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Prevalence of Virulence Genes and Biofilm-Forming Ability Among Multidrug-Resistant *Klebsiella pneumoniae* Isolates from Intensive Care Unit Patients

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Abstract: Background: Multidrug-resistant *Klebsiella pneumoniae* is an important cause of healthcare-associated infection, and its persistence is enhanced by biofilm formation and virulence-associated genes. This study investigated antimicrobial susceptibility, biofilm-forming ability, and the prevalence of *mrkA* and *luxS* among clinical *K. pneumoniae* isolates recovered in Mosul, Iraq. Methods: Ten non-duplicate isolates were obtained from Al-Salam Teaching Hospital, Al-Jumhuriya Teaching Hospital, and the Specialized Burn Center. Isolates were identified phenotypically and confirmed using the VITEK 2 system and 16S rRNA PCR. Antimicrobial susceptibility was assessed by the Kirby-Bauer disc diffusion method. Biofilm formation was quantified using the crystal-violet microtiter plate assay. Endpoint PCR was used to detect *mrkA*, which encodes the major subunit of type 3 fimbriae, and *luxS*, which is involved in autoinducer-2 quorum sensing. Results: Burn swabs accounted for 50% of isolates, wound swabs for 30%, and sputum and urine for 10% each. All isolates were resistant to the ten antimicrobial agents tested. The 16S rRNA target was detected in all isolates. *mrkA* was present in 10/10 isolates (100%), whereas *luxS* was detected in 4/10 isolates (40%). All isolates were classified as moderate biofilm producers. Conclusion: The isolates showed extensive antimicrobial resistance together with a high prevalence of *mrkA* and consistent biofilm formation. The lower frequency of *luxS* suggested that quorum-sensing capacity varied among isolates. These findings support continued surveillance of resistant *K. pneumoniae* and virulence-associated traits in hospital settings.

Keywords: *Klebsiella pneumoniae*; multidrug resistance; biofilm; *mrkA*; *luxS*.

Introduction

Klebsiella pneumoniae is a major opportunistic Gram-negative pathogen in hospitals and is frequently recovered from severe burn and wound infections. Reports from Mosul have highlighted the importance of nosocomial pathogens in damaged tissues and their capacity to persist in clinical environments [1]. The growing burden of antimicrobial resistance has further complicated treatment, particularly with the spread of multidrug-resistant (MDR) and extensively drug-resistant (XDR) organisms. Resistance determinants such as extended-spectrum beta-lactamases and carbapenemases contribute substantially to this problem [2]. *K. pneumoniae* can also reduce antimicrobial susceptibility through beta-lactam hydrolysis, reduced outer-membrane permeability, and mutations affecting antimicrobial targets [3].

The pathogenic potential of *K. pneumoniae* depends not only on antimicrobial resistance but also on virulence traits that promote colonization and persistence. A prominent polysaccharide capsule protects bacterial cells from host defenses, while adhesion and biofilm development allow organisms to remain attached to biotic and abiotic surfaces [4]. Type 3 fimbriae are particularly important during the early stages of surface attachment. Their major structural subunit is encoded by *mrkA*, a gene closely associated with biofilm initiation in clinical *K. pneumoniae* isolates [5].

Cell-to-cell communication can further influence the organization of bacterial communities. The LuxS/autoinducer-2 (AI-2) system is one of the best-described quorum-sensing pathways in Gram-negative bacteria. The *luxS* gene contributes to production of the AI-2 signal molecule and has been linked to biofilm-associated behavior in clinical isolates [6]. The distribution of *mrkA* and *luxS* may therefore provide useful information about the virulence potential of resistant *K. pneumoniae* recovered from hospital settings.

Accordingly, this study evaluated clinical *K. pneumoniae* isolates recovered from hospitals in Mosul, Iraq, with three objectives: to characterize their antimicrobial susceptibility profiles, to determine their biofilm-forming ability, and to assess the prevalence of the virulence-associated genes *mrkA* and *luxS* by polymerase chain reaction (PCR) [7].

