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Molecular Investigation of Metallo B-Lactamase genes in Klebsiella Pneumoniae Bacteria from Clinical Isolates

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Abstract

Klebsiella pneumoniae is a significant nosocomial bacteria, that causes a large range of infections e.g. bacteremia, pneumonia, meningitis, and urinary tract infections. The current study intends to detect the existence of specific genes (blaNDM-1, blaNDM-2, blaVIM, blaIMP) associated with metallo-beta-lactamase (MBL) production in multidrug-resistant *Klebsiella pneumoniae* isolates attained from various clinical isolate. These genes are responsible for conferring resistance to a wide range of antibiotics, making an infection caused by such bacteria difficult to treat. Two hundred and seventy eight samples are collected from patients in Baquba Teaching Hospital during the period between December 2023 and May 2024. The samples included urine, sputums, burn, and wound swabs in addition to blood samples. The isolates are diagnosed microscopically and biochemically and the VITEK-2 compact system was used for diagnosis confirmation. It is found that 39.4%; n=69 is *K. pneumoniae* with 26%; n=18 of isolates are Multidrug resistant. Also, this study ascertains that multidrug-resistant (MDR) *K.pneumoniae* has the highest resistance against different types of antibiotics, including: AMP: 100%, AMC73: 36%, PIP: 81.16%, ATM: 72.46%, FEP: 71.01%, CAZ: 62.32%, CRO: 56.5%, IPM: 27.53%, MEM: 26.19%, AK: 47.82%, TOB: 43.47%, GM: 36.23%, LEV: 31.82%, OFX: 28.98%, CIP: 24.63% and SXT: 65.22%. Referring to phenotypic detection of MBL, 11 from 18 of MDR K. pneumoniae (61.11%) indicate a positive result. Also, during the molecular detection of Metallo B-lactamase genes, it was found that (81.81%; n=9) isolates were positive for blaNDM-1, blaNDM-2, and blaVIM gene, while no isolates showed positive results to blaIMP.

Objective: Molecular detection of metallo-beta-lactamase genes in the bacteria *Klebsiella pneumoniae.*

Keywords. Klebsiella pneumonia; Multidrug-resistant (MDR); Metallo-beta-lactamase (MBL) Genes; blaNDM-1, blaNDM-2, blaVIM, blaIMP; Phenotypic and molecular detection.

1. Introduction

Klebsiella pneumoniae is a significant nosocomial bacterium, which belongs to the family Enterobacteriaceae, Gram – negative straight rods bacteria [1], that causes a high range of infections e.g. bacteremia, pneumonia, meningitis, and urinary tract infections [2]. *K. pneumoniae* genome allowing for the expression of capsule, siderophores, adhesions, and antimicrobial determinants [3]. Antibiotics are a cornerstone of modern medicine and have significantly reduced the burden of infectious diseases [4]. The prevalence of antimicrobial-resistant bacteria has attained an

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incongruous level worldwide and threatens global public health as a silent pandemic, necessitating urgent intervention [5]. The antimicrobial resistance problem is demonstrated by a recent elevated carbapenem-resistant *K. pneumoniae*, which was highly driven by the appearance and spread of mobile genetic element that carries carbapenemase resistance gene such as the metallo beta lactamases [6].

Against MDR pathogens, carbapenems (Imipenems and Meropenems) have been considered the last resort drug for a long time. Carbapenem, and in general β-lactams, act by inhibiting cell wall biosynthesis and are the most used class of antimicrobial agents in the clinic armamentarium for infectious diseases [7]. The production of carbapenem hydrolyzing B-lactamase is one of the main mechanisms of carbapenem resistant in this pathogen, and these specific B-lactamase groups are classified into class B Metallo B-lactamase (MBL) which include New Dalhi metallo beta lactamase (NDM), Verona integrin encoded metallo beta lactamase (VIM) and Imipenemse (IMP) [8]. The bacteria with NDM-1 gene are known as superbug and the public health should pay more attentions to it [9]. Several genotypic, phenotypic, phylogenic as well as molecular techniques were utilized for the detection of enzyme production by bacteria. Those bacteria are responsible for drug resistance, that results in high mortalities and morbidities in patients infected with such bacteria, therefore they increase healthcare costs because of the long periods of hospital stays [10].

