MOLECULAR DETECTION OF VIRULENCE GENES IN KLEBSIELLA PNEUMONIAE ISOLATES FROM WASIT PROVINCE, IRAQ

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Abstract

The most common kind of nosocomial infection is an infection of the urinary tract (UTI), and K. pneumonia is the second most common Gram-negative bacterial cause of UTIs. The rise of bacterial pathogens that are resistant to several drugs is a serious global health concern. Overuse of antibiotics has reduced our treatment choices for K. pneumoniae and made appropriate management of this bacterial illness more challenging.

The ability to produce biofilm was examined by using microtiter plate (MTP) and modified Congo red agar (MCRA), in MCRA method, among the 40 clinical strains 35(87.5%) were biofilm producers.33 (82.5%) were strong biofilm-producers with black color, 2(5%) were classified as weak biofilm-producer in orang(grey) colony, whilst 5 (12.5%) were pink isolates of Klebsiella pneumonia none producers biofilm. In MTP method , the 40 K. pneumonia isolates tested, there were 36 (90%) isolates as biofilm producer and 4 (10%) isolates that were not biofilm producers. Among biofilm producers, there were 32 (80%) isolates as strong, 2 (5%) isolates as moderate, and 2 (5%) isolates identified as weak biofilm producers.

16S rRNA confirms the molecular identification of k pneumonia isolates. Utilizing the NanoDrop instrument, the concentration and purity were determined. Purity was between 1.6 and 2.0, while concentration ranged from 50 to 360 ng/l. The fimH gene, with 550 bp was found in 82% of the isolates, 226 bp mrkD gene gave 70% positive results.

The mrkD primer nucleotide sequences of six samples were analyzed and compared to the NCBI database. Three samples had a perfect match, as determined by the nucleotide sequence analysis, while the remaining samples had a match rate of between 97 and 99 percent. Transition A/G, Transition T/C, Transversion A/C, and Gaps were among the several genetic variations found.

Keyword: *Klebsiella Pneumoniae, Biofilm, MDR, Virulence genes.*

1. INTRODUCTION

Urinary tract infections (UTIs) are among the most prevalent illnesses seen in modern medicine (1). UTIs affect 150 million individuals annually worldwide (2). Among the most common bacterial infections, both in nosocomial infections and outpatient facilities are the infections (UTI). Enterobacteriaceae are commonly responsible for urinary infections (3). Because of the length of the urethra and its proximity to the anus, which enable fecal bacteria entering the urinary tract, females are more susceptible to urinary tract infections (4). Urinary tract infections typically manifest themselves with urgency, increased frequency, and discomfort during urinating, and a strong unpleasant odor. Urethritis, or urethral inflammation, is a common precursor to these illnesses. E. coli, a type of Enterobacteriaceae, is responsible for 75%-90% of all cases of urinary tract infections (2). There are many different types of uropathogens, but E. coli continues to be the most common. Other common uropathogens include Klebsiella pneumoniae, Enterobacter, Proteus spp., and Enterococcus spp. (5).

The kidney, ureters, bladder, and urethra are all impacted by urinary tract infections (UTIs). The three main forms of a urinary tract infection are pyelonephritis (kidney inflammation), urethritis (urethral inflammation), and cystitis (inflammation or infection in bladder). Klebsiella species are members of the family Enterobacteriaceae; these bacteria are Gram-negative, non-motile, encapsulated, able to ferment lactose, and are facultative anaerobes. In the gastrointestinal tracts of both humans and animals, Klebsiella spp. are significant opportunistic pathogens that are part of the natural flora. However, in favorable circumstances, this bacterium causes a number of illnesses in both people and animals (5). Including pneumonia, urethral infections, and mastitis. Several lipopolysaccharides (LPS), fimbrial and non-fimbrial adhesins, and iron-scavenging systems are among the numerous virulence factors found in Klebsiella species (siderophores) (5). Additionally, Klebsiella's capacity to produce biofilms increases its pathogenicity (6)

The biofilm is an aggregation of adherent microbial cells that are inextricably linked to abiotic and/or biotic surfaces and embedded in an extracellular matrix made of polysaccharides, proteins, and DNA (7).

