Molecular Analysis and Phylogenetic Classification of Uropathogenic Escherichia coli Isolated from Patients with Urinary Tract Infections

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Abstract: Urinary tract infections are one of the most prevalent bacterial infections all over the world, with *Escherichia coli* responsible for a large number of clinical cases. Bacterial pathogens employ multiple mechanisms to invade the host. A major mechanism in these strategies is the presence of secretion systems, including secretory system VIII, where the *csgG* gene encodes the outer membrane channel of the system. Study aimed to consider the importance of the class VIII secretion system in E. coli bacteria for biofilm formation; this study investigates the prevalence of the *csgG* gene encoding the outer membrane channel of this system. This study involved collecting 378 midstream specimens of urine from patients with urinary tract infections. Some virulence genes were detected and the phylogenetic groups were determined. In addition, detection of the *csgG* gene. Results show *E. coli* isolates were identified using an *Enterobacteriaceae* T-Kit *and trpA* gene (used as an internal control following the Clermont method). All 82 isolates were confirmed as *E. coli*. Phylogenetic groups were determined for all isolates: B2, D, A, C, B1, and F, representing 25.61%, 18.29%, 17.07%, 14.63%, 14.63%, and 9.77%, respectively. The present study showed detection of the genes: *fimH, papGII, afa and sfa* at 82, 79, 20 and 13 isolates, representing 100%, 96.34%, 24.39% and 15.85% respectively. The results also showed that 44 isolates that were selected were all found to contain the *csgG* gene 100 %. In conclusion, all 44 isolates selected *for csgG* gene detection carried this gene, indicating their ability to produce *Curli fibers* via the T8SS. These results indicate a possible association between the presence of the *csgG* and biofilm production in UPECs, suggesting that the gene plays a pivotal role in adhesion to urinary tract surface structures and in protecting bacteria from host defences.

1 INTRODUCTION

Urinary tract infections (UTIs) are one of the most prevalent bacterial infections all over the world, with *E. coli* responsible for a large number of clinical cases [1]. UTI are a major health concern [2]. *E. coli* is recognized as the main etiological factor of UTIs, distinguished by capacity to harbor many of the virulence factors and its genetic diversity [3].

E. coli is part of a family Enterobacteriaceae, which are Gram-negative, rod-shaped, it is a non-spore-forming, motile, ferments lactose, catalase-positive, oxidase-negative [4]. Pathogenic *E. coli* is known as intestinal pathogenic *E. coli* (InPEC) or extra-intestinal pathogenic *E. coli* (ExPEC) [5].

ExPEC, such as Uropathogenic *E. coli* (UPEC) are the highly frequent cause for the majority of UTIs. UPEC is a highly adaptable pathogen [6]. There are several ways in which adaptive evolution of UPEC has been observed, including changes in adhesion, colonization and invasion, to penetrate the urinary epithelial cells [7]. That have many virulence factors that cause infection. One of the important virulence factors of UPEC is their ability to form a biofilm, which allows them to persist and survive, and which causes most cases UTIs [8]. In many clinical and natural settings, bacteria are associated with surface that enables them to form biofilms[9]. Bacterial interactions rely with surfaces on the expression of a repertoire of surface adhesins by a set of virulence genes such as *fimH*, which coding for the α-d-mannose (adhesin of type 1 fimbriae) a major *E. coli* virulence factor [10]. Other important adhesion genes include the *papG*, which is often associated with pyelonephritis [11],the *sfa* gene and the *afa* gene, which code for Adhesins or specialized proteins

with sticky ends, which help to break the urinary bladder mucosa and help to attach to them [12].

Molecular studies have shown that there is genetic diversity within *E. coli* bacteria, but rather as a spectrum of phylogenetic-groups that differ in their pathological and biological characteristics [13]. In 2000, Clermont and his colleagues developed a method using PCR that enabled researchers to classify any *E. coli* isolate into four phylo-groups. In 2013, he was able to classify *E. coli* isolates into eight groups: A, B1, B2, D, F, E, C, and UP [14].

