests Motility, Methyl red, Indole production and Oxidase as illustrated in the table -3 [15,16].

Table 3- Biochemical tests of *K. pneumonia.*

Test	Result
Citrate utilization	+
VP	+
Urease production	+
TSI	A/A-+ (NegativeH2S,positive gas)
Motility	-
MR	-
Indole	-
Oxidase	-

(+)positive result,(-)negative result, A=acidic

Antibiotic susceptibility of *K. pneumoniae* isolates:

Disk diffusion test was employed to determine antibiotic susceptibility of forty six isolates on Muller Hinton agar following the clinical and Laboratory Standard Institute (CLSI) guideline (CLSI,2013), by using eleven antibioics (Imipenem, Meropenem Cefepime, Ceftazidime, Ciprofloxacin, Nitrofurantoin ,Cefotaxime, Amoxicillin -Clavulanic acid, Amikacin, Gentamicin and Piperacillin). As illustrated in figure -1 and table -4 the results revealed that high sensitivity of isolates to Imipenem and Meropenem and only six isolates (9.3%) were resistant against these two antibiotics and these results are confirmed with [17].On the other hand the isolates showed 23.9% resistant against Ciprofloxacin ,while some isolates shown highly resistant against several antimicrobial agents such as 65.2%, 69.0% for Amikacin and Cefepime consequently , 71.1%, 71.7 % for Amoxicillin -Clavulanic acid and Gentamicin, 82.6% against Piperacillin , Nitrofurantoin and Ceftazidime. The isolates appeared high level of resistance against Cefotaxime at a percentage 91.3% this result was confirmed with [1,18] . Depending on the obtained results 6 isolates were selected (K21, K32,K 33, K37,K 38, K43) for detection of *blaTEM*, *blaSHV*, *blaKPC* and *blaAmpC* because of their resistance of almost chosen antibiotics. The results elicited were elevation in resistance against antimicrobial agents .The antibiotic Imipeneme and Meropenem were highly effected against *K. pneumoniae* .The development of multidrug-resistant *K. pneumoniae* has been a worldwide concern. Carbapenem antibiotics are the drug of choice for treating severe infections caused by Enterobacteriaceae bacteria because of their high stability to ESBLs, as well as having a strong bacterial activity [19].

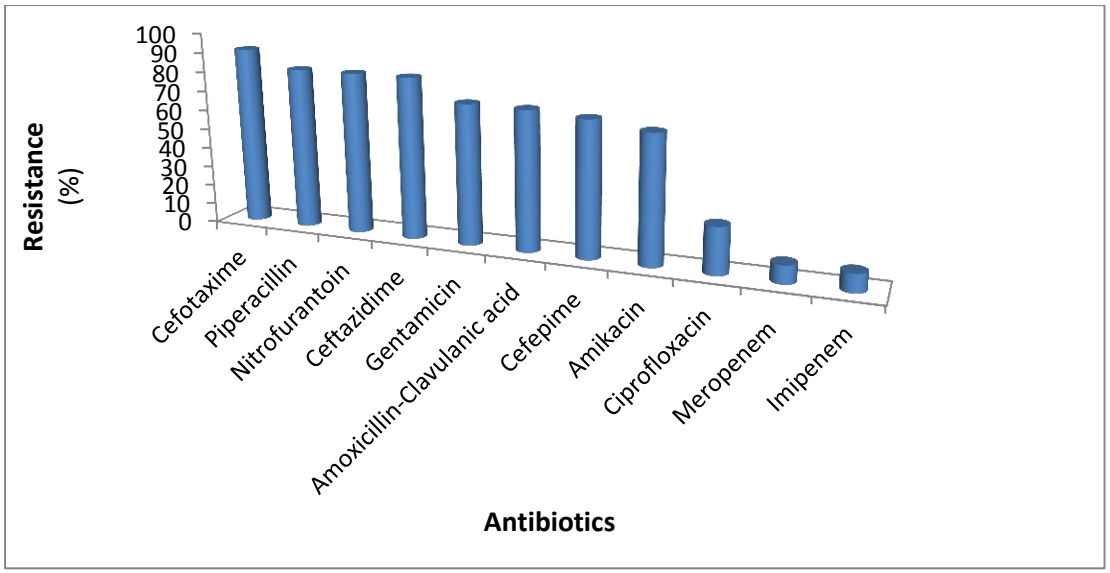

Figure 1- Percentages of susceptibility of *K. pneumoniae* isolates to antibiotics

Table 4 -The antibiotics susceptibility results of the *K.pneumoniae*.

