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Detection of bla_{KPC} and bla_{NDM} Genes in Carbapenems Resistant Strains of *Klebsiella pneumoniae* Isolated from some Egyptian Hospitals Patients

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Abstract

Background: **Treatment of Klebsiella pneumoniae (K. pneumoniae) infections has become very difficult especially in the last decades as the levels of antibiotic resistance are becoming increasingly a serious problem throughout the world.** ***Aim:*** **The growing number and rapid increase in carbapenems resistance which are the last resort of treatment of K. pneumoniae infections has urged for investigating some of these possible resistance mechanisms.** ***Materials and Methods:*** **A total of 202 K. pneumoniae isolates were selected from 593 specimens collected from patients attended 4 hospitals during the period from October 2013 to August 2014. Fifty three multi-drug resistant (MDR) K. pneumoniae isolates which showed resistance to imipenem and/or meropenem were tested phenotypically and gynotypically for the producibility of Klebsiella pneumoniae carbapenmase (KPC) and New Delhi metallo β-lactamase (NDM) and the presence of bla_{KPC} and bla_{NDM} genes encoding for these enzymes respectively.** ***Results:*** **The rate of production of both potential KPC and NDM was 35/53 (66.04%), whereas only 8 of these 53 MDR K. pneumoniae isolates (15.1%) were positive for NDM but not KPC production. The rates of detection of potential bla_{KPC} and bla_{NDM} genes among the 35 MDR K. pneumoniae positive isolates for the production of both KPC and NDM were 10/35 (28.57%) and 5/35 (14.28%) respectively.** ***Conclusion:*** **bla_{KPC} and bla_{NDM} were detected in some MDR isolates. However, the presence of isolates devoid of both genes suggests that resistance against carbapenems could be due to mechanisms other than the production of KPC and NDM-β-lactamases.**

Keywords: KPC carbapenemase and NDM β-lactamase

Introduction

K. pneumoniae is clinically the most important member of genus *Klebsiella*. It has been identified as an important common pathogen which causes many diseases worldwide including community acquired pneumonia which remains one of the major causes of mortality even in developed countries. It is also a major nosocomial pathogen causing pneumonia, burn, wound infections, septicemia and urinary tract infections[1-3]. Treatment of *K. pneumoniae* infections has become

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very difficult especially in the last decades as the levels of antibiotic resistance are becoming increasingly very serious problem throughout the world. The levels of antibiotic resistance among *K. pneumoniae* isolates have been rapidly changing for many classes of antimicrobial agents especially the successive generations of β-lactams and carbapenems, and *K. pneumoniae* has been considered as an important source of transferable antibiotic resistance[4-5]. The most common mechanism by which *K. pneumoniae* clinical isolates resist a wide range of antibiotics is the production of inactivating enzymes such as the β-lactamases. Carbapenemases, as β-lactamases, are of increasing concern as they confer resistance to carbapenems and to most β-lactam antibiotics. Carbapenem antibiotics such as imipenem and meropenem have been considered as the corner stone or the resort of drug treatment for serious infections caused by Gram-negative bacteria. However, the development of a novel mechanism of resistance in the last few years known as KPCs and NDM resulted in the decrease of the efficacy of this class of antibiotics[1,6].

Materials and Methods

Bacterial isolates

A total of 202 *K. pneumoniae* isolates were obtained from 593 specimens (34.06%) including (urine, blood, sputum, endo-tracheal aspirate and wound) collected from in- and out-patients who attended (ICU, surgery, out-clinics and chest departments) at Dar-El Fouad private hospital, El-Sheikh Zayed private hospital, Kasr Al-Ainy teaching hospital, and Sayed Galal teaching hospital (Greater Cairo/Egypt) during the period from October 2013 to August 2014 (Table 1). The isolates were identified by the conventional methods according to Collee et al, Koneman et al, Brooks et al[7-9] and by using the API 20E system kits, Biomerieux, France.

