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Original Article
Bangladesh Journal of Infectious Diseases
June 2025, Volume 12, Number 1, Page 116-124
ISSN (Online) 2411-670X
ISSN (Print) 2411-4820
NLM ID: 101761093
DOI: https://doi.org/10.3329/bjid.v12i1.84768

Detection of Surface Virulence Factor Genes and Molecular Typing of *Klebsiella pneumonia* by Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction

Sahar Hussain Rashid[1], Ali Jaffar Saleem[2]

[1]Post Graduate Student, Department of Biology, College of Education for Pure Sciences, University of Diyala, Iraq; [2]Professor, Department of Biology, College of Education for Pure Sciences, University of Diyala, Iraq

Abstract

Background: *Klebsiella pneumoniae* possesses virulence genes (*fimH*, mrkD, wabG, *wcaA*, *magA*) that contribute to its high pathogenicity, ability to cause infections in the community and hospitals, as well as its antibiotic resistance and difficulty in treatment. **Objective:** This study investigated the prevalence of some virulence genes in *Klebsiella pneumoniae* in local clinical isolates in Baqubah, Iraq and identified local strains and their relationship to each other using ERCI-PCR. **Methodology**: A total of 285 clinical samples (urine, wound swabs, sputum, vaginal swabs, burn swabs, and blood) were collected from patients attending health centers and outpatient clinics in Baqubah, Iraq, between March to May 2024, Samples were transported to the laboratory within 2 hours and processed according to standard microbiological procedures. Ethical approval was obtained from the Institutional Review Committee of the University of Diyala. PCR was used to detect virulence genes. ERIC-PCR was used to identify local isolates' molecular profiling. Genetic relationship was determined by Past Software. **Results**: Twenty-four (8.42%) *Klebsiella pneumoniae* isolates were identified by morphological, microscopic, and biochemical characteristics. The frequency of *fim*H, *mrk*D, *wab*G, *wca*A and *mag*A genes was 58.3%, 91.6%, 37.5%, 4.1% and 0.0% respectively. Molecular typing of 24 *Klebsiella pneumoniae* isolates using ERIC-PCR revealed 11 different Geno patterns, including four clons and seven single isolates. **Conclusions**: In conclusion, *Klebsiella pneumoniae* isolates carrying high levels of the *fimH* and mrkD genes, which contribute to biofilms production, increase their resistance to antibiotics. ERIC-PCR showing genetic relationships between local isolates and helps in epidemiological surveillance to identify the spread of strains. [*Bangladesh Journal of Infectious Diseases, June 2025;12(1):116-124*]

Keywords: *Klebsiella pneumoniae; fimH; mrkD; wabG; wcaA; magA;* Enterobacterial Repetitive Intergenic Consensus; Polymerase Chain Reaction

Correspondence: Sahar Hussain Rashid, Department of Biology, College of Education for Pure Sciences, University of Diyala, Iraq; Email: sahar.hussain.rasheed@uodiyala.edu.iq; ORCID: https://orcid.org/0009-0008-8365-1453

Introduction

Klebsiella pneumoniae is a member of the Enterobacteriaceae family found as a normal flora in the intestines of healthy people and turns into an opportunistic bacterium when several factors are present, such as repeated exposure to antibiotics, environmental stress, and the health status of people. Its members are usually in the form of rods, Gram-negative, and have a wide range of virulence factors such as toxins, enzymes, capsule, flagellum, and others[1]. It is isolated from a variety of clinical specimens, such as wound and burn infections[2]. Urinary, respiratory, and gastrointestinal tract infections, sepsis, and female reproductive tract infections[3]. *Klebsiella pneumoniae* possesses multiple virulence factors, such as a capsule, cilia, and biofilm, which enhance its pathogenicity and antibiotic resistance and help it adhere to mucosal surfaces and animal tissues[4].