Materials and Methods

Ethical approval

The study was approved by the Scientific and Ethical Research Committee of the Nineveh Health Directorate.

Study isolates and phenotypic identification

Ten non-duplicate clinical isolates of *K. pneumoniae* were obtained from diagnostic microbiology laboratories at three healthcare institutions in Mosul, Iraq: Al-Salam Teaching Hospital, Al-Jumhuriya Teaching Hospital, and the Specialized Burn Center. Initial identification was based on conventional microbiological criteria, including Gram staining, colony morphology on MacConkey agar, catalase production, and oxidase activity. The identification of the isolates was subsequently confirmed using the VITEK 2 automated system.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the Kirby-Bauer disc diffusion method on Mueller-Hinton agar in accordance with Clinical and Laboratory Standards Institute recommendations. Bacterial suspensions were adjusted to the 0.5 McFarland standard before inoculation. Ten antimicrobial agents were tested: ciprofloxacin, levofloxacin, chloramphenicol, imipenem, amikacin, ceftazidime, cefepime, tetracycline, trimethoprim/sulfamethoxazole, and piperacillin/tazobactam. Plates were incubated at 37°C for 18-24 h, inhibition-zone diameters were measured, and isolates were categorized as susceptible, intermediate, or resistant according to CLSI interpretive criteria.

Quantification of biofilm formation

Biofilm formation was evaluated using the crystal-violet microtiter plate assay. Bacterial suspensions adjusted to a 0.5 McFarland standard were prepared in Brain Heart Infusion broth, dispensed into sterile 96-well polystyrene plates, and incubated without agitation at 37°C for 24 h. Planktonic cells were removed and the wells were washed three times with phosphate-buffered saline

(PBS; pH 7.2). Adherent biomass was fixed with 99% methanol for 15 min, stained with 0.1% crystal violet for 20 min, and rinsed with distilled water. The bound dye was solubilized in 95% ethanol, and absorbance was measured at 630 nm using a microplate reader.

Biofilm production was classified according to the optical-density cut-off (OD_c) as follows: non-producer, OD ≤ OD_c; weak producer, OD_c < OD ≤ 2×OD_c; moderate producer, 2×OD_c < OD ≤ 4×OD_c; and strong producer, OD > 4×OD_c.

Molecular identification and detection of biofilm-associated genes

Genomic DNA was extracted from overnight bacterial cultures using a commercial bacterial genomic DNA extraction kit according to the manufacturer's instructions. The extraction approach followed previously described laboratory procedures [7]. Species identity was assessed by PCR amplification of the 16S rRNA gene, and confirmed isolates were screened by endpoint PCR for *mrkA* and *luxS*. The *mrkA* gene encodes the major structural subunit of type 3 fimbriae, whereas *luxS* encodes an enzyme involved in AI-2-mediated quorum sensing.

Each PCR was prepared in a final volume of 25 µL containing 12.5 µL of 2X Master Mix, 1 µL of each primer (10 pmol/µL), 2 µL of template DNA (approximately 50 ng), and nuclease-free water. Thermal cycling consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing for 45 s (55°C for 16S rRNA, 58°C for *mrkA*, and 54°C for *luxS*), and extension at 72°C for 60 s (90 s for 16S rRNA). A final extension was performed at 72°C for 10 min.

PCR products were separated on 1.2%-1.5% (w/v) agarose gels prepared in 1X TAE buffer, stained with ethidium bromide, and visualized under UV transillumination with an appropriate DNA marker. Expected amplicon sizes were approximately 1500 bp for 16S rRNA, 105 bp for *mrkA*, and 477 bp for *luxS*. Purified 16S rRNA amplicons were subjected to bidirectional Sanger sequencing, and the resulting sequences were compared with the NCBI database using BLAST for species verification. Primer sequences are listed in Table 1; the *luxS* primers were based on a previously reported assay.

Table 1. Oligonucleotide primer sequences used for PCR amplification.