Table 1: Clinical specimens collected from different Egyptian hospitals patients

Hospital	Urine	Blood	Sputum	Wound swabs	ET aspirate	Total
Dar El-Fouad						
Total specimens	40	25	13	11	21	110
Klebsiella isolates	20 (50%)	12 (48%)	5 (38.4%)	5 (45.4%)	10 (47.6%)	52 (47.2%)
El-Sheikh Zayed						
Total specimens	48	39	32	38	55	212
Klebsiella isolates	12 (25%)	14 (35.9%)	8 (25%)	9 (23.6%)	12 (21.8%)	55 (25.9%)
Kasr Al-Ainy						
Total specimens	39	18	15	20	5	97
Klebsiella isolates	19 (48.7%)	10 (55.5%)	5 (33.3%)	12 (60%)	0	46 (47.4%)
Sayed Galal						
Total specimens	78	11	50	21	14	174
Klebsiella isolates	19 (24.3%)	4 (36.3%)	15 (30%)	4 (19%)	7 (50%)	49 (28.1%)
All hospitals						
Total specimens	205	93	110	90	95	593
Klebsiella isolates	70 (34.1%)	40 (43%)	33 (30%)	30 (33.33%)	29 (30.5%)	202 (34%)

ET=Endo-tracheal

Antimicrobial susceptibility test:
The antimicrobial susceptibility tests for the isolates were performed by the disc diffusion method according to Kirby-Bauer protocol[10]and the interpretation of the zones of inhibition was achieved according to CLSI[11]. The tested antimicrobial discs (concentration/disc) included: Amikacin (30µg), amoxycillin/ clavulnic acid "20/10" (30µg), ampicillin (10µg), cefaclor (30µg), cefepime (30µg), cefotaxime (30µg), cefoxitin (30µg), ceftazidime (30µg), ceftriaxone (30µg), cephalothin (30µg), ciprofloxacin (5µg), doxycycline (30µg), gentamicin (10µg), imipenem (10µg), levofloxacin (5µg), meropenem (10µg), minocycline (30µg), nitrofurantoin (300µg), piperacillin (100 µg), and sulfamethoxazole/Trimethoprim "1.25/23.75" (25µg) *(Oxoid, UK).*

Primers:

Table 2: Primers used in the present study

Gene	Primers used
bla_{KPC}	F: 5'-GCTCAG GCGCAACTG TAAG-3' R: 5'-AGCACAGCGGCAGCAAGAAAG-3'
bla_{NDM}	F: 5'-TGCCCAATATTATGCACCCGG-3' R: 5'-AGCACAGCGGCAGCAAGAAAG-3'

(Invetrogen, UK).

Phenotypic detection of carbapenmases
Fifty three isolates that were found resistant to three or more antimicrobial classes (MDR) and resistant to imipenem and/or meropenem were selected for further testing of carbapenemases production phenotypically by a modified Hodge test (MHT) and EDTA disc synergy test[12-13].

Modified Hodge Test (MHT) for detection of KPC carbapenemase
An overnight culture of *Escherichia coli* (*E. coli*) ATCC 25922 on Mueller Hinton agar was suspended to the turbidity of 0.5 McFarland in 5mL of saline. From this preparation 500µL were streaked for a confluent growth on a Mueller Hinton agar plate. A meropenem disc (10µg) was placed in the centre of the test area. Single colonies of overnight cultures of the tested *K. pneumoniae* strains on Mueller Hinton agar were streaked, each in a straight line from the edge of the disc to the edge of the plate. The plates were incubated overnight at 37°C in ambient air for about 18 hours. After incubation, MHT positive test results showed a clover leaf-like indentation of the *E. coli* ATCC 25922 growing along the test organism growth streak within the disc diffusion zone[12].

EDTA disc synergy test for detection of NDM β-lactamase
An overnight culture of the test *K. pneumoniae* strain on Mueller Hinton agar was suspended to the turbidity of 0.5 McFarland in 5mL of saline. From this preparation 500µL were streaked for a confluent growth on a Mueller Hinton agar plate. After drying, two discs of imipenem (10µg) were placed in the plate centre 10mm apart edge to edge. An aliquot of 10µL of 0.5M EDTA solution in distilled water (pH 8.0) was then added to one of the two imipenem discs. This resulted in approximately 1.5mg EDTA/ disc. After an overnight incubation at 37°C, the presence of an enlarged zone of inhibition by more than 4mm around the disc which contains EDTA was interpreted as a positive test for NDM production[13].

DNA extraction
Plasmids from *K. pneumoniae* isolates to be tested were extracted using QIAprep spin miniprep kits, Qiagen, Hilden, Ger-

many, according to the manufacturer procedures.

Detection of bla$_{KPC}$ and bla$_{NDM}$

1-Preparation of the specimens: The lyophilized primers (Table 1) were reconstituted in nuclease free water and the concentration of the primers was adjusted to 10 picomole/µL. The Polymerase Chain Reaction (PCR) was set up in a PCR tube (total volume 25.0µL), by adding 12.5µL of the master mix (Qiagen), 1.0µL of each of the forward and reverse primers and 1.0µL of template DNA (containing 100-200ng/µL) and the volume was completed to 25.0µL using nuclease free water. The PCR reaction was performed in GeneAmp thermal cycler[14-15].

