The relationship between biofilm formation and presence of *fimH* and *mrkD* genes among *E. coli* and *K. pneumoniae* isolated from patients in Mosul

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ABSTRACT

Background and objectives: Biofilms have a major medical significance as they decrease susceptibility to antimicrobial agents. The decreased susceptibility to microbial agents within a biofilm arises from multiple factors, including physical impairment of diffusion of antimicrobial agents, reduced bacterial growth rates, and local alterations of the microorganisms (M.O) that may impair activity of the antimicrobial agent. The aim of the study was to investigate the biofilm formation among coliforms and study the relationship between biofilm formation and the presence of *fimH* and *mrkD* genes among *E. coli* and *K. pneumonia* isolates respectively .

Materials and method: One hundred and seventy three clinical samples were collected from both gender who visited or admitted to AL- Salam General Teaching Hospital and AL- Wafa'a center for diabetic patients in Mosul city from April 2013 to February 2014, (110) urine samples from patients suffering from urinary tract infections (UTIs) and (63) from patients with diabetic foot infections (DFIs). All samples were cultured on selective media as MacConkey and blood agar aerobically. Coliform isolates are identified to species level depending on biochemical and physiological tests and using Rapid ™ ONE panel kit to be conformed to the diagnosis. The ability of coliforms spp. for biofilm formation was assessed using qualitative and quantitative assay. The Congo red agar (CRA) method was used in the qualitative biofilm assay.

Results: Out of (85) bacterial spp. , 44(51.8%) species were biofilm producers. The tissue culture plate (TCP) method was used in the quantitative biofilm formation assay. The results showed that 46(54.1%) spp. produced biofilm strongly. All biofilm producing *E. coli* and *K. pneumoniae* spp. which gives strong biofilm formation by CRA method and non-biofilm producer spp. were subjected to PCR for determining the relation between biofilm formation and the presence of *fimH* gene in *E. coli* isolates and *mrkD* gene in *K. pneumoniae* isolates. The results revealed that all biofilm producing *E. coli* 15(100%) isolated from UTI samples were positive for *fimH* gene. While all biofilm producing *K. pneumoniae* 9(100%) and 5(100%) isolated from UTIs and DFIs respectively were positive for *mrkD* gene.

Key words: Biofilm, fimH and mrkD genes, E. coli, K. pneumonia**.**

INTRODUCTION

Infection caused by pathogenic *E. coli* and other coliforms spp. are often initiated by binding of the bacteria to the host cell surface via specific bacterial adhesins with fimbriae . Fimbrial adhesins are thread-like structures that reach out from the bacterial surface enabling bacteria to adhere to host cells (Heydari *et al*., 2013). Most clinical isolates of coliforms express type 1 (mannose-sensitive) and type 3 (mannose-resistant) fimbrial adhesins. Among adhesions of uropathogenic *E. coli* ,the adhesive subunit of type 1 fimbriae ,FimH, is a major determinant ,which has high tropism for urinary tract receptors ;thus, FimH adhesion is important in colonizing different niches of *E. coli* (Hojati *et al*., 2015). Type 3 fimbrial adhesins are able to mediate the binding of *K. pneumonia* to various human cells , such as endothelial cells , epithelial cells of the respiratory tract and urinary tract . MrkD protein is an important factor in binding of the microorganism to collagen molecules (Melo *et al*.,2014).

The presence of adhesins (*fimH* and *mrkD*) related to biofilm formation were also investigated (Bellifa *et al*., 2013 and Jarjees , 2014). Different microorganisms combine together to form micro-communities within a matrix of extracellular polymeric substances (EPS) and are called biofilm . The ability of a microorganisms to form biofilm is an important virulence factor and such biofilms are the main cause of many chronic infections (Swarna *et al*., 2012). Biofilm formation is common in chronic wounds such as diabetic foot infections (DFIs), and urinary tract infections (Zubair *et al*., 2011 and Poovendran and Ramanathan , 2014).

Biofilms have a major medical significance as they decrease susceptibility to antimicrobial agents. The decreased

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susceptibility to microbial agents within a biofilm arises from multiple factors , including physical impairment of diffusion of antimicrobial agents , reduced bacterial growth rates , and local alterations of the microorganisms(M.O) that may impair activity of the antimicrobial agent (Pramodhini *et al*., 2012). The aim of the study was to investigate the biofilm formation among coliforms and study the relationship between biofilm formation and the presence of *fimH* and *mrkD* genes among *E. coli* and *K. pneumonia* isolates respectively .

