

Tracking Extended Spectrum B -Lactamase and Carbapenemase Resistance Genes in *Klebsiella pneumoniae* Clinical Strains in Al-Diwaniyah, Iraq

Mohanad Jawad^{1*}

¹College of Biotechnology, University of Al-Qadisiyah, Iraq

*Corresponding Author: Mohanad Jawad
College of Biotechnology, University of Al-Qadisiyah, Iraq

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Abstract: The present cross-sectional study addresses the molecular epidemiology of *Klebsiella pneumoniae* clinical strains isolated from pneumonia patients, recruited from Al-Diwaniyah teaching hospital from January 2025 to May 2025, regarding the presence of some carbapenemase and extended spectrum β -lactamase (ESBL) resistance genes using conventional polymerase chain reaction. Biochemical profile (VITEK2 system) and molecular identification (*16S rRNA* gene sequence analysis) of the isolated clinical strains confirmed their affiliation to *K. pneumoniae* sensu stricto with 20% prevalence among the cohort study patients. ESBL resistant genes showed frequency of occurrence 55, 70, and 75% for *blaCTX-M8*, *blaTEM*, and *blaSHV*, respectively. Serine Carbapenemase (Class A) exhibited 5 and 50% frequency of occurrence for *blaKPC* and *blaIMI*, respectively. MBL (Metallo- β -lactamase, Class B) – Carbapenemase displayed 5, 5, 10, and 50% for *blaIMP*, *blaGIM*, *blaVIM*, and *blaNDM*, respectively. Class D Carbapenemases showed 60% frequency of occurrence for *blaOXA-48*. Finally, the *rCSA* carbapenemase exhibited 90% frequency of occurrence. The current findings evidence an alarming increase regarding the circulation of ESBL and carbapenemase resistant genes harbored by *K. pneumoniae* sensu stricto clinical strains in the Al-Diwaniyah region. These findings underline a crucial obligation to re-evaluate the practical antimicrobial therapy approaches targeting pulmonary diseases caused by *K. pneumoniae* clinical strains in the Al-Diwaniyah region, to fight the growing circulation of ESBL and carbapenemase producing *K. pneumoniae* clinical strains.

Keywords: Cross-Sectional Study; *Klebsiella pneumoniae* sensu stricto; Triple Carbapenemase resistant genes; Extended Spectrum β -lactamase resistance genes; Polymerase chain reaction.

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INTRODUCTION

Klebsiella pneumoniae, a Gram-negative bacterium belonging to the Enterobacteriaceae Family, have displayed implications in numerous infectious diseases such as urinary tract infections, pulmonary infections, bloodstream infections, and necrotizing liver abscesses worldwide (Podschun and Ullmann 1998). It predominantly influences newborns, the elderly, and immuno-compromised individuals (Bengochea and Pessoa 2019). *K. pneumoniae* impacts are not only restricted to the pathogenicity and virulence but also extends to the emergence of multidrug resistance strains harboring carbapenemases and beta-lactamases resistance genes that would infer multidrug resistance against carbapenems and Beta-lactam ring containing antibiotics (Al-Agamy *et al.*, 2009; Tawfik *et al.*, 2011;

Somily *et al.*, 2014; Jiang *et al.*, 2025; Aldali JA 2025). The emergence of Beta-lactamases and carbapenemases that have the capability to hydrolyze Beta-lactam ring antibiotics and carbapenems totally compromised the efficiency of these lifesaving antibiotics (Perez *et al.*, 2007). Extended Spectrum Beta-lactamases (ESBL), synonymously called β -lactamases, and carbapenemases are plasmid-mediated enzymes that confer resistance to 1st, 2nd, 3rd, and 4th generation cephalosporins, aztreonam, and carbapenem (Bhaskar *et al.*, 2019).

ESBL is mainly existed in *Klebsiella* spp. and *Escherichia coli* (Kang *et al.*, 2004). The most predominant ESBLs are encompassed in three groups: TEM, SHV and CTX-M (Lin *et al.*, 2010). Acquired carbapenemases assign to group A (IMI, NMC, SME

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GES, and *Klebsiella pneumoniae* carbapenemase (KPC), group B metallo- β -lactamase (MBLs of VIM, IMP, GIM, NDM, SIM, and DIM), and group D (carbapenem hydrolyzing oxacillinases e.g. OXA -48) (Nordmann *et al.*, 2011). The multidrug resistance against multiple sets of antibiotics, directed by *K. pneumoniae*, has left a limited number of antibiotics for treatment purposes.

The objective of the present work is to unveil the dominant etiological agent of clinical strains isolated from pneumonia patients in Al-Diwaniyah Province, Iraq. The aim is extended to track their genetic make-up regarding some antibiotic resistance genes targeting these antibiotic groups and underscoring the antimicrobial resistance phenomenon: ESBL-resistant genes (*blaTEM*, *blaSHV*, and *blaCTX-M8*), carbapenemase-resistant genes (*blaKPC*, *blaIMI*, *blaIMP*, *blaGIM*, *blaVIM*, *blaNDM*, *blaOXA-48*, and *rCSA*) using conventional PCR and gene-specific primer sets.

MATERIALS AND METHODS

Sampling

During the period from January to May 2025, 100 transtracheal wash samples were collected from patients diagnosed with pneumonia at Al-Diwaniyah Hospital, Iraq. No demographic patient data was collected or presented in this study. Furthermore, no demographic patient data was subjected to correlation analysis with the laboratory data on antibiotic resistance genes. Patients' names were anonymized. The laboratory data/samples were irrevocably stripped of all identifiers by a party independent of the researchers (meaning the researchers never had access to the direct identifiers). Moreover, the laboratory collected data regarding the antibiotics resistance genes had not indirect identifiers except the diagnosis with pneumonia. The type of samples collected from the patients is categorized as secondary data not primary data (e.g. blood samples to isolated individuals' genomic DNA). Consequently, specific additional ethics approval was not required.

Bacteriological Media

MacConkey agar (HiMedia, Canada) was used in the course of bacterial culturing from the collected transtracheal wash samples.

Gram -staining

Gram-staining procedure was applied to each clinical strain enrolled in this study to unravel the Gram stain type of the quest strain. Gram-staining procedure was conducted using kit (HiMedia Laboratories, India) according to the manufacturer instructions.