Virulence factors are under the control of genes, including the *fimH* gene fimbriae, which helps these bacteria adhere to cell surfaces and capillaries. Also helps in the formation of biofilms and plays a fundamental role in their ability to resist treatments[5]. The *mrkD* gene acts as a regulator of biofilm formation in *Klebsiella pneumoniae*. Isolates containing the *mrkD* gene can form biofilms and increase their virulence, and enhance their ability to acquire resistance to multiple antibiotics[6]. The *wabG* gene is considered one of the capsule-coding genes in *Klebsiella pneumoniae*, which helps in antibiotic resistance[7]. The *wcaA* gene in *Klebsiella pneumoniae* plays a role in the formation of the outer surface or surface layer that protects bacteria from harmful environmental factors. It also affects the ability of bacteria to resist some antibiotics and works to increase virulence factors such as adhesion and attachment to surfaces, which increases the likelihood of infection and causes diseases[8]. When the *magA* gene is present in *Klebsiella pneumoniae*, it is highly virulent and generally associated with a mucoviscosity phenotype. It encodes the mucoviscosity-associated protein (MAVP), which is a clinically important pathogen responsible for severe disseminated infections, such as pyogenic liver abscesses, osteomyelitis, and endophthalmitis, in patients of different ages[9].

Genomic tools offer powerful discrimination; they are often expensive and require significant expertise. In contrast, ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus PCR), a rapid and cost-effective technique, helps differentiate *Klebsiella pneumoniae* isolates based on their DNA fingerprint patterns[10]. ERIC-PCR is a widely accessible technique for bacterial genome fingerprinting. By amplifying regions between ERIC sequence repeats, it effectively distinguishes between bacterial strains, including closely related species[11]. Its accuracy, simplicity, and speed make it a valuable tool for pathogen tracking, providing more sensitive classification than basic phenotypic methods[12-13]. *Klebsiella pneumoniae* causes serious health problems and can spread rapidly in areas with poor sanitary conditions, also associated with severe hospital-acquired and community-acquired infections, which may cause major health problems for the community if new resistant strains spread.

Given the importance of *Klebsiella pneumoniae* in causing many diseases, increasing its resistance to antibiotics, and possessing many virulence factors that help it spread and adapt to new environmental conditions, this study aimed to detect surface virulence genes (*fimH, mrkD, wabG, wcaA* and *magA*) in *Klebsiella pneumoniae* clinical isolates and molecular typing of *Klebsiella pneumoniae* using ERIC-PCR.

Methodology

Sample Collection: A total of 285 clinical samples (urine, wound swabs, sputum, vaginal swabs, burn swabs, and blood) were collected from patients attending health centers and outpatient clinics in Baqubah, Iraq, between March to May 2024, Samples were transported to the laboratory within 2 hours and processed according to standard microbiological procedures. Ethical approval was obtained from the Institutional Review Committee of the University of Diyala.

Bacterial Isolation and Identification

Cultural Diagnosis: The bacteria were diagnosed by studying the general cultural characteristics of the colonies growing on MacConkey agar and blood agar at 37°C for 24 hours, and observing the colony shapes that appeared through the color, edges, and mucous texture[14], then cultured on Eosin methylene blue (EMB) agar selective medium for *Klebsiella pneumoniae*.

Microscopic Diagnosis: Colonies of the growing bacteria were taken and placed on a glass slide and mixed with normal saline solution, left to dry and then quickly passed over a flame two to three times to fix the colonies, then stained with Gram stain and examined under a microscope to observe the color, shape and clusters of bacteria[15].

Biochemical Test: Three biochemical tests were performed to diagnose the bacteria. These are the oxidase test, catalase test and indole test.

Oxidase Test: 2 to 3 drops of oxidase reagent were added to the filter paper and then a colony of bacteria was transferred with a wooden stick and mixed well with the reagent on the filter paper. The filter paper acquired a purple color after (20-30) seconds, indicating that the test was positive[16].

Catalase Test: A 24-hour-old pure colony was taken from the surface of MacConkey agar using a wooden stick and placed on a glass slide. Then, drops of 2% hydrogen peroxide were added and mixed well with the colony. The appearance of bubbles after one minute indicates a positive test[17].

Indole Test: The colony to be diagnosed was added to the tubes containing a peptone water medium and then incubated at a temperature of 37°C for 24 hours. Then, (5) drops of Kovacs reagent were added. The appearance of a red ring at the top of the medium was evidence that the test was positive. This test is used to detect the presence of the enzyme tryptophanase, which breaks down tryptophan into indole and pyruvic acid[18].

