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Evaluation of the Presence of *fimH* and *bfpA* Genes in *Escherichia coli* Isolated from Pregnant Women

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Authors
Fatemeh Keshavarzi, *phD*[1*]
Rozhin Rahmani, *MSc*[1]
Farouq Karimpour, *MSc*[2]

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[1] Department of biology, Sanandaj Branch, Islamic Azad University, Sanandaj, Iran
[2] Department of biology, Kurdistan Science & Research Branch, Islamic Azad University, Sanandaj, Iran

**** Correspondence***
Address: Department of Biology, Sanandaj Branch, Islamic Azad University, Sanandaj, Iran.
F.keshavarzi@iausdj.ac.ir

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ABSTRACT

Background: *Escherichia coli* (*E. coli*) is one of the most abundant bacteria in human and animal infections. Many virulence genes in *E. coli* intensify its infectivity. This study explored the presence of two pathogenic genes, including *fimH* and *bfpA,* in *E. coli* strains isolated from pregnant women.

Materials & Methods: From autumn 2016 to spring 2017, a total of 100 *E. coli* isolates were collected from clinical samples (116) of pregnant women. The strains were identified using biochemical tests (catalase, Simmons citrate, indole, mobility, H_2S, MR, VP, TSI, and urease). The presence of pathogenic genes in these isolates was examined using colony PCR method. Finally, the relationship between the gene and the site of infection was analyzed in SPSS-23 software.

Findings: PCR results indicated that out of 100 *E. coli* samples, 15 were *bfpA* positive (15%), and 64 were *fimH* positive (64%). A significant relationship was found between the presence of bfpA gene and samples taken from blood (p=.049), and stool (p<.001).

Conclusion: None of the urinary strains harbored the *bfpA* gene, while the strains isolated from stool had a significant relationship with the presence of *bfpA* gene (OR = 18.667), which confirms that this gene is of great importance for EPEC (enteropathogenic *E. coli*). There was also a significant relationship between blood-isolated strains and the presence of *bfpA* gene. A significant relationship was also found between the *fimH* gene and strains isolated from urine samples (OR=36.733), while no relationship was observed between the presence of *fimH* gene and blood-isolated strains.

Keywords: *Escherichia coli*, *fimH*, *bfpA*, EPEC.

CITATION LINKS

[1] Abbasi M, Aslani MM, Mostafavi E, Alikhani MY, Nikbin VS. Determination of adhesion virulence factors of enteropathogenic Escherichia coli (eaeA-, bfpAA-) isolates from asymptomatic individuals compared to... [2] Abdi A, Rashki A. Comparison of virulence factors distribution in uropathogenic E. coli isolates from... [3] Abed ZA, Habib KA, Abass ZN. Genotypic study of two virulence factors fimH and kpsMTII in uropathogenic Escherichia coli isolates from children patients with urinary tract infections. Baghdad Sci J. 2014;11(4):1475-80... [4] Bieber D, Ramer S, Wu C, Murray W, Tobe T, Fernandez R, et al. Type IV pili, transient bacterial aggregates, and virulence of... [5] Chen HD, Frankel G. Enteropathogenic Escherichia... [6] Choudhury D, Thompson A, Stojanoff V, Langermann S, Pinkner J, Hultgren SJ, et al. X-ray... [7] Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay B. B. Recent advances in understanding enteric... [8] Hyland RM, Sun J, Griener TP, Mulvey GL, Klassen JS, Donnenberg MS, et al. The bundlin pilin protein of... [9] Khursigara C, Abul-Milh M, Lau B, Giron J, Lingwood C, Foster DB. Enteropathogenic Escherichia coli virulence factor bundle-forming pilus has... [10] Klemm P. Fimbriae adhesion, genetics... [11] Klemm P, Schembri MA. Bacterial adhesins: Function ... [12] Giron JA, Ho A, Schoolnik GK. An inducible bundle-forming pilus of... [13] Foxman B. The epidemiology of... [14] Rono SJ, Kakai R, Esamai F, Mibei E, Odundo E. Pathotypes and virulence markers in Escherichia coli associated with diarrhea among HIV seropositive and seronegative children... [15] Saldana Z, Erdem AL, Schüller S, Okeke IN, Lucas M, Sivananthan A, et al. The Escherichia coli common pilus and the bundle-forming pilus act in concert during the formation of localized adherence by enteropathogenic... [16] Schembri MA, Klemm P. Heterobinary adhesins based... [17] Sidhu JP, Ahmed W, Hodgers L, Toze S. Occurrence of virulence genes associated with diarrheagenic pathotypes in Escherichia coli isolates from... [18] Tarchouna M, Ferjani A, Ben-Selma W, Boukadida J. Distribution of uropathogenic virulence genes in Escherichia coli isolated from patients with urinary tract... [19] Thumbikat P, Berry RE, Zhou G, Billips BK, Yaggie RE, Zaichuk T, et al. Bacteria-induced... [20] Tiba MR, Yano T, Leite DD. Genotypic characterization of virulence factors in... [21] Tobe T, Sasakawa C. Role of bundle-forming pilus of enteropathogenic Escherichia coli in host cell adherence and in microcolony...