There is a remarkable mortality rate, nearly (50%), in pneumonia patients infected with *K. pneumoniae* [6]. These infections may raise the mortality rate of critically deliberated and ill patients who are hospitalized in intensive care unit (ICU) and may negatively affect the financial costs of their hospitalization in all parts of the world [11]. The occurrence of multidrug-resistant (MDR) *Klebsiella pneumoniae* strains, predominantly those creating metallo-beta-lactamases (MBLs), poses a noteworthy threat to public health. MBLs deliberate resistance to a broad spectrum beta-lactam antibiotics, comprising carbapenem, making infection caused by these bacteria exceptionally difficult to treat. This research intends to determine the incidence and molecular properties of MBL-producer MDR *K. pneumoniae* isolate from a tertiary Iraqi care hospitals. More specifically, this research attempts to detect the metallo B-lactamase gene (blaNDM-1, blaNDM-2, blaVIM, blaIMP) among MDR *K. pneumoniae* isolated from many clinical sources. Expectedly, by recognizing the specific MBL genes existed in MDR *K. pneumoniae* isolates, clinicians can make more well-versed and knowledgeable decisions concerning antibiotic therapy. This in turn can help to optimize treatment outcomes and decrease the risk of treatment failure.

2. Material and method

A. Sample collection and identification

Two hundred and seventy eight samples were collected from different sources (urine from UTI patients, sputum, swab from wound and burn and blood), from Baquba Teaching hospital in Iraq, between December 2023 to May 2024. The specimens were streaked on blood, MacConkey and Eosin methylene blue (EMB) agars then incubated for 24 hours at 37 ℃. The bacterial isolates showed mucoid colonies with typical colors, growth as well as hemolysis. Then, they were recognized as *K. pneumoniae* using manual biochemical investigations including Gram stain, oxidase test, catalase test, Voges-Proskaur test, Indole test, methyl red test, Kligler Iron test, Simmon citrate test as well as urease test. The biochemical investigations were embedded in VITEK-2 compact system (Biomerieux / USA) for eventual affirmation of diagnosis.

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B. Antimicrobial Susceptibility Test

The following (16) antibiotics were used to test the resistance of (69) isolates of K. pneumoniae: Amoxicillins/Clavulanic acid (AM/C), Ampicillins (AMPs), Piperacillins (PIPs), Azetronams (ATMs), Cefepimes (FEPs), Ceftazidimes (CAZs), Cefriaxones (CROs), Imppenems (IPMs), Meropenems (MEMs), Gentamicins (GMs). Amikacins (AKs), Tobramicins (TOBs), Levofloxacins (LEVs), Ofloxacins (OFXs), Ciprofloxacins (CIPs), and Trimethoprime /salfa methouxazok (SXT). The Kirby–Bauer diffusion method was used for antibiotic resistance determination. For obtaining approximate numbers equal to (1.5 X 108) cell / ml [12], we used the turbidity of McFarland standard as well as the Viteck2 sensitive compact system from (Biomerieux / USA).

C. Screening of some Metallo B-lactamase (MBL)

The double disk Meropenem –EDTA method was carried out via the use of disks that contained 1900µg of EDTA plus 10µg of meropenem disks which were put on the inoculated Muller Hinton agar dishes following 24 hour incubation, there was an increased 7 mm in the zone diameter in presence of 1900µg EDTA in comparison with meropenem disks alone, which was regarded as MBL-producing *K. pneumoniae* (positive result) [13].

