Association of *bcsA* and *draE* genes with biofilm formation on urinary catheter in uropathogenic *Escherichia coli* isolates

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ABSTRACT

The production of biofilm by *E. coli* strain can cause serious problem for human health. Catheter is considered to be a suitable place for colonization of microorganisms, so that in 5-10% of patients who used the catheter for only one day and also in all patients who used it for over 28 days, the colonization of bacteria is observed on catheter. The aim of this study was to compare biofilm formation in *E. coli* and its relationship with pathogenic factors. In this study 144 isolates of uropathogenic *E.coli* were used in order to compare their potential of biofilm formation on urinary catheter. In order to do this, a piece of the catheter was sliced and put in the tubes containing *E. coli* suspension, as well as in broth medium (as witness). After incubation at 37ºC for 24 hours, the number of live bacteria involved in biofilm formation was counted by surface cultivation method. The result showed that among the 144 studied *E.coli* isolates, 130 (89.7%) had cellulose synthesized gene (*bcsA*), and 22(15.2%) had Dr adhesine gene (*draE*). There was a significant relationship between *bcsA* gene and biofilm formation potential on urinary catheter.

1. Introduction

Uropathogenic *E.coli* (UPEC) is the main cause of urinary tract infection (UTI) in developing countries (Luke et al., 2011) which has numerous pathogenic factors such as biofilm formation (in the form of external biofilm like colonies as well as intercellular ones in bladder). The produced biofilm by *E.coli* strains, result in the stability increase and bacterial colonization in the host body, causing serious problem such as resistance against antibiotics and phagocytes and other comparative innate of body inflammatory defense system, prostatitis formation, biliary tract infection, bladder inflammation and also expression intensity factor (Dacheng Ren et al., 2005; Khurosh et al., 2007). Moreover, the risk of being confined to bed, its duration and the treatment period with antibiotic for patients using catheter is 3 times more than that of patients not using that. CAUTI, the most common hospital infections, is more than one million cases per year or over 40% of all hospital infections in homes and nursing homes (Jacobsen et al., 2008). Catheter is suitable place for colonization of microorganism. In 5-10% of patients using catheters for only one day to more than 28 days, the colonization of bacteria is seen on catheters (Ro¨mling et al., 2009). Also, catheters used by patients with resistant urosepsis, who haven't been treated by antibiotics, have a biofilm layer stuck to them (Hatt and Rather, 2008).

Biofilm formation in *E.coli* is mainly affected by numerous factors such as movement, fimbria, adhesines, protein auto transporter, exopoly saccharides such as cellulose, F pili and curli fimbria which are expressed by various genes

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such as *upa*, *fim*, *fli*, *csg*, *dra* and *bcs* (Hoiby, 2011).

Dr adhesine genes cluster which is expressed by uropathogenic *E.coli* strains, causing bacterial connection and invasion. This adhesines linked to IV collagen and integrin which increases the recognition and linkage of this microorganism to kidney, renal interstitial compartment, neutrophils and red cells (Zalewska et al., 2005). The Dr operon consists of six genes encoding the main structural subunit DraA, the chaperone DraB, the usher DraC, the potential invasion DraD, DraP, and the adhesion DraE. The structural adhesin DraE determines the receptor-binding specificity of Dr adhesins (Khurosh et al., 2007). Cellulose is an important combination on the outside of *E.coli* which expresses it broadly as an extra cellular saccharide, causing binding cell-bacterial interaction to abiotic surface (Reisner et al., 2006). Cellulose expression requires 2 operons: *bcsABZC,* which codes structural genes for cellulose formation and *bcsEFG*, which are express contrarily. The cellulose synthase catalytic subunit, which dose polymerization and regulation of cellulose formation, is encoded by *bcsA* (Bokranz et al., 2005).

The aim of this research was to study the biofilm formation frequency of *bcsA* and *draE* genes.

2. Materials and Methods

In this study, 144 isolates of *E.coli* were collected from patients with urinary infection symptoms (32 male patient and 112 female patients), confirmed by customary methods as *E.coli* bacteria in Gorgan medical faculty. Different cultivation media (TS1, MRVP Broth, SIM, EMB) (Merk, Germany) have been used for revival, purification, movement specification and biofilm formation of *E.coli* species. The isolates were used to compare the biofilm formation on urinary catheter. The patients were divided in 2 groups: A) inpatients and B) outpatients.

