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Article

Detection of Biofilm Formation Among the Clinical Isolates of *Klebsiella pneumoniae*: Phenotypic and Genotypic Methods

Fatimah E. Alquraishi [1]* , Zainab N. AL-Saadi[1] and Jalal A.T. Al-Azzawi[2]
[1]Department of Biology, College of Science, University of Wasit, Iraq.
[2]Ministry of Health, Wasit Health Directorate, Wasit Governorate, Iraq.
*Correspondence: fatimaenad6@gmail.com; Tel.: (+09647726878654)

Abstract

Infections caused by biofilm-embedded pathogens decrease the efficacy of traditional treatments and increase antibiotic tolerance. Most of the human bacterial infections are biofilm-associated. Therefore, this study aimed to detect the biofilm formation among the clinical isolates of *Klebsiella pneumonia* collected from different hospitals in Wasit province-Iraq by phenotypic and genotypic methods. 525 clinical samples were used to isolate 77 *K. pneumoniae* strains from clinical specimens for five months. They were identified by microbiological method as *K. pneumoniae*. The microtiter plate method is used to detect the biofilm formation. Results showed that out of 77 *K. pneumonia* isolates, 76 (98.7%) isolates were biofilm producers with three different categories; 12 (15.6%) were weak-biofilm producers, while other isolates 63 (81.8%) and 1 (1.3%) were moderate and vigorous producers, respectively. However, 1 (1.3%) isolates were identified as non-biofilm producers. Amplification of genes by multiplex PCR technique was done for 77 isolates of *K. pneumonia* to detect biofilm production genes, *mrkD* and *FimH*. Results showed that out of 77 isolates, there were 74 isolates (94.8%) positive to *mrkD* and 33 isolates (42.8%) to *fimH*.

Keywords: *K. pneumonia*; Microtiter plate method; *mrkD*; *fimH*; Iraq.

Introduction

Klebsiella pneumoniae, a member of the family *Enterobacteriaceae*, is a part of the flora and is isolated as the causative agent in complicated infections. It is a microorganism that causes severe diseases such as pneumonia, septicemia, bacteremia, wound infections and purulent abscesses at different sites in humans. The bacterium is widely distributed in healthy people's urinary, respiratory, and gastrointestinal tracts. Most *K. pneumoniae* are hospital-associated with a high fatality rate if incorrectly treated [1]. *Klebsiella* spp. is among the five gram-negative pathogens most usually encountered in hospital-acquired infections. [2]. *Klebsiella* was found in two general habitats: the first habitat is the mucosal surface of mammals such as humans, swine or horses, which they colonize, and the other habitat is the environment, where they are found in surface water, soil and on plants [3]. *Klebsiella pneumonia* can produce several virulence factors necessary for the infection's colonization, adherence, invasion and progress. These include lipopolysaccharide presence, biofilm production, capsule production, adherence factors and siderophore activity. In addition, further virulence factors such as

hemolysins, protein tyrosine kinase, heat-stable endotoxins and heatlabile exotoxin [4,5,6].

Filament formation is a typical response in which bacteria replicate but incompletely divide, leading to long, slender chains. Many bacteria have existed in biofilm for prolongation. Biofilms preserve persistent bacteria from antibiotic effects, leading to the emergence of persistent bacterial cells. It should be noted that persistent pathogens lead to chronic diseases, resulting in the overuse of antibiotics and reduced antibiotic efficacy [7].

Materials and Methods

Specimens collection

This study was carried out in Al-Haj Jalal Hospital in Wasit Province-Iraq. 525 specimens from patients clinically diagnosed with UTI from 1st October to 30th February 2021. Of these patients, 442 were females (aged 25 days to 80 years), and 83 were males (aged 1 month to 80 years). All urine samples were collected in sterile screw-capped test tubes.

Isolation and identification

Clinical samples were cultured onto MacConkey and Blood agar plates for 18-24 h at 37°C. Morphologic characteristics tested all lactose-fermenting isolates according to MacFaddin [8]. The collected isolates were identified biochemically according to Forbes et al. [9] and methods described by MacFaddin [8]. Confirmation of K. pneumoniae was conducted using the API20E system. The test was done according to the manufacturer's instructions (BioMeriux, France). Bacterial isolates were stored in BHI broth containing 20% glycerol at -20°C [10].

