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Biofilm formation in uropathogenic *Escherichia coli*: association with adhesion factor genes

Hojjatolah ZAMANI[1], Ali SALEHZADEH[2,*]
[1]Department of Biology, Faculty of Science, University of Guilan, Rasht, Iran
[2]Department of Biology, Rasht Branch, Islamic Azad University, Rasht, Iran

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Background/aim: Biofilm formation is a major determinant factor in the development of urinary tract infections (UTIs) by uropathogenic *Escherichia coli* (UPEC). Several adhesion factors are involved in attachment of bacterial cells to the urinary tract and biofilm development.

Materials and methods: The possible relationship between different adhesion factor genes (AFGs) and biofilm formation among UPEC isolates was investigated. Prevalence of different AFGs including *fimA*, *fimH*, *papAH*, *papC*, *papEF*, *sfa-S*, *foc/G*, and *bmaE* and their association to biofilm formation were investigated. Phenotypic expression of type 1 and P fimbriae was also investigated.

Results: Our results showed that 84% of UPEC were moderate to strong biofilm producers. Prevalence of the type 1 and P fimbriae associated genes (*fimA*, *fimH*, *papEF*, *papC*) as well as the F_1C fimbriae gene (*foc*/G) did not show a difference among different biofilm-producing groups, while higher prevalence of *papAH*, *bmaE* (M fimbriae), and *sfaS* (S-fimbriae) was observed for the strains producing moderate to strong biofilms. Phenotypic expression of type 1 fimbriae was associated with biofilm formation, while no association was observed for P fimbriae.

Conclusion: Different AFGs (especially M and S fimbria) and their expression levels affect the biofilm formation ability of UPEC.

Key words: Biofilm, virulence factors, *Escherichia coli*, urinary tract infections

1. Introduction

Urinary tract infection (UTI) is regarded as a serious worldwide health threat. Uropathogenic *Escherichia coli* (UPEC) is the predominant cause of community-acquired and nosocomial UTIs (1,2). The ability of bacteria to persist and grow in a biofilm seems to be the major factor for pathogenesis and therapeutic failure. Biofilm formation by UPEC is considered a determinant factor in long-term persistence of bacterial cells in the urinary tract and causing inflammatory reactions associated with UTIs (3,4). Furthermore, increased drug resistance among the strains associated with biofilm could significantly increase the difficulty of treatment.

Biofilm formation consists of initial bacterial adherence to the surface, followed by multiplication and production of extracellular polymeric matrix, which cause cell aggregation (5). UPEC strains can produce a variety of adhesions for attachment to solid surfaces and biofilm formation. Type 1 fimbriae, which may promote bacterial adhesion and biofilm formation, recognize mannose-oligosaccharides naturally presented on glycoprotein molecules of the host cell surface. Type 1 fimbriae are encoded by the *fim* gene cluster and consist of a major protein, *Fim*A, associated with ancillary proteins *Fim*F and *Fim*G and the adhesion protein *Fim*H. P fimbriae are expressed by UPEC and are strongly associated with the ability of the bacteria to colonize the kidney and cause pyelonephritis. The *pap* gene cluster consists of 11 genes encoding the main component of the fimbria rod (PapA), papEF, which encode adaptor subunits, and a terminal adhesion PapG (1). M fimbria, which is encoded by *bmaE*, recognizes blood group M-specific determinants of glycophorin A and has been reported to be associated with UTIs. The S fimbriae are mannose-resistant adhesions, encoded by the *sfa* operon of UPEC. The presence of S fimbria is also correlated with pathogenicity of *E. coli* strains. F_1C fimbria has been described as a nonhemagglutinating adherence factor and is expressed by pathogenic *E. coli* strains. A cluster of eight genes (*foc*) is necessary for the biogenesis of F_1C fimbriae (1,6).

Epidemiological studies suggest these adhesion factors to be associated with UTIs (1,2–7). However, few studies investigated the association between adhesion factor genes (AFGs) and biofilm formation potential of UPEC. Thus,

* Correspondence: salehzadeh@iaurasht.ac.ir

the present study was conducted to investigate the possible association between AFGs and biofilm formation among UPEC strains.