2.1. Bacterial suspension preparation

At first, Luria-Bertani (LB) broth medium was made for all the 144 isolates. As much as 2-3 ml was transferred to test tubes and then were put in autoclave for 15 minutes, at $121^{o}C$, under the pressure of 15 Ibs.

In the next stage, 1-2 colonies with 24-hour cultivation in agar medium, was infused to this medium (BHI) and were incubated at 37 ^{o}C, for 2 hours. Then the tubes turbidity was compared by means of 0.5 MC-farland tube. The turbidity equal to MC-farland tube specifies the active presence of 100,000,000 bacteria (CFU/ml $10^{5}*10^{8}$) in broth medium. In order to ensure about the obtained concentration, the OD of the samples were also studied.

2.2. Biofilm production on catheters

In order to form biofilm on 40 isolates the urinary catheter was sliced into equal 1.5 cm pieces in sterile condition. Then, it was put in the tubes containing 10 ml of bacterial suspension with density of 0.5 MC-farland or control tubes containing BHI broth medium. After 24 hours of incubation at $37^{o}C$ the bacteria not attached to the catheter surface, were washed (3times) by 200 ml sterile distilled water. Then the catheters were transferred to tubes containing 5 ml of PBS medium and vortex for 5 minutes with high speed. At this stage, the bacteria forming biofilm were separated from catheters sample and entered into PBS medium. Then different dilutions (1 to10^{-7}) of biofilm bacteria samples were prepared. 30λ of each dilution was given superficial cultivation at Muller-Hinton agar and number of colonies was counted after 24 hours of incubation at $37^{0}C$. The intensity of biofilm formation was calculated on the basis of Log cfu/cm^{2} (Costerton, 2004; Hachem et al., 2009; Movassagh and karimi, 2010). All processes were repeated 3 times to increase study's accuracy and *E.coli* (ATCC1399) were used as controls.

2.3. Molecular analysis

DNA extraction: to do this, the bacteria sample were cultivation in tubes for 24 hours at $37^{o}C$ in Brain Heart infusion broth. Then the tubes were centrifuged at the speed of (g*1000); the surface liquid was discarded and only 100ml of each were kept for further analysis. Then, in exchange for each 30 ml of the sample, 200 ml sterile distilled water was added to them and was mixed by vortex. The samples were then heated for 15 minutes at $56^{o}C$ and mixed by vortex. The samples were kept for 8 minutes at $100^{o}C$, were then rapidly put in ice water for 5 minutes. At

the end of the experiment, they were centrifuged for 10 minutes at the speed of 14000 rpm; the top liquid was taken and transferred to a new micro tube. The extracted solution in the micro tube was kept at -20^0C before being used.

PCR: To trace *bcsA* gene, the polymerase chain reaction was done. Combination of this reaction includes sample DNA (2µl), for runner and follower primer oligonucleotide (each one in the amount of 20 Pmole) $MgCl_2$ (50nM), 0.5 µl of dNTP, PCR buffer 1X and tag polymerase enzyme (unit2), with final volume of 25 µl, was used in sterilized distilled water. The final steps of temperature include primary denaturation of 90^oC for 3minutes; 30 cycles in the form of 1 minutes of 94^oC denaturation; 30 seconds connection at 58^oC; 1 minute multiplication stage at 72^oC for 5 minutes.

To trace *draE* gene, the mixture of the polymerase chain reaction was also prepared with the final volume of 25µl. This mixture is made up of sample DNA (5µl), for runner and follower primer oligonucleotide (each one, as much as 10 Pmole) $MgCl_2$ (25nM), 0.5 µl of dNTP, PCR buffer 1X and tag polymerase enzyme (2unit), and the rest of the sterile distilled water (12.8 µl). The temperature program was as follow: the primary denaturation of 95°C for 3minutes; 30 cycles in the form of 1 minutes of 94^oC denaturation; 45 seconds connection at 59^oC; 1 minute multiplication stage at 72^oC for 5 minutes and finally the last multiplication steps at 72^oC for 5 minutes. The polymerase chain reaction products were then electrophoresed in 1.5% agarose gel, with exclusive size markers (100bp, MBI, Lithuania DNA ladder, Fermentase). They were observed Gel Doc machine after being dyed with ethidium bromide.

The characteristic of the used primers in this study are given in Table1.