MATERIAL AND METHOD

Isolation and identification of coliforms bacteria

One hundred and seventy three clinical samples were collected from both gender who visited or admitted to AL- Salam General Teaching Hospital and AL- Wafa'a center for diabetic patients in Mosul city from April 2013 to February 2014 , (110) urine samples from patients suffering from urinary tract infections (UTIs) and (63) from patients with diabetic foot infections (DFIs). All samples were cultured on selective media as MacConkey and blood agar aerobically. Coliform isolates are identified to species level depending on biochemical and physiological tests and using Rapid ™ ONE panel kit to be confirmed to the diagnosis**.**

Table (1): Biofilm formation by TCP method

Biofilm degree	Absorbance at 570 nm
Non / Weak	< 0.120
Moderate	0.120 - 0.240
Strong	> 0.240

Phenotypic detection of the type 1 and 3 fimbriae among coliforms

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Table (2):Primer used for the amplification of *fimH* & *mrkD* gene.

Genes	Oligonucleotides (5`→ 3`)	Product size	Reference
Type 1 Pili *fimH*	F GAGAAGAGGTTTGATTTAACTTATTG R AGAGCCGCTGTAGAACTGAGG	559 bp	Karimian *et al* .,(2012)
Type 3 pili *mrkD*	F CCACCAACTATTCCCTCGAA R ATGGAACCCACATCGACATT	228bp	Melo *et al* ., (2014)

Table (3): The PCR reaction components (25µl) for genes amplification .

Component	Volume (µl)
GoTaq Green Master Mix (2X)	12.5
Nuclease Free Water	9.5
DNA Template	2
Forward Primer (10 picomoles)	0.5
Reverse Primer (10 picomoles)	0.5
Total volume	25

Table (4) . Program for amplification of the *fimH* gene .

Stage	Temperature °C	Time	Cycle number
Initial denaturation	94	3 min.	1
Denaturation	94	1 min.	40
Annealing	58	70 sec.	
Extension	72	70 sec.	
Final extension	72	6 min.	1
Hold	4	3 min.	1

Table (5) . Program conditions for amplification of the *mrkD* gene .

Stage	Temperature °C	Time (min.)	Cycle number
Initial denaturation	95	5	1
Denaturation	94	1	33
Annealing	62	1	
Extension	72	2	
Final extension	68	2	1
Hold	4	3	1

Table (6): Number and percentage of coliforms spp. isolated from two types of infection

Coliforms	Type of infection		Total No.(%)
	UTIs No. (%)	DFIs No. (%)	
E. coli	28 (50.9)	17 (56.7)	45 (52.9)
K. pneumoniae	17 (30.9)	8 (26.7)	25 (29)
Ent. cloacae	2 (3.7)	0 (0)	2 (2.4)
Ent. aerogenes	5 (9.1)	0 (0)	5 (5.9)
C. freundii	0 (0)	2 (6.7)	2 (2.4)
Serr. marcescens	2 (3.7)	3 (10)	5 (5.9)
Serr. fonticola	1 (1.8)	0 (0)	1 (1.2)
Total	55 (64.7)	30 (35.3)	85 (100)

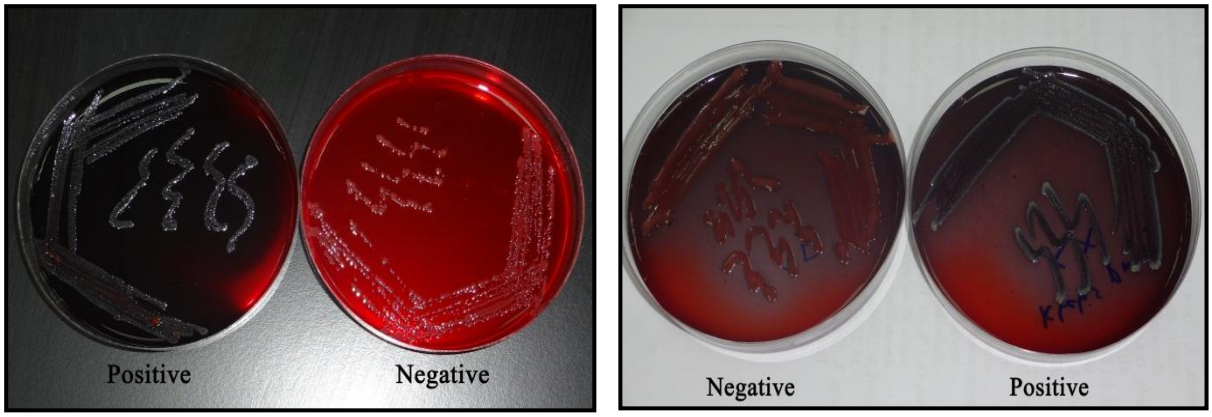
Table (7): Qualitative biofilm formation by CRA method among coliform bacteria

