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Molecular detection of fimH& mrkDgenes of strong biofilm producers & MDR Klebsiella pneumoniae

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Abstract*---Klebsiella pneumoniae*** is an adaptable pathogen that forms biofilms on a variety of surfaces. This study's objective was to identify the presence of fimbrial genes (types 1 and 3) in *K. pneumoniae* strains isolated from various clinical sources based on their antibiotic resistance and ability to form biofilms. According to identification utilizing the vitek 2 technology and confirmation by molecular identification targeting the 16S rRNA gene with a particular primer, forty isolates were identified from clinical specimens. The vitek 2 compact system was utilized to evaluate the antibiotic susceptibility of all the isolates. The findings revealed a range of resistance percentages, including 52.5% for Penicillin, 40.5% for Trimethoprim/Sulfamethoxazole, 34.5% for Cephalosporins, 6.25 % for Fluoroquinolones, and 2.5% for each of Carbapenem, Aminoglycoside, Tetracycline, and Nitrofurantoin. The 96-well microtiter plate technique was utilized to generate biofilms. The results demonstrated that all 40 *Klebsiella pneumoniae* isolates (100%) produced potent biofilms. In order to identify the genes involved in biofilm formation (fimh & mrkd) and the genes responsible for adhesin in type 1& type 3 fimbriae using traditional PCR method, eleven isolates were chosen for molecular analysis that are powerful biofilm makers and MDR. The findings revealed that eight out of eleven isolates (72.7%) have either a *fimh* or *mrkd* gene.

***Keywords*---**Klebsiella pneumoniae, adaptable pathogen, biofilms.

Introduction

The genus *Klebsiella* contains facultative, anaerobic, non-motile, Gram-negative rods with a conspicuous polysaccharide capsule (1). *K. pneumoniae* may colonize and infect both plants and animals and is widely present in the environment. One of the multidrug resistant organisms that has been recognized as a serious hazard to human health, infections from it are especially problematic in the hospital context for newborns, the elderly, and immunocompromised patients (2). Because it is difficult to cure and might result in chronic infection, *K. pneumoniae* causes severe nosocomial infections. Owing to the capacity of infections associated with biofilm development to resist antibiotic therapy due to decreased medication penetration within the biofilm in the presence of extracellular material, these infections are highly challenging to treat. Antibiotics from different classes were utilized to treat infections caused by *K. pneumoniae*. When treating severe infections brought on by the enlarged spectrum of *K. pneumoniae*, carbapenems are the drug of choice, along with cephalosporins and trimethoprim-sulfamethoxazole. Aminoglycosides, fluoroquinolones, and isolates that produced beta-lactamase (3) were often utilized to inhibit the development of enterobacteriaceae family members (4). Antibiotics may, however, only kill bacteria that are floating freely; they will not kill germs that are entrenched in a biofilm.

The production of the extended-spectrum beta-lactamase (ESBL), one of the major causes of increased infection in hospitals, the alteration of the permeability barrier, the target site represented by penicillin binding protein, or the alteration of outer membrane protein are the main causes of *K. pneumoniae* resistance to antibiotics, including beta lactam antibiotics (5). Furthermore, *K. pneumoniae* has a large number of Efflux Pumps that discharge the antibiotic outdoors. Penicillins, cephalosporins, carbenems, and monobactam are among the antibiotics of the class of beta-lactams (6).

A biofilm is an organized community of bacterial cells that is regulated by two phases of biological processes, including cell-to-cell communication through quorum sensing and surface adhesions to biotic or abiotic surfaces, to grow in multilayered clusters on protected or coated substrate (7). Fimbrial adhesions aid in adhesion to certain tissue surfaces (both biotic and abiotic). *K. pneumoniae* expresses type 1 and type 3 fimbriae of two fimbrial adhesions (8). All members of the Enterobacteriacae family have type 1 fimbriae, which are necessary for *K. pneumonae* to produce urinary tract infections (UTI). The Fim H adhesion, which is found on the tip of the fimbriae and is encoded by operon fim, helps them to exert their sticky qualities (9). FimA subunits dominate Type 1 fimbriae, which are filamentous, membrane-bound, sticky structures containing a FimH subunit at the tip. These fimbriae play a part in the invasion of bladder cells by *K. pneumoniae* as well as the development of biofilms on abiotic surfaces and in the bladder. On the surface of *K. pneumoniae*, type 3 fimbriae are membrane-bound, sticky structures that resemble helix-like filaments. MrkA subunits make up the majority of them, with MrkD subunits making up the tip. It has been discovered that type 3 fimbriae are essential for the formation of *K. pneumoniae* biofilms and their attachment to medical equipment. The current study's objective was to

determine if clinical isolates of *K. pneumoniae* with high biofilm-producing capabilities and multidrug resistance included the fimh and mrkd genes.

