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Evaluation of *pap* and *sfa* Genes Relative Frequency P and S Fimbriae Encoding of Uropathogenic *Escherichia coli* Isolated from Hospitals and Medical Laboratories; Yasuj City, Southwest Iran

Mostafa Boroumand [1], Asghar Sharifi [2,*], Leila Manzouri [3], Seyed Sajjad Khoramrooz [2] and Seyed Abdolmajid Khosravani [2]

[1] Student Research Committee, Yasuj University of Medical Sciences, Yasuj, Iran
[2] Cellular and Molecular Research Center, Yasuj University of Medical Sciences, Yasuj, Iran
[3] Social Determinants of Health Research Center, Yasuj University of Medical Sciences, Yasuj, Iran

[*] *Corresponding author*: Professor of Microbiology, Cellular and Molecular Research Center, Yasuj University of Medical Sciences, Yasuj, Iran. Email: asgharsharifi@yahoo.com

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Abstract

Background: Urinary tract infections (UTI) are one of the common infections worldwide. *Escherichia coli* is the most common bacteria causing UTI.

Objectives: The current study aimed at investigating the susceptibility pattern, biofilm formation, and the relationship between *pap* and *sfa* virulence genes and biofilm formation in *E. coli* strains isolated from patients with UTI from 2017 to 2018 in Yasuj City, Iran.

Methods: In the current cross-sectional study, 130 *E. coli* strains were isolated from patients with UTI. Antimicrobial susceptibility testing was performed by disk diffusion method. The presence of *pap* and *sfa* genes was investigated by multiplex polymerase chain reaction (PCR). Biofilm formation was tested using microtiter plate assay and ELISA (enzyme-linked immunosorbent assay) reader for *E. coli* isolates.

Results: Out of 130 *E. coli* strains isolated from UTI samples, 98 (75.38%) were positive for the biofilm formation, (14% strong, 31% moderate, and 53% weak). The *pap* and *sfa* genes were detected in 50% and 29% of the isolates, respectively. Majority of the isolates were resistant against ampicillin (80%) and susceptible to amikacin and meropenem (100%); the *pap* and *sfa* genes were detected in 92.85% and 85.71% of the biofilm-forming isolates, respectively. These genes in Uropathogenic *E. coli* (*UPEC*) have a significant relationship with strong biofilm formation ($P < 0.0001$).

Conclusions: The obtained results indicated that the frequency of *pap* gene was higher than that of *sfa* in *E. coli* strains isolated from patients with UTI in Yasuj. The *pap* and *sfa* genes in *E. coli* strains had a significant relationship with strong biofilm formation. In addition, the frequency of biofilm formation in antibiotic-resistant *E. coli* was higher than that of antibiotic-sensitive strains. According to the results of the current study, it is recommended to administer Amikacin or Meropenem in Yasuj to treat UTI.

Keywords: Antibiotic Resistance, Biofilm, *Escherichia coli*, Genes, Urinary Tract Infection, Uropathogenic, Virulence

1. Background

The urinary tract infection (UTI) is one of the most common community- and hospital-acquired infections with the huge economic burden (1). The presence of microbial pathogens in the urethra, kidney, ureter, and bladder is called UTI (2). It is accompanied by inflammatory responses to the urinary tract (3). One of the most abundant (about 90%) bacteria that cause UTI and asymptomatic bacteriuria (ABU) in humans is the common uropathogenic *Escherichia coli* (UPEC) (2, 4). It causes sickness among both genders and all age groups, but its prevalence is higher among females due to hormonal effects and anatomical conditions of the urinary tract (5). The severity of UTI depends on two factors: bacterial virulence factors and host susceptibility. The virulence factors of *E. coli* such as fimbriae and toxins in the iron acquisition systems by overcoming the host immune system, biofilm formation, and tissue damage can contribute to colonization and pathogenicity (6). UPEC can produce various adhesion types of fimbriae to diagnose and bind to epithelial cellular receptors of the urethra, including fimbriae P and fimbriae S (7, 8). *P* fimbriae is composed of six subunits as PapG, PapF, PapE, PapK, PapA, and PapH proteins encoded by a bunch of genes called *pap* (pyelonephritis-associated