Molecular Detection

Extraction of Bacterial DNA by the Boiling Method: DNA was extracted from bacteria using the boiling method[19]. The bacterial culture was boiled in a small tube using a water bath containing sterile distilled water for 10 minutes at 100^0 C. Then the tubes were placed in a cooled state for 5 minutes, after which they were centrifuged at 12,000 rpm for 5 minutes. The floating part was transferred to new tubes, and then the concentration and purity of DNA were measured by the Nanodrop device at 260 nm and 280 nm[20].

Primer Dilution: In this study, the primers shown in Table 1 were prepared as a stock solution by dissolving the initiator in deionized distilled water to obtain a concentration of 100pmol/µL and stored at -20°C. A working solution was prepared to avoid repeated freezing and thawing, several small aliquots were prepared at a concentration of 10pmol/µL and stored at -20°C.

PCR Components: Polymerase chain reaction (PCR) amplification of the (*fimH, mrkD, wabG, wcaA, magA)* genes was performed using specific primers (Table 1). Each reaction (25µl) contained 12.5µl of PCR master mix, 1µl of each primer (10µM), 2µl of DNA template, and 8.5µl of nuclease-free water. The cycling conditions were as follows: initial denaturation at 94°C for 1 min; 35 cycles of 94°C for 45 s, (54, 58 and 59°C) for 1 min (Table 1), 72°C for 1 min; final extension at 72°C for 2 min 2). The reaction components were placed in sterile tubes of 0.2 ml size, then mixed using a Vortex device for 3 seconds, and then transferred to the PCR device. The PCR reaction mix was prepared using the Go Tag ® G2 Green Master Mix kit, supplied by the American company Promega, PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized by Transilluminator.

Table 1: Primers and amplified PCR products used in the Study

Gene	Primer sequences (5' 3')	Annealing	Product size (bp)	Reference
fimH	F: TGCTGCTGGGCTGGTCGATG R: GGGAGGGTGACGGTGACATC	58^0 C/1min	550	Liao et al[21]
mrkD	F: TTCTGCACAGCGGTCCC *R: GATACCCGGCGTTTTCGTTAC*	59^0 C/45 sec	340	Yadav et al[22]
wabG	F: ACCATCGGCCATTTGATAGA R: CGGACTGGCAGATCCATATC	58^0 C/1 min	683	Jiang et al[23]
wcaA	F: GGTTGGGTCAGCAATCGTA R: ACTATTCCGCCAACTTTTGC	54^0 C/45 sec	169	Safoura et al[24]
magA	F: GGTGCTCTTTACATCATTGC R: GCAATGGCCATTTGCGTTAG	58^0 C/1min	1282	Song et al[25]
ERIC	F: ATGTAAGCTCCTGGGGATTCA R: AGTAAGTGACTGGGGTGAGCG	58^0 C/1min	-	Bilung et al[26]

Dendrogram: The genetic relationship between all bacterial isolates under study was found by using the Dendogram analysis plot using Past Software through the Dice option.

Results

Identification of *Klebsiella pneumoniae* Isolates: Twenty-four isolates of *Klebsiella pneumoniae* (8.42%) were obtained after morphological, microscopic, and biochemical identification. Table 2 shows the details of the required sources, their number, the number of isolates, and their ratios to the sources.

Table 2: Number and Percentage of Isolates according to their Sources

Sources	Frequency	Percent
Wounds (n=75)	4	5.33
Urine (n=67)	6	8.95
Sputum (n=60)	5	8.3
Vagina (n=43)	3	6.97
Burns (n=25)	4	20.0
Blood (n=15)	2	13.3
Total (n=285)	**24**	**8.42**

Cultural Identification: *Klebsiella pneumoniae* exhibited the ability to ferment lactose when cultured on MacConkey agar, resulting in pink colonies as a result of the neutral red indicator turning pink due to the acidic environment created by lactose hydrolysis. MacConkey agar is a differential medium for these bacteria, as pale or colorless colonies indicate a negative result for lactose fermentation. *Klebsiella pneumoniae* does not exhibit hemolysis on blood medium because it lacks the enzyme hemolysin. EMB selective medium is used to differentiate *Klebsiella pneumoniae* from *Escherichia coli*, as *Klebsiella pneumoniae* appears pink and mucoid, while *Escherichia coli* appears bright green[27].

Microscopic Diagnosis: Microscopic examination of the colonies after staining with Gram stain showed the appearance of straight, short, Gram-negative rods, sometimes in pairs arranged singly or in pairs, and stained pink.

