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Original Article

Investigation of Biofilm Virulence Genes Prevalence in *Klebsiella pneumoniae* Isolated from the Urinary Tract Infections

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Abstract

Klebsiella pneumonia is a pathogen and an agent that causes hospital-acquired infections. *Klebsiella pneumonia* is the first and most common causative agent in community-acquired infections and urinary tract diseases. This study aimed to detect common genes, (i.e., *fimA*, *mrkA*, and *mrkD*) in the isolates of *K. pneumoniae*, isolated from urine specimens using the polymerase chain reaction (PCR) method. The isolates of *K. pneumoniae* were collected from urine specimens in health centers in Wasit Governorate, Iraq, and diagnosed using Analytical Profile Index 20Eand 16S rRNA techniques. The microtiter plate (MTP) method was used to detect biofilm formation. A total of 56 isolates were identified as *K. pneumonia* cases. The results led to the detection of biofilms; accordingly, all *K. pneumoniae* isolates showed biofilm production by MTP, however, at different levels. The PCR method was employed to detect biofilm genes and showed that 49 (87.5%), 26 (46.4%), and 30 (53.6%) of isolates carried *fimH*, *mrkA*, and *mrkD*, respectively. Furthermore, susceptibility tests for different antibiotics revealed that *K. pneumoniae* isolates were resistant to amoxicillin-clavulanic acid (n=11, 19.5%), ceftazidime (n=13, 22.4%), ofloxacin (n=16, 28.1%), and tobramycin (n=27, 48.4%). It was also found all *K. pneumonia* isolates were sensitive to polymyxin B (92.6%), imipenem (88.3%), meropenem (79.4%), and amikacin (60.5%).

Keywords: Biofilm, *Klebsiella pneumoniae*, Urinary tract infections, Virulence factors

1. Introduction

Urinary tract infections (UTIs) are considered important human diseases. These infections are the causes of about more than 20-25% of death cases worldwide (1). As Kumar and Das (2) have mentioned, according to their UTIs survey report, 15% of adults and 50% of women are affected by these infections at least once in their lives. Furthermore, the high consumption of antibiotics and incorrect prescription of several antibiotics have been found to be the main causes of antibiotic resistance and the high rate of UTIs prevalence in human beings (3). *Klebsiella pneumoniae* is also considered an infectious microorganism because of causing severe infections, being antibiotic-resistant, having epidemiological risk factors, and having a role in virulence factors (4). In addition, Paczosa and Mecsas (5) demonstrated that *K. pneumoniae* has recently been the second prevalent cause of Gram-negative bacteremia and the main pathogen in nosocomial infections, especially in immunocompromised patients. Moreover, Ronald (6) and Singhai, Malik (7) reported that the risks of *K. Pneumonia* infections highly increased in the presence of the medical indwelling devices, including respiratory support tools, different catheters used in the neonatal suites, and UT catheters used for a long period during illness. By the way, the bacterium relative to *K.*

pneumoniae infections has the capability of biofilm formation and is resistant to treatment (8).

Clegg and Murphy (9) stated that a diversity of virulence factors in *K. pneumoniae*, such as particularly lipopolysaccharide (LPS), fimbriae, anti-phagocytic capsule (CPS), siderophores, and membrane transporters, allow surviving of *K. pneumoniae*. The capability of *K. pneumonia* in biofilm production protects it from the host immune system responses and antibiotics (10) and promotes its insistence on the epithelial tissues and external surfaces of indwelling devices. Some of the detected virulence in *K. pneumonia*, related to the biofilm genes, are also included in biofilm production. Indeed, several genes, namely CPS cluster genes (11), type 3 fimbriae genes (*mrk*) (12), *wbbM*, and *wzm* genes, which are pertinent to 6 genes, create LPS (11, 13). All of these genes participate in biofilm production along with *K. pneumonia*, as well as type two quorum sensing regulatory system and *pgaABCD* operon that participate in synthesis and translocation poly-beta-1,6-N-acetyl-D-glucosamine (PGA) adhesion (14, 15).

