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The isolation and analysis of K. pneumoniae258 virulence factor genes at the molecular level

Zainab Mahdi Kadhom Al-Sendi[1], Ilham A. Bunyan[2*]

[1]*Department of Microbiology, College of Medicine, University of Babylon, Iraq*
[2]*Department of Microbiology, College of Medicine, University of Babylon, Iraq*
Email: Ilhamalsaedi2008@gmail.com

***Corresponding author** Ilham A. Bunyan (Ilhamalsaedi2008@gmail.com)

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Abstract

This cross-sectional study collected 100 clinical specimens from Hilla Teaching Hospital and Marjan Medical City patients aged 3 to 55, 65% of which were male and 35% female. Forty strains of K. pneumonia were isolated from 100 specimens from various infection locations by morphological, microscopical, biochemical, and Vitek II system testing. Eleven (27.5%) of the forty isolates were from sputum, nine (20%) from urine, seven (17.5%) from wound swabs, six (15%) from burn swabs, five (12.5%) from burn tissue, and three (7.5%) from ear swabs. K. pneumonia was isolated from 26 men (65%) and 14 women (35%). All of the possible K. pneumonia isolates had their DNA taken and ran through a standard PCR for pilv-l gene primer amplification using the sequences, the results revealed that only 16(40%) of the 40 K. pneumonia isolates were related to K. pneumonia258 by sharing the same 320 bp DNA fragment with the allelic ladder. Some virulence genes were detected in 16 K. pneumoniae258 isolates. The results showed that FimH all 16(100%) were positive to FimH gene of K. pneumoniae258 isolates at (688bp). However, mrkD gene were detected 13/16(81.2%) were positive to mrkD gene of K. pneumoniae258 isolates at (240bp). Objective: The aim of present study is to isolation of the K. pneumoniae 258 and the detection of K. pneumoniae 258 to some virulence genes.

Keywords

K. pneumoniae258, virulence factor genes, molecular level, PCR

Klebsiella pneumoniae is a type of gram-negative bacteria that can cause infections in humans, particularly in individuals with weakened immune systems. K. pneumoniae infections can range from mild, such as urinary tract infections, to severe, such as pneumonia or bloodstream infections (Cillóniz et al., 2019).

K. pneumoniae is commonly found in the environment, including soil and water, and can also be present in the digestive tract of healthy individuals without causing harm (Choby et al., 2020). However, when the bacteria enter the bloodstream or other parts of the body where they should not be, they can cause infections. It is known for its ability to develop resistance to antibiotics, which can make it difficult to treat. This has led to an increase in the number of infections caused by multi-drug resistant strains of K. pneumoniae, particularly in healthcare settings (Paczosa & Mecsas, 2016).

Extended-spectrum beta-lactamase (ESBL) enzymes and multi-drug resistant (MDR) K. pneumoniae have increased drug resistance. K. pneumoniae also has virulence factors include LPS O-side chain (endotoxin), capsular polysaccharide, adhesions, and sidrophores (Riwu et al., 2020).

K. pneumoniae is divided into 77 serological types (K) based on its capsule polysaccharide (CPS) pathogenicity. Bacterial caps hinder phagocytosis. K. pneumoniae capsular antigens K1 and K2 are particularly significant. Capsular polysaccharides, type 1 and type 3 pili, and biofilm formation are K. pneumoniae main virulence factors. These bacteria generate a thick extracellular biofilm as a virulence factor to cling to living or abiotic surfaces and resist antimicrobial treatments. Infections require biofilm-producing bacteria (Dai & Hu, 2022).

The genome of K. pneumoniae is approximately 5.5 million base pairs in size and contains around 5,000 to 6,000 genes. The genome of K. pneumoniae is composed of a single circular chromosome that contains the bacterium's genetic information. This chromosome is divided into several different regions, including a core genome that is conserved across different strains of K. pneumoniae, as well as variable regions that are unique to specific strains (Morgenroth-Rebin, 2023).

K. pneumoniae is known for its ability to acquire and transfer genes through horizontal gene transfer, which allows it to rapidly adapt to different environments and acquire resistance to antibiotics. As a result, the genome of K. pneumoniae can be quite diverse and can vary widely between different strains (Michaelis & Grohmann, 2023).