D. Extractions of DNA

The AllianceBio (ABIO) pureTM total DNA from (ABIO pure / USA) was used to extract the genomic DNA from the isolate which cultured on brain heart infusion broth medium and incubated for 24 hours. The absorbance Quants Fluorometer was used to measure the purity and concentration of DNA extract. PCR primers were used to detect the capsular polysaccharides for MDR *K. pneumoniae.* The polymerase chain reaction (PCR) was used to detect 18 isolates out of 69 whose sequence type of Multidrug resistant (MDR) existed in the city of Baquba.

To detect of some metallo B-lactamase genes for *K. pneumoniae*, the primers were prepared in accordance with the manufacture's instructions. To be used in the PCR mixture, the diluted stock solution was prepared by addition of 10ML of the original stock solution to 90µl of the deionized distal water to produce ultimate concentration (10pmo/µl) then kept in the deep freeze until use. Thermocycter conditions of PCR was done as follows: 95 °C for 5minutes, followed by 30 cycles of 95 °C for 30seconds, annealing temperatures in table (1) for 30seconds, 72 °C for 1min 30s and final extension at 72 °C for 7 min.

Table 1. The primers sequencing of metallo B-lactamase genes

Reference	Gene	Seq	Ann. Temp.	Product size (bp)
[14]	*NDM-1* F R	ATGGAATTGCCCAATATTATGC CGAAAGTCAGGCTGTGTTG	60	500
	NDM-2 F R	CACCTCATGTTTGAATTCGCC CTCTGTCACATCGAAATCGC	55	1000
	VIM F R	TTATGGAGCAGCAACCGATGT CAAAACTCCCGCTCCAACGA	55	700
	IMP	TGACAAGTTATCTGTATTC	55	740

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	F R	TTAGTTGCTTGGTTTTTGATG		

3. Results and discussion

Two hundred and seventy-eight samples revealed 175 (62.9%) positive cultures and 103 (37.1%) negative cultures. Only 69 (39.4%) of the 175 isolates were *K. pneumoniae* and 18 (26%) were multidrug-resistant *K. pneumoniae.* These results were consistent with findings of [15]. The majority of *K. pneumoniae* isolates 42 (32.31%) were obtained from urine samples followed by sputums 17 (20.5%), wounds 7 (19.44%), burn 2 (13.33%) and blood 1 (7.17%). The age range of the patients was (1–80) years. The *K. pneumoniae* and its pathogenic significance arises in the urinary and respiratory tract infection events. This may be because this bacterium is an opportunistic intestinal normal flora that can adhere to epithelial cell surfaces.

A. Antibiotic susceptibility Tests

From results of the current study, different susceptibility levels to various antibiotics within the isolates are exhibited in table (2). The results show that isolates of *K. pneumoniae* highly to beta lactam antibiotic groups: Ampicillins (100%), Piperacillins (81.16%), Amoxicillins+Clavulanic acid (73.36%), Azetronam (72.46%) Cefepims (71.01%), Ceftazidimes (62.32%), Ceftriaxones (56.51%), Imipenems (27.53%), Meropenems (26.19%). Aminoglycoside group: Amikacins (47.82%), Tobramycins (43.47%), Gentamicins (36.23%). Quinolone groups: Levofloxacins (31.88%), Ofloxacins (28.98%), Ciprofloxacins (24.63%) in addition to Trimethoprim–Sulfamethoxazole (65.22%).

Table 2. Resistance of *K. pneumoniae* isolates to various antibiotics.

Antibiotic	(%)
Ampicillins	(100%)
Amoxicillins + clavulanic acid	(73%)
Piperacillins	(81%)
Azetronames	(72%)
Cefepimes	(71.01%)
Ceftazidimes	(62.32%)
Ceftriaxones	(56.5%)
Imipenems	(27.53%)
Meropenems	(26.19%)
Gentamicins	(36.23%)
Amikacins	(47.82%)
Tobramycins	(43.47%)
Levofloxacins	(31.88%)
Ofloxacins	(28.98%)
Ciprofloxacins	(24.63%)
Trimethoprim-sulfamethouxazole	(65.22%)