2. MATERIALS AND METHODS

2.1 Specimens Collection and Culture

Between January 2022 and April of the same year, 336 samples were taken from patients with urinary tract infections at Al-Karama and Al Zahraa teaching hospitals, Al Kut hospital, and private clinics in Wasit province. The patients' ages ranged from 18 to 98 years old.

2.2 Isolation and identification of bacteria

The samples (urine and urethral swab) were culture on MacConkey agar, Blood agar, and incubated aerobically at 37°C for 24 hours (7). The isolated bacteria were identified according to morphological, biochemical tests and Vitek2

2.3 Quantitative biofilm assay using microtiter plate

2.3.1 Crystal violet assay to detect biofilm

Each bacterial strain was inoculated in a separate microtiter plate at a concentration of 107 CFU/ml, which required a broth culture volume of 250 ml. Nutrient broth was used as a control in the inoculations. Plates were covered and incubated at 37 degrees Celsius for 24 hours. When the incubation period had ended, the bacterial culture broth was discarded and the wells were washed three times with 300 µl of sterile PBS to get rid of any bacteria that had not attached to the wells.

Biofilms were removed by treating each well with 250 ml of 96% ethanol for 15 minutes. After the ethanol was wiped away, the plate was set aside to dry. Crystal violet solution (2 % w/v) was added to each well for 5 minutes, and the excess washed away with molecular grade water. Quantitative analysis of biofilm formation was performed by adding 200 µl of 33% glacial acetic acid (v/v) to each well, incubation for 15 minutes, and then measuring absorbance at 570 nm using a DIA Reader ELX8OOG. (8)

Table 1 Classification of bacterial adherence and biofilm formation by CVA method

Mean OD value at 630nm	Biofilm production
< 0.120	NO adherence
0.120 - 0.240	weak
> 0.240	strong

2.3.2 Congo red agar

Congo red (CR) agar was produced in the following manner for qualitative detection of biofilm-producing microorganisms: A flask containing 990 ml of cold water was used to dissolve 40 grams of trypticase soy agar and 50 grams of sucrose; the mixture was then heated to 100 degrees Celsius and autoclaved at 121 degrees Celsius for 15 minutes. Autoclaved at 121°C for 15 minutes, the CR solution was made by dissolving 0.8 g of CR powder in 10 milliliter of cold water in a separate flask.

Then, CR solution was added to other components after cooling 50°C, mixed well and transferred into sterile Petri-dishes and left to solidify at room temperature (8).

2.4 Extraction of DNA from Klebsiella isolates.

Using a genomic DNA micro kit, genomic DNA was extracted from all K. pneumoniae isolates (40). (2-2-9-1). Extraction was done according to the manufacturer's instructions of Geneaid Company after cultured on the Luria-Bertani broth. DNA was extracted to provide a PCR template for amplification.

Table 2 Primers' sequence of 16S rRNA, fimH -1, and mrkD.

Primer names	Sequences (5'-3')	Tm (℃)	Amplicon size (bp)	Reference
16S rRNA	F: GTATCTAAACCAGTTCGCACC R: TGCATATCTGCTGTTGCATC	58	145	(9)
fimH -1	F: TGCTGCTGGGCTGGTCGATG R: GGGAGGGTGACGGTGACATC	61	550	(10)
mrkD	F: CCACCAACTATTCCCTCGAA R: ATGGAACCCACATCGACATT	54	226	(11)

2.5 Statistical Analysis

(SAS) Statistical Analysis System, version 9.1 was used for the statistical analysis of the data. A Chi-square analysis was performed to compare the percentages. When comparing two groups, if the difference is less than 5% (P 0.05), it is deemed significant.

3. RESULT

3.1 Isolation and Identification of Klebsiella pneumoniae

A total of 336 clinical samples (urine & urethral swab) about 105 of these sample are no growth and 40 (17,3%) of bacterial culture belong to Klebsiella pneumonia were collected from patient suspected UTIs from different age groups (18-98) years old and gender admitted to :AL-Zahraa teaching hospital, AL-Karama teaching hospital, AL-Kut hospital for gynecology obstetrics and pediatrics ,and from private clinics in Wasit province, during the period from December 2021 until the end April 2022. K. pneumonia

was determined according to conventional cultural and microscopic characteristics as well as biochemical tests. In addition, the identification is confirmed using API 20 System and Vitek 2 System and it was confirmed using 16s rRNA.