Table1. Specific primers of *bcsA* and *draE* genes

E.coli Strain		Primer sequence	Gene
K-12	forward	5' AGAGTACGTCGACTGGGTGA3'	*bcsA*
	reverse	5' CCCACACCATACTGACGACC3'	
IH11128	forward	5' CCTGACCGTGGCTAAGACTC3'	*draE*
	reverse	5' GTCGTCCAGGCGGAGTTATC3'	

3. Results

In this study, the frequency of the *draE* and *bcsA* genes in 144 *E.coli* isolates was evaluated and their frequency in tow group of isolates with and without biofilm was compared.

3.1. The frequency of draE and bcsA genes

cellulose exopolysaccharide is composed of a 7 gene operon. No study was done on the frequency of *bcsA* gene so far. In this study, the exclusive primer for the major gene in the operon, cellulose synthesis, was designed.

The result of this study showed that among the 144 *E.coli* isolates studied, 130 (89.9%) has cellulose synthase gene (*bcsA*); 22(15.2%) has Dr adhesine gene (*draE*) and 21(14.5%) has *bscA* and *draE* gene (Figure1, 2).

3.2. Biofilm formation on Urinary Catheter

Experiments, considering the potential of biofilm formation in BHI broth medium containing 1% saccharose, showed that among 40 *E.coli* isolates, 38 (95.5%), disregarding the gene types had the potential to form biofilm: 19 (47.5%) showed strong biofilms, 12 (30%) moderate biofilm and 7 (17.5%) weak biofilm. only 2 (0.5%) didn't show the potential pf biofilm formation on the catheter. The standard strain had moderate biofilm (Table 2-Figure 2).

