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Phenotypic and Genotypic Detection of PER β-Lactamase among Carbapenem Resistant *Klebsiella pneumoniae* in Hilla Hospitals.

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Abstract

This study investigates the prevalence of *bla*$_{PER}$ gene 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. Of these,117 isolates were specified as *K.pneumoniae* . High prevalence of *K.pneumoniae* isolates were detected in stool samples 38 (27%) followed by sputum 19 (15%). All 117 *K.pneumoniae* isolates were primarily screened for β- lactams resistance, 91 (78%) were found to be screen positive. 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) with rates of resistance of (99%) and (94.5%),respectively .Carbapenem resistance was reported in 17 (18.7%) of *K. pneumoniae* isolates. Phenotypic detection of extended spectrum β- lactamase by CHROMagar technique identified a proportion of 17(100%) as ESBL producers. The presence of *bla*$_{PER}$ gene was checked by Polymerase Chain reaction (PCR) and confirmed in 10 (82.3%) of isolates.

Key word: *Klebsiella pneumoniae* ,extended spectrum beta lactamase , *bla*$_{PER,}$ PCR, Carbapenem resistance .

Introduction

Extended spectrum β-lactamase (ESBL) producing *K.pneumoniae* have spread rapidly worldwide and pose a serious threat in heleathcare –associated infections [1].Since the first report of an ESBL-producing organism in 1980s,there has been a growing interest due to their wide spread and constant evolution, becoming increasingly resistant to most of the commonly used antibiotics [2].

ESBLs are mutant , plasmid mediated enzymes which derived from the older, broad spectrum β-lactamases and able to hydrolyze penicillins, first -, second cephalosporin and also third generation oxyimino cephalosporins and monobactams [3,4] but they are not active against cephamycins and carbapenems, generally they are inhibited by clavulanic acid , tazobactam and sulbactam [5].

ESBLs have evolved from point mutations, thus altering the configuration of the active site of the original and long known β- lactamases [6]. Another important epidemiological characteristic is the possibility of these enzymes being transferred to other species via plasmid or transposons [7].

ESBLs are undergoing continuous mutation, causing the development of new enzymes showing expanded substrate profiles. At present, there are more than 300 different ESBL variants , and these have been clustered into nine different structural and evolutionary families based on amino acid sequence. TEM and Sulphhydryl variable SHV were the major types but the group also includes enzymes that are not TEM or SHV derivatives [3,6].PER one of these enzymes has about 25-27% amino acid homology with the known TEM and SHV type ESBLs. PER-1 β- lactamase

hydrolyzes penicillins and cephalosporins efficiently , but is susceptible to clavulanic acid inhibition. The PER-1 β- lactamase was first detected in strains of *P. aeruginosa*, later, it was found among isolates of *S. enterica* serovar *Typhimurium* and *Acinetobacter* spp. The PER- type ESBL can be found worldwide, but is most frequent in Europe [8].

The present study was conducted to evaluate the antibiotic susceptibility pattern of clinical isolates of *K. pneumoniae* among different hospitals in Hilla City, and detect the prevalence of PER ß -lactamase gene by phenotypic and genotypic (PCR) method.

Materials and Methods

Bacterial isolates

In the present study, a total of 801 clinical samples (stool , sputum, vagina, burn, urine, wound ,blood, ear, eye and throat) were collected during the period of five months from April to 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 Holt *et al.*;Baron and Finegold ;Collee *et al.* and MacFaddin [9,10,11,12] ,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 [13]. 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 [14]. 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 [15].*E. coli* ATCC 25922 was used as the reference strain for antibiotic susceptibility testing.

