Sequence-Based Characterizationof *blaNDM* and *mrkD* in Clinical *Klebsiella pneumoniae* Isolates from Babylon, Iraq: Sanger Sequencing and Phylogenetic Analysis

Abstract

Background:*Klebsiella pneumoniae* is An important opportunistic pathogen, it is linked to hospital-acquired infections and antimicrobial resistance. The blaNDM gene produces New Delhi metallo-β-lactamase, helping the bacterium resist carbapenems. By contrast, mrkD encodes the tip adhesin of type 3 fimbriae, which supports bacterial attachment. Molecular detection paired with sequence analysis offers more than conventional PCR screening: it confirms the target's identity and measures sequence similarity.**Aim:** This study aimed to investigate the occurrence of *blaNDM* and *mrkD* among clinical *K. pneumoniae* isolates from Babylon, Iraq, and to characterize representative amplicons using Sanger sequencing and phylogenetic analysis.**Methods:**Researchers examined 35 clinical K. pneumoniae isolates with polymerase chain reaction (PCR) to detect blaNDM and mrkD. They selected representative PCR products for Sanger sequencing, then compared the resulting nucleotide sequences with reference sequences and assessed their phylogenetic relationships through sequence-analysis procedures.**Results:**Among the 35 isolates, blaNDM appeared in 33 (94.28%), while mrkD occurred in all of them (100%). The sequenced fragments measured 286 bp for blaNDM and 244 bp for mrkD, with each showing 100% identity to its matching reference sequence. The mrkD sequences grouped with K. pneumoniae references. All three blaNDM sequences formed a single cluster and were close to reference sequences from other bacterial species.**Conclusion:**Among the isolates examined, blaNDM appeared frequently, while mrkD was detected in every isolate. Sequence analysis confirmed the identity of both PCR targets and offered an initial view of their genetic relationships. The evidence remains limited, though: only a small number of representatives were sequenced, and the analysis covered short gene fragments. As a result, broader diversity within the population, transmission routes, and the genomic location of these genes cannot yet be determined..

Keywords:*Klebsiella pneumoniae*; *blaNDM*; *mrkD*; Sanger sequencing; phylogenetic analysis; carbapenem resistance.

Introduction

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*Klebsiella pneumoniae*is an opportunistic pathogen linked to several clinical infections, especially in healthcare facilities. Its ability to cause disease depends partly on genetic factors that support antimicrobial resistance and bacterial attachment. Two clinically significant genes are blaNDM and mrkD: blaNDM confers resistance to carbapenems, while mrkD promotes adhesion and persistence within biofilms (Paczosa & Mecsas, 2016; Bassetti et al., 2022).

The blaNDM gene produces New Delhi metallo-β-lactamase (NDM), a class B metallo-β-lactamase that can hydrolyse a wide range of β-lactam antibiotics, including carbapenems. Concern is heightened by the gene's frequent presence on transferable genetic elements, which can move it between bacterial species. When blaNDM appears in clinical K. pneumoniae isolates, antimicrobial resistance surveillance and infection-control efforts face a serious challenge. Molecular testing identifies isolates that carry the determinant, while nucleotide sequencing confirms the gene and permits comparison with reference sequences (Boyd et al., 2022; Rawson et al., 2022).

The mrkD gene, by contrast, encodes MrkD adhesin at the tip of type 3 fimbriae. It helps bacteria attach to host-associated surfaces and extracellular matrix components, especially collagen-containing structures. That attachment can aid colonization and biofilm formation, allowing bacteria to persist in clinical environments. Characterizing mrkD at the molecular level and analyzing its nucleotide sequence can confirm the gene's identity and assess its similarity to previously reported K. pneumoniae sequences (Paczosa & Mecsas, 2016; Kaur et al., 2020; Struve et al., 2022; Alcántar-Curiel et al., 2023).

This study examined two genetic targets, blaNDM and mrkD, in clinical K. pneumoniae isolates collected in Babylon Governorate, Iraq. PCR screening was combined with Sanger sequencing and phylogenetic analysis of representative amplicons to determine how often these targets occurred and to characterize the sequence relationships shown by the available data..

Materials and Methods

Study Design and Bacterial Isolates

A total of 35 clinical *Klebsiella pneumoniae*This study examined isolates from urine, sputum, wound, and burn samples gathered at Marjan Teaching Hospital, Imam Al-Sadiq Teaching

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Hospital, and Al-Hilla Teaching Hospital in Babylon Governorate, Iraq. Researchers identified the bacteria through standard microbiological methods and the VITEK 2 Compact system.

Molecular Detection: DNA Extraction and PCR Thermal Conditions

Bacterial genomic DNA was extracted from overnight bacterial cultures of all 35 *Klebsiella pneumoniae*isolates usinga Magen bacterial DNA kit(Magen Biotech, China) according to the manufacturer's instructions.

