Assessment of mrkD and T1 fim Genes of K. pneumoniae and Their Association with Biofilm Formation in Community-Acquired Infections

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KEYWORDS

Klebsiella pneumoniae, Community-acquired infections, mrkD, T1 fim, Biofilm

ABSTRACT

Background: Klebsiella pneumoniae is a pathogenic bacteria that can form biofilms on abiotic and biotic surfaces. The constraints of the therapeutic choices against K. pneumoniae arise from its innate capacity to develop biofilm and harboring determinants of multidrug resistance. Objective: The current study aimed to assess the prevalence of some fimbrial genes (type 1 and type 3) of K. pneumoniae strains in community-acquired infections obtained from various clinical sources on their ability to produce biofilm. Methods: A total of 256 samples (106 male and 150 females) were taken from various clinical sources including Urine, wound swabs, sputum, burn swabs, bronchial wash, diabetic foot ulcer, and higher vaginal swabs) from outpatients in Kirkuk City hospitals. The isolates were identified on the Blood agar, MacConkey agar and Eosin Methylene Blue agar based on the cultural, morphological, in addition to biochemical assays, and confirmed using the automated VITEK 2 system. The Microtiter plate (MTP) method was employed to detect Biofilm formation. Subsequently, a polymerase chain reaction was performed to determine the presence of the T1 fim and mrkD genes. Result: Out of 256 only 38 (14.8%) isolates belonged to Klebsiella pneumoniae. Of all isolates, 38(100%) were biofilm producers. The PCR results revealed that all 38 isolates showed positive (100%) for T1 fim and 37 isolates (97.4%) for mrkD.

1. Introduction

A gram-negative, non-motile Klebsiella pneumoniae is an encapsulated, rod-shaped, and facultative anaerobic member of the family Enterobacteriaceae [1]. This bacterium is one of the most opportunistic microorganisms, which is frequently related to infections acquired in the community and healthcare settings. Additionally, it has been found to play a significant role in developing healthcare-related infections [2,3]. K. pneumoniae is a clinically significant species within the Klebsiella genus, accounting for approximately 86% of human infections. This makes it the most prominent pathogen within this genus [4]. This bacterium is the causative agent for various infections in human beings including urinary tract infections(UTIs), pneumonia, wound infections, pyogenic liver abscesses, burn infections, bacteremia, and meningitis [5,6,7]. Infections caused by K. pneumoniae primarily affect vulnerable populations such as newborns, the elderly, and persons with weakened immune systems. However, it is also responsible for a growing prevalence of infections acquired within the community [8]. Klebsiella species, found in various environments; can establish itself in the nasopharynx and gastrointestinal system, acting as an opportunistic pathogen in humans. Gastrointestinal colonization is probably a prevalent and significant reservoir regarding the risk of transmission and infection among body sites [9]. The pathogenicity of K. pneumonia principally comes from an array of virulence characteristics, which allow it to bypass the host's innate immune system and develop a persistent infection in the mammalian host [8]. These virulence factors including (fimbrial adhesions, capsule, lipopolysaccharides, biofilm formation, siderophores, hemolysis, gelatinase, protease, haemagglutination and hypermucoviscosity) contribute to its ability to survive in various environmental settings and hence facilitate the establishment of infection in the human body. Each of them can cause a diverse range of diseases in both hospitalized patients and individuals in the community [10,11,12].

