

Phenotypic and Genotypic Detection of Aminoglycoside-Resistant *Klebsiella pneumoniae* Isolated from Sputum Samples of Patients with Respiratory Tract Infections in Iraq

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A major source of respiratory tract infections, especially in hospitalized patients, the elderly, and people with weakened immune systems, *Klebsiella pneumoniae* is a clinically significant opportunistic pathogen. Antimicrobial resistance in *K. pneumoniae* is becoming more common, which is a major global health problem since it restricts treatment options and raises rates of morbidity and mortality. Since aminoglycoside antibiotics are commonly used to treat severe Gram-negative bacterial infections, resistance to these medicines is particularly significant among the several resistance mechanisms. Thus, the goal of the current investigation was to examine the genotypic and phenotypic traits of aminoglycoside-resistant *K. pneumoniae* that were isolated from sputum samples taken from patients suffering from respiratory tract infections. Standard microbiological techniques were used to process 40 sputum samples that were obtained from patients at Baqubah Teaching Hospital. Biochemical tests and the VITEK 2 automatic identification system was used to provide additional validation. In addition, polymerase chain reaction (PCR) targeting the *rpoB* gene was employed to ensure accurate molecular confirmation of the bacterial isolates. The antimicrobial susceptibility testing of *Klebsiella pneumoniae* isolates against aminoglycoside antibiotics revealed variable resistance patterns. The highest resistance rate was observed against streptomycin (72.7%), followed by neomycin (68.18%), while amikacin showed comparatively lower resistance (22.7%). Genotypic analysis was subsequently performed using PCR to detect aminoglycoside resistance genes, with particular emphasis on the *aph* (3')-VI gene. The results revealed that 22 (55%) of the 40 sputum samples were identified as *K. pneumoniae*. Among these isolates, 10 (45.5%) exhibited resistance to aminoglycoside antibiotics and were selected for molecular investigation. PCR analysis demonstrated that all 10 aminoglycoside-resistant isolates (100%) carried the *aph* (3')-VI resistance gene, indicating a strong association between this gene and the observed aminoglycoside resistance phenotype. In conclusion, aminoglycoside-resistant *K. pneumoniae* represents a significant therapeutic and epidemiological challenge in respiratory tract infections. The integration of phenotypic identification methods with molecular diagnostic techniques provides a reliable approach for the accurate detection and surveillance of resistant strains. Continuous monitoring of resistance genes and antimicrobial susceptibility patterns is essential for guiding appropriate antibiotic therapy, improving patient outcomes, and strengthening infection control strategies in healthcare settings.

Keywords: Aminoglycoside-resistant gene, Aminoglycoside antibiotics, Gram-negative bacterial infections, *Klebsiella pneumoniae*, Sputum.

One of the main causes of infections linked to healthcare, especially respiratory tract infections like pneumonia in hospitalized and

immunocompromised patients, is *Klebsiella pneumoniae*, a Gram-negative opportunistic bacterium. Because of its capacity to colonize

mucosal surfaces and endure in hospital settings, it is accountable for a sizable percentage of nosocomial infections globally.¹ Both upper and lower respiratory tract infections are closely associated with morbidity and mortality, especially in older adults, those with weakened immune systems, and those with a history of lung disease.² Due to its ease of collection and lack of intrusive treatment procedures, sputum is the sample most frequently used to diagnose lower respiratory tract infections.³ The increasing clinical impact of *K. pneumoniae* is largely attributed to its remarkable capacity to acquire and disseminate antibiotic resistance determinants.⁴

A variety of GNB, including *Acinetobacter baumannii* (*A. baumannii*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Escherichia coli* (*E. coli*), and *Klebsiella pneumoniae* (*K. pneumoniae*), are susceptible to the bactericidal effects of aminoglycosides like tobramycin, gentamicin, and amikacin.^{5,6} Aminoglycoside resistance in *K. pneumoniae* is primarily mediated by aminoglycoside-modifying enzymes like acetyltransferases, nucleotidyltransferases, and phosphotransferases. By chemically altering antibiotics, these enzymes render them inactive and lessen their ability to bind to bacterial ribosomes. Horizontal gene transfer across bacterial populations is facilitated by the presence of genes encoding these enzymes, such as the *aph* (3')-VI, *aac*, and *ant* families, on mobile genetic elements such plasmids and transposons.⁷