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Introduction

Among all bacterial uropathogens, *Escherichia coli* (*E. coli*), as a Gram-negative bacillus of the normal intestinal flora, is the most prevalent organism isolated from about 75 to 90% of outpatients with UTI (urinary tract infection) [13]. Due to the relatively high prevalence of this bacterium in urine specimens as well as the high number of *E. coli* infections with or without clinical symptoms, it is necessary to investigate the virulence factors of this bacterium in urinary tract infections.

Enteropathojenice *E. coli* (EPEC) has been introduced as one of the most important causes of diarrhea in children in developed countries. EPEC bacteria attach to the mucous membranes of enterocytes, destroying them and causing diarrhea. The disease is rare in older children and adults, probably because of their immunity. Diarrhea is manifested by malabsorption due to the destruction of microvilli. *E. coli* diarrhea in children under one year of age is mainly caused by enteropathogenic isolates [5][7] .

FimH is an adhesive protein that binds to mannose-containing glycoprotein receptors, which are known as uroplakins and located on the surface of bladder epithelial cells (BECs). This attachment exposes BECs to attacks [19]. Indeed, type 1 fimbriae are the most well-known bacterial adhesion organelles sensitive to mannose. At the first, this bacterium synthesizes a protein(proenzyme) with 300 amino acids, then by separating several peptides, finally a protein with 179 amino acids will remain. Mature FimH is positioned on the surface of the bacterial cell as part of type 1 fimbriae organelle [11].

In 1999, the structure of FimH was identified via X-ray crystallography. FimH comprises two domains: the N-terminal lectin domain deals with surface identification, and the C-terminal pilin domain plays the major role in completing the fimbriae structure. A ring-type tetrapeptide connects these two domains [6, 16]. Adhesins could be used to make vaccines, and anti-adhesins could be made for FimH protein [10].

Bundle-forming pili (BFP) belong to type IV pili. This cluster includes *bfpA-bofL, bfpAP,* and *bfpAU* genes and is encoded on EPEC adherence factor (EAF) plasmid. The EAF plasmid also contains a plasmid-encoded regulator (PER) whose products help regulate the BFP operon and many genes in LEE using the LEE-encoded_regulator (LER). Structurally, they belong to class IV-B pili that are produced by ETEC (enterotoxigenic *E. coli*), *Salmonella typhi,* and *Vibrio cholera*. In general, all genes in the BFP cluster are important for bacteria, as they are required for the adhesion of bacterial cells as well as the establishment of spontaneous aggregation phenotype. In other words, BFP is responsible for microcolony formation and bacterial-bacterial interaction [12, 21]; BFP has also been shown to be necessary for pathogenicity [4].

The BFP operon is required for microcolony formation, which is the early stage of biofilm formation. There is evidence that type IV BFP contributes to the formation of microcolony, triggers bacterial-bacterial interactions, and is a virulence factor in human infections. Some studies have suggested that initial binding to host cells is mediated by BFP and EspA filament, but data on cellular receptors and their relationship with BFP are inconclusive [15]. Khursigara et al. (2001) reported that *bfp* gene cluster mediates adhesion to host cells by identifying phosphoethanolamine in host cells [9]. Various studies have considered BFP gene cluster as a virulence factor for EPEC. Hyland et al. (2008) observed that alpha *bfpA* of EPEC strains had lectin-like properties that could mediate EPEC initial adhesion to N-acetyllactosamine receptors on host cells [8]. Thus, evidence supports that the *bfpA* gene plays a major role in the binding and initiation of biofilm formation.