Screening for ESBLs enzyme production

Screening for ESBLs was determined using CHROMagar technique. Freshly prepared extended spectrum β -lactamase CHROMagar (CHROMagar) plates were streaked by overnight growth of *K. pneumoniae*. The plates were incubated at 37 C˚ for 24 hrs according to manufactured procedure. Growth of blue colonies indicated to ESBL producer. The reference strain of *E. coli* ATCC 25922 was inhibited and used as negative control**.**

Genotypic detection of *bla*$_{PER}$ gene

DNA preparation

DNA preparation from bacterial cells was performed by salting out method as described by Pospiech and Neuman with some modification and used as a template for PCR reaction [16].

PCR amplification of *bla*$_{PER}$ gene.

Polymerase chain reaction was used to amplify the entire sequence of *bla*$_{PER}$ gene. The primer (Bioneer) used for the amplification of this gene was : PER/F (5-AGTCAGCGGCTTAGATA -3) and PER/R (5- CGTATGAAAAGGACAATC-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*$_{PER}$ were as follows: an initial denaturation at 93 C˚ for 3 min, followed by 40 cycles of denaturation at 93 C˚ for 1min, anneling at 55 C˚ for 1 min, extension at 72 C˚ for 1 min. and a find extension step of 72 at 7 min. 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

Bacterial isolates

A total of 117 *K.pneumoniae* isolates were obtained from 801 clinical samples over a period of five months. The distribution of *K. pneumoniae* isolated from various clinical specimens was 38 (32.5 %) were obtained from stool, 19 (16.2%) from sputum, 18 (15.4%) from vagina and burn, 10 (8.5%) from urine, 8 (6.8%) from wound, 3 (2.5%) from blood, 2 (1.7%) from ear, 1(0.9%) from eye and 0 (0%) from throat. (Table- 1).

Primary Screening Test of β - Lactam Resistant Isolates

All 117 *K. pneumoniae* obtained from different clinical samples were primarily screened for β - lactams resistance. Results from Table (2) revealed that a total of 91/117(78%) *K. pneumoniae* isolates were able to grow normally in the presence of ampicillin and amoxicillin.

Antibiotic susceptibility test

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 higher resistance was observed for penicillins (carbenicillin and ampicillin) with rates of resistance of 90(99%) and 86(94.5%),respectively, whereas 75(82.4%) of isolates were resistance to piperacillin. a higher resistance was also detected with 79(86.8%) of isolates being resistant to ceftazidime , 76(83.5%) to cefotaxime ,75(82.4%) to ceftriaxone and 73(80.2%) to cefepime. The results also revealed that were high resistant rates for amoxi-clav 74(81.3%) and cefoxitin 71(78%). A 72(79.1 %) and 72(79%) resistance were noticed to cefaclor ,cefprozil and aztreonam antibiotics, respectively.

Among the carbapenem ,imipenem displayed a lower resistance rate 9(10%),than meropenem 16(17.6%) and ertapenem 17(18.7%). Aminoglycosides resistance was variable ,46(50%) to kanamycin , 37(40.6%) to gentamicin and 26 (28.6 %) to amikacin. The resistance to quinolones, nalidixic acid ,ciprofloxacin and levofloxacin was detected 39 (42.8%), 30(33%), 26(28.5%), respectively. Percentages of resistance of isolates to the remaining antibiotics were as follows : tetracycline 57(62.6%), doxycycline and nitrofurantoin 54(59.3%) each, trimethoprim-sulfamethoxazole 51(56%) and chloramphenicol 36(39.6%).

Phenotypic detection of extended spectrum ß-Lactamases

Detection of ESBLs were performed by CHROMagar technique. All 17 (100%) carbapenem resistance *K. pneumoniae* isolates were ESBL producers. All these

isolates showed overnight growth with blue colonies on the ESBL supplemented CHROMagar orientation medium (Figure -2), which was interpreted as a phenotypic evidence of ESBL production.

