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ORIGINAL ARTICLE

# A comparative Study between Different Laboratory Tests for Detection of Carbapenem Resistance in *Klebsiella Pneumoniae*

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***Key words:***

***Klebsiella pneumonia, carbapenemase KPC, Modified Hodge test , blaPC gene***

## ABSTRACT

***Background****: Klebsiella pneumoniae is among the most common gram-negative bacteria encountered by physicians worldwide. It is a common hospital-acquired pathogen, causing urinary tract infections, nosocomial pneumonia, and intra abdominal infections. K. pneumoniae is also a potential community-acquired pathogen.* ***Objectives****: were to determine carbapenem resistance among Klebsiella pneumonia isolates by routine disc diffusion test and E test, determine carbapenemase production by modified Hodge test and to study the presence of bla KPC gene in carbapenem-resistant and carbapenem sensitive Klebsiella pneumoniae isolates.* ***Methodology****: Fifty klebsiella pnumoniae isolates were identified and subjected to antimicrobial susceptibility testing by disk diffusion test against three carbapenem antibiotics (Imipenem, Ertapenem, and Meropenem) and were subjected to determination of minimum inhibitory concentration (MIC) to ertapenem using E-test. Modified Hodge test was carried out for strains included in the study. Twenty-six K. pneumoniae isolates were subjected to PCR for detection of $bla_{kpc}$gene. Thirteen confirmed carbapenem resistant isolates by disc diffusion, E-test and MHT and 13 confirmed carbapenem sensitive isolates by the same tests.* ***Results:*** *13/50 (26%) isolates were resistant to both meropenem and ertapenem while only 11/50(22%) isolates were resistant to imipenem. Ertapenem sensitivity tests revealed that there was a discrepancy between the results of E test and disc diffusion method in two strains. The two strains were reported resistant by disc diffusion method and intermediate sensitive by E test. All carbapenem resistant strains 13 out of 50 strains showed positive modified Hodge test. The 26 isolates tested for $bla_{kpc}$gene were negative.* ***In conclusion*** *resistance of K.pneumoniae to carbapenems was detected in 26% of isolates . The disc diffusion susceptibility did not report major errors in comparison to E test for ertapenem. Expression of carbapenemase was detected in all carbapenem resistant isolates by modified Hodge test while bla KPC gene was not detected among either carbapenem resistant or susceptible strains.*

## INTRODUCTION

*Klebsiella pneumoniae* is now recognized as an urgent threat to human health because of the emergence of multidrug-resistant strains associated with hospital outbreaks and hyper virulent strains associated with severe community-acquired infections[1]**.** In healthcare settings, *Klebsiella pneumoniae* infections commonly occur among sick patients who are receiving treatment for other conditions. Patients whose care requires devices like ventilators (breathing machines) or intravenous (vein) catheters, and patients who are taking long courses of certain antibiotics are most at risk for *Klebsiella* infections [2]

*Klebsiella* bacteria have developed antimicrobial resistance, to the class of antibiotics known as carbapenems[3]**.** One report cites that carbapenem resistant strains can contribute to death in up to 50% of patients who become infected[1]**.** Treatment of these infections is particularly worrisome as the carbapenems are often agents of the last resort for resistant Gram-negative infections. The optimal treatment of such infections is not well established and clinical outcome data remain sparse. Thus, in order to limit the spread of these serious KPC-producing pathogens, rapid detection, followed by implementation of adequate infection control methods, is essential[4].

Two mechanisms may be responsible for acquired carbapenem resistance in Gram-negative bacteria including *Klebsiella*: (i) reduced outer membrane permeability by porin; and (ii) production of beta-lactamases capable of hydrolyzing carbapenems (carbapenemases**)** [5]**.**

Among these carbapenemases is *Klebsiella pneumoniae* carbapenemase (KPC; Ambler class A) which are prevailing in large geographic regions [6]

*Klebsiella pneumoniae* carbapenemasesis a plasmid-mediated carbapenem- hydrolyzing β-lactamase enzyme produced by certain strains of enteric

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bacilli (e.g., *Klebsiella sp., E. coli, Enterobacter sp.*) allowing for increased resistance to the carbapenem and cephamycin groups of antimicrobial agents, in addition to the extended-spectrum cephalosporins [7].

The acquisition of $bla_{KPC}$ by different bacterial species may be attributed to its location on transferable plasmids or to dissemination by mobile genetic elements [8].