In recent years, nearly all the developed countries have reported some diseases caused by multidrug-resistant *Enterobacteriaceae*, such as *K. pneumoniae* bacteria (16, 17). In addition, multidrug-resistant and extensively drug-resistant *K. pneumoniae* bacteria displayed resistance to β-lactams, such as penicillin, third and fourth-generation carbapenems, cephalosporins, and monobactam, because of plasmid, encoded β-lactamases, and carbapenemases. Moreover, *K. pneumoniae* bacteria displayed resistance to other classes of different antibiotics, such as fluoroquinolones, sulphonamides, and amino-glycosides (18).

Therefore, this study aimed to detect common genes, including *fimA*, *mrkA*, and *mrkD*, in isolates of *K. pneumoniae*, isolated from the urine specimens using the PCR method.

2. Materials and Methods

2.1. Specimens Collection and Culture

A total of 130 clinical samples were collected from different infection wards as swabs from the participants hospitalized in two health centers, namely Al-Karameh and Al-Zahra teaching hospitals in Al-Kut City, Wasit Governorate, Iraq. All of these specimens were cultivated on numerous media, including traditional and rich ones. At first, bacteria were cultured on the blood agar and then on the selective media, such as MacConkey, and eosin methylene blue agars were used to detect Gram-negative and *K. pneumoniae* isolates. Cultures of bacteria were incubated at 37°C for 18-20 h. Conventional and molecular methods were used to diagnose the isolates of different bacteria. Furthermore, numerous lab techniques, such as Analytical Profile Index (API) and molecular methods (e.g., PCR), were employed to detect isolates. Additionally, Mueller Hinton agar was used to detect antibiotic sensitivity against different isolates of *K. pneumoniae*.

2.2. Extraction of *Klebsiella pneumoniae* DNA and Molecular Assay

The DNA of *K. pneumonia* isolates was extracted by Geneaid Genomic DNA Extraction Kit (USA) according to the manufacturer's guidelines. *Klebsiella pneumoniae* specimens were centrifuged, and then pellets were re-suspended in a 0.2 ml buffer for 10 min. A total of 0.2 ml from the GD buffer was tested for 10 min. After that, 0.2 ml absolute ethanol was added to the lysate. A 2-ml tube was used, and collection and centrifuge were conducted using GD columns. A buffer of W1 was added to the column GD and centrifuged. At the next step, wash buffer and elution was added and left for 3 min to ensure that pure DNA was obtained. Several genes responsible for biofilm formation were recognized using the PCR method.

Primer of 16S rRNA was used to detect *K. pneumonia* isolates, and *mrkA*, *mrkD*, and *fimH* were synthesized using Eurofins MWG Operon (MWG, Germany) (Table 1).

The concentration and quality of DNA specimens were estimated by Nanodrop. All DNA specimens with minimum purity were discarded, and extractions were repeated.

Table 1. Primers tested for *Klebsiella pneumoniae* in the present study and selected 16S rRNA gene primers

Gene		Primer sequence	Product size (bp)	Accession No.
fimH	F	CGATCACTGACTACGTCACC	143	MH818548.1
	R	CCGTGAATCGTAAACCACC	143	
mrkD	F	GCCTTAATGCTGATGCCATTAC	180	LN558592.1
	R	AACCACTGACACTGACTCCC	180	
mrkA	F	CACCAAACAGGATGATGTGAG	262	M55912.1
	R	CGCATAGCCGACGTAGTAAG	262	
16S rRNA	F	GTATCTAAACCAGTTCGCACC	145	LN558592.1
	R	TGCATATCTGCTGTTGCATC	145	

2.3. Antibiotics Susceptibility against *Klebsiella pneumoniae*

The antibiotic susceptibility was examined using the disc diffusion method for *K. pneumoniae* isolates. Different antibiotics were used, including amoxicillin/Clavulanic acid (10 µg), meropenem (10 µg), polymyxin B (200 U), ceftazidime (30 µg), ofloxacin (5 µg), tobramycin (10 µg), and imipenem amikacin (30 µg).

2.4. Statistical Analysis

The collected data were analyzed in Statistical Package for the Social Sciences (SPSS). The significance level for all tests was considered at < 0.05.

