

RESEARCH ARTICLE

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SEQUENCE-BASED CHARACTERIZATIONOF BLANDM AND MRKD IN CLINICAL KLEBSIELLA PNEUMONIAE ISOLATES FROM BABYLON, IRAQ: SANGER SEQUENCING AND PHYLOGENETIC ANALYSIS

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

Abstract

Background: Klebsiella pneumoniaeis 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..

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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.

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Introduction:-

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

The blaNDM gene encodes New Delhi metallo-β-lactamase (NDM), a class B metallo-β-lactamase capable of hydrolyzing a broad spectrum of β-lactam antibiotics, including carbapenems. Clinical concern is heightened by the frequent association of blaNDM with mobile genetic elements, which facilitate its horizontal dissemination across different bacterial species. Consequently, the emergence of blaNDM in clinical K. pneumoniae isolates poses a substantial challenge to antimicrobial resistance surveillance and infection-control programs. While conventional molecular assays identify isolates carrying this resistance determinant, nucleotide sequencing confirms the target's identity and enables comparative phylogenetic analysis against reference sequences (Boyd et al., 2022; Rawson et al., 2022).By contrast, the mrkD gene encodes the MrkDadhesin located at the tip of type 3 fimbriae. This adhesin mediates bacterial attachment to host epithelial surfaces and extracellular matrix components, particularly collagen-rich structures, thereby promoting colonization and biofilm formation in clinical environments. Molecular characterization and sequence analysis of mrkD verify amplicon specificity and evaluate genetic conservation relative to previously reported K. pneumoniae strains (Paczosa&Mecsas, 2016; Kauret al., 2020; Struve et al., 2022; Alcántar-Curielet al., 2023).

Although previous studies in Iraq have investigated antimicrobial resistance or virulence factors in K. pneumoniae, most have relied solely on conventional PCR detection or examined these traits independently. Data combining sequence-based characterization and phylogenetic analysis of both carbapenem resistance (blaNDM) and fimbrial adhesion (mrkD) determinants within the same clinical cohort in Babylon Governorate remain limited. Building upon our recent phenotypic evaluation of carbapenem resistance, hypermucoviscosity, and biofilm-forming capacity in this clinical collection (Hameed& Radhi, 2026), the present study aimed to determine the molecular prevalence of blaNDM and mrkD among clinical K. pneumoniae isolates from Babylon, Iraq, and to characterize representative amplicons using bidirectional Sanger sequencing and phylogenetic analysis.

Materials and Methods:-

Study Design and Bacterial Isolates:-

A total of 35 non-duplicate clinical Klebsiella pneumoniae isolates were recovered from 425 clinical specimens collected between September 2025 and January 2026 at three hospitals in Babylon Governorate, Iraq: MarjanTeaching Hospital (n = 21), Imam Al-Sadiq Teaching Hospital (n = 10), and Al-Hilla Teaching Hospital (n = 4). The isolates were distributed across clinical specimen types as follows: urine (n = 16; 45.7%), sputum (n = 8; 22.9%), burn swabs (n = 5; 14.3%), wound swabs (n = 5; 14.3%), and blood (n = 1; 2.8%). Bacterial identification was performed using standard microbiological procedures and confirmed via the VITEK 2 Compact system. Phenotypic profiles of these isolates—including antimicrobial susceptibility testing, modified Carba NP results, hypermucoviscosity, and biofilm formation—have been previously established in our study (Hameed& Radhi, 2026).

Molecular Detection: DNA Extraction and PCR Thermal Conditions:-

Bacterial genomic DNA was extracted from overnight bacterial cultures of all 35 Klebsiella pneumoniaeisolates usinga Magen bacterial DNA kit(Magen Biotech,China) according to the manufacturer's instructions.Monoplexpoly merase chain reaction (PCR) assays were performed to detect the presence of blaNDM 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) agarosegel 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 blaNDM and mrkD

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

Table 2. Optimized PCR thermal cycling profile for blaNDM 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	1
Hold	4		

Sanger Sequencing:-
Three representative PCR amplicons each forblaNDM and mrkD(S1-S3) were selected for bidirectional Sanger sequencing by Macrogen Inc. (Geumcheon, Seoul, South Korea). Sequence chromatograms were examined, and sequences fragments covering the target regions (including 286 bpfragment forblaNDMand a 244 bp fragment for mrkD) were analyzed.