2- Detection of bla$_{KPC}$: The specimens were subjected to an initial denaturation at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72° C for 5 minutes. Gel electrophoresis was then carried out using 1.5% agarose gel. The gel was viewed in an UV trans-illuminator where the bands pattern was observed[14].

3-Detection of bla$_{NDM}$: The specimens were subjected to an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 50 seconds, annealing at 60°C for 40 seconds, extension at 72°C for 1 minute and a final extension at 72° C for 5 minutes. Gel electrophoresis was then carried out using 1.5% agarose gel. The gel was viewed in an UV trans-illuminator where the bands pattern was observed[15].

Results

Antimicrobial susceptibility testing of K. pneumoniae isolates

Of 202 tested *K. pneumoniae* isolates, 53 (26.2%) were MDR plus resistant to imipenem and/or meropenem (49 MDR isolates were resistant to imipenem plus meropenem and 4 MDR isolates were resistant to meropenem only, Figure.1, Table 3). The antibiogram of the tested *K. pneumoniae* isolates is shown in Table 4.

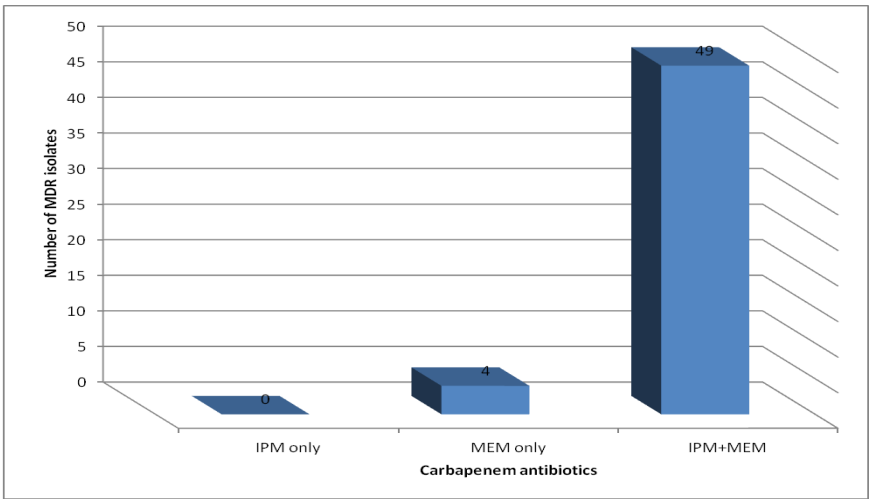


Figure 1: Resistance to carbapenems among 53 MDR *K. pneumoniae* isolates

Phenotypic detection of carbapenemases: Modified Hodge Test (MHT) for detection of KPC carbapenemase

Among the 53 MDR *K. pneumoniae* isolates which were resistant to carbapenems (imipenem and/or merope-nem), 35 isolates (66.04%) were positive for MHT (10 isolates from blood, 10 from sputum, 7 from urine, 5 from wound and 3 from endo-tracheal aspirate) and 18 isolates (33.96%) were negative for this test (Figures. 2 & 6; Table 5).

EDTA disc synergy test for NDM detection

Among the 53 MDR *K. pneumoniae* isolates which were resistant to carbapenems (imipenem and /or meropenem), 43 isolates (81.13%) were positive for EDTA disc synergy test (11 isolates from blood, 12 from sputum, 8 from urine, 8 from wound and 4 from endo-tracheal aspirate including the 35 MHT positive isolates) whereas 10 isolates (18.87%) were negative for this test (Fig. 3 and 6; Table 5).

Table 3: Prevalence of carbapenems resistant *Klebsiella* isolates in different clinical specimens

Specimens	Carbapenems resistant *Klebsiella* isolates No (%)
Blood	15 (28.3%)
Sputum	13 (24.5%)
Urine	10 (18.9%)
Wound swabs	7 (13.2%)
ET aspirate	8 (15.1%)
Total	53 (100%)

ET= Endo-tracheal

PCR detection of blaKPC gene:

The 35 carbapenems resistant isolates which showed positive MHT and EDTA disc synergy tests were further tested for the presence of bla_{KPC} gene using the PCR technique. Ten of these isolates 28.57% (4 isolates from sputum, 3 from blood, 1 from urine, 1 from wound and 1 from endo-tracheal aspirate) showed the presence of bla_{KPC} gene at 150 bp (Fig. 4 A and B, "isolates number 3, 21, 25, 41, 42, 89, 98,115, 121 and 122"; Figure 6; Table 5).