Number of isolates	Imipenem	Meropenem	Cefepime	Cefazidime	Ciprofloxacin	Nitrofurantion	Cefotaxime	Amoxicillin-Clavulanic acid	Amikacin	Gentamicin	Piperacillin
	IMP	MEM	FEP	CAZ	CIP	F	CTX	AMC	AK	GEN	PRL
K1	S	S	I	R	S	R	R	S	S	S	R
K2	S	S	I	R	S	R	R	S	S	S	R
K3	S	S	R	R	S	R	R	R	R	R	R
K4	S	S	R	R	S	R	R	R	R	R	R
K5	S	S	I	R	S	R	R	R	R	R	R
K6	S	S	R	R	S	R	R	I	S	R	R
K7	S	S	R	R	S	R	R	R	R	R	R
K8	S	S	R	R	R	R	R	R	R	R	R
K9	S	S	I	R	S	R	R	R	R	R	R
K10	S	S	S	R	S	S	S	S	R	R	R
K11	S	S	S	R	S	R	R	S	R	R	R
K12	S	S	R	R	S	R	S	S	S	S	R
K13	S	S	R	R	R	R	R	R	R	S	R
K14	S	S	R	R	S	R	R	R	S	S	R
K15	S	S	R	R	S	R	R	R	R	R	R
K16	S	S	R	R	S	R	R	R	R	R	R
K17	S	S	R	R	S	R	R	R	R	R	R
K18	S	S	R	R	S	R	R	R	R	R	R
K19	S	S	R	R	S	R	R	R	R	R	R
K20	S	S	S	R	R	R	R	R	R	R	R
K21	R	R	R	R	R	R	R	R	R	R	R
K22	S	S	R	R	S	R	R	R	R	R	R
K23	S	S	R	R	S	R	R	R	R	R	R
K24	S	S	R	R	S	R	R	R	R	R	R
K25	S	S	S	R	R	R	R	R	R	R	R
K26	S	S	S	R	R	R	R	R	R	R	R
K27	S	S	S	S	S	R	R	R	S	R	R
K28	S	S	S	R	S	R	R	R	R	R	R
K29	S	S	R	R	S	R	R	R	R	R	R
K30	S	S	S	R	S	R	S	S	S	R	S
K31	S	S	S	R	S	R	R	R	S	R	R
K32	R	R	R	R	R	R	R	R	R	R	R
K33	R	R	R	R	R	R	R	R	R	R	R
K34	S	S	S	R	S	R	R	R	S	S	R
K35	S	S	R	R	R	R	R	R	R	R	R
K36	R	R	R	R	R	R	R	R	R	R	R
K37	R	R	R	R	S	R	R	R	R	R	R
K38	R	R	R	R	S	R	R	R	R	R	R
K39	S	S	S	S	S	S	R	S	S	S	S
K40	S	S	S	S	S	S	R	S	S	S	S
K41	S	S	S	S	S	S	R	S	S	S	S
K42	S	S	S	S	S	S	R	S	S	S	S
K43	R	R	R	R	R	R	R	R	R	R	R
K44	S	S	S	S	S	S	R	S	S	S	S
K45	S	S	S	S	S	S	R	S	S	S	S
K46	S	S	S	S	S	S	S	S	S	S	S
Percentages	9.3%	9.3%	69.0%	82.6%	23.9%	82.6%	91.3%	71.1%	65.2%	71.7%	82.6%

R:resistant ,S:sensitive

Molecular detection of *blaTEM,* bla*SHV,* bla *KPC* and *AmpC*:

The selected isolates of *K. pneumoniae* (K21, K32,K33,K 37, K38,K 43) were amplified with PCR for diagnosis *blaTEM*, bla*SHV*, *blaKPC* and *AmpC* using specific primers that depend on locally designed were tested to explored about these genes which responsible for *K. pneumoniae* resistance of β-lactam . The isolates (K21, K32, K33,K 37, K38,K 43) were PCR-positive for *bla TEM* which showed bands in 209 bp . Primer annealing temperature gradient within the range 50ºC-60ºC, revealed an optimal annealing temperature 56^0Cfor *blaTEM* as illustrated in figure -2 ,on the other hand *blaSHV* gene revealed that the K33,K32 isolates appeared in size509bp,in PCR amplification of isolates using primers *blaSHV* and primer annealing temperature gradient within the range 50-60Cº , revealed an optimal annealing temperature50 ,52 ^{0}C for K32, K33 isolates(33%) (figure-3) , as reported with [20],al 12 isolates of *K. pneumoniae* product *blaTEM* sequences by using PCR technique in regard to *blaSHV*, our finding is confirmed to [21] who indicated that the percentage of this gene was 34.8% in *K. pneumoniae* , the other study in detection of *blaTEM* and *blaSHV* confirmed that 25 ESBL positive *K. pneumoniae* isolates harboured15 *blaTEM* and 18 harboured *blaSHV*. More than 100 ESBL variants from different types are known .The most abundant types are TEM, SHV, CTX-M, OXA. The prevalent genotype varies in different countries with TEM and SHV enzymes are the most frequently observed. Mutations in the genes encoding these enzymes expand the spectrum of their activity to include extended spectrum cephalosporines [21 ,22] In regard to *blaKPC* only 37 (16%) gave 811bp in optimal annealing temperature 60ºC (figure – 4).The result is confirmed with [23] who reported that number of *K. pneumoniae* producing *blaKPC* gene is 2 among K37 isolates in south Taiwan, the other study detected 73% (*blakpc*) in multi β-lactam resist of *K. pneumoniae* [24] . All selected isolate gave negative results to *AmpC* and these are comparable with [25], the other research of the same gene revealed that 30% isolates produce *AmpC* [26]. The difference in result may be due to different plasmids or production variable enzymes of *blaKPC* and *AmpC* .