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.

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.

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).

Results:-

PCR Detection of blaNDM and mrkD:-
PCR screening was performed on 35 clinical Klebsiella pneumoniae isolates to detect the presence of the blaNDM and mrkD genes. The blaNDM 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 blaNDM 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. pneumoniae isolates (94.28%), while two isolates (5.72%) showed no detectable amplification of the target gene (Figure 1).

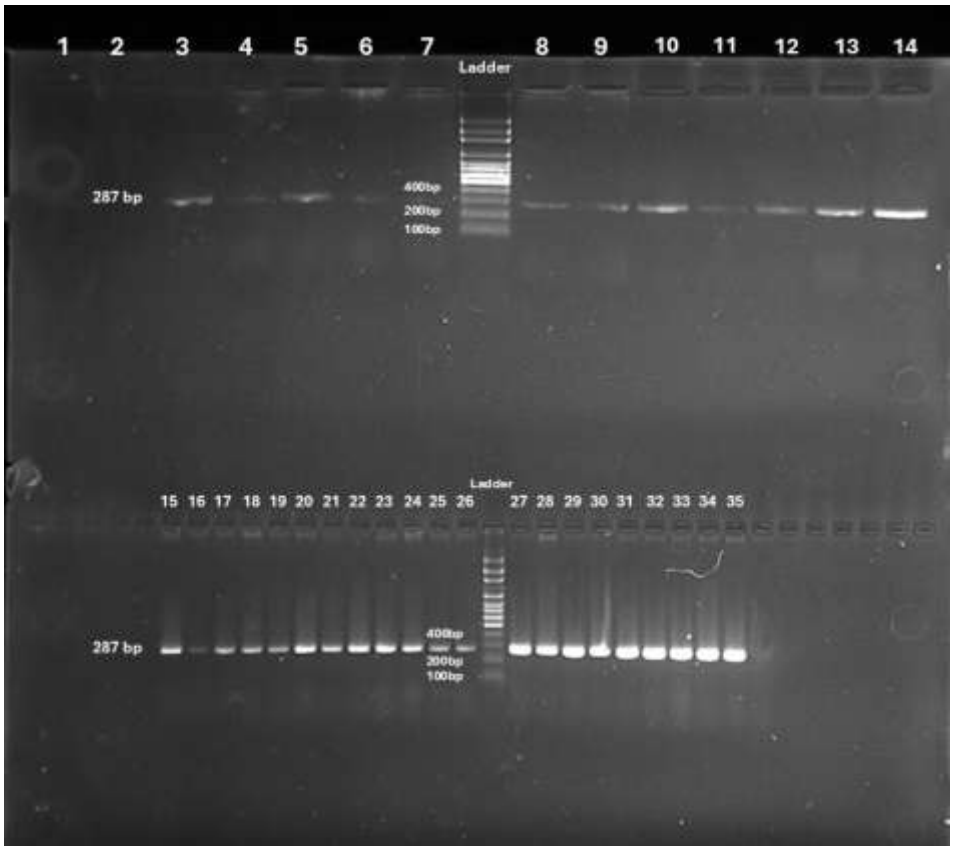


Figure 1.Agarose gel electrophoresis of blaNDM gene amplification.M: DNA marker (100 bp ladder); Lanes (1–35): clinical isolates of Klebsiella pneumoniae. Positive isolates showed bands at the expected size of blaNDM gene.