Biochemical Identification

The clinical strains enrolled in this study underwent biochemical identification using VITEK2 system (BioMerieux, France). The steps were directed according to the instructions of the manufacturer. Shortly, a pure young culture from each clinical strain

(18-24 hrs) was prepared. A bacterial suspension in saline (0.45% NaCl solution) was prepared, adjusted to a turbidity of 0.5 MacFarland standard tube using an ATB 1550 densitometer (bioMérieux). The time elapse between preparation of the suspension and card filling should be not exceeding 25 min. The identification card for Gram-negative bacilli (GN card) for the VITEK 2 system is a 64-well plastic card containing 47 colorimetric biochemical tests, including carbon source utilization (fermentation/assimilation), enzymatic activities, and resistance/inhibition. The carbon source utilization tests include the fermentation of the following sugars: D-glucose, D-mannitol, saccharose/sucrose, G-maltose, D-sorbitol, D-trehalose, adonitol, citrate, and malonate, L-histidine, and 5-keto-D-gluconate. Whilst the enzymatic tests include beta-galactosidase, urease, ornithine decarboxylase, lysine decarboxylase, beta-acetyl glucosaminidase, L-proline arylamidase, phosphatase, and gamma glutamyl transferase. However, the miscellaneous/resistance tests include H₂S production, Ellman, O/129 resistance, and fermentation glucose. The card was automatically filled by a vacuum device and automatically sealed. It was manually inserted in the VITEK2 reader-incubator module (incubation temperature, 35.5 °C), and every card was automatically subjected to colorimetric measurement every 15 min. The results were interpreted by the GN database after the incubation period of 3 h. For reporting of the results, there are five identifications that could be obtained as follow: (i) correctly identified (correctly identified to the species level). (ii) low discrimination resolved (iii) low discrimination not resolved (iv) misidentified; (v) not identified meant that no identification was given at all.

Genomic DNA isolation

Genomic DNA was extracted from each clinical strain isolated in this study using GeneJET™ Genomic DNA Purification Kit (Thermo Fisher Scientific Co., USA) according to the manufacturer's instructions. The concentration of the isolated genomic DNA in terms of ng/ μ L was determined using the Nanodrop-Spectrophotometer (Thermo Fisher Scientific Co., USA). Nevertheless, the quality of the isolated genomic DNA was assessed using UV-Transilluminator (Clever, UK) after running on 1% agarose gel electrophoresis and measuring the absorbance ratios Ab260/Ab280 and Ab260/Ab230. The ideal absorbance ratios should be 1.8 to <2.0 and 2.0-2.3 for Ab260/Ab280 and Ab260/Ab230, respectively.

Molecular identification and bioinformatics analyses

The *16S rRNA* gene sequence analysis was employed in this study to identify the clinical strains enrolled in this study. The full length of *16S rRNA* gene (1500 bp) was isolated from each strain enrolled in this study using polymerase chain reaction (PCR) and 16S rRNA universal primer set designated F27 and R1492 (Eden *et al.*, 1991) (Table 1). The PCR mixture contained 50 μ L: 30 ng genomic DNA, 0.3 μ M forward primer, 0.3

μM reverse primer, 25 μL PCR Master Mix(2X) (Promega Co., France), and the final volume was completed to 50 μL with nuclease-free water. The thermocycler conditions were established (Eden *et al.*, 1991) as mentioned in Table 1. The PCR products were subjected to Thermo Scientific™ GeneJET PCR Purification Kit, Thermo Fisher Scientific Co., USA) according to the manufacturer's instructions. DNA sequencing (Eurofin DNA Co., Luxembourg) for each amplified 16S rRNA PCR product was achieved in two directions: forward and reverse, using the aforementioned primer set. The obtained nucleotide sequences were subjected to Geneious R 8.0 software to

analyze the nucleotide sequences by trimming the overlapped non-sharp peaks. The cleaned 16S rRNA nucleotide sequences were subjected to BLASTN similarity sequence analysis search to identify the nucleotide sequences in question. The last step included the submission of each defined 16S rRNA nucleotide sequence in the GenBank database under certain accession numbers after assigning each speciated clinical strain on a genus and species level to a strain designation. A phylogenetic tree was constructed using MEGA 12.0 to demonstrate the genetic relatedness among the present 16S rRNA nucleotide sequences and other closely related sequences.

Table 1: Primer sequences and PCR product lengths for amplified genes in this study.

Gene	Primer sequence	PCR product (bp)	PCR conditions	References
Ribosomal RNA				
<i>16S rRNA</i>	F:8-27:5'- AGAGTTTGATCCTGGCTCAG-3' R:1510-1492: 5'-GGTTACCTTGTTACGACTT-3'	1500	Annealing temp.:58 °C Extension time.: 1.5 min	Eiden <i>et al.</i> , 1991
Carbapenem resistant genes, Class B, Metallo-β-lactamase				
<i>blaNDM</i>	F:5'- GCCGACACTGAGCACTAC-3' R: 5'- GGGAACGCCCGACCAAAC-3'	53	Annealing temp.:58 °C Extension time.: 10 sec	Samadi <i>et al.</i> , 2023
<i>blaVIM</i>	F:5'- GATTGCCGATGGTGTGGTGG -3' R: 5'- GAGAAGTGCCGCTGTGTT -3'	145	Annealing temp.:58 °C Extension time.: 20 sec	Samadi <i>et al.</i> , 2023
<i>blaIMP</i>	F: 5'- TTGACACTCCATTTACTG-3' R: 5'- ATTAAGCCACTCTATTCC-3'	131	Annealing temp.:57 °C Extension time.: 20 sec	Samadi <i>et al.</i> , 2023
<i>blaGIM</i>	F: 5'- GTCAATTCCTACATACATCAGA-3' R: 5'- CCTAAGCCTTCCCACTCA-3'	231	Annealing temp.:58 °C Extension time.: 20 sec	Samadi <i>et al.</i> , 2023
Carbapenem resistant genes, Class A, Serine Carbapenemase				
<i>blaKPC</i>	F: 5'- GGTCACCCATCTCGGAAA-3' R: 5'- GCGGCGTTATCACTGTATTG-3'	88	Annealing temp.:58 °C Extension time.: 10 sec	Samadi <i>et al.</i> , 2023
<i>blaIMI</i>	F: 5'- TGAACGATTTCCATTATGTAGT -3' R: 5'- TATTGTAAAGCGGCAGCA-3'	198	Annealing temp.:57 °C Extension time.: 15 sec	Samadi <i>et al.</i> , 2023
Carbapenem resistant genes, Class D, Carbapenemase				
<i>blaOXA-48</i>	F: 5'-AGAATAAGCAGCAAGGAT-3' R: 5'- CCGAATAATATAGTCACCATT-3'	473	Annealing temp.:58 °C Extension time.: 20 sec	Samadi <i>et al.</i> , 2023
Carbapenem resistant genes, <i>Rhodoferox</i> Carbapenemase				
<i>rCSA</i>	F: 5'-GCTGTTTGTATCTTTATGTC-3' R: 5'- AATCACCTGCTTATTATGC-3'	133	Annealing temp.:57 °C Extension time.: 15 sec	Samadi <i>et al.</i> , 2023
β-lactam resistant genes, ESBL				
<i>blaTEM</i>	F: 5'-GAGTATTCAACATTTCCGTGTC-3' R: 5'- TAATCAGTGAGGCACCTATCTC-3'	861	Annealing temp.:55 °C Extension time.: 45 sec	This study
<i>blaSHV</i>	F: 5'- TGTATTATCTC(C/T)CTGTTAGCC(A/G)CCCTG-3' R: 5'-GCTCTGCTTTGTTATTCGGGCCAAGC-3'	868	Annealing temp.:55 °C Extension time.: 45 sec	This study
<i>blaCTX-M-8</i>	F: 5'- AACGCACAGACGCTCTACC-3' R: 5'-GGGTAGCCCAGCCTGAAT-3'	517	Annealing temp.:60 °C Extension time.: 25 sec	This study