3. Results

3.1. Isolation and Identification of *Klebsiella pneumoniae*

A total of 56 *K. pneumoniae* were obtained from the urine samples. These isolates were identified according to the conventional and molecular techniques, such as culture and microscopic examination, biochemical tests, API 20E kit, and PCR. Afterward, all the results were confirmed using the molecular method (PCR). All isolates of bacteria had the same results using the API 20E kit. Moreover, several tests were conducted to confirm the characterization of *K. pneumoniae* by 16S rRNA after analysis by API 20E kit and Vitek2 system. The DNA for all isolates of *K. pneumoniae* was extracted and subjected to PCR. The final results confirmed that all isolates were recognized as *K. pneumoniae.*

3.2. Estimation of Biofilm Production in Isolates of *Klebsiella pneumoniae*

Biofilm formation in bacterial cells is complex with an adhesion capability to different surfaces, including plastics, metals, medical implant materials, and tissues. Biofilm formation enhances the survival of microorganisms, such as bacteria, and strengthens them against damage (22). A total of 56 isolates of *K. pneumoniae* were evaluated by the microtiter plate (MTP) method. According to the MTP method, the biofilm productions were detected, and the results demonstrated that all *K. pneumoniae* isolates produced 10 (18%), 14 (25%), and 32 (57%) weak, moderate, and strong biofilms, respectively (Figure 1).

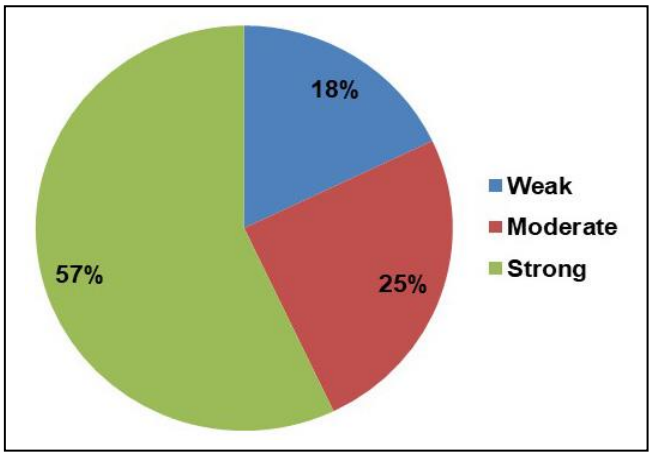

Figure 1. Different results by the MTP method to detect biofilm production in *K. pneumoniae*

3.3. Identification of *Klebsiella pneumoniae* and Biofilm Production Genes Using PCR

Various *K. pneumoniae* isolates were identified by 16S rRNA as PCR-positive, as depicted in figure 2.

The PCR method was used to detect the existence of different genes forming a biofilm, such as *fimH*, *mrkA*, and *mrkD*, in *K. pneumoniae* isolates. The *fimH* gene was detected in 49 (87.5%) isolates out of all isolates of *K. pneumoniae* as PCR-positive (Figure 3).

The *mrkA* and *mrkD* genes were detected in 26 (46.4%) and 30 (53.6%) isolates as PCR-positive, respectively, as shown in figures 4 and 5, respectively.

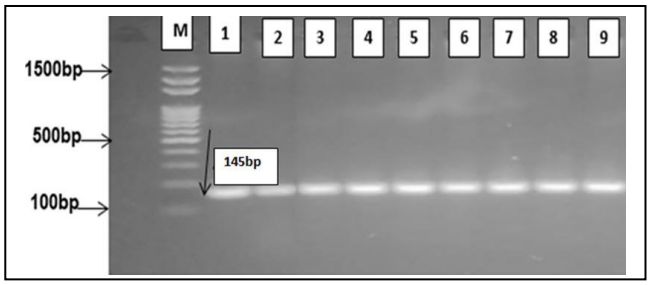

Figure 2. Agarose gel electrophoresis and genomic DNA isolated from *K. pneumoniae* observed in PCR product analysis for16S rRNA gene in *K pneumoniae*
M: Marker (1,00-1,500 bp). All lines (1-9) were PCR-positive at a PCR product size of 145 bp