Detection of the mrkD Gene:-

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

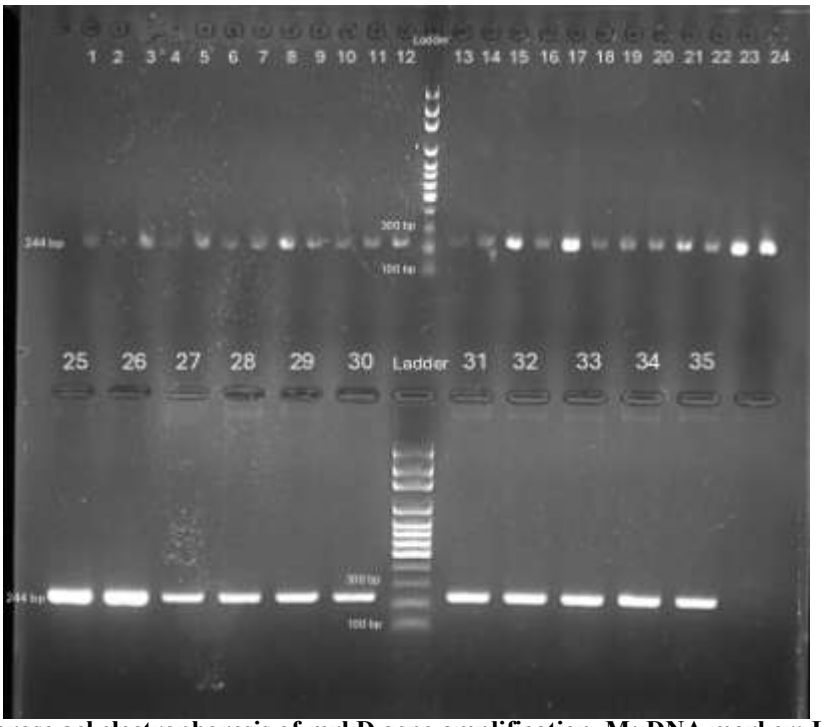


Figure 2.Agarose gel electrophoresis of mrkD gene amplification. M: DNA marker; Lanes (1–35): all isolates showed positive amplification bands of mrkDgene

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 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-Aissaniet al., 2013; Koet al., 2023; Ragupathiet 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.Because sequencing and phylogenetic analysis were restricted to three representative isolates (S1-S3) per gene, these phylogenetic relationships apply to the sequenced subset and are not generalized to all 35 clinical isolatesBidirectional sequencing provided full coverage across the analyzed fragments (244 bp for mrkD) with 100% identify to reference sequences. GenBank accession numbers were not deposited in GenBank at this stage.

Table (4): identified nucleotide sequence with its corresponded reference (mrkD).

Sample	Nucleotide sequence	Size bp
>ref	CCACATCGACATTCATATTTTTCCCGCTCAGCACCGAACAGGAGGGCGAG ACCACGGTGATGGCGTTGGCGCTCAGATAGGTTTCGAGGATCGGGTTACC	244

	GCCGCTCTCCCAGTCGTAGGAGGTGTACTTACCCGCCGCCAGGGTGCCGC TGCCGGTGGTTGCCGCGGTTTTGATAATCTGCAGCGTAAAGCGCGACCCT TCGAGGGAATAGTTGGTGGTGTTATATACCCGGGACGAAAAGAC	
>S1	CCACATCGACATTCATATTTTTCCCGCTCAGCACCGAACAGGAGGGCGAG ACCACGGTGATGGCGTTGGCGCTCAGATAGGTTTCGAGGATCGGGTTACC GCCGCTCTCCCAGTCGTAGGAGGTGTACTTACCCGCCGCCAGGGTGCCGC TGCCGGTGGTTGCCGCGGTTTTGATAATCTGCAGCGTAAAGCGCGACCCT TCGAGGGAATAGTTGGTGGTGTTATATACCCGGGACGAAAAGAC	244
>S2	CCACATCGACATTCATATTTTTCCCGCTCAGCACCGAACAGGAGGGCGAG ACCACGGTGATGGCGTTGGCGCTCAGATAGGTTTCGAGGATCGGGTTACC GCCGCTCTCCCAGTCGTAGGAGGTGTACTTACCCGCCGCCAGGGTGCCGC TGCCGGTGGTTGCCGCGGTTTTGATAATCTGCAGCGTAAAGCGCGACCCT TCGAGGGAATAGTTGGTGGTGTTATATACCCGGGACGAAAAGAC	244
S3	CCACATCGACATTCATATTTTTCCCGCTCAGCACCGAACAGGAGGGCGAG ACCACGGTGATGGCGTTGGCGCTCAGATAGGTTTCGAGGATCGGGTTACC GCCGCTCTCCCAGTCGTAGGAGGTGTACTTACCCGCCGCCAGGGTGCCGC TGCCGGTGGTTGCCGCGGTTTTGATAATCTGCAGCGTAAAGCGCGACCCT TCGAGGGAATAGTTGGTGGTGTTATATACCCGGGACGAAAAGAC	244

