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ORIGINAL ARTICLE

MrkD Gene as a Regulator of Biofilm Formation with Correlation to Antibiotic Resistance among Clinical *Klebsiella pneumoniae* Isolates from Menoufia University Hospitals

Asmaa M. Elbrolosy, Naira A. Eissa, Nahed A. Al-Rajhy, Esraa El-Sayed A. El-Mahdy*, Rasha G. Mostafa

Department of Medical Microbiology and Immunology, Faculty of Medicine, Menoufia University, Egypt

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****Corresponding Author:***
Esraa El-Sayed Abdel-Maksoud El-Mahdy
Department of Microbiology & Immunology, Faculty of Medicine, Menoufia University, Egypt.
Tel: 00201095604989
dr_esraa_elmahdy87@yahoo.com

ABSTRACT

Background: *Klebsiella pneumoniae (K. pneumoniae) is a common pathogen involved in a diverse array of life-threatening infections. Increasing frequent acquisition of antibiotic resistance by K. pneumoniae has given rise to multidrug-resistant pathogen mostly at the hospital level.* ***Objectives****: To assess the prevalence and antibiotic resistance pattern of the clinical K. pneumoniae isolates at Menoufia University Hospitals (MUHs) as well as to explore the role of mrkD gene as a regulator of biofilm formation.* ***Methodology****: A total of 340 different clinical samples were obtained from 270 patients who were admitted to MUHs and those from Outpatient clinics during the period from April 2018 to September 2019. 84 K. pneumoniae isolates were identified by the standard microbiological methods and vitek-2 system. The antimicrobial resistance pattern was determined by disk diffusion method. The biofilm-forming ability of all K. pneumoniae isolates was demonstrated phenotypically by the modified Congo red agar method (MCRA) and PCR assay verified the presence of mrkD gene as a genetic determinant of biofilm formation.* ***Results:*** *Klebsiella spp. represented 34.7% of the collected isolates and the predominant spp. was K. pneumoniae (91.3%). The highest resistance rates were for ceftriaxone (69%) followed by aztreonam (67.9%), 66.7% for each of piperacillin and ceftazidime, while the least resistance rate was for fosfomycin (8.3%). Biofilm production was detected among 83.3% of the isolates by MCRA method. A highly significant statistical difference was noted between biofilm- and non- biofilm - producing K. pneumoniae isolates regarding resistance to cefepieme and amikacin (P <0.001) and similarly regarding resistance to aztreonam, imipenem, meropenem, ertapenem and tobramycin (P<0.05). Conventional PCR assay showed that, 92% of the isolates harbored mrkD gene with a highly significant association with biofilm formation.* ***Conclusion****: The increasing prevalence and remarkable ability to acquire antibiotic resistance among K. pneumoniae isolates together with biofilm formation should alert even more regarding the hazard of this pathogen in hospital settings.*

INTRODUCTION

Klebsiella pneumoniae is one of the most clinically relevant species in immunocompromised individuals responsible for serious infections e.g. pneumonias, urinary tract infections and bacteremias[1]. *K. pneumoniae* species are ubiquitous in nature and have two common habitats; one is the environment and the other is mammalian mucosal surfaces. In humans, *K. pneumoniae* can be present in the intestinal tract and nasopharynx[2].

K. pneumoniae is a member of the so-called 'ESKAPE' group that involves six of the most significant antimicrobial- resistant pathogens (*Enterococcus faecium, Staphylococcus aureus, K. pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.) responsible for nosocomial infections world wide[3].

The misuse of antibiotics has revealed a considerable increase in outbreaks caused by microorganisms resistant to antimicrobial drugs, *Klebsiella pneumoniae* is rapidly becoming known for its resistance properties to almost all clinically important antibiotics which are especially problematic in hospitals settings[4].