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Objectives: The present study aimed to determine the abundance of *fimH* and *bfpA* pathogenic genes in *E. coli* strains isolated from clinical samples of pregnant women hospitalized at maternity wards.

Materials and Methods

Sample collection: In this descriptive laboratory study, a total of 100 *E. coli* isolates were collected from clinical samples (116 samples) of pregnant women with suspected *E. coli* infection from autumn 2016 to spring 2017. In order to better determine the prevalence of the desired genes, in addition to urine samples, stool and blood samples were also collected.

Phenotypic bacterial identification: Phenotypic identification was carried out by culturing each sample on three culture media, including EMB, Blood agar, and McConkey agar. Furthermore, biochemical tests were performed to identify samples, including catalase, citrate, mobility, indole in the SIM culture medium, MR-VP, TSI, and urease tests.

Maintenance of isolates: The identified specimens were stored in Luria Bertani (LB) broth. LB medium was prepared, of which 800 µL was poured into 1.5 µL vials and autoclaved for 10 min at 121 °C and a pressure of 15 psi. Then a few pure colonies were taken from each positive sample on solid medium, inoculated into sterile medium, completely dissolved, and kept at 37 °C for 2 hours. Then 200 µL of glycerol was poured in vials containing 800 µL of the LB medium wherein bacteria were grown. Using this method, these specimens could be stored for at least 6 months at -20 °C, then they could be transferred to a new subculture.

Identifying the desired virulence genes

Active culture preparation: The prepared LB medium was placed at ambient temperature. A full loop of bacterial colonies cultured on the nutrient agar, was removed and injected into the LB liquid medium. The LB medium was incubated at 37 °C for 24 hours in a shaking incubator at 130 rpm. The grown bacterial colonies in this culture medium were used for colony-polymerase chain reaction (PCR) method. Colony-PCR is an efficient method for screening intact bacteria regarding the presence of the desired DNA sequences. This method makes the use of PCR even more applicable in automation, screening, and detection processes.

Molecular experiments: Colony-PCR Table 1 shows the sequence of primers designed using Primer 3 software (*fimH* GenBank: JF289169.1, *bfpA* GenBank: KJ641933.1). The colony PCR was performed for each sample (including negative control) using a thermocycler (Germany, 96 Primus) with a final volume of 25 µL containing 1.5 U of Taq DNA polymerase (CinnaGen, Iran), 0.4 µM F and R primers each, 2.5 µL of 10× buffer containing 15 mM $MgCl_2$, 2.5 µL of dNTP, 0.2 mM nucleotides each, and some bacterial colonies. The PCR programs for each gene are presented in Table 2. To ensure the correct replication of the desired pieces, the

Table 1) Primer sequence of *FimH* & *bfpA* and PCR products length

Gene	Primer Sequence		PCR Product Length	Tm °C
fimH	Forward	5´- CGCCGCGGGTTGTTTATAAT -3´	358 bp	58
	Reverse	5´- AAAATCGAGTTGCCCGCATC -3´		
bfpA	Forward	5´- GGTTGGATAAAGCGGCATGT -3´	124 bp	57
	Reverse	5´- GCCTGATCAGCGCTATTACC -3´		

Table 2) PCR proliferation conditions

	Cycling Conditions				
Genes	**Initial Denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final Extension**
fimH	95°C 7 min	94°C 60 s	58°C 60 s	72°C 45 s	72°C 5 min
Repeated for 34 cycles					
bfpA	95°C 7 min	94°C 60 s	57°C 60 s	72°C 60 s	72°C 5 min
Repeated for 35 cycles					

PCR products were loaded on 3% agarose gel, and their quality was determined using a gel doc.

Statistical analysis: The obtained data were analyzed by SPSS software Ver. 23 using logistic regression. The results were calculated at a confidence interval of 95%. A *p* value <.05 was considered as statistically significant.

Findings

Based on the results of biochemical tests (positive for catalase, indole, mobility, and MR and negative for Summon citrate, H2S, VP, and Urease), among 116 clinical specimens collected from pregnant women, a total of 100 specimens were *E. coli* positive. Figure 1 shows the frequency of strains based on their isolation site.