Biochemical Tests: *Klebsiella pneumoniae* isolates were diagnosed using the biochemical tests shown in Table 3, the results of which were consistent with those reported[28].

Table 3: Biochemical Tests for Diagnosing *Klebsiella pneumoniae*

Tests	Results
Oxidase Test	Negative
Catalase Test	Positive
Indole Test	Negative
Methyl Red Test	Negative
Voges-Proskauer Test	Positive
Citrate Utilization Test	Positive
Urea Utilization	Positive

Molecular Detection of Virulence Factor Genes of *Klebsiella pneumoniae*: Polymerase chain reaction (PCR) revealed the presence of virulence genes (*fimH*, *mrkD*, *wabG*, *wcaA*, *magA*) at 58.0%, 91.6%, 37.5%, 4.16% and 0.0% respectively.

Table 4: Distribution of Genes in *Klebsiella pneumoniae* Local Isolates

Source	*magA*	*wcaA*	*markD*	*wabG*	*fimH*
Urine			+	+	+
Urine		+			+
Urine					+
Urine					+
Burns					+
Burns					+
Burns					+
Burns					+
Wounds				+	
Wounds				+	
Wounds			+		+
Wounds				+	
Sputum				+	
Sputum					+
Sputum			+		
Sputum					
Vagina				+	+
Vagina				+	+
Vagina					+
Vagina				+	
Blood				+	
Blood					
Blood					+
Blood					
Total	**0.0**	**4.1**	**8.3**	**37.5**	**58.3**

The detection results in the current study showed the presence of the fimH gene in (14) isolates and at a rate of 58.3% in *Klebsiella pneumonia* (Table 4). The study revealed the presence of the *mrkD* gene in 2 (8.3%) isolates as shown in Figure II. The study results showed that the *wabG* gene was found in 37.5% of *Klebsiella pneumoniae* (9 isolates) (Figure III).

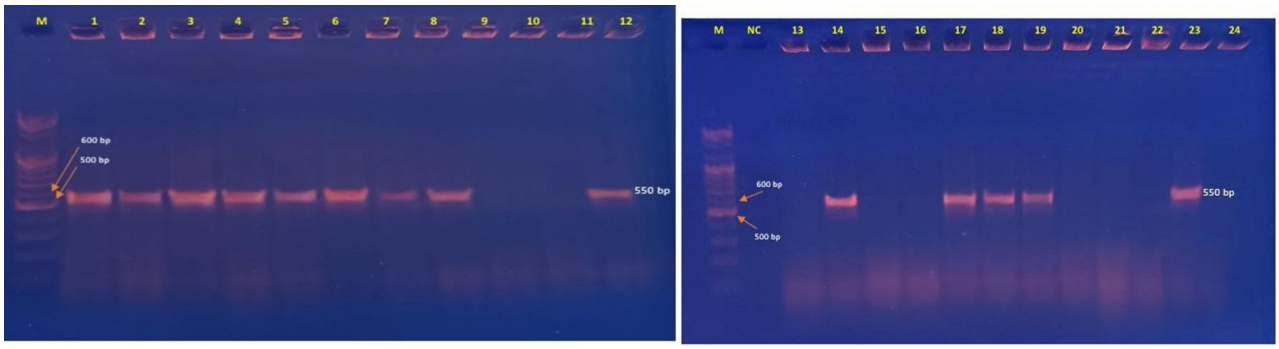

Figure I: Electrophoresis of the PCR reaction product for the gene (550bp) fimH using 1.5% agarose gel with Ethidium bromide dye (60 min, 7 V/cm2). Lane M: Standard size indicator 100 base pairs (ladder 100bp DNA