- All extension and annealing steps among different protocols were conducted at 72 °C and 30 secs, respectively.
 - All denaturation steps among different protocols were conducted at 94 °C for 30 sec.
 - Classification of β-lactamases is based on Ambler, SRP (1980).

PCR amplification of antibiotics-resistance genes

Eleven β-lactam antibiotics-resistant genes were detected in the isolated clinical strains using PCR directed with gene specific primer sets. These genes

encoded β-lactam containing antibiotics resistance genes, classified into four classes of carbapenemase resistance genes and a EBSL group (Ambler RP 1980). These genes were designated *blaNDM*, *blaIMP*, *blaVIM*,

and *blaGIM* for Carbapenem resistant genes, Class B, Metallo-*B*-lactamase, *blaKPC* and *blaIMI* for Carbapenem resistant genes, Class A, serine carbapenemase, *blaOXA-48* for Carbapenem resistant genes, Class D, Carbapenemase, rCSA for Carbapenem resistant genes, *Rhodoferox* Carbapenemase, and *blaTEM*, *blaSHV*, and *blaCTX-M* for *B*-lactam resistant genes, EBSL. The PCR mixture for the amplification of each gene contained 25 μ L (30 ng genomic DNA, 15 pmole of each forward and reverse primer, 12.5 μ L PCR 2X Master Mix (Promega Co., USA) and the reaction volume was completed to 25 μ L with nuclease-free

water. The PCR conditions were settled as shown in Table 1.

RESULTS

Klebsiella pneumoniae clinical strains

Preliminary identification

Preliminary isolation of pathogenic bacteria from 100 transtracheal wash clinical samples on MacConkey agar revealed pink colonies due to lactose utilization with production of acid (Figure 1A). Finally, microscopic examination of Gram-stained cells proved that the strains exhibited Gram-negative appearance visualized as short rods (Figure 1B).

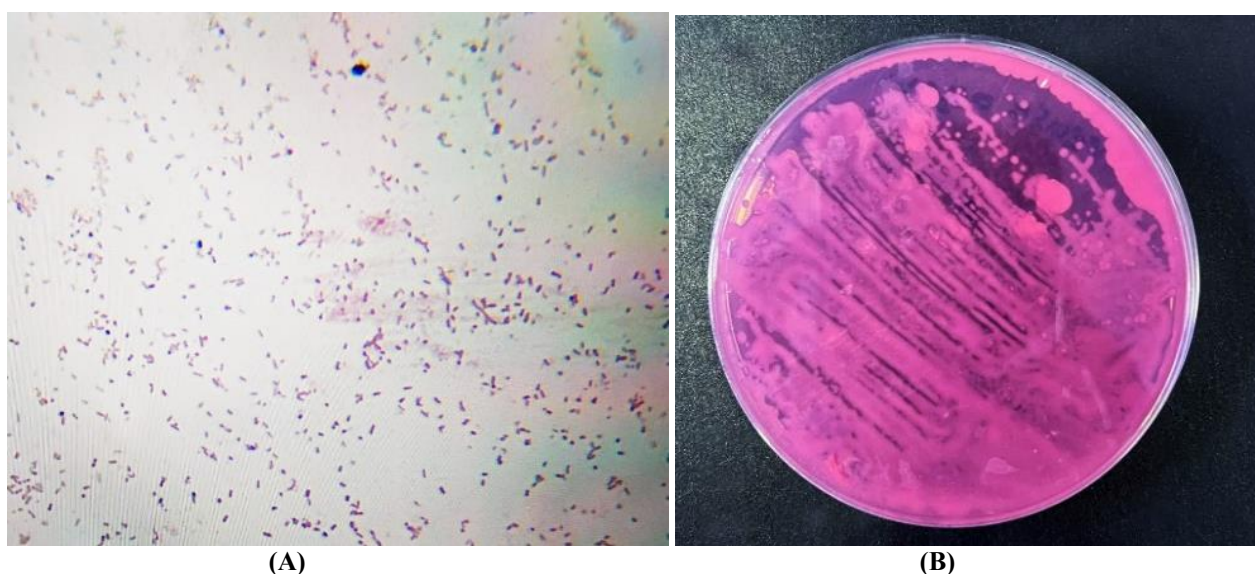


Figure 1: Colonial and cell morphological features of pathogenic bacteria isolated from transtracheal clinical samples for one strain, a representative example. A: MacConkey agar plate, B: Gram-stained bacterial film visualized under light microscope using oil immersion lens (100X).

Biochemical profile of *Klebsiella pneumoniae* on VITEKS for identification

The twenty clinical strains biochemical profile on VITEK2 system exhibited typical biochemical profile of *Klebsiella pneumoniae*.

16S rRNA gene sequence analysis

BLASTN similarity sequence analysis revealed that the obtained 16S rRNA nucleotide sequences of the twenty clinical strains were affiliated to *Klebsiella pneumoniae*. These 16S rRNA nucleotide sequences were submitted in GenBank database under the accession numbers: PX909930-PX909949. A phylogenetic tree constructed with MEGA 12.0 was depicted in Figure 2. This Neighbor-Joining phylogenetic tree revealed that

the twenty study *K. pneumoniae* strains could be grouped into two clades namely clade I and clade II. Clade I encompassed 9 study strains namely MQBA1, MQBA15, MQBA8, MQBA16, MQBA7, MQBA14, MQBA4, MQBA11, and MQBA19 under the accession numbers: PX909930, PX909944, PX909937, PX909945, PX909936, PX909943, PX909933, PX909940, and PX909948, respectively. However, clade II included 11 study strains namely MQBA1, MQBA13, MQBA3, MQBA18, MQBA10, MQBA2, MQBA9, MQBA17, MQBA5, MQBA12, and MQBA20 under the accession numbers: PX909935, PX909942, PX909932, PX909947, PX909939, PX909931, PX909938, PX909946, PX909934, PX909941, and PX909949, respectively.