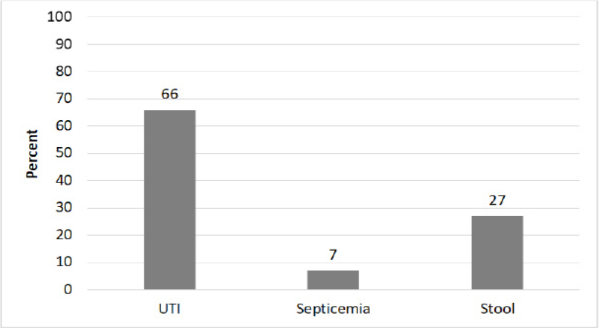

Figure 1) The frequency of strains based on their isolation site

Out of 116 clinical samples, 100 *E. coli* strains were collected and studied. Specifically, 66, 7, and 27% were isolated from urine, blood, and stool samples, respectively (Figure 2). Among which, 15 (15%) and 64 (64%) isolates harbored *bfpA* and *fimH* genes, respectively. Because virulence genes are related to uropathogens, the prevalence rate of *bfpA* and *fimH* virulence genes was 44.44 and 93.93% among urinary isolates, respectively; no strain harbored these two genes simultaneously. Figures 3 and 4 show images of PCR products related to *bfpA* and *fimH* genes, belonging to a number of samples, on 3% agarose gel and compare *E. coli*-negative and positive strains, respectively. The bands created for *bfpA* and *fimH* genes were 124 and 358 bp compared to molecular weight marker 100bp, respectively.

The relationship between the presence of genes and the site of infection and isolation was investigated using logistic regression analysis. The results demonstrated that the *bfpA* gene had a significant relationship with septicemia (*p*=.049) and stool samples (*p*<.001) (Table 3). The relationship in UTI samples was not analyzed using logistic regression, because they lacked the *bfpA* gene. Rather, this relationship was studied by Pearson's Chi-square test. The results also showed that the *fimH* gene had a significant relationship with UTI (p0.001<) and stool samples (p0.001<), but not with septicemia (Table 4).

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Table 3) The relationship between infection site and the presence of *bfpA* gene was analyzed by SPSS software Ver. 20 using logistic regression. The results demonstrated that the *bfpA* gene had a significant relationship with septicemia (*p* =.049) and stool samples (*p*<.001).

Site of Infection	Exp(B)	CI (95%) for EXP(B)		*P*-Value
		Upper	Lower	
UTI	------	----	-----	<0.001
Septicemia	5.062	25.455	0.007	.049
Stool	18.667	74.391	4.684	<0.001

Table 4) The relationship between infection site and the presence of *fimH* gene was analyzed by SPSS software Ver. 20 using logistic regression. The results showed that the *fimH* gene had a significant relationship with UTI (*p*0.001<) and stool samples (p0.001<), but not with septicemia.

Site of Infection	Exp(B)	CI (95%) for EXP(B)		sig.
		Upper	Lower	
UTI	36.733	119.675	11.275	<0.001
Septicemia	0.805	3.808	0.170	.784
Stool	0.008	0.061	0.001	<0.001

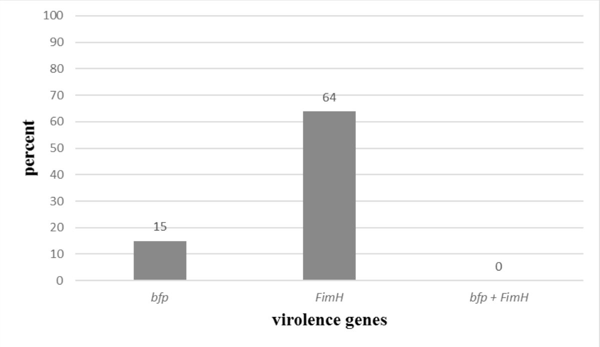

Figure 2) Frequency of virulence genes studied in this study in terms of percentage: among the isolates, 15 (15%) and 64 strains (64%) harbored *bfpA* and *fimh* genes, respectively. No strain harbored these two genes simultaneously.