Aminoglycoside resistance genes are highly prevalent in clinical isolates of *K. pneumoniae*, according to recent molecular research. These genes are frequently linked to multidrug-resistant phenotypes and co-existence with other resistance determinants. For example, resistant isolates frequently have genes like *aph* (3')-Ib, *ant* (2'')-Ia, and *aac* (3)-IIa, which contribute to high levels of resistance to aminoglycosides such as amikacin, gentamicin, and tobramycin.^{8,9} Acetyltransferases (*aac*), nucleotidyltransferase (*ant*), and phosphotransferases (*aph*) are aminoglycoside-modifying enzymes (AMEs) that inactivate medicines and are often carried on mobile genetic elements that allow rapid spread. These enzymes are the main cause of aminoglycoside resistance in *K. pneumoniae*.¹⁰ The prevalence and content of AME genes vary significantly between

locations and healthcare settings, according to international studies; frequently found genes include *aac*(62)-Ib, *aac*(32)-II, *ant*(22)-Ia, and *aph*(3'')-Ia; reported rates range greatly (H²20–80% or higher) based on antibiotic selection pressures and local epidemiology.^{11,12}

The purpose of this study is to use the *rpoB* gene to identify *Klebsiella pneumoniae* isolates that were recovered from sputum samples. Additionally, it seeks to identify the *aph* (32)-VI gene that causes aminoglycoside resistance by PCR and to assess their resistance to aminoglycoside antibiotics using phenotypic techniques. Additionally, the study aims to determine the frequency of resistant strains and their function in respiratory infections, as well as the relationship between phenotypic and genotypic resistance patterns.

MATERIALS AND METHODS

Samples and bacterial isolation

Forty sputum specimens were collected from patients with respiratory tract infections attending Baqubah Teaching Hospital, Diyala, Iraq, between October 2025 and the end of December 2025 for the isolation and identification of *Klebsiella pneumoniae*. The sputum samples were collected aseptically in sterile containers and transported immediately to the laboratory for microbiological processing. The study protocol was approved by the Scientific and Ethical Committee of the College of Science, University of Diyala, Iraq (Ethical Approval No. 26-ASJ-Sci-189).

Identification of Bacteria

Conventional biochemical tests, such as growth on MacConkey (MAC) agar (Merck, Darmstadt, Germany), blood agar plates, assessment of colony morphologies and Gram stain characteristics, oxidase test, catalase test, citrate utilization test, nitrate reduction test, indole production, motility, lactose fermentation, H₂S production, urease activity, Methyl Red (MR) test, and Voges Proskauer (VP) test and capsule test.¹³ The VITEK 2 automatic identification system was used to provide additional validation. Following identification, molecular analysis targeting the *rpoB* gene.¹⁴

Antibacterial susceptibility test

The Kirby-Bauer disc diffusion technique was used to determine the isolates' susceptibility

to aminoglycoside medications. Amikacin (30 ig/disk), gentamicin (10 ig/disk), tobramycin (10 ig/disk), kanamycin (30 ig/disk), Neomycin (30 ig/disk), and streptomycin (10 ig/disk) were tested for antibacterial sensitivity. Isolates resistant to at least one of these aminoglycoside agents were classified as aminoglycoside-resistant. Sterilized cotton swabs were used to inoculate Mueller-Hinton agar plates with bacterial cultures that showed a 0.5 McFarland turbidity standard. The plates were incubated at 37 °C after antibacterial discs were placed. The Clinical and Laboratory Standards Institute's guidelines were followed in the analysis of the millimetre-scale measurements of the inhibitory zones (CLSI 2024).¹⁵