3.3. The relationship of biofilm formation potential with bcsA and draE genes

Considering the potential of *bcsA* and *draE* genes in biofilm formation, the relationship of colonization rate in 40 different strains (different, regarding having or not having *bcsA* and *draE* genes) was studied by T-test and X^2 statistic test.
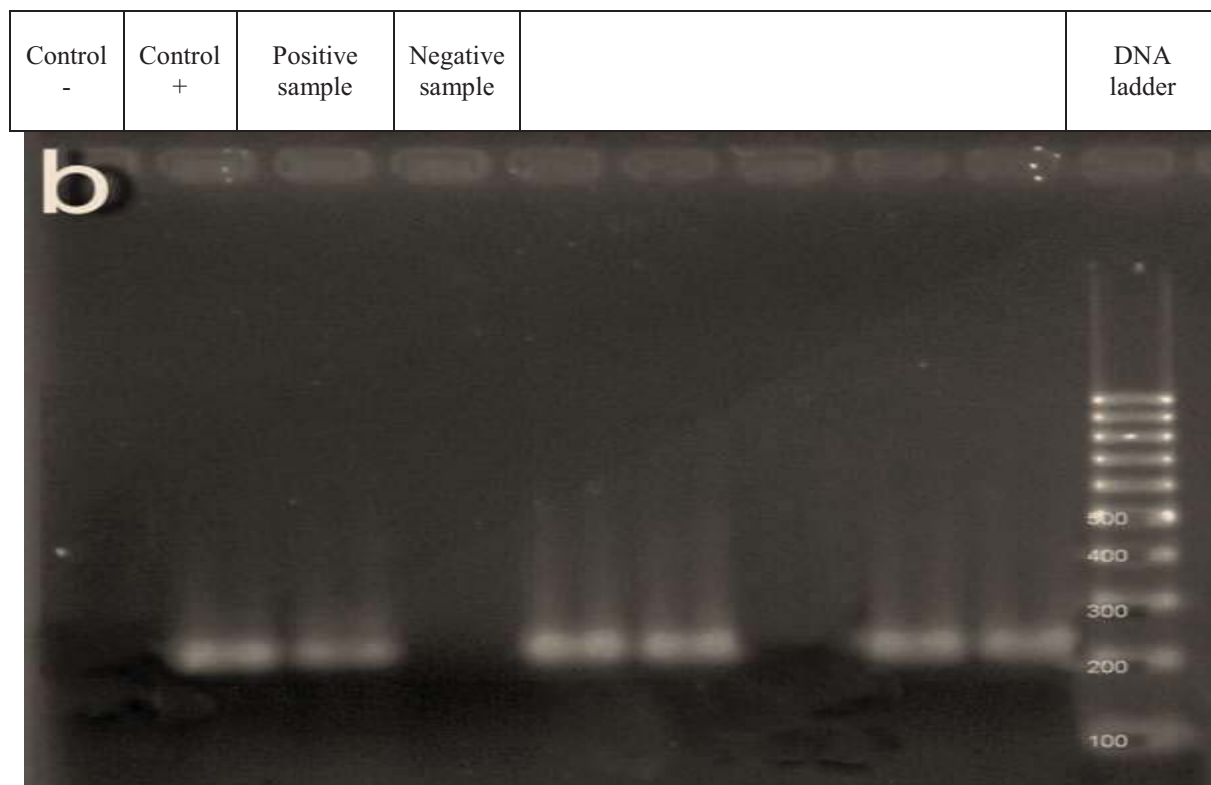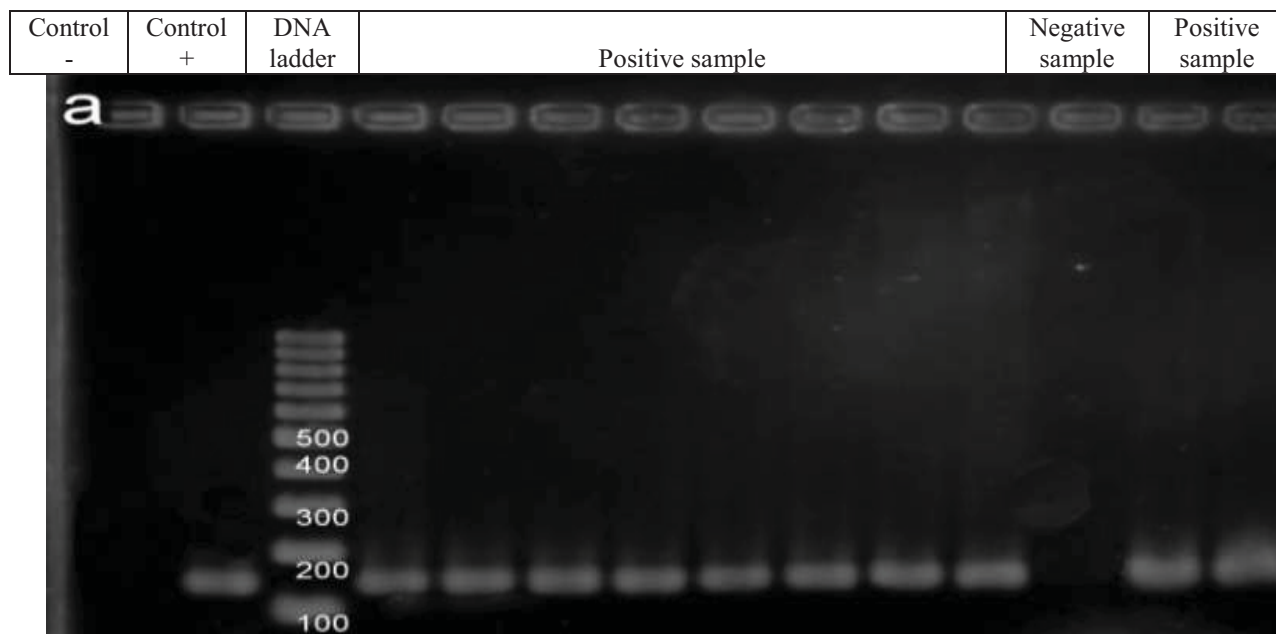
Control -	Control +	DNA ladder	Positive sample										Negative sample	Positive sample

Control -	Control +	Positive sample	Negative sample				DNA ladder

Figure 1. a; *bcsA* gene on agarose gel. B; *draE* gene tracing on agarose gel.

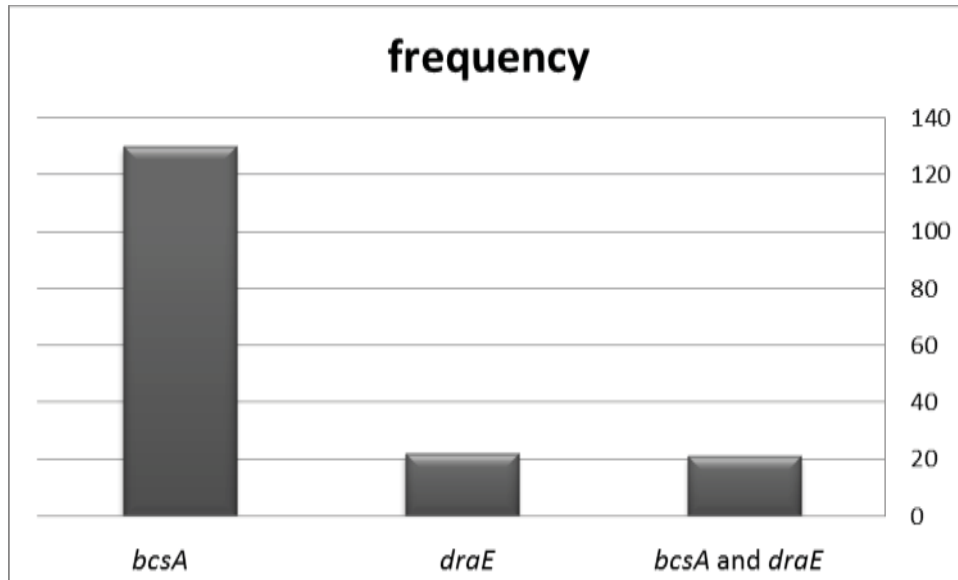**Figure 2.** Surface culture of *E.coli* which form biofilm