PCR detection of bla_{NDM} gene

The 35 carbapenems resistant isolates which showed positive MHT and EDTA disc synergy tests were further tested for the presence of bla_{NDM} gene using the PCR technique. Five of these isolates 14.28% (2 isolates from blood, 2 from urine and 1 from sputum) showed the presence of bla_{NDM} gene at 621 bp (Fig. 5 A, B, C, "isolates number 1, 87, 88, 99 and 135"; Figure 6; Table 5).

Discussion

The emergence of antibiotic resistance is a global public health problem[16-17]. Gram-negative bacterial resistance is of particular importance as there is a dearth of novel antibiotics such as carbapenems, the agents of last resort against MDR *Enterobacteriaceae* including *K. pneumoniae*. The clinical utility of carbapenems is under threat with the growing incidence of resistance among K. pneumoniae especially with the emergence of acquired carbapenemases, particularly Ambler class A (i.e. KPC) and class B

metallo-β-lactamases. Such enzymes have emerged in many geographical locations[4,12,13,18,19]. In this study; 53 isolates (26.24%) were MDR plus resistant to imipenem and/or meropenem (49 isolates were resistant to imipenem plus meropenem and 4 isolates were resistant to meropenem only).

Table 4: Antimicrobial resistance profile of *K. pneumoniae* isolates

Antibiotics	Resistant isolates[1] (%)	Intermediate isolates[1] (%)	Susceptible isolates[1] (%)
Ampicillin	≤13 mm 184 (91.09%)	14-16mm 12 (5.94%)	≥17mm 6 (2.97%)
Piperacillin	≤17mm 154 (76.24%)	18-20mm 18 (8.91%)	≥21mm 30 (14.85%)
Amoxicillin/ clavulnic acid	≤13mm 86 (42.58%)	14-17mm 36 (17.82%)	≥18mm 80 (39.6%)
Cefaclor	≤14mm 146 (72.27%)	15-17mm 13 (6.44%)	≥18mm 43 (21.29%)
Ceftriaxone	≤22mm 147 (72.77%)	23-25mm 8 (3.96%)	≥26mm 47 (23.27%)
Cefotaxime	≤19mm 147 (72.77%)	20-22mm 10 (4.95%)	≥23mm 45 (22.28%)
Ceftazidime	≤17mm 135 (66.83%)	18-20mm 25 (12.38%)	≥21mm 42 (20.79%)
Cephalothin[2]	≤14mm 32 (45.71%)	15-17mm 22 (31.43%)	≥18mm 16 (22.86%)
Cefipime	≤14mm 108 (53.47%)	15-17mm 24 (11.88%)	≥18mm 70 (34.65%)
Cefoxitin	≤14mm 75 (37.13%)	15-17mm 16 (7.92%)	≥18mm 111 (54.95%)
Amikacin	≤14 mm[4] 52 (25.74%)	15-16mm 17 (8.42%)	≥17mm 133 (65.84%)
Gentamicin	≤12mm 57 (28.21%)	13-14mm 21 (10.4%)	≥15mm 124 (61.39%)
Minocycline	≤12mm 50 (24.75%)	13-15mm 69 (34.16%)	≥16mm 83 (41.09%)
Doxycyclin	≤10mm 59 (29.21%)	11-13mm 44 (21.78%)	≥14mm 99 (49.01%)
Ciprofloxacin	≤15mm 72 (35.64%)	16-20mm 17 (8.42%)	≥21mm 113 (55.94%)
Levofloxacin	≤13mm 71 (35.15%)	14-16mm 10 (4.95%)	≥17mm 121 (59.9%)
Trimethoprie/ sulphamethoxazole	≤10mm 127 (62.87%)	11-15mm 17 (8.42%)	≥16mm 58 (28.71%)
Imipenem	≤19mm 49 (24.26%)	20-22mm 18 (8.91%)	≥23mm 135 (66.83%)
Meropenem	≤19mm 53 (26.24%)	20-22mm 13 (6.43%)	≥23mm 136 (67.33%)
Nitrofurantoin[2]	≤14mm 14 (20%)	15-16 mm 11 (15.71%)	≥17mm 45 (64.29%)

[1] Degree of susceptibility to the tested antimicrobial agents (CLSI 2013).
[2] Cephalothin and nitrofurantoin were used with isolates from urine specimens only (70 isolates)