The results coincided with the findings of [15-16] who reported that the resistance percentages of *K. pneumoniae* was as follows: (to Ampicillins 97%, to Amoxicillins+ Clavulanic acid 97.5%, to Cefepimes 74%, to Azetronams 78%, to Piperacillins 92%, to Ceftazidimes and Ceftriaxones 84% for both antibiotics). In this regard, the resistance rate to Aminoglycoside antibiotics was different and agreesd well with the findings of [17] who showed the resistance rate *K. pneumoniae* was to

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(Gentamicins 51%, Tobramicins 49% and to Amikacins was 31.4%). Furthermore, our results were in agreement with those of [18] who found a resistance rate of (Levofloxacins 38.59%; Ciprofloxacins (44.73%), ofloxacins 27%.

In regard to quinolone antibiotics, and for Cephalosporin antibiotics, our results were in agreement with those revealed by [19] who detected that the resistance rate of *K. pneumoniae* to Trimethoprim– sulfamethouxazole was 60%. All Gram – negative bacilli harbor a series of antibiotic resistance genes that could be transferred to other bacteria horizontally and can cause difference nosocomial infections in hospitals [20]. The different in the resistance rate can be attributed to the different isolate numbers, variable working conditions as well as patients' health in addition to the antibiotics' offense.

B. Screening of Metallo B-lactamase (MBL)

The Meropenem–EDTA double disk method was used to detect the metallo B-lactamase (MBL). Several carbapenem-resistant isolates of *K. pneumoniae* were MBL producers. Eleven of eighteen of the MDR *K. pneumoniae* isolate (61.11%) revealed positive results while 7 (38.99%) showed negative results as displayed in Fig. 1. These results agreed with the results of [21] who demonstrated that the resistance rate of *K. pneumoniae* producer to MBL was (66.6%).

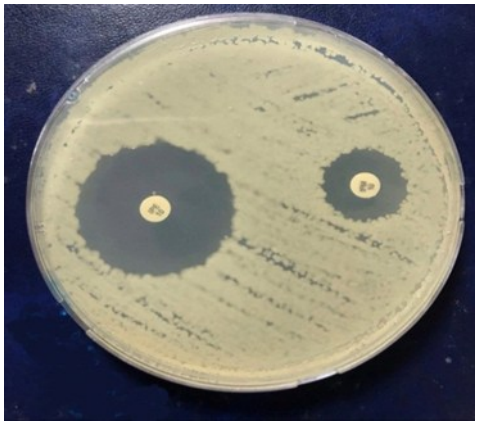

Fig. 1 Phenotypic detection of MBL producing *K. pneumoniae.*

C. Detection of metallo B-lactamase gene

The molecular detection for *blaNDM-1*, *blaNDM-2* , *blaVIM* and *blaIMP* gene using PCR, 11 isolates of *K. pneumonia,* indicated that (81.81%; n=9) were positive for *blaNDM-1*, *blaNDM-2* and *blaVIM*. however, no isolates showed a positive result to *blaIMP* (Fig. 2).