Figure 3. frequency of the *draE* and *bcsA* genes in 144 *E.coli* isolates

Table 2. Compare of the relative and absolute frequency of biofilm genes associated with biofilm

Gene		Without biofilm	Have biofilm	Have biofilm			P value
				strong	moderate	weak	
DR adhesine (*draE*)	Have	0	7 (100%)	3 (42.9%)	3 (42.9%)	1 (14%)	0.5
	Not have	2 (6.1%)	31 (93.9%)	16 (48.5%)	9 (27.3%)	6 (18.2%)	
Cellulose synthase (*bcsA*)	Have	1 (2.7%)	36 (97.3%)	17 (45.9%)	12 (32.4%)	7 (18.9%)	0.01
	Not have	1 (33.3%)	2 (66.7%)	2 (66.7%)	0	0	
Total (Regardless of the gene type)		2 (5%)	38 (95%)	19 (47.5%)	12 (30%)	7 (17.5%)	

The result of this study showed that among the 40 isolation studied, only 7 (17.5%) had *draE* gene, all of which had the potential to form biofilm (3 ones formed strong biofilm, 3 ones formed moderate biofilm and 1 formed weak biofilm).

Among the other 33 isolates (82.5%) (Lacking *draE* gene), 31(93.9%) formed biofilm (16 ones, strong biofilm; 9 ones moderate biofilm and 6 ones weak biofilm). Statistically, no significant relationship was observed between *draE* gene and biofilm formation potential (P=0.5) (Table 2).

Also among the 40 isolates studied, 37 (92.5%) had *bcsA* gene, among which only 1 isolate (2.7%) didn't form biofilm and 36 (97.3%) formed biofilm (17 had strong biofilm; 12 moderate biofilm and 7 had weak biofilm). Among the 3 isolates lacking *bcsA* gene (7.5%), 2 (66.7%) formed strong biofilm. χ^2 statistical test showed that the significant relationship with biofilm formation (P=0.01) (Table 2).

Synchronous consideration of the two genes showed that 7 isolates (18.9%) had both *bcsA* and *draE* genes and all of them formed biofilm. Among the 3 isolates (9.1%) lacking both genes, 1 isolate didn't form biofilm (Table 2).

The χ^2 statistical test showed that the presence of cellulose synthase gene (*bcsA*) can significantly (P= 0.01) increase biofilm formation potential in *E.coli* isolates, whereas no significant relationship was seen between Dr adhesin gene (*draE*) presence and increaseed biofilm formation (P= 0.5).

4. Discussion

Various factors, including adhesion, fimberia, flagella, F pili and extra cellular polysaccharide (cellulose, curli, colonic acid), have role in biofilm formation.

In this study, the amount of biofilm formation, of *E.coli* separated from patients having urinary infection, on urinary catheter, was studied on the basis of colony count method. Our finding showed that the presence of *bscA* gene, cellulose synthase-encoding factor, with *E. coli* potential, is significantly effective in biofilm formation on urinary catheters.

In this survey, cultivation method on urinary catheter was used to study biofilm formation and the frequency of 95.5% was obtained, while by using micro-titer plate method on samples and similar media, low frequency (61.8%) has been recorded (Samet et al., 2013) the antibiotic resistances in all isolates were specified, 61 (42.6%) isolates having resistance against third generation cephalosporin antibiotics and ESBL antibiotic (non-ESBL) (Bagheri et al., 2008)

Considering the significant difference (P< 0.01) it seems that in studying biofilm formation culture method on urinary catheters is a more appropriate method.

According to other studies, the frequency average of biofilm formation in *E.coli* which are urinary infection causative agent is variable from 36% to 16%, by microtiter plate method in common media.