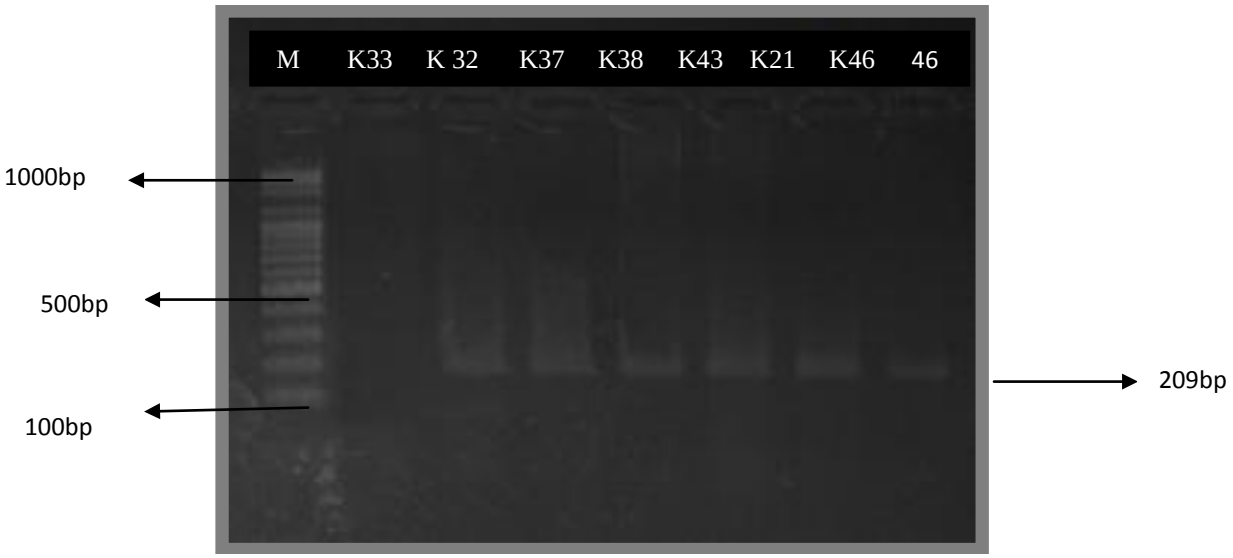

Figure 2- Gel electrophoresis for amplification products of *blaTEM K.pneumoniae* on 2% garose at 90 V for 1 hour .K32,K33,K37,K38,K43,K21 are resistance isolates to Imipenem,Meropenem, Cefepime , Ceftazidime , Nitrofurantion , Cefotaxime, Piperacillin , Amoxicillin-Clavulanic acid, Amikacin and Gentamicin , K46 is sensitive isolate (annealing temperature50,25^{0}C) .M is a marker (1000bp).