Microorganisms		NO. of isolates	CRA method	
			Biofilm producers (%)	Biofilm non-producers (%)
UTIs	*E. coli*	28	15 (53.6)	13 (46.4)
	K.pneumoniae	17	9 (52.9)	8 (47.1)
	Ent. cloacae	2	1 (50)	1 (50)
	Ent.aerogenes	5	3 (60)	2 (100)
	Serr. marcescens	2	0	2 (100)
	Serr. fonticola	1	0	1 (100)
DFIs	*E. coli*	17	10 (58.8)	7 (41.2)
	K.pneumoniae	8	5 (62.5)	3 (37.5)
	C. freundii	2	0	2 (100)
	Serr. marcescens	3	1 (33.3)	2 (66.7)
Total		85	44 (51.8)	41 (48.2)

Table (8): Quantitative biofilm formation by TCP method among coliform bacteria

Microorganisms		NO. of isolates	Biofilm formation (Absorbance at 570nm)			P value
			Strong NO.(%)	Moderate NO.(%)	Weak NO.(%)	
UTIs	*E .coli*	28	14 (50)	9 (32.1)	5 (17.9)	0.113
	K .pneumoniae	17	10 (58.8)	5 (29.4)	2 (11.8)	0.056
	Ent. cloacae	2	1 (50)	1 (50)	0	1.000
	Ent. aerogenes	5	1 (20)	3 (60)	1 (20)	0.449
	Srre. marcescens	2	0	2 (100)	0	1.000
	Serr. fonticola	1	0	1 (100)	0	0.564
DFIs	*E. coli*	17	11 (64.7)	4 (23.5)	2 (11.8)	0.019*
	K. pneumoniae	8	5 (62.5)	3 (37.5)	0	0.264
	C. freundii	2	2 (100)	0	0	1.000
	Serr. marcescens	3	2 (66.7)	1 (33.3)	0	0.779
Total		85	46 (54.1)	29 (34.1)	10 (11.8)	0.000*

* Sig. : $P \leq 0.05$.



A- *E. coli* **B- *K. pneumoniae***

Figure (1) : Qualitative biofilm formation by CRA method

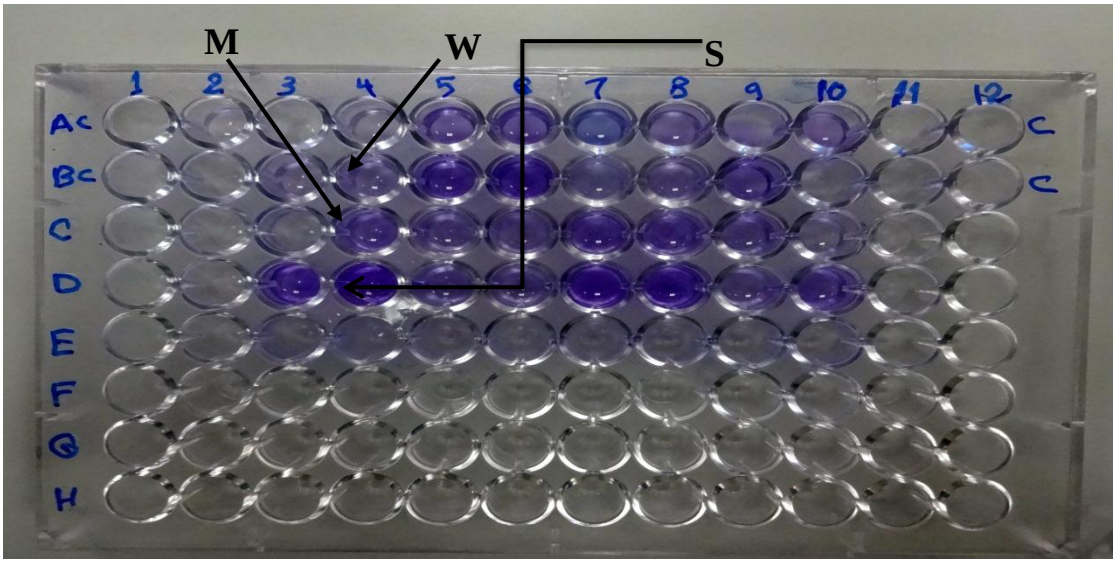


Figure (2): Quantitative biofilm formation of coliforms spp. by TCP method. S= Strong , M= Moderate ,W= Weak