pili) (9). This fimbria helps bacterial pathogenicity by facilitating colonization, preventing bacterial removal by urine filtration, and increasing the proliferation and invasion of kidney tissues (10). P fimbria is a mannose-resistant adjuvant and a predominant phenotype encoded by the pap gene associated with pyelonephritis in adolescents and children infected with *E. coli* caused by pyelonephritis (11). The fimbria S is encoded by the *sfa* gene. *S* fimbriae mediate interaction with receptors containing sialic acid, bladder epithelium, and urethral ducts, and facilitate pathogenicity at the host tissue surface (11, 12). Sepsis and meningitis are caused by common UPEC (13). Biofilm formation is the pathogenic process that allows *E. coli* to be maintained in the urinary system and prevents the loss of bacteria (14). Bacterial biofilm is a consortium of sticky bacteria growing on inanimate or animate surfaces surrounded by a polysaccharide matrix. Biofilms reduce the sensitivity of bacteria to antibiotics and are involved in the food exchange, as well as the exchange of genetic materials such as the plasmid from one bacterium to another, and plasmid exchange leads to antimicrobial resistance development (15). The development of bacterial biofilms leads to a major challenge for the improvement of patients due to increased resistance to antimicrobial agents and host defense system (16). Among the members of the Enterobacteriaceae family, especially *E. coli*, the emergence and spread of antimicrobial resistance is increasing worldwide. The antimicrobial resistance of *E. coli* reduces therapeutic options and leads to increased treatment costs and mortality (7). In each geographical region, the differences in the type of antibiotics administered can be attributed to the different resistance patterns and antibiotic susceptibility across pathogens (17).

2. Objectives

Regarding the high prevalence of UTI and the role of fimbriae and the formation of biofilms in the development of this infection, to the current study aimed at investigating the association of biofilm formation in strains isolated from uropathogenic *E. coli* with *pap* and *sfa* genes and also its antibiotic resistance pattern in patients with UTI in Yasuj, southwest of Iran. It is hoped that the findings of the current study could be effective in the planning of screening and appropriate therapeutic interventions for healthcare providers in choosing the appropriate treatment option and reducing the cost of treatment.

3. Methods

3.1. Sample Size and Sampling Method

Regarding the frequency of *fim A* and *pap C* genes, 43% and 94% in the *E. coli* isolated from urine samples (6), the sample size was determined 130 based on the prevalence formula and considering P = 43%, α = 0.05 and d = 0.2, n = 128.9.

$$n = \frac{(z_1 - \alpha/2)^2 P(1-P)}{d^2} \quad (1)$$

In the current cross sectional study, suspected *E. coli* strains isolates from urine specimens obtained from patients with UTI (10^5 CFU/mL of urine) in Imam Sajjad and Shahid Beheshti hospitals, as well as Pasteur diagnostic, Azizi, and Dr. Yazdanpanahe labs in Yasuj, Southwest of Iran were randomly selected, based on the number of urine cultures present in each medical center in the period of five months from July to November 2017; the proportion of the sample was multiplied. Since in each laboratory, urinary cultures were checked by more than one expert to ensure *E. coli* growth, the specimens were finalized and confirmed by the researcher and entered into the study. Based on the study design, systematic random sampling was used to determine the sample size. The population of the study included outpatients with UTI referring to medical labs for urine culture; the growth of *E. coli* was considered as a positive result. Exclusion criteria were having an indwelling urinary catheter, being pregnant, having genitourinary abnormalities, and antibiotic therapy within the last two weeks. Suspected *E. coli* isolates were transferred to the Microbiology Lab of the Yasuj University of Medical Sciences for diagnosis and confirmation. They were cultured on eosin methylene blue (EMB) agar. After 24 hours of incubation at 37°C, the colonies were confirmed with biochemical complementary tests such as triple sugar iron agar (Merck, Germany), indole production, Simmons' citrate, methyl red and Voges-Proskauer, and urea hydrolysis. The isolates were subcultured in vials containing trypticase soy broth (Merck, Germany) and after 18 to 24 hours incubation and growth of the bacteria, one to two drops of 20% sterilizer glycerol were added to the culture and the stock was stored at -20°C (18).

