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Molecular Analysis of FimH Gene in Biofilm-Producing and Multidrug Resistant Klebsiella pneumoniae

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ORIGINAL STUDY

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Molecular Analysis of FimH Gene in Biofilm-Producing and Multidrug Resistant *Klebsiella pneumoniae*

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Abstract

Background: *Klebsiella pneumoniae* is a significant gram-negative, opportunistic bacterium. Among the many infectious disorders it can cause are infections of the bladder, bacteremia, pneumonia, and hepatic abscesses. Biofilms slow down the rate of bacterial growth, prevent antibiotics from penetrating the body, encourage the creation of persister cells, and promote genetic exchange. Thus, biofilms can lead to an outbreak of superbug infections.

Objectives: This study aimed to molecularly analyze the *FimH* gene in biofilm-producing and multidrug-resistant *K. pneumoniae*.

Materials and Methods: 250 clinical samples were collected from several hospitals in Baghdad and from different clinical sources. Laboratory diagnosis was based on morphological and biochemical tests. Then, confirmation by the VITEK 2 system compact. About 68 (27.2%) isolates of *K. pneumoniae* were obtained. Disk agar diffusion testing was used to screen for antibiotic susceptibility in accordance with CLSI 2023. Consequently, 44 (64.7%) MDR bacterial isolates were detected that were resistant to one or more of three classes of antibiotics. Biofilm production was detected for these MDR strains using the Congo red method.

Results: The number of black, crystalline-dry colonies regarded as test-positive was 32 (72.7%). DNA was extracted from 24 strains, which were MDR and biofilm producers. Following this, PCR technology was used to amplify the FimH gene using specific primers. *FimH* was detected in all these isolates (100%). The products of PCR were sent to Macrogen Corporation in Korea for sequencing. The analysis of sequences, carried out using bioinformatics software, and the drawing phylogenetic trees between the locally isolated strains showed genetic variation among them.

Conclusion: There is a relationship between biofilm formation and the spreading of drug resistance in *K. pneumoniae* as well as an increase in the expression of *FimH* among biofilm-forming strains.

Keywords: Biofilm, *FimH*, Type 1 fimbriae, MDR, *K. pneumoniae*

1. Introduction

Klebsiella pneumoniae is the primary cause of the majority of infections in humans and one of the most important multidrug-resistant (MDR) bacteria on the globe [1]. Multidrug-resistant (MDR) *K. pneumoniae* (resistance to three or more antimicrobial families) and extensive drug resistance (XDR)—that is, being sensitive to two or fewer antimicrobial families are prevalent, causing severe infections including pneumonia and bloodstream infections caused by classical *K. pneumoniae* strains that are more likely dangerous [2]. Compared to pneumococcal pneumonia, the mortality rate from *K. pneumoniae* is significantly higher: 21% in the general population and 64% in alcoholics [3]. The infection known as ventilator-associated pneumonia (VAP) is commonly seen in immunocompromised patients undergoing mechan-

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ical ventilation for longer than 48 hours. It is caused by *K. pneumoniae* and can have fatality rates as high as 70% in some circumstances [4]. The generation of several virulence factors by *K. pneumoniae* contributes to its pathogenicity, enabling the germs to evade the immune system and cause a variety of illnesses [5] such as lipopolysaccharides (O antigen), capsular polysaccharides (K antigen) [6], fimbriae (pili) and iron transfer elements [7].

A crucial aspect of the pathogenesis of *K. pneumoniae* disease is the creation of biofilms, which enhance resistance to environmental conditions and act as a reservoir for the spread of genes linked to antibiotic resistance [8]. Biofilms are bacterial communities that are highly organized and exhibit heightened resistance against host defenses and antibacterial components, such as phagocytosis [9], the complement system, and antimicrobial peptides [8]. Microbes adhere to inert or living surfaces through the production of extracellular polymeric compounds (EPC), a process known as biofilm formation. Proteins, fatty acids, nucleic acids, polysaccharides, and genetic material from other cells make up the majority of the EPC [10]. Since biofilms contain persistent cells, dense EPS layers, and improved efflux pump expression, the bacteria within them are a thousand times more resistant to drugs than planktonic cells [11].

