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Investigation of Some Carbapenemase Resistance Genes of *Klebsiella pneumoniae* Isolates and their Role in the Antibiotics Resistance

[1]Zahraa R. Shamsee, [2]Aida H. Ibrahim

[1,2] Department of Biotechnology, College of Science, University of Baghdad

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Abstract: *Klebsiella pneumoniae* is ubiquitous gram-negative encapsulated pathogen that has long been associated with variety of infections. The aim of the current study was to determine the presence of carbapenemase genes in *K. pneumoniae* isolates obtained from different sources and their role in the antibiotics resistance. A total of 210 clinical samples were collected from patients of both sexes ranging between 10 to 80 years old from different sources; (urine, sputum, wound swabs, blood, and liver abscess) who admitted from different hospitals in Baghdad from (March, 2022 to July, 2022). Results appeared that only 90 isolates were identified as *K. pneumoniae* based on differential and selective media, biochemical tests followed by Vitek 2 systems and molecular detection using *16S rRNA*. Antibiotic susceptibility test of *K. pneumoniae* isolates against 13 antibiotics were established using disc-diffusion technique. Result appeared that all isolates were 100% resistant to Vancomycin. While, Colistin had the lowest resistance rate 25.55% manifested that was the most effective antibiotic. Carbapenemase genes; New Delhi metallolactamase *NDM-1*, Verona integron-encoded metallo β-lactamase *VIM* and imipenem-resistant carbapenemases *IMP* were detected using polymerase chain reaction PCR technique. It was concluded that antibiotics resistance rate was high in *K. pneumonia* isolates. Moreover, Colistin recommended for the treatments of carbapenemase-producing *K. pneumonia*. The PCR results revealed that *IMP* gene was not detected, while the percentage of *NDM-1* and *VIM* genes were 23% and 3% respectively.

Keywords: Antibiotics resistance, carbapemase genes, *K. pneumoniae*, polymerase chain reaction.

Corresponding author: (Email: zahraa.reda1106a@sc.uobaghdad.edu.iq).

Introduction

Antimicrobial resistance is one of the most significant issues increased infection in hospitals. The *K. pneumoniae* accountable among one-third of all Gram-negative infections in the hospitals (1). Furthermore, that makes it the second most significant opportunistic *Enterobacterium* in nosocomial and society infections followed *Escherichia coli* involved pneumonia, meningitis, liver abscess, UTIs and others. Moreover, bacteria can even result in high rates of illness and mortality when treated improperly (2). The *K. pneumoniae* isolates can develop several mechanisms that results in antibiotics resistance include; Carbapenem resistance due to production of carbapenemases, the overuse of carbapenems antibiotics and the horizontal gene transfer of plasmids carry on multi resistance genes would be the reason of carbapenems to resistance and leads to limitations in the treatment options against these drug-resistant bacteria (3,4). Other mechanisms such as alteration outer

membrane permeability, ESBL, over expression of AmpC that hydrolyze many beta-lactam rings of antibiotics, mutations of porins and penicillin-binding proteins (PBP), alteration of metabolic pathways, the presence of efflux pumps and biofilm formation capacity (5).

According to the Ambler classification which depends on sequence and structural homology, class B/ metallo-β-lactamases that hydrolyze carbapenem in a zinc dependent manner involved: imipenem-resistant carbapenemases *IMP*, Verona integron-encoded metallo β-lactamase *VIM* and New Delhi metallolactamase *NDM*. They are encoded by *blaIMP*, *blaVIM* and *blaNDM*, respectively (6). The NDM has been detected in carbapenem-resistant *K. pneumoniae* as well as in *E. coli* in a patient who has been relocated in 2009 from India (7). Multi-drug resistant *Enterobacteriaceae* carrying *NDM-1*gene has been recognized worldwide, bacteria with *NMD-1* may be responsible and more dissemination for urinary tract infections (8). Otherwise, the *VIM* and *IMP* families share certain similarities and both are susceptible to all β-lactam inhibitors and hydrolyze all β-lactams except monobactams (9). Hence, aim of this study: isolation and identification of *K. pneumoniae* isolates from different clinical sources as well as determination of multi-drug resistant (MDR) isolates using antibiotic susceptibility test, molecular detection of some carbapenemase genes including; *NDM-1*, *IMP* and *VIM*.