3.2. Antimicrobial Susceptibility Testing

For this purpose, antibiotic discs (BD-BBL Company, American) were used. Antibiotic susceptibility testing was performed by the Kirby-Bauer the disk diffusion method using a bacterial suspension with standard 0.5 McFarland turbidity and according to 2018 CLSI clinical and laboratory standards. The 0.5 McFarland standard turbidity was prepared by solving the bacteria in normal saline. The suspension was cultured on Muller-Hinton agar and then standard antibiotic discs were placed on the culture with standard spacing and incubated at 37°C for 24 hours. *E. coli* ATCC 25922 was used as a quality control strain. The standard antibiotic discs used in the study were amikacin (30 μg), ampicillin (10 μg), imipenem (10 μg), cefotaxime

(30 μg), ceftriaxone (30 μg), gentamicin (10 μg), nitrofurantoin (300 μg), nalidixic acid (30 μg), meropenem (10 μg), cotrimoxazole (25 μg), tetracycline (30 μg), aztreonam (30 μg), ciprofloxacin (30 μg), and ceftizoxime (30 μg) (18, 19). The study was approved by the Ethics Committee of Yasuj University of Medical Sciences (ethical code: IR.YUMS.REC.1396.81).

3.3. Detection of Biofilm Formation by Microtiter Plate Method

Biofilm formation in UPEC was investigated by microtiter plate assay, according to previous methods with some modifications that was repeated in triplicate. At first, 24-hour culture of *E. coli* was prepared by inoculation of a bacterial colony into 5 mL of Tryptic soy broth and incubation at 37°C for 24 hours. A bacterial suspension adjusted to 0.5 McFarland standard turbidity was prepared; 200 μL of diluted suspension was added to each well of the microplate (Maxwell, China) and incubated for 24 hours at 37°C. After washing with normal saline for three times, 200 μL of 96% ethanol was added to the wells to fix the cells; then the contents of the wells were drained (after 15 minutes) and the plate was dried at room temperature. The staining step was performed by adding 200 μL of crystal violet 2% for five minutes. After removing the color of the crystal with water, 200 μL of 33% acetic acid was added to each well and incubated at 37°C for 15 minutes; then the optical density of wells stained with 2% crystal violet was read by the ELISA Plate Reader at 492 nm (20). The biofilm-formation ability of *E. coli* strains was interpreted according to the criteria introduced by Stepanovic et al. (21).

3.4. DNA Extraction

DNA genome extraction was performed using a boiling method described by Yu et al. with some modifications (22). For this purpose, first, several colonies of *E. coli* were dissolved in 300 μL of distilled water and boiled for 10 minutes at 95°C. The centrifugation (Sigma, Germany) was carried out for 10 minutes at 12,000 rpm. Then 100 μL of supernatant was stored after centrifugation as a DNA template at -20°C for multiplex PCR. In the present research, *pap* and *sfa* genes were detected by multiplex PCR with primers described by Tajbakhsh et al. (23). The specific primers are shown in Table 1.

3.5. Multiplex PCR Assay

To perform multiplex PCR, first, the reaction mixture was prepared in a 1.5-μL microtube. Then the number of required samples was calculated according to the samples size. The reaction mixture was prepared in a total volume of 25 μL containing 12.5 μL of master mix (Ampliqon, Denmark), 1 μL (10 pmol/L) of each primer (forward and reverse), 5 μL of bacterial DNA, and 3.5 μL of nuclease-free water. In the current study, strains harboring *pap* and *sfa* genes were used as the positive control in order to check the accuracy of each test and sterile distilled water was used as the negative control. Gene amplification was performed in a thermocycler device (Bio-Rad T100, Singapore); the multiplex PCR program was as follows: initial denaturation one cycle at 94°C for one minute, followed by 30 cycles; denaturation at 94°C for one minute; annealing at 63°C for 30 seconds; extension, 72°C for 90 seconds and the final extension, one cycle at 72°C for five minutes (23). The PCR products were electrophoresed (Major Science MP300, Taiwan) on 1% agarose gel (Pishgam, Iran). Then the final products were stained with SafeStain (Pishgam, Iran) and visualized by Gel Documentation (Major Science, Taiwan). Electrophoresed PCR products are shown in Figure 1.