The K. pneumoniae genome has been extensively studied in recent years, and a number of virulence factors, antibiotic resistance genes, and other important features have been identified. This knowledge has helped researchers to better understand the biology of K. pneumoniae and to develop new strategies for diagnosing and treating infections caused by this bacterium. The K. pneumoniae capsule genome has gene clusters cps, magA, and rmpA. K1-specific capsular polymerase gene magA (35-Kbp) trans-acts to biosynthesize cps. MagA is identical to LPS glycosylation, transfer, and biosynthesis genes (Wang et al., 2020).

rmpA can increase colony mucoidy of different K. pneumoniae serotypes and regulate additional capsular polysaccharide synthesis via plasmid. Pilli, protein, and bacterial adhesion are adhesives. MrkD encodes type 3 fimbria adhesion and extracellular matrix binding. K. pneumonia uses enterobactin, yersiniabactin, and hydroxamate siderophores to get iron from host transport proteins transferrin and lactoferrin. EntB, ybtS, kfu, and iutA genes produce enterobactin, yersiniabactin, iron-uptake system, and hydroxamate siderophore) (Walker & Miller, 2020).

The strain Klebsiella pneumoniae 258 is a multi-drug resistant strain that has become a cause for concern in healthcare settings, as it is often associated with infections that are difficult to treat. This strain is resistant to multiple antibiotics, including carbapenems, which are often used as a last resort for treating bacterial infections. Infections caused by Klebsiella pneumoniae 258 can be severe and potentially life-threatening, especially in people with weakened immune systems or underlying health conditions. Preventive measures such as good hand hygiene, infection control practices, and appropriate use of antibiotics are crucial to prevent the spread of this strain and reduce the risk of infection (Ahn et al., 2021).

Materials and methods

Study Design

A Cross-sectional study was designed that include 100 clinical specimens of sputum, urine, wound swabs, burn swabs, burn tissue and ear swabs obtained from patients aged 3 to 55 years, there were (65) samples from male patients and (35) from female patients attending to Hilla teaching hospital and Marjan medical city. The period extended from October 2021 to April 2022.

Ethical Approval

Getting the required ethical approval from the hospital's ethical review board, patients, and their supporters is essential. In addition, all participants are verbally informed, and consent for the research and publishing of this work is sought from each individual before any samples are collected.

Clinical specimens

This is a description of how to properly collect samples for a bacteriological study. These samples were carefully gathered to eliminate the risk of contamination (Collee et al., 1996).

Urine samples

Patients with urinary tract infections (UTIs) were the usual subjects for collecting the samples. Urine

samples were taken from the middle of the stream and placed in sterile screw-cap containers before being inoculated into culture media and cultured aerobically at 37°C for 24 hours (Vandepitte et al, 1991).

Sputum samples

For culturing, sterile screw cups were used for collecting samples, which were then transferred to the lab, inoculated into culture media, and incubated aerobically at 37°C for 24 hours (Kennedy et al., 1999).

Wound, burn swab and soft tissue swab

Normal saline should be used to keep the sterile cotton swabs moist until they can be transported to the laboratory, where they will undergo the culture process. During 24 hours, the swab was incubated at 37 degrees Celsius in an aerobic environment (Ramsay et al., 2016).

Ear swab samples

Normal saline should be used to keep the sterile cotton swabs moist until they can be transported to the lab, where they will be cultured. Aerobic incubation at 37 degrees Celsius for 24 hours followed by inoculation on culture media (Sillanpää et al., 2017).

Identification of K. pneumoniae with Vitek2 System

Vitek 2 medical microbiology used as an automatic identification (ID) instrument device.

DNA Extraction

This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company Geneaid, (Korea).

Diagnosis of K. pneumoniae 258 by specific primer gene

K. pneumoniae 258 was diagnosed with PCR by using the primer specific for pilv-l gene (Table 1).

Table 1: The sequence of primers that used this study.

