Prevalence of VIM Metallo ß –Lactamase among Clinical Isolates of *Klebsiella pneumoniae* in Hilla Hospitals

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Abstract

This study investigated the presence of *bla*$_{VIM}$ 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 diagnosed 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%) 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. Phenotypic detection of metallo β-lactamase by imipenem-EDTA disk identified a proportion of 65% as metallo β-lactamase producers. The presence of *bla*$_{VIM}$ gene was checked by Polymerase Chain reaction (PCR) and confirmed in 14(82.3%) of isolates.

Keywords: *Klebsiella pneumoniae* ,VIM metallo β-lactamase, Polymerase Chain Reaction.

الخلاصة

تهدف الدراسة الحالية الى التحري عن موروث البيتالاكتام نوع VIM بين عزلات بكتيريا الكليبسيلا الرئوية.تم الحصول على ١١٧ عزلة بكتيرية تابعة للنوع *K. pneumoniae* من مجموع ٨٠١ عينة سريريه ومن مختلف مستشفيات مدينة الحلة وللفترة من بداية نيسان إلى نهاية آب ٢٠١١.أظهرت النتائج إن أكثر عزلات *K. pneumoniae* تم عزلها من عينات البراز ٣٨ (٢٧%) تلتها عينات القشع١٩ (١٥%) . وقد اظهر المسح الأولي لحساسية العزلات لمضادات البيتالاكتام ان 91 عزلة (٧٨%) كانت مقاومة لمضادي الامبسيلين والاموكسيسلين.

اختبرت حساسية العزلات المقاومة لمضادات البيتالاكتام للمضادات الحيوية بطريقة انتشار القرص(كيربي– باور) وتبين إن أعلى نسبة للمقاومة كانت لمضادات البنسلين،(٩٩%) لمضاد الكاربنسلين و(٩٤.٥%) لمضاد الامبسلين. .فيما أبدت ١٧(١٨.٧%) عزلة من بكتيريا *K. pneumoniae*مقاومة لمضادات الكاربابنيم وقد تم اختبار قابلية هذه العزلات على انتاج انزيمات البيتالاكتام المعدنية مظهريا بطريقة قرص ال EDTA مع الاميبنيم وتبين إن ٦٥% من العزلات كانت منتجة لهذه الانزيمات .تم التحري عن مورث *bla*$_{VIM}$ بتقنية سلسلة تفاعلات البلمرة (Polymerase Chain Reaction) وكشفت النتائج إن ١٤ (٨٢.٣%) من العزلات كانت حاملة للمورث*bla*$_{VIM}$.

Introduction

Although a ubiquitous pathogen ,yet capable of causing both community and healthcare-associated infections that range from mild urinary tract infection to sever bacteremia and pneumonia, *Klebsiella pneumoniae* has a remarkable ability to up regulate or acquire resistance determinants, making it one of the most feared organisms threatening the current antibiotic era [1,2,3].It has recently emerged as a major

cause of hospital- acquired infections because of its propensity to accumulate mechanisms of antimicrobial resistance leading to pandrug resistance , and causing outbreaks that often involve multiple facilities in hospitals [4,5].

Antimicrobial resistance is a growing threat worldwide. The foundation of modern medicine is built on the availability of effective antibiotics, especially in economically deprived areas of the world where the disease burden due to bacterial infections remain high .Antibiotic resistance is predominantly fueled by antibiotic use[6].With their broad spectrum activity for Gram-positives , Gram-negatives and anaerobic bacteria ,carbapenems are frequently used as a last line of therapy [7,8].