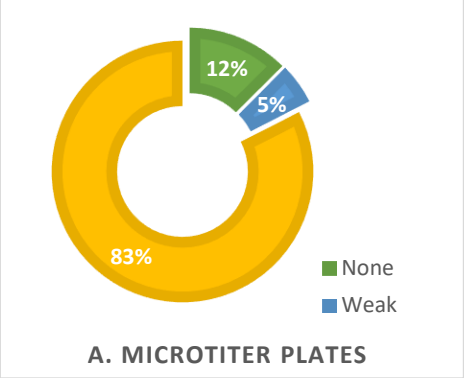
3.2 Frequency and Percent of biofilm

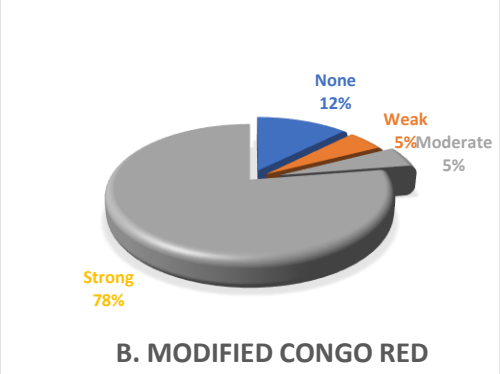
40 isolates was assessed for its ability to produce biofilms using both aquantitative method using microtiter plates (MTP) and aqualitative method using modified Congo red (MCRA)

As for (MTP) method, 83% of samples were strong producer of biofilm, while 5 and 12% of samples were weak and non-producer of biofilm, respectively.

As for MCRA method, 78% of samples were strong producer of biofilm, while 5, 5, 12% of samples were moderate, weak and non-producer of biofilm, respectively.

Figure 1 Percentage of biofilm A. MTP method B. MCRA method





The MTP approach was used to identify biofilm development in 40 K. pneumoniae isolates, with 36 (90%) of the isolates being biofilm producers and 4 (10%) not being biofilm producers. 32 (80%) of the isolates that produced biofilms were classified as strong biofilm producers, 2 (5%), as moderate biofilm producers, and 2 (5%), as weak biofilm producers. Table (4-5).

Table 3 Frequency and Percent of biofilm

Biofilm		
	Frequency	Percent
None	4	10%
Weak	2	5%
Moderate	2	5%
Strong	32	80%
Total	40	100%

40 isolates was assessed for its ability to produce biofilms using both aquantitative method using microtiter plates (MTP)

Table 4 Distribution of Biofilm producing K. pneumoniae by MCRA vs MTP

		MCRA				Total	
		-		+			
MTP	-	1	2.5%	3	7.5%	4	10.0%
	+	4	10.0%	32	80.0%	36	90.0%
Total		5	12.5%	35	87.5%	40	100.0%
CHI-SQUARE		0.635					
P-Value		0.426					

P-Value < 0.05 (significant)

3.2.1 Identification of K. pneumoniae and biofilm production genes using PCR:

All of the isolates 40 were submitted to molecular identification utilizing PCR amplification of the 16S rRNA using K 16S-F and K 16S-R primers, which are specific primers for the PCR amplification of the 16S rRNA of K. pneumonia. The amplified fragments were around 130 bp in size,

All 40 isolates underwent molecular detection by PCR amplification of fimH gene using specialized primers that are specific primers for PCR amplification of K. pneumonia fimH gene. Figure from the data demonstrates that the amplified fragments were 550 bp in size (4-13). All of the isolates 33 of 40 (82.5%) produced favorable findings (550 bp).

The mrkD gene was amplified by polymerase chain reaction (PCR) in all thirty-four isolates using primers designed specifically for this purpose. The size of the amplified fragments was determined to be 226 bp (Figure) (3-7). Results were positive for 28 out of 40 isolates (70.5%). (226 bp).