Laboratory identification of KPC-producing clinical isolates will be important for limiting the spread of this resistant strain [9]. Detection of KPC based strictly on susceptibility testing is challenging due to heterogeneous expression of ß-lactam resistance [10]. A PCR assay, offers the advantage of faster genotyping and a shorter turnaround time (4 hours for the PCR, compared with >24 hours for the MHT) and potentially a higher sensitivity than that offered by culture. Because these assays are specific for the particular target sequence, they cannot be used to monitor the emergence of novel variants. Furthermore, the presence of the target *bla*KPC genes does not always correlate with the phenotypic expression of KPC, nor is it able to quantify the level of expression. These assays are not able to detect mechanisms of carbapenem resistance apart from KPC [11].

The aim of this study was to determine carbapenem resistance among *Klebsiella pneumonia* isolates by routine disc diffusion test and E test, to determine carbapenemase production by modified Hodge test and to detect the presence of *bla KPC* gene in carbapenem-resistant and carbapenem sensitive *Klebsiella pneumoniae* isolates.

## METHODOLOGY

This study included 50 *Klebsiella pneumoniae* isolates obtained from routine samples in the laboratory of National institute of diabetes and endocrinology during the period from September 2012 to June 2013. Isolates were identified according to Collee *et al.* [12] and Cheesbrough [13]. Conventional identification methods based on colony morphology, microscopic examination of Gram stained films and biological activity of the isolated strains were done.

**1- Detection of carbapenem resistance in *K.pneumoniae* strains by disk diffusion**

All *K.pneumoniae* isolates were tested for carbapenem resistance by disk diffusion method, by commercially prepared meropenem (MEM), imipenem (IPM) and ertapenem (ETP) disks (Oxoid). Susceptibility testing and results were interpreted according to the recommendations of the CLSI, 2012[14] as shown in table 1.

**Table 1: Zone diameter interpretive standard for the tested antibiotics**

| | Disk Diffusion (mm) | | |
|---|---|---|---|
| | **Susceptible** | **Intermediate** | **Resistant** |
| **Ertapenem** | >22 | 19-21 | <18 |
| **Imepenem** | >23 | 20-22 | <19 |
| **Meropenem** | >23 | 20-22 | <19 |

**Detection of carbapenem resistance in *K.pneumoniae* strains by E test**

The 50 isolates were subjected to determination of minimum inhibitory concentration (MIC) to ertapenem using E-test. MIC value would be the value at which the zone convenes the strips. If there was no zone of inhibition, the MIC was reported as higher than the highest concentration of the strip.

If the zone of inhibition was less than the lowest concentration, MIC was reported as lower than the lowest concentration. The values of the MIC of ertapenem strips were interpreted according to CLSI, 2012 [14]as shown in table 2

**Table 2: MIC values of ertapenem**

| | MIC (µg/ml) | | |
|---|---|---|---|
| | **Susceptible** | **Intermediate** | **Resistant** |
| **Ertapenem** | **<0.5** | **1** | **>2** |

**2- Modified Hodge test** for detection of carbapenemase was done for all 50 isolates, A quality control strain *E.coli* ATCC 25922 was obtained from Naval Medical Research Unit (NAMRU) and used for quality control of Mueller Hinton agar. This test was performed as recommended by the Clinical and Laboratory Standards Institute (CLSI) 2012 guidelines [14]. A 0.5 Mc Ferland turbidity standard suspension of previously isolated carbapenem sensitive *Escherichia coli* in sterile saline was prepared and was diluted 1:10 in sterile saline. This was inoculated on a Mueller Hinton agar plate, as for the routine disk diffusion testing. The plate was dried for 5 minutes and a disk of ertapenem (10 g) was placed in the center of the agar plate. Using a swab 3-5 colonies of the test organism were picked up and inoculated in a straight line, from the edge of the disk, up to a distance of at least 20mm from the edge of the plate. Quality control strains were used with each plate (MHT Positive *Klebsiella pneumoniae* ATCC1705 and MHT Negative *Klebsiella pneumoniae* ATCC1706). The plate was incubated overnight at 35±2°C in ambient air for 16–24 hours, and they were examined next day. They were checked for growth of Carbapenem sensitive *E coli* around the disk of ertapenem. The presence of an enhanced growth of *E. coli* indicated Carbapenemase production by the tested organism that inactivated ertapenem.

Distorted or cloverleaf shaped inhibition zone which was interpreted as positive for carbapenemase producing isolates [14] (Figure 1).