Pathogens employ multiple mechanisms to invade the host. A major mechanism in these strategies is the presence of secretion systems in pathogens such as *E. coli*. These systems play a significant role in bacterial virulence [15]. Secretion systems are one of the secrets to the important determinants of bacterial pathogenicity. They act through secreted effector proteins that act as signaling signals, allowing them to overcome their competitors and providing them with high adaptability [16]. *E. coli*, have developed numerous and diverse secretion systems [17]. Among the secretion systems of Gram-negative bacteria, including *E. coli*, are: the type (class) 1 secretion system, T3SS, T4SS and T6SS, or two-step, are: the T2SS and T5SS [18]. In addition, another specialized pathway known as the class VIII secretion system (T8SS) is mediates for the biosynthesis of curli fibers in *E. coli*. This system has an outer membrane channel that is encoded by a *csgG* gene, facilitates the secrete of unfolded protein subunits across the outer membrane. It is crucial for biofilm formation and survival in harsh environments [19]. The *csgG* gene is a vital component of the curli fimbriae system in *E. coli* bacteria. It encodes a structural protein that acts as a specialized secretory channel in the outer membrane to export curli subunits to the cell surface. Its essential importance lies in its role as a key factor in biofilm formation, which enhances the ability of pathogenic bacteria to adhere to the host, resist the immune system, and survive in harsh environmental conditions [20].

Considering the importance of the T8SS in *E. coli* bacteria for biofilm formation through the secretion of Curli fibers via the outer membrane channel encoded by the *csgG* gene, this study aimed to investigate the presence this gene and classify *E. coli* isolates according to the Clermont method.

2 MATERIALS AND METHODS

2.1 Sample Collection and Phenotyping Identification

This study involved collecting 378 midstream specimens: female 196 and male 182 of urine from patients with urinary tract infections of both sexes with an age range from 28 days to 70 years from November 2025 to February 2026, sampling was performed randomly. The broad age range (28 days to 70 years) was intentional, reflecting the study's objective to assess the prevalence of T8SS and virulence factors across the general UTI patient population rather than within a specific age group, thereby providing a comprehensive epidemiological picture of the study area. Samples were collected from hospitals in Diyala Governorate, Iraq (Baqubah Teaching Hospital, Al-Batoul Teaching Hospital, and Al-Khalis General Hospital), and several private clinical laboratories.

Samples were cultured on Eosin Methylene Blue (EMB) agar, MacConkey agar, and blood agar plates (HiMedia, India). Samples were considered UTI - positive if they showed bacterial growth of at least 10^5 CFU/mL.

Bacterial phenotyping identification was performed using biochemical tests by Enterobacteriaceae T-Kit (Shenzhen Render Bio-tech Co., Ltd).

2.2 Detection of Biofilm

Detecting the ability of bacteria to synthesize biofilms using the microplate assay (Quantitative Assay) [21].

2.3 Extraction of DNA and Molecular Identification

Phenotyping identification *E. coli* isolates were cultured in Nutrient Broth media and cultured for 24 h at 37 °C. DNA was extracted using the PureLink™ DNA Mini Kit (Thermo Fisher Scientific, USA), following manufacturer's protocol. DNA extracts were eluted in 90 µL of buffer and store at −20 °C.

After the initial biochemical identification by Enterobacteriaceae T-Kit and subsequent using the Clermont (2013) method, the *trpA* gene was employed as an internal control. As a conserved housekeeping gene, *trpA* served to confirm the species identity of *E. coli* and differentiate it from closely related taxa, thereby enhancing the identification reliability [14]. Thermo cycling conditions were: initial denaturation 94 °C for 5min; 30 cycles of denaturation 94 °C for 5sec, annealing 57 °C for 20sec, and extension 72 °C for 1min; followed by a final extension step 72 °C for 5 min. Sequences of primer are listed in Table 1[22].

2.4 Phylogenetic Group Determination

The phylogenetic groups for *E. coli* isolates was determined by Clermont [14]. Sequences of primer are shown in Table 1. amplifications were carried out on a PCR under the conditions: initial denaturation 95°C for 5min and 40 cycles for denaturation 95°C for 60 sec, annealing 68°C for 60 sec, amplification 72°C for 1 min, and final extension 72°C for 10 min [22].