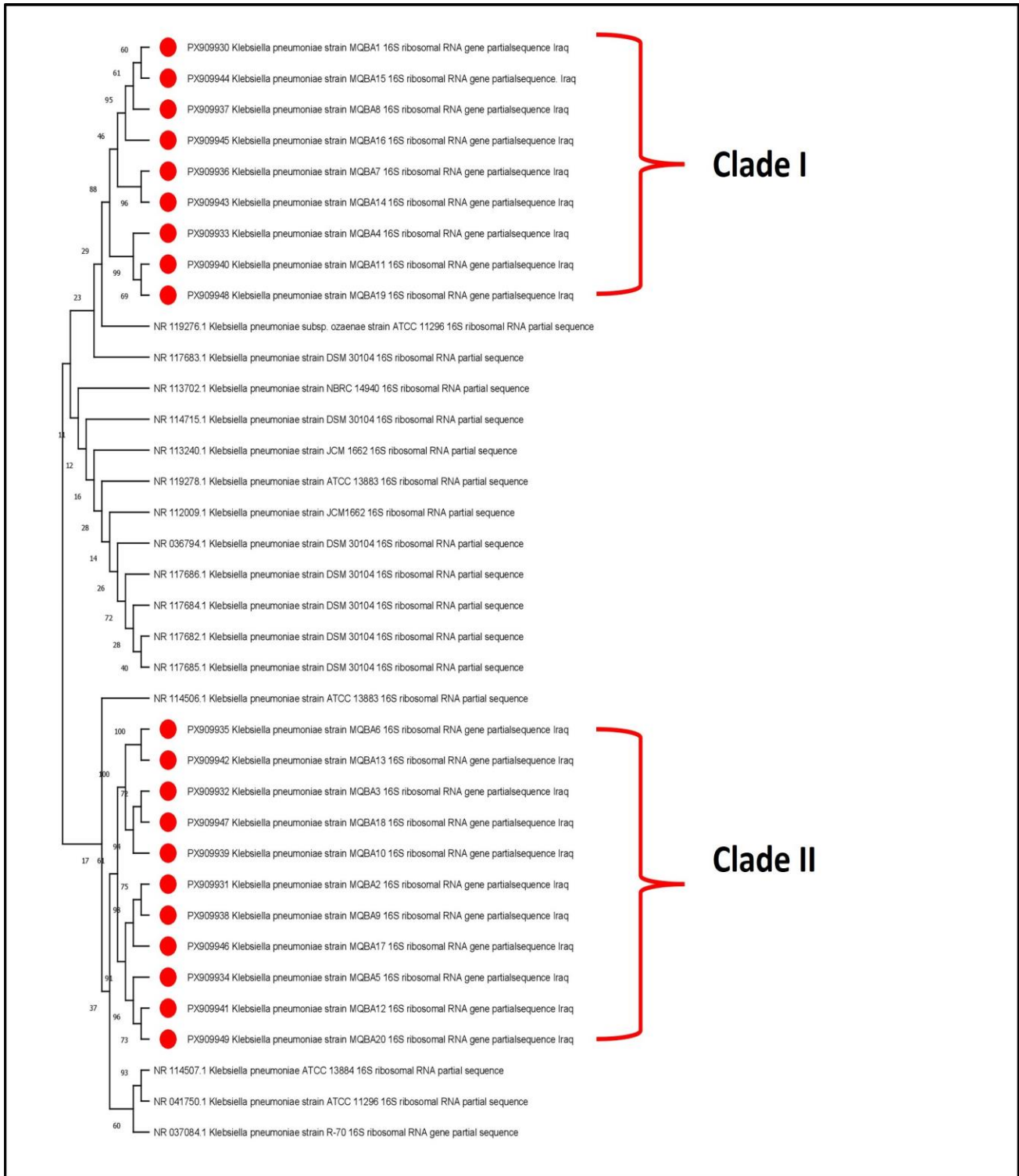


Figure 2: A Neighbor-Joining tree constructed by MEGA 12.0 software illustrating the phylogenetic relationship among the twenty study clinical strains of *K. pneumoniae* and other closely related strains worldwide. Red Bullets: the 16S rRNA nucleotide sequences of the twenty study clinical strains of *K. pneumoniae*. Numbers on branches: Bootstrapping values derived from 1,000 re-samplings. The twenty study clinical strains of *K. pneumoniae* were distributed in two clusters or clades namely Clade I and Clade II.