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

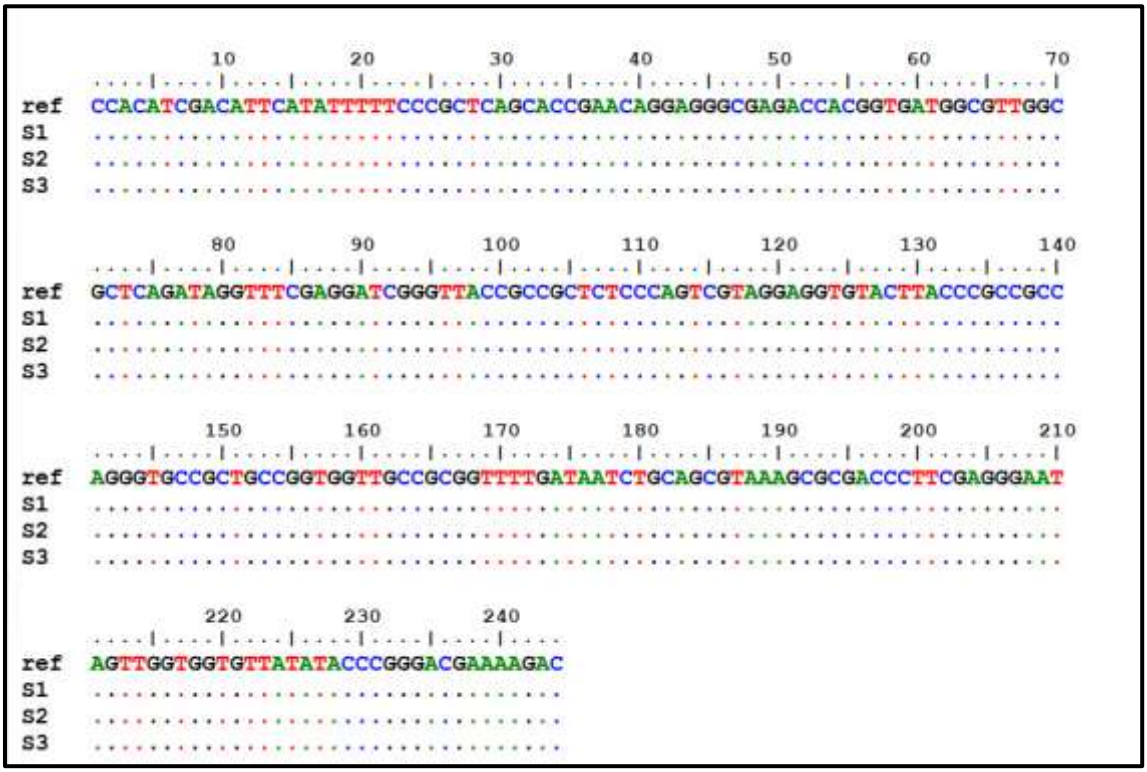


Figure (3) : nucleotide alignment of 3 samples with reference sequence (mrkD).