K. pneumoniae exhibits significant virulence through biofilm formation, a polymerization process in which bacteria adhere to inert or active surfaces via extracellular polymeric substances [13]. The biofilms formation on the inner surfaces of catheters and other implanted devices are of medical

significance, which can, also result in the colonization of the respiratory, urinary, and gastrointestinal tract, which in turn contributes to the occurrence of invasive diseases, especially in individuals with weakened immune systems [14]. This capability of K.. pneumoniae enables the protection of strains against the immune response of the hosts' and results in drug resistance. The development of biofilms is influenced by a variety of genes, such as mrk (which codes for type 3 fimbriae) and T1 fim (which codes for type 1 fimbrial adhesion) [15]. The T1 fim gene is responsible for bacterial adherence. Approximately 90% of K. pneumoniae express Type 1 fimbriae, they serve a key function in adhering to various types of epithelial cells, particularly those found in the bladder [16]. The gene MrkD acts as an adhesin and is located at the fimbrial tip. Studies have revealed that Type 3 fimbriae increases K. pneumoniae adherence to the extracellular matrix.

Furthermore, they have the ability to adhere to human endothelium and bladder cells, as well as enhance the production of biofilms on both living and non-living surfaces [17]. These virulence genes, either individually or in combination, contribute to different extents to the initiation, invasion, spread, severity, and outcome of K. pneumoniae infection [16]. Therefore, the current study aimed to evaluate the occurrence of some fimbrial genes (namely type 1 and 3) in community-acquired strains of K. pneumoniae obtained from different clinical samples and identify their ability to produce biofilm.

2. Methodology

Study design and study period

This cross-sectional study was carried out in Kirkuk City, focusing on four major hospitals: Kirkuk General Hospital, Azadi Teaching Hospital, AL-Naser Hospital and General Pediatrics Hospital, and was conducted between September 2023 and December 2023.

Study subjects and collection of clinical samples

The study included 256 outpatients from four main Hospitals. Those individuals had community-acquired infections (CAIS). Thirty-eight (38) clinical isolates of *K. pneumoniae* from 256 samples were taken from different body sites (urine, wound, sputum, burn, bronchial wash, diabetic foot ulcer, and vagina) of 256 outpatients. The patients were within the age range of less than 1-80 years for both genders (21 male and 17 female). The samples collected from outpatients were classified as infected based on the clinical manifestations in each patient. Urine and sputum samples were collected using a clean, sterile, well-labeled, and leak-proof container, without any apparent signs of contamination; other clinical samples were obtained using sterile transport swabs and then transferred to the Microbiology laboratory for additional processing.

Identification of bacterial isolates

All clinical samples were cultured on the Blood agar, Nutrient agar, and MacConkey agar (Scharlau, Barcelona, Spain), with incubation at 37◦C for 24 hours. Large, lactose fermenting mucoid colonies were then subcultured on Eosin methylene blue agar (Neogen, USA) for differentiation between *K. pneumoniae* and *E.coli.* The isolated colony was Gram-stained and biochemically characterized using oxidase, catalase, urease, indole, motility, kligler iron agar, methyl red, citrate utilization, and Vogues-Proskauer. All 38 isolates of *K. pneumoniae* were confirmed using the Vitek 2 system (BioMeriux, France) following the manufacturer's recommendations. All isolates were stored in Brain heart infusion broth in a frozen state (Scharlau, Barcelona, Spain) supplemented with 20% glycerol at a temperature of -20◦C for further investigation [15].

Detection of the biofilm production

The ability of *K. pneumoniae* isolates to produce biofilm was evaluated using the microtiter plate method (MTP). Thirty-eight *K. pneumoniae* isolates were inoculated in trypticase soy broth (TSB) supplemented with (1% glucose), incubated for 18-24 hours at 37 ˚C, and then diluted 1:100 in a new TSB medium. Every strain was tested three times. Three wells, one in each well of a 96-well flat-bottomed microplate, were applied as a negative control (containing only 200 µl of sterile TSB).

Following 48 hours of incubation at a temperature of 37◦C, the plate was gently washed three times with phosphate buffer saline (PH=7.2) to remove planktonic cells. Subsequently200 µl of (0.1%) crystal violet was applied to all wells. Following incubation for 15 minutes at room temperature, then supernatant was discarded, and consequently, the MTP was rinsed three times with distilled water. After that, MTP was inverted for several hours to facilitate drying. The biofilm-bound dye was re-solubilized by adding 200 µl of a 95% ethanol solution and covering microtiter plate with the lid and kept at room temperature for 30 minutes. The optical densities (OD) of the stained films that adhered to the surface were quantified using an ELISA reader (BioTek, USA) at a wavelength of 630 nm[18]. Results were evaluated as in Stepanović et al., 2007[18].