Genotyping of *K. pneumoniae* clinical strains

i) Carbapenem resistant genes, Class B, Metallo-*B*-lactamase prevalence

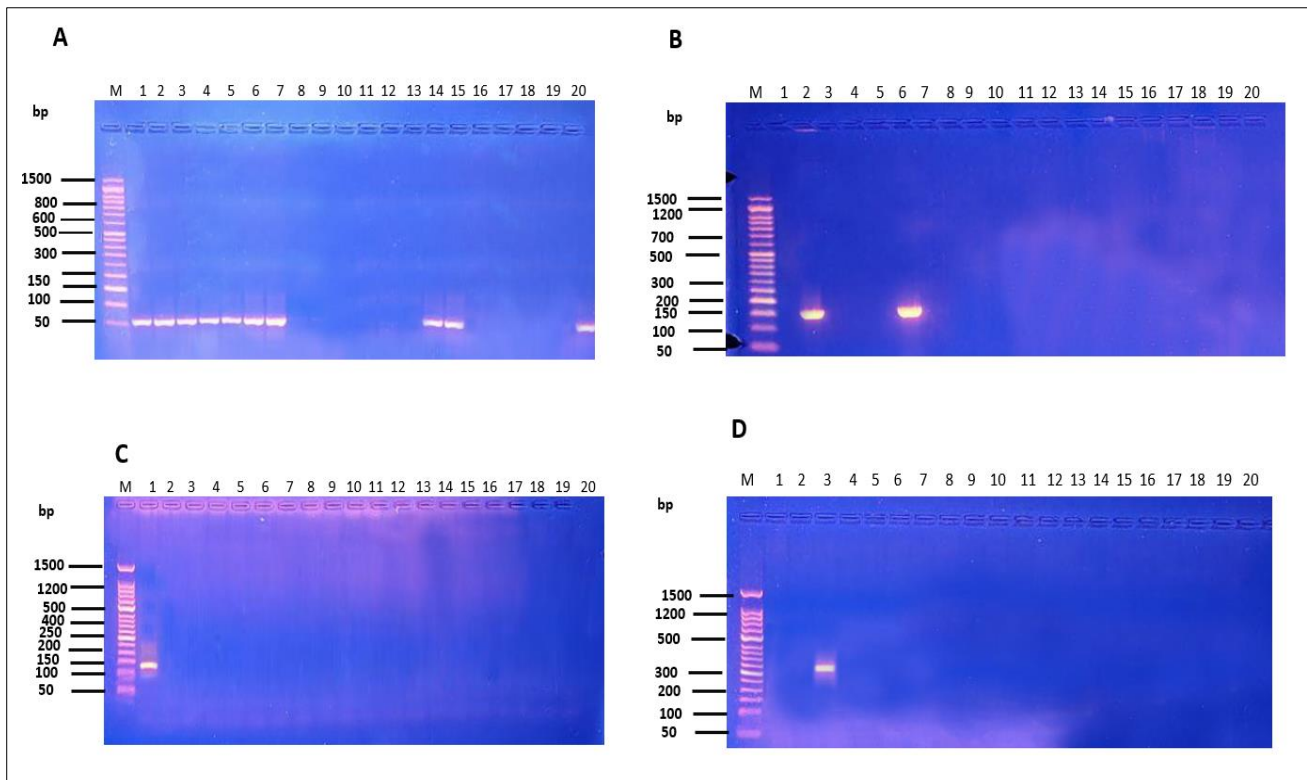


Figure 3: 1% agarose gel electrophoresis showing the amplification of partial fragments of four carbapenem resistant genes, Class B, Metallo-*B*-lactamase: *blaNDM* (A), *blaVIM* (B), *blaIMP* (C), and *blaGIM* (D). Lane M: DNA ladder. Lanes 1-20 in each panel: PCR products for a partial fragment amplified from the relative carbapenem resistant encoding gene among the twenty *K. pneumoniae* clinical strains.

The prevalence of four carbapenem resistant genes, Class B, Metallo-*B*-lactamase (*blaNDM*, *blaVIM*, *blaIMP*, and *blaGIM*) was recorded among the twenty *K. pneumoniae* clinical strains as demonstrated in Figure 3. A partial fragment of 53 bp of *blaNDM* gene was successfully amplified from 10 out of 20 (50%) clinical strains (Figure 3A), however, 2 out of 20 (10%) strains harbored the *blaVIM* gene (Figure 3B), deciphered from a partial fragment of 145 bp. Only 1 out of 20 (5%) clinical strain showed a partial fragment of 131 bp encoding *blaIMP* gene (Figure 3C). Similarly, 1 out of 20 (5%) clinical strain exhibited a partial fragment of 231 bp encoding *blaGIM* gene (Figure 3D)

ii) Carbapenem resistant genes, Class A, Serine Carbapenemase prevalence

The prevalence of two carbapenem resistant genes, Class A, Serine Carbapenemase was recorded among the twenty *K. pneumoniae* clinical strains as demonstrated in Figure 4. A partial fragment of 198 bp of *blaIMI* gene was successfully amplified from 10 out of 20 (50%) clinical strains (Figure 4A), however, 1 out of 20 (5%) strain harbored the *blaKPC* gene (Figure 4B), deciphered from a partial fragment of 88 bp.

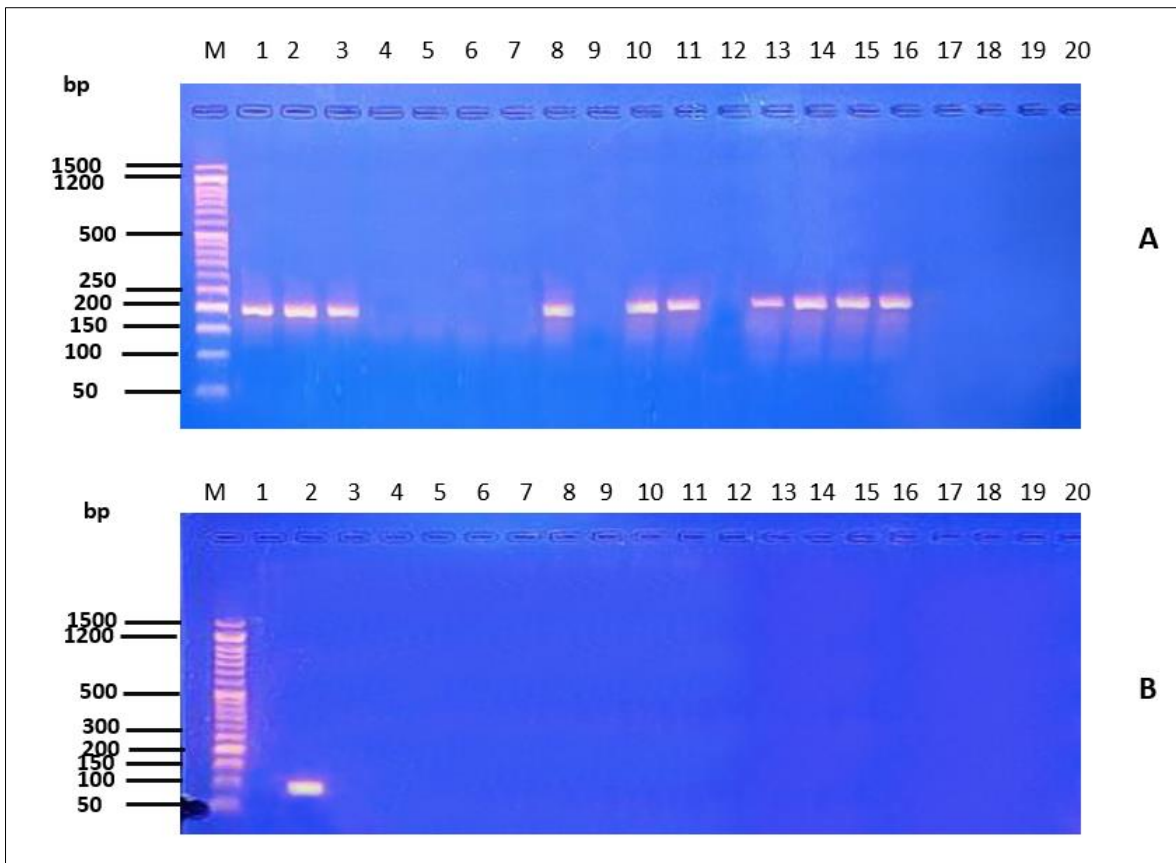


Figure 4: 1% agarose gel electrophoresis showing the amplification of partial fragments of two carbapenem resistant genes, Class A, Serine Carbapenemase: *bla_{IMI}* (A) and *bla_{KPC}* (B). Lane M: DNA ladder. Lanes 1-20 in each panel: PCR products for a partial fragment amplified from the relative carbapenem resistant encoding gene among the twenty *K. pneumoniae* clinical strains.

iii) Carbapenem resistant genes, *Rhodoferax* Carbapenemase (*rCSA*) prevalence

The prevalence of carbapenem resistant genes, *Rhodoferax* carbapenemase (*rCSA*) was studied among

the twenty *K. pneumoniae* clinical strains as demonstrated in Figure 5. A partial fragment of 133 bp of *rCSA* gene was successfully amplified from 18 out of 20 (90%) clinical strains (Figure 5).

