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First identification of NDM-1 Metallo ß –Lactamase among clinical isolates of *Klebsiella pneumonia* isolates in Hilla hospitals, Iraq

Fatima Moeen Abbas[1]* and Eman Mohammad Jarallah[2]
[1]College of science for women, Babylon university and [2]College of science, Babylon university, Iraq.
***Corresponding author: Fatima.abas99@yahoo.com**
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Abstract

This study analyzed the prevalence of bla_{NDM-1} 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 toward 26 antibiotics by Kirby-Bauer disk diffusion methods .Higher 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%) *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_{NDM-1} gene was checked by Polymerase Chain reaction (PCR) and confirmed in 3(17.6%) of isolates.

Keywords: *Klebsiella pneumonia*, NDM-1 Metallo ß –Lactamase, Clinical isolates, PCR.

Introduction

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 (Goossens *et al*., 2005) .With their broad spectrum activity for Gram-positives , Gram-negatives and anaerobic bacteria ,carbapenems are frequently used as a last line of therapy (Ejikeuqwu *et al*., 2012 and Kosmidis *et al*., 2012).

Carbapenems have the broadest spectrum of all β-lactam antibiotics and are increasingly used to treat infections caused by otherwise multidrug –resistant Gram-negative bacteria. Consequently, emerging resistance to carbapenems is the major public health concern ,especially when it involves acquired ,horizontally transmissible carbapenemases (Queenan and Bush, 2007). Carbapenem -resistance isolates are difficult to control because they spread easily within and between hospitals (Kochar *et al*.,2009),and treatment options for infections caused by these isolates are limited and are associated with mortality rates upwards of 50% (Patel *et al*.,2008 and Ben-David *et al*., 2012).

Metallo β- lactamase (MBLs) are resistant to well-known β - lactamase inhibitors like clavulanic acid, sulbactam, and tazobactam and confer resistance to all β - lactam antibiotics except monobactam (Mirsa, 2012). 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 (Walsh *et al*.,2005;Queenan and Bush, 2007; Dugal and Fernandes, 2011 and Mirsa, 2012). These integron structures may then associate with transposons and plasmids which then can be easily transferred between bacteria (Tailor, 2011).

The NDM-1 is a new molecular class B enzymes that were recently recognized from *K.pneumoniae* isolate from a patient in Sweden who seems to have

imported from India. Particularly NDM-1 is endemic to India but due to international travel ,its emerging as an important clinical threat worldwide (Patel *et al*., 2011). NDM-1 expressed high level of resistance to all β- lactam antibiotics but remained susceptible only to colistin and tigecycline. It shares very little identity with other metallo β-lactamases, maximum identity has been observed to VIM-1 /VIM-2 (32.4%). Compared to VIM-2, NDM-1 displayed tight binding to most cephalosporins and , in particular to cefuraxime cefotaxime, and cephalothin and also to the penicillins (Shakil *et al*., 2011).However, it does not bind to carbapenem as tightly as IMP-1 or VIM-2 (Yong *et al*., 2009).

This study aimed to determine carbapenem – resistance isolates of *K. pneumoniae* isolated from various clinical specimens in Hilla city and to detect bla_{NDM-1} 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, Iraq 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*.(1994),Baron and Finegold (1994),Collee *et al.* (1996) 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 antibiotic 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 (100µg), 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 (30µg), Ciprofloxacin (5µg); Levofloxacin (5µg); Trimethoprim- Sulfamethoxazole (25µg) Nitrofurantion (30µg), Chloramphenicol (30µg), Tetracycline (30µg) and Doxycycline (30µg). The cultures were incubated at 37 C° 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 reference 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*.(2003). A 10 µg imipenem disk was placed in the center of a Mueller- Hinton agar plate inoculated with a 0.5 McFarland's tube dilution of the test isolate. An EDTA disk (1,900 µg) was placed at a distance of 15mm center to center from the imipenem disk. The plate was incubated at 37 C° overnight. The zone around the imipenem disk would be extended on the side is a metallo-β-lactamase producer.

Genotypic detection of bla_{NDM-1} 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_{NDM-1} gene: Polymerase chain reaction was used to amplify the entire sequence of bla_{NDM-1} gene. The primer (Bioneer) used for the amplification of this gene was : NDM-1/F (5`-F: GGTTTGGCGATCTGGTTTTC -3`) and NDM-1 / R (5`-CGGAATGGCTCATCACGATC-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_{NDM-1} were as follows: an initial denaturation at 94 C˚ for 1 min, followed by 30 cycles of denaturation at 94 C˚ for 1 min, anneling at 55 C˚ for 1 min, extension at 72 C˚ for 2 min. and a final extension step of 72 at 10 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 and Discussion

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 amoxicilli

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 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).