PCR detection of *bla*$_{PER}$ gene

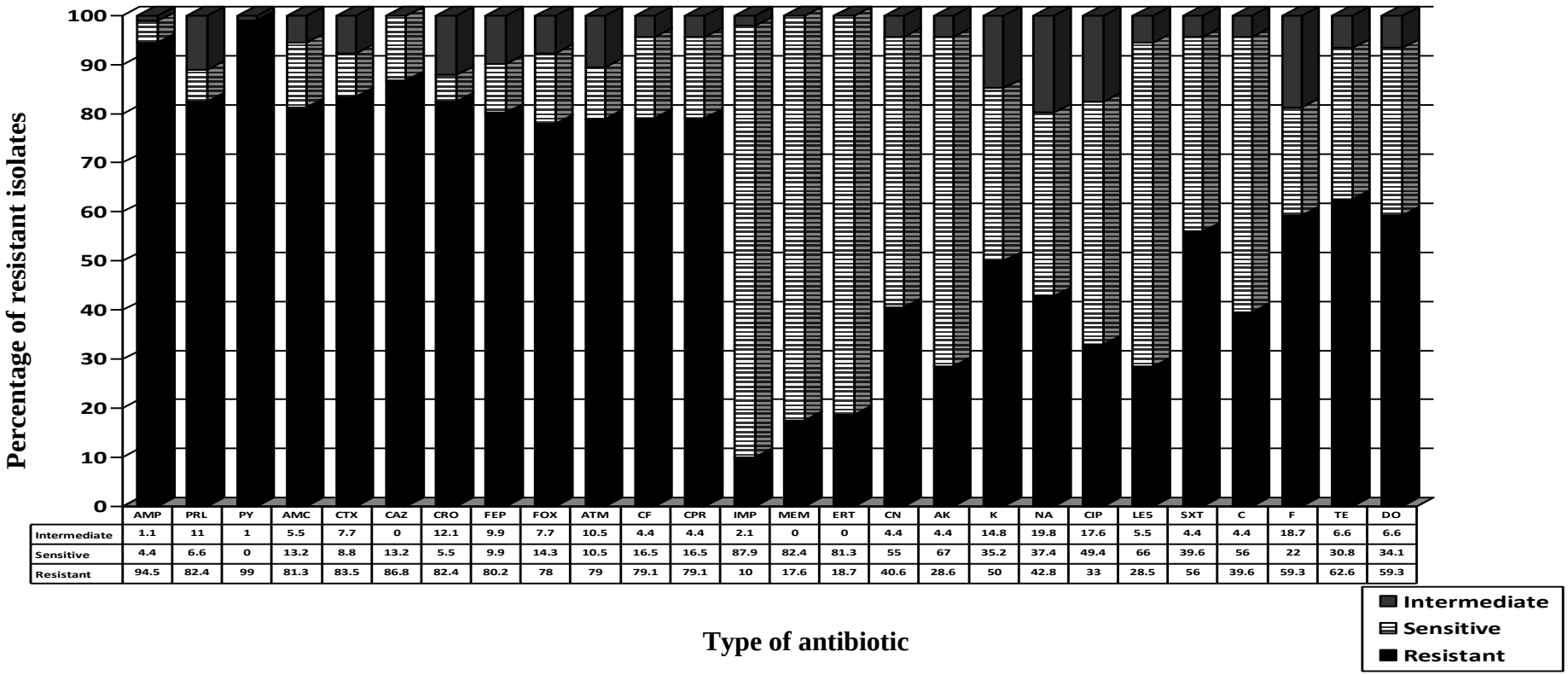
PCR was carried out on the DNA of 17 carbapenem resistance *K. pneumoniae* isolates for *bla*$_{PER}$, using specific primer for *bla*$_{PER}$ forward and *bla*$_{PER}$ reserve, PCR revealed amplification of 978 bp fragment corresponding to the *bla*$_{PER}$ gene among the majority of isolates 10(58.8%) (Fig.3).

Table (1): Number and percentage of *Klebsiella pneumoniae* isolates among different clinical samples.

