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Original article

Virulence determinants associated with biofilm formation by *Klebsiella pneumoniae* causing hospital-acquired bloodstream infection

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ABSTRACT

Background: *Klebsiella pneumoniae* (*K. pneumoniae)* significantly contributes to hospital-acquired bloodstream infections with high morbidity and mortality, especially biofilm-producing strains. It has been noticed that ability to produce biofilm by *K. pneumoniae* is linked to presence of some virulence genes, but this relationship needs further investigation. This work aimed to investigate ten virulence genes that may contribute to biofilm formation in *K. pneumoniae* strains causing bloodstream infections. **Methods:** This cross-sectional study included 108 *K. pneumoniae* isolates obtained from cases of hospital-acquired bloodstream Infections. The sensitivity to different antibiotics was tested by the disc diffusion method. Ability to form biofilm was detected by the tissue culture plate method. All isolates were tested by polymerase chain reaction (PCR) to detect ten virulence genes suggested to be linked to biofilm formation ability. **Results:** The ability of biofilm formation was detected in 55.6% of the studied strains. Biofilm formation was more prevalent among *wcaG*, *fimH*, *wabG*, and *mrkD* positive isolates in comparison to negative isolates for the same genes. However, only *wcaG* and *fimH* genes have been found to be significantly associated with biofilm formation ($P < 0.05$). **Conclusion:** The association between the ability to form biofilm and the existence of *wcaG* and *fimH* genes in *K. pneumoniae* bacteremia isolates suggests these genes as promising therapeutic targets.

Introduction

Klebsiella pneumonia (*K. pneumoniae)* is a Gram-negative rod that attracts worldwide attention due to marked increase in the rate of opportunistic and severe infections caused by the organism, as well as the growing antibiotic resistance with marked limitation in effective treatment options [1].

Klebsiella pneumoniae significantly contributes to hospital-acquired bloodstream infections with a significant mortality rate (27–37%), especially in cases caused by multi-drug resistant (MDR) strains [2].

Infections caused by biofilm-producing *K. pneumoniae* are more resistant to treatment than others. Biofilms act as shields for bacterial communities giving them more ability to escape the host defense mechanisms. Moreover, they prevent the access of different antibiotics giving the bacteria more resistance power. In addition to protecting the bacterial from harsh conditions such as altered pH, shear forces and deficient nutrients. Therefore,

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biofilms cause emergence of challenging bugs infections [3].

Previous studies noticed that ability of *K. pneumoniae* strains to produce biofilm is associated with presence of some virulence factors [4,5], but the authors recommended further evaluation of this relationship, especially in different geographic regions.

This study investigated *K. pneumoniae* strains causing hospital-acquired bloodstream infections isolated from a tertiary hospital in Egypt aiming to detect the genetically encoded virulence factors that may be linked to biofilm formation.

Patients and Methods

This cross-sectional study was carried out over 18 months (March 2019 - September 2020) in Tropical Medicine Department and Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, Egypt. This study included 430 patients admitted to the intensive care unit (ICU) of the Tropical Medicine Department., Hospital-acquired bloodstream infection was suspected by developing at least one of the following signs or symptoms: fever (>38ºC), chills, or hypotension after at least 48 hours of hospitalization without evidence that it was already in the incubation phase at the time of admission, according to definitions of Centers for Disease Control and Prevention (CDC) [6].

This study has been approved by the Institutional Review Board (IRB) – Faculty of Medicine, Zagazig University (IRB number: 5797). It was conducted according to the revised Declaration of Helsinki. Informed consents were obtained from all study participants or the relatives of the unconscious ones.

Bacterial isolates

A total of 430 blood specimens were collected under complete aseptic conditions only from those who had not received antibiotics, yet. Blood cultures were done followed by subcultures using blood and MacConkey agar plates every 48 hours.