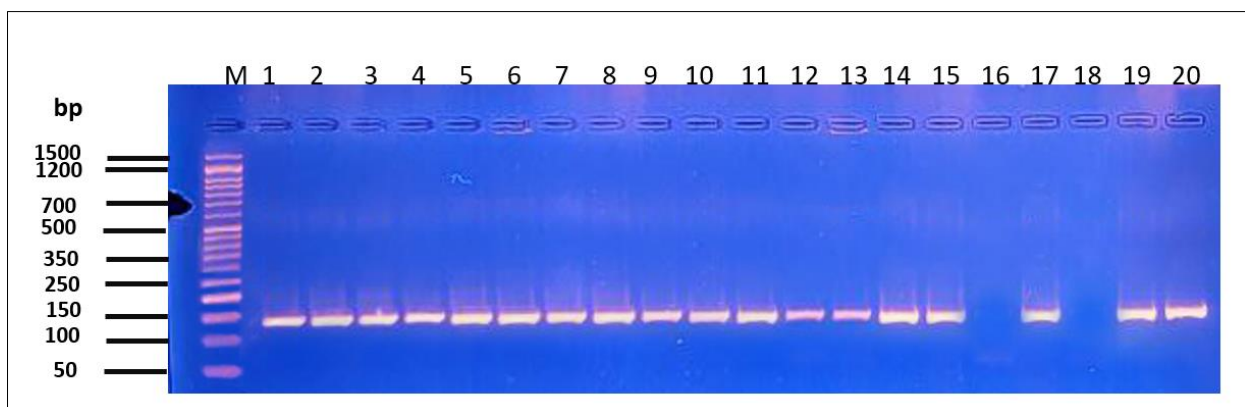


Figure 5: 1% agarose gel electrophoresis showing the amplification of a partial fragment of carbapenem resistant genes, *Rhodoferax* carbapenemase (*rCSA*). Lane M: DNA ladder. Lanes 1-20 in each panel: PCR products for a partial fragment amplified from the relative carbapenem resistant encoding gene among the twenty *K. pneumoniae* clinical strains

iv) Carbapenem resistant genes, Class D, Carbapenemase prevalence

The prevalence of carbapenem resistant genes, Class D, Carbapenemase (*bla_{OXA-48}*) was investigated

among the twenty *K. pneumoniae* clinical strains as demonstrated in Figure 6. A partial fragment of 473 bp of *bla_{OXA-48}* gene was successfully amplified from 12 out of 20 (60%) clinical strains (Figure 6).

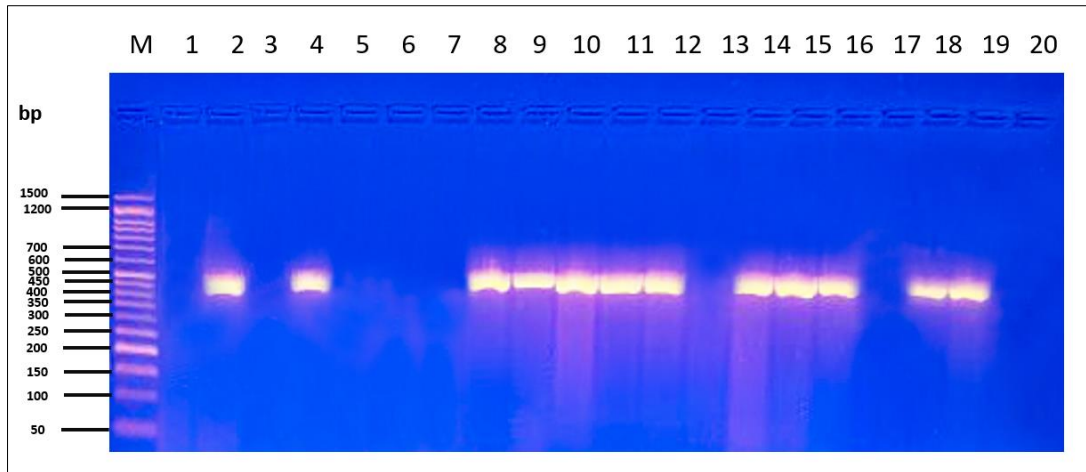


Figure 6: 1% agarose gel electrophoresis showing the amplification of a partial fragment of carbapenem resistant genes, Class D, carbapenemase, *blaOXA-48*. Lane M: DNA ladder. Lanes 1-20 in each panel: PCR products for a partial fragment amplified from the relative carbapenem resistant encoding gene among the twenty *K. pneumoniae* clinical strains

v) β -lactam resistant genes, EBSL prevalence

The prevalence of three β -lactam resistant genes (EBSL): *blaTEM*, *blaSHV*, and *blaCTX-M* was investigated as shown in Figure 7. A partial fragment of 861 bp of *blaTEM* gene was successfully amplified from 14 out of 20 (70%) clinical strains (Figure 7A),

however, 15 out of 20 (75%) strains harbored the *blaSHV* gene (Figure 7B), deciphered from a partial fragment of 868 bp. Only 11 out of 20 (55%) clinical strain showed a partial fragment of 517 bp encoding *blaCTX-M8* gene (Figure 7C).