Monoplexpolymerase chain reaction (PCR) assays were performed to detect the presence of *bla*NDM and *mrkD* genes using specific primer sets (Table 1). PCR reactions were prepared in a total volume of 25 µL containing Master Mix, 1 µL of each forward and reverse primer, template DNA, and nuclease-free water.The thermal cycling conditions were carried out in a thermal cycler with the following profile (Table 2).

Amplified PCR products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel stained with ethidium bromide in 1$\times$ TAE buffer. Amplicons were visualized under ultraviolet (UV) transillumination to confirm the presence and expected molecular weight of the amplified gene fragments (Table 1).

Table 1. Primers used for PCR amplification and sequencing of *bla*NDM and *mrkD*

Target Gene	Primer Sequence (5' → 3')	Amplicon Size (bp)	Reference
***bla*NDM**	**F:** GGCGGAAGGCTCATCACGA **R:** CGCAACACAGCCTGACTTTC	286	Xiang *et al*. (2020)
mrkD	**F:**GTCTTTTCGTCCCGGGTATATAAC **R:**CCACATCGACATTCATATTTTTCC	244	Bakhtiari *et al*. (2021)

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Table 2. Optimized PCR thermal cycling profile for *bla*NDM and *mrkD* amplification

Step	Temperature (°C)	Time	Number of Cycles
Initial Denaturation	95	5 min	1
Denaturation	95	30 s	35
Annealing	55	30 s	35
Extension	72	30 s	35
Final Extension	72	5 min	35
Hold	4		

Sanger Sequencing

Representative PCR amplicons of *blaNDM* and *mrkD* were subjected to bidirectional Sanger sequencing by Macrogen Inc. (Geumcheon, Seoul, South Korea). Sequence chromatograms were examined, and suitable sequences were selected for subsequent analysis.

Sequence Analysis

The obtained nucleotide sequences were edited and aligned with corresponding reference sequences using BioEdit Sequence Alignment Editor, version 7.1. Sequence annotation was performed using SnapGene Viewer, version 4.0.4. Reference sequences were identified using NCBI BLASTn. The accession numbers of the reference sequences and the sequence identity and coverage values should be provided for reproducibility.

Phylogenetic Analysis

Phylogenetic analyses were performed separately for *blaNDM* and *mrkD*. Study sequences and homologous reference sequences were compared, and phylogenetic trees were constructed using the neighbour-joining method and visualized in Geneious software. The software version, alignment settings, evolutionary model, and branch-support method and values, where applicable, should be reported according to the analyses actually performed.

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Ethical Approval

Ethical approval was obtained from the Ethics Committee of the College of Science, University of Al-Qasim Green, Iraq (Approval No. QGEC/56/2026), subject to confirmation against the official approval document.