Gene	Primer	Sequence (5'→3')	Product size (bp)	Reference
16S rRNA	27F (Forward)	AGAGTTTGATCMTGGCTCAG	~1500	[8]
16S rRNA	1492R (Reverse)	TACGGYTACCTTGTTACGACTT		
<i>mrkA</i>	<i>mrkA</i> -F (Forward)	AAGCAAAAGGCAGTGACAGC	105	[5]
<i>mrkA</i>	<i>mrkA</i> -R (Reverse)	CACCATCACGTTTGCCTTAG		
<i>luxS</i>	<i>luxS</i> -F (Forward)	GTTCTGCTGGATGATTTC	477	[9]
<i>luxS</i>	<i>luxS</i> -R (Reverse)	AATTCGTCGGACATTGACC		

Results

Isolation and phenotypic identification

Ten non-duplicate *K. pneumoniae* isolates were recovered from clinical specimens collected between October and December 2025 at the three participating healthcare institutions. Conventional microbiological identification was consistent with *K. pneumoniae* and was confirmed using the VITEK 2 system [8].

Burn swabs were the most frequent source, accounting for five isolates (50.0%), followed by wound swabs with three isolates (30.0%). One isolate (10.0%) was recovered from sputum and one (10.0%) from urine. On MacConkey agar, the isolates produced pink, mucoid to hypermucoid colonies. On blood agar, the colonies were smooth and non-hemolytic, with no visible zone of erythrocyte lysis [9]. The source distribution and phenotypic features are summarized in Table 2, and representative colony morphologies are shown in Figure 1.

Table 2. Source distribution and phenotypic characteristics of the 10 clinical *Klebsiella pneumoniae* isolates.

Specimen source	Isolate (n=10)	count	Relative frequency (%)	Colony morphology on MacConkey agar	Hemolytic pattern on blood agar
Burn swabs	5		50.0	Large, pink, hypermucoid colonies	Gamma-hemolytic (non-hemolytic)
Wound swabs	3		30.0	Large, pink, mucoid colonies	Gamma-hemolytic (non-hemolytic)
Sputum	1		10.0	Pink, mucoid colonies	Gamma-hemolytic (non-hemolytic)
Urine	1		10.0	Pink, moderately mucoid colonies	Gamma-hemolytic (non-hemolytic)
Total	10		100	Lactose fermenter / mucoid	Gamma hemolysis

**Figure 1.** Diagnostic morphological traits of clinical *K. pneumoniae* isolates: (A) large, pink, mucoid coalescent colonies on MacConkey agar; (B) gamma-hemolytic (non-hemolytic) colonies on blood agar [10].

Antimicrobial susceptibility

All 10 isolates were resistant to each of the ten antimicrobial agents tested: ciprofloxacin, levofloxacin, chloramphenicol, imipenem, amikacin, ceftazidime, cefepime, tetracycline, trimethoprim/sulfamethoxazole, and piperacillin/tazobactam. Thus, the isolates displayed a broad multidrug-resistant phenotype across the antimicrobial panel used in this study [11].

Biofilm-forming ability

All 10 clinical isolates (100%) were classified as moderate biofilm producers by the crystal-violet microtiter plate assay. This consistent phenotype indicated that biofilm formation was common among the resistant isolates examined in the study. The biofilm-forming result is summarized in Table 3.

Table 3. Biofilm-forming ability of the 10 clinical *Klebsiella pneumoniae* isolates.

Assay	Total isolates (n)	Biofilm category	Isolates, n (%)
Crystal-violet microtiter plate assay	10	Moderate biofilm producer	10 (100%)

Molecular identification and prevalence of *mrkA* and *luxS*

PCR amplification of the 16S rRNA target was positive in all 10 isolates, supporting molecular confirmation of *K. pneumoniae*. The *mrkA* gene was detected in all isolates (10/10; 100%), whereas *luxS*

was detected in four isolates (4/10; 40%). The molecular prevalence of the tested targets is presented in Table 4. Representative agarose-gel electrophoresis images are shown in Figures 2-4.

Table 4. Molecular prevalence of the target genes among the 10 clinical *Klebsiella pneumoniae* isolates.