Molecular detection of aminoglycoside-resistant genes

The PCR experiment involved the extraction of DNA using the DNA Extraction Kits (Qiagen; Germany). The purity of the isolated DNA was assessed utilising a Nanodrop spectrophotometer (Sigma; USA). The PCR technique was employed to investigate the existence of the *rpoB* and *aph (3')-VI* genes in

a PCR mixture of 1 µl of template DNA at a concentration of 50-100 ng/ml, along with 1 µl of forward and reverse primers from Bioneer, South Korea. 9.5 µM DDW and 12.5 µL of Green Master Mix 2X (Ampliqon Company, Denmark) in a total volume of 25 µL.¹⁶ The specific primers with PCR conditions are listed in Table 1. The PCR reaction was performed on a thermal cycler (Eppendorf, Mastercycler Gradient; Eppendorf, Hamburg, Germany) under the following condition: 1 cycle at 95 °C for 3min, followed by 35 cycles at 94 °C for 1 min, annealing at 55 °C for 40 s, and then extension step at 72 °C for 1 min followed by a final extension step at 72 °C for 5 min. PCR products were analyzed on a 1.5% agarose gel, stained with DNA-safe stain (SinaClon, Tehran, Iran), and visualised under (UV) light (UVItc, Cambridge, UK).¹⁷

Statistical analysis

Data were analyzed using statistical software (e.g., SPSS). Differences in resistance rates among the tested aminoglycoside antibiotics were analyzed using (e.g., Chi-square test), with p-value < 0.05 considered statistically significant.

Table 1. Primers utilised in this work from Macrogen/ Korea

Primer	Primer sequence	Product Size	Annealing temperature	Reference
<i>rpoB</i>	F: CAACGGTGTGGTTACTGACG R: TCTACGAAGTGGCCGTTTTC	108 bp	55	14
<i>aph(3)-VI</i>	F: ATGGAATTGCCCAATATTATT R: TCAATTCAATTCATCAAGTTT	780 bp	55	18

Table 2. Biochemical identification profile of clinical *Klebsiella pneumoniae* isolates

Test	Result
Gram stain	-ve
Catalase	+ve
Oxidase	-ve
Indole	“ve
Methyl Red	“ve
Voges-Proskauer	+ve
Citrate utilization	+ve
Urease	+ve
Lactose fermentation	+ve
Motility	“ve
Hydrogen sulfide (H, S)	“ve
Capsule	+ve

RESULTS

A total of 40 sputum samples were collected from patients with respiratory tract infections. Out of these, 22 (55%) isolates were identified as *Klebsiella pneumoniae* based on cultural, morphological, and biochemical characteristics, as shown in Table 2. Identification of *Klebsiella pneumoniae* was confirmed using the VITEK 2 system for biochemical analysis. Molecular confirmation was performed by PCR targeting the *rpoB* gene Figure (1), and the amplified products were verified by agarose gel electrophoresis based on the expected band size.

The antimicrobial susceptibility testing of *Klebsiella pneumoniae* isolates against

aminoglycoside antibiotics revealed variable resistance patterns. The highest resistance rate was observed against streptomycin (72.7%), followed by neomycin (68.18%), while amikacin showed comparatively lower resistance (22.7%) as shown in (Table 3).

The resistance rates to aminoglycoside antibiotics ranged from 22.7% to 72.7%. The highest resistance was observed for streptomycin (72.7%), followed by neomycin (68.2%) and kanamycin (54.5%), whereas the lowest resistance was detected for amikacin (22.7%). Statistical analysis using the Chi-square test showed no significant difference in resistance rates among the tested aminoglycosides

PCR analysis revealed that the *aph(3')-VI* gene was detected in all ten (100%) *Klebsiella pneumoniae* isolates examined, indicating the

widespread distribution of this aminoglycoside resistance determinant among the selected isolates. Similarly, the *rpoB* gene was successfully amplified in all isolates (10/10, 100%), confirming its suitability as a reliable molecular marker for the identification of *K. pneumoniae*. These findings are presented in Figures 1 and 2.

Overall, the results highlight a considerable prevalence of aminoglycoside-resistant *Klebsiella pneumoniae* in sputum samples, supported by both phenotypic and genotypic findings.

DISCUSSION

The present study demonstrated that *Klebsiella pneumoniae* was isolated from sputum samples of patients with respiratory tract infections, which is consistent with its well-established role

Table 3. Aminoglycosides Susceptibility Pattern of *Klebsiella pneumoniae* isolated from sputum (n=22)

Antibiotic disc	Disc Potency	No. of Resistant Isolates	Resistance (%)
Amikacin	30 ig/disk	5	22.7%
Gentamicin	10 ig/disk	10	45.4%
Tobramycin	10 ig/disk	10	45.4%
Streptomycin	10 ig/disk	16	72.7%
Kanamycin	30 ig/disk	12	54.5%
Neomycin	30 ig/disk	15	68.18%



Fig. 1. The amplification results using the *rpoB* primer in *K. pneumoniae* fractionated on 1.5% agarose stained with Eth.Br. M: 100bp ladder marker. Lanes 1-10 resemble 108 bp PCR product.