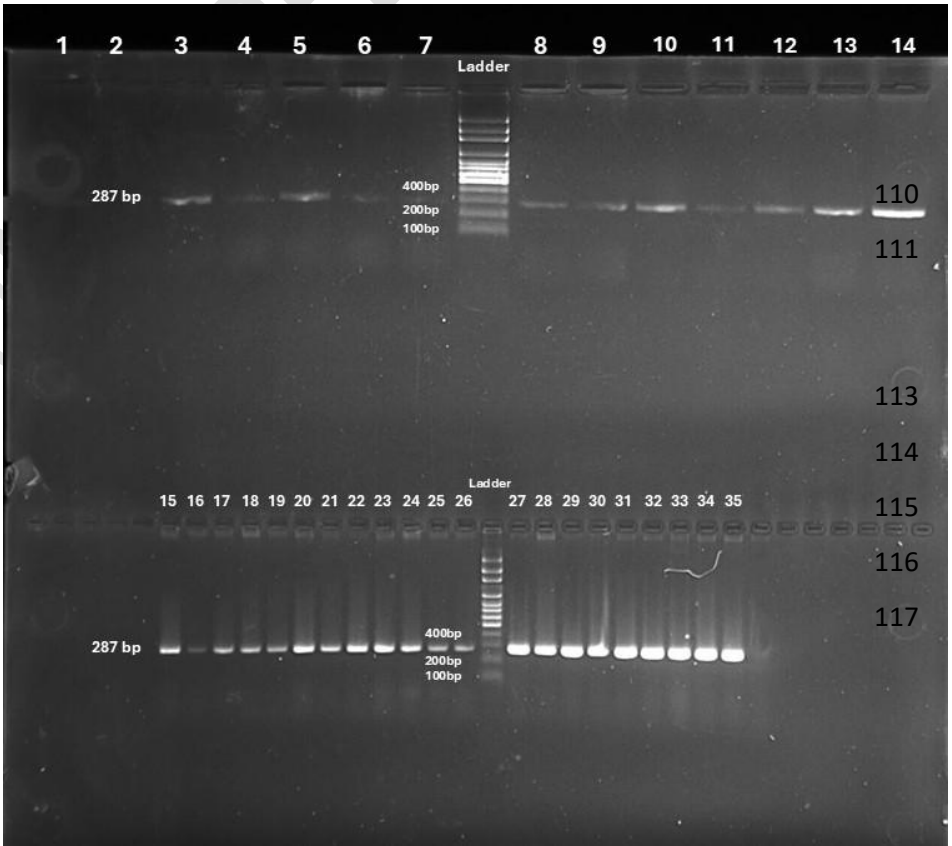
Results

PCR Detection of *bla*NDM and *mrkD*

PCR screening was performed on 35 clinical *Klebsiella pneumoniae* isolates to detect the presence of the *bla*NDM and *mrkD* genes. The *bla*NDM gene was detected in 33 isolates (94.28%), whereas two isolates (5.72%) were negative. In contrast, the *mrkD* gene was detected in all 35 isolates (100%) (Table 3).

Table 3. PCR detection of *bla*NDM and *mrkD* among clinical *K. pneumoniae* isolates

Genetic target	Positive, n (%)	Negative, n (%)	Total
blaNDM	33 (94.28%)	2 (5.72%)	35
mrkD	35 (100%)	0 (0%)	35



Detection of the *blaNDM* Gene

PCR amplification revealed that the *blaNDM* gene was present in 33 of the 35 clinical *K.*

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pneumoniae isolates (94.28%), while two isolates (5.72%) showed no detectable amplification of the target gene (Figure 1).

Figure 1.Agarose gel electrophoresis of *blaNDM* gene amplification.M: DNA marker (100 bp ladder); Lanes (1–35): clinical isolates of *Klebsiella pneumoniae*. Positive isolates showed bands at the expected size of *blaNDM* gene.

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Detection of the *mrkD* Gene

PCR analysis demonstrated that the *mrkD* gene was detected in all 35 clinical *K. pneumoniae* isolates (100%) (Figure 2).

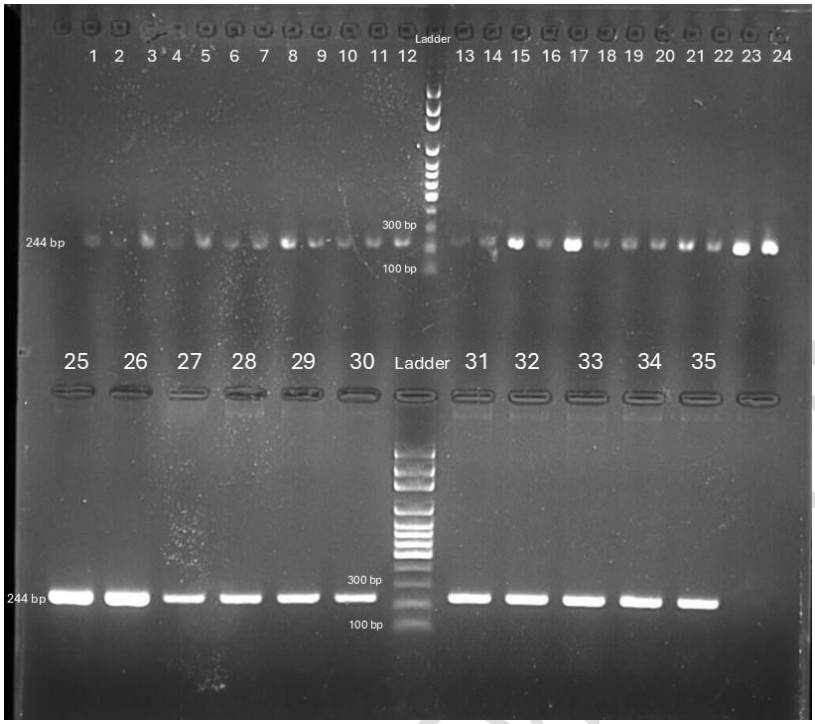

Figure 2.Agarose gel electrophoresis of *mrkD* gene amplification. M: DNA marker; Lanes (1–35): all isolates showed positive amplification bands of *mrkD*gene

Sanger Sequencing and Phylogenetic Analysis

Sequence Analysis and Phylogenetic Relationships of *mrkD*

Sanger sequencing of the *mrkD* gene was performed to confirm the identity of the amplified target and investigate its genetic relationship with reference sequences available in the NCBI database. Sequence analysis revealed 100% identity with the corresponding reference sequence. Phylogenetic analysis further indicated that the study sequences clustered with *K. pneumoniae* reference sequences, demonstrating a close genetic relationship between the local sequences and the corresponding reference sequences.

