International Journal of
Enteric Pathogens

Int J Enteric Pathog. 2022 August;10(3):98-104 Original Article

http://enterpathog.abzums.ac.ir

doi 10.34172/ijep.2022.5536

Possible Relationship of Novel Phylogenetic Structure With Antimicrobial Resistance, Biofilm Formation, and Hemolytic Activity in Uropathogenic *Escherichia coli* (UPEC)

Batoul Rahimifard[1], Vahid Soheili[2], Gholamreza Hashemitabar[1*], Mahdi Askari Badouei[1*]

[1]Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
[2]Department of Pharmaceutical Control, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

Received: June 19, 2022
Revised: August 8, 2022
Accepted: August 19, 2022
epublished: August 28, 2022

***Corresponding Authors:**
Gholamreza Hashemitabar,
Email: hashemit@um.ac.ir;
Mahdi Askari Badouei,
Email:askari.m@um.ac.ir

Abstract

Background: Due to the increase of multidrug resistance (MDR) in organisms that cause urinary tract infections (UTIs), we need to investigate anti-microbial resistance (AMR) in these pathogens on a regular basis.

Objectives: The main purpose of this cross-sectional study was the use of updated phylotyping method to evaluate the possible correlations between biofilm production, hemolysin production, and antibiotic resistance among uropathogenic *Escherichia coli* (UPEC).

Materials and Methods: A total of 138 UPEC isolates were evaluated for biofilm formation, hemolysin production, and antimicrobial susceptibility to five classes of antibiotics, including quinolones, β-lactams, tetracyclines, and sulfonamides. The phylogenetic structure was determined using the original and recently updated protocols.

Results: Our results demonstrated that of 138 UPEC isolates, the majority belonged to phylogenetic group B2 (34.7%), followed by F (13.7%). Ninety-four (68%) isolates showed hemolytic activity but hemolysis had no correlation with antibiotic resistance while a correlation was observed between the hemolytic activity and biofilm formation. Moderate to strong biofilm production was observed in 34.7% of the isolates. Additionally, 73% of them showed hemolytic activity and most of them belonged to B2 phylogroup (37.5%). In this study, increasing rates of phylogroup F were detected compared to the old method that indicates the possible importance of this phylotype in UTI. Additionally, detecting the novel phylogroup G provides more precise data which can only be obtained by the new method.

Conclusion: The findings of the present study showed that more precise phylotyping results can be obtained when evaluating different aspects of UPEC in epidemiological studies using the new complementary method.

Keywords: *Escherichia coli*, UPEC, Phylogenetic group, Biofilm, AMR

Background

Urinary tract infection (UTI) caused by uropathogenic *Escherichia coli* (UPEC) strains is one of the most commonly occurring bacterial infections worldwide.[1] UPEC strains are a genetically heterogeneous group that exhibits various virulence properties in the urinary tract.[2] The identification of UTI is of great importance due to its high prevalence and the dangerous consequences including cystitis and pyelonephritis that may follow.[2]

Escherichia coli exhibits extensive phylogenetic sub-structures, which emphasizes the need to reliably identify the *E. coli* phylogroups.[3] Clermont et al devised a polymerase chain reaction (PCR) based method in 2013 that classified the 8 phylogenetic groups of *E. coli* as A, B1, B2, C, D, E, and F, and Clade 1.[4] In 2019, they analyzed the accumulation of whole genome sequence data and found that some strains belonged to an intermediate group between the F and B2 phylogroups, named as phylogroup G, that interestingly showed higher rates of resistance.[3] In the study of Jaureguy,[4] the B2 phylogenetic group has been suggested to be a sister group to phylogenetic group F, but in the study of Nielsen et al, by analyzing the relationship between each phylogenetic group and biofilm formation, a substantial difference in biofilm formation was observed between strains of B2 and F groups. In this way, biofilm formation in the B2 group was relatively high in three media compared to the F phylogroup.[5] Therefore, an investigation of the genetic differences between phylogenetic groups of B2 and F was warranted and a complementary phylotyping method was developed in order to identify the intermediate group as phylogroup G.[5]