Figure 2 PCR product of 16S rRNA with band size 145 bp. The product was electrophoresis on 1.5% agarose at 70 volt/cm2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100)

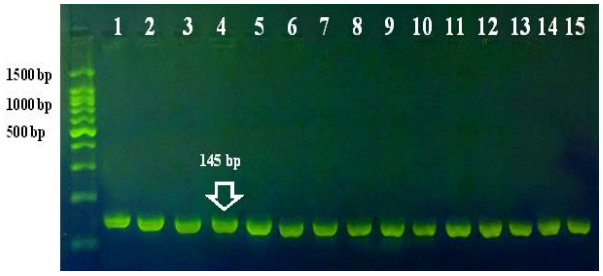


Figure 3 PCR product of fimH gene with band size 550 bp. The product was electrophoresis on 1.5% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100).

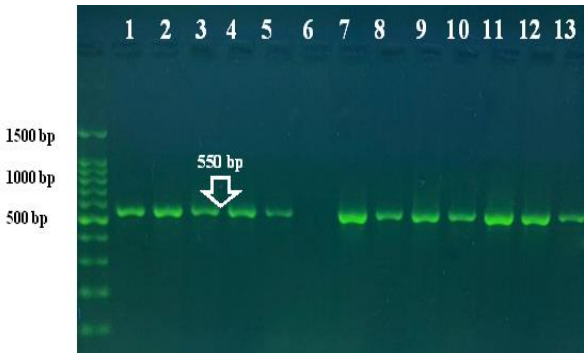
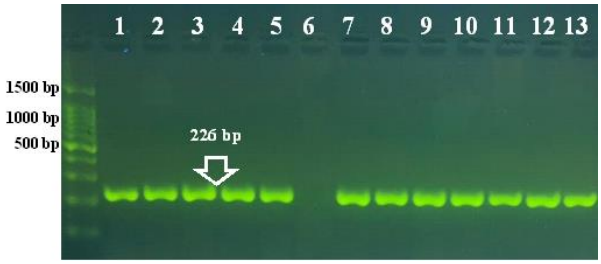


Figure 4 PCR product of mrkD gene with band size 226 bp. The product was electrophoresis on 1.5% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100).



3.3 Sequence analyzing of mrkD

PCR results for six isolates were sent to determine the nucleotide sequence of the mrkD gene. The gene size was 226 base pairs. Nucleotide sequencing results were analyzed using the NCBI website. Figures (3-16) (3-17) (3-18) (3-19) (3-20) (3-21)

The results of the nucleotide sequencing analysis of the studied samples showed that the match percentage for the studied samples was (98, 100, 100, 100, 99, 97)% in the results of the polymerase chain reaction of the mrkD gene for the six studied samples, respectively. The results showed that the match rate in the first sample was 98%, as A/G Transition was found in both sites 841292 and 841357, while there was an A/C Transversion in site 841312.

The isolate sequence was recorded with the ID number OW995950.1. As for isolates 2, 3, and 4, they were 100% identical and recorded with ID number OW995950.1 as well.

Isolate No. 5 showed a match rate of 99%, as the results of the analysis showed the presence of Gap at site 841252 by adding one nucleotide base (Adenine). As for isolate No. 6, it was the least identical isolate with a matching rate of 97%, as it was found that there was a Transition in each of the sites 841240 and 841255 of the bacterial genome, and it was also found that there was a Gap in each of the sites 841242, 841244 and 841253. **Table (4-9)**