The 100% sequence identity indicates that no nucleotide differences were detected between the analyzed study sequence and the reference sequence across the compared region. This finding supports the molecular identification of the sequenced *mrkD* fragment. However, this result

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applies to the analyzed sequence region and does not establish complete genomic identity among the isolates.

The *mrkD* gene encodes an adhesin associated with type 3 fimbriae and contributes to adhesion-related processes in *K. pneumoniae* (Paczosa &Mecsas, 2016). The high prevalence of this gene in the present collection, together with the observed sequence identity, is consistent with its widespread distribution among the investigated isolates. Previous studies have also investigated the prevalence and functional importance of this gene in *K. pneumoniae* (El Fertas-Aissani *et al.*, 2013; Ko *et al*., 2023; Ragupathi *et al*., 2024).Nevertheless, the presence and sequence conservation of *mrkD* do not, by themselves, demonstrate increased virulence or biofilm production. Functional assessment would require additional evidence, such as gene-expression analysis or phenotypic assays. Therefore, the present findings support the presence and sequence similarity of the investigated *mrkD* fragment and provide a basis for further investigation of its functional significance.

Sequencing and Phylogenetic Analysis

Sequencing and phylogenetic tree of the *blaMrkD* gene

Sangersequencing of the *mrkD* gene was conducted in this study to confirm the gene's identity and to examine the genetic relationship between the study isolates and the reference isolates recorded in the NCBI database. The analysis results showed a complete (100%) match with the reference sequence, and the phylogenetic tree indicated that the study isolates clustered only with *K.pneumoniae*isolates, demonstrating a significant genetic similarity between the local isolates and the reference isolates, thus confirming the accuracy of the molecular diagnosis of the studied isolates.

Table (3): identified nucleotide sequence with its corresponded reference (*mrkD*).

Sample	Nucleotide sequence	Size bp

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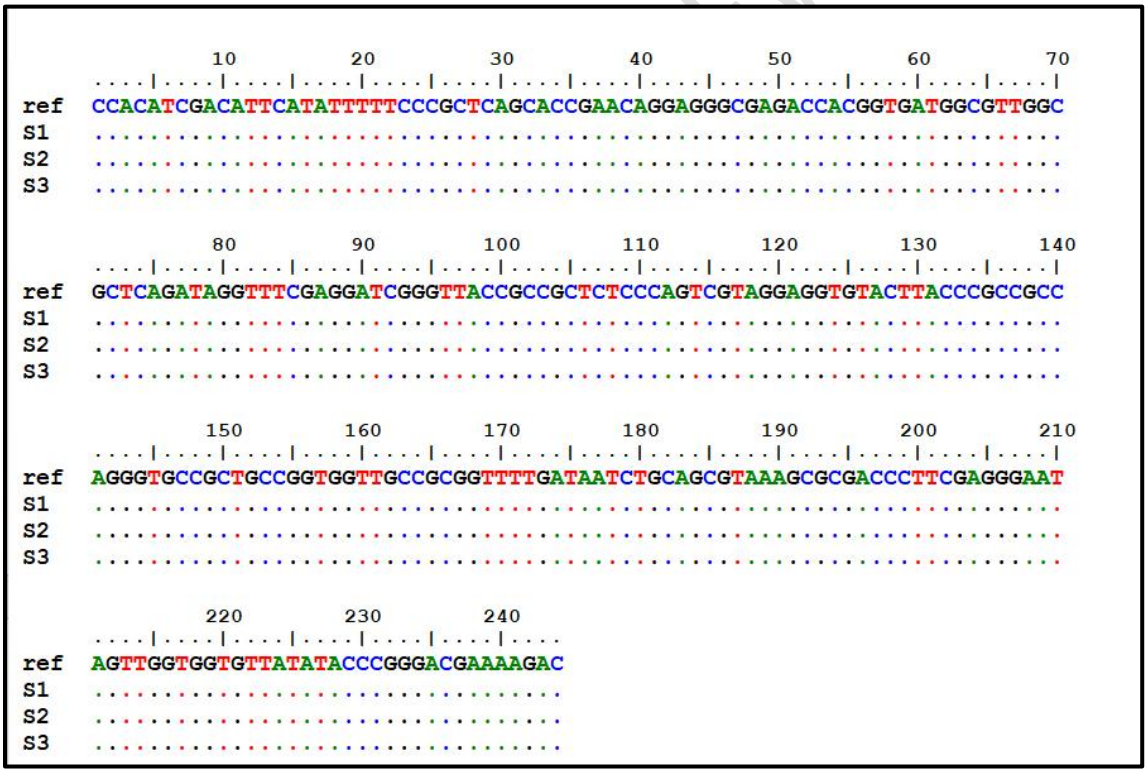
>ref	**CCACATCGACATTCATATTTTTCCCGCTCAGCACCGAACAGGAGGGCGAGACCACGGTGATG GCGTTGGCGCTCAGATAGGTTTCGAGGATCGGGTTACCGCCGCTCTCCCAGTCGTAGGAGGT GTACTTACCCGCCGCCAGGGTGCCGCTGCCGGTGGTTGCCGCGGTTTTGATAATCTGCAGCG TAAAGCGCGACCCTTCGAGGGAATAGTTGGTGGTGTTATATACCCGGGACGAAAAGAC**	**244**
>S1	**CCACATCGACATTCATATTTTTCCCGCTCAGCACCGAACAGGAGGGCGAGACCACGGTGATG GCGTTGGCGCTCAGATAGGTTTCGAGGATCGGGTTACCGCCGCTCTCCCAGTCGTAGGAGGT GTACTTACCCGCCGCCAGGGTGCCGCTGCCGGTGGTTGCCGCGGTTTTGATAATCTGCAGCG TAAAGCGCGACCCTTCGAGGGAATAGTTGGTGGTGTTATATACCCGGGACGAAAAGAC**	**244**
>S2	**CCACATCGACATTCATATTTTTCCCGCTCAGCACCGAACAGGAGGGCGAGACCACGGTGATG GCGTTGGCGCTCAGATAGGTTTCGAGGATCGGGTTACCGCCGCTCTCCCAGTCGTAGGAGGT GTACTTACCCGCCGCCAGGGTGCCGCTGCCGGTGGTTGCCGCGGTTTTGATAATCTGCAGCG TAAAGCGCGACCCTTCGAGGGAATAGTTGGTGGTGTTATATACCCGGGACGAAAAGAC**	**244**
S3	**CCACATCGACATTCATATTTTTCCCGCTCAGCACCGAACAGGAGGGCGAGACCACGGTGATG GCGTTGGCGCTCAGATAGGTTTCGAGGATCGGGTTACCGCCGCTCTCCCAGTCGTAGGAGGT GTACTTACCCGCCGCCAGGGTGCCGCTGCCGGTGGTTGCCGCGGTTTTGATAATCTGCAGCG TAAAGCGCGACCCTTCGAGGGAATAGTTGGTGGTGTTATATACCCGGGACGAAAAGAC**	**244**