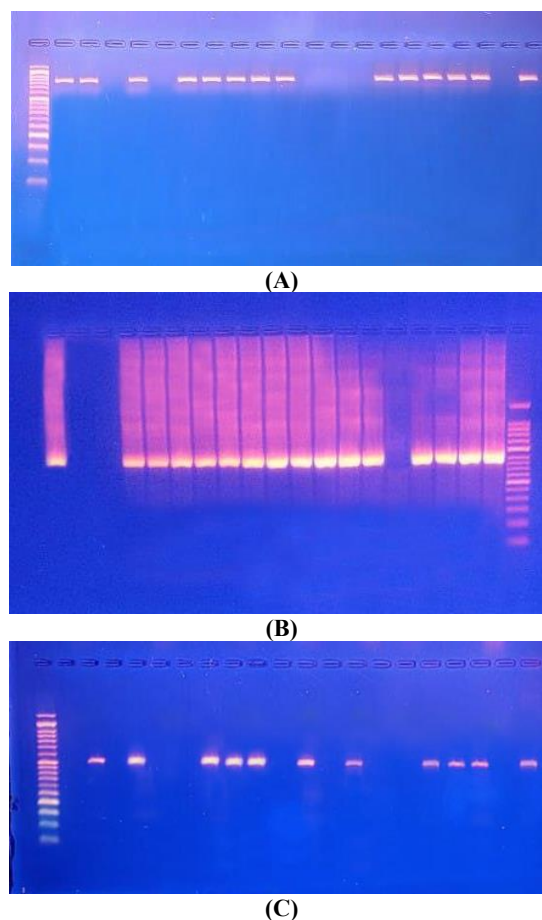


Figure 7: 1% agarose gel electrophoresis showing the amplification of a partial fragment of ESBL genes (*blaTEM*, *blaSHV*, and *blaCTX-M*). *blaTEM* (A), *blaSHV* (B), and *blaCTX-M-8* (C). Lane M: DNA ladder. Lanes 1-20 in each panel: PCR products for a partial fragment amplified from the relevant ESBL encoding gene among the twenty *K. pneumoniae* clinical strains

DISCUSSION

The global burden of multidrug-resistant (MDR) Enterobacteriaceae is characterized by significant rates of morbidity and mortality. Clinicians relied heavily on carbapenems as the first-line therapy for resistant gram-negative bacterial infections (Walsh TR 2010). These pathogens possess the capacity for rapid dissemination both within nosocomial settings and on a transcontinental scale (Nagaraj *et al.*, 2012). Nevertheless, the emergence of carbapenem resistance has become a global public health burden (Giske *et al.*, 2008). Carbapenem resistance mainly stems from carbapenemase enzymes inactivating these and other β -lactam agents. Furthermore, the prevalence of Extended-Spectrum Beta-Lactamases (ESBLs) is most notable in *Klebsiella* species and *E. coli*, though it is also observed across other Enterobacteriaceae. The emergence of CRKP (carbapenem resistant *K. pneumoniae*) is a critical global health issue, primarily because these strains exhibit high-level resistance to nearly all β -lactam agents and their associated inhibitors (Wyres *et al.*, 2020). In the light of these facts and alarms, the molecular mechanisms mediating the carbapenem and β -lactam antibiotics resistance in *K. pneumoniae* clinical strains isolated from pneumonia patients in Al-Diwaniyah Province was decoded in this study.

There is agreement between the biochemical identification via VITEK®2 system and the molecular approach using *16S rRNA* gene sequence analysis. This greatly underscores the potential of using VITEK® 2 system for rapid identification in diagnostic laboratories. The twenty study clinical strains of *K. pneumoniae* were grouped into only two clades or clusters (Figure 2). This greatly underpin the likelihood of distinct features of these study strains compared to other closely related *K. pneumoniae* clinical strains worldwide. Moreover, the study twenty strains were categorized into two clades with 9 strains (clade I) and 11 strains (clade II). This would in turn evidence that these 9 out of 20 study strains (MQBA1, MQBA15, MQBA8, MQBA16, MQBA7, MQBA14, MQBA4, MQBA11, and MQBA19) were distally related to the 11 study strains (MQBA1, MQBA13, MQBA3, MQBA18, MQBA10, MQBA2, MQBA9, MQBA17, MQBA5, MQBA12, and MQBA20). *16S rRNA* gene sequence analysis fundamentally lacks the discriminatory capability to differentiate among closely related strains; accordingly, it failed to resolve the genetic relationships among the 9 study strains clustering within Clade I, as well as the 11 study strains encountered in Clade II. Therefore, future prospective studies would inevitably necessitate the adoption of molecular approaches with superior discriminatory power at the strain level, such as Multi-Locus Sequence Typing (MLST) and Ribosomal Multi-Locus Sequence Typing (rMLST). Not only would these advanced typing methods provide higher resolution at the strain level, but they would also facilitate the unveiling and tracking of circulating epidemic, pandemic, or high-risk clones of *K. pneumoniae*, whether

within the same country or across different countries, thereby contributing to a more comprehensive understanding of the global dissemination dynamics of this clinically significant pathogen.

The prevalence of β -lactamase genes among ESBL-producing *K. pneumoniae* strains varied widely across different studies conducted worldwide. Unlike our findings, regarding the prevalence of *bla*CTX-M8, *bla*TEM, and *bla*SHV encoding genes among *K. pneumoniae* clinical strains, the prevalence of *SHV*, *TEM* and *CTX-M* β -lactamase genes among *K. pneumoniae* clinical strains isolated from two hospitals in Riyadh, Saudi Arabia was 97.3, 84.1 and 34.1%, respectively recently (Aldali JA 2025). A previous study conducted in Riyadh from July 2011 to October 2012 revealed that *bla*CTX-M8, *bla*TEM, and *bla*SHV encoding genes exhibited the following prevalence rate across 4250 clinical strains of *K. pneumoniae* and *E. coli* isolated from various body fluids (sputum, blood, and wounds) 63.5, 58, and 27.3%, respectively (Marie *et al.*, 2013). A previous study conducted in India revealed that the prevalence rate was 66.66, 31.9, 1.43, and 0.0 % for *bla*SHV, *bla*TEM + *bla*SHV, *bla*TEM, and *bla*CTX-M8, respectively (Bhaskar *et al.*, 2019). A previous study conducted in China revealed that the prevalence of ESBL genes harbored by clinical strains of *K. pneumoniae* was 31.8%, however, *bla*SHV and *bla*CTX-M genes exhibited 19.1 and 90.8% prevalence rate, respectively (Zhang *et al.*, 2016). Another study performed in Germany demonstrated 71% prevalence rate of ESBL genes (*bla*SHV, *bla*TEM, and *bla*CTX-M) harbored by *K. pneumoniae* clinical strains (Müller-Schulte *et al.*, 2020). A recent study conducted in 2025 in Egypt revealed the occurrence of *bla*SHV, *bla*TEM, and *bla*CTX-M genes among *K. pneumoniae* clinical strains (Badran *et al.*, 2025).