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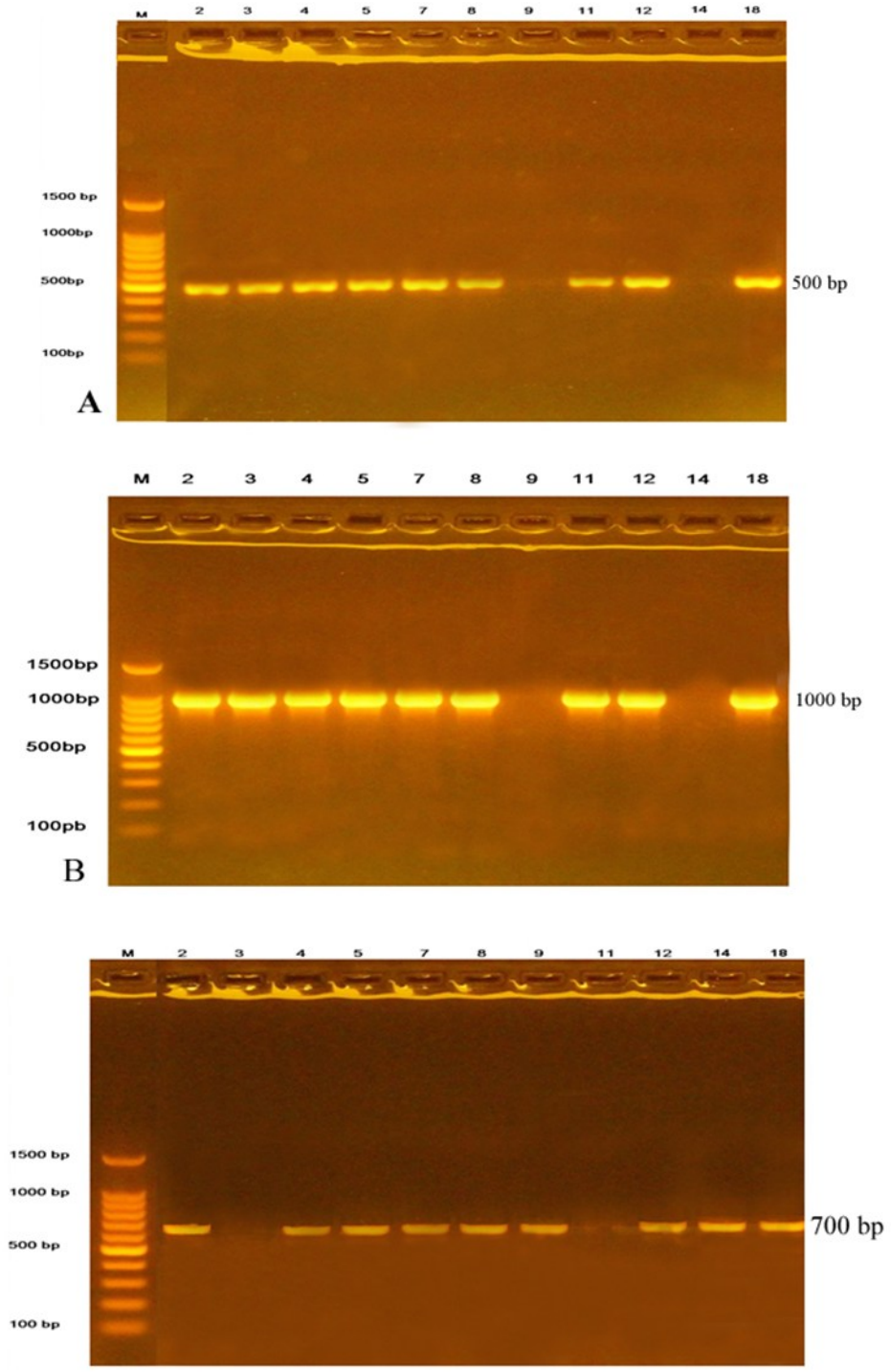

Fig. 2 Gel electrophoresis of amplified PCR products.it was run on 1% agarose (90 minutes at 100 volts) stained by ethid. bromid. M: Marker DNA ladders (100 bp). A: Detection of *blaNDM-1* genes, B: Detection of *blaNDM-2* genes, C: Ddetection of *blaVIM* genes.

The results agreed with the result of another study which showed that the percentage prevalent of blaNDM-1 was (92.85%). to assess the percentage of blaNDM and ensure it as (75%)[22]. Another study was conducted by [21] and ascertained that all isolates of *K. pneumoniae* gave positive results with blaNDM-1 gene (100%) and showed (70%) for blaNDM-2). There are several blaNDM gene types located commonly on the conjugative plasmids that belong to many incompatible groups [23].