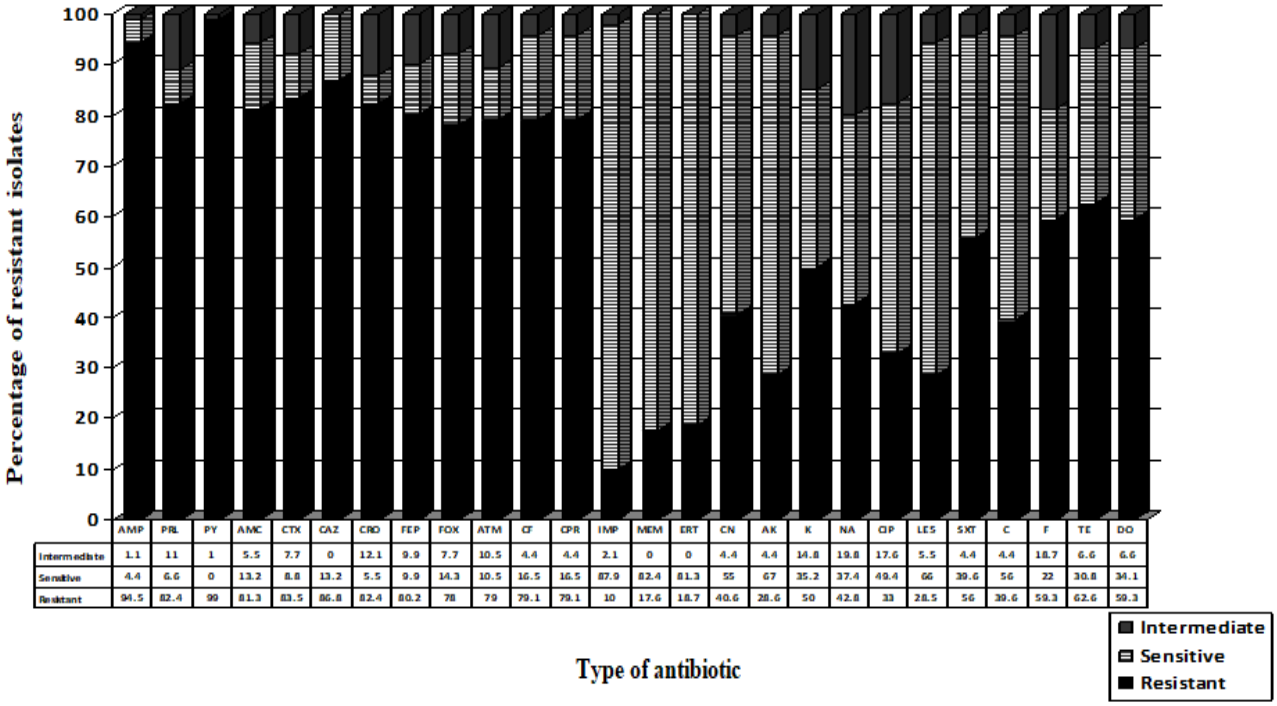
PCR detection of bla_{NDM-1} gene: PCR was carried out on the DNA of 17 carbapenem resistance *K. pneumoniae* isolates for bla_{NDM-1}, using specific primer for bla_{NDM-1} forward and bla_{NDM-1} reserve, PCR revealed amplification of 621 bp fragment corresponding to the bla_{NDM-1} gene among 3(17.6 %) of the isolates (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	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.

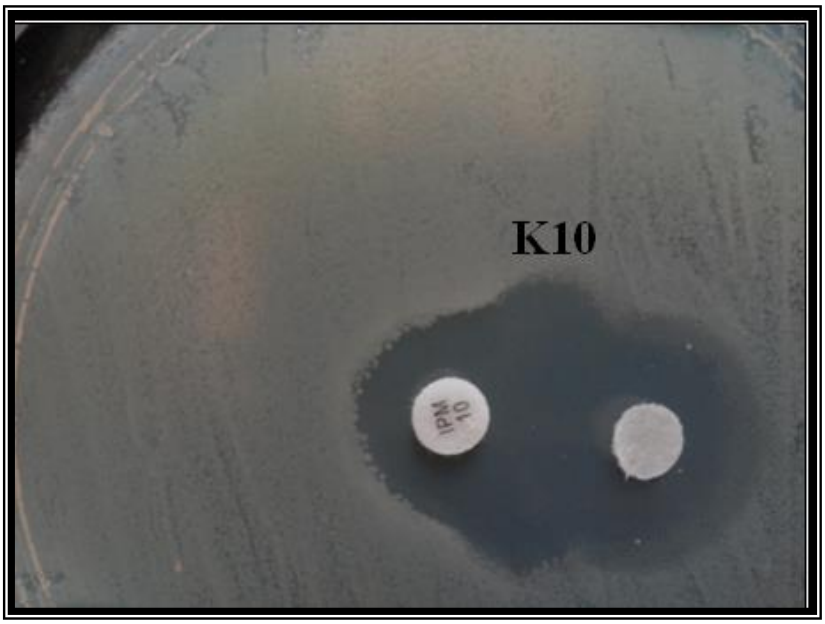


Figure (2): Phenotypic appearance of MBL – producing *K. pneumoniae* by imipenem – EDTA double disk synergy test. K10 test isolate showing large synergistic inhibition zone between IMP disk and EDTA disk (positive result).