Clinical sample	No. of samples	No. (%) of *K. pneumoniae* isolates
Stool	141	38 (27%)
Sputum	128	19 (15%)
Vagina	116	18 (15.5%)
Burn	153	18 (11.7%)
Urine	97	10 (10%)
Wound	60	8 (13.3%)
Blood	58	3 (5%)
Ear	30	2 (6.6%)
Eye	8	1 (12.5%)
Throat	10	0(0%)
Total	801	117(14.6%)

Table (2): 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%)



	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.

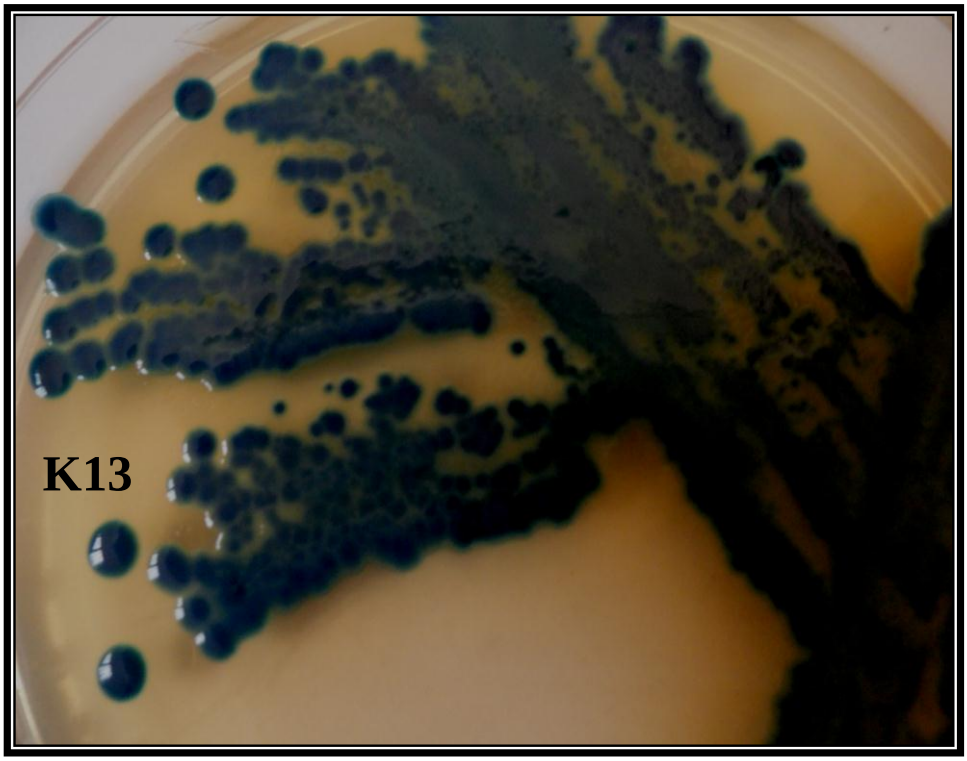
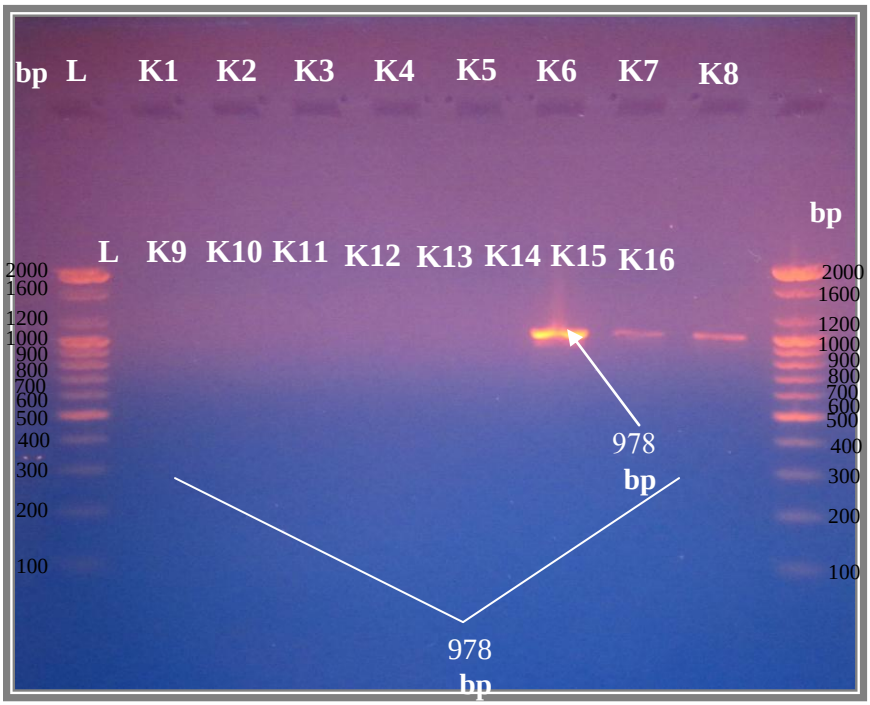