Identification of suspected colonies by Gram stained smears and classical biochemical tests, including Indole, Methyl red, Voges-Proskauer, and Citrate utilization (IMViC) tests, and growth on Triple Sugar Iron agar (TSI), followed by API 20E systems (Biomérieux, France).

Antimicrobial susceptibility testing

Susceptibility to different antimicrobials was tested by the disc diffusion method (Modified Kirby-Bauer) using Muller Hinton agar according to guidelines of Clinical and Laboratory Standards Institute (2019) [7]. The following commercial antimicrobial discs were used; piperacillin (10 µg), amoxicillin–clavulanic acid (20, 10 µg); gentamicin (10 µg), amikacin (30 µg), imipenem (10 µg), meropenem (10 µg), cephalosporins: cefoperazone (75 µg), ceftriaxone (30 µg), cefipeme (30 µg) and ceftazdime (30 µg), fluoroquinolone: levofloxacin (5µg), (Oxoid, Basingstoke, UK). As quality control strains, *Pseudomonas aeruginosa* ATCC® 27853™ and *Escherichia coli* ATCC® 25922™ were used (American Type Culture Collection Global Bioresource Center, USA).

Multidrug-resistant (MDR) *K. pneumoniae* showed resistance to one or more antibiotics in at least three groups [7].

Assessing biofilm formation

The capability of *K. pneumoniae* isolates to form biofilm was assessed by the tissue culture plate method [8]. Briefly, overnight cultures of the tested strains were adjusted in trypticase soy broth to 0.5 McFarland. Two hundred µl of the formerly prepared suspensions were added to the wells of a polystyrene plate (Costar, New York, USA). Each strain was tested in triplicate. Three wells -in each plate- were utilized as a negative control (contained 200 µl of TSB only). After 24h of incubation at 37◦C, staining with crystal violet (0.1%) was done for all wells. After 15 min incubation, the supernatant was removed, followed by rinsing of the plate by phosphate-buffered saline. With 95% ethanol, the biofilm-bound dye was then eluted. The optical densities (OD) of the stained adherent films were determined with microplate reader (BioTek ELX800, USA) at 630 nm. Results were interpreted according to **Stepanovic´ et al.** [9].

Genotypic detection of virulence determinants

All *K. pneumoniae* isolates were investigated by three different multiplex polymerase chain reactions (PCRs) to detect 10 virulence genes; *iutA* (Aerobactin siderophore system), *mrkD* (the type 3 fimbrial adhesion), *allS* (associated with allantoin metabolism), *k2A* (specific to K2 capsule serotype), *rmpA* (the regulator of mucoid phenotype A), *wabG* (capsule associated gene), *cnf-1*(other virulence factors that enable to overcome host defense), *wcaG* (associated with capsular fucose

production), *magA* (mucoviscosity-associated gene A), and *fimH* (type 1 fimbriae).

Preparation of DNA templates was carried out by suspending two or three bacterial colonies in PCR-quality water followed by boiling for 10 minutes. Centrifugation was done and the supernatant was collected for PCRs [10].

Analysis of the virulence genes was carried out using three multiplex PCRs, the first for *iutA*, *allS*, *mrkD,* and *k2A;* the second for *wabG*, *rmpA*, *cnf-1* and *wcaG;* while the third for *magA* and *fimH*. Primers are listed in **table (1)**.

Each of the first and second multiplex PCRs were carried out in a 50 ul volume. The final reaction mixture contained 5 ul of DNA template, PCR buffer with 20 pmol of each of the primers, and 2.5U of Taq polymerase.

The third multiplex PCR was carried out in a 25 ul volume. The final reaction mixture contained 2.5 ul of DNA template, PCR buffer with 20 pmol of each primer, and 2.5U of Taq polymerase.

Thermal cycling conditions of the first multiplex PCR included 15 min at 95°C, followed by 30 amplification cycles over 30 sec at 94°C, 90 sec at 60°C, and 60 sec at 72°C. Finally, 10 min at 72ºC for the final extension [11].