Regarding the relationship between the presence of *bfpA* gene in blood samples, the odds ratio (OR) was 5.062 (Table 3 and 4). Therefore, it could be inferred that strains isolated from blood samples were 5.062 times more likely to have the *bfpA* gene. In the case of the relationship between the presence of the *bfpA* gene and stool samples, the OR value was 18.667 (Table 3), which indicates that strains isolated from stool samples were 18.667 times more likely to have the *bfpA* gene. Regarding the relationship between the presence of the *fimH* gene and urine samples, the OR value was 36.733 (Table 4), indicating that strains isolated from urine samples were 36.733 times more likely to have the *fimH* gene. However, regarding the relationship between the presence of the *fimH* gene and stool samples, the OR value was 0.008 (Table 4), which reflects a different kind of relationship; thus, strains isolated from stool samples had a very low probability of coexisting with the *fimH* gene.

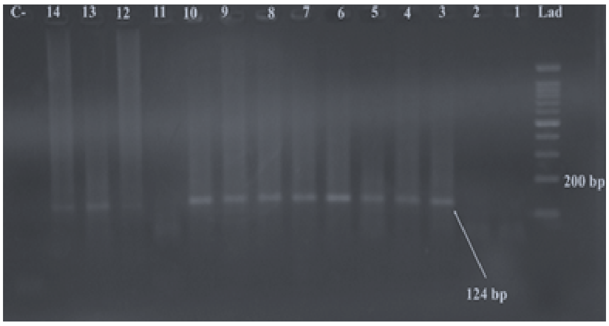

Figure 3) The electrophoretic pattern image of PCR products related to *bfpA* gene. The length of amplified fragment by primers used is 124 bp compared to molecular weight marker 100bp. Lane ladder: molecular weight marker (100 bp), Lane 1 and 2: negative control (water), Lane 3: positive control, Lane 4 to 14: a number of samples, and Lane C: an E. *coli* negative strain, respectively. As the picture shows, except samples 1, 2, and 11, all other samples are positive for *bfpA* gene.

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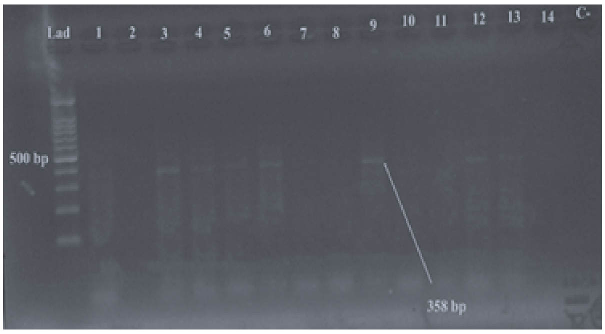

Figure 4) The electrophoretic pattern image of PCR products related to *fimH* gene. The length of amplified fragment by primers used is 358 bp compared to molecular weight marker 100 bp, Lane ladder: molecular weight marker (100 bp), Lane 1 and 2: negative control (water), Lane 3: positive control, Lane 4 to 14: a number of samples, Lane C: an *E. coli* negative strain, respectively. As the picture shows, except samples 7, 8, 10, 11, and 14, all other samples are positive for *fimH gene*.

Discussion

In various studies and sources, BFP has been considered as a virulence factor in enteropathogenic *E. coli*. In this study, the *bfpA* gene was not found in strains isolated from urine samples, and the observed significant difference in urinary strains was due to the fact that none of the strains harbored the *bfpA* gene. However, biofilm formation is very important for those bacteria causing urinary tract infections. Europathogenic *E. coli* isolates that do not have the bfp gene may use other biofilm formation mechanisms; it should be considered that the receptors required for BFP binding to urinary tract cells may be absent, and binding via BFP may not be possible.

The strains isolated from the stool samples of patients with intestinal infection had a significant relationship with the presence of *bfpA* gene (OR = 18.667), which confirms that *bfpA* is of great importance for EPEC.

On the other hand, the results showed a significant relationship between blood-isolated strains and the presence of *bfpA* gene. Blood does not have any germs unless the patient has septicemia, which allows the germs to enter the blood. In this case, bacteria from various infections in different parts of the body could enter the blood. Therefore, blood-isolated strains could be related to any tissue such as intestine, which justifies the significance of blood samples data. It could be inferred that strains enter the blood through the intestine due to factors such as intestinal injuries and internal surgeries that involve bleeding. In general, *bfpA*-carrying strains isolated from the blood are associated with EPEC. Since the main mechanisms of EPEC are binding and degradation, those isolates that lack *bfpA* take advantage of other mechanisms. In this regard, some studies have reported a high incidence of *bfp* gene in these isolates [1].