Molecular method for detection of *T1 fim* and *mrkD* genes of *K. pneumoniae* isolates

DNA extraction: Using FavorPrep™ Blood Genomic DNA Extraction Mini Kit (Favorgen FABGK100, Taiwan) for the extraction of *K. pneumoniae* genomic DNA (chromosomal and plasmid) from all isolates from overnight cultures. Each kit comes with enough supplies to perform 100 genomic DNA isolations. The Quantus fluorometer was employed to quantify the extracted DNA concentration, which was then used to assess the quality of the samples for further applications. A mixture was prepared by combining 1 µl of DNA with 200 µl of diluted Quantifluor dye. Following incubation for 5 minutes at room temperature, the concentration of DNA was determined and ranged from 2.1 to 84 ng/µl. Then DNA solution was kept at -20◦C until perform PCR assay.

Primers for Genes Amplification: The researcher designed the primer dedicated to *T1 fim* and *mrkD* genes via NCBI web primer-designing tools, as listed in Table (1). To reconstitute the primers, a stock solution was made (Macrogen DNA Technologies, Korea) by adding 250 µl of ddH2O to each vial of lyophilized primers. This yielded a stock solution of 100 pmol, which was then diluted to 20 pmol/l and kept at (-20 °C)

Table 1. Specific primers for type 1 and type 3 fimbriae

Primer Name	Primer sequence (5′ → 3′)	Product Size (bp)
T1 fim-F	GCTGGTCGATGAACGCCTGG	458bp
T1 fim-R	GATGAACTGGAAGGAGTCGC	
mrkD-F	CTGGGAACCACCTCGTCCTG	442bp
mrkD-R	CGTAGGAGGTGTACTTACCC	

To acquire the best PCR results, the temperature for the amplification technique was tuned. This was done by running the PCR on a gradient run until distinct bands were formed.

PCR amplification and Agarose gel electrophoresis

The Polymerase Chain Reaction was conducted with a Thermal Cycler to determine some virulence genes: fimbriae (*T1fim*, *mrkD*). The PCR reaction mixtures were prepared by combining 12.5 µl of GoTaq® G2 Green Master Mix 2X (Promega, USA), 5 µl of DNA template, 1 µl each of forward and reverse primers, and nuclease-free water to complete the volume of 25 µl. The thermal cycler was programmed under the optimum conditions as listed in Table (2). Note: The annealing temperature for the *mrkD* gene was set at 61°C, while the annealing temperature for the *T1 fim* gene was set at 56°C.

Table 2. The optimal condition to detect *T1 fim* and *mrkD* genes

Amplification steps	Temperature (◦C)	Time(sec)	Number of cycles
Initial Denaturation	95	300	1
Denaturation	95	30	35
Annealing	61,56	30	

Extension	72	45	
Final extension	72	300	1
Holding	4	300	1

PCR product was running on the 1.5% agarose gels containing ethidium bromide stain and electric current was allowed at 80 volts for 45 minutes. DNA bands were observed using a UV-Transilluminator system and photographed with a camera. The PCR product size was determined using a 100bp DNA ladder from Promega, USA.

Statistical analysis: The present study's data results were statistically analyzed using the Minitab program. Chi-square (X2) and probability (p-value) are utilized for data comparison. The probability level at (p-value) of 0.05 or less is referred to as statistically significant, a (p-value) of 0.01 or less is referred to as highly significant, whereas (p-value) greater than 0.05 is referred to as non-significant.