Fimbriae are adhesins that facilitate attachment to both abiotic surfaces [12], such as healthcare devices, where bacteria build biofilms, and biological surfaces (leading to tissue invasion) [8]. In addition to colonizing the pulmonary, digestive, and urinary tracts, *K. pneumoniae* biofilm can cause invasive infections, particularly in patients with compromised immune systems [13]. *K. pneumoniae* adheres to tissue cells primarily through two main adhesion pili systems: the mannose-sensitive type 1 pili (T1P) and the mannose-resistant type 3 pili (T3P). T1P consists of a fimbrial FimA subunit and an adhesin FimH subunit, while T3P is made up of MrkA and MrkD subunits [14]. Type 1 fimbriae, especially the FimH subunit are found in many members of Enterobacteriaceae and play an important role in UTI [15]. *FimH* is one of the genes in *K. pneumoniae* that encodes for virulence factors responsible for the majority of damage caused by bacteria [16]. Fimbriae are expressed by the fim genes, which represent all the genes necessary for fimbrial construction and assembly [17]. The FimH adhisin, located on the tip of the fimbria, is encoded by operon fim and aids in the fimbriae's ability to cling together and form sticky structures [18].

In several bacteria belonging to the Enterobacteriaceae family, type 1 fmbriae are among the best-described fmbrial adhesins. Phase variation is the means by which type 1 fimbriae in *K. pneumoniae* are controlled, just as it is in *Escherichia coli* [19]. Ninety percent of *K. pneumoniae* express type 1 fimbriae, which mediate adhesion to many epithelial cell types, particularly the urinary epithelium [20]. The objective of this study is to examine the *FimH* gene molecularly in biofilm-forming, multidrug-resistant *K. pneumoniae*.

2. Materials and methods

2.1. Isolation and identification

Two hundred fifty specimens in all were drawn from various clinical sources, such as the foly tip, urine, blood, sputum, bodily fluids, injury, ear swab, and tissue biopsy. These samples were taken throughout December 2023 to March 2024 from hospitals in Baghdad, Iraq, and covered males and females. Therefore, *K. pneumoniae* was identified using morphological and biochemical testing following culture on MacConkey, blood agar media, and a 24-hour incubation period at 37°C. Using the VITEK 2 system compact, colonies with mucoid and glossy characteristics were chosen and identified. As a result, 68 isolates of *K. pneumoniae* were acquired.

2.2. Antimicrobial susceptibility testing

Most clinical specimens were evaluated for resistance to antibiotics in order to identify MDR strains using the disk diffusion approach, which was developed in coordination with standards from the Clinical and Laboratory Standards Institute (CLSI). Antibiotics were chosen in the following order, per the CLSI 2023 standard and prior studies: Cefotaxime (30 μg), amoxicillin (20 μg), gentamicin (10 μg), cefepime (30 μg), imipenem (10 μg), meropenem (10 μg), azatreonam (30 μg) and amikacin (30 μg). The bacteria grown on Mueller-Hinton agar plates were treated with the aforementioned antibiotics, and they were subsequently incubated for eighteen hours at 37 Celsius. Using a ruler, the diameter of the inhibition growth zones was measured.

2.3. Detection of biofilm production

MDR Strains of *K. pneumoniae* were cultured for a period of 24 to 48 hours at 37°C in Brain Heart Infusion Medium, which contains 5% (w/v) sucrose and 0.08% (w/v) congo red [21]. The Congo red test was used to identify biofilm growth phenotypically.

Table 1. Primers used for the detection of K. pneumoniae FimH gene.

Primers		Sequence 5′-3′	Annealing temp. (°C)	Product size (bp)	Reference
FimH	F	TGCTGCTGGGCTGGTCGATG	58	680	[2]
	R	GGGAGGGTGACGGTGACATC			

2.4. Detection of FimH

2.4.1. Extraction of DNA

The genome's DNA was extracted from the bacterial culture using the ABIOpure Extraction procedure. After centrifuging the NB culture overnight for two minutes at 13,000 rpm, the supernatant was discarded, and the precipitate was used as a source of genetic material. After completing the steps in the ABIOpure Extraction kit, DNA was obtained from the microbe. To assess the quality of the samples for use in subsequent processes, the concentration of extracted DNA was measured using a quantus fluorometer. 200 μl of diluted Quantifluor dye was combined with 1 μl of DNA. DNA concentration readings were obtained following a 5-minute incubation period at room temperature.