2.5 Amplification of Virulence Genes (VGs)

Four virulence genes linked to UPEC have been identified. These genes were grouped into two categories 1: Fimbriae attachment genes: *fimH* and *papG*-II [23]. 2: Adhesion genes: *afa* and *sfa*. Genes detection via multiplex PCR the protocol: initial denaturation 94 °C for 5 min; 30cycles of denaturation 94 °C for 5 sec, annealing 63 °C for 60 sec, and extension 72 °C for 1 min; final extension 72 °C for 5 min. sequences of primer are listed in Table 1 [24], [25].

2.6 Amplification of the *csgG* Gene

From the total of 82 *E. coli* isolates, a representative sample of 44 was selected for *csgG* gene detection. The selection was strategically based on their phylogenetic distribution and the presence of virulence genes: *fimH*, *papG* and *afa* or *sfa*, This approach ensured that the selected isolates encompassed genetically distinct lineages with a specific virulence profile. By targeting these diverse and functionally significant isolates, the study aimed to investigate the T8SS across the most clinically relevant lineages, thereby strengthening the robustness of the correlation between genetic background and secretion system prevalence.

The *csgG* gene was amplified using PCR. A primer designed specifically for this study to target a specific region of the gene was used. The reaction cycle consisted of the following phases: initial denaturation 95°C for 5 min, 35 cycles of denaturation 95°C for 30 sec, annealing 57°C for 30 sec, extension 72°C for 45 sec, and final extension 72°C for 5 min. Primer sequence used in the amplification designed for this study and listed in Table 1.

Table 1: Primer sequences used in the study.

Gene	Oligo sequence 5'-3'	References
fimH	F:TGCAGAACGGATAAGCCGTGG	23
	R:GCAGTCACCTGCCCTCCGGTA	
papG	F:GGGATGAGCGGGCCTTTGAT	
	R:CGGGCCCCCAAGTAACTCG	
afa	F:GCTGGGCAGCAAACTGATAACTCTC	24
	R:CATCAAGCTGTTTGTTCGTCCGCCG	
sfa	F:CTCCGGAGAACTGGGTGCATCTTAC	25
	R:CGGAGGAGTAATTACAAACCTGGCA	
trpA	F:CGGCGATAAAGACATCTTCAC	14
	R:GCAACGCGGCCTGGCGGAAG	
chuA	F:ATGGTACCGGACGAACCAAC	
	R:TGCCGCCAGTACCAAAGACA	
arpA	F:AACGCTATTCGCCAGCTTGC	
	R:TCTCCCCATACCGTACGCTA	
TspE-4.C2	F:CACTATTCGTAAGGTCATCC	
	R:AGTTTATCGCTGCGGGTCGC	
yjaA	F:CAAACGTGAAGTGTCAGGAG	
	R:AATGCGTTCCTCAACCTGTG	
csgG	F:TGTCATTCTGCCGTTCTGCT	This Study
	R:GCTGGTCACGGCATTGAAAG	

3 RESULTS

3.1 Isolation and Identification of *E. coli*

The study included collecting 378 urine samples from patients with urinary tract infections of both sexes, with the number of samples divided into 196 samples for females and 182 samples for males. The total number of samples that showed positive growth on culture media (MacConkey agar, Blood agar, and Eosin-Methylene Blue (EMB) agar) was 241 samples, representing 63.76%, of which 141 samples were from females 58.42% and 100 samples were from males 41.49%. The total number of samples that did not show growth on culture media was 137 samples, representing 36.24%. The total number of samples that showed positive growth on blood agar alone was 55, representing 22.80% of the total samples that showed positive growth on the culture media. The total number of samples that showed positive growth on the all three-culture media was 186, representing 77.20%, of which 159 lactose-fermenting isolates 85.48% and 27 isolates were not lactose- fermenting 14.52%.

E. coli isolates were identified in the first stage based on morphological properties after being cultured on the above media. The results indicated that 82 isolates were lactose fermenter and produced bacterial colonies with a bright pink color and a well-define margins. On EMB agar, they showed bright metallic green colonies, which is a characteristics feature of *E. coli*.