3. Results and Dicussion

Study samples: A total of 256 different clinical samples; 106 male samples and 150 females were collected at four main hospitals in Kirkuk City. All samples were cultured, of which 108 (42.2%) exhibited no bacterial growth and 148 (57%) exhibited bacterial growth. Among the total samples, *K. pneumoniae* accounted for 38 (14.8%) of them and was the most prevalent among other bacterial growth, which is highly significant (p value= 0.0009) (Table 3).

Table 3. Culture results of clinical samples

Bacterial growth		**No. bacterial growth**	**Total**	**P value**
k. pneumoniae	Other bacteria	108 (42.2%)	256 (100%)	Chi-Square = 49.109 P-Value = 0.0009
38 (14.8%)	110 (43%)			
** represent p-value ≤ 0.01 highly significant; Number (No.)				

Isolation and Identification of *K. pneumonia*

Among the 38 isolates of *K. pneumoniae*, 21 (55.3%) were detected in males and 17 (44.7%) were found in females. The prevalence of infection with *K. pneumoniae* was highest in urine samples, followed by wound swabs, sputum, burn swabs, bronchial wash, diabetic foot ulcer swabs, and higher vaginal swabs (47.4%, 21.1%, 13.2%, 10.5%, 2.6%, 2.6%, and 2.6%, respectively). The isolation sources and the frequency of *K. pneumoniae* infections were significantly associated as listed in [Table 4].

Table 4. Isolation sources and percentage of *K. pneumoniae*

Isolation Sources	**No. of samples**	**No. of *K. pneumoniae* Isolates**	**% of *K. pneumoniae***
Urine	118	18	47.4%
Wound swab	53	8	21.1%
Sputum	26	5	13.2%
Burn swab	39	4	10.5%
Bronchial wash	2	1	2.6%
Diabetic foot ulcer swab	13	1	2.6%
Higher vaginal swab	5	1	2.6%
Total number	256	38	100%
Chi-Square = 28.509, P-Value = 0.0008**			
** represent p-value ≤ 0.01 highly significant;% (percentage)			

The *K. pneumoniae* colonies exhibited a prominent, viscous, and pink appearance on MacConkey agar due to lactose fermentation. Conversely, they exhibited white, large, and mucoid colonies on blood agar without any destruction of red blood cells. Eosin methylene blue agar is used frequently for distinguishing between *Klebsiella* species and *E. coli* bacteria [Figure 1]. Thus, *Klebsiella* colonies exhibit a pink to purple color, while colonies of *E. coli* are dark and surrounded by a green metallic shine.