Target gene	Biological function / target subunit	Amplicon size (bp)	Positive isolates (n=10)	Prevalence (%)
<i>mrkA</i>	Major structural subunit of type 3 fimbriae	105	10	100
<i>luxS</i>	Autoinducer-2 synthase involved in quorum sensing	477	4	40
16S rRNA	Molecular identification target	~1500	10	100

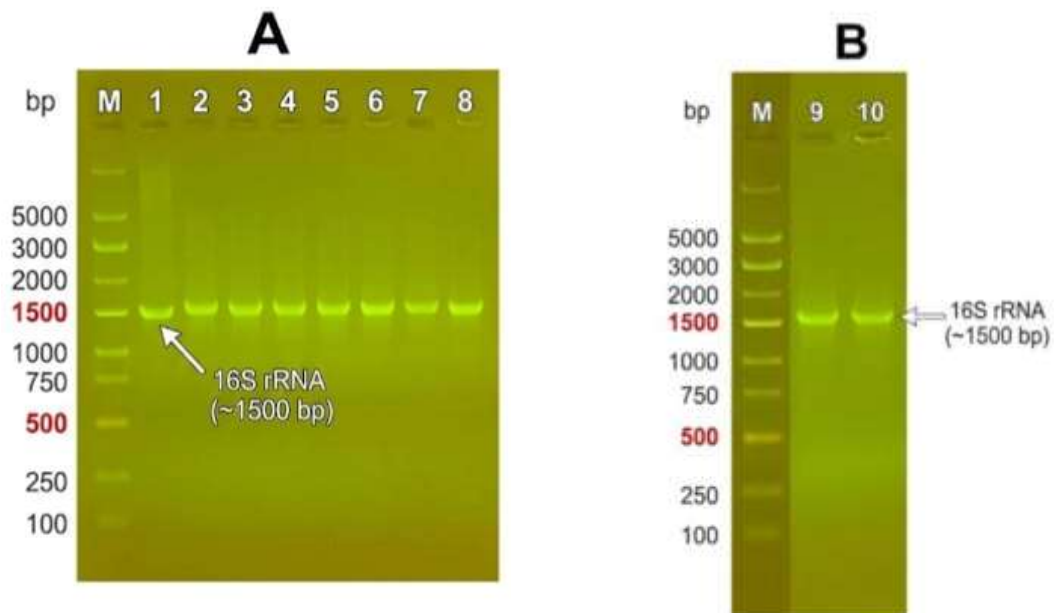


Figure 2. Agarose gel electrophoresis of the 16S rRNA gene from *K. pneumoniae* isolates. (A) Initial screening of eight isolates (lanes 1-8). (B) Retesting of two additional isolates (lanes 9-10). Lane M indicates the DL5000 DNA marker; the amplified product was approximately 1500 bp.

