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Original Article

Evaluation of Biofilm Formation and Frequency of Genes Encoding Curli Fiber, Colanic Acid Capsule and F1c Fimberia Among Uropathogenic *Escherichia coli* Isolates With Strong Cell Surface Hydrophobicity

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Abstract

Background: The uropathogenic *Escherichia coli* (UPEC) is an important bacterium that colonizes the bladder mucous membrane as biofilm and causes urinary tract infection (UTI). In addition, UPEC favors long-term persistence and leads to relapses in untreatable UTI. Further, bacterial hydrophobic interactions play a role in bacterial adherence to the surface and facilitate biofilm formation due to adhesion. Similarly, cell surface hydrophobicity (CSH), fimbriae, curli fiber, and colanic capsule allow UPEC isolates to initiate infections. Considering the above-mentioned explanation, this study evaluated the association between genes encoding curli fimbriae, colanic acid (CA) capsule, and f1c fimbriae with biofilm formation and CSH among UPEC isolates.

Methods: To this end, 100 *Escherichia coli* strains were isolated from the urine samples of the patients and were diagnosed by biochemical tests. Furthermore, a tissue culture plate method was used to determine the capacity of biofilm formation, followed by conducting microbial adhesion to hydrocarbons method for CSH determination. Finally, the presence of *csgA, csgD, rcsA, rcsC*, and *foc* genes was determined by applying polymerase chain reaction.

Results: Totally, 40, 22, and 28 isolates had strong, moderate, and weak biofilm formation capacity, respectively. Moreover, 42 and 38 isolates had strong and moderate CSH. Similarly, among the isolates with strong CSH, 32, 13, and 5 isolates had strong, moderate and weak biofilm formation capacity and the prevalence of *csgA, csgD, rcsA*, and *foc* genes was 33, 35, 35, 16, and 29, respectively. Based on the findings, no significant difference was observed between the frequency of *csgA, csgD, rcsA, rcsC*, and *foc* genes among the strong, moderate, and weak biofilm producers.

Conclusions: In general, there is an association between CSH and the biofilm formation of UPEC isolates. This result showed the role of CSH as an effective factor on bacterial adhesion for the first stage of biofilm formation. However, differentiating the strains is not confirmed regarding their ability to form biofilms and their CSH and the presence of all studied genes.

Keywords: Uropathogenic *Escherichia coli*, Biofilm, Cell surface hydrophobicity, Curli fiber, Colanic capsule, Fic fimbriae

Background

Uropathogenic *Escherichia coli* (UPEC) creates a specific biofilm as a complex intracellular bacterial community within the superficial cells of the bladder that causes urinary tract infection (UTI) (1,2). UPEC bacteria tend to colonize the bladder mucous membrane as a biofilm and causes UTI. In addition, it favors the long-term persistence in host tissue (3), is impermeable to many antibiotics, and leads to the occurrence of bacterial multidrug-resistance which is responsible for relapses in untreatable UTI, recurrent UTIs, chronic cystitis, and prostitutes (4). Due to the growing resistance of bacteria to many antibiotics, preventing the formation of biofilms by blocking the first step bacterial adhesion is considered as one promising approach to treat UTI, especially catheter-associated UTI infections. Bacterial biofilm is also dependent on environmental conditions and extracellular polymers that are secreted by bacteria (5,6) but it is mainly governed by the physicochemical properties such as electrostatic, van der Waals, hydrophobic, and contact interactions, namely, cell surface hydrophobicity (CSH). CSH plays an important role in bacterial colonization on distinct materials (6,7) and there are many different reasons for its importance. For example, it is conducive to adhesion to abiotic and biotic surfaces (8-10). Further, medical implants such as catheters, mechanical heart valves, or pacemakers are constructed from hydrophobic materials (e.g., silicon, stainless steel, and Teflon) that hydrophobic