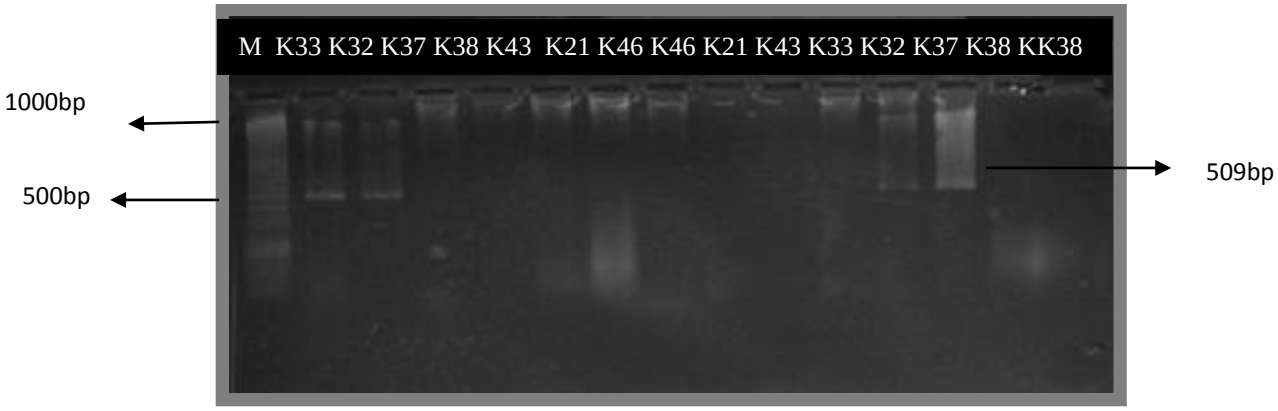

Figure 3- Gel electrophoresis for amplification products of *blaSHV* gene *K. pneumoniae* on 2% at90 V for 1 hour .K32,K33,K37,K38,K43,K21 are resistance isolates to Imipenem, Meropenem , Cefepime , Ceftazidime , Nitrofurantion , Cefotaxime , Piperacillin , Amoxicillin-Clavulanic acid , Amikacin and Gentamicin , K46 is sensitive isolate (annealing temperature50,25^{0}C) .M is a marker (1000bp).

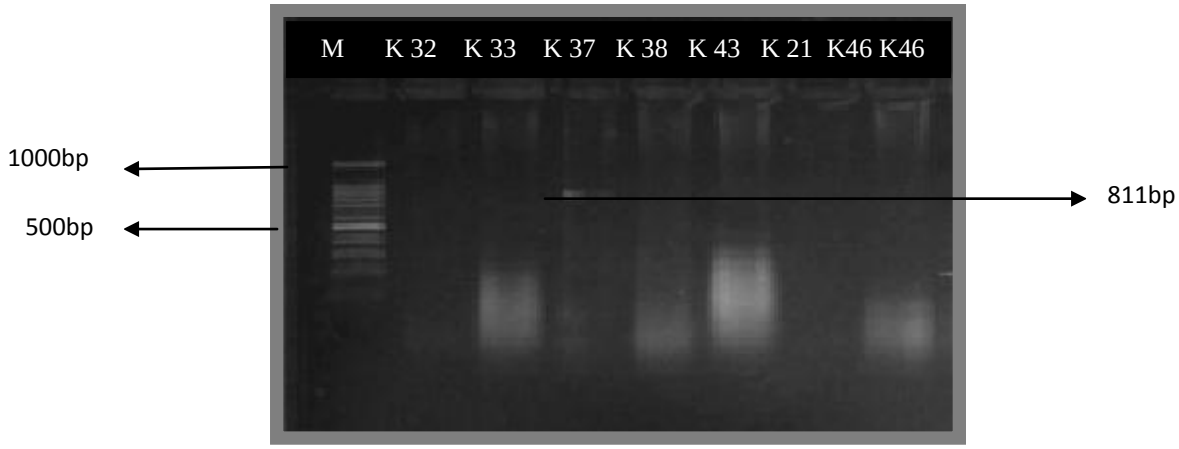

Figure 4- Gel electrophoresis for amplification products of *bla KPC*gene *K. pneumoniae* on 2% garose at90 V for 1 hour .K32,K33,K37,K38K43K21are resistance isolates to Imipenem, Meropenem , Cefepime , Ceftazidime, Nitrofurantion, Cefotaxime , Piperacillin , Amoxicillin-Clavulanic acid , Amikacin and Gentamicin , K46 is sensitive isolate(annealing temperature60 ^{0}C). M is a marker (1000bp).

Detection of β-lactamae by using acidimetric tests (tube method):

The selected isolates were detected of beta-lactamase production by using acidimetric tests (tube method) all isolates gave positive result (yellow color) . Hydrolyze of the beta-lactam ring generates a carboxyl group ,the resulting acidify can be examined in tubes(14) . Our finding is confirmed with [27] who indicated that 50% of *K.pneumoniae* were Blactamase producing by using rapid standard Iodometric assay and capillary tubes method . According to high ratio of resistance to more than antibiotics that related to beta – lactam group this showd that these β-lactamase-producing isolates have an enzymatic mechanism of resistance represented by the production of β-lactam.

In recent study [28], SHV genes were the dominant genotype while TEM genes were the major genotype of ESBL-KP in the other research [21] . ESBL continue to be the leading cause of resistance to β lactam antibiotics among gram negative bacteria .There has been an increased incidence and prevalence of extended spectrum β lactamase that show a wide spread in hospital sitting worldwide.

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