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The ability of UPEC to form biofilms is considered the main attribute of urinary tract pathogens, and similarly, the biofilm formation capability of UPEC are the most crucial factors for the establishment, persistence, recurrence, and treatment in complicated cases of UTIs.[6] In the urinary tract, biofilm protects the pathogens against harmful conditions, antimicrobial agents, and the host's immune system, which is an important challenge in UTI treatment.[7] To the best of our knowledge, no prior study has been done to investigate the relationship of the new phylogenetic classification with biofilm formation and anti-microbial resistance (AMR).

Nowadays, resistance to antimicrobial agents is one of the most important challenges worldwide and the rising prevalence of AMR among different microorganisms causes failure in antimicrobial treatment and an increase in mortality and morbidity, which are the alarming consequences of AMR.[8] It is certainly the case for uropathogens, such as UPEC strains, where multidrug-resistant strains are emerging and causing treatment failure around the world.[9] In some of the previous studies, phylotyping analysis demonstrated that certain *E. coli* phylogenetic groups showed resistance to special types of antibiotics.[10] There is still a need for further evidence to establish a connection between phylogroup G strains and AMR, despite the primary observation that antimicrobial resistance determinants were common among phylogroup G strains.[3]

UPEC releases toxins such as hemolysin, a pivotal virulence factor of UPEC which targets several host pathways to facilitate infection and optimize the host environment for colonization in the host tissues.[11] UPEC strains that express genes related to hemolysin production (e.g., HlyA) cause tissue damage in urinary tract system.[12] Therefore, evaluating the presence of hemolysin production and determining its correlation with phylogenetic groups, biofilm production, and AMR in UPEC isolates could potentially lead researchers to a better investigation, prevention, and treatment.

The aim of the study was to determine a new classification of phylogenetic groups according to the Clermont method and finally investigate the possible correlation of this new phylogenetic classification with biofilm formation ability, multidrug resistance (MDR), and hemolysin production.

Materials and Methods

A total of 138 *E. coli* isolates were obtained from the urine samples of patients with UTIs. At first, the identification of isolates was done according to the colony morphology on MacConkey agar. Then, gram-staining and standard biochemical tests were performed and interpreted according to the standard microbiological diagnostic procedures.[13]

Molecular Phylotyping of UPEC

All isolates were cultured on Luria Bertani (LB) agar and incubated overnight at 37°C. The boiled bacterial lysate was used as template in PCR reactions.[14] The phylogenetic origin of samples was determined using two protocols described by Clermont et al in 2013 and 2019.[3,4]

The first step was a quadruplex PCR on the isolates to detect four genes including *arpA*, *chuA*, *yjaA*, and *TspE4.C2*. Then, based on the quadruplex genotype obtained, an isolate either was immediately assigned to a phylogroup or required supplementary PCRs to be assigned to phylogroups E or D.[4] Another multiplex PCR corresponding to the presence of the three genes, including *ybgD*, *cfaB*, and *trpA*, was performed on all isolates that were assigned to phylogroups B2 and F in the first test.

The amount of each forward and reverse primer used was 1 µM except for *arpA* and *trpA*, which was 0.5 µM. PCR amplification was done under the following conditions: denaturation for 2 minutes at 95°C, 31cycles of annealing for 10 seconds at 95°C and 30 seconds at 59°C (quadruplex PCR and group G), and a final extension for 5 minutes at 72°C. All PCR tests were performed in a volume of 20 µL containing 10 µL of ready-to-use PCR Master Mix 2x (Ampliqon, Denmark), 3 µL of bacterial lysate, and the appropriate primers as described before. In Table 1, primer sequences and the size of amplicons are shown in detail. The analysis of PCR products was performed by electrophoresis using 1.5% (w/v) agarose gel and DNA Green Viewer which is a safe nucleic acid stain (0.01 v/v).