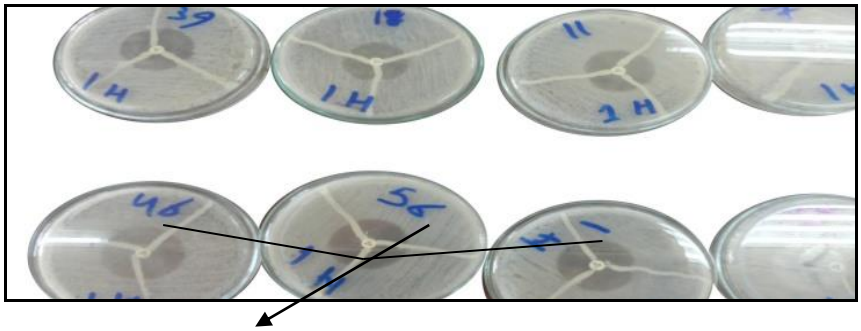


Figure 2: Positive MHT of some isolates.

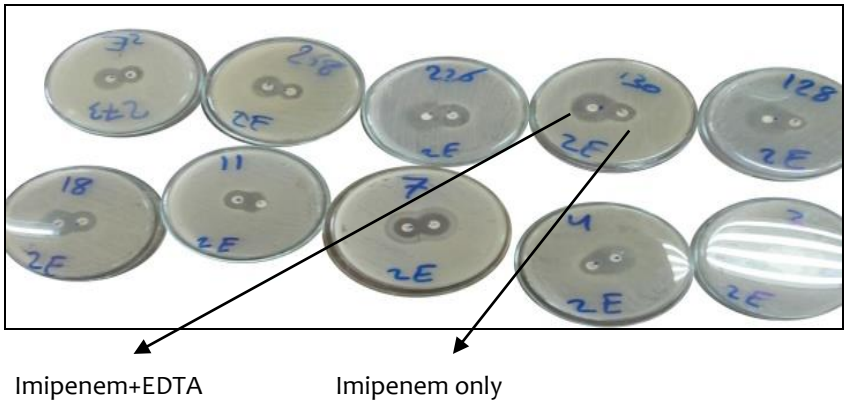


Figure 3: Positive EDTA disc synergy test of some isolates.

In Pakistan, Jamil et al investigated 92 *K. pneumoniae* strains isolated from 230 urine specimens. The incidence rate of resistance against meropenem among these *K. pneumoniae* isolates was 14.1%[20]. In a study in India, Chaudhary & Murthy investigated 60 *K. pneumoniae* strains isolated from 1000 urine specimens. The incidence rate of resistance against both imipenem and meropenem was 6.66%[21]. In an earlier study in Pakistan Ullah et al investigated 92 *K. pneumoniae* isolates collected from urine. The incidence rates of resistance against imipenem and meropenem were 13.04% and 6.52% respectively[22]. The results in the current study indicate a marked increase in resistance rate against carbapenems in comparison to other studies which may be due to the increased disaster of antibiotic misuse in Egypt. In the present study among the 53 MDR *K. pneumoniae* isolates which were resistant to carbapenems (imipenem and/or meropenem), 35 isolates (66.04%) were positive for MHT and 18 isolates (33.96%) were negative for this test. In India, Shanmugam et al investigated 46 carbapenem resistant isolates (22 *K. pneumoniae*, 21 *Escherichia coli*, 2 *Citro-bacter* species and 1 *Proteus mirabilis*) for KPC production by MHT. The results were positive in 38 isolates (82.6%)[14]. In Italy, Mosca et al investigated 38 *K. pneumoniae* isolates. The results were positive for KPC production by MHT in 32 isolates (84%)[23]. In addition, in a study in Egypt, Metwally et al investigated 20 *K. pneumoniae* isolates for KPC production by MHT. The results were positive in 14 isolates (70%)[24]. These results show a fair agreement with the results of the current study. The current study illustrated that among the carbapenems resistant 53

MDR *K. pneumoniae* isolates, 43 isolates (81.13%) were positive for EDTA disc synergy test and 10 isolates (18.87%) were negative for this test.