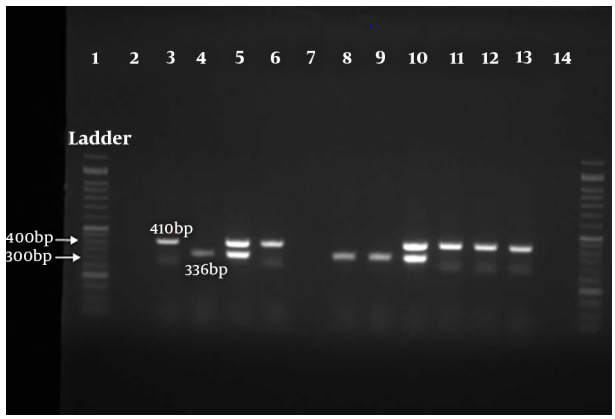

Figure 1. Multiplex PCR Results for *pap* and *sfa* Genes in Uropathogenic *E. coli* Isolates. Lane 1, Ladder (50 - 1000 bp); Lane 2, negative control; Lane 3, positive control for the 410-bp *sfa* gene; Lane 4: positive control for the 336-bp *pap*; Lanes 5 and 10, positive controls for *pap* and *sfa* genes; Lanes 6, 11, 12 and 13, harbored *sfa* genes, Lanes 8 and 9, harbored *pap* gene.

3.6. Statistical Analysis

Data analyses were performed using SPSS Statistics for Windows, version 16.0 (SPSS Inc., Chicago, Ill., USA). Nominal variables were expressed as mean or frequency and compared by chi-square test. All statistical tests were based on two-tailed probability. P value < 0.05 was considered statistically significant.

4. Results

The current study was conducted on 130 strains of *E. coli* isolated from patients with UTI referring to hospitals and medical laboratories in Yasuj from 2017 to 2018. Age range of the patients with UTI in the current study varied from one month to 87 years (mean: 33.05 $\pm$ 27.67). In terms of gender, 30 (23.07%) and 100 (76.9%) isolates were

Table 1. The Primers Used for *pap* and *sfa* Genes Detection in Strains Suspected of Uropathogenic *Escherichia coli*

Target Gene	Primer Sequence (5' → 3')	AmpliconLength, bp	Reference
***pap* F**	GCAACAGCAACGCTGGTTGCATCAT	336	(23)
***pap* R**	AGAGAGAGAACTGGGTGCATCTTAC		
***Sfa* F**	CTCCGGAGAACTGGGTGCATCTTAC	410	(23)
***Sfa* R**	CGGAGGAGTAATTACAAACCTGGCA		

obtained from male and female subjects, respectively (Figure 2). In terms of the prevalence of virulence genes in the 130 *E. coli* isolates by multiplex PCR, 66 strains (50.76%) harbored operon *pap* and 38 (29%) isolates carried the *sfa* gene. In Table 2 the molecular analysis results of *pap* and *sfa* genes are shown. Antibiotic susceptibility and resistance pattern showed that the highest resistance was found against ampicillin (79.23%) in 103 isolates and the lowest resistance was observed to nitrofurantoin (2.04%) and imipenem (0.76%).