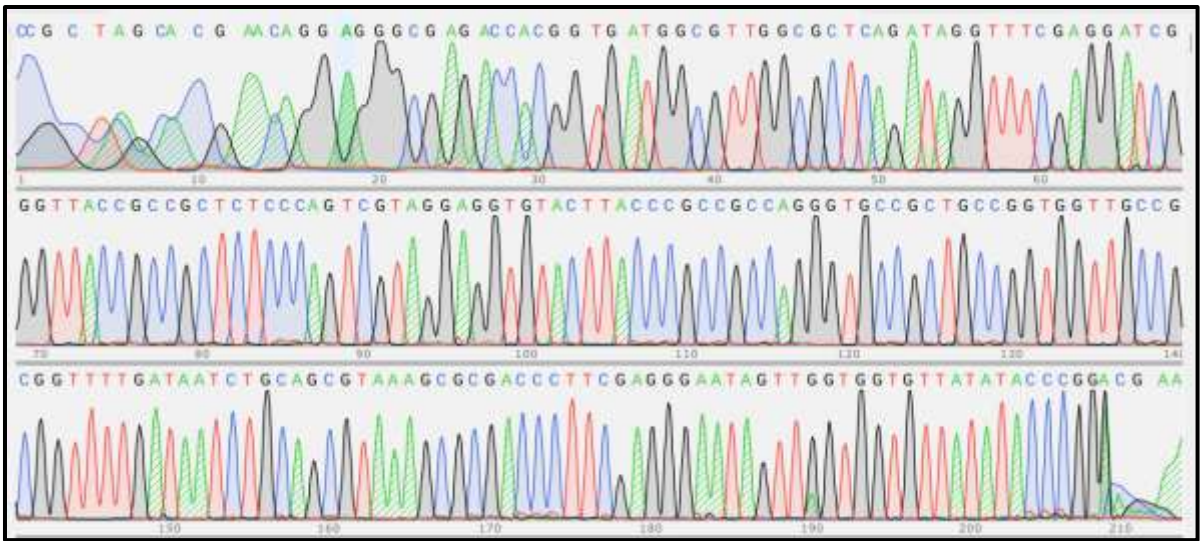


Figure (4) sample of sanger chromatogram of identified samples(mrkD).

Sequencing and phylogenetic tree of the blaNDMgene:-

Sangersequence analysis of the blaNDMgene 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 blaNDMgene 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 blaNDMgene, 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, andProvidencia spp. This is not unexpected, as the blaNDMgene 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 blaNDMgene 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 blaNDMgenes are not limited to a single bacterial species. Because sequencing and phylogenetic analysis were restricted to three representative isolates (S1-S3) per gene, these phylogenetic relationships apply to the sequenced subset and are not generalized to all 35 clinical isolates

Table (5): identified nucleotide sequence with its corresponded reference (blaNDM).

Sample	Nucleotide sequence	Size bp
>ref	CGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACC AGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTACCCC GGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACG GCACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAA GGCCAAGTCGCTCGGCAATCTCGGTGATGCCGACACTGAGCAC TACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGG CCAGCATGATCGTGATGAGCCATCCGCC	286
>S1	CGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACC AGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTACCCC GGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACG GCACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAA GGCCAAGTCGCTCGGCAATCTCGGTGATGCCGACACTGAGCAC TACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGG CCAGCATGATCGTGATGAGCCATCCGCC	286

>S2	CGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACC AGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTACCCC GGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACG GCACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAA GGCCAAGTCGCTCGGCAATCTCGGTGATGCCGACACTGAGCAC TACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGG CCAGCATGATCGTGATGAGCCATCCGCC	286
>S3	CGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACC AGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTACCCC GGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACG GCACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAA GGCCAAGTCGCTCGGCAATCTCGGTGATGCCGACACTGAGCAC TACGCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGG CCAGCATGATCGTGATGAGCCATCCGCC	286