2. Materials and methods

2.1. Bacterial isolates

In this study 100 UPEC isolates were collected from patients presenting to clinical laboratories in the city of Rasht (Iran) during January to July 2016. Bacterial identification was performed by conventional biochemical methods and all the isolates were stored at –70 °C in tryptic soy broth (TSB) containing 20% glycerol for subsequent analysis.

2.2. In vitro biofilm assay

The biofilm formation assay was performed in triplicate in 96-well microtiter plates, according to the method described previously with some modifications (8). Briefly, isolated strains were grown overnight at 37 °C in Luria broth (LB) medium. The optical density at 600 nm (OD_{600}) of the culture was adjusted to 1.0 and the cultures were then diluted 1:100 in fresh LB. Then 100 µL of the diluted cultures was added to each well. The plates were incubated at 37 °C for 48 h under static condition. Following incubation, the plates were washed three times using sterile phosphate buffered saline (PBS) and 125 µL of 0.1% crystal violet solution was added to the wells. The plates were incubated for 15 min at room temperature and washed with PBS three times. Biofilm formation ability could be assessed based on color intensity. In order to quantify biofilm formation ability, the plates were left a few hours to dry and then 125 µL of 30% acetic acid was added to solubilize the dye. Finally, the OD_{550} was recorded using a microplate reader (Bio-Rad, USA) and the strains were classified into two groups including no or weak biofilm producers (Group I) and moderate to strong biofilm producers (Group II) according to their OD value (9). Biofilm formation was scored as follows:

Strong biofilm (S) = OD ≥ 0.3, Moderate biofilm (M) = 0.2 ≤ OD ≤ 0.299, Weak biofilm (W) = 0.1 ≤ OD ≤ 0.199, Negative (N) = OD < 0.1. The negative control wells contained broth only and *E. coli* ATCC 10798 was used as positive control (10).

2.3. Genotypic detection of AFGs

All UPEC isolates were screened for the presence of different AFGs by polymerase chain reaction (PCR) assay. Prevalence of the genes corresponding to the following adhesion factors was investigated: P fimbria associated genes (*papAH*, *papC*, and *papEF*), S fimbria (*sfaS*), F_1C fimbria (*foc/G*) and M blood group antigen-specific M fimbria (*bmaE*). In addition, the mannose specific type 1 fimbria genes including *fim A* and *fim H* were targeted.

DNA extraction was performed using a CinnaGen DNA extraction kit according to the manufacturer's instructions. The master mix for the PCR was prepared as follows: 3 µL of 10X PCR buffer, 3 µL of 25 mM $MgCl_2$, 3 µL of 10 mM dNTP mix, 0.5 µL of Taq DNA polymerase, 9.5 µL of Milli-Q water, and 1 µL of each of the forward and reverse primers. Finally, 4 µL of each DNA template was added to the corresponding tubes to make up the final reaction volume of 25 µL. Amplification of *papAH*, *sfaS*, *bmaE*, and *foc/G* genes was performed using multiplex-PCR. The PCR primers and condition for each gene are presented in Tables 1 and 2. Finally, PCR products were mixed with 3 µL of PowerLoad DNA stain and were visible after electrophoresis in a 1% agarose gel in TBE buffer and under UV illumination. In order to ensure the accuracy of the PCR products, the amplified genes were sequenced, submitted to GenBank (NCBI), and blasted with other published sequences from the GenBank database. The aggregate AFG score was also defined as the median number of the eight investigated AFGs detected among UPEC isolates.

2.4. Phenotypic detection of type 1 and P fimbriae

Expression of type 1 fimbria was evaluated by mannose sensitive agglutination assay. Briefly, a drop of 0.5% suspension of *Saccharomyces cerevisiae* cells was mixed with and one without a drop of 2% mannose solution. UPEC were grown overnight at 37 °C in LB broth and 5 µL of bacterial suspension (approximately 10^6 CFU/mL) was added to the drops. The isolates were interpreted as positive for type 1 fimbria when showing precipitation with yeast cells and no precipitation with yeast in the presence of mannose (2).

To detect P fimbria, UPEC were grown overnight at 37 °C in LB agar and 5 µL of bacterial suspension (approximately 10^6 CFU/mL) was added to a drop of O type human RBCs suspension (8% v/v) on a slide and monitored for hemagglutination (11).

2.5. Statistical analysis

The chi-square test was used to establish the results' significance and P values of <0.05 were considered statistically significant.