Detection of biofilm formation

Phenotypic method

Biofilm formation test was done by microtiter plate method as described by Al-Timimi [11]. Bacterial isolates were cultured on brain heart infusion agar and incubated at 37ºC for 24 h. then, a few (3-5) colonies, suspended in 5 ml of normal saline in test tubes, were mixed by vortex. Twenty microliters of bacterial suspension overnight culture were used to inoculate 96 wells of a bottomed microtiter plate containing 180 µl of brain-heart infusion broth with 2% sucrose. Control wells contained 200 µl of BHI broth with 2% sucrose; triplicate was done for each isolate. The microtiter plate was covered with a lid during incubation at 37ºC for 24 h. After incubation, the content of each well was removed, and the wells were carefully washed three times with PBS (pH 7.2) and then left to dry. The plates were dried at room temperature for 15 minutes. Crystal violet (1%) was added to the wells for 15 minutes. After removing the crystal violet solution, wells were washed three times with PBS (pH 7.2) to remove the unbounded dye and allowed to dry at room temperature. Dye bound to the adherent cells was disbanded with 200 µl ethanol. The absorbance of each well was measured at 630 nm using an ELISA reader. The control well's optical density (OD) value was deducted from the entire test, and each assay was performed in triplicate. The adherence capabilities of the bacterial isolates were classified into four categories; above, the mean optical density of the negative control (contained broth only) was considered as the CUT-OFF, and isolates were classified according to Mathur et al. [12] as follows:

OD value Biofilm ranking

$OD \leq ODc$ …… None

$ODc < OD \leq 2 \times ODc$ …… Weakly

$2 \times ODc < OD \leq 4 \times ODc$ …… Moderate

$4 \times ODc < OD$ …… Strong

Genotypic method

DNA was extracted from 77 *K. pneumonia* clinical isolates using a commercial purification system Easy Pure® Bacteria Genomic DNA Kit. The extraction of genomic DNA was performed according to the company's manufacturing. Aseptically prepared the PCR reaction mix using Taq Ready master mix Kit according to the manufacturer's instructions for a final reaction volume of 50 µl with 7 µl of DNA extract. Multiplex PCR of each primer was performed with Taq GreenMaster Mix PCR Kit. Reaction mix 50 µl consisted of 25 µl of 1X PCR Master Mix, 7.5 µl of biofilm formation genes (3.75 µl from mrkD, and 3.75 µl from fimH) of each primer, and 7 µl (10–100 ng) of template DNA and then complete the volume into 50 µl by nuclease-free water. DNA amplification was carried out with the following thermal cycling: an initial denaturation of DNA at 95°C for 15 min. was followed by 35 cycles of amplification (95°C for 40 sec, 52°C for 30 sec, and 72°C for 45sec), ending with a final extension at 72°C for 5 min, and soak at 4°C for 5 min. The sequence of oligonucleotide primers was used to detect *fimH* [F (5′-CGGAAACGATCACCGACTAC-3′) and R (5′-CACGTCGTTATTGGCGTAGA-3′)] and *mrkD* [F (5′-CCACCAACTATTCCCTCGAA-3′) and R (5′-GGCCGACGGTGTATTTCTTA-3′)] at a product size of 489bp and 317bp, respectively.

Electrophoresis results were identified using a UV-Transilluminator system. The DNA bands were measured according to the ladder DNA. The positive results were distinguished when there was a DNA band equal to the target product size and then photographed using a camera

Statistical analysis

This study's data results were analyzed using Graph Pad Prism 8 software and Microsoft Excel 2013 for each biological replicate. The probability level at P values below ≤ 0.05 was used to identify a significant difference [13,14,15].

Results

Culture

Of 525 patients clinically diagnosed with UTI, only 233 (44.38%) were positive for bacterial culture (only one specimen was selected per patient).

Morphological characteristics

For identification of *K. pneumonia* isolates based on morphological characteristics of the colonies on MacConkey agar and blood agar, the isolates appeared as large, mucoid and pink on MacConkey agar due to lactose fermenting. In contrast, they appeared white, large, and mucoid colonies on blood agar without hemolysis.

Biochemical tests

The biochemical tests were used to identify bacterial isolates (Table 1). It showed that all isolates of *K. pneumonia* revealed a positive catalase test indicated by bubbles formation of O_2. While the results of IMViC differentiate them from other lactose fermenter genera showed negative results for indole. A positive result for the citrate utilization test utilization of citrate is an important physiological test. *Klebsiella* showed positive reactions to citrate. (Figure 1).