Biochemical Identification was performed using the Enterobacteriaceae T-Kit, and the biochemical test results confirmed that all 82 bacterial isolates were identified *E. coli*. The biochemical test results are shown in Table 2.

After initial biochemical identification by using Enterobacteriaceae T-Kit and according to the Clermont method (2013), the *trpA* gene was used as an internal control gene. As a conserved housekeeping gene, this gene confirmed the identity of all 82 *E. coli* isolates, showing a 489 base pair band in all isolates (Fig. 2).

3.2 Formation of Biofilm

The capacity of *E. coli* to form biofilms was detected using the Microtiter plate method. The findings revealed that 80 isolates were biofilm-producing, which represents 97.56%. 45 isolates, which represents 56.25%, had weak biofilm production, 18 isolates, which represents 22.50%, had moderate biofilm production, 17 isolates, which represents 21.25%, had strong biofilm production, and only two isolates, which represents 2.44%, were non-biofilm-producing.

3.3 Phylogenetic Group Determination

The Clermont method was applied to classifying *E. coli* bacterial isolates into evolutionary groups, and the results are presented in Figure 1 and 3.

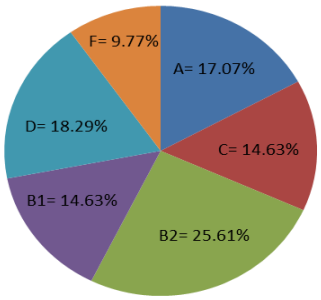

Figure 1: Phylogenetic grouping of *E. coli* isolates by Clermont method.

Table 2: Biochemical identification results of *E. coli*.

Test Name	Symbol	Result
Arginine Dihydrolase	ADH	Negative
Lysine Decarboxylase	LDC	Positive
Ornithine Decarboxylase	ODC	Positive
Citrate Simmons	C	Negative
Urease	URE	Negative
Glucose Fermentation	GLU	Positive
Hydrogen Sulfide Production	H2S	Negative
Esculin Hydrolysis	ESC	Negative
Gelatin Liquefaction	GEL	Negative
Tryptophan Deaminase	TDA	Negative
Indole Production	IND	Positive
Voges-Proskauer	VP	Negative
Mannitol- Fermentation	MAN	Positive
Fermentation of Sorbitol	SOR	Positive
Inositol-Fermentation	INO	Negative
Melibiose-Fermentation	MEL	Positive
Fermentation of Saccharose	SAC	Positive
Fermentation of Rhamnose	RHA	Positive
Fermentation of Amygdalin	AMY	Positive
Arabinose-Fermentation	ARA	Positive
Lactose Fermentation	LAC	Positive
Cellobiose Fermentation	CEL	Positive
Citrate - Alternative	CIT	Negative
Beta-galactosidase	ONPG	Positive

3.4 Molecular Detection of Virulence Factor Genes

The detection results by PCR in the current study showed presence of the genes (*fimH*, *papGII*, *afa* and *sfa*) at 82, 79, 20 and 13 representing 100%, 96.34%, 24.39% and 15.85% respectively (Fig. 4 and 5).

3.5 Detection of the T8SS in UPEC Isolates via the *csgG* Gene

The results for the 44 UPEC isolates, which were selected based on their phylogenetic distribution and the presence of virulence genes: *fimH*, *papG* and *afa* or *sfa,* showed that all isolates carried the *csgG* gene 100 % (Fig. 6).

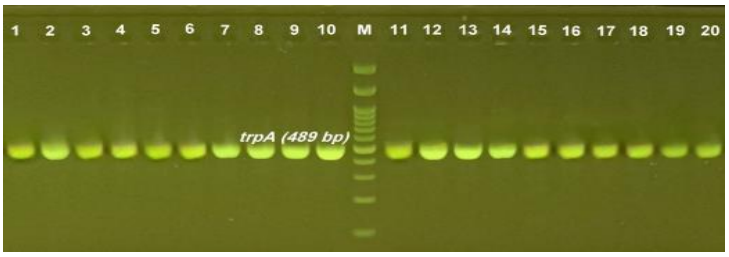

Figure 2: PCR product for detecting the trpA gene 489 bp by use 1.5% agarose with red safe stain for 60 min. Lane M: size indicator (DNA ladder 100-3000 bp).