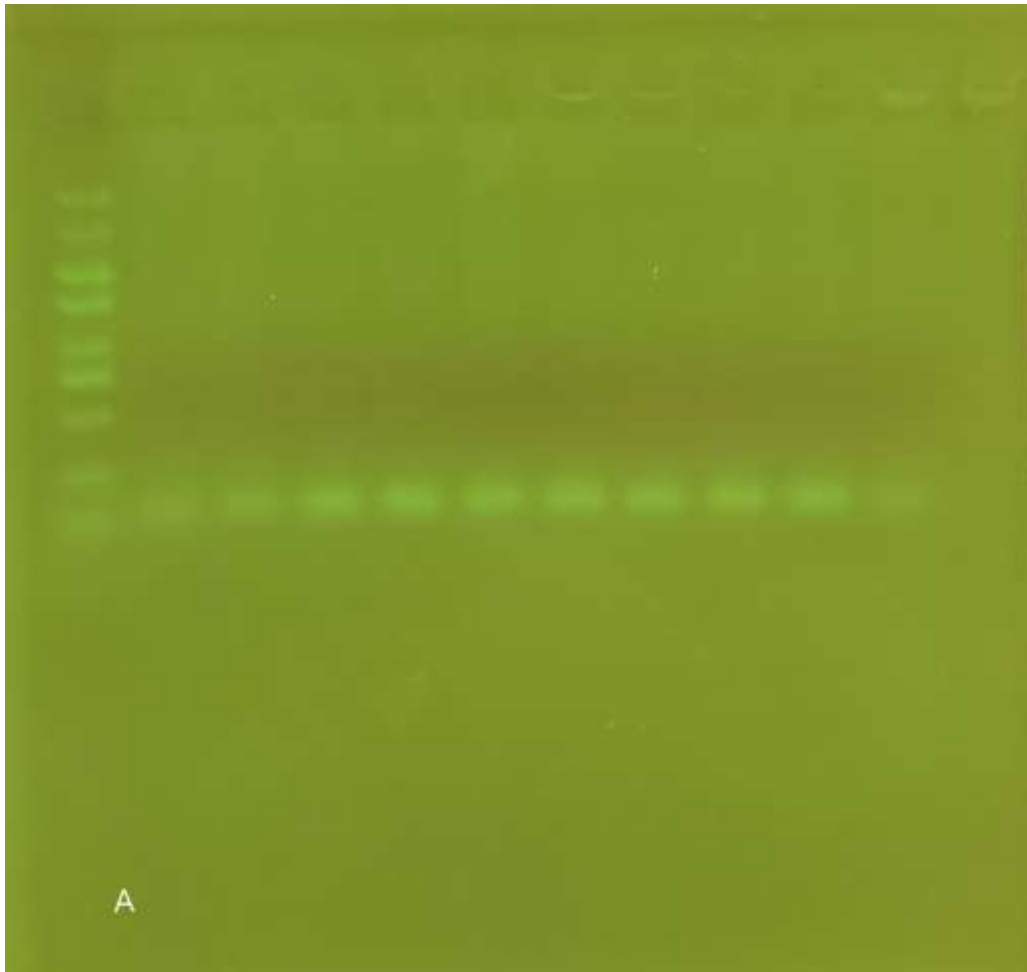


Figure 3. Agarose gel electrophoresis of the *mrkA* gene from *K. pneumoniae* isolates; the expected amplicon size was 105 bp.

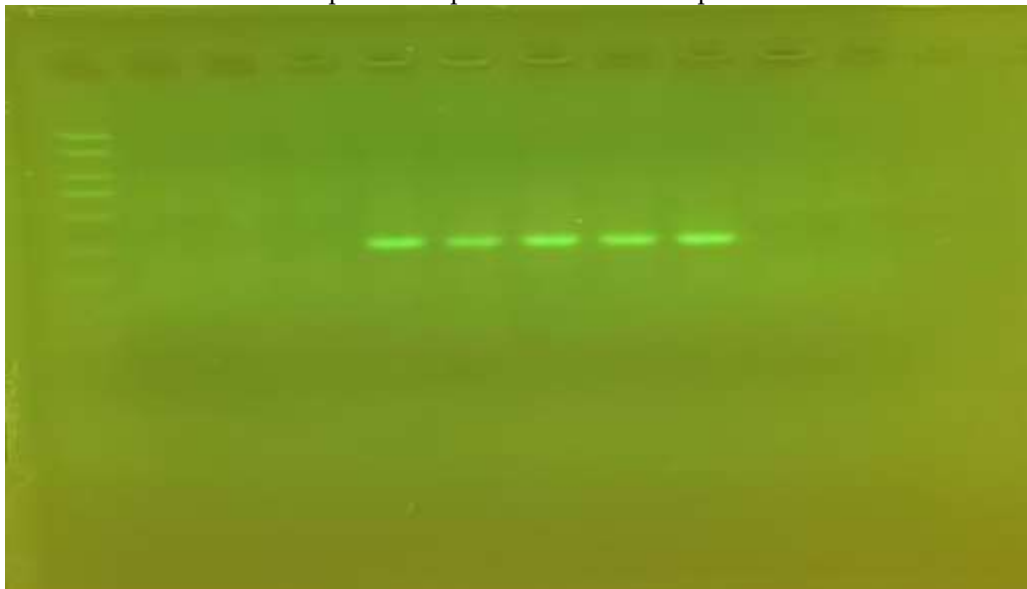


Figure 4. Agarose gel electrophoresis of the *luxS* gene from *K. pneumoniae* isolates; the expected amplicon size was 477 bp.