A critical step in progression to infection is for bacteria to adhere to host surfaces. Biofilm is formed of complex, sessile communities of microbes attached to a surface or buried firmly in an extracellular matrix as aggregates. The biofilm matrix makes the bacteria tolerant to harsh conditions and resistant to antibiotics[5], as they play a role in antibiotic trapping and impairment

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and plasmid exchange. Therefore, they can lead to persistent infections of many pathogenic microbes[6].

There are many genes that are involved in *K. pneumoniae* virulence including fimbrial and non-fimbrial adhesion genes such as *mrkD* gene. *MrkD* is believed to function as the type 3 fimbrial adhesin involved in *K. pneumoniae* biofilm. Biofilm formation is an important virulence factor that provides greater ability to cause of many chronic infections[7].

We aimed in this study to determine the prevalence and antibiotic resistance pattern of the clinical *K. pneumoniae* isolates at MUHs, to detect biofilm production phenotypically and genotypically and also to clarify the relation between biofilm formation and antimicrobial resistance.

METHODOLOGY

This study was conducted at the Medical Microbiology and Immunology Department, Faculty of Medicine, Menoufia University during the period from April 2018 to September 2019. Clinical samples were collected from 270 patients (1 month-74 years old) admitted to different departments of MUHs. The local ethics committee of Menoufia University approved the study protocol and informed consents were obtained from all participants.

- **Specimens collection and isolation of *K. pneumoniae*:**

A total of 340 clinical samples (95 blood, 64 sputum and 12 bronchial aspirate, 53 pus swabs, 37 drain samples, 32 ascetic fluid, 22 urine samples, 17 burn swabs and 8 CSF) were collected, processed, and cultured on different bacteriological media.

K. pneumoniae isolates were identified by standard methods and Vitek 2 system. The strains were preserved on tryptic soy broth with 16% glycerol and frozen at -80°C[8].

- **Antimicrobial susceptibility pattern:**

Antimicrobial susceptibility was performed for all *K. pneumoniae* isolates by the disk diffusion method on Muller Hinton agar plates (Oxoid, UK) against different antimicrobial agents (Oxoid, UK) and interpreted according to Clinical Laboratory Standard Institute (CLSI, 2019)[9] for the following antimicrobial agents: piperacillin (PRL, 100µg), piperacillin-tazobactam (TZP, 100/10µg), cefoxitin (FOX, 30µg), ceftriaxone (CRO, 30µg), ceftazidime (CAZ, 30µg), cefepime (FEP, 30µg), aztreonam (ATZ, 30µg), amikacin (AK, 30µg) , gentamicin (CN, 10µg), imipenem (IMP, 10µg), meropenem (MEM, 10 µg), ertapenem (ETP, 10µg), tobramycin (TOB, 10µg), ciprofloxacin (CIP, 5µg), levofloxacin (LEF, 5µg) and fosfomycin (Fos, 200µg).The isolates were subjected to:

Phenotypic detection of biofilm production by the modified Congo red agar method (MCRA): The medium is composed of Congo red dye 0.4 g/L, blood base agar-2 (BAB-2) 40 g/L, glucose 10g/L and 1000 ml water. Congo red stain was prepared as concentrated aqueous solution and autoclaved separately from other medium components then was added when the agar had been cooled to 55°C. Inoculated agar plates were incubated for 48 hrs at 37°C. Growth of black colored colonies was interpreted as positive biofilm producers. Meanwhile, red colonies were interpreted as negative biofilm producers[10].

Genotypic detection of *mrkD* gene by PCR:

DNA extraction: Bacterial DNA of 50 *K. pneumoniae* strains were extracted and purified using the gene JET™ genomic DNA purification kit (Thermo Fisher Scientific, UK). The used primers for *mrkD* gene were: F: AAGCTATCGCTGTACTTCCGGCA and R: GGCGTTGGCGCTCAGATAGG[7]. Amplification was performed in a thermal cycler (Applied biosystem, Singapore) and consisted of an initial denaturation at 95°C for 15 min, followed by 30 cycles [(DNA denaturation at 94°C for 30 sec), primer annealing at 60°C for 90 sec, primer extension at 72°C for 1 min and final extension at 72°C for 10 min]. Electrophoresis was performed with agarose gel 1.5% (Fermentas, Lithuania) stained with ethidium bromide (Sigma, USA) for 20 minutes. The amplified products were visualized by UV trans-illuminator and compared with a 100 bp DNA ladder (226 bp[7]) (figure-5).