3. Results

3.1. Biofilm formation

Biofilm formation capability of the UPEC isolates was investigated using microtiter plates. Our results showed that 36 isolates were strong biofilm producers, while 48 and 10 isolates were moderate and weak biofilm producers, respectively. In addition, six UPEC isolates did not show biofilm formation capability (Figure 1).

3.2. Prevalence of AFGs

Prevalence of different AFGs among UPEC isolates was investigated and high prevalence (more than 50%) was observed for *fimA*, *fimH*, *papEF*, and *papC*. According to the results, *papC* was the most prevalent AFG among all

Table 1. PCR primers for each adhesion factor gene.

Gene	Primers (5'→3')	Size of product (bp)	Reference
fimH	F: TGCAGAACGGATAAGCCGTGG R: GCAGTCACCTGCCCTCCGGTA	508	(21)
fimA	F: GTTGTTCTGTCGGTCGGCTCTGCT R: ATGGTGTTGGTTCCGTTATTC	447	(19)
papEF	F: GCAACAGCAACGCTGGTTGCATCAT R: AGAGAGAGCCACTCTTATAGGACA	336	(21)
papC	F: GTGGCAGTATGATGAATGACCGTTA R: ATATCCTTTCTGCAGGGATGCAATA	200	(21)
papAH	F: ATGGCAGTGGTGTCTTTTGGTG R: CGTCCCACCATACGTGCTCTCTTC	720	(22)
sfaS	F: GTGGATACGACGATTACTGTG R: CCGCCAGCATTCCCTGTATTC	244	(22)
bmaE	F: ATGGCGCTAACTTGCCATGCTG R: AGGGGGACATATAGCCCCTTC	507	(21)
foc/G	F: CAGCACAGGCAGTGGATACGA R: GAATGTCGCCTGCCCATTGCT	364	(22)

Table 2. PCR conditions for each adhesion factor gene.

Gene	Initial denaturation (°C/min)	Denaturation (°C/s)	Annealing (°C/s)	Extension (°C/s)	Final extension (°C/min)	Cycles
fimH	95/4	94/30	54/60	72/60	72/8	35
fimA	95/4	94/30	55/45	72/45	72/8	35
papEF	95/4	94/30	55/30	72/60	72/8	35
papC	95/4	94/30	60/55	72/45	72/8	33
papAH[b]	95/4	94/30	60/45	72/60	72/8	33
sfaS[a]	95/4	94/30	60/45	72/60	72/8	33
bmaE[a]	95/4	94/30	60/45	72/60	72/8	33
foc/G[b]	95/4	94/30	60/45	72/60	72/8	33

[a & b] gene amplification was performed using multiplex-PCR.

isolates (92%) followed by *fimH*, *papEF*, and *fimA* with prevalence of 86%, 86%, and 76%, respectively. Moderate prevalence for *papAH* and *sfaS* (42% for both genes) was observed while low prevalence was recorded for *foc/G* (20%) and *bmaE* (17%) (Figure 2; Table 3).

3.3. Association between AFGs and biofilm formation ability

Distribution of different AFGs among UPEC isolates according on their biofilm formation capability was determined. In order to evaluate the association between biofilm formation ability and AFGs, isolated UPECs were classified into two groups, including group I (no or weak biofilm producers) and group II (moderate to strong biofilm producers). According to the results, prevalence of *fimH* among biofilm producing groups I and II were 87% and 86%, respectively, which was not significantly different (P = 0.605). In addition, no significant difference was observed for *fimA* (P = 0.253), *papEF* (P = 0.166), *papC* (P = 0.198), or *foc/G* (P = 0.419). In contrast, higher prevalence of *sfaS* and *bmaE* was recorded for the strains able to produce moderate to strong biofilm (50% and 20%, respectively) while no strain belonging to the group

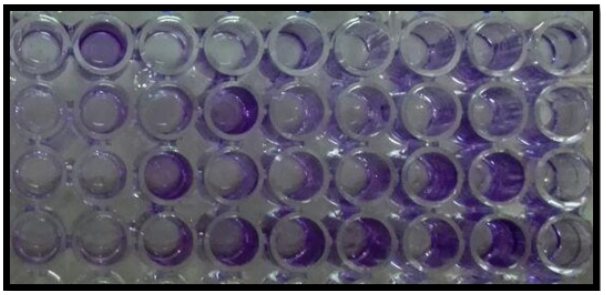

Figure 1. Biofilm formation ability of UPEC isolates in microtiter plate after staining with crystal violet.