Primer	Sequence	Primer sequence	Tm (°C)	GC%	Size of Product (bp)
pilv-l	F	5'- TGATGCTGATGGCAGACTGA - 3'	60.6	50	320
	R	5'- TGTAGTCACACCCTGCCCA - 3'	64.4	58	

Detection of K. pneumoniae 258 virulence gene

DNA (extract from bacterial cells) was used as a template in specific PCRs for the detection of Fim H and mrkD virulence gene. DNA was purified from bacterial cells by using the Geneaid DNA extraction Kit. The primers used for the amplification of a fragment gene were listed in Table (2, 3).

Table 2: the primers, sequences, and PCR conditions

Primer	Sequence	Primer sequence5'- - 3'	Tm (°C)	GC%	Size of Product (bp
Fim H	F	TGCTGCTGGGCTGGTCGATG	67.9	65	688
	R	GGGAGGGTGACGGTGACATC	67.4	65	
mrkD	F	TTCTGCACAGCGGTCCC	63.8	65	240
	R	GATACCCGGCGTTTTCGTTAC	60.7	52	

Table 3: The optimum condition of detection of virulence genes

No.	Name of gene	Phase	Tm (°C)	Time	No. of cycle
1.	fimH	Initial Denaturation	95°C	3 min	1 cycle
		Denaturation -2	95°C	45 Sec	35 cycle
		Annealing	52°C	45 Sec	
		Extension-1	72°C	45 Sec	
		Extension -2	72°C	7 min.	1 cycle
2.	magA (K1)	Initial Denaturation	95°C	3 min	1 cycle
		Denaturation -2	95°C	30 Sec	30 cycle

		Annealing	55°C	30 Sec	
		Extension-1	72°C	1 min	
		Extension -2	72°C	10 min.	1 cycle

Results and Discussion

For this cross-sectional study, it was collected 100 clinical specimens, including sputum, urine, wound swabs, burn swabs, burn tissue, and ear swabs (Figure 1), from patients at Hilla Teaching Hospital and Marjan Medical City, ranging in age from 3 to 55 years (Figure 2), of these, 65% were from male patients and 35% were from female patients (Figure 3). In this investigation, K. pneumonia was isolated from a total of (100) specimens obtained from various infection sites by aerobic culture on various media. Forty of the isolated strains were positively recognized as K. pneumonia (Table 4). Nonetheless, it was found that 60% of the isolates contained bacteria that could be challenging to culture, possibly because of differences in sample size and composition. Eleven of the forty isolates (27.5%) were taken from sputum samples, nine (20%) from urine samples, seven (17.5%) from wound swabs, six (15%) from burn swabs, five (12.5%) from burn tissue, and three (7.5%) from ear swabs. Nevertheless, K. pneumonia was only found to be isolated from men at a rate of 26 (65%), while females were only isolated at a rate of 14 (35%). Table (5) displays the outcomes. Furthermore, K. pneumonia was isolated from a wide age range, as indicated in Table (6).

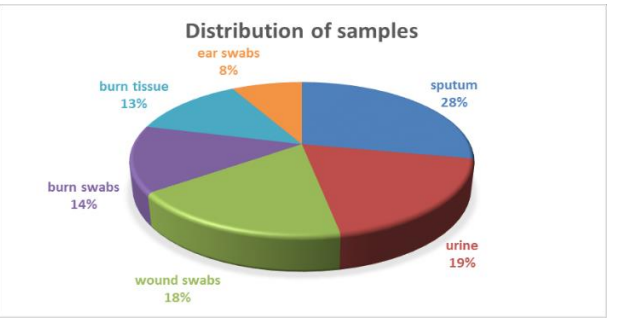

Figure (1): Distribution of samples according to site of infections

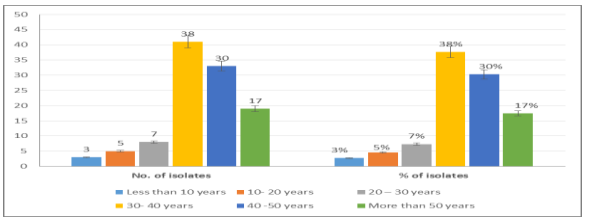

Figure (2): Distribution of the patients according to the age

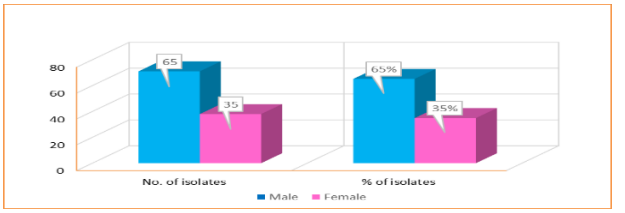

Figure (3): Frequency of patients according to gender

Table (4): Types of Bacterial isolates recovered from sample

Bacterial isolates	No.	%
Klebsiella pneumoniae	40	40%
Others	60	60%
Total	100	100%