Metallo β- lactamase are resistant to well- known β- lactamase inhibitors like clavulanic acid , sulbactam, and tazobactam and confer resistance to all β- lactam antibiotics except monobactam [9].Of greater importance are the acquired or transferable families of MBLs which include IMP (active on imipenem), VIM (Verona integron – encoded metallo β- lactamase) , GIM (German imipenemase), SIM (Seoul imipenemase), SPM (Sao Paulo MBL) and NDM (New Delhei Metallo β- lactamase) which are located within gene cassettes as a part of integron structures[9,10,11,12]. These integron structures may then associate with transposons and plasmids which then can be easily transferred between bacteria [13].
VIM class 1 integron associated MBL was first observed in a *P. aeruginosa* isolate in Verona , Italy in 1997 [14].It is closely related to BCII from *Bacillus cereus* sharing 39% amino acid identity [15].These types are mainly borne on integrons on plasmid, they are the second most dominant group of transferable MBLs. VIM enzymes are resistant to a number of beta lactams like piperacillin, cefazidime, imipenem and aztreonam. These enzymes are dependent on metal ions, which is indicated by loss of activity on addition of EDTA and restoration up on addition of Zn^{++} [12].

The current study aimed to determine carbapenem –resistance isolates of *K. pneumoniae* isolated from various clinical specimens in Hilla city and to detect bla_{VIM} 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 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* and MacFaddin [16,17],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 [18].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 [19]. 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 [20]. *E. coli* ATCC 25922 was used as the standard strain for antibiotic susceptibility testing.

Phenotypic detection of Metallo β-lactamase

Imipenem – EDTA Double Disk Synergy Test

Metallo β-lactamases detection was performed by double disk synergy method according to lee *et al* .[21].

Genotypic detection of *bla*$_{VIM}$ 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 [22].

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

Polymerase chain reaction was used to amplify the entire sequence of *bla*$_{VIM}$ gene. The primer (Bioneer) used for the amplification of this gene was : VIM/F (5`-AGTGGTGAGTATCCGACAG-3`) and VIM/R (5`-ATGAAAGTGCGTGGAGAC-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*$_{VIM}$ were as follows: an initial denaturation at 94 C˚ for 5 min, followed by 30 cycles of denaturation at 94 C˚ for 25 sec, anneling at 52 C˚ for 40 sec, extension at 72 C˚ for 50 sec. and a final extension step of 72 at 6 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(27%), 19(15%),18(15.5%), 18(11.7%), 10(10%) ,8 (13.3%), 3(5%) ,2(6.6%) and 1(12.5%)were recovered from stool ,sputum, vagina , burn , urine, wound, blood, ear and eye samples , respectively. (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 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. Isolates showed high resistance to ceftazidime 79(86.8%) ,

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%), trimethoprim-sulfamethoxazole 51(56%) and chloramphenicol 36(39.6%).

Phenotypic detection of metallo β-lactamase

Results of the present study revealed that out of 17 carbapenem – resistant isolates, 11 (65%) showed an increase in the zone diameter around the IMP – EDTA disk, suggesting the production of metallo β-lactamases (Fig -2 **)**

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

PCR was carried out on the DNA of 17 carbapenem resistance *K. pneumoniae* isolates for *bla*$_{VIM}$, using specific primer for *bla*$_{VIM}$ forward and *bla*$_{VIM}$ reverse, PCR revealed amplification of 261 bp fragment corresponding to the *bla*$_{VIM}$ gene among the majority of isolates 14 (82.3%) (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%)