2.4.2. PCR amplification

DNA extract from the 24 MDR bacterial isolates was utilized. A polymerase chain reaction (PCR) analysis using specific primers (Table 1) was done for the purpose of looking for *FimH* genes in MDR *K.pneumoniae*. The PCR reactions were prepared in a total volume of 25 μL and amplification was performed in a thermal cycler (Eppendorf Master Cycler®, MA) as follows: 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 58°C, 30 s at 72°C, 10 min at 72°C for detection of the *Fim*H gene.

2.4.3. Gene sequencing

The *FimH* genes of ten bacterial strains were sent to Macrogen Corporation in Korea, which performed Sanger sequencing for the PCR products using an automated DNA sequencer, the ABI3730XL. Emails with the results were sent out. Following that, the results were analyzed using in-house software. Creating a phylogenetic tree connecting geographically distant strains was the final stage.

3. Results and discussion

3.1. Identification of bacteria

This study was conducted on 250 patients, both male and female, of different ages. Following laboratory diagnosis by traditional morphological (Fig. 1) and biochemical tests, along with confirmation using the VITEK 2 system compact, there were 68 (27.2%) bacterial isolates identified as *K. pneumoniae* for patients aged between 1 and 65 years, with 29 (42.6%) present in males and 39 (57.3%) in females.

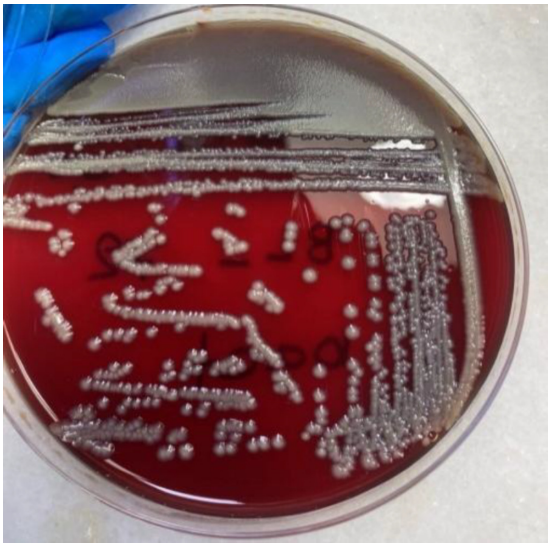

Fig. 1. Morphological identification of K. pneumoniae on blood agar media.

K. *pneumoniae* is one of the medically important pathogens that has raised serious problems for public health (23). Nowadays, it is known that *K. pneumoiae* is a serious bacterium that causes around 20% of infections in hospitals worldwide (24). In this research, the prevalence of *K. pneumoniae* was estimated to be 27.2%, although in earlier research, it was reported to be 32.48% and 22.94% (25, 26). In females, the number of specimens (57.3%) was slightly larger than the specimens collected from males (42.6%); therefore, the high percent of isolated microbes originated from females. This is due to the fact that the female patients made up the majority of patients who took part in this study. Compared to isolates from other clinical sources, a larger percentage of *K. pneumoniae* isolates were found in urine. This was consistent with the findings of another study by Ibrahim (27). These isolates from clinical samples were distributed as shown in Table 2.

3.2. Antibiotic susceptibility testing

Phenotypically, multi-drug resistance is indicated through patterns of antimicrobial susceptibility testing by disk diffusion (Fig. 2), as utilized by [22].

Table 2. Frequency of K. pneumoniae depending on the source of the sample.

Source of specimens	No. of samples	%
Urine	17	25.00%
Sputum	14	20.58%
Blood	10	14.70%
Body fluid	8	11.76%
Wound swab	6	8.82%
Pus	5	7.35%
Foly tip	4	5.88%
Ear swab	3	4.41%
Tissue biopsy	1	1.47%
Total	68	100%

The results of this test exhibited varying degrees of antibiotic resistance. The highest rates were (92.81%), (89.12%), (86.20%), (83.98%) and (77.31%) for Cefotaxime, amoxicillin-clavulanic acid, cefepime, azetronam and gentamicin respectively, while the intermediate resistances were for meropenem, imipenem and amikacin (Table 3). A total of 68 isolates were 44 (64.7%) isolates categorized as multi-drug resistance (MDR), 14 (20.58%) isolates categorized as extensively drug resistant (XDR) and the residual 10 (14.7%) isolates categorized as sensitive (S).