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The occurrence of isolates contains blaNDM in Baquba Teaching hospital can result from plasmid transfer among resistant bacteria present in medical care units that can help in NDM gene acquiring as several Iraqi patients traveled to India and other countries to seek medical care. The results of the recent study are agreed with [24] who revealed that the prevalence rate of blaVIM in *K. pneumoniae* was (82.3%). Other study of [25] was evaluated all *K. pneumoniae* isolates and assured (100%) for blaVIM. The location of blaVIM gene was in class 1 integron as gene cassettes and was recognized on the plasmid with various types of replicons; this increases the dissemination chance and linkages with other genes of antibiotic resistance [26]. Referring to the current molecular detection of blaIMP, the results showed that all isolates of *K. pneumoniae* were negative for blaIMP gene as shown in Fig. 3. These results agree with the results shown by [27, 28].

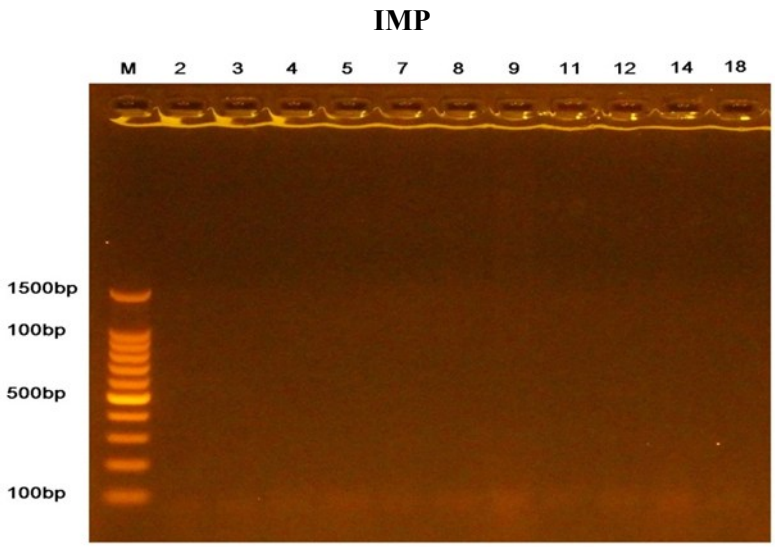

Fig. 3 Gel electrophoresis of amplified PCR products to detect *baIMP* genes. It was run on 1% agarose (90 minutes at 100 volts) stained by eth. Brom. M: Marker DNA ladder (100 bp).

4. Conclusions

Our present study demonstrated the spreading of *blaNDM*, *blaVIM K .pneumoniae* isolate among patients in Baquba Teaching hospital. In Iraq, the fast spread of *K. pneumoniae* MBL genes in patients, posed high threats to the hospitalized patients. More importantly, avoiding misuse, overuse of antibiotics may converse the undesired effects of multidrug resistant and MBL producing bacteria. The most important findings of the current study are listed below:

1- *Klebsiella pneumoniae* was shown to remain among the most prevalent multidrug-resistant bacteria that cause health care-related diseases.
2- The highest percentage of isolation for Klebsiella pneumoniae isolates in clinical samples was from urine.
3- There was merely a restricted number of sensitive drugs for these bacteria, and the drug of choice was Ampicillin.
4- The study revealed the spread of blaNDM, blaVIM K. pneumoniae isolates in patients in Baquba Teaching hospital.
5- The rapidly spreading K. pneumoniae MBL genes among the patients.

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6- Isolates were positive for blaNDM-1, blaNDM-2 and blaVIM gene, while no isolates show positive results to blaIMP.

The current research provided a detailed molecular characterization of MBL genes (blaNDM-1, blaNDM-2, blaVIM, and blaIMP) in MDR *K. pneumoniae* isolates. It is fair to accept that the present findings can be essentially used to understand the mechanisms of resistance that would aid to develop novel therapeutic strategies. However, this research ascertained that avoiding misuse in Iraq hospitals, because antibiotic overuse can result in unwanted impacts of multidrug resistant bacteria as well as MBL and the production of bacteria.

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