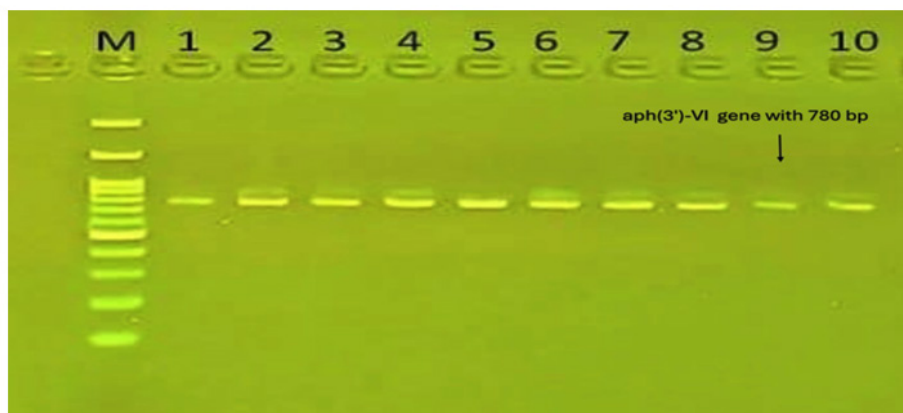


Fig. 2. The amplification results using *aph (3) -VI* primer in *K. pneumoniae* fractionated on 1.5% agarose stained with Eth.Br. M: 100bp ladder marker. Lanes 1-10 resemble a 780 bp PCR product.

as a major opportunistic pathogen associated with pulmonary infections, particularly in hospitalized and immunocompromised patients. Recent studies confirm that *K. pneumoniae* remains one of the leading causes of healthcare-associated respiratory infections and contributes significantly to morbidity and mortality worldwide.^{14,18}

The use of phenotypic methods, including culture characteristics and biochemical tests, remains essential for primary identification. However, these methods alone may not always provide sufficient accuracy, especially in distinguishing closely related Enterobacteriaceae. Automated systems such as VITEK 2 have improved the speed and reliability of bacterial identification and antimicrobial susceptibility testing, making them widely used in clinical laboratories.¹⁹ Molecular confirmation using PCR targeting the *rpoB* gene significantly enhanced diagnostic accuracy in the current study. The *rpoB* gene is considered a highly conserved housekeeping gene and has been widely used for precise bacterial identification. Recent molecular studies have highlighted that PCR-based techniques offer superior sensitivity and specificity compared to phenotypic methods, particularly in detecting clinically relevant pathogens directly from clinical isolates.¹⁴

The present study revealed variable resistance patterns among *Klebsiella pneumoniae* isolates against the tested aminoglycosides. The overall resistance rates indicated a high level of resistance to several commonly used antibiotics,

reflecting the growing challenge of antimicrobial resistance in clinical settings. Among the tested antibiotics, streptomycin exhibited the highest resistance rate (72.7%), followed by neomycin (68.18%) and kanamycin (54.5%). These findings suggest that these antibiotics may have limited clinical effectiveness against *K. pneumoniae* isolates in this study. The high resistance rates could be attributed to the widespread use of these antibiotics over time, which has facilitated the selection of resistant strains, particularly those harbouring aminoglycoside-modifying enzyme genes.¹⁴

Gentamicin and tobramycin showed moderate resistance levels (45.4% each), suggesting some efficacy but also a worrying degree of resistance. In contrast, amikacin demonstrated the lowest resistance rate (22.7%), indicating that it is still the most effective aminoglycoside among those tested. These results are in line with earlier research that documented rising resistance to these drugs among Gram-negative bacteria, including *K. pneumoniae*. It is a more dependable alternative for treating infections brought on by resistant strains because of its structural resistance to enzymatic modification by typical aminoglycoside-modifying enzymes. The generation of aminoglycoside-modifying enzymes, such as acetyltransferases, phosphotransferases, and adenylyl transferases, which render the antibiotics inactive, is largely responsible for the observed resistance patterns. The resistance seen in these isolates may potentially be caused by additional mechanisms, such as