I harbored the mentioned genes. In addition, *papAH* was significantly more prevalent among group II strains compared to group I (P = 0.040). The median of AFG scores for groups I and II were 3 and 4, respectively, and no strain harbored all AFGs. Table 3 presents the distribution of different AFGs among isolated UPEC considering their biofilm production ability.

3.4. Phenotypic expression of type 1 and P fimbriae

Phenotypic expression of type 1 and P fimbriae was monitored by agglutination assays. According to the results, 74% (62 out of 86) of the group II biofilm forming UPEC expressed type 1 fimbriae, which was significantly higher than the strains with poor biofilm formation ability (44%). In addition, evaluation of phenotypic expression the P fimbria showed no significant difference between different biofilm-producing groups (69% for group I versus 76% for group II strains).

4. Discussion

A better understanding of microbial pathogenesis would facilitate the development of new therapeutic strategies. The present study was conducted to elucidate the possible association between different AFGs with biofilm formation among UPEC strains isolated from UTIs. Ability to adhere to different surfaces and biofilm formation have been highlighted as important features associated with *E. coli* virulence (3,4).

Surface virulence factors of the pathogens including different adhesion factors may promote bacterial adhesion and biofilm development (12). Our study indicated that the majority of UPEC isolates were moderate to strong biofilm producers (84%), while the remaining isolates either did not produce biofilm or showed weak biofilm formation potential. Thus, biofilm formation could be considered an important virulence determinant possessed by UPEC. Association of biofilm formation ability and UTIs has been reported previously (13,14). Biofilm formation promotes bacterial persistence in the urinary tract by protecting bacteria from the clearing out effect of hydrodynamic forces, antibacterial agents, and host defense mechanisms. Evaluation of the factors contributing to biofilm formation could be helpful to conceive new therapeutic solutions to treat these infections.

We found that the UPEC isolates able to form a moderate to strong biofilm had a higher prevalence of three AFGs, indicating that these adhesion factors may play a role in biofilm development. Evaluation of the prevalence of several AFGs among UPEC isolates showed that the majority of the isolates harbored P and type 1 fimbriae associated genes (except for *papAH*), regardless of their biofilm formation capability. High prevalence of type 1 and P fimbriae associated genes among UPEC strains was reported previously (15,16).

Association between a higher biofilm formation potential and some virulence genes including P and type 1 fimbriae genes was reported earlier (15,17). However, no significant association was observed between prevalence of *fimA*, *fimH*, *papC*, and *papEF* and biofilm formation potential in our study. Five genes of *pap* operon were screened and only the *papAH* gene was significantly associated with biofilm formation. This finding suggests the unequal importance of P fimbria associated genes in biofilm formation. However, further studies will need to be done to interpret the significance of this finding.

In addition, our findings suggest that although type 1 and P fimbriae are important adhesion factors for bacterial initial attachment to the biological surfaces presence of the mentioned genes are not the only determinant for biofilm development in UPEC strains and several environmental and genetic factors may be involved with expression of these genes (18). Similarly, Fattahi et al. (19), in a study on *E. coli* strains causing UTIs from northwest Iran, found no correlation between

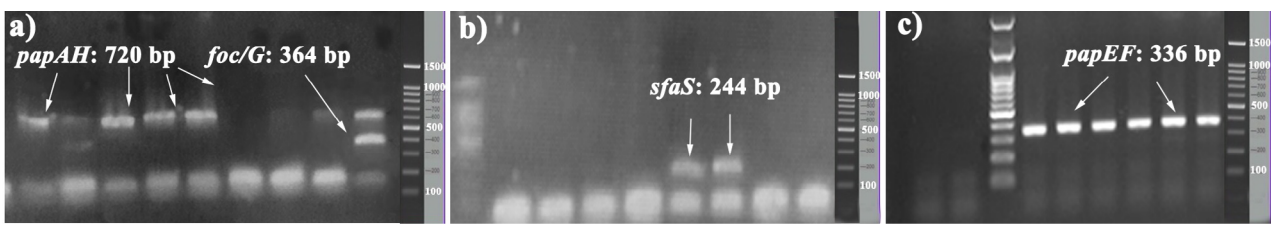

Figure 2. Agarose gel electrophoretic detection of some VFGs among UPEC strains.