Statistical analysis

Data were collected, tabulated and analyzed by statistical package for the social sciences (SPSS, version 20; SPSS Inc., Chicago, Illinois, USA) software. Chi-square test (χ^2) was done at 5% level of significance. Accuracy was represented using the terms sensitivity, specificity, positive predictive value, negative predictive value, and overall accuracy.

RESULTS

A total of 265 isolates were obtained from 340 collected clinical specimens. *Klebsiella* spp. represented the most frequent isolated organism (34.7%) followed by *E. coli* (29.4%), *Staph. aureus* (9.8%), *Pseudomonas* (8.3%), *Enterobacter* spp. (6.8%), *Acinetobacter* spp. (5.7%) and *Proteus* spp. (2.3%). *Candida* spp. and CoN staphylococci had the least isolation rate (1.5% for each) as shown in figure 1.

K. pneumoniae was the most predominant *Klebsiella* species identified by Vitek -2 system representing 91.3% (84/92) followed by *K.oxytoca* (6.5%, 6/92) and finally *K. ozaenae* (2.2%, 2/92) (figure- 2).

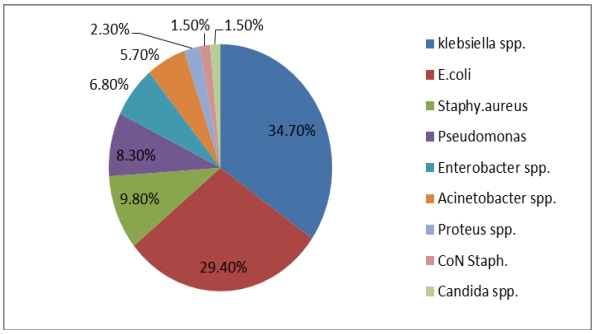

Fig. 1: Distribution of the isolated microorganisms among the studied patients

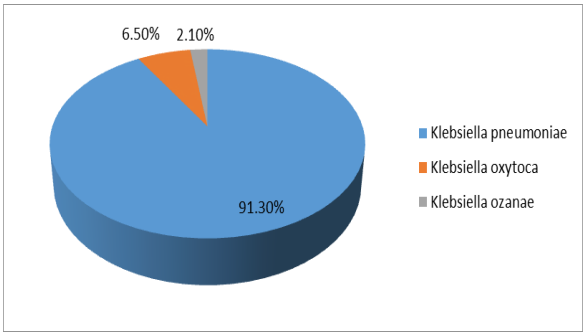

Fig. 2: Number and percentage of *Klebsiella* species identified by Vitek -2 system

Distribution of the isolated *K. pneumoniae* among different clinical samples was as follows: 29 (34.5%) from blood samples, 25 from respiratory secretions (29.8%), 9 from pus swabs (10.7%), 9 from ascetic fluid samples (10.7%), 6 from urine specimens (7.2%), 4 from surgical drain specimens (4.8%) and 2 isolates from CSF samples (2.4%) as shown in figure 3.