Similarly, the emergence of carbapenem resistance among *K. pneumoniae* clinical strains (CRKP) has posed a major public health crisis and financial burden for patients and governments worldwide particularly in developing countries (Aghamohammad *et al.*, 2023). Reportedly, the genes encoding carbapenem resistance (carbapenemases) (e.g. *bla*KPC, *bla*NDM, *bla*OXA-48-like, and *bla*GES) are harbored on plasmids and these plasmids play a major role in the dissemination of these genes among CRKP clinical strains in clinical settings (Aghamohammad *et al.*, 2023). A recent retrospective study conducted in Iran in 2025 has revealed that carbapenemase-encoding *bla*OXA and *bla*NDM in *K. pneumoniae* clinical strains isolated from intensive care units (ICU) between 2014-2025 exhibited a prevalence rate of 47 and 33.6%, respectively (Halimi and Siroosi 2025). Since the isolation of *Klebsiella pneumoniae* strains co-harboring triple carbapenemase genes remains globally common, their emergence represents a catastrophic therapeutic challenge, often resulting in pan-drug resistance that leaves clinicians with virtually no effective treatment options. Our

findings conferred the presence of triple carbapenemases encoding genes, which is of paramount importance from health issue point of view. Our data revealed that the prevalence of the following triple carbapenemases encoding genes among the twenty CRKp strains were 5, 5, 5, 5, 5, and 15% for (*bla*IMI, *bla*NDM, *bla*IMP), (*bla*IMI, *bla*NDM, *bla*GIM), (*bla*OXA-48, *bla*IMI, *bla*NDM), (*bla*OXA-48, *bla*KPC, *bla*NDM), and (*bla*VIM, *bla*KPC, *bla*OXA-48), respectively (Figures 3-6). However, the prevalence of quintuple carbapenemases encoding genes (*bla*OXA-48, *bla*IMI, *bla*VDM, *bla*KPC, *bla*VIM) was 5% (Figures 3-6). A recent longitudinal study conducted in India between 2014 and 2025 reported a 6.66% prevalence rate for triple carbapenemase-encoding genes (*bla*OXA-48, *bla*KPC, *bla*NDM) (Halder *et al.*, 2025), a figure that closely aligns with the findings of the present study. *K. pneumoniae* strains were reported to co-harbor (*bla*KPC-2/*bla*VIM-24) in Colombia (Rojas *et al.*, 2013), (*bla*KPC-2/*bla*NDM-1) in Pakistan (Sattar *et al.*, 2016), (*bla*KPC-2/*bla*VIM-1) in Greece (Giakkoupi *et al.*, 2009), (*bla*KPC-2/*bla*VIM-1) in Italy (Munoz-Price *et al.*, 2013; Richter *et al.*, 2012), (*bla*KPC-2/*bla*NDM-1, *bla*KPC-2/*bla*IMP-26 and *bla*KPC-2/*bla*IMP-4) in China (Dong *et al.*, 2015; Liu *et al.*, 2015; Liu *et al.*, 2014). The prevalence of the triple carbapenemases *bla*NDM-1, *bla*VIM and *bla*OXA-48 were 58.6%, 10.3% and 6.9%, respectively in the respective CRKP clinical strains. Co-existence *bla*NDM-1/*bla*VIM and *bla*NDM-1/*bla*OXA-48 were noted to be 6.9 and 3.4% in the clinical strains, respectively in Nepal (Neupane *et al.*, 2025). A recent study conducted in Ethiopia in 2025, 100% prevalence of *bla*NDM was reported across *K. pneumoniae* clinical strains (Kindu *et al.*, 2025). The occurrences of strains harboring a single carbapenemase gene were 39 and 19% for *bla*OXA-48 and *bla*NDM, respectively. Remarkably, the occurrence of the carriers of both genes was as high as 29% (Homkaew *et al.*, 2025). A recent study conducted in Iran revealed a much higher prevalence percentage of carbapenemase genes across *K. pneumoniae* strains as follow: *bla*IMI/*bla*IMP (91%), *bla*OXA-48 (19%), *bla*KPC (11%), and *bla*NDM (2%) using PCR (Samadi *et al.*, 2026). However, a much higher sensitive percentages of prevalence were reported *bla*KPC (91.67%), class B carbapenemases (100%), and class D carbapenemases (86.4%), with specificities of 97.8%, 86.7%, and 75%, respectively, with specificities of 97.8%, 86.7%, and 75%, respectively upon using flow cytometry (Samadi *et al.*, 2026). A previous study addressed that the prevalence of *bla*KPC, *bla*OXA, *bla*NDM, and *bla*IMP were 57, 29, 16, and 1%, respectively (Cox *et al.*, 2025). A recent study approved 43.4% of CRKP strains carried carbapenemase genes (*bla*NDM, *bla*KPC, *bla*IMP, *bla*OXA-48/181/232, *bla*VIM and *bla*GES) from 2000-2023 (Zhang *et al.*, 2025). A previous study conducted in Jordan showed a prevalence of 46 % *bla*KPC, 23 % *bla*IMP, 19 % *bla*OXA-48, and 11.5 % *bla*NDM (Gharaibeh *et al.*, 2022). The findings of the present study emphasize the urgent need to strengthen hospital infection control

measures and surveillance programs to effectively detect and mitigate the spread of *K. pneumoniae* strains carrying triple or quintuple carbapenemase genes all over Iraq. Future studies would include larger sample sizes and cover multiple provinces in Iraq.

CONCLUSION

The present investigation documented a critically alarming prevalence of ESBL and carbapenemase-producing *K. pneumoniae* clinical strains among pneumonia patients in Al-Quadysia, Iraq, with the exceptionally high frequency of rCSA carbapenemase (90%) and *bla*OXA-48 (60%), alongside the co-harboring of multiple carbapenemase classes (A, B, and D) within the same clinical strains, representing an unprecedented therapeutic catastrophe, as such co-existence of diverse carbapenemase classes virtually abolishes all available β -lactam therapeutic options, ultimately forcing dependency on colistin and polymyxins as the absolute last therapeutic resort. The clinical and public health significance of these findings is profound, as carbapenemase-producing *K. pneumoniae* is globally recognized as a critical-priority pathogen by the World Health Organization (WHO), and its unchecked circulation within pulmonary infection settings in Al-Diwaniyah signals an urgent need for immediate implementation of comprehensive antimicrobial stewardship programs, enhanced infection control protocols, and mandatory microbiological surveillance within healthcare facilities. For prospective investigations, whole genome sequencing (WGS)-based approaches combined with MLST are strongly warranted to fully characterize the resistome, virulome, and mobilome of these strains, elucidate plasmid-mediated horizontal gene transfer mechanisms driving resistance dissemination, and identify circulating high-risk epidemic clones of carbapenemase-producing *K. pneumoniae* at both national and regional levels across the Middle East.

STUDY LIMITATIONS

Small sample size is the main limitations in this study.

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