It could be said that the *fimH* gene is related to uropathogenic strains. According to the results, there was a significant relationship between the *fimH* gene and strains isolated from urine samples (OR = 36.733). Also, the *fimH* gene was present in one strain isolated from stool. It should be said that the subject's stool might have been contaminated with uropathogenic strain due to the specific physiology of women (i.e., proximity of the urethra to the rectum). The OR obtained for fecal strains (0.008) indicated that the *fimH* gene and fecal strains were inversely correlated.

Abdi and Rashki (2014) compared the prevalence of pathogenic factors in two phylogenetic groups (i.e., B2 and D) of uropathogenic *E. coli* and reported a 100% frequency for *fimH* gene in both groups. The lowest prevalence was related to *troN* gene, which had a frequency of 40 and 9% in groups B2 and D, respectively [2]. In the present study, the frequency of *fimH* gene in uropathogenic strains was 93.93%, while Abdi reported a 100% frequency for this gene. This slight difference could be related to the differences in time and geographical location of sampling.

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Tarchouna et al. (2013) examined the spread of pathogenic genes in uropathogenic *E. coli* isolated from patients with urinary tract infections. They observed a frequency of 68, 41, and 34% for *fimH, pap,* and *sfa/foc* genes, respectively. The prevalence of *fimH* in their study was lower than in the present study, which could be explained by geographical variations [18].

Rono et al. (2014) explored seven pathogenic genes in *E. coli* isolated from diarrhea of people with HIV-positive and HIV-negative serology in Kenya. Their results revealed that the frequencies of *ipaH* and *bfpA* genes were 66.7 and 33.3% among HIV-positive cases and 33.3 and 66.7% among HIV-negative cases, respectively [14]. The prevalence of *bfpA* gene in the present study was 44.44%, which is close to that reported by Rono.

Abed and colleagues examined the prevalence of two pathogenic genes in uropathogenic *E. coli* strains isolated from children. Their results suggested a prevalence rate of 90 and 72% for *fimH* and *kpsMTII* genes, respectively [3]. These results are similar to the current study results, indicating a 93.93% prevalence for *fimH* gene.

Tiba and colleagues (2008) identified various virulence factors in *E. coli* strains isolated from patients with cystitis. They found that *fimH, cnf1, usp, hly,* and *sfa* genes had a frequency of 97.5, 18.5, 22.2, 25.3, and 27.8%, respectively [20]. This is compatible with this study results as far as the *fimH* gene is concerned.

Sidhu et al. (2013) investigated virulence genes associated with diarrhea-causing *E. coli* strains isolated from surface water. They isolated 300 *E. coli* samples from six regions and examined 11 pathogenic genes in them. *ipaH* and *bfpA* genes were reported to have a frequency of 14 and 24%, respectively [17]. The frequency of *bfpA* in the present study was higher than that found by Sidehu et al. (2013), which is reasonable because the prevalence of genes varies across different regions.

Conclusion

The observed significant difference in urinary strains was due to the fact that none of the strains harbored the *bfpA* gene. The strains isolated from stool samples had a significant relationship with the presence of *bfpA* gene (OR = 18.667), which confirms that this gene is of great importance for EPEC. There was also a significant relationship between blood-isolated strains and the presence of *bfpA* gene. Also, the results showed a significant relationship between the *fimH* gene and strains isolated from urine samples (OR = 36.733). The *fimH* gene was present in one strain isolated from stool. The OR obtained for fecal strains (0.008) indicated that the *fimH* gene and fecal strains were inversely correlated. However, no relationship was found between the presence of *fimH* gene and blood-isolated strains.

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Ethical Permission: The study was ethically approved by Research Committee of Department of Biology, Sanandaj Branch, Islamic Azad University, Sanandaj, Iran(P-8688).

Conflicts of Interests: The authors declare that there is no conflict of interests with the organization that sponsored this research and publications arising from this research.

Authors Contribution: Conceptualization: FK; Data curation:RR; Formal analysis: FK; Funding acquisition: RR; Investigation: RR, FK; Methodology: RR;
Project administration: FK; Resources: FK; Software:FK; Supervision: FK; Writing of the original draft: FK; writing review and editing: FK.

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