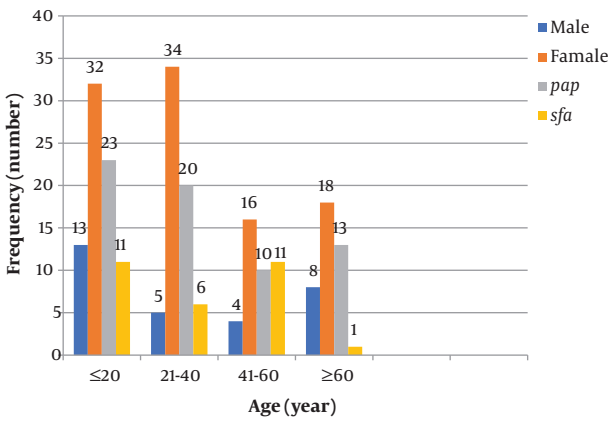

Figure 2. Distribution of the genes based on age and gender

According to the results of the study, 79 isolates (60.8%) were resistant against tetracycline, 78 (60%) against cotrimoxazole, 73 (56%) against aztreonam, 64 (49.2%) against nalidixic acid, 57 (43.8%) against ceftriaxone and cefotaxime, 58 (44.6%) against ceftizoxime, 48 (36.92%) against ciprofloxacin, 16 (8.16%) against gentamicin, and the highest antibiotic susceptibility (100%) was to amikacin and meropenem. In the current study, there was no significant relationship between gender and biofilm formation ($P > 0.744$). The current study results showed that using microtiter plate assay, 98 (75.38%) *E. coli* strains isolated from patients with UTI were biofilm producer. Biofilm-production ability based on OD was classified into three groups of strong, moderate, and weak. Of the 98 biofilm-producing *E. coli* strains, 14 (14%) were strong, 31 (31%) moderate, and 53 (53%) weak biofilm producers. However, 32 (24.61%) isolates did not show biofilm formation. A statistically significant association was observed between the formation of strong biofilms and *pap* and *sfa* genes ($P < 0.0001$). The results of microtiter plate assay and the prevalence of *pap* and *sfa* genes in the isolates are shown in Table 2. Among the positive biofilm isolates, the highest resistance was observed against ampicillin (80%), followed by cotrimoxazole (63.63%), tetracycline (60.2%), aztreonam (58%), nalidixic acid (52%), ceftriaxone, cefotaxime (46.9%), and ceftizoxime (45.9%). In the current study, the correlation between biofilm production and antibiotic resistance was found statistically significant (P value < 0.05) in the antibiotics such as cefotaxime and ceftriaxone. But the correlation between biofilm production and other antibiotics wasn't found statistically significant ($P > 0.05$).The pattern of antibiotic resistance in the studied biofilm-producing *E. coli* is shown in Figure 3.

5. Discussion

Among UTI-inducing bacteria, *E. coli* is the most important and common agent in ambulatory and hospitalized patients. The most important stage in *E. coli* pathogenicity is the bacterial adherence to the uroepithelial cells. This connection and colonization is handled by the fimbriae. The most important fimbriae of UPEC are *P*, *S*, *fis*, and *Dr*. Further understanding and recognizing the pathogenicity of microorganisms could help physicians to predict the infection behavior. Today, various molecular studies are conducted to identify the frequency of these virulence factors in *E. coli* (1, 24). In the current study, the prevalence of *E. coli* was higher in females than males, which could be due to hormonal effects and anatomical conditions of the urinary tract in females. These results were consistent with those of the studies by Jadhav et al., (25) and Tabasi et al. (26). The current study indicated that the prevalence of *pap* and *sfa* adhesive genes among *E. coli* strains isolated from urine samples obtained from patients with UTI in hospitals and medical laboratories of Yasuj were 50.76% (n = 66) and 29% (n = 38), respectively, using multiplex PCR. High frequency of fimbriae P in the current

Table 2. Biofilm Formation Results and the Prevalence of *pap* and *sfa* Genes in the Studied Isolates[a]

Virulence Gen	Biofilm Reaction				P Value
	High Biofilm 14	Moderate Biofilm 31	Weak Biofilm 53	Non Biofilm 32	
pap	13 (92.85)	21 (67.74)	24 (45.28)	8 (25)	< 0.0001
sfa	12 (85.71)	13 (41.93)	8 (15.09)	5 (15.62)	< 0.0001

[a]Values are expressed as No. (%).