No.	Test	Result
1	Vogesproskauer	+
2	Indol test	-
3	Citrate utilization test	+
4	Methyl red	-

5	TSI	A/A/G+/H2S-
6	Oxidase	-
7	Catalase	+
8	Urease test	+

Positive result (+) ; Negative result (-); A (Acid); G+ (Gas production); H_2S (No black sediment).

Table 1. Biochemical tests used for confirming the identification of *K. pneumoniae* isolates.

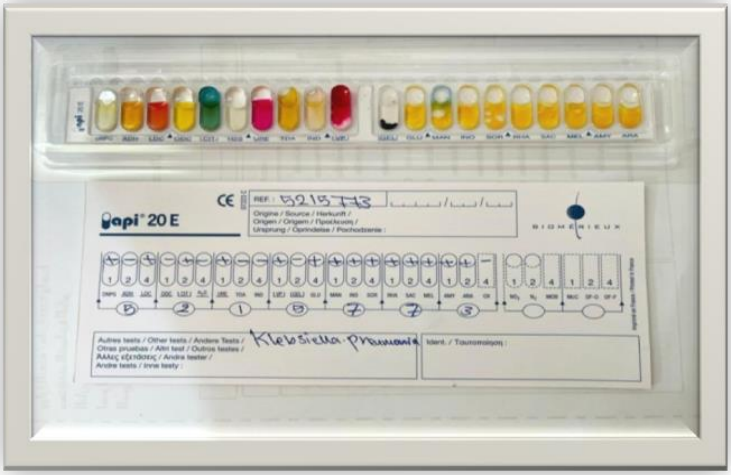

Figure 1. API 20E system for *K. pneumoniae* characterization.

Of 233 patients with positive bacterial culture, isolated *E. coli* from 79 (33.05%), followed by *K. pneumonia* 77 (32.22%), *Staphylococcus aureus* 65 (27.2%), *Citrobacter* 6 (2.51%), *Proteus* spp. 5 (2.09%), *Enterobacter* 3 (1.26%), and *P. aeruginosa* 3 (1.26%), and *Streptococcus Pneumoniae* 1 (0.42%) (Table 2). The results of this study agreed with other local studies, such as Essa et al. [19] performed a study in Baghdad and Arbil on pregnant women, isolated *E.coli* from 49.1% followed by *Acinetobacter baumannii* (21.3%), *K. pneumoniae* (13%), *P. mirabilis* (11.1%), and *P. aeruginosa* (5.6%).

No.	Test	Result [No. (%)]
1	*Escherichia coli*	79 (33.05%)
2	*Klebsiella pneumonia*	77 (32.22%)
3	*Staphylococcus aureus*	65 (27.2%)
4	*Citrobacter*	6 (2.51%)
5	*Proteus*	5 (2.09%)
6	*Enterobacter*	3 (1.26%)
7	*Pseudomonas aeruginosa*	3 (1.26%)
8	*Streptococcus Pneumoniae*	1 (0.42%)
9	Total	239

Table 2. Positive bacterial culture distribution among 233 patients with UTI.

The highest frequency of UTI was among young women (15-40 years), as shown in Table (4).

Gender	Age group of patients	Result [No. (%)]
Female	1month-14 years	15 (19.5%)
	15-40 years	(50.6%) 39
	>40 years	6 (7.7%)
Total		60 (77.8%)
Male	1month-14 years	8(10.4%)
	15-40 years	7 (9.1%)

	>40 years	2 (2.6%)
Total		17 (22.1%)

Table 3. Gender and age distribution of K. pneumoniae isolates from patients with UTI.

Biofilm formation

Phenotypic method

Klebsiella pneumonia forms biofilms on surfaces, whether biotic or abiotic, such as catheters and other medical devices, contributing to antibiotic resistance. Biofilms facilitate persistence, leading to persistent UTIs, which can lead to stone formation. Virulence factors like adhesions. From this point, the microtiter plate method tested the ability of *K. pneumonia* isolates to produce biofilms (Table 4, Figure 2).