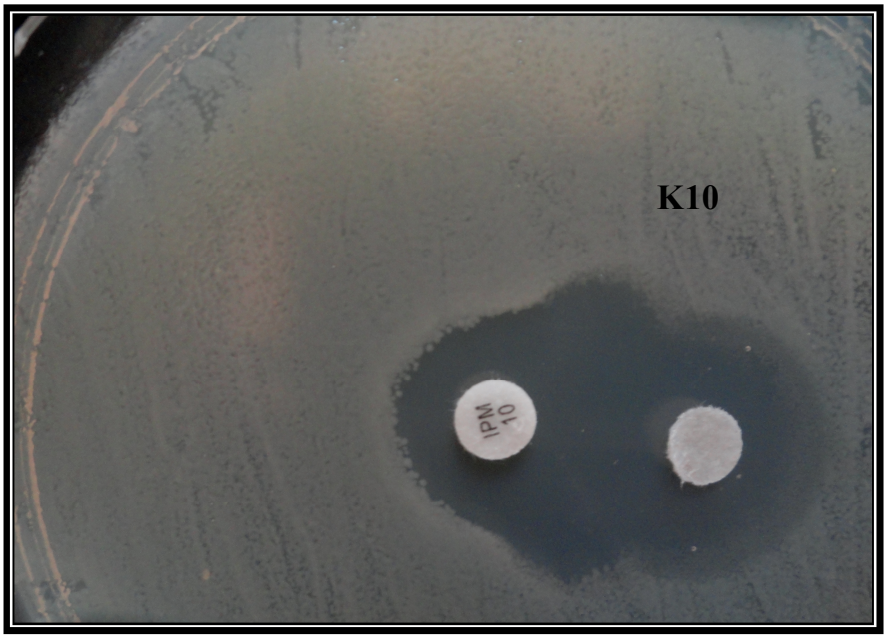
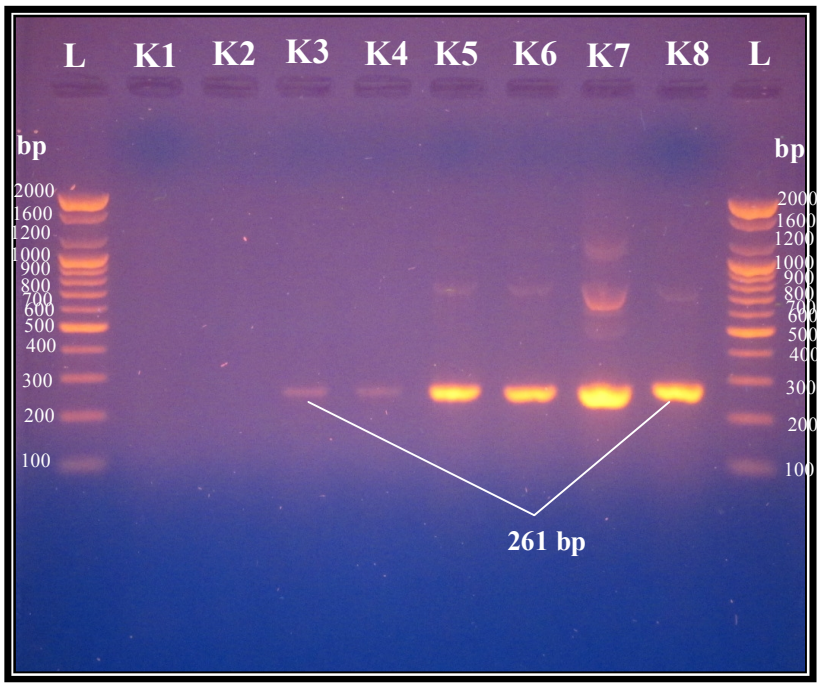


Figure (2): Phenotypic appearance of MBL – producing *K. pneumoniae* by imipenem – EDTA double disk synergy test. K10 test isolate showing large synergistic inhibition zone between IMP disk and EDTA disk (positive result).