Materials and methods

Samples collection and bacterial identification

The project was approved by Baghdad College of Science's ethics committee **(**CSEC/0523/0042). In this study two hundred ten clinical samples were obtaining from patients including both sex, their age ranging from (10 to 80) years with different sources (urine, sputum, blood, wound swabs and liver abscess) who admitted from different hospitals in Baghdad (Central Children and Medical city / Educational lab, Teaching Laboratories /Medical city, Al-Yarmuk Hospital, Al-Karama teaching hospital and Al - Kadhimiya hospital), throughout the period from March, 2022 to July 2022. All isolates were identified using selective and differential culture media, biochemical tests, followed by Vitek 2 system and molecular detection using the *16S rRNA* gene.

Antibiotics susceptibility of *K. pneumoniae* isolates

Disk -diffusion method usually test the susceptibility of thirteen antibiotics disc. Disks containing; Ampicillin AMP (10 µg), Cefixime CFM (5 µg), Cefotaxime CTX (30 µg), Chloramphenicol CHL (30 µg), Dorepenem DOR (10 µg), Imipenem IMP (10µg), Nalidixic acid NAL (30 µg), Tetracycline TET (30 µg), Vancomycin VA (30µg), Streptomycin STR (10 µg), Nitrofurantoin NIT (300 µg), Amoxicillin-clavulanate AMC (10/20 µg). Otherwise, Colistin CST (10 µg) was examined using the minimum inhibitory concentration (MIC) by the broth microdilution method. All disks were obtained from (Liofilchmem\ Italy), after the period of incubation at 37ºC. The results were interpreted in accordance with the Clinical Laboratory Standards Institute.

DNA extraction

Extraction of genomic DNA using Monarch® Genomic DNA Purification Kit (NEB, England). The concentration of the extracted DNA was

examined using Qubit 4.0 (Invitrogen/USA).

Polymerase chain reaction (PCR)

Primers design

The specific primers of *NDM-1*, *IMP* and *VIM* genes of *K. pneumoniae* isolates were check for the right annealing temperature using software called Geneious Prime bioinformatics. The primers were further evaluated using a variety of internet applications, including Oligo Analyzer by IDT (Integrated DNA Technology). The primers sequences were showed in (Table 1). Traditional PCR technique started with denaturation phase at 95 °C/ 5 minutes, followed by 30 cycles of denaturation at 95 °C. After that reaction mixture annealed at at 35 cycles for 55 C/ 45 seconds. The reaction mixtures are then subjected to 35 cycles of the extension stage over the course of one minute. The amplification ultimately ceases after seven minutes of the final extension stage at 72 °C. Then PCR product have been visualized using an agarose gel (2%) containing RedSafe dye and an ultraviolet (UV) light.

Table (1): The Designed primers sequences utilized in this study

Primer name		Sequence (5´-3´)	Ambilicon size(bp)	References
16S RNA	F	CGGTCTGTCAAGTCGGATGT	172	This study
	R	AGCGTCAGTCTTTGTCCAGG		
IMP	F	ATTCTCAATCCATCCCCACGT	150	This study
	R	TGTGTCCTGGGCCTGGATAA		
VIM	F	TCATGGCTATTGCGAGTCCG	127	This study
	R	GCGATATGCGACCAAACACC		
NDM-1	F	GCATTAGCCGCTGCATTGAT	146	This study
	R	TAGGAAGTGTGCTGCCAGAC		

Statistical analysis

The Statistical Analysis System SAS program (10) was employed to determine the impact of various factors on study's parameters. The Least Significant Difference (LSD) test (Analysis of Variation, ANOVA) was used in order to considerably compare between the means. In this study, the Chi-square test was used to compare percentages with $p<0.05$.