Table (9): The relation between biofilm formation and presence of *fimH* gene in *E. coli* isolates according to type of infection

Source of isolates	Biofilm formation by CRA method in *E. coli*	Test		P values
		fimH by PCR N. (%)	(MSHA) assay N. (%)	
UTIs	Biofilm producing (n=15)	15(100)	11(73.3)	0.885
	non-biofilm producing (n=13)	9(69.2)	6(46.2)	
DFIs	Biofilm producing (n=10)	7(70)	6(60)	0.876
	non-biofilm producing (n=7)	3(42.9)	3(42.9)	

* Sig. : $P \leq 0.05$.

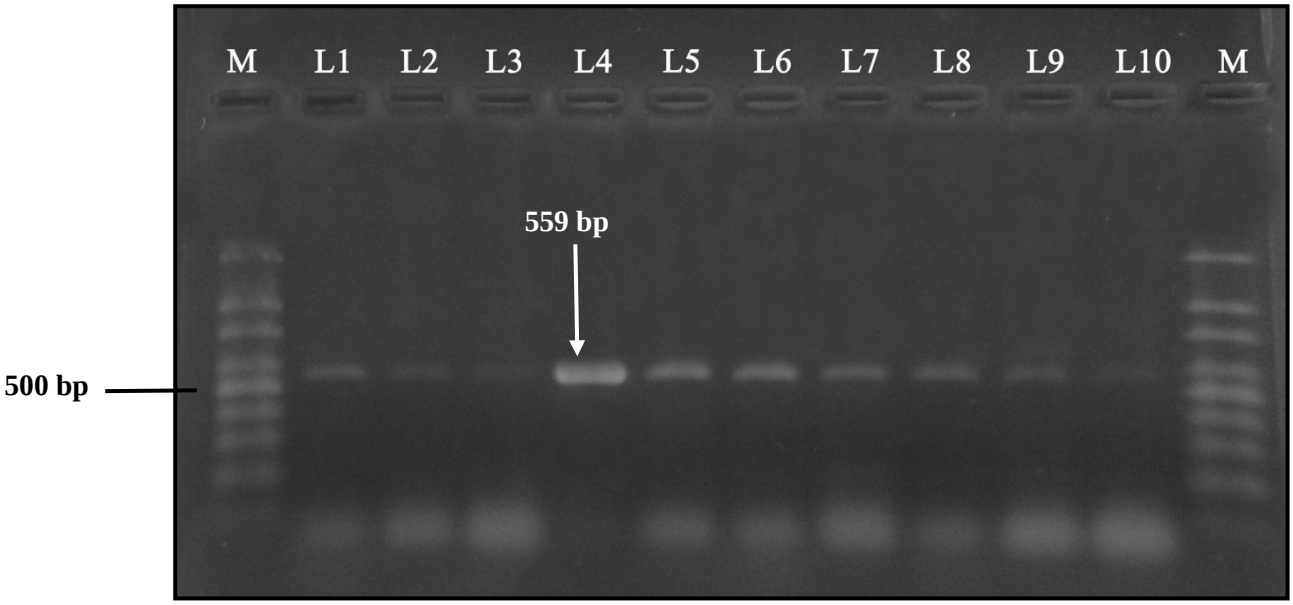
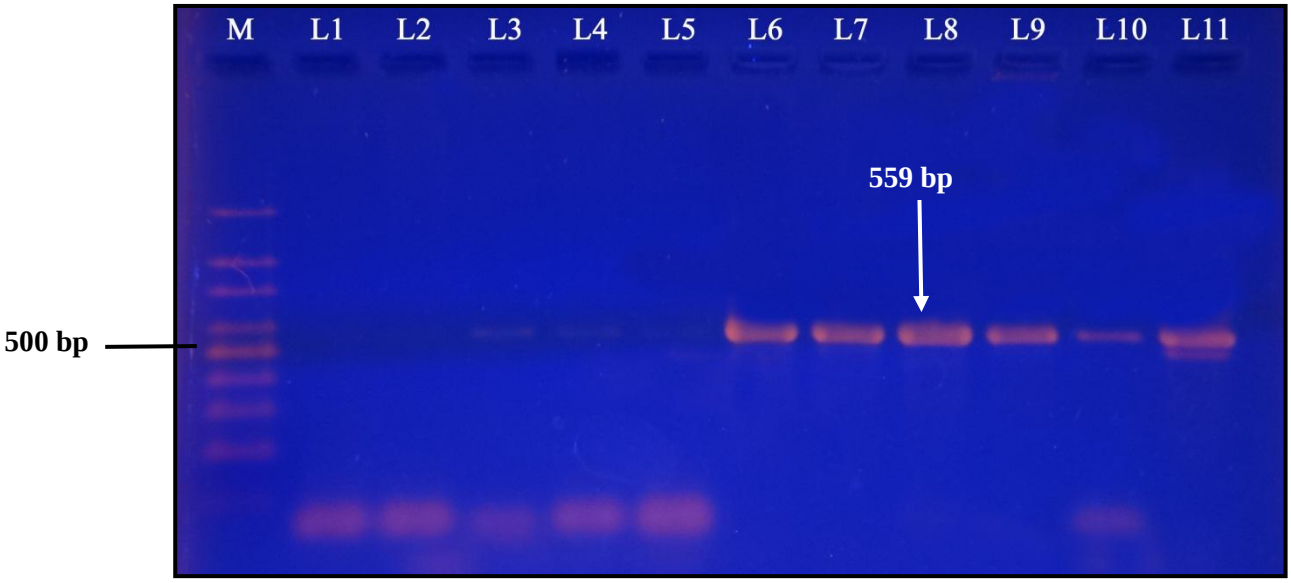