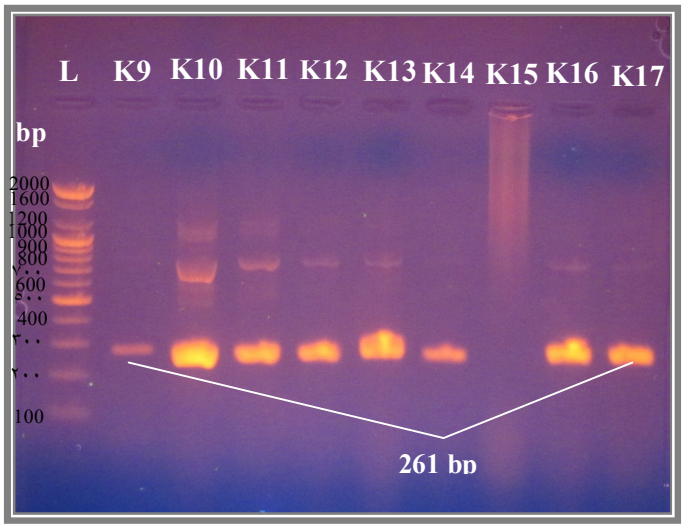


Figure (3): Agarose gel electrophoresis (1.5%agarose,70 % volt for 2-3 hrs) for *bla*$_{VIM}$ gene product (ampilified size 261 bp) using DNA template of carbapenem-resistant *K. pneumoniae* isolates extracted by using salting out method. Lane (L), DNA moleculer size marker (100- bp Ladder).Lanes (K3, 4, 5, 6, 7, 8, 9,10,11,12,13,14, ,16 and 17) of *K. pneumoniae* isolates show positive results with *bla*$_{VIM}$ gene , lanes (K1,2and 15) show negative results with *bla*$_{VIM}$ 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 (2000) who found that *K. pneumoniae* isolates comprised (15.3%) from 725 clinical samples [23]. In another study, Al-Sehlawi reported that the detection rate of *K. pneumoniae* was (14%) among all pathogens isolated from clinical samples in Najaf hospitals [24].

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%) [23 ,25,26]. 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%) [24,27].

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 [28]. In Najaf, Al- Muhannak found that 98.2% of *K. pneumoniae* were resistant to both antibiotics [27] . 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 pencillin binding proteins (PBPs), permeability changes in the outer membrane, and active efflux of the antibiotic [29].

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 (2009) who found that all 15 (100%) β-lactam resistant *Enterobacteriaceae* isolates were resistant to ampicillin , piperacillin and carbencillin [30]. Al –Hilli stated that all *K.pneumoniae* isolates were resistant to carbenicillin (100%) and (81%) to piperacillin [31]. 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%) [27, 30,,31,32] . Pathak *et al* demonstrated 2% resistance to imipenem by *K.pneumoniae* in a surveillance study in two hospitals In India [33]. Reasons behind resistance may be due to inappropriate duration of antibiotic therapy and subtherapeutic concentrations of the drug [34,35].

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 [36].Beside these , problem of renal metabolism of imipenem , and risk of seizures [37],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 [38,39].Specificity is questioned because enterabacteria with ESBL and porin mutations are also resistant to ertapenem [40].

All carbapenem –resistant isolates were screened by phenotypic test for carbapenemase production. The present study showed that 11(65%) of the isolates gave positive results by imipenem- EDTA disk test . Different studies which have used the IMP-EDTA to detect MBL production in *K.pneumoniae* reported a very wide range of prevalence varying from 2.97% to 50% [24,41]. However, there are six isolates which gave negative results with EDTA disk synergy test. This means that 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 produce other enzymes (Like IMI,GES and KPC) that were not inhibited by EDTA. Moreover , they may have other mechanisms of carbapenem resistance like hyperproduction of AmpC β-lactamases associated with a loss of outer membrane proteins , efflux pumps, and mutations that alter the expression and /or function of porins and PBPs [42,43].

Results revealed that among 17 carbapenem –resistant *K. pneumoniae* isolates , 14(82.3%) had a bla_{VIM} gene (Figure-3) . One study preformed in India, Shahid *et al.* found that among 16 carbapenem- resistant *E.coli* and *K.pneumoniae* , bla_{VIM} was detected in only 3(18.6%) isolates, all were *K.pneumoniae* [44].In another study in Greece, Giakkoupi *et al*. stated that all *K.pneumoniae* isolates carried bla_{VIM} that also harbored KPC carbapenemase [45]. Birgy *et al*. reported the first case of imported health care - associated fecal carriage of an Egyptian infant VIM-1-producing *K.pneumoniae* strain in France [46]. The bla_{VIM} – genes are located in class 1 integrons as a gene cassttes and have been identified on plasmid with different replicon types [47], increasing the possibility of dissemination and linkage to other antibiotic resistance genes. However, this constitutes the first report on prevalence and detection of bla_{VIM} in Hilla hospitals.