Results and discussion

Two hundred ten clinical samples were collected from various sources (urine, sputum, blood, wound swabs and liver abscess). Samples were transferred to the lab and streaked on sterile Macconkey and CHROMagar media after samples incubation for 24 hrs. Only 90 isolates showed pink and mucoid colonies on Macconkey agar due to lactose fermentation ability and metallic blue color on CHROMagar Orientation medium which is define as a chromogenic, differential, and highly selective media for detecting *K. pneumoniae* in remarkably green colonies which was agreement with (11) as mentioned in (Figure 1). Biochemical tests also showed positive result for Urease, Citrate, Catalase and Vogues-Proskauer. Otherwise, negative for Indole, Oxidase and Methyl Red. Vitek 2 System results were utilized to confirm the identity of *K. pneumoniae* isolates. Distribution of *K. pneumoniae* isolates were illustrated in (Table 2), which represented that UTIs (57.7%) and sputum (24.4 %) have the highest percentage value, followed by blood (10%), wound swabs (5.5%) and liver abscess that represented the lowest percentage (2.2%). This is because of the UTI a relatively prevalent justification in current practice for consultation and antibiotic prescription.

Results agreement with local study of high percentage for urine 42.26% followed by sputum 26.70% (12). Furthermore, result was consistent with the findings of Tehran hospital and found that *K. pneumoniae* was obtained from different sources including; urine, sputum, and blood with percentages of 61.7%, 11.7%, and 8.5%, respectively (13).

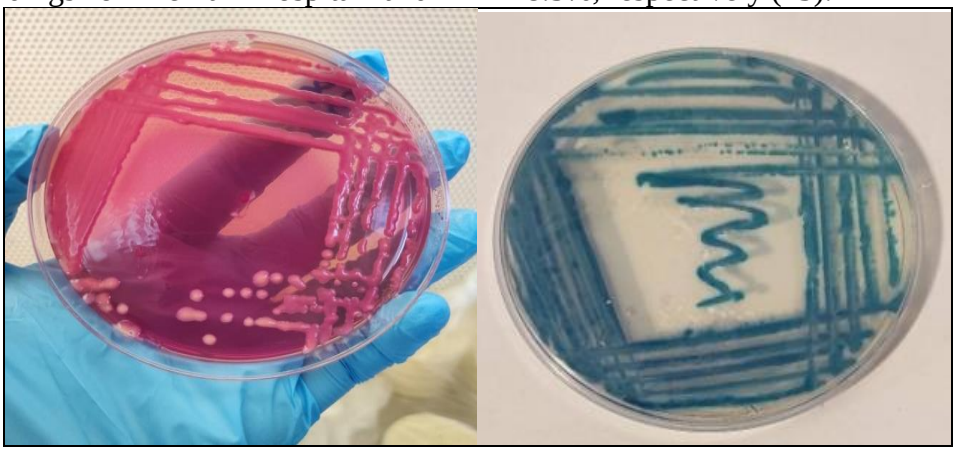

Figure (1): Colonies of *K. pneumoniae* on: A: Macconkey agar, B: CHROMagar after 24 hours of incubation at 37 C.

Table (2): The number and percentage of *K. pneumoniae* isolated from different sources.

Types of Samples	Total Samples N.O	Bacterial strain N.O	Percentage (%*)	Percentage (%**)
Urine	105	52	50%	57.7%
Sputum	41	22	19.5%	24.4 %
Wound	31	5	14.7%	5.5%
Blood	27	9	12.8%	10%
Liver abscess	6	2	2.8%	2.2%
Total	210	90	100%	100%
P-value	---	---	0.0001 **	0.0001 **
** (P≤0.01).				

Percentage (%*) distribution of *K. pneumoniae* relative to the samples, Percentage (%) distribution of *K. pneumoniae* relative to the overall no. of *K. pneumoniae* strain.**

Antibiotic susceptibility test of *K. pneumoniae*

Disk diffusion method was employed for antibiotics susceptibility of 90 *K. pneumoniae* isolates against thirteen antibiotics including; (Ampicillin, Cefixime, Cefotaxime, Chloramphenicol, Colistin, Dorepenem, Imipenem, Nalidixic acid, Tetracycline, Vancomycin, Streptomycin, Nitrofurantoin, Amoxicillin-clavulanate), as mentioned in (Figure 2). The emergence of antimicrobial drug resistant strains has been reported in *K. pneumonia* isolated from community and hospital acquired infections (14). The results revealed that high resistance of isolates against Vancomycin (100%), Ampicillin (97.77%), Amoxicillin-clavulanate (96.66%) followed by Cefixime (94.44%) and Cefotaxime (93.33%) these results are confirmed with a local study in Al-kufa, Iraq (15). On the other hands, isolates showed (35.33%) for Nalidixic acid and (25.55%) for Colistin. From the present study colistin represented high sensitivity that were approval with a study by Abdelhamid *et al*. (16) who notified that colistin was