The findings of the antibiotic susceptibility test in this study demonstrated the pathogen's high level of resistance to several antibiotics, and the proportion of MDR bacteria (64.70%) was larger than that of XDR and sensitive strains. This Findings consistent with current studies, where MDR was confirmed in 60% and 65% of isolates (2, 23). The pathogen has numerous resistance mechanisms, including the ability to build biofilms, which can prevent antibiotics

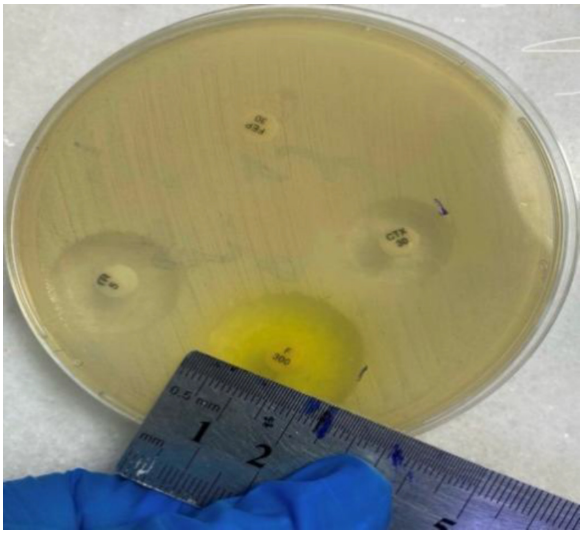

Fig. 2. Antimicrobial susceptibility test for detection of MDR K. pneumoniae.

Table 3. Antibiotic susceptibility test results.

Antibiotic agent	Resist	Intermediate	Susceptible
CTX	No. 63 % (92.64)	No. 0 % (0)	No. 5 % (7.35)
FEp	No. 58 % (85.29)	No. 0 % (0)	No. 10 % (14.70)
IMI	No. 44 % (64.70)	No. 13 % (19.11)	No. 11 % (16.17)
MEM	No. 41 % (60.29)	No. 4 % (5.88)	No. 23 % (33.82)
AMC	No. 60 % (88.23)	No. 1 % (1.47)	No. 7 % (10.29)
GEN	No. 52 % (76.47)	No. 2 % (2.94)	No. 14 % (20.58)
AZT	No. 57 % (83.82)	No. 3 % (4.41)	No. 8 % (11.76)
AK	No. 45 % (66.17)	No. 1 % (1.47)	No. 22 % (32.35)
Total	No. 68 % (78.20)	No. 68 % (3.81)	No. 68 % (17.99)

from being absorbed, as well as the production of the enzyme carbapenemase (24). Antibiotic resistance can also arise due to genetic mutations on chromosomal DNA, which are caused by the emergence of antibiotic-resistant genetic material and spread to younger generations (25). The misuse and excessive consumption of drugs are two factors contributing to the emergence of antibiotic resistance (26). Another mechanism of resistance is plasmid transfer, which is arguably the most significant since it can provide the host organism and its offspring with more genetic information expressing antibiotic resistance (27).

3.3. Congo red method

The findings indicated that Congo red was positive for 32 (72.7%) *K. pneumoniae* strains that were resistant to imipenem and meropenem, generated dark, crystalline-dry colonies, and were assumed to be exopolysaccharide producers. *K. pneumoniae* can create a thick coating of extracellular biofilm that facilitates the microbe's adhesion to biological or inert surfaces, in order to protect antibiotic penetration and lessen its effects (23). Congo red agar method is effective and makes the bacterium simple to replicate. Therefore, the method was chosen in an effort to improve its ability to identify whether biofilm growth is present in *K. pneumoniae*. The process is easy to do, and the color of the colony that forms typically indicates the outcome. For strains that do not form biofilm, this color can be red; for bacteria that do, it can be black (24).

According to the results of this method, most MDR strains (72.7%) were capable of biofilm production.