microorganisms adhere to them relatively. Hydrophobic microorganisms are more invasive and cause infections which is difficult to treat (11). Previous research showed that molecules expressed on the bacterial cell surface and hydrophobicity influence the physiochemical interactions between bacteria and substrates during reversible adhesion (12). Polymeric molecules (e.g., pili, fimbriae, lipopolysaccharides, or capsular polysaccharides) on the bacterial cell surface are believed to medicate the specific binding of bacteria to substrates (13,14). Based on genetic analysis, surface structures such as flagella and specific outer membrane adhesions, as well as type 1 and the curli fimbriae of *Escherichia coli* might be important for biofilm formation (15,16). Colanic acid (CA) is a negatively charged polymer of glucose, galactose, fucose, and glucuronic acid that forms a protective capsule surrounding the bacterial cell surface (17). According to Danese et al (18), there is a complex signal transduction pathway, namely, the Rcs phosphorelay that involves a histidine kinase (RcsC), a response regulator (RcsB), a phospho-transfer protein (RcsD), a signal transductor (RcsF), and an auxiliary activator protein (RcsA). Some studies showed that the synthesis of CA is up-regulated in biofilms (19,20). Similarly, curli fimbrium is the bacterial produced extracellular fiber that is required for biofilm formation and other community behaviors (21). Its synthesis is co-regulated by a complex regulatory network in which CsgD plays a key role as well (19). Furthermore, Lund et al (22) indicated that CsgD stimulates the production of curli through the transcriptional activation of the *csgABC* operon, *csgAB* and *csgDEFG* (*csg*, curli specific genes) (22). Moreover, Ideses et al reported that a curli-producing strain of *E. coli* O157:H7 was significantly more hydrophobic than its non-curli-producing counterpart (20). Additionally, they further found that UPEC isolates express F1C fimbriae that are encoded by *foc* gene in addition to S, P, and type 1 fimbriae. All these structures are needed to ascent the UPEC to the bladder in the urinary tract (23,24). Considering the importance of UPEC cell surface hydrophobicity and the presence of adhesion molecules for bacterial pathogenesis, the current study aimed to determine the association between biofilm formation and the presence of genes encoding CA capsule (*csgA, csgD*), curli fimbriae (*rcsA, rcsc*), and F1C pili (*foc*) in UPEC isolates with strong cell surface hydrophobicity.

Methods

Patients and Samples

This descriptive study was performed on 2185 urine samples obtained from the patients who were admitted to the Clinical Laboratory of Milad Hospital, Tehran during September 2015 and June 2016. The midstream urine samples of the patients were collected in sterile bottles and those samples with significant bacteriuria (more than 10^5 CFU/mL) were selected for the study.

Cultivation and Bacterial Isolation

The samples were directly inoculated on MacConkey agar and eosin methylene blue (EMB) agar plates and after overnight incubation at 37°C, the biochemical identification was performed by bacterial culturing on triple sugar iron agar, sulfide indole motility medium, Simmons citrate agar, and conducting methyl-red and Voges-Proskauer (MR-VP) tests.

In Vitro Biofilm Assay

The biofilm formation of all UPEC isolates was detected by the tissue culture plate method in trypticase soy broth (TSB) on round bottom 96-well microtiter plate (SPL Life sciences, Korea) as described previously (25). An overnight culture was grown in TSB (Merck, German) at 37°C, adjusted to 0.5 McFarland, and finally, diluted to1:100 in TSB with 2% (w/v) glucose. A total of 200 µL of these cell suspensions was transferred in a U-bottomed well. The plates were then incubated aerobically at37 °C for 24 hours. Furthermore, the culture was removed and the plates were washed three times with 200 µL of phosphate-buffered saline (pH=7.4; Sigma, USA) to remove non-adherent cells, followed by drying in an inverted position. Then, the adherent biofilm was fixed with95% ethanol and stained with 0.1% crystal violet (Merck, German) for 5 minutes. Next, unbound crystal violet was removed and the wells were washed three times with sterile distilled water, and then cleared and the microtiter plate was air-dried as well. The crystal violet from the stained biofilm was resuspended in 200 microliters of glacial acetic acid (21) and the optical density (OD) of each well was measured at570 nm using an ELISA Plate Reader (BioTek cytation3, USA). The cut-off OD for a tissue culture-plate is defined as three standard deviations above the mean OD of the negative control. In this study, each strain was tested in triplicate and wells with sterile TSB were alone served as controls. The biofilm production was interpreted according to the criteria of Stepanovic et al (26).