188 **Not:** Green shaded sequence represent the forward and reverse primer location



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190 **Figure (3) : nucleotide alignment of 3 samples with reference sequence (*mrkD*).**

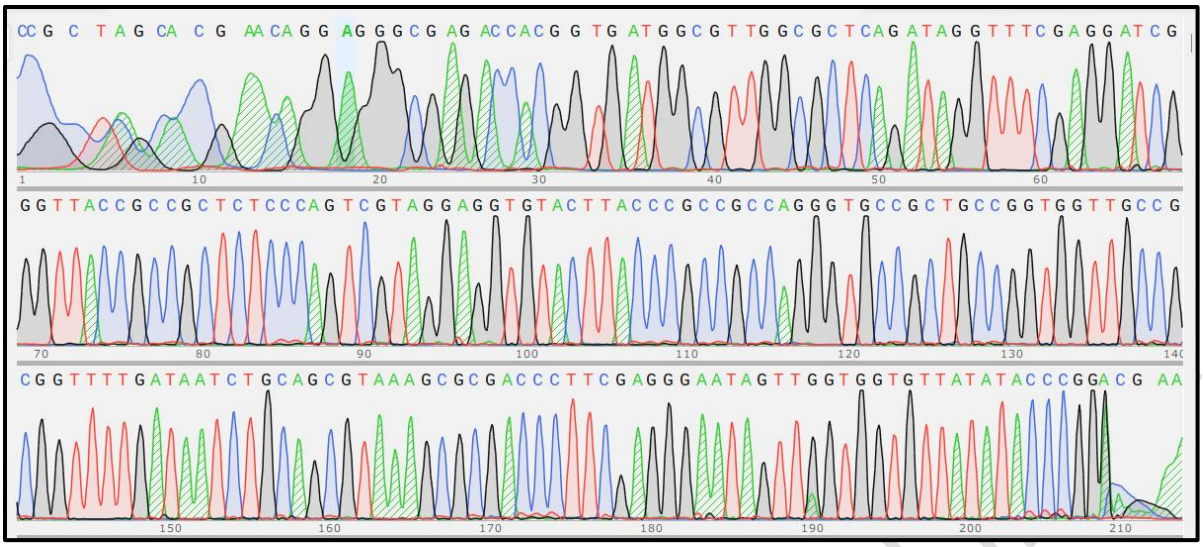

Figure (4) sample of sanger chromatogram of identified samples(*mrkD*).