Table (5): Distribution of K. pnemoniae according to samples types & pateints gender

Sample type	No. (%)	K. pneumoniae	
		No.	%
Sputum	28 (28%)	11	27.5
Urine	19 (19%)	8	20
Wound swap	18 (18%)	7	17.5
Burns swap	14 (14%)	6	15
Burns Tissue	13 (13%)	5	12.5
Ear swap	8 (8%)	3	7.5
Total	100 (100%)	40 (100%)	
Gender	No. (%)	K. pneumoniae	
		No.	%
Males	65 (65%)	26	65%

Females	35 (35%)	14	35%
Total No.	100 (100%)	40 (100%)	

Table (6): Distribution of K.pneumoniae according to pateints age

Age groups	No. of isolates	% of isolates
Less than 10 years	1	2.5%
10- 20 years	2	5%
20 – 30 years	3	7.5%
30- 40 years	7	17.5%
40 -50 years	12	30%
More than 50 years	15	37.5%
Total	40	100%

Recent results indicated that K. pneumonia were highly isolated from sputum samples at percentage (27.5%) followed by urine at percentage (20%) wound at percentage (17.5%), burns swap at percentage (15%), burns tissue at percentage (12.5%), and ear swap at rate (7.5%). Bunyan and Al-Salem, (2016) observed that, the prevalence of K. pneumonia is around 45.4% in sputum, 21.1% in urine, and 3.0% from otitis media, however our results were not consistent with these numbers. Fifty K. pneumonia isolates were identified by Kateete et al., (2016), with 46% of those being obtained from urine and 54% coming from clinical cases (32% from wound, 12% from sputum, and 10% from otitis media). Many factors, including sample size, location, isolation and identification methods, and the influence of environmental and patient health factors, likely contributed to the wide range of reported K. pneumonia isolation rates across investigations (Moon et al., 2022). All K. pneumoniae bacterial isolates were put through a multitude of morphological, Microscopical, biochemical, and Vitek II system testing in this investigation. The ability of bacterial isolates to consume citrate as the only source of carbon resulted in a clear positive result for the Citrate utilization test when applied to the K. pneumoniae isolates. In addition, the isolates produced urease enzyme, which turned the yellow Urea agar slant pink. Infections caused by Klebsiella are distinguished by the presence of urease enzyme production (Yaqoob et al., 2022). Because it catalyzes the formation of kidney and bladder stones or to encrust or obstruct indwelling urinary catheters, this enzyme is also considered one of the most significant virulence factors of K. pneumoniae, which has been involved in the pathogenesis of several diseases, including pyelonephritis and the development of infection-induced urinary stones. They failed the motility test, so we know they're not mobile. As a result of glucose and lactose fermentation, they also gave favorable results to the Vogas Proskauer test and the Kligler iron test by creating an acidic slant / acidic bottom with gas generation but without H_2S gas production (Yuan et al., 2021).

When the PCR process is complete, billions of copies of the target sequence will have been produced thanks to DNA polymerase's ability to synthesize a new strand of DNA that is complementary to the provided template strand (Amplicon). All of the possible K. pneumonia isolates had their DNA taken and ran through a standard PCR for pilv-l gene primer amplification using the sequences and software in Table (1). Figure (4) shows the results of the gel electrophoresis analysis, which revealed that only 16(40%) of the 40 K. pneumonia isolates were related to K. pneumonia258 by sharing the same 320 bp DNA fragment with the allelic ladder.