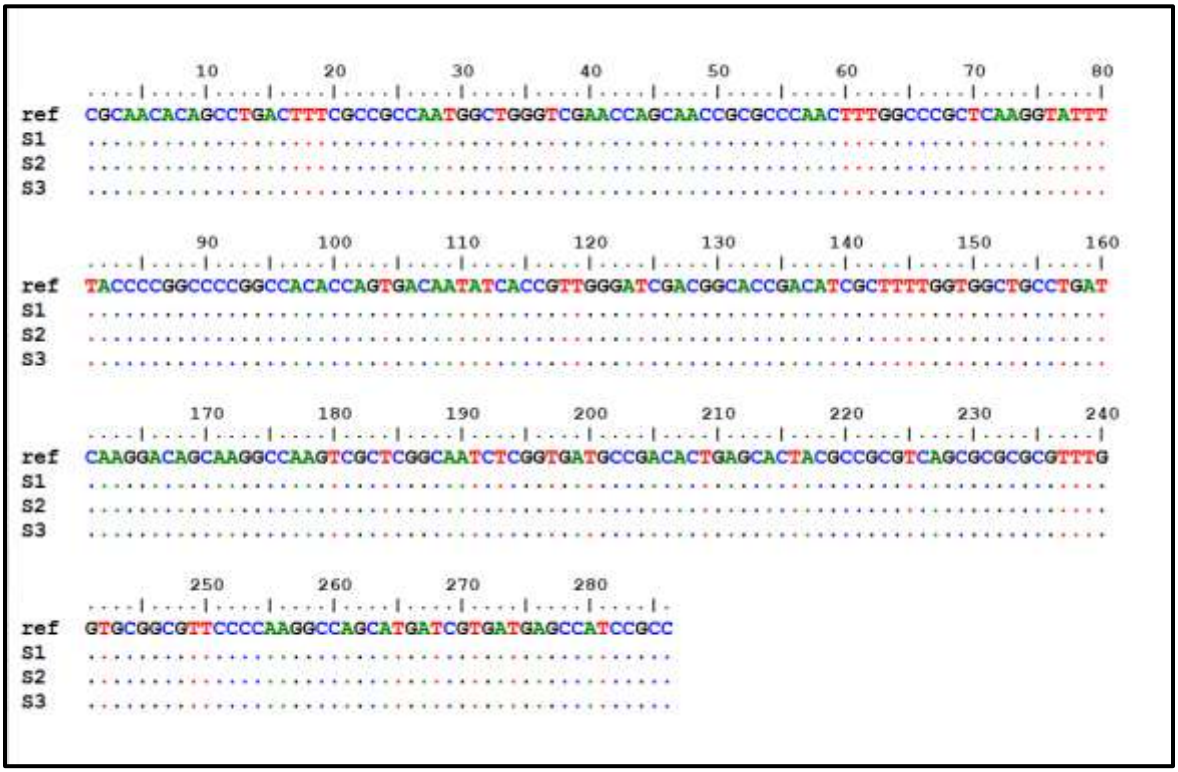


Figure (5) : nucleotide alignment of 3 samples with reference sequence (blaNDM).

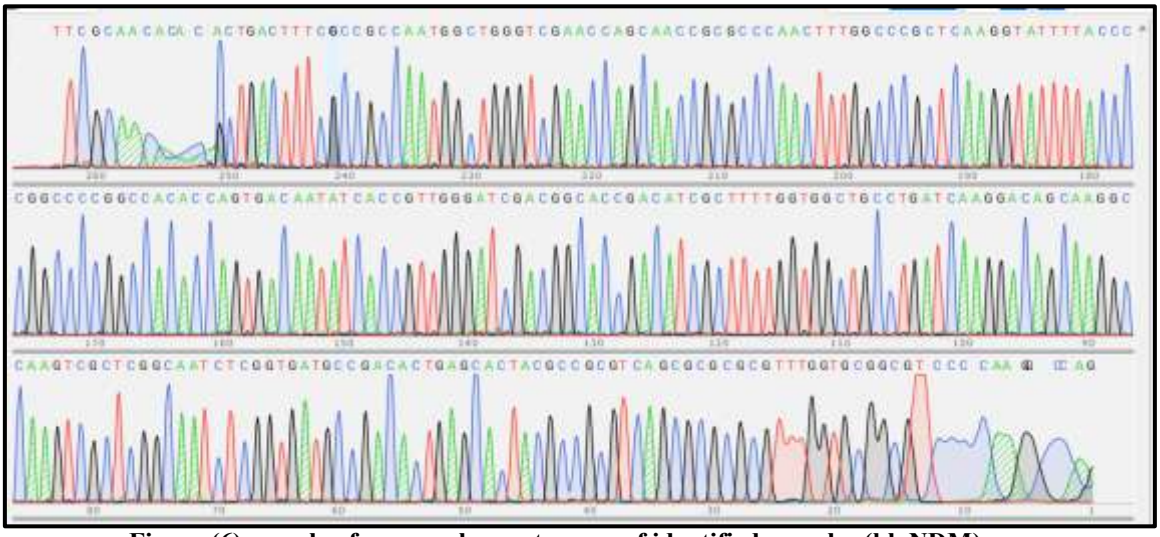


Figure (6) sample of sanger chromatogram of identified samples (blaNDM)