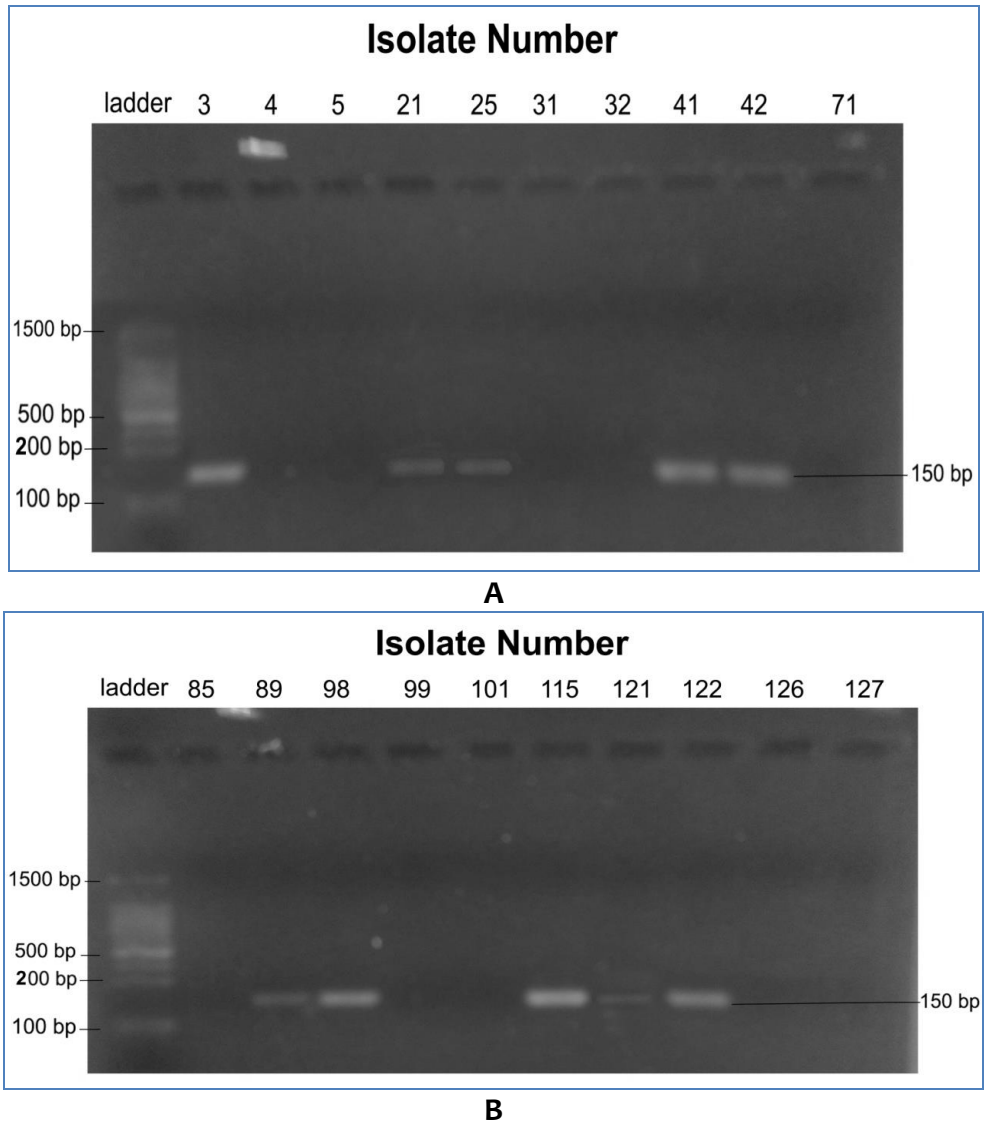
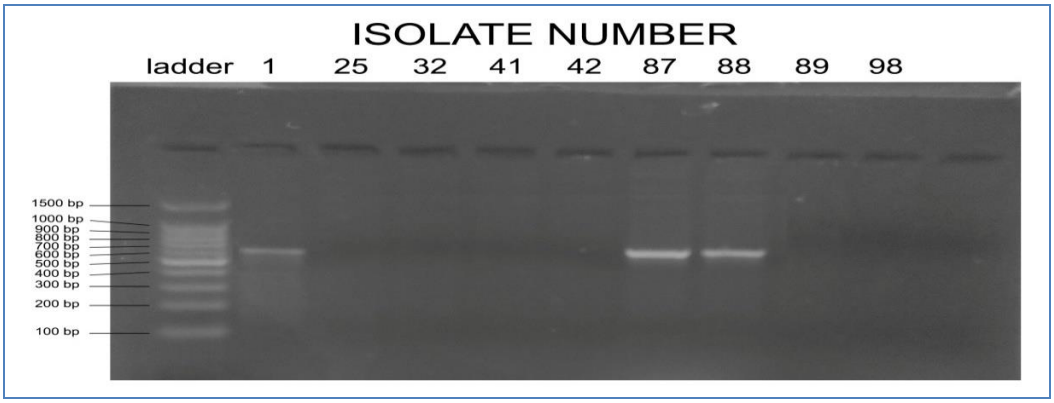