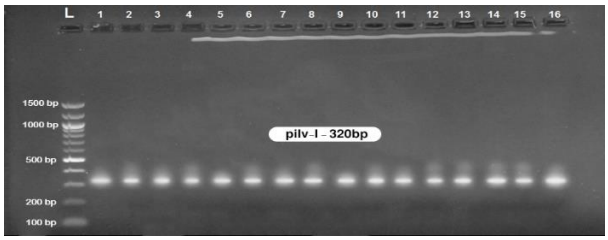

Figure (4): Agarose gel electrophoresis (1.5%) of RCR amplified of pilv-l gene (320bp) of K. pneumonia258 for (55) min at (70) volt L: ladder (DNA marker). Number (1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16) positive K. pneumonia258 isolates.

These findings corroborated those of Mendes et al., (2022), who discovered that polymerase chain reaction (PCR) amplification of a certain gene or area of the genome allows for molecular identification of K. pneumoniae 258, using specific primers.

A study of Gato et al., (2020) found the 16S rRNA gene and the pilv-l gene were frequently utilized for molecular identification of K. pneumoniae258. Reyes Chacón, (2019) found, primers utilized for amplification of this gene were tailored to recognize only K. pneumoniae 258 and exclude all other bacteria.

A comparison of the amplified product to previously established K. pneumoniae258 sequences will be served to verify the bacterium's identification. For further insight into the processes of virulence and antibiotic resistance in K. pneumoniae 258, sequencing the amplified product can provide much more about the bacterium's genetic make-up, which can aid in epidemiological investigations (Shankar et al., 2020). Detecting Klebsiella pneumoniae strain 258 in a biological sample by the use of a molecular biology methodology that focuses on the pilv-l gene (Mandras et al., 2020). The protein encoded by this gene was a type IV pilus, which plays a role in bacterial adhesion to host cells and the development of biofilms. Marking K. pneumoniae strain 258 for detection, the pilv-l gene was unique to this strain. K. pneumoniae 258 can be detected molecularly by amplifying the pilv-l gene according to study of Moore, (2017), which requires the extraction of DNA from a biological sample (such as blood, urine, or tissue) and subsequent amplification using polymerase chain reaction (PCR) or other molecular techniques. Gel electrophoresis or sequencing can be used on the amplified DNA to confirm the presence of K. pneumoniae 258.

In this study, FimH gene were detected in all 16 K. pneumoniae258 isolates. The results showed that all 16(100%) were positive to FimH gene of K. pneumoniae258 isolates. PCR product was roughly (688bp) in size, when they used the primer, the results were shown in Figure (5). However, mrkD gene were detected in all 16 K. pneumoniae258 isolates. The results showed that 13/16(81.2%) were positive to mrkD gene of K. pneumoniae258 isolates. PCR product was roughly (240bp) in size, the results were shown in Figure (6).

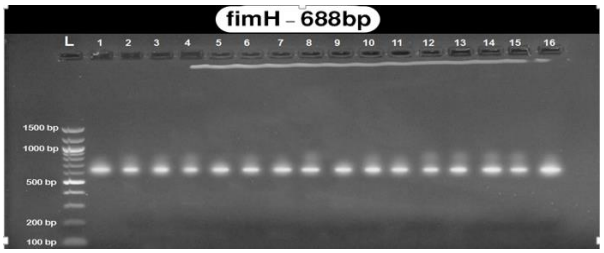

Figure (5): Agarose gel electrophoresis (1.5%) of RCR amplified of FimH gene (866bp) of K. pneumonia258 for (55) min at (70) volt L: ladder (DNA marker). Number (1, 2, 3, 4 ...16) positive K. pneumonia258 isolates,

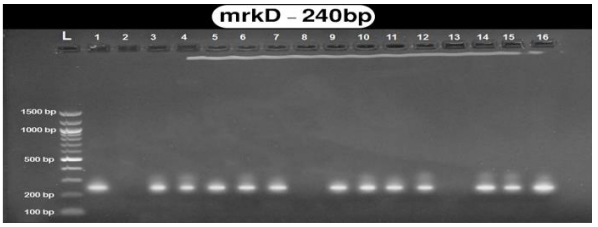

Figure (6): Agarose gel electrophoresis (1.5%) of RCR amplified of mrkD gene (240bp) of K. pneumonia258 for (55) min at (70) volt L: ladder (DNA marker). Number (1,2,3,4,5,6,7,9,10,11,14,15,16) positive K. pneumonia258 isolates.