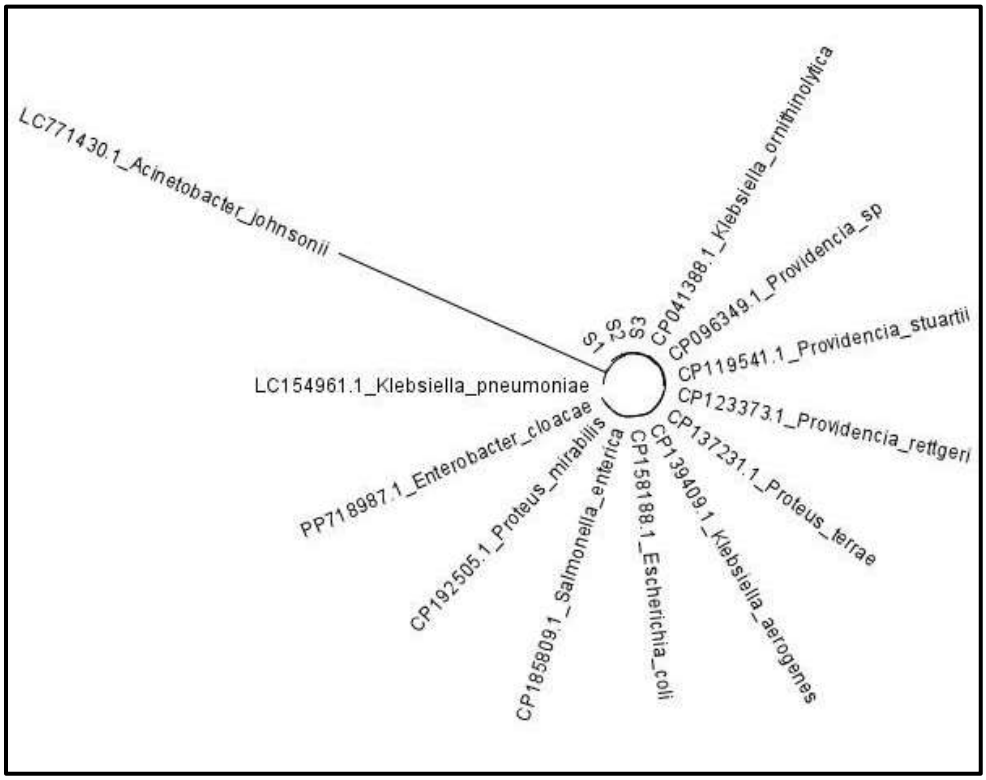


Figure (7)phylogenetic tree of the blaNDMgene

Discussion:-

The high detection rate of blaNDM (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 (Baralet al., 2024; Pattolathet al., 2023). A high occurrence of blaNDM has also been reported among clinical isolates in Asian settings (Santajitet al., 2024). The high frequency of blaNDM in the present collection may reflect the circulation of isolates carrying this resistance determinant in the clinical setting. The dissemination of blaNDM 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; Ragupathiet al., 2024).

In addition, a study by Kotet 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-Aissaniet al., 2013; Koet al., 2023; Kotet 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; Ragupathiet 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.

Integrating these molecular findings with our companion phenotypic evaluation of the same 35 isolates (Hameed& Radhi, 2026), blaNDM was detected in 94.28% (33/35) of the isolates, phenotypic carbapenemase production via the modified Carba NP assay was observed in 77.14% (27/35), and resistance to imipenem and meropenem reached 54.28% and 60%, respectively (Hameed& Radhi, 2026). This discrepancy between genotypic carriage and phenotypic expression suggests that silent or low-level blaNDM expression, regulatory variations, or outer membrane permeability changes may modulate observable carbapenem resistance in clinical settings. Similarly, although mrkD was universally present (100%) and highly conserved in the sequenced representatives, 60% of these isolates exhibited only weak biofilm formation and 37.15% were non-producers phenotypically (Hameed& Radhi, 2026), reinforcing that mrkD carriage alone does not guarantee strong biofilm production without coordinated expression of the full mrk operon and regulatory pathways

Conclusion:-

The blaNDMgene was detected in 94.28% of the 35 clinical Klebsiella pneumoniaeisolates, while mrkD was detected in all isolates (100%). Sequencing revealed 100% identity between representative gene fragments (286 bp for blaNDM 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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