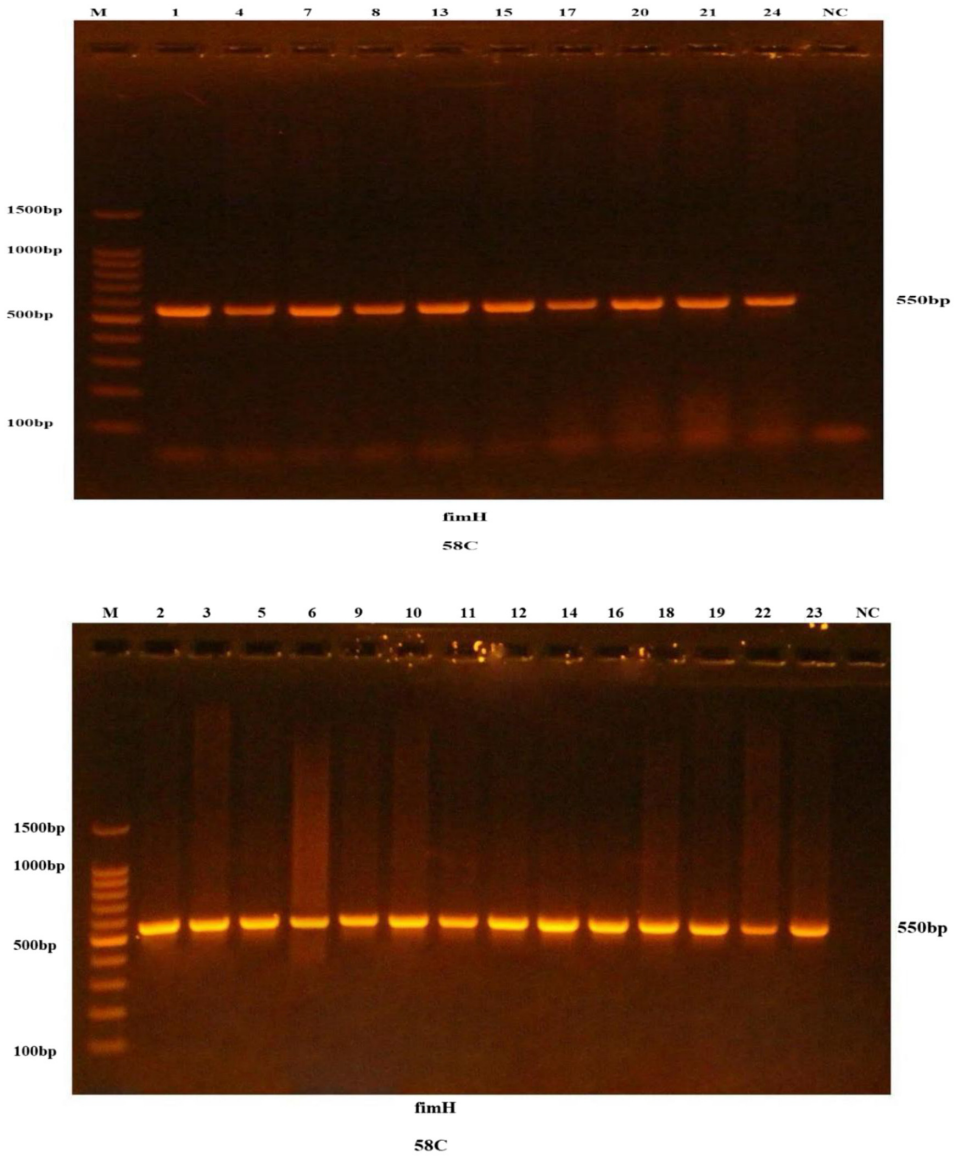

Fig. 3. FimH's results by polyerase chain reaction technology.

The finding of this study agreed with those of a recent study by Shadkam *et al*., who discovered that 75% of isolates form biofilm (25). In other recent research, 49.10% of MDR isolates were positive for the biofilm test. Therefore, it was concluded that the majority of *K. pneumoniae* isolates exhibited resistance to a variety of antibiotics and produced biofilm in various manners (26). The drug resistance mechanism of biofilms is influenced by various factors, including gene transfer and the impermeability of antimicrobial drugs (27). Additionally, each isolate has a distinct ability to create biofilms depending on the temperature, pH, type of surface the biofilm adheres to, and the physical interactions that exist among components (28).