Antimicrobial Susceptibility Testing

The Kirby-Bauer disc diffusion method was utilized according to the standard protocols to determine the resistance of isolates to 9 antibiotics belonging to 5 antibiotic classes.[15] The following antibiotics were used: nitrofurantoin (300 µg/disc), nalidixic acid (30 µg/disc), ciprofloxacin (5 µg/disc), norfloxacin (10 µg/disc), cefalexin (30 µg/disc), ceftriaxone (30 µg/disc), cefotaxime (30 µg/disc), doxycycline (30 µg/disc), and sulfamethoxazole (25 µg/disc). All examinations and also the interpretation of the findings were performed according to the instructions and guidelines of the 2021 Clinical and Laboratory Standard Institute (CLSI).[16] *Escherichia coli* ATCC 25922 was used as a control organism. Finally, isolates resistant to at least three antimicrobials belonging to three classes of antibiotics were designated as MDR (Multidrug-resistant).[17]

Biofilm Formation Ability

For detecting in vitro biofilm formation ability, the Tissue Culture Plate method was used, which has been described by Christensen et al as the gold standard quantitative technique.[18] *E. coli* strains were cultured

Table 1. The Primers Used in Two Phylotyping Methods in this Study

Target Group	Target Gene	Primer Designation	Primer Sequences	Size of PCR Product (bp)	Reference
PCR quadruple	*arpA*	AceK.f	5′-AACGCTATTCGCCAGCTTGC-3′	400	4
		ArpA1.r	5′-TCTCCCCATACCGTACGCTA-3′		
	chuA	chuA.1b	5′-ATGGTACCGGACGAACCAAC-3′	288	
		chuA.2	5′-TGCCGCCAGTACCAAAGACA-3′		
	yjaA	yjaA.1b	5′-CAAACGTGAAGTGTCAGGAG-3	211	
		yjaA.2b	5′-AATGCGTTCCTCAACCTGTG-3′		
	TspE4.C2	TspE4C2.1b	5′-CACTATTCGTAAGGTCATCC-3′	152	
		TspE4C2.2b	5′-AGTTTATCGCTGCGGGTCGC-3′		
E group	*arpA*	ArpAgpE.f	5′-GATTCCATCTTGTCAAAATATGCC-3′	301	3
		ArpAgpE.r	5′-GAAAAGAAAAAGAATTCCCAAGAG-3′		
G group	*ybgD*	ybgD.1	5′-TATGCGGCTGATGAAGGATC-3′	177	3
		ybgD.2	5′-GTTGACTAAGCGCAGGTCGA-3′		
F group	*cfaB*	cfaB	5′-CTAACGTTGATGCTGCTCTG-3′	384	3
		cfaB.2	5′-TGCTAACTACGCCACGGTAG-3′		
Control	*trpA*	trpBA.f	5′-CGGCGATAAAGACATCTTCAC-3′	489	4
		trpBA.r	5′-GCAACGCGGCCTGGCGGAAG-3′		

on LB plates in aerobic conditions overnight. Some colonies were selected and cultured in 10 mL of LB liquid medium for 18 hours. The turbidity of cultured LB broth was compared with the 0.5 McFarland standard to reach 10^8 CFU/mL. Then, 1 mL of cultured LB broth was added to 9 mL of LB broth to reach a concentration of approximately 10^7 CFU/ mL. Afterwards, 20 µL of each culture sample was transferred to a well of a 96-well plate and diluted to 200 µL with LB supplemented with 2.5% glucose. Each sample had three repeats in three wells. After incubation at 37°C for 24 hours, the LB culture was removed and the plate was washed with PBS. Afterwards, biofilms were stained with 200 µL of 1% (v/v) crystal violet (CV) staining solution and incubated for 10 minutes at room temperature. Then, CV was discarded and the plate was washed with distilled water until the excess CV was completely removed. Then, biofilms formed in the plate wells were dissolved in 30% acetic acid, and finally, OD600 was recorded using a microplate reader (Bio-Rad, USA). The negative control wells contained only broth and *E. coli* ATCC 25922 was used as the positive control.[19,20] Biofilm forming capacity was classified as follows: non-producer (OD ≤ ODc), weak biofilm producer (ODc < OD ≤ 2 ODc), moderate biofilm producer (2 ODc < OD ≤ 4 ODc), and strong biofilm producer (4 ODc < OD).[18]