Materials & Methods

Isolation & identification

From September 2021 to January 2022, 120 specimens (blood, urine, sputum, and wound) were collected in sterile containers from Baghdad Teaching Hospital, Ghazi Al- Hariri Hospital for Surgical Specialties, and Teaching Laboratories at Medical City in Baghdad, Iraq. All of these specimens were grown overnight at 37^{o}C on MacConkey agar plates (Himedia/ India). Later, the isolates were identified using biochemical tests in vitek- 2 system and validated with a molecular approach.

Antimicrobial sensitivity test

The susceptibility test for 40 *K. pneumoniae* isolates to sixteen antibiotics belong to (eight groups) of antibiotics was determined by using Vitek -2 compact system. The antibiotics utilized in this study are shown in table (1).

Table (1) Antibiotics utilized in this study

ANTIBIOTIC GROUP	ANTIBIOTIC
Penicillin	Ampicillin piperacilin/tazobactam
Cephalosporins	cefazolin cefoxitin ceftazidim ceftriaxone cefepime
Carbapenem	Imipenem ertapenem
Aminoglycoside	amikacin gentamicin
Fluoroquinolones	ciprofloxacin levofloxacin
Tetracyccline	tigecycline
Nitrofurantoin	nitrofurantoin
Trimethoprim/ Sulfamethoxazole	trimethoprime/sulfamethoxazole

Biofilm formation assay

As stated by(10), biofilm was conducted in a 96-well microtiter plate with modifications. Briefly, *K. pneumoniae* isolates were subcultured overnight at 37 °C in brain heart infusion broth (Himedia/ India). In triplicate, 200µl of bacterial cultures were added to each well and cultured at 37°C for 24 hours; the negative control contained just medium. The plates were stained with 200 µl of 1% crystal violet solution at room temperature for 10 minutes. The crystal violet was then removed and rinsed with distilled water three times. The crystal violet inside the cells was dissolved in 200 µl of 100% ethanol, and the absorbance at 630nm was measured using an ELISA reader. After comparing the optical density (O.D) of biofilm to that of the control, and based on the readings, the isolates were categorized as weak, moderate, and strong.

Molecular study

Extraction of Genomic DNA

The total DNA (chromosomal and plasmid) was extracted from all isolates from overnight cultures utilized to extract the genomic DNA of bacteria ,the protocol of ABIOpure Extraction Kit was used .

Molecular identification of *K.pneumoniae* using *16SrRNA* gene

The clinical *K. pneumoniae* isolates that were diagnosed by Vitek2 system identified by *16S rRNA*gene. The primer sequence, annealing temperature& product size were listed in table (2) and the thermal programm shown in table (3).

Table (2): Specific Primers for *16S rRNA*

Primer Name	Seq.	Annealing Temp. (°C)	Product Size (bp)
16SrRNA -F	5'- GCAAGTCGAGCGGTAGCACAG-3'	58	260
16SrRNA -R	5'-CAGTGTGGCTGGTCATCCTCTC-3'		

Table (3): Thermal programme utilized to amplify 16srRNA

Steps	°C	m:s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	58	00:30	
Extension	72	01:00	
Final Extension	72	07:00	1
Hold	10	10:00	

Detection of *fimh & mrkd* genes in *K.pneumoniae* isolates

FimH and *mrkD* genes were detected by PCR using specific primers as shown in table (4). The PCR mixture set up in 20 µl total volume consisting of 10 µl FIREPol® Master Mix Then complete the volume to 20 microliters of distilled water does not contain cutting enzymes and ions. The reaction mixture contain 2 µl of each primer and 2 µl for DNA's template. The thermal program was optimized and executed on a master cycler (Eppendorf, Germany) as shown in the table below (5). Note: 62°C is used as the annealing temperature for *mrkd* gene while 57°Cused as annealing temperature for *fimh* gene .