K1 K2 K3 K4 K5 K6 K7 K8 K9 K10 K11 K12 K13 K14 K15 K16 K17 L

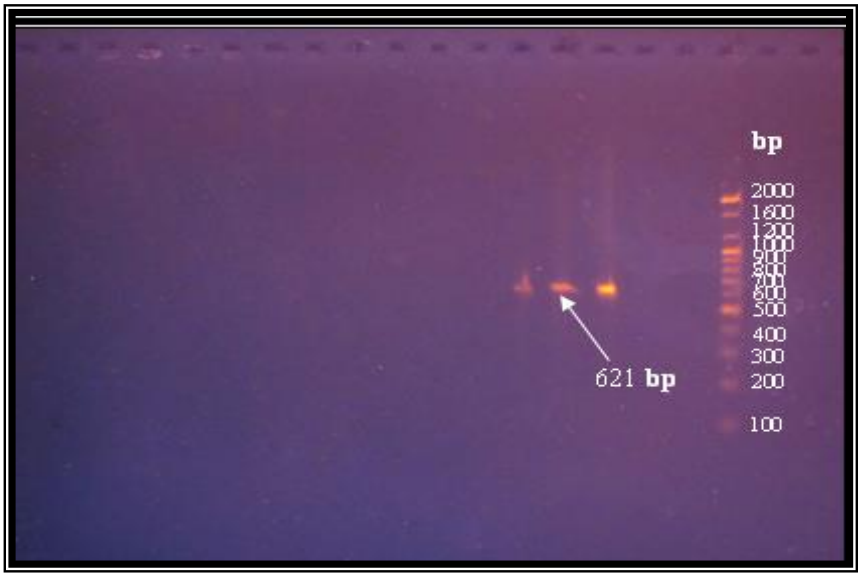


Figure (3): Agarose gel electrophoresis (1.5%agarose,70 % volt for 2-3 hrs) for *bla*$_{NDM-1}$ gene product (ampilified size 621 bp) using DNA template of carbapenem-resistant *K. pneumoniae* isolates extracted by using salting out method. Lane (L), DNA moleculer size marker (100-bp Ladder).Lanes(K13,14 and 15) of *K. pneumoniae* isolates show positive results with *bla*$_{NDM-1}$ gene , lanes (K1,2,3, 4, 5, 6, 7, 8, 9,10,11,12, 16 and 17) show negative results with *bla*$_{NDM-1}$ gene.

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 . In another study, Al-Sehlawi (2012) reported that the detection rate of *K. pneumoniae* was (14%) among all pathogens isolated from clinical samples in Najaf hospitals.

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 (2000) in Hilla, (14%), Ali *et al*. (2010) in Jordon, (20%) and Sarojamma and Ramakrishna (2011) in India, (50%). 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%).

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 (2005) who stated that 73.8% *Klebsiella* isolates obtained from clinical samples were resistant to both ampicillin and amoxicillin. In Najaf, Al- Muhannak (2010) found that 98.2% of *K. pneumoniae* were resistant to both antibiotics. High percentage of resistant for these antibiotics could be attributed not only to the production of β- lactamases, but also other resistance mechanisms , Amyes (2003) 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.

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. Al –Hilli (2010) stated that all *K.pneumoniae* isolates were resistant to carbenicillin (100%) and (81%) to piperacillin. 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%) (Hadi,2008; Al-Asady, 2009; Al- Muhannak, 2010 and Al-Hilli, 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 (Baquero *et al*.,1997 and Philippe *et al*.,1999) .

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 (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.

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 (Overturf , 2010 and Thomson , 2010). Specificity is questioned because enterabacteria with ESBL and porin mutations are also resistant to ertapenem (Flonta *et al.*, 2011)

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% (Deshmukh *et al*., 2011 and Al-Sehlawi, 2012). 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 (Cao *et al*., 2000 and Papp-Wallace *et al*., 2011) .

In this study, the overall prevalence of bla_{NDM-1} possessing *K.pneumoniae* was found to be 3 (17.6%) (Figure 3). In one study, Bora and Ahmed (2012) observed that all *K.pneumoniae* with reduced susceptibility to carbapenem carried bla_{NDM-1} gene. In another study ,among 22 NDM-1 producing *Enterobacteriaceae* ,10 *Klebsiella* spp. were found to be positive for NDM-1 at a tertiary care unit in Mumbi (Deshpande *et al*., 2010). This is the first report on prevalence of NDM-1 in Hilla hospitals.

NDM-1 is carried on plasmid or on chromosomes (Chen *et al*., 2012). The rapid emergence of NDM-1 has been related to a moveable plasmid which can be transferred from one bacteria to another, from man to man and even from country to country (Fallah *et al*., 2011). In recent years, many Iraqi patients were travelled to India and to other countries for medical care purpose which may helped in acquiring this gene.

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