effective against all 50 Egyptian isolates that were examined. While, other isolates shown resistance to Nitrofurantoin (66.66%), Tetracycline (56.66%), Streptomycin (53.33%), Chloramphenicol (41.11%). production of specified enzymes, reduced cell permeability due to Omps depletion and over expression of efflux pumps are the main mechanisms that contribute to antibiotic resistance. Carbapenemase play an important role in MDR resistant mechanism which hydrolyze β-lactam antibiotics (17).

Colistin has become more widely used to treat Gram-negative bacteria that are resistant to carbapenem and multidrug resistant (MDR) as a rescue therapy, either by itself or in conjunction with one or more antimicrobials (18). The mechanism of action of colistin is based on the electrostatic interaction between the cationic residues of the drug's di-aminobutyric acid, and the anionic phosphate groups of lipid A presence in bacterial lipopolysaccharide (LPS). It destabilizes the LPS, resulting in raised cell membrane permeability and causes cell leakage and death. So it has been used as a first-choice medication, particularly in critical care units, due to high rates of carbapenem-resistance among KPC- producing *K. pneumonia* (19).

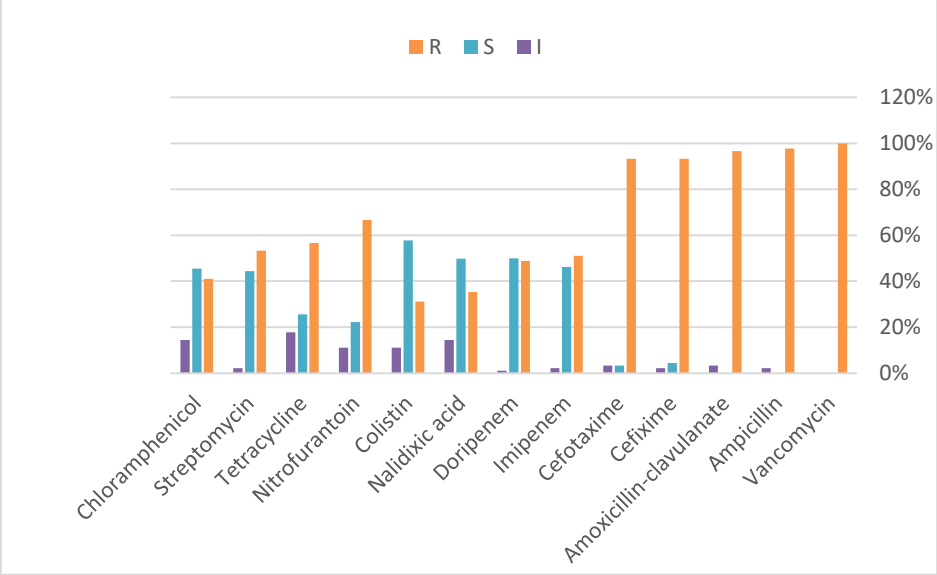

Figure (2): Antibiotics sensitivity test of 90 *K. pneumoniae* isolates obtained from various sources.

Molecular detection of *K. pneumoniae* using *16S rRNA*

The *16S rRNA* gene, a gene used to identify *K. pneumoniae*, has been tested using a polymerase chain reaction on each extracted DNA sample. The results of the PCR revealed positive *16S rRNA* gene which appeared in single band with molecular base 172 bp by comparing the molecular size of the bands to a 100bp DNA ladder (Figure3). The findings presented here are in agreement with previous study by Ghaima and Tamara (20), who collected 260 clinical samples from urinary tract infection patients, and the results showed that only 76 of the 260 bacterial isolates belonged to *K. pneumoniae* by combining traditional methods with a molecular diagnostic approach based on the *16S rRNA* gene (159 bp).