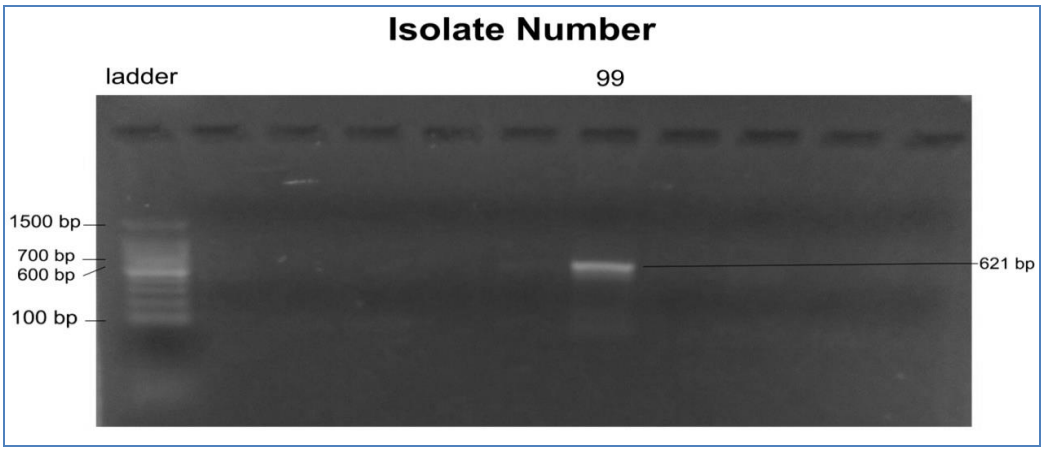
Figure 4: A & B: PCR amplification of bla$_{KPC}$ in some *K. pneumoniae* isolates

In India, Kumar et al investigated 97 *Klebsiella* isolates for NDM production by EDTA disc synergy test. The results were positive in 21 isolates (21.6%)[25]. In Slovakia, Lovayova et al investigated 4 *K. pneumoniae* isolates for NDM production by EDTA disc synergy test. All the isolates (100%) were positive for NDM production[26]. On the other hand, Balan et al investigated 32 *K. pneumoniae* isolates from various clinical specimens (pus, pleural fluid, sputum, blood). Only one of those isolates (3.12%) was positive for NDM production by EDTA disc synergy test[27]. The differences especially in the results of EDTA disc synergy test between the current study and the other studies may be due to strain differences. The presence of isolates with negative results for both MHT and EDTA disc synergy tests suggests that resistance against carbapenems could be due to mechanisms other than the production of KPC and NDM-β-lactamases. In this

study, among the 35 MHT and EDTA disc synergy tests- positive *K. pneumoniae* isolates, 10 isolates (28.57%) gave positive results for detection of bla_{KPC} by PCR. In India, Shanmugam et al results were positive in 31 out of the 46 isolates of *K. pneumoniae*, *Escherichia coli*, *Citrobacter* species and *Proteus mirabilis* mentioned above (67.4%) for bla_{KPC} gene detection[14].



A



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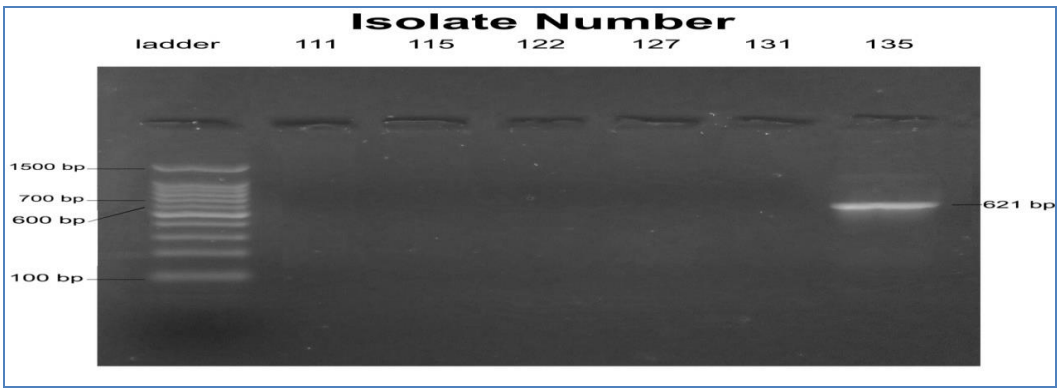