Detection of Hemolysin Production

The hemolysis test was performed on blood agar with 5% sheep blood agar and the plates were incubated overnight at 37°C. Plates were also kept at 4°C for 16-24 hours to clearly observe hemolysis. Afterwards, hemolysin production was detected by complete hemolysis of the erythrocytes around the colony.[21]

Statistical Analysis

Data analysis was performed using SPSS version 16.0. The chi-square test was used to compare the occurrence of described factors and their association with UPEC isolates. *P* values of less than 0.05 were considered statistically significant.

Results

Antibiotic Resistance Pattern of UPEC Isolates

In this study, we analyzed the susceptibility of 138 isolates to 9 different types of antibiotics from 5 classes. Out of 138 *E. coli* isolates, 80 (58%) were MDR. Figure 1 shows the antibiotic resistance of 138 UPEC isolates to 9 antibiotics used in this research. Forty-six (33%) isolates were resistant to nitrofurantoin. Three antibiotics were chosen from the quinolone class of antibiotics, including nalidixic acid, ciprofloxacin, and norfloxacin, and it was found that 101 (72%), 82 (59%), and 81 (58%) of isolates were resistant to them, respectively. Three antibiotics including cephalexin, ceftriaxone, and cefotaxime were chosen from the beta-lactam class of antibiotics, and 134 (96%), 87 (62%), and 96 (69%) of isolates were resistant to them, respectively. Additionally, from tetracycline and sulfonamide classes of antibiotics, doxycycline and sulfamethoxazole were selected, and 87(62.5%) and 78 (56%) isolates showed resistance to them, respectively.

Phylogenetic Distribution in UPEC Isolates

According to the Clermont methods (2013 and 2019), phylotyping of 138 UPEC isolates revealed that most strains belonged to the phylogroup B2 with 48 isolates (34.7%), followed by F with 19 (13.7%), D with 16 (11.5%), and B1 with 14 (10.1%) isolates. Some phylotypes were not predominant including A with 5 (3.6%), Clade I with

4 (2.8%), G with 3 (2.17%), and E with 1 isolates (0.7%), respectively. Additionally, 28 (20.2%) isolates were not phylotyped according to the applied methods (Table 2). Of 70 isolates that were assigned to phylogenetic groups B2 and F according to 2013 method, 14 isolates moved from phylogroup B2 to phylogroups F (n = 11) or G (n = 3) using the 2019 method.

Phylogenetic Grouping in MDR UPEC Strains

The phylotyping based on 2013 and 2019 methods revealed that MDR strains belonged to 7 phylogroups as follows: A, B1, B2, D, F, G, Clade I, and unknown (non-typeable) (Table 2; Supplementary file 1). The majority of MDR isolates belonged to phylogroup B2 with 34 isolates (42.5%), followed by D with 12 isolates (15%), F with 10 isolates (12.5%), B1 with 5 isolates (6.2%), A, Clade I, and G each one with 2 isolates (2.5%).

Biofilm-Related Phylogenetic Groups

Detection of biofilm formation in UPEC shows that out of 138 UPEC isolates, 87 (63%) were found to be weak biofilm producers, 39 (28%) isolates were moderate biofilm producers, and 9 (6.5%) isolates were strong biofilm producers whereas 3 (2%) isolates were found to be non-biofilm producers.