Table 5 mrkD gene sequencing of Klebsiella pneumoniae

***Klebsiella* pneumoniae**						
No.	**Type of substitution**	**Location**	**Nucleotide**	**Sequence ID with compare**	**Source**	**Identities**
1	**Transition**	841292	**A/G**	**ID: OW995950.1**	***Klebsiella pneumoniae* isolate 307 genome assembly**	**98%**
	Transversion	841312	**A/C**			
	Transition	841357	**A/G**			
2				**ID: OW995950.1**	***Klebsiella pneumoniae* isolate 307 genome assembly**	**100%**
3				**ID: OW995950.1**	***Klebsiella pneumoniae* isolate 307 genome assembly**	**100%**
4				**ID: OW995950.1**	***Klebsiella pneumoniae* isolate 307 genome assembly**	**100%**
5	**Gap**	**841252**	**A**	**ID: OW995950.1**	***Klebsiella pneumoniae* isolate 307 genome assembly**	**99%**
6	**Transition**	841240	**T/C**	**ID: OW995950.1**	***Klebsiella pneumoniae* isolate 307 genome assembly**	**97%**
	Gap	841242	**T**			
	Gap	841244	**G**			
	Gap	841253	**A**			
	Transition	841255	**T/C**			

4. DISCUSSIONS

A bacterial colony that is encased in a polymeric matrix that is made up of slime is known as a biofilm (12). exopolysaccharide, nucleic acids and lipids. Because it was discovered that Congo red turned a colony black when it interacted with bacterial slime, Congo red agar has become an extremely popular screening medium for the presence of biofilm.

The screening for biofilm-positive bacteria was done using a modified version of the Congo red agar technique. Strains that produced biofilm were colored black, whereas strains that did not produce biofilm were colored red (13).

Among the 40 clinical strains 35(87.5%) were biofilm producers.33 (82.5%) were strong biofilm-producers with black color, 2(5%) were classified as weak biofilm-producer in orang (grey) colony, whilst 5 (12.5%) were

pink isolates of Klebsiella pneumonia none producers biofilm, whereas observed that orange and red colony in 24 hr became black after 48 hr to 4 days

According to another study that was conducted by Lijuan Chu and colleagues, out of 45 clinical strains, 28 of them (62.2 percent) were biofilm-producers, whereas 17 of them (37.8 percent) were categorized as biofilm-negative (13).

In the modified congo red method, the penetration of the dye CR is dependent on the following: cell surface physicochemical characteristics were investigated in Klebsiella pneumoniae. These characteristics included surface charge, surface hydrophobicity as measured by a variety of techniques, and accessibility of the lipid fraction of the outer membrane. Hydrophobic and negatively charged, the bacterial surface was made possible by the capsular polysaccharide and the O-antigen repeating units of the lipopolysaccharide (LPS), and the lipid-binding dye congo red was prevented from penetrating the outer membrane (OM) (14).

In this investigation, 36 (90%) of the 40 isolates produced biofilm. According to similar research, of the 110 K. pneumoniae examined, 70 isolates (64.7%) were found to be strong or moderate biofilm builders, while 40 isolates (35.3%) were found to be weak biofilm producers (15). According to Mirzaie and Ranjbar's findings, 89 percent of K. pneumoniae biofilm-forming strains and 77% of K. pneumoniae strains were MDR (16). Nirwati et al. demonstrated that 148 (85.63%) of their isolates were biofilm producers, with 45 (26.95%) being a strong biofilm producer, 48 (28.74%) being a moderate biofilm producer, and 50 (29.94%) being a weak biofilm producer (17). Investigation of Klebsiella pneumoniae isolated from clinical samples in Indonesia by Nirwati et al., 2019. The 167 isolates of K. pneumoniae that were evaluated had 143 (85.63%) isolates that produced biofilm and 24 (14.37%) isolates that did not. There were 50 (29.94%) isolates classified as weak biofilm producers, 48 (28.74%) isolates as intermediate, and 45 (26.95%) isolates as strong biofilm producers. (17). 37.6 percent of K. pneumoniae strains were biofilm makers, according to Cepas et alfindings .'s in another investigation.. (Cepas et al., 2019) According to Yang and Zhang, 62.5% of the K. pneumoniae isolated from urine, sputum, wound swabs, and blood produced biofilms (18). According to Seifi et al., the majority of K. pneumoniae (93.6 %) produced biofilms whereas just 6.4 percent did not. (Seifi et al., 2016). According to Haghighifar et al., of K. pneumoniae, 67.2 percent produced weak biofilm, 18.8 percent produced intermediate biofilm, and 14.1 percent produced strong biofilm (19).