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Figure (5) A, B &C: PCR amplification of bla_{NDM} in some *K. pneumoniae* isolates

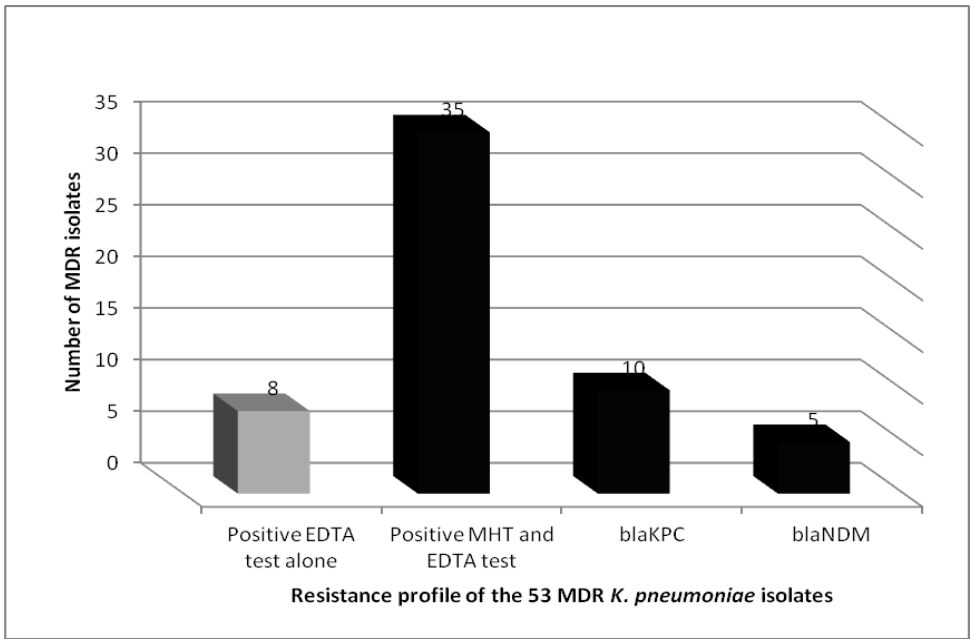

Figure 6: Phenotypic and genotypic tests results

Table 5: Comparison between phenotypic (conventional) methods and molecular technique results for detection of carbapenemases

Methods of carbapenemases detection.	Tested Isolates No.	Positive isolates No. (%)
Phenotypic(conventional) methods		
– MHT for detection of KPC.	53	35 (66.04%).
– EDTA disc synergy test for detection of NDM- metalo-β-lactamase.	53	43 (81.13%).
Molecular technique		
– bla$_{KPC}$ detection	35	10 (28.57%).
– bla$_{NDM}$ detection	35	5 (14.28%).

In Italy, Mosca et al investigated 38 *K. pneumoniae* isolates and found that all of the isolates (100%) were positive for bla$_{KPC}$ gene detection[23]. In Egypt, Metwally et al investigated 20 *K. pneumoniae* isolates; 14 isolates of which (70%) were found positive for bla$_{KPC}$ gene detection[24]. In the present study, among the 35 *K. pneumoniae* isolates which gave positive results for both EDTA disc synergy test and MHT, 5 isolates (14.28%) were positive for detection of bla$_{NDM}$ by PCR. In Saudi Arabia, Shibl et al investigated 60 *K. pneumoniae* isolates from different clinical specimens (blood, sputum, urine and wounds) for bla$_{NDM}$ and detected it in only 12 (20%) isolates[28]. In India Chaudhary & Payasi investigated 150 *K. pneumoniae* isolates for bla$_{NDM}$ detection and the results were positive in 24 isolates (16%)[29]. In another study in India, Bhaskar et al investigated 59 blood isolates of *Klebsiella* where 40 of those isolates (67.8%) were bla$_{NDM}$ positive[30]. The differences in the results of the present study and the other studies suggest that resistance against carbapenems could be due to mechanisms

of resistance other than the production of bla_{KPC} and bla_{NDM} genes.

Conclusion

bla_{KPC} and bla_{NDM} genes were detected in some MDR *K. pneumoniae* isolates. The presence of isolates devoid of both genes suggests that resistance against carbapenems could be due to mechanisms of resistance other than the production of KPC and NDM-β-lactamases.

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