The second and third reactions were performed under conditions of pre-denaturation at 95°C for five min followed by 30 cycles: one minute at 94°C, one minute at 58°C, one minute at 72°C and 10 minutes of final extension 72°C [12]. The produced amplicons were analyzed using 1.5% agarose gel by electrophoresis **(Figure 1)**.

Workflow of the study is illustrated in **(Figure 2)**.

Table 1. The primer sets used for the detection of virulence genes.

Virulence Genes	**Primer sequence (5'-3')**	**Product size (bp)**	**Reference**
magA	F:GGTGCTCTTTACATCATTGC	1282	**10**
	R:GCAATGGCCATTTGCGTTAG		
k2A	F:CAACCATGGTGGTCGATTAG	531	**9**
	R:TGGTAGCCATATCCCTTTGG		
rmpA	F:ACTGGGCTACCTCTGCTTCA	516	**10**
	R:CTTGCATGAGCCATCTTTCA		
wabG	F:ACCATCGGCCATTTGATAGA	683	**10**
	R:CGGACTGGCAGATCCATATC		
allS	F:CATTACGCACCTTTGTCAGC	764	**9**
	R:GAATGTGTCGGCGATCAGCTT		
fimH	F:TGCTGCTGGGCTGGTCGATG	550	**10**
	R:GGGAGGGTGACGGTGACATC		
mrkD	F:AAGCTATCGCTGTACTTCCGGCA	340	**9**
	R:GGCGTTGGCGCTCAGATAGG		
*wcaG**	F:GGTTGGKTCAGCAATCGTA	169	**10**
	R:ACTATTCCGCCAACTTTTGC		
iutA	F:GGGAAAGGCTTCTCTGCCAT	920	**9**
	R:TTATTCGCCACCACGCTCTT		
cnf-1	F:AAGATGGAGTTTCCTATGCAGGAG	498	**10**
	R:CATTCAGAGTCCTGCCCTCATTATT		

* K= G or T

Figure 1. Multiplex PCR for amplification of virulence genes. lane M: DNA marker. Strains positive for amplified genes show DNA bands; *mrkD* gene (340 bp) and *iutA* gene (920 bp amplicon) in Figure 1a, *wabG* gene (683 bp) and *wcaG* gene (169 bp) in Figure 1b, and , *fimH* gene (550 bp) in Figure 1c