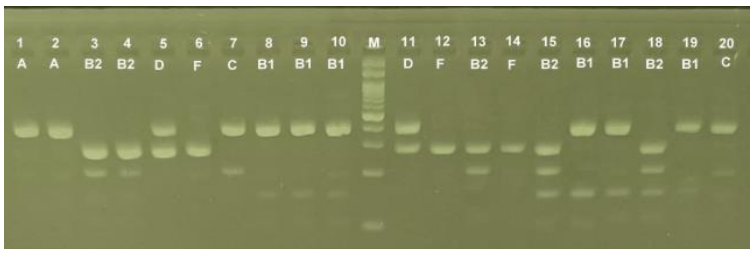

Figure 3: Electrophoresis of the Quadruplex PCR products for phylogenetic grouping, genes: arpA 400 bp, chuA 288 bp, yjaA 211 bp, and TspE4.C2 152 bp by use 1.5 % agarose with red safe stain for 60 min. Lane M: size indicator (DNA ladder 100-3000 bp).

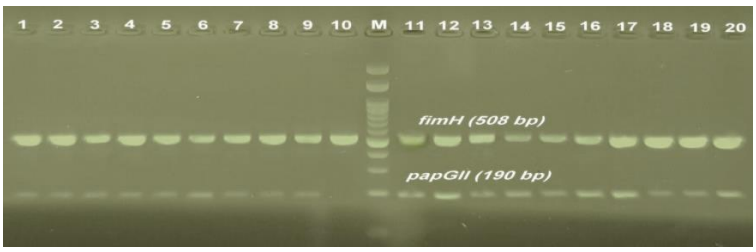

Figure 4: PCR product for detecting the papGII gene 190 bp and fimH 508 bp by use 1.5% agarose with red safe stain for 60 min. Lane M: size indicator (DNA ladder 100-3000 bp).

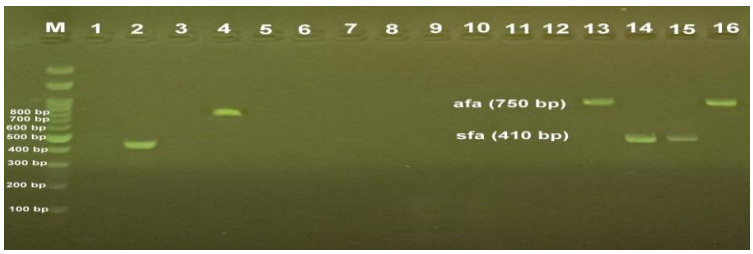

Figure 5: PCR product for detecting the *sfa* gene 410 bp and *afa* gene 750 bp by use 1.5% agarose with red safe stain for 60 min. Lane M: size indicator (DNA ladder 100-3000 bp).

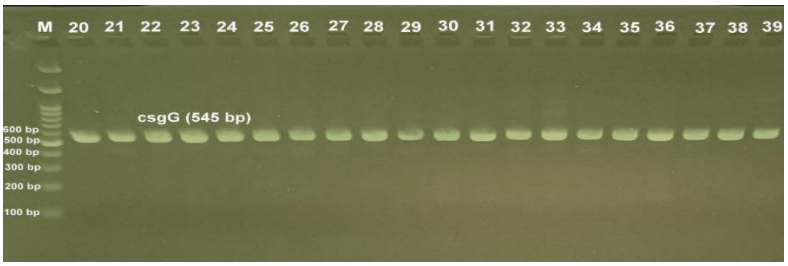

Figure 6: PCR product for detecting the csgG gene 545 bp by use 1.5% agarose with red safe stain for 60 min. Lane M: size indicator (DNA ladder 100-3000 bp). This figure for 20 representative isolates from the rest of the isolates.