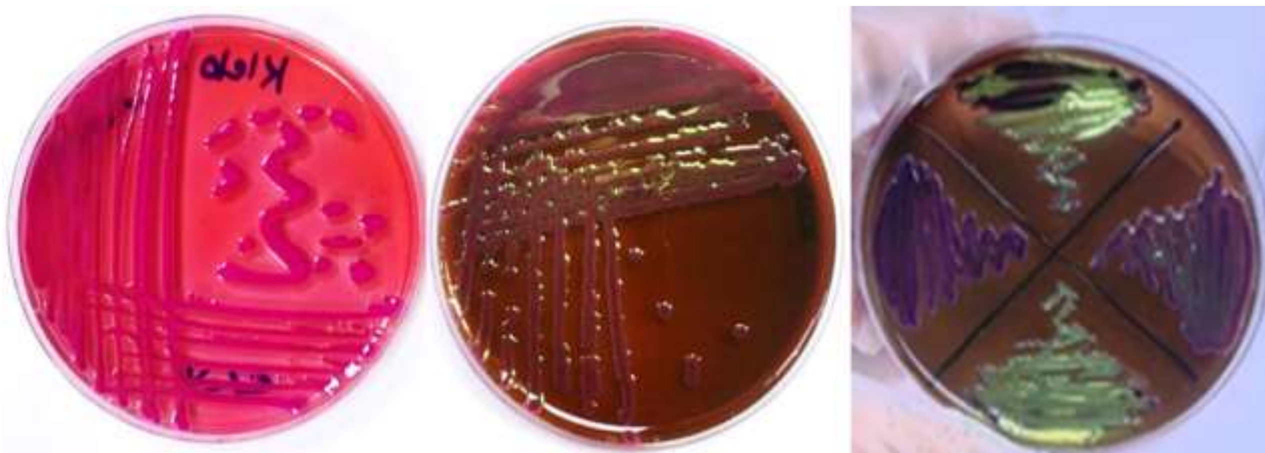

Figure 1. K. pneumoniae colonies on A: MacConkey agar, B: Eosin Methylene Blue agar, C: Metallic shine of E. coli colonies and pink to purple color of K. pneumoniae on Eosin Methylene Blue agar.

Quantification of Biofilm Formation

K. pneumoniae have the ability to form biofilms, which is a complex process including adhesion to various surfaces such as plastics, metals, medical implant materials, and tissues. Biofilm development augments the viability of microorganisms, such as bacteria, and strengthens them against damage [19]. The microtiter plate method [MTP] assessed the biofilm production capability of *K. pneumonia* isolates, the results showed that all isolates produced 25 (65.8%), and 13 (34.2%) weak and moderate biofilms, weak biofilm is the most predominant and statistically high significance. There are no strong biofilms. The results obtained for biofilm production are listed in [Table 5].

Table 5. Detection of biofilm-producing *K. pneumoniae*

Biofilm-forming ability	NO.	%	Mean±SD
Strongly adherent	0	0	0
Moderately adherent	13	34.2	0.1196 ± 0.198
Weakly adherent	25	65.8	0.0666 ± 0.0105
Non-adherent	0	0	0
Total	38	100	
Chi square = 60.772, P-value = 0.00003 **, SD= Standard deviation, ** represent p-value ≤ 0.01 highly significant			

Molecular method for detection of *T1fim* and *MrkD* genes of *K. pneumoniae* isolates

PCR technique was used for amplification of genes for all *K. pneumonia* isolates to detect biofilm production-related genes: *T1 Fim* and *mrkD*. Results showed that all 38 isolates (100%) were positive for *T1 Fim* and 32 isolates (97.4%) to *mrkD* [Figure 2]

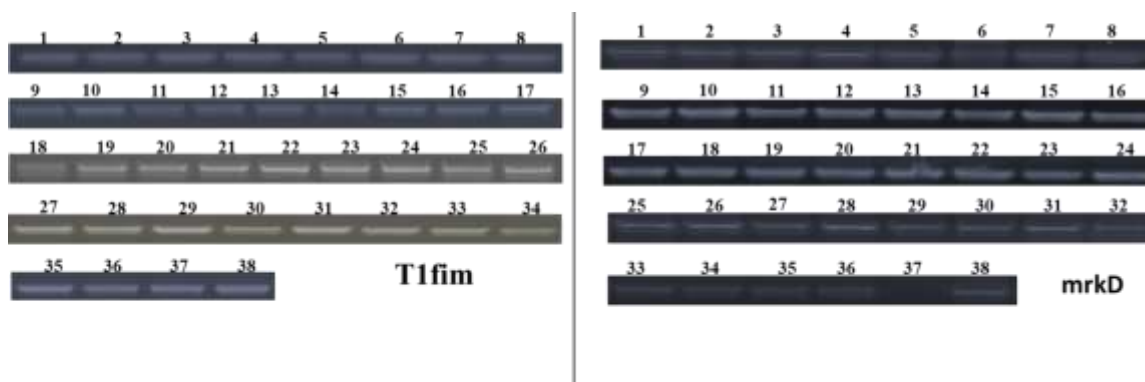

Figure 2. The T1fim *and mrkD* gene amplification results of *K. pneumoniae* fractionated on 1.5% agarose gel electrophoresis stained with ethidium bromide. Ladder marker: 100bp. Lanes 1-38 resemble 458 bp (T1fim) 442 bp (mrkD) PCR product size.