Sequencing and phylogenetic tree of the *blaNDM*gene

Sangersequence analysis of the *blaNDM*gene was conducted in this study to confirm the identity of the PCR reaction products and to examine the genetic relationship between the study isolates and the reference sequences recorded in the GenBank database. The analysis results showed a complete match (100%) with the reference sequence recorded in the database, confirming the accuracy of the amplification and sequencing results, and supporting the precise identification of the *blaNDM*gene in the studied isolates.

The phylogenetic tree also showed that the three study isolates (S1, S2, S3) clustered within a single branch, indicating a high degree of genetic similarity among them, and suggesting that the study isolates carry a very similar sequence of the *blaNDM*gene, on the other hand, the phylogenetic tree showed the study isolates' proximity to recorded sequences of other bacterial species, including Escherichia coli, *Enterobacter cloacae*, *Proteus mirabilis*, and*Providencia* spp. This is not unexpected, as the *blaNDM*gene is one of the genes associated with mobile genetic elements (Cuzon *et al*., 2013; Chen *et al*., 2020).The results of the current study are consistent with what Chen *et al*. (2020) mentioned, who explained that the *blaNDM*gene spreads among many Gram-negative bacteria due to the transfer of plasmids carrying it between different species, which contributes to maintaining a high degree of similarity in the sequence of this gene. Cuzon *et al*. (2013) also indicated that *blaNDM*genes are not limited to a single bacterial species.

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211 **Table (4): identified nucleotide sequence with its corresponded reference (*blaNDM*).**

Sample	Nucleotide sequence	Size bp
>ref	CGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGC CCAACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACA ATATCACCGTTGGGATCGACGGCACCGACATCGCTTTTGGTGGCTGCCTGATCA AGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCGACACTGAGCAC TACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGAT CGTGATGAGCCATCCGCC	286
>S1	CGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGC CCAACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACA ATATCACCGTTGGGATCGACGGCACCGACATCGCTTTTGGTGGCTGCCTGATCA AGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCGACACTGAGCAC TACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGAT CGTGATGAGCCATCCGCC	286
>S2	CGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGC CCAACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACA ATATCACCGTTGGGATCGACGGCACCGACATCGCTTTTGGTGGCTGCCTGATCA AGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCGACACTGAGCAC TACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGAT CGTGATGAGCCATCCGCC	286
>S3	CGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGC CCAACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACA ATATCACCGTTGGGATCGACGGCACCGACATCGCTTTTGGTGGCTGCCTGATCA AGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCGACACTGAGCAC TACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGAT CGTGATGAGCCATCCGCC	286

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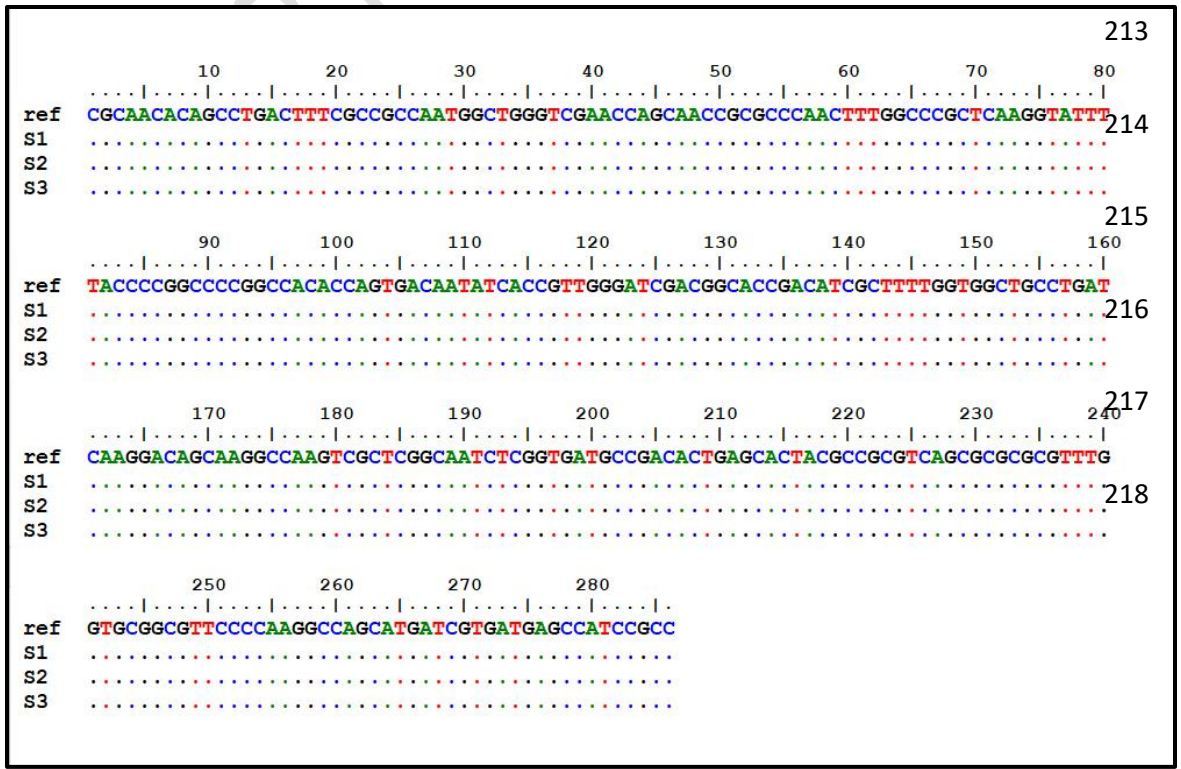