4 DISCUSSION

The results of the current study indicate that the percentage of samples that were positive for bacterial growth in this study is 63.76%, which is a relatively high percentage, reflecting the prevalence of urinary tract infections among the patients in this study. This may be attributed to the nature of the community and to the excessive and indiscriminate use of antibiotics, which is consistent with what studies have indicated, which confirm that urinary tract infections are still among the most common bacterial infections globally [26].

The results showed a higher infection rate among females, at 58.42%, compared to males, whose infection rate was 41.49%. This is consistent with what was mentioned in many epidemiological studies, which showed that women are more susceptible to urinary tract infections due to anatomical and hormonal factors [27].

The identification of *E. coli*, the results showed that 82 isolates were primarily identified based on morphological properties, exhibiting shiny pink colonies on MacConkey agar and metallic green colonies on EMB agar-characteristics of this bacterial species.

The diagnosis using biochemical tests (Enterobacteriaceae T-Kit), with all isolates 100% showing results consistent with *E. coli*. This strengthens the reliability of the initial diagnosis and confirms the accuracy of the laboratory procedures used in this study[28]. Overall, these results confirm that *E. coli* is the leading cause of urinary tract infections.

After initial biochemical identification by using Enterobacteriaceae T-Kit and according to the Clermont method (2013), the *trpA* gene was used as an internal control gene. As a conserved housekeeping gene, this gene confirmed the identity of all 82 *E. coli* isolates, showing a 489 bp band in all isolates, which contributed to enhancing the reliability of species identification and differentiation from closely related species [29].

The results showed that the majority of *E. coli* isolates 97.56% were capable of biofilm formation, which reflects the ability of the bacteria to adhere to surfaces and form a community protected from host defenses and antimicrobial agents. Among the biofilm-producing isolates, production was weak in 56.25%, moderate in 22.50%, and strong in 21.25%, while only 2.44% of the isolates did not produce a biofilm. These findings are consistent with several studies that have shown that the majority of *E. coli* isolates associated with urinary tract infections possess biofilm-forming capabilities. Research conducted in 2023 indicated that the percentage of biofilm-producing isolates ranged from 90% to 98%, with varying levels of biofilm production among the isolates [30].

Phylogenetic group analysis of urinary tract infection (UTI) isolates of *E. coli* is a tool for understanding the relationship between genetic characteristics and pathogenicity. The results of the current study, using the Extended Clermont method, showed significant genetic diversity, with group B2 was the most prevalent at 25.61%, followed by group D at 18.29%, and then group A at 17.07%. As for the remaining genetic groups, the study showed the presence of groups B1, C, and F in low and similar proportions. This results is relatively agreement with the study Halaji *et al.* [31]. The proportion of B2 and D in the current study is relatively lower than in some international studies where it can reach 50%, which is likely due to geographical variation or the nature of the samples. One of the striking results of this study is the appearance of Group A at a similar rate to Group D. Group A is classified as commensal isolates, but its appearance in urinary tract infections indicates an genomic adaptation; recent studies indicate that Group A isolates have begun to acquire virulence genes through horizontal gene transfer, or that they compensate for the lack of virulence by

increasing their resistance to antibiotics, which allows them to remain in the urinary tract [32].

In the current study, and to increase the probability that the isolates under study are UPEC bacteria, four virulence genes (*fimH*, *papG*, *sfa* and *afa*) were included, in addition to isolating them mainly from patients with urinary tract infections, where 49 isolates 59.76% possesses two genes, 29 isolates 35.37% possesses three genes, and only two isolates 2.44% possesses all four of the above genes. This reflects the existence of variation in the genetic content of virulence factors among the isolates. The presence of multiple isolates with two or three genes indicates the pathogenicity of these bacteria. This does not depend on the presence of all virulence factors together but can be demonstrated on presence of varying numbers of these genes, particularly those associated with adhesion to urinary tract epithelial cells. Furthermore, the limited number of isolates possessing all four genes suggests that the presence of the full set of virulence factors is not a prerequisite for infection. Therefore, the presence of two or more genes, in addition to the source of the clinical isolate, is a reasonably acceptable presumption for classifying these isolates as belonging to UPEC.