Figure (2): ESBL production by *K. pneumoniae* K13 test isolate exhibit deep blue colonies on the ESBL supplemented CHROMagar medium after incubation at 37C° for 24 hrs.



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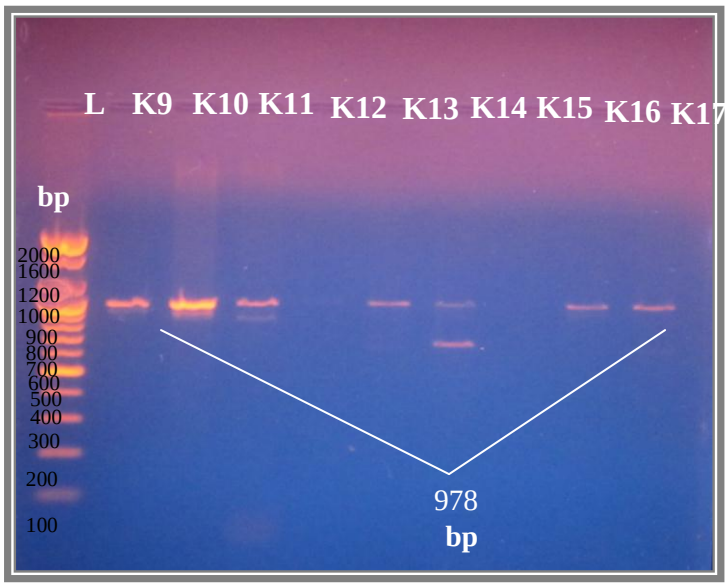

Figure (3): Agarose gel electrophoresis (1.5%agarose,70 % volt for 2-3 hrs) for *bla*$_{PER}$ gene product (ampilified size 978 bp) using DNA template of carbapenem-resistant *K. pneumoniae* isolates extracted by using salting out method. Lane (L), DNA molecular size marker (100-bp ladder). Lanes (K 6, 7, 8, 9, 10, 11, , 13, 14, 16 and 17)of *K. pneumoniae* isolates show positive results with *bla*$_{PER}$ gene. Lanes (K1, 2, 3, 4, 5, 12 and 15) show negative results with *bla*$_{PER}$ gene.

Discussion

Results from Table (1) showed that 117 (14.6%) isolates were identified as *K. pneumoniae* .This result is in agreement with a previous local study in Hilla by Al-Saedi who found that *K. pneumoniae* isolates comprised (15.3%) from 725 clinical samples [17]. Another study reported that the detection rate of *K. pneumoniae* was (14%) among all pathogens isolated from clinical samples in Najaf hospitals [18].