Figure (3): Electrophoresis of PCR amplification of *fimH* gene in biofilm producers *E. coli* with expected product length 559bp in (1.2%) agarose gel. M lane, left and right (100bp ladder). Lane 1 – 10 poitive *fim H* gene.

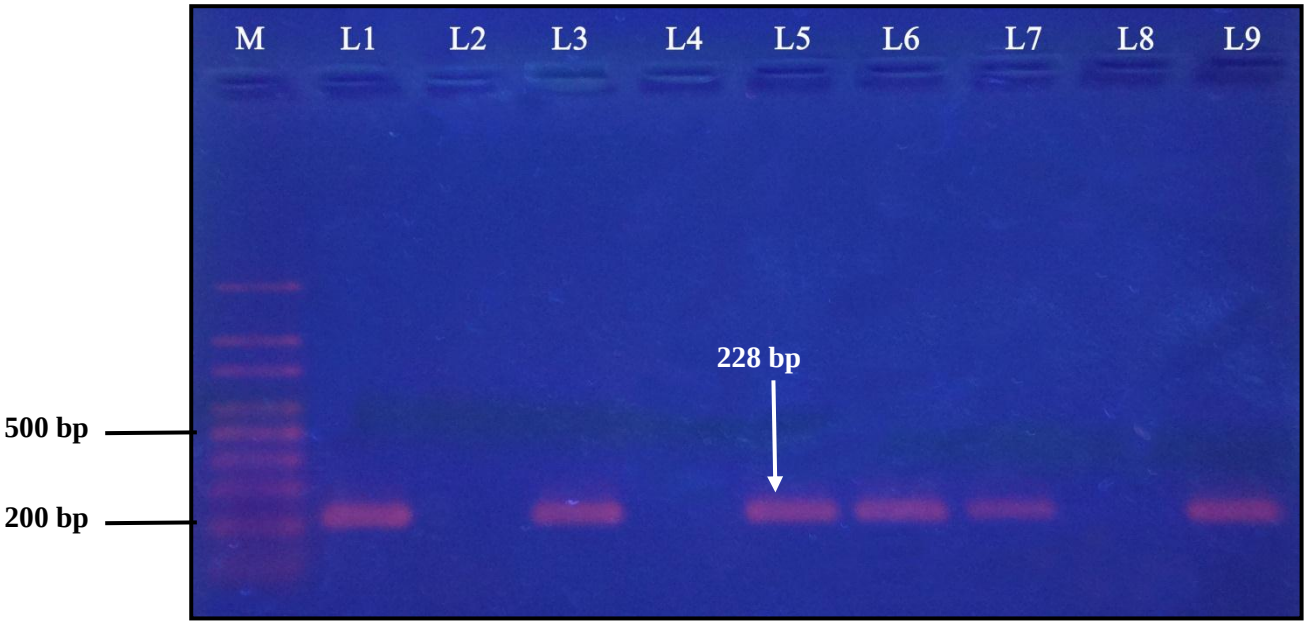


Figure(4): Electrophoresis of PCR amplification of *fimH* gene in non-biofilm producers *E. coli* with expected product length 559bp in (1.2%) agarose gel. M lane (100bp ladder). Lane 1, 2 negative, lane 3-11 positive *fimH* gene.