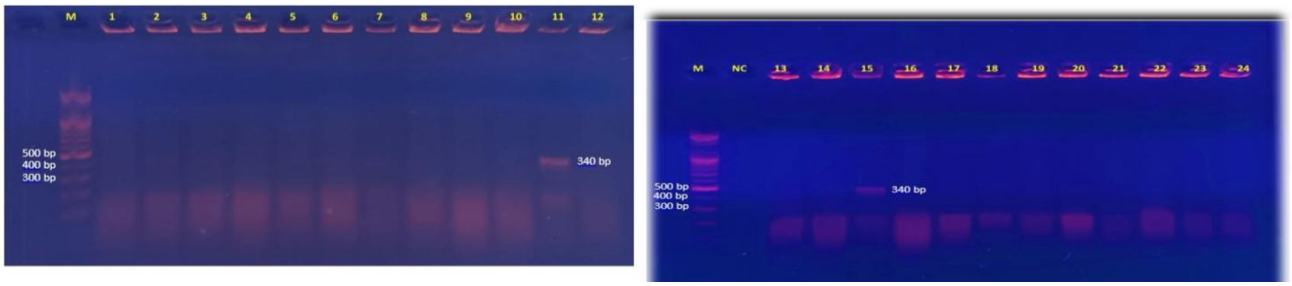

Figure II: Electrophoresis of the PCR reaction product for the gene (340bp) mrkD using 1.5% agarose gel with Ethidium bromide dye (60 min, 7 V/cm2). Lane M: Standard size indicator 100 base pairs (ladder 100bp DNA)

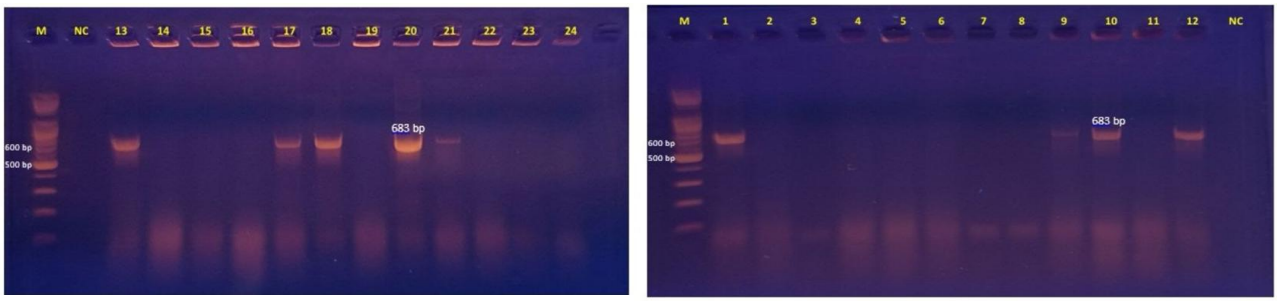

Figure III: Electrophoresis of the PCR reaction product for the (683bp) wabG gene using 1.5% agarose gel with Ethidium bromide dye (60 min, 7 V/cm2). Lane M: Standard size indicator 100 base pairs (ladder 100bp DNA)

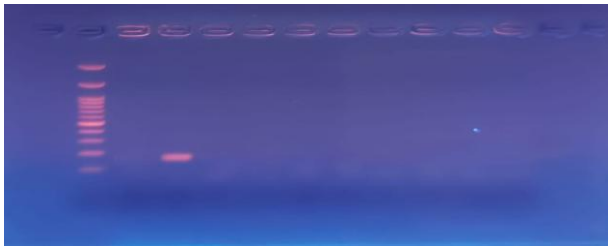

Figure IV: Electrophoresis of the PCR reaction product for the gene (169Pb) wabG using 1.5% agarose gel with Ethidium bromide dye (60 min, 7 V/cm2). Lane M: Standard size guide 100 base pairs (ladder 100bp DNA

The wcaA gene was detected in 4.1% of the studied Klebsiella pneumoniae isolates (one isolate), as shown in Figure IV. The current study showed the absence of the magA gene in the studied isolates.

Molecular Typing of *Klebsiella pneumonia* using ERIC-PCR: The results shown in Figure V the similarity percentage between the genetic patterns according to the ERIC-PCR method, that there were 11 different genetic patterns for the twenty-four isolates under study, distributed into four clones and seven single isolates. The largest of these clones included ten isolates from urinary tract infections (UTIs), burns, and blood. The isolates with unique genetic patterns were distributed among several sources: three from sputum, two from wounds, and one from blood and vagina. The

dendrogram analysis (Figure VI) showed the presence of diversity and differences among the twenty-four isolates under study, as it appeared that the percentage of similarity between single isolates and clones ranged between 0-40% for single isolates with clones, as it appeared that the percentage of similarity between the single isolates and the clones ranged between 0-40% for the single isolates with the clones, while twenty isolates were similar at a percentage ranging between 50-100%. It is noted in the results of our current study that there is a correlation between the intensity of biofilm formation and the genetic patterns of ERIC-PCR.