All isolates in the phylogenetic group E produced moderate biofilm. In the phylogenetic group B1, 1 isolate (6.6%) formed strong biofilm and 5 (33%) and 8 (53.3%) isolates produced moderate and weak biofilms, respectively. Three (6.3%) isolates in the phylogenetic group B2 were strong biofilm producer. Besides, in this group, 15 (32%) isolates produced moderate biofilm and 29 isolates (61.7%) produced weak biofilm. The majority of the isolates in the phylogenetic group D were weak biofilm producers with 11 isolates (78.5%), and only 3 isolates (21.4%) were moderate biofilm producers.

In phylogroup F, the rate of moderate and strong biofilm formation was 42% (n = 8), which was one of the highest rates of biofilm formation among all phylogroups. Only 3 isolates were assigned to group G, all of which produced weak biofilm (100%). Moreover, in the strong + moderate biofilm producers, phylogenetic group B2 was the most prevalent group.

Our data demonstrated that the ability to form a strong and moderate biofilm was absent in phylogroup A, and all isolates (100%) were weak biofilm producers. Besides, out of 138 isolates, 28 isolates were not assigned to any of the phylogenetic groups. In addition, 2 (6.6%), 8 (26.6%), and 18 (64%) isolates were strong, moderate, and weak biofilm producers, respectively. Table 2 shows the correlation between antimicrobial resistance, biofilm production, and phylogenetic groups of 138 isolates.

Correlations Between Hemolysin Production and other Parameters

Out of the 138 *E. coli* isolates, 94 isolates (68%) produced hemolysin. The distribution of hemolysin in each phylogroup is represented in Table 2. There is a significant correlation between hemolysin and biofilm formation and 100% hemolysin production was observed

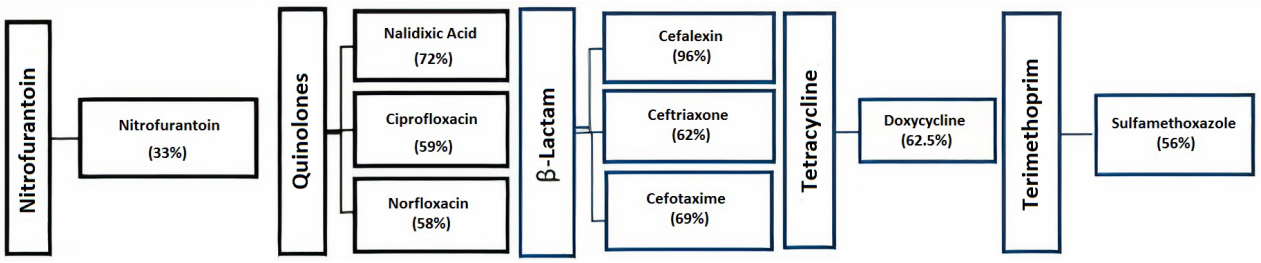


Figure 1. Frequency of Resistance of Uropathogenic *E. coli* Isolates (N = 138) to Different Antibiotic Classes

Table 2. Relationship Between Antimicrobial Resistance, Biofilm Production, and Phylogenetic Groups of 138 UPEC Isolates

Phylotype Based on Clermont 2013/2019	MDR	Non-MDR	Strong	Intermediate	Weak	No Biofilm	Hemolysin Positive
A	2 (2.5%)	3 (5.1%)	0	1	4	0	2 (50%)
B1	5 (6.25%)	9 (15.5%)	1	4	8	1	8 (53%)
B2	34 (42.5%)	14 (24.1%)	3	16	29	0	36 (75%)
D	12 (15%)	4 (6.8%)	0	5	10	0	9 (64%)
E	0 (0%)	1 (1.7%)	0	1	0	0	1 (100%)
F	10 (12.5%)	9 (15.5%)	3	5	9	2	15 (78%)
G	2 (2.5%)	1 (1.7%)	0	0	3	0	1 (33%)
Clade I	2 (2.5%)	2 (3.4%)	0	2	2	0	4 (100%)
Unknown	13 (16.25%)	15 (25.8%)	2	6	20	0	18 (63%)
Total	80	58	9	41	85	3	94