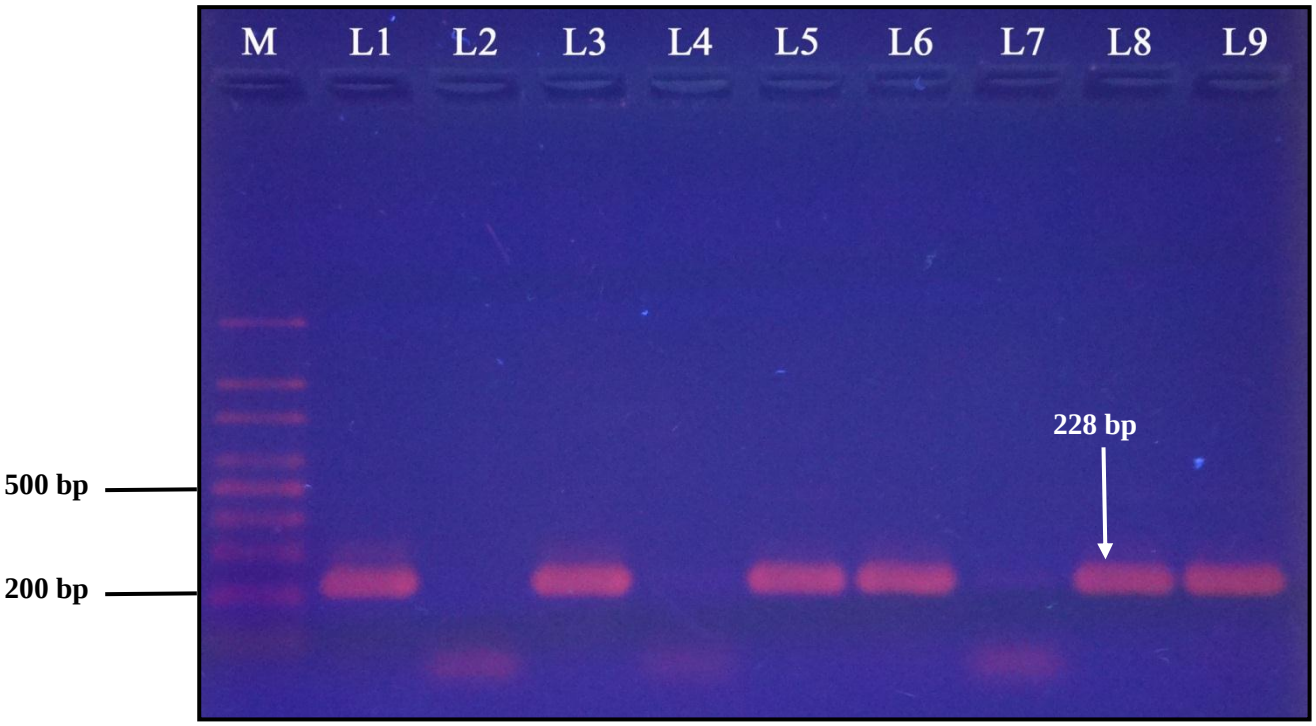
Table (10): The relation between biofilm formation and presence of *mrkD* gene in *K. pneumoniae* isolates according to type of infection

Source of isolates	Biofilm formation by CRA method in *K. pneumoniae*	Test		P values
		mrkD by PCR N. (%)	(MRHA) assay N. (%)	
UTIs	Biofilm producing (n=9)	9(100)	3(33.3)	0.350
	non-biofilm producing (n=8)	2(25)	2(25)	
DFIs	Biofilm producing (n=5)	5(100)	3(60)	0.524
	non-biofilm producing (n=3)	1(33.3)	0(0)	

* Sig. : P ≤ 0.05 .



Figure(5): Electrophoresis of PCR amplification of *mrkD* gene in *K. pneumoniae* (UTI isolates) with expected product length 228bp in (1.2%) agarose gel. M lane (100bp ladder). Lane 1,3,5,6,7 and 9 poitive *mrkD* gene.



Figure(6): Electrophoresis of PCR amplification of *mrkD* gene in *K. pneumoniae* (DFI isolates) with expected product length 228bp in (1.2%) agarose gel. M lane (100bp ladder). Lane 1,3,5,6,8 and 9 poitive *mrkD* gene.

RESULTS AND DISCUSSION

Biofilm formation assay

Qualitative assay of biofilm formation by Congo Red agar method:

Plates containing a specially prepared Congo Red agar medium are inoculated and incubated aerobically for 24 – 48 hours at 37°C . A positive result was indicated by Black colonies with a dry crystalline constancy. (Pramodhini *et al* ., 2012) .