3.4. FimH genes detection

Products of PCR reactions for extracted DNA 23–25 ng/μl) of MDR bacterial isolates electrophoresed on 2% agarose gel revealed positive results (100%) for the *FimH gene, approximately* 550 bp. In a similar article, *FimH* was found in 91% of the isolates that were antibiotic-resistant (MDR, XDR, and PDR) (Fig. 3).

3.5. Gene sequencing of FimH gene

The gene sequencing results of 10 FimH genes in MDR *K. pneumoniae* isolates revealed genetic variation in nucleotide Sequence compared to other local isolates as explained in the phylogenetic trees.

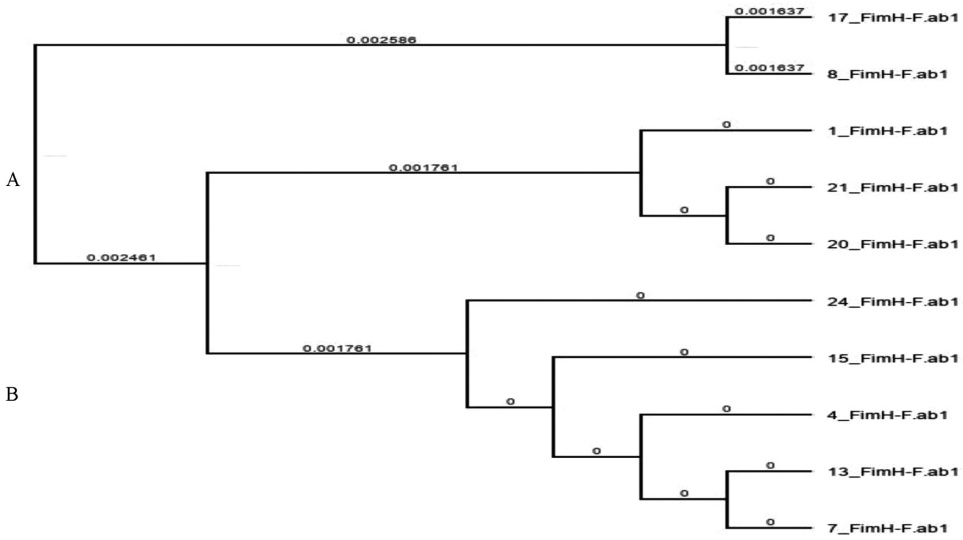

Fig. 4. Phylogenetic tree of FimH in comparison with locally K. pneumoniae isolates.

3.6. phylogenetic tree of sequencing the FimH gene

In the phylogenetic tree of *FimH* genes, there are two main groups, A and B. There are genetic variations between our locally isolated groups A (17_FimH-F.ab1 and 8_FimH-F.ab1) and B (1_FimH-F.ab1, 21_FimH-F.ab1, 20_FimH-F.ab1, 24_FimH-F.ab1, 15_FimH-F.ab1, 4_FimH-F.ab1, 13_FimH-F.ab1 and 7_FimH-F.ab1) as shown in Fig. 3.

In the present study, most biofilm-producing and MDR isolates showed positive PCR for FimH gene (100%). The results of a similar study coincide with the present study (23). Another study by Reham *et al*. in 2021 showed fimH (86.7%) of biofilm-formed strains. There is a relationship between FimH, biofilm formation and drug resistance in *K. pneumoniae*. According to the most recent studies, biofilm FimH played a significant role in the infections' ability to persist (24). The binding to D-mannose is carried out by the protein that is encoded by FimH. It has been demonstrated to control type 1 fimbriae's structure and functionality (25). In recent research, in 89% of the type 1 fimbriae-encoding FimH gene was found. It was found to be most prevalent in urine isolates (91.1%), indicating that type 1 fimbriae play a role in the pathophysiology of urinary tract infections [20].

4. Conclusion

K. pneumoniae isolates that form a biofilm are resistant to many drugs, although not all antibiotic-resistant isolates can develop it. This could be related to other virulence factors. Even multidrug-resistant *K. pneumoniae* isolates may not have to be biofilm-producers. *FimH* has shown elevated expression among biofilm-forming strains. Finally, type 1 fimbriae actively enhance biofilm formation in *K. pneumoniae*.

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