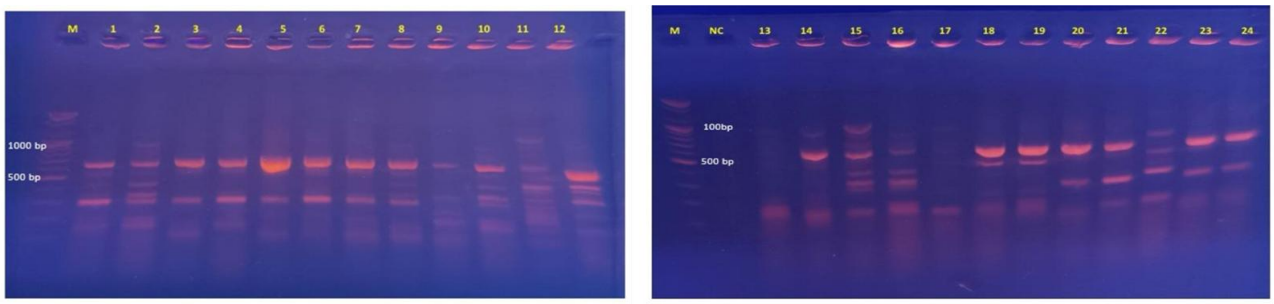

Figure V: Electrophoresis of the PCR reaction product for the ERIC using 1.5% agarose gel with Ethidium bromide dye (60 min, 7 V/cm2). Lane M Standard size guide 100 base pairs (ladder 100bp DNA

Table 5: Shows the Similarity Percentage Between Genetic Patterns According to the ERIC-PCR Method

1000 bp	650	500	400	350	300	Source
	+				+	1
	+		+	+	+	2
	+				+	3
	+				+	4
	+				+	5
	+				+	6
	+				+	7
	+		+	+	+	8
	+					9
	+		+	+	+	10
	+		+			11
		+	+		+	12
						13
	+					14
+	+		+		+	15
	+		+		+	16
						17
	+	+				18
	+	+				19
	+				+	20
	+				+	21
+		+		+		22
	+				+	23
	+				+	24

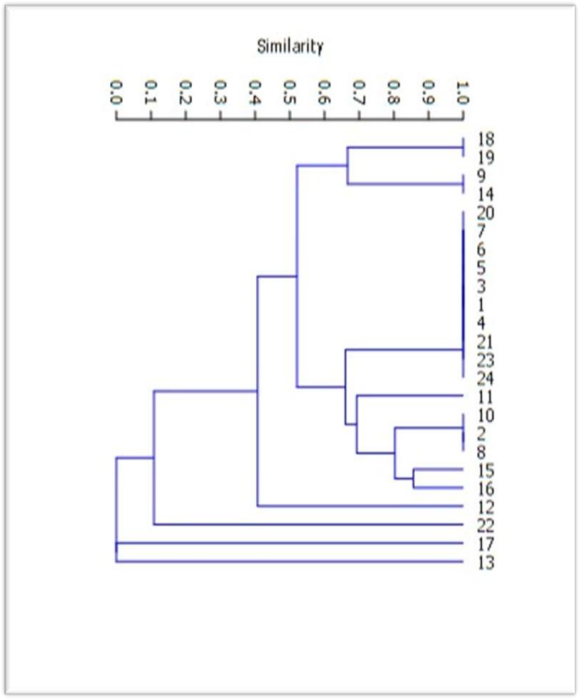

Figure VI: Cluster of Genetic Relationships among Local *Klebsiella pneumonia* isolates

Discussion

The results of the current study are consistent with a study conducted in Saudi Arabia by Booq et al[29] where the frequency of the *fim*H gene reached 65.0% in *Klebsiella pneumoniae* isolates. However,

they differ from the results of other studies in Diyala conducted by Mohamed et al[30] and Al-aajam[31], where the gene frequency was 80.0% and 86.6% respectively. This gene codes for fimbriae, which contribute to the adhesion of bacteria to host cells and the occurrence of infection, as well as their role in contributing to the formation of biofilm.