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Figure (5) : nucleotide alignment of 3 samples with reference sequence (*blaNDM*).

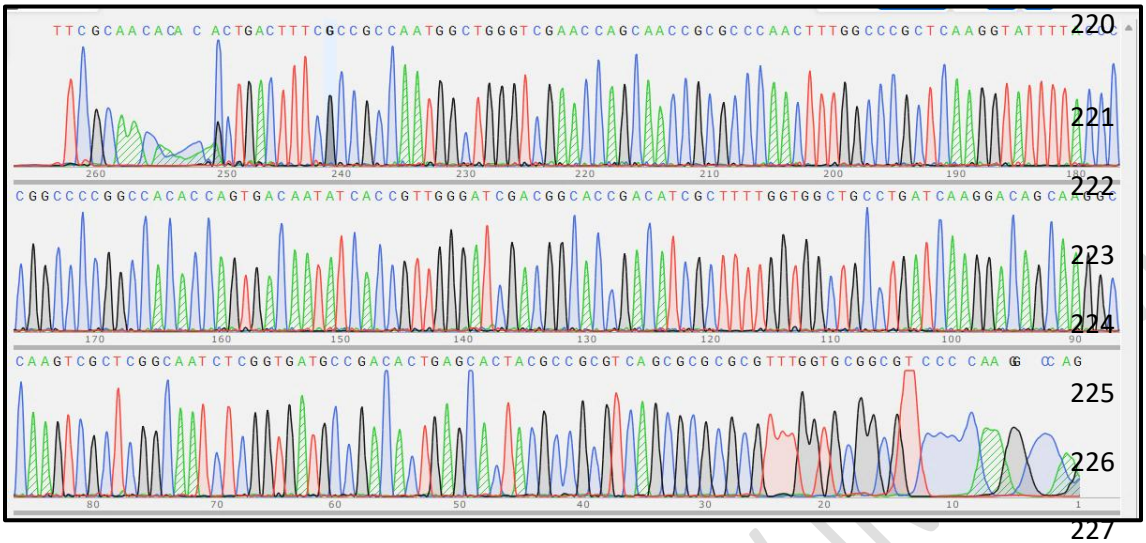

Figure (6) sample of sanger chromatogram of identified samples (*blaNDM*)