Schroll et al., (2010) demonstrated, the gene for type 1 fimbrial adhesin (FimH gene) in bacteria was encodes for the FimH protein. For many dangerous bacteria, like K. pneumonia258, this protein served as a crucial virulence component. Polymerase chain reaction and other methods of molecular biology could be used to detect the FimH gene. Primers designed to bind to the DNA sequence of interest were employed in the PCR procedure to kick off the amplification process (Monesh Babu et al., 2021). Several infections might be better diagnosed and treated when the FimH gene was discovered. The ability of Klebsiella pneumonia to adhere to and infiltrate host cells is essential for infection to take hold, and this ability was found to be substantially correlated with the presence of the FimH gene. More precise diagnoses and treatments of infection could be implemented if the FimH gene could be detected in patient samples (Aljanaby et al., 2016). Molecular biology methods can be used to detect the FimH gene in K. pneumoniae, which can reveal important details regarding the strain and virulence of the infecting bacteria (Mirzaie & Ranjbar, 2021). These findings corroborated those of Surgers et al., (2019), who reported that the mrkD gene, which codes for virulence factors, was present in K. pneumoniae isolates at a rate of 60.5%. The mrkD gene was discovered to be responsible for the production in 91.66 percent of K. pneumoniae isolates, as reported by Liu et al., (2019). Klebsiella pneumoniae type 3 fimbriae consist of the primary fimbrial subunit (MrkA) and an adhesion (MrkD) that has been proven to mediate binding to collagen. Using continuous-flow chambers, we tested the biofilm-forming potential of K. pneumoniae adhesive and non-adhesive derivatives on collagen-coated surfaces (Abozahra et al., 2020). In contrast to biofilm formation on abiotic plastic surfaces, growth on collagen-coated surfaces required the presence of the MrkD adhesion. Strains of fimbriate bacteria missing the MrkD adhesion have trouble sticking to and

colonizing these surfaces (Clegg & Murphy, 2017). While direct attachment to the respiratory cells was not detected during MrkD-mediated biofilm formation, both purified human extracellular matrix and the extracellular matrix produced by human bronchial epithelial cells cultured in vitro served as acceptable substrates (Willsey, 2018). This suggests that type 3 fimbriae play a dual role in the establishment of adhesion and subsequent growth on long-term implants like endotracheal tubes. Small molecules called siderophores can scavenge iron from their host proteins (Bunyan et al., 2018). Further characterization of the function of the mrkD gene in Klebsiella pneumoniae type 3 fimbriate attachment was performed. Two copies of the mrkA fimbrial subunit gene and one copy of the mrkD adhesion subunit gene were discovered in a K. pneumoniae clinical isolate. Both mrkA and mrkD were present in bacteria, but only one was on the chromosome. mrkA was connected with mrkD on a plasmid (Hu et al., 2020). Adhesions of type 1 and type 3 fimbriae, encoded by the genes fimH and mrkD in K. pneumoniae, mediate adherence to the extracellular matrix; promote biofilm production; and may play a crucial role in colonization, invasion, and pathogenicity (Panjaitan et al., 2019). Both the fimH-1 and mrkD virulence genes were present in the vast majority of the MRD K. pneumoniae isolates (Luo et al., 2017). One of the most crucial steps in the development of a K. pneumoniae infection is related to its ability to adhere to host surfaces and demonstrate persistent colonization, even though studies have reported that many clinical K. pneumoniae isolates normally express both type 1 and type 3 fimbrial adhesions (de Arajo et al., 2019). MrkD mediates attachment to the extracellular matrix specifically, allowing Klebsiella pneumoniae to stick to injured tissue and coat indwelling devices such urinary catheters and endotracheal tubes (Martin & Bachman, 2018). K. pneumoniae type 3 fimbriae have been shown to bind to endothelium cells and epithelial cells in the respiratory and urinary systems, and play a crucial role in biofilm formation (Lin et al., 2017; Marques et al., 2019), it is known that K. pneumoniae isolates were more invasive, resistant to the normal bactericidal effect of human serum, and able to create more fimbrial adhesions (Sofiana et al., 2020).

Conclusion

K. pneumoniae258 was important findings infection that discovered, and evidence of the creation of some virulence factor gene across isolates was shown.

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