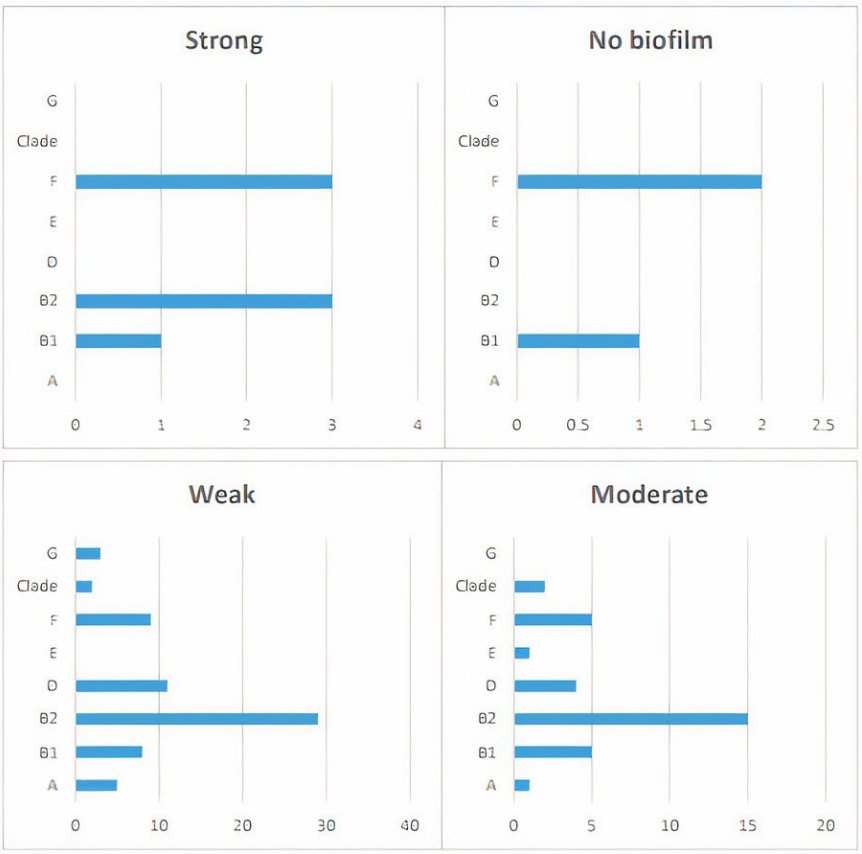


Figure 2. Relationship between the Biofilm Formation and Number of Isolates in Each Phylogenetic Group

in strains with the ability of strong and intermediate biofilm formation ($P<0.05$).

Discussion

Escherichia coli exhibits extensive phylogenetic substructure. Accordingly, it is possible to better understand the characteristics of the genetic nature of pathogenic or resistant strains using a more detailed classification, which can facilitate the development of an effective treatment strategy.[3] In the current study on 138 uropathogenic *E. coli*, group B2 had the highest number of MDR isolates among the phylogenetic groups, which is similar to the findings of studies conducted in other areas using the Clermont method (2013) including Ethiopia,[22], Mexico,[10] and Iran.[23] One explanation for this finding is that UTI isolates are mostly derived from phylogroup B2 and phylogroup D,[24] which is consistent with our study.

Overall, implementing the novel complementary phylotyping method showed advantages over the original one as a high percentage of strains moved from phylogroup B2 (14; 23%) to phylogroups F and G. This finding was reported for the first time, indicating that phylogroup F can be considered as a cause of UTI, which was previously reported as B2.