Optical density cut-off (ODc) value = the average OD of negative control + 3x standard deviation (SD) of negative control

Bacterial Cell Surface Hydrophobicity Assay

According to the procedure by Rosenberg et al, the hydrophobicity of the above-mentioned bacterial cell suspensions was determined using microbial adhesion to hydrocarbons assay, as a measure of their adherence to the hydrophobic hydrocarbon (noctane) (25). After 24 hours of incubation, microbial cells were concentrated and harvested during the exponential growth phase by centrifugation (5000 x rpm for 20 minutes, TGL–16M, PR China). Then, they were washed twice with phosphate-buffered saline (PBS: 7.6g NaCl, 1.9g Na2HPO4.7H2O, 0.7 g NaH2PO4.2H2O per liter, and a pH of 7.2, which is a hydrophilic solution) and resuspended in the same

buffer and then the absorbance was measured at 660 nm (*A*1). In addition, 5 mL of microbial suspension and 1 mL n-octane were mixed for 120 seconds by vortexing and then incubated for 1 hour without shaking to ensure that both solutions were separated into the biphasic state. The absorbance of the lower hydrophilic (aqueous) layer was calculated again (*A*2) by recording the changes in the absorbance of microbial suspensions due to microbial adhesion to noctane at 660 nm using a spectrophotometer. Microbial CSH was expressed as percentage adherence (*%Adh*) and calculated using the following formula (26):

Percentage adherence = [(A1- A2)/A1] × 100

The degree of hydrophobicity was assigned as strongly hydrophobic, moderately hydrophobic, and hydrophilic within percentage adhesion values of >50%, 20%-50%, and <20%, respectively.

Molecular Identification of *rcsA, rcsC, csgD, csgA*, and *foc* Genes by Polymerase Chain Reaction

The DNA of all isolates was extracted using a genomic DNA isolation kit (Gene Transfer Pioneers, Iran). Before DNA extraction, *E. coli* strains were cultured in Luria broth at *37*°C for 18 hours. The nucleotide sequences of primers that were used in this study are listed in Table 1.

PCR was conducted in a volume of 25 µL containing 2.5 µL of 10× PCR buffer (Sinaclon, Iran), 2 mM $MgCl_2$, 1 µm of each dNTP (Sinaclon, Iran), 2 U of Taq DNA polymerase (Sinaclon, Iran), 1 picomol of each primer, and 10 ng of bacterial DNA. In addition, amplification was performed in 30 cycles as 1 minute at 94°C, 1 minute of annealing at 59°C, 1 minute at 72°C, and a final extension at 72°C for 10 minutes. Finally, the PCR products were electrophoresed by gel agarose (Sinaclon, Iran) and visualized by UV transilluminator (Ultraviolet Transilluminator, UVT-20M, KIGEN).

Statistical Analysis

Statistical analysis was performed applying SPSS, version 20.0. The chi-square test, also written as χ^2 test, was used to study the correlation between hydrophobicity, biofilm formation, and the frequency of *rcsA, rcsc, csgD, csgA*, and *foc* genes. A value of $P<0.05$ was considered statistically significant.

Table 1. The Nucleotide Sequences of Primers

Genes	Nucleotide Sequences of Primers	Size (bp)
rcsA-F	TGGATTTATCTAGTTACACCCGAC	587
rcsA-R	ACCATTAGTCACATTATCCGTCAG	
rcsC-F	TCGTGAGGAATTTAATCTGAGTTC	706
rcsC-R	GTACCCTTCCGTATAGCCAAAC	
focA-F	ATTCGCATTCGTCTTCTATATCAC	450
focA-R	ACCATAATGAACGCTTTGTCC	
csgA-F	ATTTGCAGCAATCGTATTCTCC	400
csgA-R	GCCATCCTGAGTCACGTTGAC	
csgD-F	TGATCACTAGATCTTCTTTGCAGG	500
csgD-R	GAACAACGAACGAGCGATCTC	

Results

From 2185 urine samples of patients under investigation, 200 (9.1%) UTIs were diagnosed and 100 UPEC strains were isolated based on the results of biochemical tests.

The Results of Biofilm Formation

Given the standard tissue culture plate method and the evaluation of optical density cut-off (Table 2), biofilm-producing bacteria included the strong (40 isolates), moderate (22 isolates), and weak (28 isolates) biofilm producers while 10 isolates were non-biofilm-producing bacteria.