The results of the current study are not consistent with previous studies conducted in Iraq by Al-Zaidi[32], in Spain by Li[33] and China by Hu[34] where the frequency of the *makD* gene ranged between 92.2% and 100.0%. Also, the results differ from the study by Al-Ajam[31] which recorded a frequency of 76.6%. It is worth noting that the *mrkD* gene plays a role in regulating biofilm formation in *Klebsiella pneumoniae* and isolates carrying this gene exhibit an increased ability to form biofilms, which enhances their virulence, spread, and resistance to multiple antibiotics[35].

The results of the current study on the frequency of the *wabG* gene are consistent with the study of Hassani[36] at Tabriz University of Medical Sciences in Iran, where reached 29.5%, but differ from the results of the study conducted by Mohammed[37] in Baghdad, where the frequency of the *wabG* gene 84.0%. The *wabG* gene, a virulence factor gene within the bacterial chromosome, encodes the capsule, which contributes to resistance to antibiotics and resistance to immune cells and antibiotics.

Regarding the *wacA* gene, the results of the current study do not agree with a previous study by Jabar[38] in Baghdad, where the gene did not appear in his study and Derakhshan in Tehran[39], where it was present at a rate of 23.5%. It is known that this gene contributes to the formation of a surface layer that helps in adhering to the host cells and thus causing infection.

The absence of the magA gene in the studied isolates is consistent with the results of Al-Zaidi[32] and Al-Zubaidi[40], but conflicts with studies from Najaf, Iraq, Hammoud and Al-Ammar[41] and Iran, Zamani et al[42], which showed the presence of the gene in 3.7% and 3.8% of *Klebsiella pneumoniae* isolates, respectively. In this study, the magA gene, commonly associated with hypervirulent, mucoviscosity-phenotype *Klebsiella pneumoniae* strains, was not detected, possibly because samples of pyogenic liver abscess and osteomyelitis were not included. The *magA* gene encodes a mucoviscosity-associated protein frequently found in strains causing severe disseminated infections, such as pyogenic liver abscesses, osteomyelitis, and endophthalmitis[43]. The discrepancy in the percentages of the current study compared to previous studies may be attributed to differences in sample size and source, and the health and age of the patients. Furthermore, the current study utilized vaginal swabs from women with urinary tract infections, a source not previously studied.

Molecular profiling of *Klebsiella pneumoniae* using ERIC-PCR revealed the results showed diversity among the isolates under study. Understanding the spread of strains is crucial in epidemiology and requires a broad survey of pathogens and their multiple resistances[44]. Various typing and documentation tools are used to examine the genetic diversity of bacteria and antimicrobial susceptibility testing results, helping clinicians select antibiotic therapy and detect bacterial transmission and diversity early[45].

Conclusion

The results of the current study showed different levels of genes associated with surface virulence factors among *Klebsiella pneumoniae* local isolates, which may be attributed to the diversity of isolation sources and patient age groups. ERIC-PCR analysis also revealed diverse *Klebsiella pneumoniae* nonpattern, suggesting the potential presence of multiple strains of *Klebsiella pneumoniae* prevalent in hospital settings or community acquired.

Acknowledgements
None

Conflict of Interest
We declare that we have no conflict of interest.

Financial Disclosure
The authors received no specific funding for this work.

Authors' contributions
Rashid SH: conceptualization, specimen preparation, data collection, drafting of the manuscript; Saleem AJ: methodology design, statistical analysis, interpretation of results, manuscript editing; Rashid SH: supervision, validation, critical revision of the manuscript, final approval. All authors read and approved the final version of the manuscript.

Data Availability
Data are available from the corresponding author upon reasonable request and subject to approval by the relevant institutional authorities.

Ethics Approval and Consent to Participate
Ethical approval for the study was obtained from the Institutional Review Board. As this was a prospective study the written informed consent was obtained from all study participants. All methods were performed in accordance with the relevant guidelines and regulations.

How to cite this article: Rashid SH, Saleem AJ. Detection of Surface Virulence Factor Genes and Molecular Typing of Klebsiella pneumonia by Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction. Bangladesh J Infect Dis 2025;12(1):116-124

ORCID
Sahar Hussain Rashid: https://orcid.org/0009-0008-8365-1453
Ali Jaffar Saleem: https://orcid.org/0000-0001-5758-1895

Article Info
Received on: 1 March 2025
Accepted on: 20 April 2025
Published on: 1 June 2025

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