Figure 1a

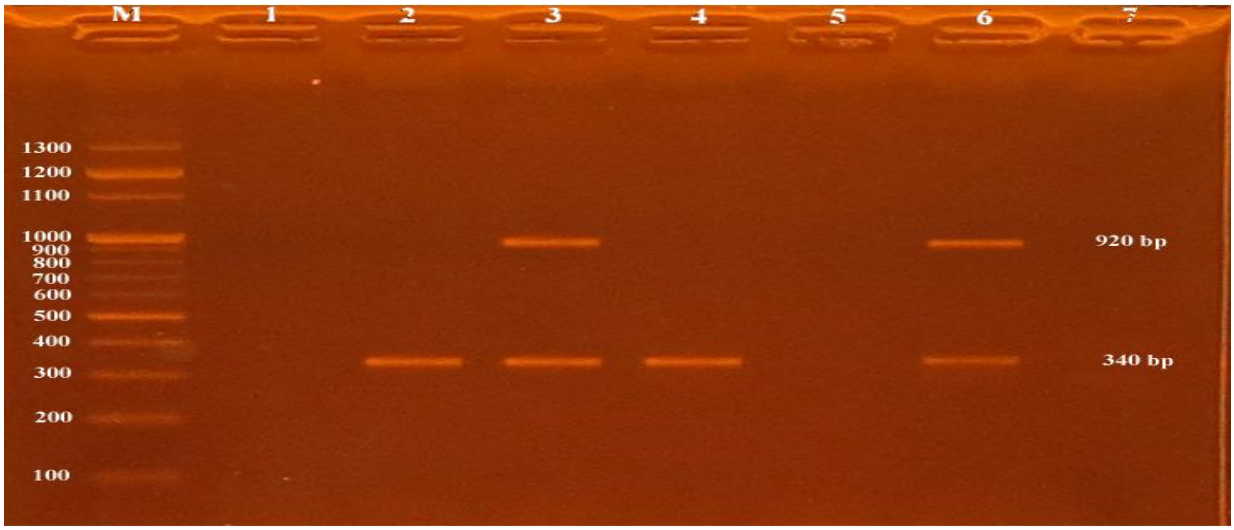


Figure 1b

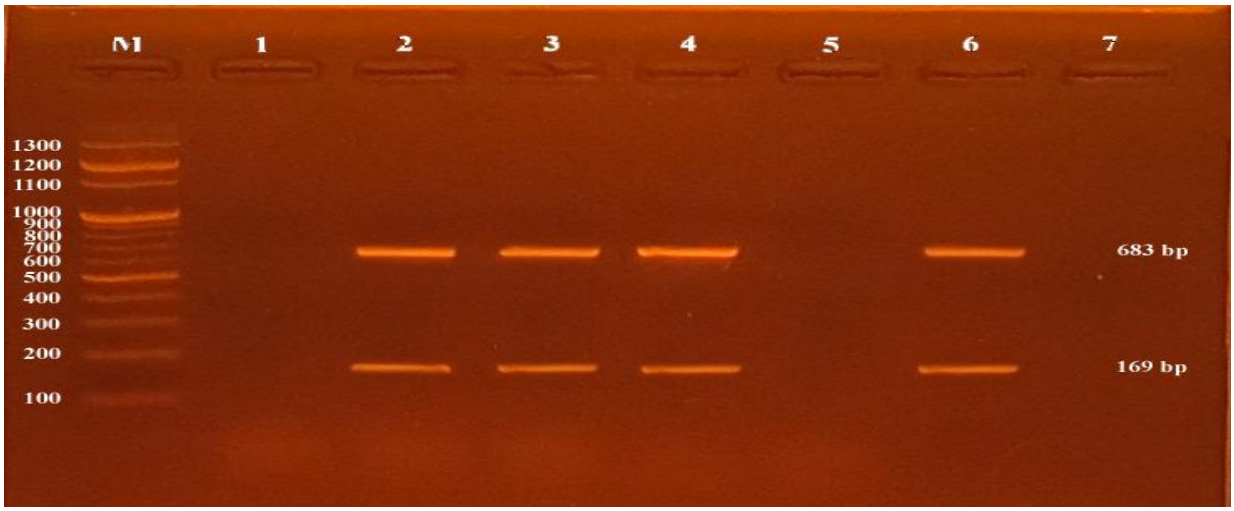


Figure 1c

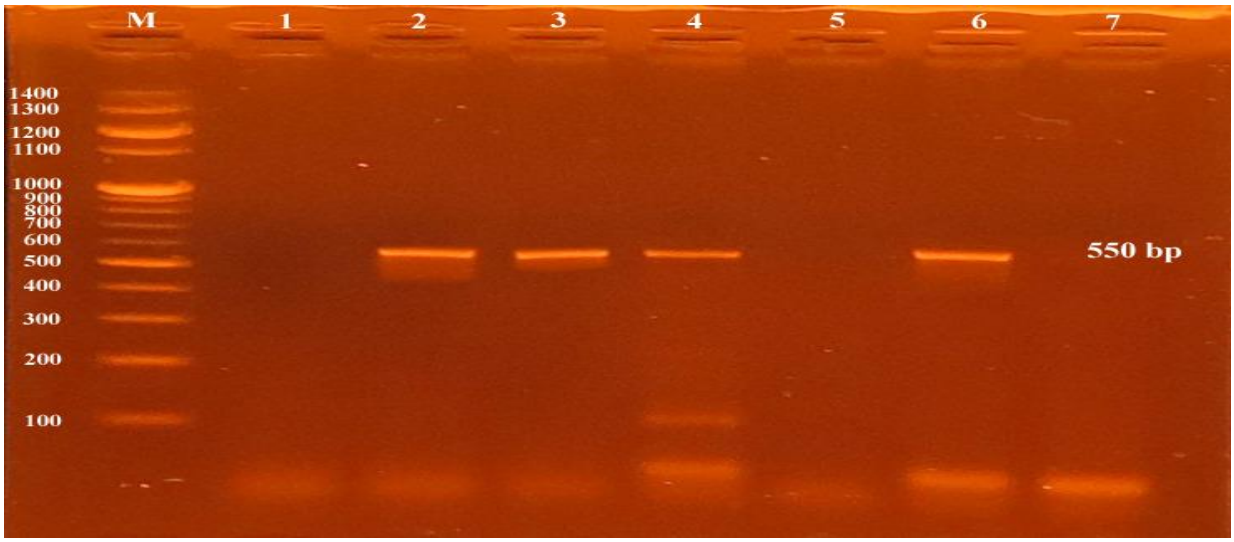
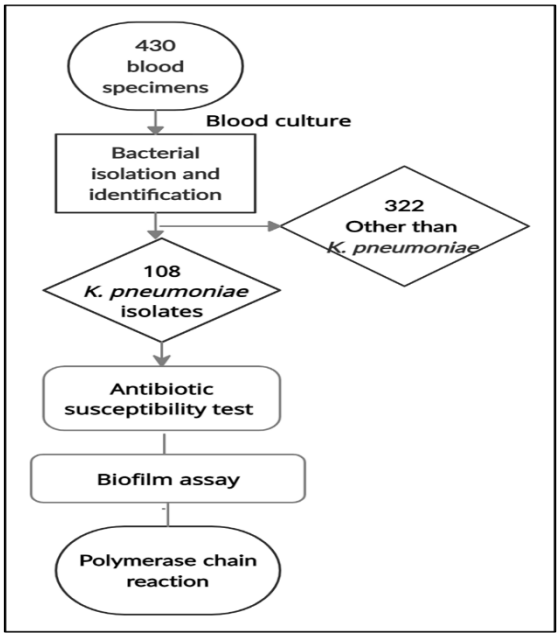


Figure 2. Workflow of the current study.



Statistical analysis

All results were analyzed using IBM SPSS 23.0 (SPSS Inc., Chicago, USA). Categorical data have been presented as frequency and percentage, while continuous data have been presented as mean ± standard deviation (SD).

Proportions between two qualitative parameters were compared using Chi-square (X2) test of significance. While Fisher Exact test was used instead of chi-square test in 2 by 2 tables. Significance was indicated by probability (*P*-value) ≤ 0.05.

Results

In this study, 60/108 (55.6%) of the studied *K. pneumoniae* isolates showed the ability of biofilm formation. No significant difference was detected between biofilm-forming and non-forming strains regarding in vitro antibiotic susceptibility **(Table 2)**.

Analysis of virulence genes showed that biofilm formation was more prevalent in *wcaG* (86.7%), *fimH* (86.7%), *wabG* (80%), and *mrkD* (80%) positive isolates with a statistically significant difference from isolates that were negative for these virulence genes **(Table 3)**. No significant difference was detected among positive and negative isolates for *IutA* gene. However, no isolate was detected containing *magA*, *allS*, *rmpA*, *cnf-1*, or *k2A* genes.

To establish the role of each virulence factor to biofilm formation, the four independent variables *wcaG*, *fimH*, *wabG*, and *mrkD* were submitted to multiple regression analysis. Only *wcaG* and *fimH* were detected as independent risk factors for biofilm formation by *K. pneumoniae* isolates ($P<0.05$), while the other factors were found to be non-significant, as shown in **table (4)**.