One of the probable reasons of increase in observing biofilm formation on catheters in BHI medium containing%1 saccharose, is the substances forming this medium. There are substances such as brain and heart extracts, dextrose and saccharose in this medium which make it very rich. Adding a step to stabilize biofilm on the surface by using saccharose, may be another reason. For instance, Mersham in 2012, by using this method, showed biofilm formation in 76% of *E.coli*, which were the causing agent of urinary infection (Meshram et al., 2012). Besides, the silicone catheters surface may be another agent to facilitating biofilm formation potential, just as Lionel Ferries and his colleagues in 2007, in Denmark, indicated that all *E. coli* strains which are urinary infection causing agent, have very high potential for biofilm formation on silicone latex catheters. The results show that this kind of (silicone) catheters is suitable for establishing, reproducing and forming biofilm by strains of *E.coli,* urinary infection causing agent (Ferrières et al., 2007). In the present study more than 90% of isolates were showed to be able to form biofilm on urinary silicon catheters.

General expectation is that bacteria isolated from patients using urinary catheters, have more biofilm formation potential. But in this study, no relationship was seen between catheter using history and biofilm formation. Although Abdallah in a study carried out in 2011 on 50 patients showed that the amount of biofilm formation in the group using catheters and the one not using, it was 43.3% and 30%, respectively. This means that there is no significant different between them (Abdallah et al., 2011).

Our survey showed that there is no significant relationship between the potential of bacteria antibiotic resistance and biofilm formation (P=0.5) (data are not revealed). 90.5% of isolates, resistant against broad spectrum antibiotics, had biofilm formation potential and all the isolates sensitive to broad spectrum beta lactamase formation biofilm. Similarity, Marhova in 2010, by studying on 50 *E.coli* isolates specified that 38 isolates had multidrug resistance and 16 ones were sensitive to broad spectrum beta lactamase. But there was not any significant relationship between high resistance and biofilm formation (Marhova et al., 2010). Also, Ri Javec in 2008, concluded that there is no significant relationship between antibiotic resistance and biofilm production (Rijavec et al., 2008). In 2006, Soto and his colleagues, by studying on 80 *E.coli* isolates (43 female patients and 33 patients having antibiotic consumption history, during the last 3 months) proved that there is no significant relationship between biofilm formation potential with antibiotic consumption history (Soto at al., 2006).

Among the 40 isolates being studied for biofilm formation potential, 97.3% ones which had *bcsA* genes and 100% ones which had *draE*, formed biofilm. 66.7% isolates lacking *bcsA* gene and 93.9% ones not having *draE* gene formed biofilm. Meanwhile, it seems that the presence of the *bcsA* gene for biofilm formation is more effective than *draE* gene, and there was

significant relationship with biofilm formation, since almost 94% of isolates lacking *draE* gene had the potential of biofilm formation on urinary catheters.

Zalewska and his colleagues in 2009, by using IH11128 *E.coli* clinical strains, which have *draA-E* operon and *E.coli* non-combination strains having *draE* or *draD* gene, studied the role of these gene in biofilm formation for first time and showed that IH11128 clinical strain is not able to form biofilm in LB medium, since the expression regulation mechanism of *dra* gene is probably similar to that of pili And fimbria (because of the homology that exist in the regulation area of these tow operons) and Lb medium prevents the expression of these genes. This researcher by induction of mutation in the regulatory area showed that *draE* and *draD* are required for surface attachment. DraE adhesine capping by DraD protein. The above action causes this adhesine to have a role in biofilm formation by confronting with hydrodynamic environment of urinary tract. This research in 2008 showed that *E.coli* strain having *draE* and *draD* genes are able to attach to Hela cells (Zalewska et al., 2009).

Anyhow further and wider studies and better methods are required in order to specify the role of this adhesine which is one of the adhesion factor in *E.coli*.

Conclusion

About 95.5% of *E.coli* isolated from patients' urine in Gorgan city, are able to form biofilm on silicon urinary catheters in BHI medium and saccharose 1%. Comparing to others' records from different parts of the world, the amount of isolates having biofilm formation potential, is almost high. In this study, the participation genes in *E.coli* biofilm formation the frequency of *bcsA* and *draE* gene were 89.7% and 15.2%, respectively.

By studying of 40 isolate of *E.coli,* it was specified that isolates having *bcsA* and *draE* genes, 97.3% and 10%0 respectively, are

able to form biofilm on urinary catheter and all isolates having both genes, formed biofilm.

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