The presence of the *fimH* gene among all isolates, confirming its important role as a conserved virulence factor in UPEC. This finding is consistent with several studies that have shown very high or complete prevalence of this gene, further supporting its status as an important molecular marker for the diagnosis of uropathogenic *E. coli* isolates [33].

The study results showed a high prevalence of the *papGII* gene >90% among UPEC isolates. This is one of the allelic variants of the *papGII* gene associated with P fimbriae, indicating its important role as a virulence factor, particularly in urinary tract infections. Most international studies have reported lower prevalence to the current study. This high prevalence can be explained by several factors, including the selective nature of the patient samples taken, as well as the geographical and epidemiological specificities of the local samples. This result reflects the importance of the *papG*II gene as a strong indicator of pathogenicity in UPEC isolates.

The current study demonstrated a low prevalence of the *afa* and *sfa* genes in UPEC isolates, both of which are associated with adhesion factors that determine the location and severity of infection. Isolates possessing the *afa* gene were associated with lower urinary tract infections, particularly cystitis, while isolates possessing the *sfa* gene were associated with upper urinary tract infections, especially pyelonephritis. This reflects the ability of these genes to determine the pathogenic localization of bacteria [24], [25]. In this study, UPEC isolates were categorized based on their clinical association with lower or upper urinary tract infections. This distribution was consistent with patient clinical data, supporting the role of these two genes as indicators of pathogenicity severity and different infection patterns in UPEC isolates.

The results above confirm the existence of a strong correlation between group B2 and the virulence genes *afa* and *sfa*, as the majority of UPEC bacterial isolates belonging to groups B2 and D possessed one of the *afa* or *sfa* genes and also carried the *fimH* and *papG*II genes. This gives them additional advantages for colonizing the urinary tract and overcoming the host's immune defenses, which is consistent with the hypothesis that specialized virulence is the dominant feature in this phylogenetic groups.

All 44 UPEC isolates tested for the *csgG* gene were positive 100%, indicating that all isolates possess the ability to form Curli fibers via the Type VIII secretory system(T8SS), a key component of biofilm formation. The study also demonstrated that these 44 isolates were biofilm producers, further supporting the possible association between the presence of the *csgG* and biofilm production in UPECs. These findings suggest that *csgG* gene often plays a pivotal role in adhesion to urinary tract surface structures and the formation of biofilms that protect bacteria from host immune defenses, thus contributing to infection persistence and recurrence. Gene presence and biofilm production, Statistical analysis to test the relationship between them using analytical tests was not possible. These results indicate a widespread presence of the csgG gene among the studied isolates, however, establishing a statistically significant relationship between the gene and biofilm formation requires data variability.

5 CONCLUSIONS

This study confirmed that Escherichia coli remains the predominant causative agent of urinary tract infections among the examined patients and demonstrated a high prevalence of biofilm-forming isolates. Phylogenetic analysis revealed considerable genetic diversity among the isolates, with phylogroups B2 and D being the most common, supporting their recognized association with uropathogenic E. coli (UPEC). The widespread distribution of the virulence genes fimH and papGII,

together with the presence of afa and sfa in selected isolates, highlights the diverse virulence profiles that contribute to bacterial colonization, persistence, and pathogenicity within the urinary tract.

Molecular analysis demonstrated that all 44 selected UPEC isolates carried the csgG gene, indicating the genetic potential to produce curli fibers through the Type VIII Secretion System (T8SS). In addition, all of these isolates exhibited biofilm-forming ability, suggesting a close association between the presence of csgG and biofilm formation. Although statistical analysis of this relationship was not feasible because no variability in csgG occurrence was observed, the findings strongly support the importance of this gene in bacterial adhesion, persistence, and recurrent urinary tract infections.

Overall, the results suggest that the csgG gene may serve as a reliable molecular marker for identifying biofilm-forming UPEC isolates and further emphasize the role of the T8SS in UPEC pathogenicity. Future studies involving a larger number of isolates, including csgG-negative strains, together with gene expression and functional analyses, are recommended to further clarify the contribution of the T8SS to biofilm development and the pathogenesis of urinary tract infections.

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