Hydrophobicity Determination

The degree of cell hydrophobicity in microbial adhesion to hydrocarbon assay varied among UPEC isolates as strong (42 isolates), moderate (38 isolates), and hydrophilic (20 isolates) (Table 3).

The results demonstrated that among 42 isolates with strongly cell surface hydrophobicity, 32, 13, and 5 cases had strong, moderate, and weak biofilm formation capacity. Eventually, there was a significant relationship between biofilm formation and strongly cell surface hydrophobicity (P = 0.048).

The Results of Molecular Identification of *rcsA, rcsc, csgD, csgA,* and *foc* Genes

The analysis of PCR amplification products by gel electrophoresis showed that the bands with different sizes as follows (Figure 1).

- 587 bp and 706 bp for *rcsA* and *rcsC* genes;
- 400 bp and 500 bp for *csgA* and *csgD* genes;
- 450 bp for *foc* gene.

Among the 100 isolates, the prevalence of *csgA, csgD, rcsA*, and *foc* genes was 33%, 35%, 35%, and 29%, respectively (Figure 2) and the frequency of *rcsC* was low (16%). Similarly, the prevalence of genes coding curli fimberia (*csgA*+*csgD*) and the frequency of genes coding

Table 2. Interpretation of the Biofilm Production of UPEC Isolates

Average OD Value	Results of Average OD Value	Biofilm Production
OD ≤ ODc	OD ≤ 0.232	Non-adherent (negative)
ODc <OD ≤2 × ODc	0.232 < OD ≤ 0.464	Weakly adherent (weak)
2×ODc <OD ≤4 × ODc	0.464 < OD ≤ 0.929	Moderately adherent (moderate)
4 × ODc <OD	0.929 < OD	Strongly adherent (strong)

Note. UPEC: Uropathogenic *Escherichia coli*; OD: Optical density; ODc: Optical density cut-off; SD: Standard deviation =0.03214, Mean=0.136, ODc=0.232.

Table 3. Degree of the Hydrophobicity of UPEC Isolates

Assay	Criteria	Hydrophobicity	No. of Bacterial Isolates (%)
MATH	>50%	Strongly hydrophobic	42
	20-50%	Moderately hydrophobic	38
	<20%	Hydrophilic	20

Note. UPEC: Uropathogenic Escherichia coli; MATH: Microbial adhesion to hydrocarbons

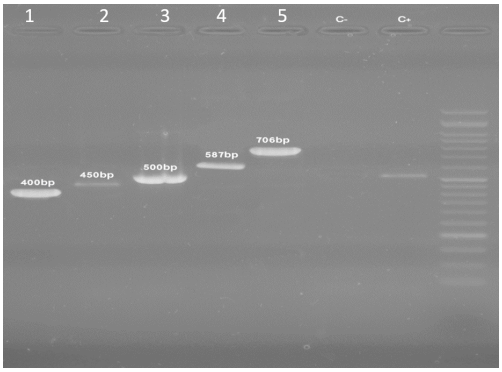

Figure 1. Gel Electrophoresis Analysis of Polymerase Chain Reaction Products. Lane 1: *csgA* gene; Lane 2: *foc* gene; Lane 3: *csgD* gene; Lane 4: *rcsA* gene; Lane 5: *rcsC* gene; Lane 6: Control negative; Lane 7: Control positive; Lane 8: Ladder 50 bp.

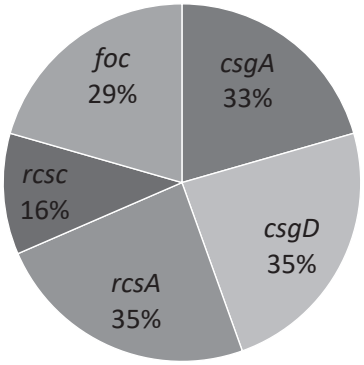

Figure 2. The Frequency of *csgA, csgD, rcsA, rcsc*, and *foc* Genes Among Uropathogenic *Escherichia coli* Isolates.

CA capsule (*rcsA+rcsc*) were 16% and 10%, respectively.