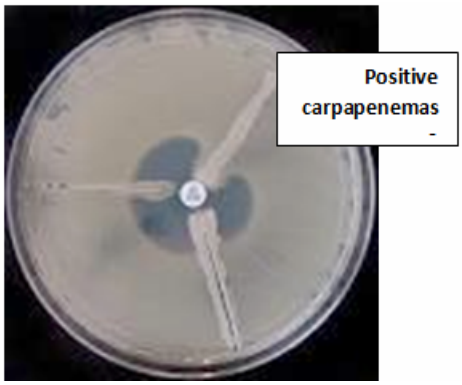


**Fig. 1: Modified Hodge test**

**3- PCR for KPC gene detection**: Twenty-six *k.pneumoniae* isolates were subjected to PCR for detection of $bla_{kpc}$gene. Thirteen confirmed carbapenem resistant isolates by disc diffusion, E-test and MHT and 13 confirmed carbapenem sensitive isolates by the same tests

***A- Extraction of DNA***

Isolated colonies were dissolved in 1.5 ml micro tube, which contain 200µL-distilled water then centrifuged at 15000 xg for 1 min to harvest the deposited cells.

The pellet was re suspended in 300 µL of cell lysis solution, then 1.5 µL of RNase A solution was added to the tube and mixed by inversion.

The mixture was incubated at 37 ºC for 20 min and then cooled on ice for 1 min. One hundred µl of protein precipitation solution were added and vortexed vigorously for 20-30 sec. This was followed by centrifugation at 15.000 xg for 5 min.

The supernatant was transferred to a clean 1.5 ml micro tube containing 300 µl isopropanol > 99%. Centrifugation at 15.000 xg for 1 min was performed (DNA was visible as a small white pellet). The supernatant was discarded by inversion of the tube in fluid waste container and the tubes were briefly drained on clean absorbent paper. 500µl ethanol 80% were added and the tubes were inverted several times to wash the DNA pellet.

The ethanol was carefully discarded by inversion of the tube in fluid waste container .100µl of DNA hydration solution were added to the dried DNA pellet and incubated at 65ºC in dry incubator for 60 min. The DNA products were stored at -20ºC.

*B- DNA amplification: Primers used in the study was as follows*

| Forward primer (5'-3') | Reverse primer (3'-5') |
|---|---|
| ATGTCACTGTATCGCCGTCT | TTTTCAGAGCCTTACTGCCC |

Dilution of each primer was done by adding 10 microns of each primer to90 microns of nuclease free water. Then thin walled PCR tubes were placed at cold bath at -4 and the following components were added to each tube to reach a final volume 25 microns; Dream taq green PCR master mix 12.5 microns, 1.5 microns of each primer, 6 microns of template DNA, 3.5 nuclease free water. The samples were vortexed. The thermocycler was programmed for PCR amplification as follows:

Initial activation (Hot-Start): 15 min at 95ºC. 38 cycles of amplification consisting of: denaturation at 94ºC for 1 min. annealing at 60ºC for 1 min.Extension at 72ºC for 1 min. Final extension at 72ºC for 10 min [15].

Positive (ATCC-1705) and negative (ATCC-1706) controls for KPC (obtained from NAMRU) were used in each run

***C- Detection of the PCR amplified products*** : by electrophoresis on agarose Gel through visualization of the separating DNA, by exposing the gel to ultraviolet (UV) light, if the sample was positive ethidium bromide will fluoresce an orange-pink color giving band corresponding to bp specific to examined gene (881 base pair) [16].

**4- Statistical Analysis:** Statistical analysis of the present study was conducted by SPSS V17 using Chi-square test and Receiver operating characteristic curve (ROC) curve. P values<0.05 were considered significant, whereas values<0.01 were considered highly significant.

## RESULTS

The fifty *Klebsiella* Isolates included in the study were obtained from 36(72%) urine samples and 14(28%) wound swabs. Antibiotic susceptibility of isolates to the three carbapenems by the Kirby-Bauer disc diffusion method showed that 13/50 (26%) isolates were resistant to both meropenem and ertapenem while only 11/50(22%) isolates were resistant to imipenem and 2 (4%) were in the intermediate zone as shown in table 3.