decreased membrane permeability, efflux pumps, and target site changes. The findings of the antimicrobial susceptibility test showed a high degree of resistance to aminoglycosides, especially to gentamicin (50%) and tobramycin (55%), but amikacin showed relatively lesser resistance (37.5%).²⁰ These results are consistent with recent worldwide patterns showing a slow reduction in aminoglycoside effectiveness as a result of resistance genes spreading widely. The findings of the current investigation are corroborated by a number of recent studies (2023–2024) that found gentamicin and tobramycin resistance rates in *K. pneumoniae* above 50%. Amikacin's limited clinical use and structural resistance to specific modifying enzymes may be responsible for its comparatively retained efficacy.

By permanently attaching to the 30S ribosomal subunit, aminoglycosides limit protein synthesis and produce nonfunctional proteins, which ultimately cause bacterial cell death. However, resistance strains have emerged as a result of the extensive and improper use of these antibiotics, particularly in clinical isolates of *K. pneumoniae*.²⁰ The results show that the synthesis of aminoglycoside-modifying enzymes, such as acetylation, phosphorylation, and adenylation, which render the antibiotic inactive and less effective, is one of the main routes of resistance. An enzyme that phosphorylates aminoglycosides and reduces their antibacterial action is encoded by the *aph (3')-VI* gene, which was found in 100% of the samples in this investigation. These findings are in line with earlier research showing that these resistance genes are widely distributed among clinical isolates.²¹ Nevertheless, some phenotypically resistant isolates lack the *aph (3')-VI* gene, indicating the presence of other resistance mechanisms. These could include alterations in membrane permeability, overexpression of efflux pumps, or more aminoglycoside-modifying enzymes. On the other hand, the identification of the gene in a limited number of isolates that are phenotypically sensitive could suggest low-level expression, silent gene carriage, or regulatory factors influencing gene expression. These differences between genotypic and phenotypic results highlight the complexity of antibiotic resistance and have been observed more frequently in recent molecular epidemiology research.²²

Crucially, the substantial association found between the existence of the *aph (3')-VI* gene and phenotypic resistance highlights the need for molecular diagnostics in verifying resistance mechanisms. Combining PCR-based detection with traditional susceptibility testing improves diagnostic precision, speeds up turnaround times, and offers vital information about the genetic foundation of resistance.²³

A substantial correlation between aminoglycoside resistance and the presence of the *aph (3')-VI* gene was found in the correlation analysis between phenotypic resistance and genotypic detection.²⁴ However, the identification of the gene in certain phenotypically susceptible isolates may indicate low-level resistance or silent gene expression that is undetectable by conventional susceptibility testing. This emphasises how crucial it is to integrate molecular and phenotypic methods for a more thorough comprehension of antibiotic resistance.

This study's high incidence of aminoglycoside-resistant *K. pneumoniae* raises concerns because it restricts treatment options and may result in treatment failure. The issue is made worse by the transfer of resistance genes via mobile genetic elements, which makes it easier for resistance to proliferate in clinical settings.

CONCLUSION

Aminoglycosides have been considered adequate therapeutic agents against both Gram-negative and Gram-positive pathogens, so simultaneous detection of resistance-creating agents to aminoglycosides in these strains is of clinical importance. Emphasis on the suitable use of antibiotics, effective infection control measures, and identification of antibiotic resistance mechanisms by molecular procedures are necessary to reduce the incidence of infections caused by antibiotic-resistant organisms. This study shows that *Klebsiella pneumoniae* isolates have a high prevalence of aminoglycoside resistance, which is mostly linked to the *aph (3')-VI* gene. To accurately identify and comprehend resistance mechanisms, phenotypic and genotypic approaches had to be integrated. To stop the spread of resistant strains, it is highly advised to conduct ongoing surveillance and use antibiotics sensibly.

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This research does not involve any clinical trials.

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Not Applicable.

Author Contributions

Naseer Hafedh Ibrahim: Data Collection, Analysis, Writing – Review & Editing; Izdehar Mohammed Jasim: Supervision, Project Administration. Funding Acquisition, Resources; Shahad Saad Alwan: Conceptualisation, Methodology, Writing – Original Draft.

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