Quantitative assay of biofilm formation by tissue culture plate (TCP) method:

Biofilm formation was conducted by growing bacteria isolates in a flat bottom tissue culture plates (96 well) as previously described by Zubair *et al* ., (2011). Biofilm production was estimated based on the Absorbance 570 nm as in (Table 1**).**

Phenotypic detection of the type 1 and 3 fimbriae among coliforms

A suspension of human blood and PBS was mixed on a clean microscopic slide with a single colony of fresh bacterial cultures . The slide was gently rotated until agglutination was visible . Agglutination assays were performed in the presence or absence of (5%) mannose (Stahlhut *et al* ., 2012) . The phenotypic expression of type 1 fimbriae was measured by the occurrence of mannose-sensitive hemagglutination (MSHA), while expression of type 3 fimbriae was detected as mannose-resistant hemagglutination (MRHA) , which not inhibited by addition of mannose(Li *et al* ., 2009)

DNA extraction

The total DNA (chromosomal and plasmid) was extracted from all isolates using Wizard Genomic DNA purification kit supplemented by (Promega \ USA) according to manufacture instructions. The purity and concentration of genomic DNA were measured using Biodrop spectrophotometer. The isolated DNA was checked by (0.7%) agarose gel electrophoresis and visualized by exposed to UV light using UV transilliuminator.

Primers used

Primers sequences were taken from previous articles(Table 2) . All primers were synthesized by Alpha DNA company , Canada .

Polymerase chain reaction (PCR) assay

PCR reaction

All PCR reactions were performed in 25µl volumes in Eppendorf tube (Table 3) . Laminar flow cabinet with UV lamp was used for the preparation of reaction mixture , all the reaction components were prepared separately in ice and used with optimum concentration .

Detection of the type 1 pili encoding gene

PCR conditions program depending on Karimian *et al* ., (2012) , was used for detection of *fimH* gene (Table 4). After the reaction was complete , the PCR products were visualized by exposed to UV light using UV transilliuminator , then photographed by using digital camera .

Detection of the type 3 pili encoding gene

Amplification of mrkD gene was done by using PCR program depending on Melo et al ., (2014) , as shown in (Table 5).

Statistical analysis

All statistical analysis was conducted using the Statistical Package for Social Sciences (S.P.S.S.) version 19 from IBM Company, USA. The $x2$ test was used for statistical comparison of groups , values < 0.05 were regarded as significant .

The result showed that the isolated bacteria belonged to five genera ; the number and percentage of coliforms isolated from UTIs and DFIs are listed in Table (6). The total number of coliform bacteria was (85) isolates , of which UTIs isolates were more frequently encountered (55; 64.7%) than the DFIs isolates (30;35.3%) . Among the isolates , *Escherichia coli* was the predominant isolates 45 (52.9%) from clinical samples, 28 (50.9%) were UTIs samples , while 17 (56.7%) were DFIs samples followed by *Klebsiella pneumoniae* which isolated from 25 (29%) samples 17 (30.9%) were UTIs samples , and only 8 (26.7%) were DFIs samples .

Biofilm formation among coliform bacteria

Several methods of biofilm formation have been developed over the years using numerous types of M.O. for different applications (Agostinho *et al*., 2011). The ability of each coliform bacteria isolated from UTIs and DFIs into biofilm formation was assessed using qualitative and quantitative assay . The Congo red agar (CRA) method is used in the qualitative biofilm assay. The results showed that out of (85) bacterial isolates , 44 (51.8%) isolates were biofilm producers which showed black colonies with crystalline appearance. While 41 (48.2%) isolates gave pink colonies indicating non-biofilm production as in (Table 7 and Figure 1).

Detection of the type 1 fimbriae gene (*fimH*) and the type 3 fimbriae gene (*mrkD*) in biofilm and non-biofilm producing species.

In the present study , the most frequent biofilm and non-biofilm producing coliforms (*E. coli* and *K. pneumoniae*) , which gives strong biofilm formation by CRA method , were subjected to PCR for determining the relation between biofilm formation and the presence of *fim H* gene in *E. coli* isolates , and *mrkD* gene in *K. pneumoniae* isolates . Moreover, type 1 fimbria (MSHA) , and type 3 fimbriae (MRHA) were investigated phenotypically as in (Tables 9 and 10).