Table (4) Specific primers for type 1 and type 3 fimbriae

Primer Name	Seq.	Annealing Temp. (°C)	Product Size (bp)	Reference
mrkD-F	5'-CCACCAACTATTCCCTCGAA-3'			Mathur *et*

mrkD-R	5'-ATGGAACCCACATCGACATT-3'	62	228	*al., 2013(11)*
FimH F	5'-TGGTGGTCGACCTCTCCACG-3'	57	169	This study
FimH R	5'-GTTTCCGTGGTGGTCGGGAA-3'			

Table(5) Thermal programme utilized in this study

Steps	°C	m:s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	**62&57**	00:30	
Extension	72	01:00	
Final Extension	72	07:00	1
Hold	10	10:00	

Results and Discussion

One hundred twenty sample that collected from different sources including:1-blood(20 sample) 2-urine (50 sample) 3-sputum (34 sample) 4-wound (16 sample) were utilized to isolate *Klebsiella pneumoniae*.Sixty isolates which gave pink mucoid colonies on MacConkey agar were identified by Vitek -2 compact device , the results revealed that(40) of the bacterial isolates were*Klebsiella pneumoniae*.

Molecular identification of *K.pneumoniae* using *16SrRNA* gene

The PCR utilized to confirm the presence of *K. pneumoniae*.The results revealed that all of isolates(40)(100%) were identified as *K. pneumoniae* which gave positive results at 260 bp domain for the *16SrRNA* gene by PCR depending on this molecular method. The PCR products were analyzed on an agarose gel as illustrated in (Figure 1).

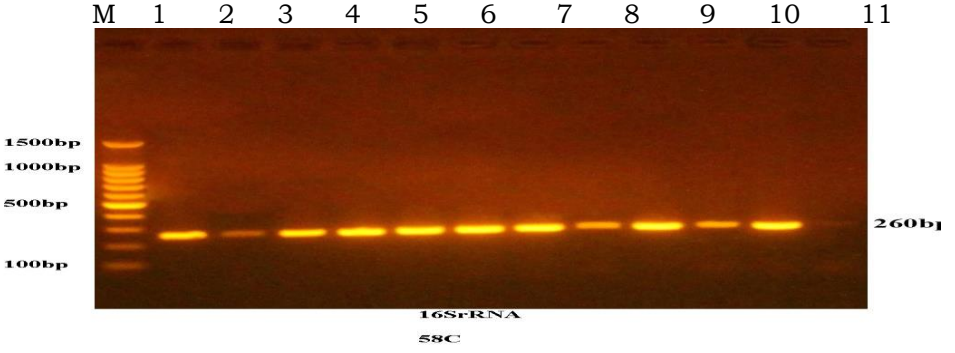

Figure (1): Agarose gel (1.5%) stained by Ethidium Bromide and 100v/mAmp for 60min. electrophoresis for 16SrRNA gene(1500bp) (Set1). Lane1-11 shows PCR product of 16SrRNA gene with an expected size of 260bp. M: DNA ladder(100-1500).

Fourty isolates of *Klebsiella pneumoniae* were tested for 8 groups of antibiotics using Vitek -2 compact system, and it was found that the highest Percent of bacterial resistance for Penicillin (52.5%) , and the lowest groups of bacterial resistance are Carbapenem, Aminoglycoside, Tetracyccline&Nitrofurantoin (2.5%) as shown in table (6).

Table (6) Antibiotics susceptibility test for *Klebsiella pneumoniae* clinical isolates

Antibiotic group	Sensitive %	Intermediate %	Resistance %
Penicillin	40	7.5	52.5
Cephalosporins	62	4	34
Carbapenem	97.5	0	2.5
Aminoglycoside	97.5	0	2.5
Fluoroquinolones	93.75	0	6.25
Tetracyccline	97.5	0	2.5
Nitrofurantoin	50	47.5	2.5
Trimethoprim/ Sulfamethoxazole	60	0	40

Also our results showed that the percentage of resistance to rest groups of antibiotics as follows:40% for Trimethoprim/ Sulfamethoxazole,34% Cephalosporins&6.25 for Fluoroquinolones,this is consistent with other researchers finding as shown below.

A local study by (12) show that all clinical and aquatic isolates of *K. pneumoniae* were resisted to Amoxiclave (128 mg/ml) and more sensitive to Imipenem and Meropenem than other beta lactam antibiotics (Ceftriaxone, Ceftazidime and Cefotaxime) and more sensitive to ceftazidme than (Ceftriaxone and Cefotaxime) and more sensitive to Cefotaxime than Ceftriaxone. Another local study(13) found that *K. pneumoniae* has a high resistance rate to most commonly used antimicrobials, and that the resistant trends of *K. pneumoniae* in China were not congruent with the isolation rate, as there were decreasing resistance trends from 2005 to 2014. Imipenem had the lowest resistance rate, while Cefotaxime had the highest prevalence (79.2 percent) Another investigation found that clinical *K. pneumoniae* isolates were resistant to Cefotaxime (97.75%) and Ceftazidime (71.87%) (14.)