Discussion

The present study characterized ten clinical *K. pneumoniae* isolates recovered from three healthcare institutions in Mosul. Burn and wound swabs together accounted for 80% of the isolates,

with burn specimens representing the largest group. This distribution is clinically plausible because disruption of the skin barrier, prolonged hospitalization, and intensive antimicrobial exposure create favorable conditions for colonization and infection by opportunistic Gram-negative bacteria. Similar concerns have been raised regarding hypermucoviscous *K. pneumoniae* and its ability to persist in severe clinical infections [12].

A notable finding was the resistance of all isolates to the ten antimicrobial agents included in the test panel. Although the small number of isolates limits broader epidemiological interpretation, the pattern points to a substantial local resistance burden. This observation is consistent with reports describing increasing difficulty in treating hospital-associated Gram-negative infections. The spread of acquired resistance determinants, including beta-lactamases and carbapenemases, together with reduced outer-membrane permeability and target modification, can collectively reduce susceptibility across several antimicrobial classes [13]. Continued local surveillance is therefore important, particularly in burn and intensive-care settings where antimicrobial exposure is frequent.

The *mrkA* gene was detected in every isolate, and all isolates were moderate biofilm producers. The parallel occurrence of *mrkA* and the biofilm phenotype supports the biological importance of type 3 fimbriae during early surface attachment. Previous work has identified *mrkA* as a key structural determinant involved in biofilm initiation by *K. pneumoniae*, while broader analyses have linked biofilm formation with persistence and antimicrobial resistance in this species [14]. The findings of the present study do not establish a causal relationship, but they indicate that the isolates commonly carried an adhesion-associated determinant alongside a measurable biofilm phenotype.

In contrast to *mrkA*, *luxS* was detected in 40% of the isolates. The lower prevalence suggests heterogeneity in the genetic basis of quorum-sensing-associated behavior within this small clinical collection. The LuxS/AI-2 pathway has been implicated in bacterial communication and biofilm regulation, including among hospital-associated Gram-negative organisms. The absence of detectable *luxS* in six isolates did not prevent moderate biofilm formation, indicating that additional adhesins, regulatory systems, and extracellular matrix components likely contributed to the phenotype observed here.

Taken together, the results show that antimicrobial resistance and virulence-associated traits coexisted in the tested isolates. The study was limited by the small sample size and by the absence of detailed molecular characterization of antimicrobial-resistance genes. Nevertheless, the combined phenotypic and PCR-based findings provide a useful preliminary picture of resistant *K. pneumoniae* circulating in the participating hospitals and support larger studies that incorporate additional virulence markers, resistance determinants, and strain-typing approaches [15].

Conclusion

Klebsiella pneumoniae recovered from the participating hospitals showed a high level of antimicrobial resistance, with all ten isolates resistant to the ten agents tested. The *mrkA* gene was detected in 100% of isolates and all isolates formed moderate biofilms, whereas *luxS* was present in 40%. These findings emphasize the coexistence of resistance and biofilm-associated virulence traits in clinical *K. pneumoniae*. Expanded surveillance with larger isolate collections and broader molecular testing is recommended to define the local epidemiology of virulence and resistance more accurately.

Consent for Publication

Not applicable.

Competing Interests

The authors declare that they have no competing financial, personal, or professional interests that could have influenced, or appeared to influence, the research reported in this manuscript.

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Authors' Contributions

All authors contributed substantially to the conception and execution of the study. The postgraduate researcher carried out isolate collection, phenotypic identification, PCR amplification, and microtiter-plate biofilm assays. The academic supervisor provided study design, methodological oversight, manuscript preparation, and critical revision. All authors read and approved the final version of the manuscript.

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