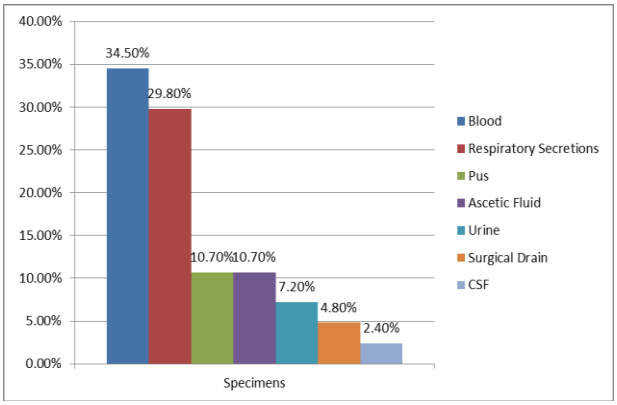

Fig. 3: Distribution of *K. pneumoniae* isolates according to the type of specimen

The antimicrobial resistance profile of *K. pneumoniae* isolates (Table1) showed that the highest resistance rates were for ceftriaxone (69%) followed by aztreonam (67.9%), 66.7% for each of piperacillin and ceftazidime, while the least resistance rate was for fosfomycin representing only 8.3%. 51.2%, 20.2% and 6% of the isolates were multidrug (MDR)-, extreme drug (XDR)- and pandrug-resistant (PDR) respectively.

Table 1: Antimicrobial resistance pattern of *K. pneumoniae* isolates by disk diffusion test

Antibiotics	Abbreviation-Disk content (µg)	*K. pneumoniae* isolates (n= 84)			
		Sensitive		Resistant	
		No.	%	No.	%
Piperacillin	Prp (100)	28	33.3	56	66.7
Piperacillin/ tazobactam	TPZ (100/10)	36	42.9	48	57.1
Cefoxitin	FOX (30)	37	44	47	56
Ceftriaxone	CRO (30)	26	31	58	69
Ceftazidime	CZC (30)	28	33.3	56	66.7
Cefepieme	FEP (30)	37	44	47	56
Aztreonam	ATM (30)	27	32.1	57	67.9
Imipenem	IPM (10)	39	46.4	45	53.6
Meropenem	MEM(10)	35	41.7	49	58.3
Ertapenem	ETP(10)	29	34.5	55	65.4
Amikacin	AK(30)	50	59.5	34	40.5
Gentamycin	CN(10)	35	41.7	49	58.3
Tobramycin	TOB(10)	42	50	42	50
Ciprofloxacin	CIP(5)	29	34.5	55	65.5
Levofloxacin	LEV(5)	32	38	52	62
Tigecycline	TGC(30)	58	69	26	31
Fosfomycin	FOS(200)	77	91.7	7	8.3
MDR		43 (51.2)			
XDR		17 (20.2)			
PDR		5 (6%)			

For biofilm production, 83.3% (70/84) of *K. pneumoniae* isolates were biofilm-producing strains while only 16.7% (14/84) were non-biofilm producers as shown in table 2 and figure 4.

Table 2: Biofilm production among *K. pneumoniae* isolates by the modified Congo red agar method

Biofilm production	*K. pneumoniae* isolates (n= 84)	
	No.	%
• Biofilm producer	70	83.3
• Non-biofilm producer	14	16.7

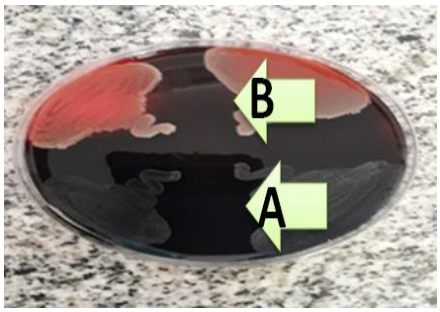

Fig. 4: Modified Congo Red agar plate (MCRA) to detect biofilm production
Letter A: Dry black crystalline colonies i.e. positive for biofilm formation.
Letter B: Red colored colonies i.e. negative for biofilm formation.

In this study, there was a highly significant statistical difference between biofilm-producing and non-biofilm-producing *K. pneumoniae* isolates (P value <0.001) regarding resistance to cefepieme and amikacin and a significant difference regarding resistance to aztreonam, imipenem, meropenem, ertapenem and tobramycin (P value <0.05) (table 3).