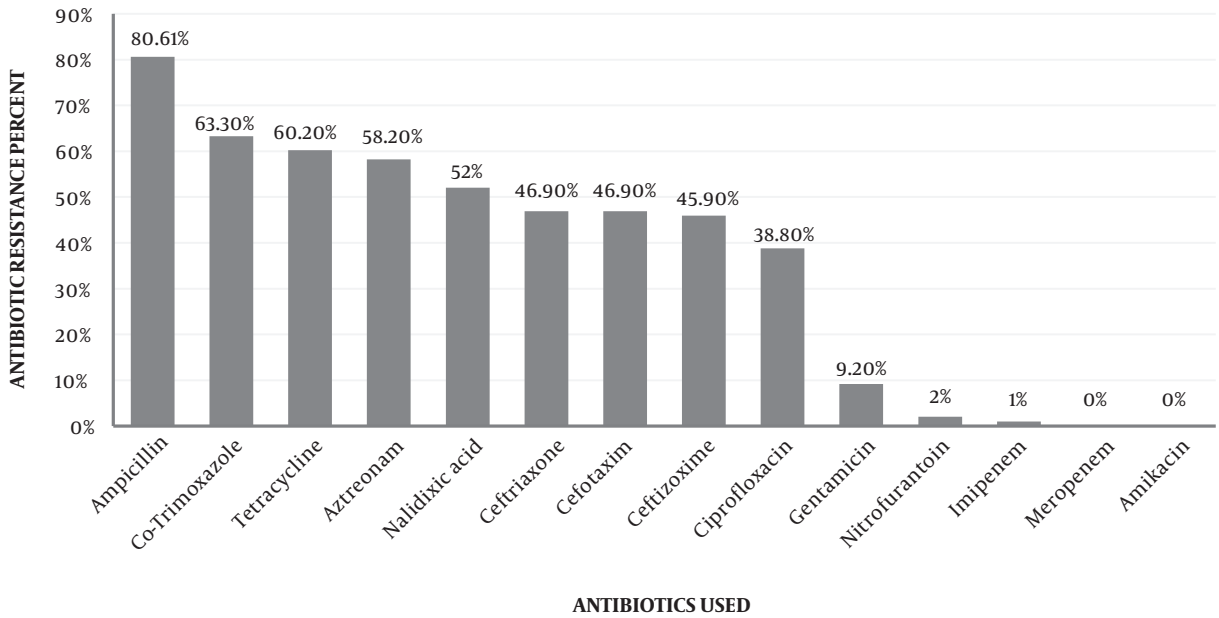

Figure 3. Antibiotic resistance pattern in biofilm-producing *Escherichia coli* Strains

study indicated the important role of this virulence factor in the development of UTI caused by *E. coli*. Also, the prevalence of fimbriae S indicated that this factor was a fimbria prerequisite for the adhesion and colonization of *E. coli* in UTI (27). Different studies conducted in various cities of Iran reported the results similar to those of the current study. The findings of the study were similar to the ones reported by Asadi et al. (7), Karimian et al. (28), Rahdar et al. (27), Jalali et al. (29), in Iran, along with reports by Kot et al. (14), Tarchouna et al. (24), and Choi et al. (30), outside Iran. It seems that the diversity in sample sources and variation in geographical regions and host clinical conditions play an important role in the frequency and type of virulence genes (31). Production of biofilm is a factor that plays a major role in the development of nosocomial infections and UTI. According to previous studies, more than 50% of bacterial infections are caused by biofilm-forming species. Identifying planktonic cells as mature biofilms causes phenotypic changes with consequences such as increased resistance to Disinfectants, antimicrobial treatment and host immune system (32). In the current study, of the 98 biofilm-forming *E. coli* strains, 14 (14%) were strong, 31 (31%) moderate, and 53 (53%) weak biofilm producers. Tajbakhsh et al. reported that out of 130 EPEC strains, 18% were strong, 25% moderate, and 56% weak biofilm producers (23). According to the results reported by Poursina et al., 80% of all *E. coli* strains isolated from patients with UTI were biofilm producers using microtiter plate assay. Among them, 29% had strong, 34% moderate, and 17% weak biofilm-producing ability (33). Bakhtiari et al. from Ahwaz, Iran, using microtiter plate assay, reported that 91.4% of *E. coli* strains were biofilm producer (20). Zamani and Salehzadeh reported biofilm formation in 84% of *E. coli* strains, of which 36% had high, 48% moderate, and 10% weak biofilm-producing abilities and 6% were non-biofilm producers (34). Tajbakhsh et al. reported that out of 130 UPEC strains, 18% had strong, 25% moderate, and 56% weak biofilm-forming abilities (23). Mittal et al. reported that of 135 isolated *E. coli*, 13.5% (n = 18) were biofilm producers (35). Ponnusamy et al. using mi-