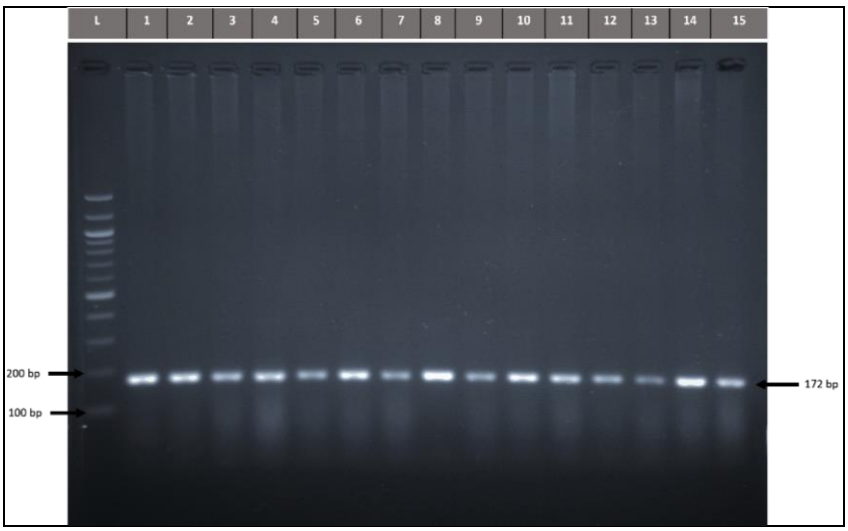

Figure (3): Gel electrophoresis of *16S rRNA* gene (172bp) amplification for *K. pneumoniae.* Lane L: 100-200 bp DNA ladder; Lanes 1-15: *K. pneumoniae* isolates positive. (2% agarose gels and stained with Red safe, 80V for 80 min.).

Molecular detection of *NDM-1, VIM, IMP* genes

According to multiple drug resistance (MDR) of *K. pneumoniae* a totally of 80 isolates were amplified with PCR for diagnosis the presence of *NDM-1*, *IMP* and *VIM* genes using specific designed primers.

The results of DNA extraction by gel electrophoresis were PCR-positive for *NDM-1* (146bp) and *VIM* (127bp) as compared with 100bp DNA Ladder as illustrated in (Figures 4, 5). Otherwise, *IMP* (150bp) gene was not detected. Results revealed that *NDM-1* was the most prevalent of Carbapenemase detect genes with (18/ 23%) *Klebsiella* isolates. While, *VIM* gene presented only in two (3%) isolates.

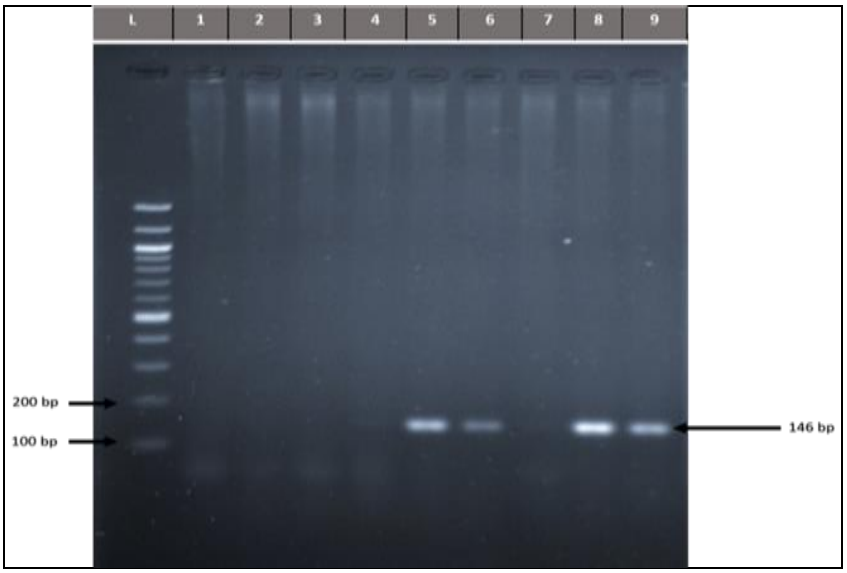

Figure (4): Agarose gel electrophoresis of PCR for the *NDM-1* gene (146bp). Lane M: 100-200 bp DNA ladder; Lanes 1-9: *K. pneumoniae* isolates. (agarose 2%, and stained with Red safe, 80V for 80 min.).