Table 3: Relation of antibiotic susceptibility to biofilm production among *K. pneumoniae* isolates

Antimicrobial agents	*K. pneumoniae* isolates (n=84)								X^2	p value
	Biofilm-producers (n=70)				Non biofilm-producers (n=14)					
	S		IS+R		S		IS+R			
	No	%	No	%	No	%	NO	%		
Piperacillin	26	37.1%	44	62.9%	2	14.3%	12	85.7%	2.74	>0.05
Piperacillin/tazobactam	33	47.1%	37	%	3	21.4%	11	78.6%	3.15	>0.05
Cefoxitin	33	47.1%	37	52.9%	4	28.6%	10	71.4%	1.63	>0.05
Ceftriaxone	22	31.4%	48	68.6%	4	28.6%	10	71.4%	0.04	>0.05
Ceftazidime	24	34.3%	46	65.7%	4	28.6%	10	71.4%	0.71	>0.05
Cefepime	35	50%	35	50%	2	14.3%	12	85.7%	6.75	**<0.001**
Aztreonam	22	31.4%	48	68.6%	5	35.7%	9	64.3%	0.09	**<0.05**
Ertapenem	21	30%	49	70%	8	57.1%	6	42.9%	3.80	**<0.05**
Imipenem	31	44.3%	39	55.7%	11	78.6%	3	21.4%	5.48	**<0.05**
Meropenem	24	34.3%	46	65.7%	11	78.6%	3	20%	9.41	**<0.05**
Amikacin	47	67.1%	23	32.9%	3	21.4%	11	78.6%	10.12	**<0.001**
Gentamycin	30	42.8%	40	57.1%	5	35.7%	9	64.3%	0.24	>0.05
Tobramycin	37	52.9%	33	47.1%	5	35.7%	9	64.3%	1.37	**<0.05**
Ciprofloxacin	24	43.3%	46	65.7%	5	35.7%	9	64.3%	0.01	>0.05
Levofloxacin	29	41.4%	41	58.6%	3	21.4%	11	78.6%	1.97	>0.05
Tigecycline	46	65.7%	24	34.3%	12	85.7%	2	14.3%	2.18	>0.05
Fosfomycin	64	91.4%	6	8.6 %	13	92.9%	1	7.1%	0.03	>0.05

Importantly, *mrkD* gene was detected in 92% (46 /50) of *K. pneumoniae* isolates by PCR assay with a positive association between *mrkD* gene and biofilm-production (P value <0.001). About 95.7% (45/47) of biofilm-producing *K. pneumoniae* isolates carried *mrkD* gene. On the other hand, 66.7% (2/3) of non-biofilm producing *K. pneumoniae* isolates were lacking *mrkD* gene (table 4 and figure 5).

Table 4: Correlation between number and percent of *mrkD* gene by conventional PCR in relation to biofilm production by MCRA method

***MrkD* gene by PCR (n=50)**	**Biofilm production by MCRA**				**χ2**	**P value**
	Positive (47)		**Negative (3)**			
	No.	**%**	**No.**	**%**		
Positive (n= 46)	**45**	**95.7**	**1**	**33.3**		
Negative (n=4)	**2**	**4.3**	**2**	**66.7**	**14.92**	**<0.001**

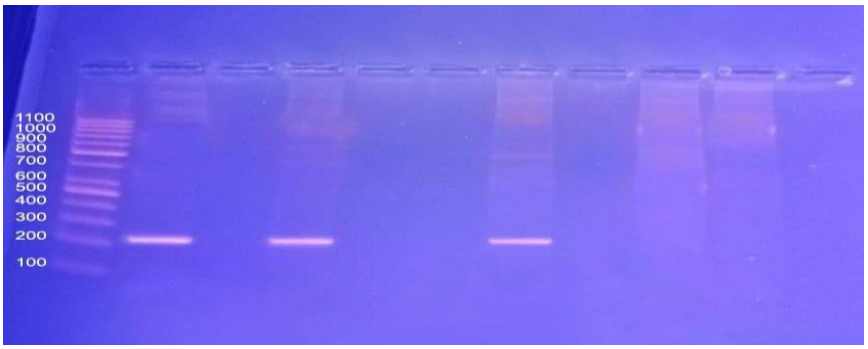

Figure 5: PCR assay for *mrkD* gene (226 bp)