Furthermore, the amount of biofilm development in these 40 isolates was examined using the microtiter plate technique. A microplate reader was used to measure absorbance at 630nm in order to assess biofilm intensity. Given this, absorbance readings reflected the intensity of biofilm thickness generated at the surface of the microtiter well by the examined isolates. Based on the limits listed in table7, the acquired findings were divided into three categories (weak, moderate, and strong)(15). The results of biofilm production were clarified in table (8).

Table(7)Categories of biofilm degrees

Biofilm degree	Absorbance at 630 nm
Non / Weak	< 0.120

Moderate	0.120 - 0.240
Strong	> 0.240

Table (8):Results of biofilm formation of *Klebsiella pneumoniae* isolates

sample	Mean ± SD	Weak	Moderate	Strong
1	0.401 ± 0.069	-	-	+
2	0.497 ± 0.023	-	-	+
3	0.279 ± 0.020	-	-	+
4	0.29 ± 0.016	-	-	+
5	0.518 ± 0.139	-	-	+
6	0.397 ± 0.033	-	-	+
7	0.858 ± 0.039	-	-	+
8	0.262 ± 0.039	-	-	+
9	0.327 ± 0.031	-	-	+
10	0.647 ± 0.057	-	-	+
11	0.853 ± 0.042	-	-	+
12	0.459 ± 0.023	-	-	+
13	0.697 ± 0.052	-	-	+
14	0.706 ± 0.039	-	-	+
15	0.849 ± 0.062	-	-	+
16	0.671 ± 0.050	-	-	+
17	0.709 ± 0.036	-	-	+
18	0.641 ± 0.043	-	-	+
19	0.847 ± 0.051	-	-	+
20	0.311 ± 0.052	-	-	+
21	0.397 ± 0.052	-	-	+
22	0.418 ± 0.028	-	-	+
23	0.551 ± 0.011	-	-	+
24	0.603 ± 0.022	-	-	+
25	0.432 ± 0.030	-	-	+
26	0.517 ± 0.033	-	-	+
27	0.65 ± 0.015	-	-	+
28	0.481 ± 0.024	-	-	+
29	0.516 ± 0.121	-	-	+
30	0.354 ± 0.005	-	-	+
31	0.459 ± 0.015	-	-	+
32	0.45 ± 0.033	-	-	+
33	0.385 ± 0.016	-	-	+
34	0.477 ± 0.042	-	-	+
35	0.457 ± 0.057	-	-	+
36	0.353 ± 0.018	-	-	+
37	0.528 ± 0.035	-	-	+
38	0.437 ± 0.073	-	-	+
39	0.49 ± 0.032	-	-	+
40	0.608 ± 0.142	-	-	+

From the above data, we can affirm that all of the examined (40) isolates (100%) were potent biofilm makers, regardless of their origin. The O.D. or absorbance ranging from 0.262 for isolate No. 8 to 0.858 for isolate No.7. These results contradict those of (16), who isolated 139 diverse clinical *K. pneumoniae* isolates with varying resistance patterns and evaluated the relationship between biofilm formation and antimicrobial resistance in weak versus strong biofilm-forming *K. pneumoniae* and identified predictors of strong biofilm formation. Multidrug-resistant isolates were more prevalent among weak (97.9%) biofilm formers than among strong biofilm formers (76 percent). *K. pneumoniae* resistant to carbapenems was 91% less likely to generate a dense biofilm.

A research by (17) indicated that imipenem-resistant *K. pneumoniae* isolates were evaluated phenotypically for biofilm formation capabilities using the microtiter plate technique. 54.54 percent of the isolates had a robust biofilm, whereas 31.81 percent of the isolates had a moderate biofilm and 13.63 percent had a weak biofilm. All mrkA and mrkD-containing isolates developed strong or moderate biofilm, and no mild biofilm was observed. Therefore, there is a correlation between the production of biofilms and the presence of the mrkD gene in the majority of isolates having these genes.

Isolates that are strong biofilm producers and MDR were taken(11 isolate) and the genes responsible for biofilm formation were detected(*mrkd &fimh*) using conventional PCR . Two genes(*mrkd, fimh*) that seem to be involved in biofilm formation of *K. pneumoniae* clinical isolates were detected by PCR techniques. The gel electrophoresis of amplified PCR product for *mrkd* gene (228bp) was showen in figure (2) and*fimh*(169 bp) was shown in figure (3). The results showed that genes (*mrkd* and *fimh*) found in8 out of 11 isolates thats mean those genes present in 72.7% of isolates.