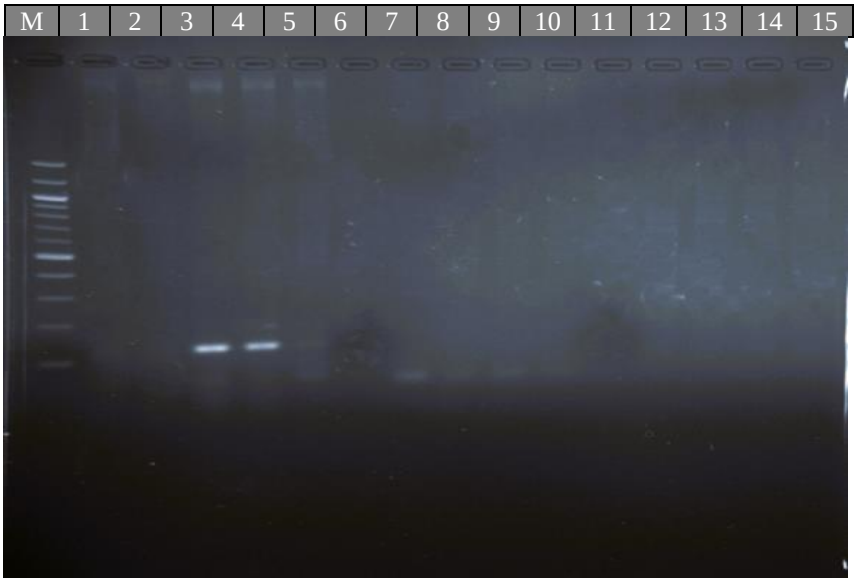

Figure (5): Agarose gel electrophoresis of PCR for the *VIM* gene (127bp). Lane M: 100-200 bp DNA ladder; Lanes 1-9: *K. pneumoniae* isolates. (agarose 2%, with Red safe stain, 80V for 80 min.).

The enzymes of the VIM, IMP, and NDM are groups in Ambler class B, that cannot be physically bound to the beta-lactam substrate and therefore escape the action of beta-lactamase inhibitors such as clavulanic acid and sulbactam and are able to bind to all beta-lactam classes, except monobactam (21). These genes responsible for the production of carbapenemase and carbapenems resistance in *K. pneumonia.* Emerging *NDM-1* provides resistance to all β-lactam antibiotics; it is primarily seen in *E. coli, K. pneumoniae,* and *A. baumannii* as hosts. It has been determined that Pakistan and India are reservoirs of NDM producers due to their *NDM-1* gene (22). The *NDM* gene which was found in 43% of the isolates in the Egyptian study may be the causes of carbapenem resistance, according to PCR data, this finding may be strongly linked to a number of resistance mechanisms that resulted in resistance to the majority of antibiotic classes (23). Study from Turkey examined 77 isolate of *K. pneumoniae* and revealed that nine isolates (10.84%) produced *NDM-1*(24). Other carbapenems genes; *VIM* and *IMP* were detected primarily in *Pseudomonas aeruginosa* and commonly observed at a shallow rate among *Enterobacteriaceae.* On the other hands, An Iraqi study of *K. pneumoniae* strains reminded no *blaVIM* gene distribution among carbapenem-resistant *K. pneumonia* and just five strains were illustrated as positive for *blaIMP* gene 5 (9.1%), while results showed the presence of a *blaNDM-1* gene in 37 (67.27%) carbapenem-resistant *K. pneumoniae* strains (25). Moreover, Mahmood (26) who revealed that Carbapenemase-producing *E. cloacae* has been reported carbapenemase genes *blaNDM* (n=4/8, 50%) and *blavim* (n=3/8, 37%).

Conclusion

In this study, all of *K. pneumoniae* isolates revealed 100% resistant to Vancomycin followed by Ampicillin, Amoxicillin-clavulanate, Cefixime. On the other hands the lowest resistance was against colistin with a percentage 25.55% that was the most

effective antibiotic applied in the present study. Molecular detection of carbapenemase genes achieved using PCR technique. The distribution of carbapenemase *NDM-1* gene was more frequent gene than *VIM*. On the other hands, *IMP* gene not detectable. All *K. pneumoniae* isolates that have carbapenemase genes are resistant to most antibiotics used.

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