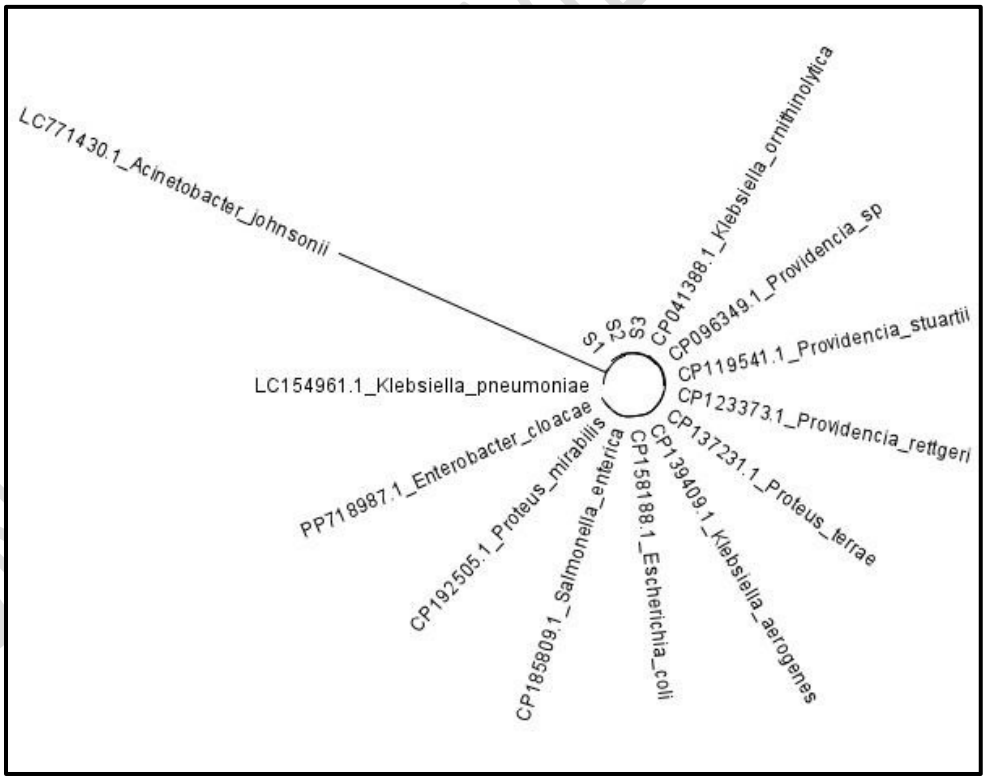

Figure (7)phylogenetic tree of the *blaNDM*gene

Discussion

The high detection rate of *bla*NDM (94.28%) observed in this study is consistent with reports identifying this gene as an important carbapenemase determinant among carbapenemase-producing and carbapenem-resistant *K. pneumoniae* isolates (Baral *et al*., 2024; Pattolath*et al*., 2023). A high occurrence of *bla*NDM has also been reported among clinical isolates in Asian settings (Santajit*et al*., 2024). The high frequency of *bla*NDM in the present collection may reflect the circulation of isolates carrying this resistance determinant in the clinical setting. The dissemination of *bla*NDM can be facilitated by mobile genetic elements, including plasmids, while antibiotic selection pressure may contribute to the persistence of resistant bacteria. However, the present findings establish the detection frequency of the gene among the investigated isolates but do not independently determine the mechanisms responsible for its dissemination.

The phylogenetic tree placed the study isolates—S1, S2, and S3—on one branch, showing that they are highly similar genetically. The local blaNDM sequences were also close to sequences reported in other bacterial species, including Escherichia coli, Enterobacter cloacae, Proteus mirabilis, and Providencia spp. That pattern is expected because blaNDM commonly travels on mobile genetic elements (Cuzon et al., 2013; Chen et al., 2020; Porretta et al., 2020). Chen et al. (2020) reported the same broad distribution, linking it to plasmids that move between Gram-negative species and help preserve high sequence identity. Cuzon et al. (2013) likewise found that blaNDM genes are not confined to one bacterial species.

The mrkD gene appeared in every isolate tested (100%), showing that it was consistently present in this collection. Sanger sequencing verified the target, with 100% sequence homology to the reference isolates, while the phylogenetic analysis placed all local isolates exclusively among the K. pneumoniae reference sequences. That exact match is consistent with the gene's function: mrkD encodes the adhesin at the tip of type 3 fimbriae, helping bacteria attach to host cells and biotic surfaces and form biofilms. Because this protein is functionally important, purifying selection acts on its coding regions, limiting mutations and preserving sequence stability across generations. (Paczosa & Mecsas, 2016; Ragupathi *et al*., 2024).

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In addition, a study by Kot *et al*. (2023) showed a very high prevalence of the *mrkD* gene among clinical isolates of *K. pneumoniae*, confirming that this gene is one of the most widely spread and conserved virulence factors among clinical isolates. This aligns with the current study's results, which demonstrated complete sequence homology and clear phylogenetic proximity (El Fertas-Aissani *et al.*, 2013; Ko *et al*., 2023; Kot *et al*., 2023). Based on this, the results of the current study confirm that the *mrkD* gene exhibits a high degree of genetic conservation among *K. pneumoniae* isolates, reflecting its status as one of the most important virulence factors associated with adhesion and biofilm formation (Zamora-Lagos *et al.*, 2013; Ragupathi *et al*., 2024).

However, while the presence and sequence conservation of *mrkD* indicate the genetic potential for MrkD-associated adhesion, gene detection alone does not establish the level of gene expression, adhesion strength, or biofilm-forming capacity of an isolate. These phenotypes are influenced by multiple bacterial genes and regulatory mechanisms (Murphy *et al.*, 2013; Clegg & Murphy, 2016). Therefore, the present findings support the presence and sequence conservation of the investigated *mrkD* fragment and provide a basis for further investigation of its functional significance.

Conclusion

The *bla*NDMgene was detected in 94.28% of the 35 clinical *Klebsiella pneumoniae*isolates, while *mrkD* was detected in all isolates (100%). Sequencing revealed 100% identity between representative gene fragments (286 bp for *bla*NDM and 244 bp for *mrkD*) and their corresponding reference sequences. Phylogenetic analysis showed close relationships with corresponding reference sequences. These findings provide preliminary molecular characterization of the two genes in clinical *K. pneumoniae* isolates from Babylon, Iraq**.**

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