The *fimH* gene was amplified using specific primers and appeared as a band of (559

bp) on agarose gel as shown in (Figures 3 and 4). It was found that all biofilm producing *E. coli* 15(100%) isolated from UTI samples were positive for *fimH* gene, while only 9(62.2%) non-biofilm producing isolates were carried *fimH* gene. Regarding *E. coli* isolated from DFI samples, only 7(70%) biofilm producing *E. coli* isolates had *fimH* gene .The prevalence of *fimH* gene was well studied by many researcher in different countries. Locally, Jarjees (2014) found that (93%) of UPEC isolates were positive for *fimH* gene in the study of patients with UTI , attended at the Hospitals of Erbil city / Iraq. The *fimH* gene was(65.9%) positive in UPEC isolated from Egyptian childrens. Hojati *et al*, (2015) described the presence of *fimH* gene in *E. coli* strains isolated from hospitalized and non-hospitalized patients with UTIs as (92.8%) in the Iranian study. Also, Arabi *et al* (2012) investigated the frequency of *fimH* and other adhesion genes in UPEC isolated from some private clinical laboratories located in Tehran / Iran , and determined the *fim H* gene frequency as(87.7%) . Another study conducted in Malaysia by Lai *et al*,(2015) revealed that (92.5%) of UPEC isolates were positive for *fimH* gene . The type 1 fimbriae is commonly found among UPEC as well as non-UPEC strains (Heydari *et al*., 2013). However, in this study the *fimH* gene was detected in *E. coli* isolated from patients with DFIs . But, we did not found any previous study about the relation between the presence of *fimH* gene and biofilm formation in patients with DFIs to compare our results.

In the present study, there was a relation between biofilm formation and the presence of *fimH* gene as shown in (Table 9). Recently, Cargole-Novella (2015) reported that biofilm formation by extraintestinal *E. coli* can be associated with the expression of different adhesins such as type 1 fimbriae, and there was significant correlation between biofilm formation on glass and the presence of *fimH* gene . Furthermore, Moreira *et al*,(2006) found that adhesive structures , such as type 1 fimbriae (*fim H*) was expressed during the initial stages of biofilm development .

As shown in (Table 9). The presence of type 1 fimbriae (MSHA) was identified in all biofilm and non-biofilm producing *E. coli* phenotypically by MSHA assays . In our results type 1 fimbriae were detected in (73.3%) and (46.2%) of biofilm and non-biofilm producing *E. coli* respectively . While, only (60%) and (42.9%) of biofilm and non-biofilm producing *E. coli* isolated from DFIs respectively were positive for test. Some isolates did not give any agglutination , they may loss their fimbriae during culture process, or some strains of bacteria lacking these fimbriae (Madigan *et al*., 2000). Another study conducted by Najar *et al*, (2007) found that type 1 fimbriae (MSHA) was seen in (71%) of isolates of UPEC and this finding is similar to our results.

Various fimbrial adhesins have been shown to play a role in biofilm formation . Most *K. pneumoniae* isolates express type 3 fimbrial adhesin (Bandeira *et al*., 2014). In this study, the *mrkD* gene was amplified by PCR and appeared as a band of 228bp on agarose gel. Electrophoresis of *mrk D* gene is shown in (Figures 5 and 6).

Many studies have revealed that type 3 fimbriae (*mrkD gene*) are important in *K. pneumoniae* biofilm formation (Jagnow and Clegg , 2003 ; Ong *et al*., 2010 and Bellifa *et al*., 2013). Therefore, the *mrkD* gene may play significant roles in forming biofilm . In the present study, all biofilm and non-biofilm producing *K. pneumoniae* were examined for the presence of *mrkD* gene . It was found that all biofilm producing *K. pneumoniae* 9(100%) and 5(100%) isolated from UTIs and DFIs respectively were positive for *mrkD* gene, thus, demonstrating a relationship between biofilm formation and the presence of *mrkD* gene as in (Table10). Same the *fimH* gene , the *mrkD* gene was also detected in *K. pneumoniae* isolated from DFIs . But, we did not found any previous study about the relation between formation of biofilm and the presence of *mrkD* gene to compare our results .

However, the presence of type 3 fimbriae (MRHA) was identified in all *K. pneumoniae* isolates phenotypically. As shown in (Table 10). not all isolates show positive *mrkD* gene by PCR gives positive test by MRHA assays, they may lose their fimbriae during culturing, or some isolates lacking these fimbriae as mention later in *fimH* gene detection.We can conclude from the results that type 1- encoding gene (*fimH*), and type 3- encoding gene (*mrkD*) are associated with biofilm formation in coliforms spp.

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