The results obtained from the relationship between antibiotic resistance and phylogenetic groups showed that all isolates of group B2 were resistant to all antibiotic agents used. In contrast, in groups A and E, there were not any MDR isolates (Table 2). A similar finding was reported by Ranjbar et al[25]; these data also suggest that the majority of MDR isolates of UPEC belonged to phylogenetic group B2, which is consistent with the outcomes reported in South Korea.[26] In the current study, the rate of MDR isolates that belonged to phylogenetic groups B2, D, F, B1, G, and Clade I was 42.5%, 15%, 12.5%, 6.2%, 2.5%, and 2.5%, respectively (Table 2).

In this study, 97.8% of UPEC isolates exhibited biofilm formation ability, and 34.7% of the isolates were moderate to strong biofilm producers. Phylogroups E, Clade I, and F had the highest percentage of strong to moderate biofilm formation with rates of 100%, 50%, and 42% respectively. As there was only one strain in phylogenetic group E and 4 strains in Clade I, it was not possible to draw a conclusion for these two phylogroups about the correlation between phylogenetic groups and biofilm. The phylogroup F with a rate of 42% ranked third in the formation of biofilm, while in other studies by the use of old phylogenetic structure conducted in Thailand[27] and Pakistan,[28] the majority of biofilm producers belonged to phylogenetic group B2. Additionally, in the study of Pompilio et al,[29] no statistically significant difference was found among phylogenetic groups in biofilm formation

capacity. The ability to form a strong and moderate biofilm was absent in phylogroup A, and all isolates (100%) were weak biofilm producers. Three isolates were assigned to group G, all of which produced weak biofilms (100%). Therefore, it can be concluded that a low biofilm production capacity was observed in this group using the microplate method. Other methods for studying biofilm production in this phylogenetic group may lead to different or similar results, which are recommended for more accurate conclusions. Moreover, for a more accurate conclusion, comparison of a larger number of phylogroup G is necessary.

Colonization with the hemolytic strains of *E. coli* is more likely to cause UTI[30] and in this study, hemolysin production was observed in 68% of all isolates. Phylogenetic groups F and B2 with 78% and 75% positive hemolysis had the highest rates of positivity. The distribution of hemolysin production was almost similar in non-MDR and MDR isolates. In other words, no correlation was observed between hemolytic activity and AMR. The present study supposed a possible correlation between biofilm formation and hemolysin production and showed that hemolytic isolates had a higher capacity to produce biofilms ($P<0.05$). Such observation was also reported before, which implies the importance of this finding.[31]

Conclusion

The majority of UPEC isolates from patients with UTIs belonged to phylogenetic group B2, which was highly resistant to most antibiotic agents. In this study, phylogroup F had higher rates compared to previous studies and showed the highest rate of moderate and strong biofilm formation. Hemolytic activity had no correlation with antibiotic resistance but showed a positive correlation with biofilm formation. Importantly, the present study showed the importance of phylogroup F in UTI that was overlooked in the old method.

Acknowledgements

We sincerely thank Prof. Kiarash Ghazvini and Mrs. Shima Hoseini for their kind support in sampling. We would also like to thank Zahra Sabeti Noghabi for her help during the laboratory processes. This research was supported by Ferdowsi University of Mashhad under the project number FUM. 53230.

Authors' Contribution

Conceptualization: Batoul Rahimifard , Gholamreza Hashemitabar, Mahdi Askari Badouei.
Data curation: All Authors.
Formal analysis: Batoul Rahimifard, Gholamreza Hashemitabar, Mahdi Askari Badouei, Vahid Soheili.
Funding acquisition: Batoul Rahimifard.
Investigation: All Authors.
Methodology: Batoul Rahimifard.
Supervision: Gholamreza Hashemitabar, Mahdi Askari Badouei.
Validation: All Authors.
Visualization: Batoul Rahimifard.
Writing – original draft: Batoul Rahimifard.
Writing – review & editing: All Authors.

Conflict of Interests

The authors declare that they have no competing interests.

Funding

The present project was supported by Ferdowsi University of Mashhad (Grant No. 53230).

Supplementary File

Supplementary file S1.

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