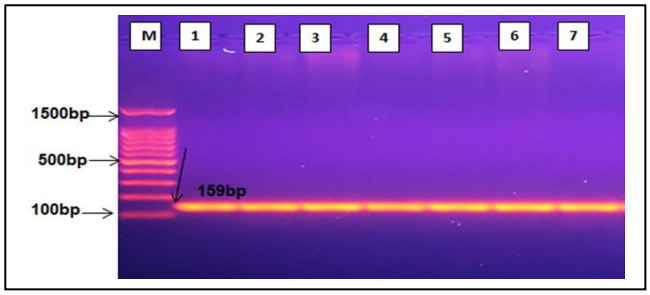

Figure 4. Agarose gel electrophoresis and genomic DNA isolated from *K. pneumoniae* observed in PCR product analysis for the *mrkD* gene in *K. pneumoniae*
M: Marker (1,00-1,500 bp). All lines (1-7) were PCR-positive at a PCR product size of 159 bp

3.4. Antibiotics Susceptibility against *Klebsiella pneumoniae* Isolates

Table 2 presents the antibiotic susceptibility and resistance *in vitro* compared to different antibiotics against *K. pneumoniae* isolates (Table 2). All isolates of *K. pneumonia* appeared to be resistant to amoxicillin-clavulanic acid (19.5%), ceftazidime (22.4%), ofloxacin (28.1%), and tobramycin (48.4%). Furthermore, all *K. pneumonia* isolates were sensitive to polymyxin B (92.6%), imipenem (88.3%), meropenem (79.4%), and amikacin (60.5%) (Table 2).

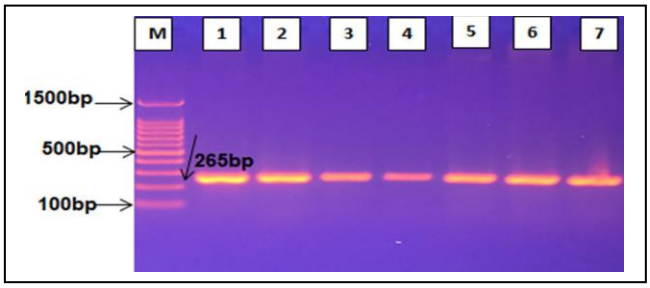

Figure 3. Agarose gel electrophoresis and genomic DNA isolated from *K. pneumoniae* observed in PCR product analysis for the *mrkA* gene in *K. pneumoniae*
M: Marker (1,00-1,500 bp). All lines (1-8) were PCR-positive at a PCR product size of 265 bp

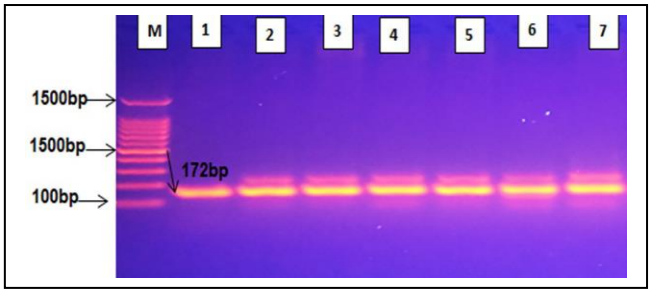

Figure 5. Agarose gel electrophoresis and genomic DNA isolated from *K. pneumoniae* observed in PCR product analysis for the *wbbm* gene in *K. pneumoniae*
M: Marker (1,00-1,500 bp). All lines (1-10) were PCR-positive at a PCR product size of 172 bp

Table 2. Antibiotic sensitivity in different isolates of *Klebsiella pneumoniae*

Antibiotics dosage (µg)	Sensitivity (%)	
	n	(%)
Amoxicillin-clavulanic acid	11	19.5
Meropenem	44	79.4
Polymyxin B	52	92.6
Ceftazidime	13	22.4
Amikacin	34	60.5
Tobramycin	27	48.8
Imipenem	49	88.3
Ofloxacin	16	28.1