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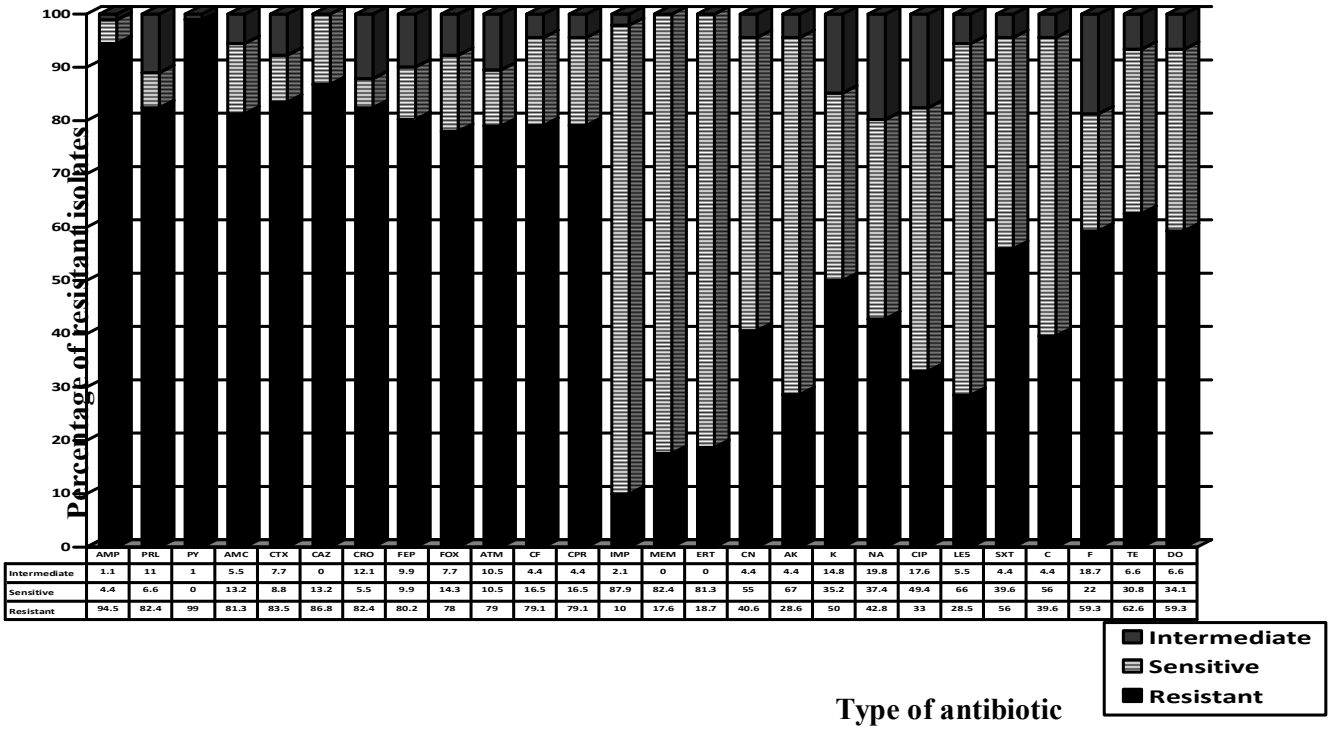

	AMP	PRL	PY	AMC	CTX	CAZ	CRO	FEP	FOX	ATM	CF	CPR	IMP	MEM	ERT	CN	AK	K	NA	CIP	LES	SXT	C	F	TE	DO
Intermediate	1.1	11	1	5.5	7.7	0	12.1	9.9	7.7	10.5	4.4	4.4	2.1	0	0	4.4	4.4	14.8	19.8	17.6	5.5	4.4	4.4	18.7	6.6	6.6
Sensitive	4.4	6.6	0	13.2	8.8	13.2	5.5	9.9	14.3	10.5	16.5	16.5	87.9	82.4	81.3	55	67	35.2	37.4	49.4	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.