DISCUSSION

Klebsiella pneumoniae is an important opportunistic pathogen that causes hospital- and community- acquired infections and contributes to significant morbidity and mortality. In the present work, *Klebsiella* spp. were the predominant isolates representing 34.7% of all the collected isolates, a result that matched with *El-Badawy et al.*[11] who found that *Klebsiella* spp. were the most frequent pathogens (38%) among the collected nosocomial isolates. While according to *Abo-State et al.*[12] *E. coli* was the most frequently isolated organism (41.89%) followed by *Klebsiella* spp. (27.2%), *Pseudomonas* spp. (18%). Also, *Kotb* and *Mowafy*[13] reported that *Enterobacteriaceae* spp. were the most frequent bacterial isolates with *E. coli* as the most prevalent (36%) followed by *Klebsiella* spp. (34.4%). However, *Staphylococcus aureus* was the predominant organism (53.3%) in another study [14].

The discrepancy in the isolation rates of the infectious agents can be attributed to geographical and epidemiological variations, regional differences in the hygienic status, criteria of the involved patients, sample size and duration of the study [11].

According to the current results, *K. pneumoniae* was the most predominant *Klebsiella* species representing 91.3% (84/92) followed by *K. oxytoca* (6/92; 6.5%) and finally *K. ozaenae* (2/92; 2.1%). Such findings came in accordance with *Biradar* and *Roopa*[15] and *Kishk et al.*[16] who found that *K. pneumoniae* was the most frequent spp. followed by *K. oxytoca*. Surveillance data in China and reports from other parts of the world also showed that *K. pneumoniae* has become the most common clinically isolated bacteria and the one of the most common bacterial pathogens that causes blood- stream infections[17].

As for distribution of the isolated *K. pneumoniae* among different clinical samples the present study revealed that, the highest rate of isolation of *K. pneumoniae* was from blood samples (34.5%) followed by respiratory secretions (29.8%) which coincided with *Li et al*[18] . Also, *Lin et al.*[19] recorded that blood was the most common source of *K. pneumoniae* (37.2%) followed by urine (30%) and others (32.8%). *Ahmed et al.* [20] reported *K. pneumoniae* as the most common organism isolated from blood culture specimens representing 34.4% of *Enterobacteriaceae* spp. On the other hand, *Wen-Liang et al.* [21] and *Yu et al.* [22] found that *K. pneumoniae* strains were mainly isolated from sputum (65.62% and 57% respectively) followed by blood (26.04% and 21.7% respectively).

The dramatic increase of antibiotic resistance in *K. pneumoniae* has become a significant and growing threat to public and environmental health[23]. Upon applying disk diffusion method for assessment of antibiotic susceptibility pattern, *K. pneumoniae* isolates

displayed higher levels of antibiotic resistance for most of the used antibiotics. These results agreed with that reported by previous studies [24,25.] The highest resistance rate was to ceftriaxone (69%). Similar result was obtained by *Ferreira et al.*[24] and Effah *et al*[4.]. Moreover, 67.9% of the current *K. pneumoniae* isolates were resistant to aztreonam and 66.7% were resistant for each of piperacillin and ceftazidime, a finding that came in parallel with *Li et al.* [26] and *Khalil et al.* [27]

The lowest resistance rates were for fosfomycin as only 8.3% (7/84) of *K. pneumoniae* isolates exhibited fosfomycin non- susceptibility. This coincided with *Vagras et al.* [28] who found that only 3% of the isolated *K. pneumoniae* strains were resistant to fosfomycin while *Berglund et al.*[29] reported higher resistance rates to fosfomycin (30%) among the studied *K. pneumoniae* isolates.