Table 2. Results of *in vitro* antibiotic susceptibility test of *K. pneumoniae* isolates.

	Biofilm forming isolates (N=60)		Non biofilm forming isolates (N=48)		X^2	*P* -value
	N	%	N	%		
Piperacillin: R	60	100	48	100	-----	------
Amoxicillin–clavulanic: R	60	100	48	100	----	------
Gentamycin: R	9	15	8	16.7	5.88	*0.529*
I	11	18.3	12	25		
S	40	66.7	14	58.3		
Amikacin: R	20	33.3	12	25.0	0.89	*0.721*
I	40	66.7	36	75.0		
Imipenem: R	56	93.3	48	100	Fisher	*0.159*
S	4	6.7	0	0.0		
Meropenem: R	52	86.7	44	91.7		*0.41*
I	8	13.3	4	8.3		
Cefoperazone: R	60	100	48	100	------	------
Ceftriaxone: R	60	100	48	100	-------	-----
Cefipeme: R	60	100	48	100	-----	------
Ceftazidime: R	60	100	48	100	-------	------
Levofloxacin: S	40	66.7	28	58.3	0.79	*0.373*
I	20	33.3	20	41.7		

R: resistant, S: sensitive, I: intermediate sensitivity

Table 3 . Association between biofilm formation and virulence genes of *K. pneumoniae* isolates.

		Biofilm forming isolates (N=60)		Non biofilm forming isolates (N=48)		X^2	*P* -value
		N	%	N	%		
magA	**-ve**	60	100	48	100	---------	------
WcaG	**-ve**	8	13.3	24	50	17.19	*<0.001*
	+ve	52	86.7	24	50		HS
allS	**-ve**	60	100	48	100	----	-----
rmpA	**-ve**	60	100	48	100	-----	-----
cnf-1	**-ve**	28	46.7	24	50.0	0.119	*0.73* NS
WabG	**+ve**	48	80	16	33.3	24.1	***<0.001***
	-ve	12	20	32	66.7		**HS**
FimH	**+ve**	52	86.7	20	41.7	24.3	***<0.001***
	-ve	8	13.3	28	58.3		**HS**
mrkD	**+ve**	48	80	12	25	32.7	***<0.001***
	-ve	12	20	36	75		**HS**
IutA	**-ve**	18	30	8	16.7	2.59	*0.105*
	+ve	42	70	40	83.3		NS
k2A	**-ve**	60	100	48	100	-----	------

+ve: Positive -ve: Negative HS: Highly significant NS: Non -significant

Table 4. Multiple logistic regression analysis of virulence genes associated with *k. pneumoniae* biofilm formation.

Virulence factors	coefficient	SE	Wald statistics	*p* value	OR	95% CI for OR	
						Lower	Upper
WcaG	0.38	2.04	0.456	***0.040****	0.252	0.115	13.7
WabG	0.742	1.76	0.178	*0.673*	2.1	0.067	66.3
fimH	0.42	2.08	0.460	***0.044****	0.257	0.118	14.0
mrkD	2.31	1.46	2.52	*0.112*	10.1	0.581	175.4

SE: Standard error, OR: Odds ratio, CI: Confidence interval, *: Significant

Discussion

Biofilm production by bacterial pathogens has gained special attention being an important virulence factor that causes difficult elimination of infection. Previous studies suggested association between presence of some virulence genes in some bacteria and ability for biofilm formation [4,5]. Therefore, the current study investigated this association in one of the most threatening hospital-acquired pathogens in Egypt; *K. pneumoniae* focusing on bloodstream infection being serious with increased morbidity and mortality.

Regarding *in vitro* antibiotic susceptibility test results, we observed no significant difference between biofilm-forming and non-forming *K. pneumoniae* isolates.