No. of isolate	Biofilm pattern	No. of isolate	Biofilm pattern
1	Moderate	**40**	Weak
2	Moderate	**41**	Moderate
3	Moderate	**42**	Moderate
4	Moderate	**43**	Weak
5	Moderate	**44**	Moderate
6	Weak	**45**	Moderate
7	Moderate	**46**	Non
8	Moderate	**47**	Moderate
9	Moderate	**48**	Weak
10	Moderate	**49**	Moderate
11	Moderate	**50**	Moderate
12	Moderate	**51**	Moderate
13	Moderate	**52**	Moderate
14	Weak	**53**	Moderate
15	Moderate	**54**	Moderate
16	Moderate	**55**	Moderate
17	Moderate	**56**	Moderate
18	Moderate	**57**	Moderate
19	Moderate	**58**	Moderate
20	Weak	**59**	Moderate
21	Moderate	**60**	Moderate
22	Moderate	**61**	Strong
23	Moderate	**62**	Moderate
24	Moderate	**63**	Moderate
25	Moderate	**64**	Moderate
26	Moderate	**65**	Moderate
27	Moderate	**66**	Weak
28	Moderate	**67**	Moderate
29	Moderate	**68**	Moderate
30	Moderate	**69**	Moderate
31	Moderate	**70**	Moderate
32	Moderate	**71**	Moderate
33	Moderate	**72**	Moderate
34	Weak	**73**	Moderate
35	Weak	**74**	Moderate
36	Weak	**75**	Moderate
37	Moderate	**76**	Moderate
38	Weak	**77**	Moderate
39	Weak	-	-

Table 4. Biofilm pattern among isolates of *K. pneumoniae*.

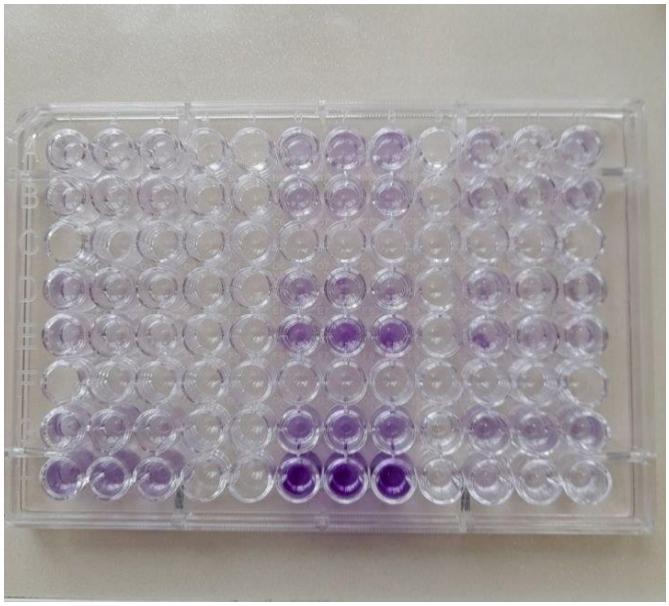

Figure 2. Microtiter plate test of biofilm-positive *Klebsiella pneumoniae*.

All 77 *K. pneumonia* isolates were detected for biofilm formation by microtiter plate assay. Results showed that out of 77 *K. pneumonia* isolates, 76 (98.7%) isolates were biofilm producers with three different categories:12 (15.6%) were weak-biofilm producers, while other isolates 63 (81.8%) and 1 (1.3%) were moderate and strong producers, respectively. However, 1 (1.3%) isolates were identified as non- biofilm producers.

Genotypic method

Klebsiella pneumonia isolates were genomic, typically using the multiplex-PCR. Amplification of genes by multiplex PCR technique was done for 77 isolates of *K. pneumonia*e to detect biofilm production genes: *mrkD* and *FimH*. Results showed that out of 77 isolates, there were 74 isolates (94.8%) positive to *mrkD* 33 isolates (42.8%) to *FimH* (Figure 3).