crotiter plate assay, identified 100% of the isolated strains as biofilm-producing bacteria, of which 6.6% were strong, 80.8% moderate, and 14.14% weak biofilm formers (36). Neupane et al., reported an increase in biofilm-formation ability of 108 (51.92%) out of 208 *E. coli* strain using Congo-red Agar method (CRA) (37). Gonzalez et al, in a study a total of 116 *E. coli* strains were tested for biofilm formation using the crystal violet microplate technique. 41.3% of the strains was produce biofilm. 16.4%, 18.1% and 6.8% formed weak, moderate and strong biofilms, respectively (38). Difference in the results biofilm formation may be due to regional differences in low hygiene status and variable resistance to antibiotics and different sources of isolation of patients with urinary tract infections. The current study investigated the antibiotic susceptibility pattern of all isolated *E. coli* strains. Antibiotic resistance in biofilm-forming isolates was higher than that of non-biofilm forming isolates. Among the biofilm forming strains showed highest resistance to ampicillin (80%), followed by cotrimoxazole (63.63%), tetracycline (60.2%), aztreonam (58%), nalidixic acid (52%), ceftriaxone, cefotaxime (46.9%). The maximum sensitivity (100%) was also observed to amikacin, imipenem, and meropenem among biofilm-forming and non-biofilm forming isolates. The current study findings were consistent with those of Awoke et al. (16), Tabasi et al. (26), Sudheendra and Basavaraj (39), Neupane et al. (37) and Makled et al. (40). Differences between the current study results with those of other studies could be due to indiscriminate use of antibiotics and different antibiotic resistance patterns in Iran compared to other countries. This may be due to the fact that bacterial biofilms are often associated with the long-term viability of the organism in different environments, the slowdown of bacterial growth in a biofilm, the expression of resistance genes and the restriction of antibiotics to the biofilm. High resistance to ampicillin may be due to its excessive administration in the treatment of UTIs, especially in hospitalized patients. It can also be said that antibiotics even in proper doses cannot penetrate the biofilm and disable its mechanism and metabolites. Lower use of imipenem, amikacin, meropenem, and nitrofurantoin can be a reason for high sensitivity of bacteria to them in the current study (40, 41). The results of the current study showed the high prevalence of *pap* and *sfa* genes in UPEC strains using multiplex PCR for both genes in biofilm-isolated strains. The prevalence of *pap* and *sfa* genes was respectively 92.85% and 85.71%, in high biofilm-producing isolates. The study showed that *pap* and *sfa* genes in UPEC had a significant relationship with strong biofilm production ($P < 0.0001$).The relationship between *P* and *S* fimbriae and the formation of biofilm displayed the role of fimbriae in the formation of biofilms and development of UTIs. Tajbakhsh et al. (23), and Naves et al. (42), reported a significant ($P < 0.05$) relationship between *pap* and *sfa* genes and strong biofilm formation in UPEC strains. Martinez-Medina et al. (43) also reported a significant relationship between fimbria S and strong biofilm formation. In the study by Zamani and Salehzadeh the relationship between biofilm formation and fimbria S and *papAH* fimbriae was significant (34). In the study by Gonzalez et al., biofilm formation was significantly associated with pili P codifying genes, whereas virulence factors were not statistically associated (38). Fimbriae P causes proliferation and invasion of kidney tissue by facilitating bacterial colonization and preventing removal of bacterial by urine filtration flow. The fimbria S is the virulence factor of UPEC that bindings to epithelial and endothelial cells of urinary tract and kidneys in humans and is one of the important factors in the biofilm formation of UPEC strains (41, 44). The limitation in the present study could be compared to those of other studies in the small number of virulence genes involved in biofilm formulation in UPEC and also the selected community for study from a particular geographic region. Awareness of biofilm formation in UTIs can help physicians decide on more effective treatment and prevention methods for multi-drug resistant strains in this geographical region.

5.1. Conclusions

The results of the current study indicated that the *pap* gene in *E. coli* strains isolated from patients with UTI in Yasuj had a higher frequency than *sfa*. Both the *pap* and *sfa* genes were associated with strong biofilm-formation. In addition, biofilm formation was more prevalent in antibiotic-resistant strains of *E. coli* than antibiotic-susceptible ones. Given to the antibiotic resistance pattern, it is recommended to administer amikacin or meropenem to treat UTI in Yasuj in order to prevent the emergence of multi-drug resistant strains.

It is suggested that other virulence factors involved in biofilm formation in *Escherichia coli* that cause UTI be investigated.

Ciprofloxacin (due to relative resistance) and gentamicin (low resistance) antibiotics can also be recommended in the treatment of UTI as an experimental drug in this geographical area.

Footnotes

Conflict of Interests: The authors declared no conflict of interest.

Ethical Approval: Ethical code number: IR.YUMS.REC.1396.81.

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