**Table 3:** Antibiotic susceptibility pattern of isolated *k. pneumonia* to three carbapenem antibiotics

| Antibiotic | Sensitive | Intermediate | Resistant |
|---|---|---|---|
| **Imepenem** | **37(74%)** | 2 (4%) | 11(22%) |
| **Meropenem** | **37(74%)** | 0 | 13(26%) |
| **Ertapenem** | **37(74%)** | 0 | 13(26%) |

The 50 isolates were subjected to determination of minimum inhibitory concentration (MIC) to ertapenem using E-test. Results were interpreted according to CLSI, 2012[14]: 11out of 50 (22%) had MIC ≥ 2µg/ml and recorded as resistant strains, 2 (4 %) had MIC ≥ 0.5 µg/ml and ≤2 µg/ml and were recorded as intermediate sensitive strains, 37 out of 50 isolates (74%) showed MIC ≤ 0.5 µg/ml so they were considered susceptible to ertapenem as shown in table 4 .

**Table 4: Minimal inhibitory concentration of the isolated *k. pneumonia* to ertapenem antibiotic by E-test**

| | **Susceptible (MIC ≤ 0.5 µg/ml)** | **Intermediate** MIC ≥ 0.5 µg/ml | **Resistant** (MIC ≥ 2µg/ml) |
|---|---|---|---|
| **No. & Percentage** | **37 (74%)** | **2 (4%)** | **11 (22%)** |

Out of the 50 *k. pneumoniae* isolates, 3 isolates had MIC = 12 µg/ml, 3 isolates had MIC = 8µg/ml, 3 isolates had MIC = 4 µg/ml, 1 isolate had MIC = 3 µg/ml, 1 isolate had MIC = 2 µg/ml, 2 isolates had MIC = 1 µg/ml, 37 isolates had MIC < 0.5 µg/ml ranging from 0.003 to 0.125 µg/ml. (Fig 2)

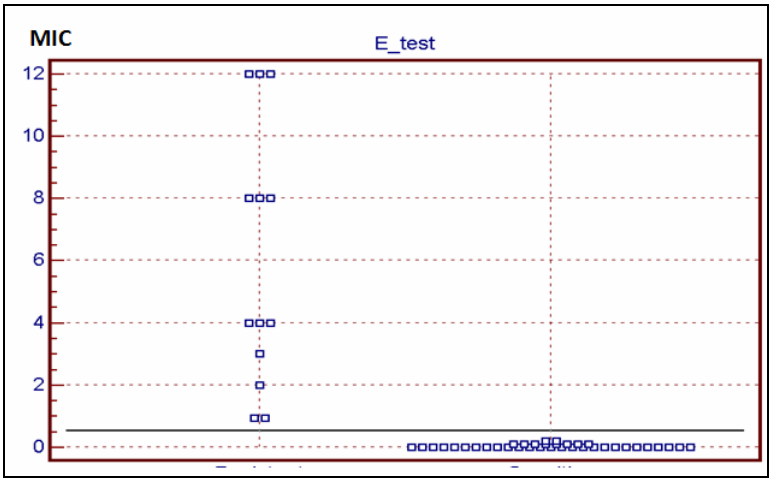


**Fig. 2: Roc curve for E-test results**

Ertapenem sensitivity tests revealed that there was a discrepancy between the results of E test and disc diffusion method in two strains. The two strains were reported resistant by disc diffusion method and intermediate sensitive by E test as shown in table 5

**Table 5: Discrepancy between results of ertapenem susceptibility by E test and disc diffusion**

| | **E test** | | **Disc diffusion method** | |
|---|---|---|---|---|
| | **No** | **%** | **No** | **%** |
| **Sensitive** | **37** | **74%** | **37** | **74%** |
| **Intermediate** | **2** | **4%** | **0** | **0%** |
| **Resistant** | **11** | **22%** | **13** | **26%** |

Modified Hodge test for detection of carbapenemase has been done for all 50 isolates, 13 out of 50 (26%) showed positive modified Hodge test while 37 out of 50 were negative (74%) as shown in table 6 .

**Table 6:** Modified Hodge test

| | **Positive MHT** | **Negative MHT** |
|---|---|---|
| **No.& Percentage** | **13 (26%)** | **37 (74%)** |

Modified Hodge test was negative for all isolates sensitive to ertapenem by disc diffusion and E- test. MHT was positive for all isolates resistant to ertapenem by disc diffusion and E- test. With100% agreement (table 7).