Although this finding is against the expected, it agreed with a previous study that found no significant difference in *in vitro* antibiotic sensitivity. However, the authors reported persistent bacteremia after treatment for 72 hours in case of biofilms producing strains [5]. This affirms difficult elimination of the bacteria in biofilms and reflects the role of biofilm matrix in giving the bacteria additional protection by preventing access of antibiotics to bacterial biofilm communities.

On the other hand, and in contrast to our finding, one study reported that 85.0% of biofilm-

producing *K. pneumoniae* strains from sputum and urine had shown less *in vitro* sensitivity to antibiotics [13]. Another study found 73% and 83 % resistance rates to cefotaxime and ampicillin among biofilm-producing *K. pneumoniae* strains isolated from urine specimens, in comparison to 35% and 60% resistance, respectively, among non-biofilm producing strains [14]. This suggests that the relationship between biofilm formation and *in vitro* susceptibility testing needs further investigation.

In the current study, no significant difference was observed for the used panel of antibiotics. A larger panel is required for better investigation of this point. In addition, determining the MIC (minimal inhibitory concentration) for these antibiotics using broth microdilution method may detect significant difference being more accurate than the disc diffusion method.

Currently, the role of virulence factors in biofilm formation by *K. pneumoniae* is still not clear. The current study found that *wcaG*, *fimH*, *wabG*, and *mrkD* positive isolates showed more biofilm-formation than isolates negative for these genes. However, only *wcaG* and *fimH* were confirmed to be independent risk factors for biofilm formation.

In a previous study, the authors reported that biofilm production was more prevalent in *magA*, *wcaG*, *iutA*, *rmpA*, and *allS* positive isolates than others negative for the same genes. While they have reported that *wcaG* only played an important role in *K. pneumoniae* biofilm formation based on multiple logistic analysis [5].

The protein encoded by *wcaG* was proved to be involved in the synthesis of fucose [15], that is an essential part of the polysaccharide capsule of *K.pneumoniae* that was found to be significantly correlated with bacterial virulence in mice [16].

The exact mechanism that enhances *K. pneumoniae* biofilm formation by *wcaG* has not been detected, yet. One study has reported that deletion mutations of *wcaG* affected most of the capsule polysaccharides [15], indicating that *wcaG* may enhance *K. pneumoniae* biofilm production by modifying the capsular polysaccharide.

On the other hand, the protein encoded by *fimH*, is responsible for the binding to D-mannose. It was confirmed to regulate the structure and function of type 1 fimbriae. Some previous studies reported insignificant association of *fimH* gene with biofilm formation [5]. Other studies reported only weak association [17].

In this study, we did not detect any isolate that was positive for *magA*, *allS*, *rmpA*, *cnf-1*, and *K2A* genes. Similar findings were reported in a previous study [11] that was carried out on 50 isolates of *K. pneumonia* among which no isolate was detected to be positive for the *allS*, *magA*, *cnf-1*or *k2A* genes. The *magA*, *k2A*, and *allS* genes are greatly involved in liver abscess pathogenesis [18, 19]. There were no liver or abscesses specimens included in that study that caused the absence of these genes.

Similar findings were reported in other studies [20-22].

Conclusion

In conclusion, this study reports that many *K.pneumoniae* bacteremia strains are biofilm-producers. Among the ten studied virulence genes, only *wcaG* and *fimH* have been found to be significantly associated with biofilm formation.

Limitation

This study is entirely descriptive. There is an association between the presence of some genes and biofilm formation, but this association does not confirm causation. To demonstrate that a gene causes a phenotype, silencing of that gene must lead to loss of the phenotype. Therefore, trials to explore the effect of silencing *wcaG* and *fimH* genes in biofilm-forming isolates are recommended. Additionally, we recommend further studies on larger scales that include a larger number of isolates and more virulence factors to be investigated.

Conflict of interest

All authors declare no conflict of interest in this work.

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