The numbers of gene combinations are shown in Figure 2. The number of isolates harboring *csgA+foc* was the highest among two gene combinations. Likewise, 15 isolates had both *csgD+foc* and *rcsA+foc* gene combinations while only 4 isolates had *rcsC+foc* gene combinations. Fic gene was almost, along with *csgA, csgD,* and *rcsA*, especially *csgA* whereas only 3 isolates, containing *csgA*, had no *foc* gene (Figure 3). Further, 3 isolates had *csgA+csgD+rcsA+rcsc* gene combination and 44 UPEC isolates had any gene as lonely or in combination.

Based on the results, no significant difference was observed between the frequency of *csgA, csgD, rcsA*, and

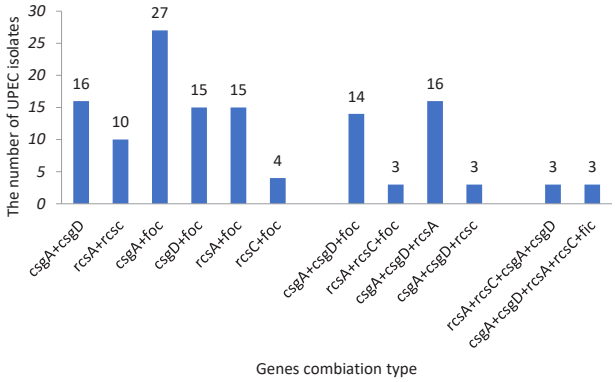

Figure 3. The Frequency of Genes Combinations Among Uropathogenic Escherichia coli Isolates.

rcsC genes among the strong, moderate, and weak biofilm producer of UPEC (P>0.05). However, the frequency of *foc* in the weak biofilm producer of UPEC was lower compared to strong and moderate biofilm producers (Table 4). In strains with no biofilm formation capacity, there was no *csgA* and *rcsA* genes. Only one isolate had *foc* gene. Furthermore, there was no association between the frequency of *csgA*, *csgD* , *rcsA, rcsC* and *foc* genes and CSH (P>0.05).

Discussion

Bacterial adhesion is governed by reversible physiochemical forces that include electrostatic, van der Waals, and hydrophobic interactions (cell surface hydrophobicity), followed by the establishment of irreversible interactions such as specific receptor ligand-binding events (27). Moreover, the adherence of UPEC isolates to the surfaces can be influenced by a wide variety of intrinsic factors such as adhesive proteins, fibers, and exopolysaccharide molecules although the carriage and expression of such factors differ from strain to strain (28).

The present study first evaluated the biofilm formation and cell surface hydrophobicity (CSH) of *E. coli* isolates obtained from patients with UTI. Among uropathogenic *E. coli* isolates, 90 cases were positive for biofilm formation. Additionally, 40, 22, and 28 isolates had strong, moderate, and weak biofilm formation capacity while only 10 isolates had no biofilm formation capacity.

Cucarella et al reported that 10 strains among 14 isolates of *E. coli* were positive for biofilm formation (29). In addition, Fattahi et al showed that 92% of the 100 *E. coli* strains isolated from UTIs were biofilm positive (30). In another study by Tajbakhsh et al, 80 (61.53%) out of 130 *E. coli* isolates were able to make biofilm including 15 (18.75%), 20 (25%), and 45 (56.25%) isolates which represented strong, medium, and weak biofilm reactions, respectively (31). Similarly, Zamani et al found that 84% of UPEC were moderate to strong biofilm producers (32). Gawad et al, measuring the degree of biofilm formation in all UPEC isolates, indicated that 44% (77/175), 10.8%

Table 4. The Association Between the Frequency of *csgA, csgD, rcsA, rcsC,* and *foc* Genes and Bacterial Hydrophobicity and Biofilm Formation

Genes	Biofilm Formation				Hydrophobicity		
	Strong (40)	Moderate (22)	Weak (28)	Negative (10)	Strong (42)	Moderate (38)	Hydrophilic (20)
csgA	15 (37.5)	9 (22.5)	6 (21.4)	0	14 (33.3)	12 (31.5)	4 (20)
csgD	18 (45)	6 (27.2)	9 (32)	2 (20)	19 (45.2)	9 (23.6)	7 (35)
rcsA	17 (42.5)	8 (36.3)	10 (35.7)	0	18 (42.8)	10 (26.3)	7 (35)
rcsC	6 (15)	6 (27.2)	2 (7.1)	2 (20)	8 (19)	4 (10.5)	4 (20)
foc	14 (35)	10 (45.4)	4 (14.2)	1 (10)	13 (30.9)	12 (31.5)	5 (25)

(19/175), 21.7% (38/175), and 23.42% (41/175) of the isolates were strong, moderate, weak, and negative biofilm producers, respectively (33).