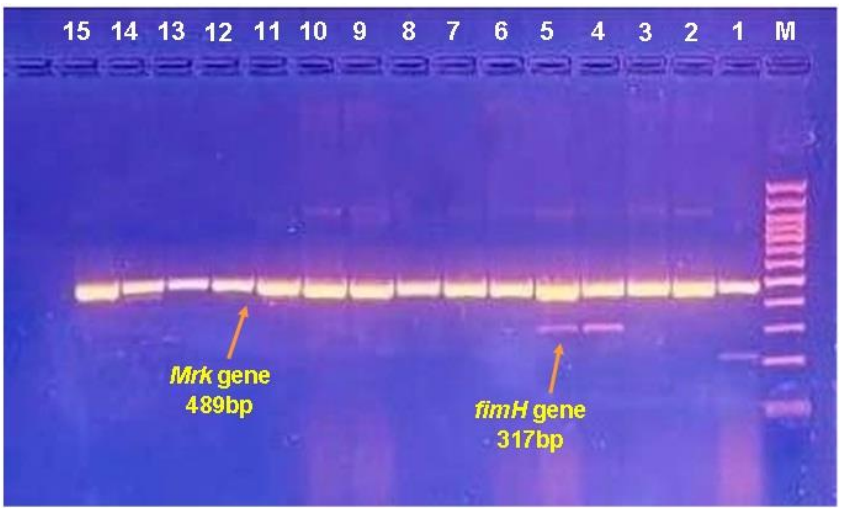

Figure 3. The agarose gel electrophoresis image showed the multiplex PCR product of two genes (*fimH* and *mrk*) of *Klebsiella pneumoniae* isolates at 317 and 489 bp PCR product size, respectively. The Lane (M): DNA marker (100-1500bp).

Discussion

This result was consistent with other Iraqi researchers. A study by Alsamarai and Ali [12] in Tikrit reported that 234 out of 563 (41.6%) gave positive cultures. In addition, Al-Jemely [16] in Baquba 135 urine samples, only 110 Samples gave bacterial growth at 81.4 %. Also, on Day [17] in Wasit, 278 out of 774 specimens (35.9%) were positive for bacterial culture. The findings of morphological characteristics were similar to those reported by another study [16]. The biochemical tests' results were identical to those of others [8,17,18,19]. Among UTIs caused by *K. pneumoniae*, females had a higher frequency than males. The results agreed with other studies. Bachay (2018) found that the highest frequency of UTI (51.7%) occurred in women aged 15-40 years in Iraq [17]. Iranpour et al. (2015) clarified that 50% of patients with UTI aged 15-45 years in Iran [20]. Bachay performed a study in Wasiton women, isolated *E. coli* from 67.20%, followed by *K. pneumoniae* (15.90%), *Proteus* spp. (6.70%), *P. aeruginosa* (6.70%) and *Enterobacter* (3.36%) [17].

Concerning the phenotypic method, our results were identical to that detected by others [21-25]. The emergence of weak and moderate biofilm phenotypes among Gram-negative bacteria was in agreement with some local studies. Al-Rubyaie [26] indicated that all clinical *K. pneumonia* isolates were able to produce biofilm 100%. Al-Timimi [11] observed that out of 50 *K. pneumoniae*, 40 isolates were biofilm producers with different categories, including 23 (46%), 14 (28%) and 3 (6%), which were weak, moderate and robust biofilm production, respectively. Also, Al-Husseini [27] observed that out of 100 *P. aeruginosa*, 85 isolates were biofilm producers with different categories, including 44% weak, 35% moderate and 6% strong biofilm production.

The genotypic result disagreed with Iraqi research, such as Al-Musawi [28], in Baghdad, which reported *mrkD* in (82.85%) and a study done by Mirzaie and Ranjbar [29] in Iran, who reported *mrkD* in 88%. Our results agreed with Ferreira et al., 30 reported *mrkD* in 96% of Brazil. The result of *FimH* genes disagreed with local studies such as Al-Aajem et al. [31] in Diyala reported *fimH* genes in rates (86.66%), and Aljanaby and Alhasani [4] indicated *fimH* genes in rates 100%. Another study, such as Alsanie [32] in Saudi Arabia, indicated *fimH* genes in rates (69.5%). In India, Remya et al. [33-34] reported *fimH* genes in rates (84%)[35].

Conclusions

This study aimed to detect the biofilm formation among the clinical isolates of Klebsiella pneumonia collected from different hospitals in Wasit province-Iraq by phenotypic and genotypic methods. The results support previous studies such as Essa et al. [19]. This section is mandatory but can be added to the manuscript if the discussion is unusually long or complex. All samples showed a positive result for urease production due to the ability to produce the enzyme urease; these enzymes converted the color from yellow to pink. All isolates were confirmed by using the API 20E system. Among UTIs caused by *K. pneumoniae*, females had a higher frequency than males regarding age and gender.

Author Contributions: for Conceptualization, FEA. and ZNA.; methodology, FEA. and JATA; software, ZNA.; validation, FEA., ZNA. and JATA.; formal analysis, FEA.; investigation, FEA. and ZNA.; resources, FEA.; data curation, FEA.; writing-original draft preparation, FEA.; writing review and editing, ZNA.; visualization, ZNA.; supervision, ZNA.; project administration, FEA. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics of the Scientific Committee of the Department of Biology in the College of Science (University of Wasit, Wasit, Iraq).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.
Conflicts of Interest: The authors declare no conflict of interest.

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