**Table 7: Agreement between MHT and ertapenem susceptibility by disc diffusion**

| **ETP** | | **MHT** | | | **Chi-square** | |
|---|---|---|---|---|---|---|
| | | **Negative** | **Positive** | **Total** | **X2** | **P-value** |
| **Sensitive** | **N** | 37 | 0 | 37 | 57.306 | <0.001* |
| | **%** | 74.00 | 0.00 | 74.00 | | |
| **Resistant** | **N** | 0 | 13 | 13 | | |
| | **%** | 0.00 | 26.00 | 26.00 | | |
| **Total** | **N** | 37 | 13 | 50 | | |
| | **%** | 74.00 | 26.00 | 100.00 | | |

Twenty six *k.pneumoniae* isolates were subjected to PCR for detection of $bla_{kpc}$ gene. Thirteen confirmed carbapenem resistant isolates by disc diffusion, E-test and MHT and 13 confirmed carbapenem sensitive isolates by the same tests. All of these 26 isolates were negative for $bla_{kpc}$ gene as shown in figure 3.

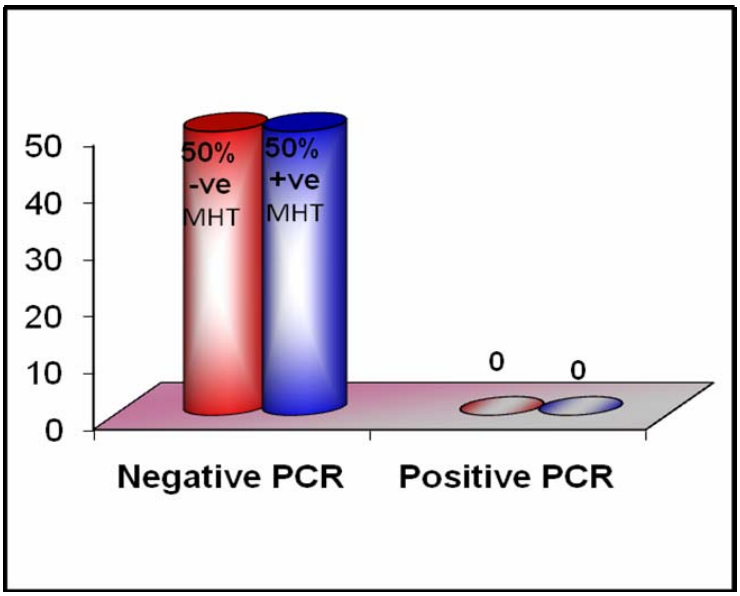


**Fig. 3: PCR results of $bla_{kpc}$gene by PCR for 26 Klebsiella pneumonaie isolates**

## DISCUSSION

Carbapenems play a critically important role in antibiotic therapy. Of the many hundreds of different β-lactams, carbapenems possess the broadest spectrum of activity and greatest potency against Gram-positive and Gram-negative bacteria. As a result, they are often used as "last-line agents" or "antibiotics of last resort" when patients with infections become gravely ill or are suspected of harboring resistant bacteria[17]. Unfortunately, the recent emergence of multidrug-resistant (MDR) pathogens seriously threatens this class of lifesaving drugs [18].

Expression of carbapenemase, porin loss or efflux pumps, which pump out any drugs or harmful chemicals that enter through the porins and alterations in PBP, are all associated with carbapenem resistance in Gram-negative rods[19]. As carbapenemase-producing *Enterobacteriaceae* are spreading worldwide, there is a need for a simple and accurate method for the detection of these bacteria [20].

The present study aimed to determine carbapenem resistance among *Klebsiella pneumoniae* isolates by routine disc diffusion test and E- test, to determine carbapenemase production by Modified hodge test and study the presence of $bla_{kpc}$ gene in carbapenem-resistant and carbapenem sensitive *Klebsiella pneumoniae* isolates. In the current study13/50 isolates (26%) were carbapenem resistant by disc diffusion method. This was in agreement with Patel and his colleagues[21] in Europe who detected carbapenem resistance in 26% of their isolates and also Hindiyeh and his coworkers [22] in Israel who detected carbapenem-resistance in25.1% of *K. pneumoniae* isolates by disc diffusion. A higher percent of carbapenem resistance was detected by Shabban and Abdel-Rahman [23] and Taha and Attalah[24] 46% (7/15) and 28% (23/67) respectively. Both carried their studies on ICU patients.

Arnold *et al.* [8] noted that ICU patients are at risk for infections caused by multidrug resistant organisms due to prolonged hospital stay and exposure to indwelling medical devices.