Based on the surface hydrophobicity, there was a high incidence of isolates with hydrophobicity so that 42, 38, and 20 strains were strong, moderate, and negative or hydrophilic, respectively. Based on the microbial adhesion to hydrocarbon method, Gogra et al (35) reported a 96.9% prevalence of *E. coli* strains with CSH compared to *Staphylococcus aureus* (78.25%) and *Aspergillus fungus* (50.30%). According to Kaira and Pai, among 123 UPEC isolates, 27.64% were positive in terms of CSH (36). In this study, there was a significant relationship between biofilm formation and CSH as the first stage of biofilm formation. Likewise, according to previous reports by Li et al and Blanco et al, biofilm formation was associated with CSH (37,38). Using comparative analysis, Mirani et al showed that cell surface hydrophobicity, growth rate, and small colony variants are correlated with each other in the biofilm consortia of *P. aeruginosa, S. aureus,* and *E. coli* isolates (39). Further, Park et al found that high CSH-expressing bacterial species show greater adherence to HeLa cells and the larger amounts of biofilm formation on polystyrene. However, strong positive correlations were observed between CSH and biofilm formation or cell adhesion in *C. albicans*. These results suggest that hydrophobic force of bacteria may play a minor role in adhesion and biofilm formation, but CSH of *C. albicans* may be an important factor for adherence on surface and biofilm-forming process (40). Adherence is considered essential for the colonization or invasion of many bacteria (41). This event is typically mediated by fibrillar structures such as fimbriae or pili (42) and capsular polysaccharides like CA (18). The findings of the present study showed that the prevalence of *csgA* and *foc* genes was equal (30%), and the frequency of both *csgD* and *rcsA* genes was 35% among 100 UPEC isolates. *rcsC* had the lowest rate (16%) as well. Based on the results of Qin, the prevalence of *csgA* genes in UPEC isolates and commensal *E. coli* isolates was 30% and 34%, respectively, indicating no significant differences between the isolates (43). In another study, Bakhtiari et al demonstrated that 33 out of 35 UPEC isolates had *csgA* gene while they found no correlation between the presence of *csg*A and biofilm production ability since most of the isolates with or without biofilm production ability had *csg*A gene (44). Similarly, Rijavec et al.observed no association between *usp*, *pap*C, and *sfa/foc* virulence genes and biofilm production in pathogenic *E. coli* (45). Finally, Adamus-Białek et al reported that all the studied *E. coli* strains possess *rcsA, csgBA, and sdiA* genes. Therefore, the presence of these genes fails to determine biofilm formation (46). The results of our study also indicated that not only not all the studied genes were present in all UPEC isolates, but there was no significance between the presence of these genes and the ability of biofilm formation and the hydrophobicity of the cell surface.

Conclusions

In summary, the findings of this research revealed a high incidence of biofilm among UPEC isolates. Biofilm production may be the key determinant for the persistence of UPEC in the vaginal reservoir, the bladder epithelial cells, or both so that it must be taken into account for the treatment of UTI associated by the biofilm producer of UPEC isolates. More interestingly, a relationship was detected between cell surface hydrophobicity (CSH) and the biofilm formation of UPEC isolates. CSH is regarded as a crucial feature of the adhesion. This result confirmed the role of CSH as an influential factor in bacterial adhesion for the first stage of biofilm formation. Conversely, the differentiation of the strains in terms of their ability to form biofilms and their CSH and the presence of *csgA, csgD, rcsA, rcsc*, and *foc* genes still require further evaluation. Therefore, the presence of other genes encoding adhesions is highly possible. On the other hand, the lack of association between the properties of UPEC isolates may result from complex mechanisms that are involved in biofilm formation. As a result, the expression of particular genes at the subsequent stages of biofilm formation is subject to further investigation. Eventually, studying factors contributing to biofilm formation may be important for conceiving new therapeutic solutions for the treatment of these infections.

Conflict of Interest Disclosures

No competing interest was declared by any of the authors.

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