At a significance level of < 0.01

4. Discussion

Biofilm productions were important to determine the pathogenicity of *K. pneumoniae* infections (19). The biofilm production ability in *K. pneumoniae* isolates had a significant association with the outcome of the respective illness. As biofilm and virulence factors were present in different microorganisms, biofilm was recognized in interaction with several communities of MO and mostly covered in an extracellular matrix, such as extracellular polymeric substances (a protein that promotes adherence to numerous surfaces) (20). Fimbria was an important part of the UTIs caused by *K. pneumoniae* (21). The biofilm formation encouraged the survival of MO and made its breakdown complex (22). According to the results of a study by Seifi, Kazemian (23), biofilm formation was considered an important factor to assess the pathogenesis and pathogenicity in *K. pneumoniae* infections. Furthermore, biofilm formation made microbial infections chronic and reduced antibiotic effectiveness. In another study conducted by Karimi, Zarei (24), the findings revealed that the ability of biofilm formation was found in 62 (75%) isolates of different clinical isolates of chronic illnesses among the UTI patients.

In the present study, the predominant growth of biofilm formation was found, and strong biofilm was observed in 32 (57%) of *K. pneumoniae* isolates. Regarding the biofilm analysis, a study was conducted in Iran by Karimi, Zarei (24), who observed that 27 (32.5%), 18 (21.6%), and 17 (20.4%) *K. pneumoniae* isolates formed biofilms weakly, moderately, and strongly. Moreover, another related study was conducted in Iran by Seifi, Kazemian (23), the results of which revealed that 93.6% of *K. pneumoniae* isolates showed the ability of biofilm formation and 33% of them could produce biofilm strongly. This discrepancy in the demonstration of biofilm formation between current results and the previous studies may be due to different proportion levels of genes, the variation in the sites of isolates, and the conditions of the study. The virulence factors of biofilm production in *K. pneumonia* infections have been reported as important agents of pathogenesis and pathogenicity determination (25). Biofilms are complex communities of microbes with the ability to adhere to different surfaces, such as plastics, metals, and indwelling devices (20). In the present study, biofilm formation (capsule and fimbria, *fimH*, *mrkA*, and *mrkD*) had an important role in the persistence of infections.

The findings of another previous study, performed by Alcantar-Curiel, Blackburn (12), showed that the *fimH* group gene was found in 100% of isolates; however, just 57% of *K. pneumonia* isolates contained *mrkA*. The different prevalence of biofilm formation in the present study compared to previous studies may be related to such factors as various proportions of genes or plasmid and different conditions of the study.

As for the antibiotic sensitivity test, the results of the current study showed that all *K. pneumonia* isolates were sensitive to polymyxin B (92.6%), imipenem (88.3%), meropenem (79.4%), and amikacin (60.5%). Nevertheless, there were numerous antibiotics that were resistant to amoxicillin-clavulanic acid (19.5%), ceftazidime (22.4%), ofloxacin (28.1%), and tobramycin (48.4%). The resistance might be attributed to gene cassettes, plasmids, and transposons. In Addition, the resistance might be due to high antibiotic consumption leading to antibiotic resistance in different pathogenic bacteria (26). Furthermore, Giacomini, Perrone (27) stated that the misuse of antibiotics could take place by professionals of health care and/or non-skilled practitioners as well as by the general public due to inadequate surveillance or the lack of information regarding routine antimicrobial susceptibility and resistivity tests, such as rotating reports that are issued from developing countries.

The results of the molecular technique using PCR revealed that the *fimH*, *mrkA*, and *mrkD* genes were associated with biofilm formation in the different isolates of *K. pneumonia*. In the current study, antibiotics were observed as significant results against different *K. pneumoniae* isolates that formed biofilm.

Furthermore, the MTP method was used to detect biofilm production, and it was revealed that isolates producing biofilm strongly were high in number, compared to other isolates. The results of sensitivity and resistivity tests of antibiotics demonstrated that the most active compounds against *K. pneumonia* were polymyxin B, imipenem, and meropenem in descending order.

Authors' Contribution

Study concept and design: S. R. A.
Acquisition of data: J. H. M.
Analysis and interpretation of data: J. H. M.
Drafting of the manuscript: B. H. O.
Critical revision of the manuscript for important intellectual content: J. H. M., S. R. A. and B. H. O.
Statistical analysis: B. H. O.
Administrative, technical, and material support: J. H. M. and S. R. A.

Ethics

The study was approved by the Wasit University, Wasit, Iraq Review Board.

Conflict of Interest

The authors declare that they have no conflict of interest.

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