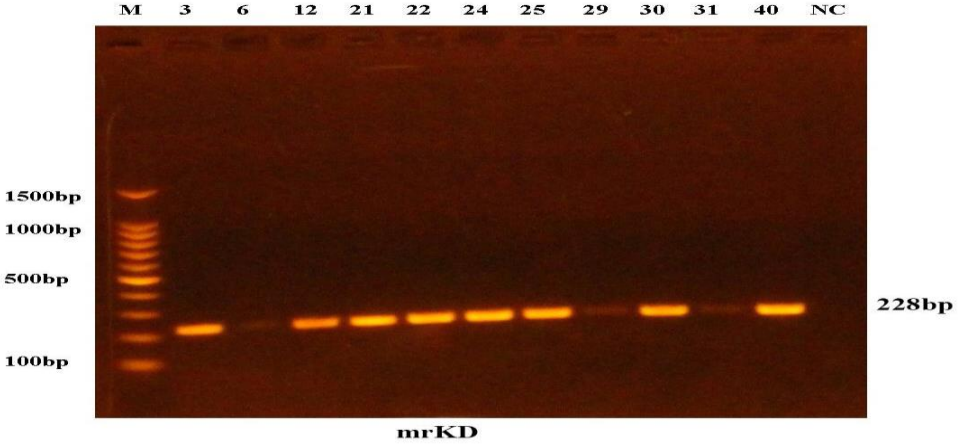

Figure (2):The amplification of mrKD gene in *Klebsiella pneumoniae* samples fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 1500bp ladder marker, NC: negative control. Numbers 3,12,21,22,24,25, 30& 40 represents positive results (228 bp).

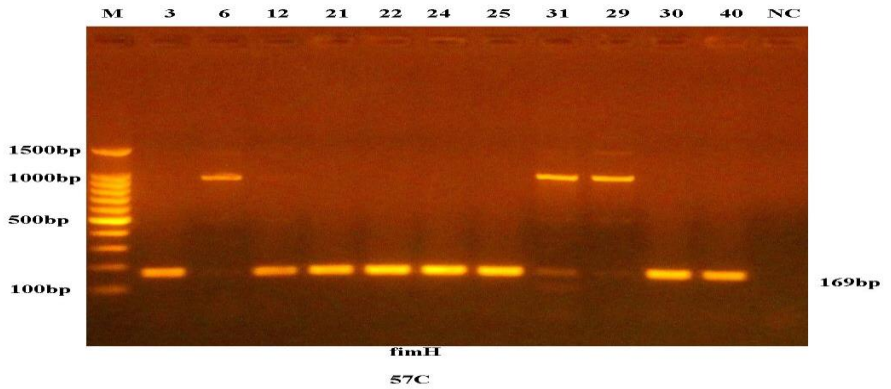

Figure (3): The amplification of fimH primers in Klebsiella pneumoniae samples fractionated on 1.5 percent agarose gel electrophoresis stained with Eth.Br. Positive outcomes are represented by the numbers 3,12,21,22,24,25,30,and 40. (169 bp.(

According to a local investigation by (18), the mrkD gene was tested in both biofilm- and non-biofilm-producing *K. pneumoniae.* A correlation between the production of biofilms and the presence of the mrkD gene was identified in all biofilm-producing *K. pneumoniae* 9 (100%) and 5 (100%) isolated from urinary tract infections (UTIs) and diabetic foot infections (DFIs), respectively. The mrkD gene, like the fimH gene, was found in *K. pneumoniae* isolated from DFIs. Our findings concur with their conclusion.

Another local investigation carried out by (19) reveals Nearly all *K. pneumoniae* isolates included the fimH gene, which encoded for adhesion factors (biofilm). In 14 out of 40 *K. pneumoniae*, the fimH gene was found. Our findings conflicted with those of (20), who discovered that only 8 of the 28 Klebsiella pneumoniae biofilm producers that they recovered from various clinical samples had the mrkD gene present (21.08 percent.) .

Conclusion

We can infer from this research that all of the examined *K. pneumoniae* clinical isolates were powerful biofilm makers, with the majority of them being MDR and having genes responsible for adhesin in a proportion of 72.7 percent for each of *fimh* and *mrkd*.

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