Discussion

This study included all Klebsiella pneumoniae isolates obtained from infections acquired in the community within the specified study period. The prevalence of K..pneumoniae among positive bacterial growth was 14.8%, which was similar to the results of [20] in which the distribution of K.. pneumoniae in clinical samples was 12.57% furthermore our results agree with [21] who mentioned that community-acquired infection accounted for 12% (15/125) of K.. pneumoniae clinical samples.

The infected patients with K.. pneumoniae in this study involved 21 males (55.3%) and 17 females (44.7%); this result agreed with the study made by [22] who also stated that the distribution of K. pneumoniae was in the frequency of 53.23 % in males and 46.77% in females. Another study done by [23] showed that K. pnemoniae infection prevalence was (53.6%) in males and 46.4% in females. In contrast, studies performed by [24, 25] revealed that females were more infected than males.

The present study has shown that K. Pneumoniae infection was higher in urine samples followed by wound swabs, sputum, burn swabs, bronchial wash, diabetic foot ulcer swab, and high vaginal swabs (47.4%, 21.1%, 13.2%, 10.5%, 2.6%, 2.6%, and 2.6%, respectively).

Furthermore, our finding was similar to Garza-Ramos U et al. [25] study, which collected samples from patients who had infections acquired in the community and reported that K. pneumoniae isolates were taken from these sources: urine, 46.1%; vaginal secretion, 25.6%; sputum, 10.2%. In addition, the local study done in Diyala [26] in which most isolates were more prevalent in urine (32.31%; n=42) followed by sputum (20.5%; n=17), wounds (19.44%; n=7), burns (13.33%; n=2) respectively, also in line the results of our study.

The dissemination of K.pneumoniae and its pathogenic significance is primarily associated with urina ry and respiratory tract infections;this is likely due to these bacteria being part of the normal intestinal flora, making them opportunistic pathogens with the capability for adherence to the surface s of epithelial cells [26].

Regarding biofilm formation, the current study revealed that all isolates (100%) were able to form a biofilm of which 25 (60.5%) were weak and 13 (34.2%) were moderately biofilm-producing. These results were similar to the results of the study conducted by Mohammed A [27] in Iraq, as well as with a study conducted by Ali et al. [28] in Iraq, which revealed that (100% of the isolates were capable of forming biofilm 40% weakly biofilm former, 44% moderately and 16% strongly biofilm former). However, our study differs from [27, 28] in that we did not observe any strong biofilm-forming isolates. The observed disparity in the biofilm formation demonstration between the present findings and the earlier research could be attributed to variations in gene percentage levels, isolates' sites, and study settings.

The genes T1 fim and mrkD were present in almost all of our isolates. This finding is consistent with the numerous studies that have reported about the ubiquity of these fimbrial genes in K.. pneumoniae [29]. In the present study, the T1 fim gene was detected in all isolates (100%) while the mrkD gene was present in (97.4%) of isolates.

Additionally, our results of fimbrial genes were in agree with a previous study conducted by Iman S et al [30] were found that the T1 fim genes among different clinical samples were detected in (100%) of isolates and mrkD in (98%), also in line with another study done by Sara H et al [31]. In contrast, a local study in Baghdad done by Eman A et al [32] reported lower detection of T1 fim and mrkD genes in (90%) and (51.6%) of isolates respectively

4. Conclusion and future scope

In the current study, biofilm production was detected by microtiter plate revealing that all community-acquired K.. pneumoniae isolates were 100% biofilm former, and molecular detection of some fimbrial genes was associated with the biofilm formation. To provide information on the distribution of these organisms in Iraq, large-scale studies involving the characterization of the community-acquired K. pneumoniae isolates from various regions of the country are required.

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