However lower percent of carbapenem resistance was detected earlier. Srinivasan and Patel [25] reported that data on healthcare-associated infections reported to the CDC from 2007 indicated that 8% of all *Klebsiella* isolates were carbapenem-resistant *K. pneumoniae* (CRKP), in comparison with <1% in 2000. The difference in results of these studies might be attributed to many factors such as the different antibiotic susceptibility pattern of organisms isolated from different countries and different antibiotic policies which may aid in selection of certain antibiotic resistant pathogen than another. There were two strains in the current study reported intermediate sensitive by Imipenem and resistant by Ertapenem. This was in agreement with Woodford *et al.* [26] who noted that imipenem was active against isolates with low-level ertapenem resistance but were less active against highly ertapenem-resistant isolates.

The present study detected MIC for ertapenem for all isolates by E-test. Ertapenem, had the highest sensitivity for detecting KPC-expressing isolates[27]. However, the specificity may be reduced due to resistance from other mechanisms, such as Amp C or ESBL production coupled with porin loss. There was a

discrepancy between the results of E-test and disc diffusion method in two strains. The two strains were reported resistant by disc diffusion method and intermediate sensitive by E-test. According to Guatam *et al.* [28] this was classified as minor error. As they noted that taking the E-test as the reference, discordance between it and the Kirby Bauer disc diffusion method was categorized as a very major error (reported susceptible when resistant), a major error (reported resistant when susceptible), or a minor error (reported intermediate when resistant or susceptible or vice versa).

In the current study The modified Hodge test (MHT) which is a phenotypic screening test for carbapenemases that is used for epidemiological purposes, and is currently proposed by the Clinical and Laboratory Standards Institute CLSI, 2012[14] was used for detecting carbapenemase production.**.** The thirteen *K. pneumoniae* carbapenem resistant isolates were positive by MHT. All Carbapenem sensitive isolates were negative by the test. Shabban and Abdel-Rahman [23] detected lower incidence of carbapenemase production by MHT (4 out of the 7 carbapenem resistant *K. pneumomniae* isolates). Arnold *et al.* [8] noted that the Modified Hodge test has been found to be 100% sensitive for the detection of a carbapenemase, although not specific for KPC production (the test cannot discriminate between KPCs and other carbapenemases)**.**

Giske *et al.* [29] added that Modified Hodge test may have false-positive results due to non-carbapenemase enzymes, such as AmpC and/or extended-spectrum beta-lactamases (ESBLs), combined with porin loss. They also noted that the subjective character of the test might cause problems of interpretation in laboratories lacking experience. When the presence of a carbapenemase is suspected, PCR is the fastest way to determine which family of β-lactamases presents [18]**.** In the current study 26 *k.pneumoniae* isolates were subjected to PCR for detection of $bla_{kpc}$gene. Thirteen confirmed carbapenem resistant isolates by disc diffusion, E-test and MHT and 13 confirmed carbapenemsensitive isolates by the same tests. The carbapenem sensitive isolates are included because the KPC carbapenemases may not confer resistance to carbapenems but only reduced susceptibility [27]**.** All of the 26 isolates were negative for $bla_{kpc}$gene. In contrast to the current study, several studies detected KPC gene in their isolates. Shabban and Abdel-Rahman [23] found $bla_{kpc}$gene in 6 out of 7 (85.7%) cabapenem resistant *Klebsiella pneumonaie* isolates.

Taha and Attalah [24] reported that $bla_{kpc}$ gene was found in 11 out of 14 *k.pneumoniae* isolates (78.5%).Qi and his coworkers 2011 [30] showed that all clinical isolates of *K.pneumoniae* with carbapenem resistance (100%) were confirmed as KPC producers by PCR. The discrepancy in results of the current study and other studies might be explained by the possibility that the carbapenem resistance was due to the production of AmpC β-lactamases, ESBLs, or non-KPC carbapenemases[31].

**In conclusion:** resistance to carbapenems in *K. pneumoniae* has started to constitute a significant problem in the national institute of diabetes and endocrinology as despite the low number of isolates in this study, 37% of the isolates were carbapenem resistant by disc diffusion method, E-test and MHT. The disc diffusion susceptibility does not report major errors in comparison to E test for ertapenem. The accurate and rapid detection of KPC-producing organisms is necessary for therapeutic considerations and for the implementation of infection control measures as antibiotic policy and isolation precaution to contain them. Phenotypic tests for detection of carbapenem resistance are good indicative for detection of carbapenemase but cannot differentiate between KPC and non KPC carbapenemases.

Expression of carbapenemase was detected in all carbapenem resistant isolates by modified Hodge test, however *blakpc* gene was not detected among either carbapenem resistant or susceptible strains .So, Further studies are needed to determine the gene responsible for carbapenemase production other than *blakpc* gene.

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