Table 3. Distribution of different adhesion factor genes among UPEC isolates.

	Prevalence of bacterial AFGs			
Adhesion factor genes (AFGs)	Group I[a] n =16	Group II[a] n = 84	Total n = 100	P values
fimH	14 (87%)	72 (86%)	86	0.605
fimA	12 (75%)	64 (76%)	76	0.253
papEF	12 (75%)	74 (88%)	86	0.166
papC	16 (100%)	76 (90%)	92	0.198
*sfaS**	0 (0%)	42 (50%)	42	0.002
*bmaE**	0 (0%)	17 (20%)	17	0.04
foc/G	2 (13%)	18 (21%)	20	0.419
*papAH**	3 (19%)	39 (46%)	42	0.040
Aggregate AFG score median (range)	3 (2–6)	4 (3–7)	4	0.003
Phenotypic expression				
Type 1 fimbriae*	7 (44%)	62 (74%)	86	0.022
P fimbriae	11 (69%)	64 (76%)	75	0.529

*shows significance at 95% confidence.
[a] Group I includes no or weak biofilm producers and group II contains moderate to strong biofilm producers.

presence of *fimA* and biofilm formation. However, they reported that biofilm formation ability was associated with presence of *papC*.

Phenotypic detection of type 1 fimbria among different biofilm-producing groups was significantly different. The isolates with moderate to strong biofilm formation ability showed higher type 1 fimbria expression level compared to the bacteria with poor biofilm formation potential. This finding indicates that phenotypic expression of the type 1 fimbria is an important determinant in biofilm formation by UPEC (13,20). Soto et al. (13) reported that

expression of the type 1 fimbria is independently associated with biofilm formation, which was in accordance with our data. Expression of type 1 fimbria could be influenced by several factors including environmental factors and genetic background of the isolates. Several environmental factors and bacterial phylogenetic group and host associated factors could regulate bacterial gene expression and thus result in different genotypic and phenotypic characteristics of UPEC strains (13).

Prevalence of P fimbria associated genes among two biofilm-forming groups was not significantly different. However, *papAH* was more prevalent among biofilm-producing UPEC isolates. In addition, phenotypic detection of P fimbria did not show significant difference either, indicating a less important role of P fimbria in biofilm development in UTIs.

Prevalence of F_1C and S fimbriae genes was also investigated in this study. Our results showed that the F_1C gene (*foc/G*) was not significantly different among the two biofilm-producing groups, while S fimbria-associated gene (*sfa*S) was significantly more prevalent among moderate to strong biofilm producers. In fact, no isolate belonging to group I harbored *sfa*S, while 42 isolates able to form moderate to strong biofilm contained the gene. This finding suggests strong association between S fimbria associated gene and biofilm formation ability among UPEC. S fimbria is a surface virulence factor with good binding efficacy to epithelial and endothelial cell lines derived from the lower human urinary tract and kidney and thus could be regarded as a determinant factor in biofilm formation by UPEC (1). In addition, the M fimbria gene (*bmaE*) was another AFG that showed a significant difference among the two biofilm-producing groups. In this study, *bmaE* was not detected in the strains with poor biofilm formation ability while 20% of the strains with good biofilm formation potential harbored the M fimbria associated gene. Thus, although the M fimbria gene was not highly prevalent among UPEC isolates, it could be regarded as another determinant gene in biofilm formation by UPEC.

Association of the aggregate virulence factor genes score (including many AFGs) and pathogenicity of UPEC has been investigated previously (13). Similarly, we found that UPEC isolates able to form moderate to strong biofilm had a higher median aggregate AFG score than those with poor biofilm formation ability.

Biofilm formation of UPEC is a major determinant in establishment of UTIs. In this study, we evaluated

the biofilm formation potential of UPEC isolates and introduced the association of *sfaS*, *papAH*, and *bmaE* genes with biofilm formation capability. AFGs associated with biofilm could be targets for prevention and treatment of UPEC-associated UTIs. Future studies must explore the function and expression level of the AFGs involved with biofilm and associate them with the genetic background of UPEC and also development of different types of UTIs.

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