Each isolate's ability to form biofilms was unique because, in general, a number of variables affect this ability, including K. pneumoniae physicochemical characteristics, the physical interactions between constituent parts, the kind of surface to which the biofilm adheres, temperature, pH, etc. (20).

A statistical analysis was conducted to look for a significant connection for each growth variable (MCRA, MTP) in order to identify the emergence of biofilms. Statistical significance is indicated by a P value of 0.05 or below.

The MTP is compared to MCRA and is regarded as a quantitative gold standard approach for detecting the production of biofilms (21).

This test reveals no overlap with MCRA, however the MTP and MCRA significantly disagree (P 0.426). Additionally, the modified Congo red agar (MCRA) demonstrated no correlation with microtiter standard techniques after 24 hours of incubation, a quick test for detecting the creation of a black or red hue. All of the clinical isolates of K. pneumoniae in

the current experiment produced slime with a consistent black color development, suggesting successful biofilm formation, when exposed to the changed composition.

Spectrophotometric evaluation of the amount of biofilm generation employed the microtiter plate test. Considering the microtiter plate method's precision and dependability in identifying and appreciating the bacteria that produce biofilm, it continues to be one of the most used assays for biofilm research.

Stable black pigmentation, which offers a complementary phenotypic and genotypic approach for the quick and precise identification, adds value to the precision of recognizing biofilm development.

as shown in figure (3-10), and Turton et al. (2010) also achieved the same size when they used the same primer (22). The findings for all forty isolates were positive (130 bp bands), identifying them as K. pneumoniae. PCR amplification results demonstrated that all isolates were K. pneumoniae and supported the earlier findings.

According to Stahlhut et al., 90% of K. pneumoniae strains from various environmental and clinical sources include the fimH gene (23). Although there isn't a lot of variation in the structural nucleotides that make up fimH alleles, these genes are often exchanged across bacterial clones. Pathogenic organisms, including K1-encapsulated liver isolates, had their clonal structure more accurately determined when the fimH gene was included to multi-locus sequence typing. Adaptive point mutations also target the FimH protein in K. pneumoniae, however not to the same extent as they target FimH in urinary pathogenic Escherichia coli or TonB in the same K. pneumoniae strains. These adaptive changes entail the loss of a single amino acid from a signal peptide, which might alter the length of the pentamer rods by altering the rate of FimH translocation to the plasma membrane. While a urinary tract pathogenic strain of K. pneumoniae was circulating endemically, a second mutation in FimH (S62A) occurred. Since this mutation is identical to one seen in a highly virulent urinary tract pathogenic strain of Escherichia coli, it's safe to assume that FimH mutants are adaptive. Given the abundance of type 1 fimbriae in Enterobacteriaceae, our present finding that fimH genes undergo adaptive microevolution establishes the importance of type 1-mediated adhesion in K. pneumoniae.

Ferreira et al. investigated the prevalence of fimH gene in Klebsiella pneumoniae isolated in a Brazilian Intensive Care Unit. The study showed that fimH was 88% of isolate (10).

The MrkD gene encodes type 3 fimbria adhesion and acts as a mediator of binding to the extracellular matrix (24).

Adhesins of fimbrial types 1 and 3 are encoded by the mrkD genes in Klebsiella pneumoniae. These adhesins enhance biofilm formation by mediating adhesion to the extracellular matrix (25), and it's possible they're crucial in colonization, invasion, and pathogenesis (26)

Ferreira et al. investigated the prevalence of mrkD gene in Klebsiella pneumoniae isolated in a Brazilian Intensive Care Unit. The study showed that mrkD was 96% of isolate (10).

Alwan & Abass (2017) investigated the prevalence of mrkD gene in Klebsiella pneumoniae isolated from clinical sources from different hospitals in Baghdad city. Their study indicated that the prevalence of the mrkD gene was 50% (27).

Clinical Klebsiella pneumoniae isolates from Iran's Kurdistan Province were studied for their virulence genes by Shakib et al. Their research showed that 65.7% of the samples tested positive for the MrkD gene (28)

Muhsin et al., (2022) indicated in their study that the prevalence of the MrkD gene in 51.6% of Klebsiella pneumoniae isolated from different disease cases in the city of Baghdad (29).

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