However, the majority of *K. pneumoniae* isolates 38/141(27%) were obtained from stool samples (Table-1). *K. pneumoniae* are Gram- negative bacteria which are part of the normal human intestinal flora and are frequently spread via fecal-oral contamination. High prevalence of *K. pneumoniae* in stool samples was demonstrated by other researchers , Al-Saedi in Hilla , (14%) , Ali *et al*. in Jordon, (20%) and Sarojamma and Ramakrishna in India, (50%) [17,19,20]. 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 (15.7%), and Al-Sehlawi ,(16%) [21,18].

Result from table (2) revealed that , 91/117 (78%) of *K. pneumoniae* isolates were resistant to ampicillin and amoxicillin. This result is in accordance with a previous study in Hilla by Al- Charrakh who stated that 73.8% *Klebsiella* isolates obtained from clinical samples were resistant to both ampicillin and amoxicillin [22]. In Najaf, one study found that 98.2% of *K. pneumoniae* were resistant to both antibiotics [21] . High percentage of resistant for these antibiotics could be attributed not only to the production of β - lactamases, but also other resistance mechanisms , Amyes ,mentioned that there are three further resistance mechanisms include conformational changes in PBPs, permeability changes in the outer membrane, and active efflux of the antibiotic [23].

Results from Figure (1) revealed that higher resistant rate was found for carbenicillin (99%) , ampicillin (94.5%) , piperacillin (82.4 %) . This result in agreement with a pervious study in Hilla by Al- Asady who found that all 15 (100%) β-lactam resistant *Enterobacteriaceae* isolates were resistant to ampicillin , piperacillin and carbencillin [24]. Al –Hilli stated that all *K. pneumoniae* isolates were resistant to penicillin (100%) and (81%)

to piperacillin [25] . High resistance to this class of antibiotics may be due to widespread use of these antibiotics in Hilla hospitals.

The resistance rate to imipenem was (10 %). 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%) [21,24,25,26]. Pathak *et al* demonstrated 2% resistance to imipenem by *K.pneumoniae* in a surveillance study in two hospitals in India [27]. Reasons behind resistance may be due to inappropriate duration of antibiotic therapy and subtherapeutic concentrations of the drug [28,29].

The present study revealed that the resistance against meropenem (17.6%) was more than imipenem . Meropenem is well –tolerated and offers several potential advantages , including greater *in vitro* activity against Gram –negative pathogens and the option of bolus administration [30] .Beside these , problem of renal metabolism of imipenem , and risk of seizures [31] , 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.

Regarding resistance to ertapenem , the resistance rate was (18.7%).Ertapenem is the least active carbapenem against most strains producing carbapenemase and therefore the first marker that indicates the likelihood of carbapenemase occurrence [32,33]. Specificity is questioned because enterabacteria with ESBL and porin mutations are also resistant to ertapenem [34].

Results also revealed high prevalence of ESBL producers among carbapenem -resistant *K. pneumoniae* isolates (100%).This may be due to chromogenic character of ESBL CHROMagar and its sensitivity and selectivity which enabled the recovery and presumptive identification of most ESBL- producing *Enterobacteriaceae* within 24hrs, while inhibiting the growth of other bacteria including those carrying AmpC β-lactamase type [35]. Thus ,this medium offers high sensitivity and high specificity combined with a short time to reporting the results , overall, it cost per single test. In Najaf , Al-Sehlawi reported that 37(47.4%) of the β-lactamase producing *K. pnenmoniae* isolates were confirmed as ESBL producers by this technique [18].

Among the 17 carbapenem –resistant *K.pneumoniae* , 10 (58.8%) isolates harbored a bla_{PER} gene as determined by PCR (Figure -3) , however this is the first report done with the detection of PER β - lactamase in Hilla . Other studies failed to detect bla_{PER} among clinical isolates of *K.pneumoniae* , in Turkey ,China and in Colmbia [36,37,38] , whereas in Korea, one study reported the prevalence of bla_{PER} in blood samples from patient with pneumonia [39].

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