Due to the extensive use and abuse of antimicrobial agents for promoting growth and treating diseases in animals, *K. pneumoniae* has gained remarkable resistance to most antibiotic agents. In particular, the emergence of PDR and MDR strains has caused great challenges for the prevention and treatment of infections caused by *K. pneumoniae*[18]. Notably, current results showed that 51.2% (43/84) of *K. pneumoniae* isolates were MDR, 20.2% (17/84) were XDR and 6% were discovered as PDR isolates. The study of *Li et al.* [18] showed a great similarity to our results as 61.4% of *K. pneumoniae* isolates were MDR, 22.0% were XDR and 1.8% were PDR isolates. Lower resistance rates were reported by *Cepas et al.* [30] in Japan, and *Nirwati et al.* [31] who found that the overall proportion of MDR *K. pneumoniae* isolates was 38%, and 34.49% respectively. The high prevalence rate of MDR and XDR *K. pneumoniae* subtypes reflects a multifactorial dissemination processes that include: the spread of high risk global multi-resistant genetic lineage; acquisition of successful multi-resistant plasmids; and acquisition of resistant genes located on successful transposons[4].

Several factors have been identified to increase the virulence of *K. pneumoniae*, of which the capacity to form biofilm is highly significant. The formation of biofilm protects *K. pneumoniae* species against the host immune responses, the action of antibiotics and enhances its persistence [6] . According to current results, about 83.3% of *K. pneumoniae* isolates were potential biofilm producers while only 16.7% did not produce biofilm. This finding was similar to the previous studies[32].

Interestingly, the current study highlighted a significant association between biofilm formation and antibiotic resistance as, there was a highly significant statistical difference between biofilm-producing and non-biofilm-producing *K. pneumoniae* isolates (P<0.001) regarding resistance to cefepieme and amikacin and a significant difference regarding resistance to aztreonam, imipenem, meropenem, ertapenem and tobramycin (P<0.05). Similar results were obtained by *Diago-Navarro et al.* [33], *Vuotto et al.* [34] and *Rahdar et al.*[35].

Type 3 fimbrial adhesins are able to mediate the binding of *K. pneumoniae* to various human cells, such as endothelial cells, epithelial cells of the respiratory tract and urinary tract. Fimbrial-related gene (*mrkD*) is an important factor in binding of the microorganism to collagen molecules. The ability of a microorganism to form biofilm is an important virulence factor and such biofilms are the main cause of many chronic infections[7].In this study, the PCR assay revealed that 92% (46/ 50) of the

K. pneumoniae isolates harbored that gene of which 95.7% also produced biofilms phenotypically by the MCRA. Similar results were reported in studies conducted by *Wu et al.* [36] and *Rastegar et al.* [37] On the other hand, *Shakib et al.*[38] reported lower rates for the prevalence of *mrkD* gene.

According to the present findings, there was a strong association between biofilm formation and the existence of *mrkD* gene as 95.7% of biofilm-producing *K.pneumoniae* isolates carried *mrkD* gene while only 33.3% of non-biofilm producing *K. pneumoniae* had the gene with a highly significant statistical difference (P<0.001). In agreement with *Mahmood* and *Abdullah*, [39] who documented 100% of biofilm- forming and only 27.3% of non- biofilm- forming isolates as *mrkD* positive isolates. Also, *Liu et al.* [40] noticed that, 95.8% of biofilm–producing *K. pneumoniae* isolates carried *mrkD* gene.

CONCLUSION

The expression of type 3 fimbrial adhesin-encoding gene (*mrkD*) was significantly associated with biofilm formation as well as resistance to variable antibiotics among *K. pneumoniae* clinical isolates. Implementation of prevention and control plans regarding biofilm-associated infections and eradication of infections particularly in the hospital settings is a must.

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Conflicts of interest:
The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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