Original Article

Evaluation of Biofilm Formation Potential and Corresponding Virulence Genes in Uropathogenic *E. coli* Isolates from Kirkuk, Iraq

Maral Abdul-Sajjad Baktash[1]*, Siham Sh. Al-Salihi[2]

[1]Department of Microbiology, Gynecology and Pediatrics Hospital, Kirkuk Directorate of Health, Kirkuk, Iraq.
Email: maral.abdul_sajjad@ntu.edu.iq
[2]Department of Medical Laboratory Techniques, College of Health and Medical Techniques, Northern Technical University, Kirkuk, Iraq.
Email: siham1968@ntu.edu.iq

Abstract

Background: Urinary tract infections (UTIs) rank among the most prevalent extraintestinal infections in humans. Escherichia coli is the principal causative agent of urinary tract infections (UTIs), belongs to Enterobacteriaceae family and exists inside the normal microbiota of humans. **Method:** A cross-sectional study was undertaken at an institution with female patients, pregnant, non-pregnant, symptomatic and asymptomatic patients. At Kirkuk General Hospital, Gynecology and Pediatric Hospital from September 2024 to December 2024. A mid-stream urine specimen was obtained using a clean-catch method and significant isolates identified by VITEK2 system. Biofilm formation phenotypically and genotypically investigated. **Result:** A total of 300 urine specimens, 49 (16.33%) was positive cultures for bacteria. Gram-negative bacteria were prevalent than gram-positive about 41 (83.67%). UPEC was the predominant gram negative pathogen 27 (55.10%), *Klebsiella pneumonia* 5 (10.20%), *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterococcus faecalis*, *Enterobacter cloacae* were 2 (4.08%). Biofilm production in UPEC estimated by micro-titer plate method, the results were 1 (3.70%) strong, 9 (33.33%) moderate, 17 (62.96%) weak biofilm producer. Molecular analysis conducted via polymerase chain reaction (PCR) demonstrated that all of 27 UPEC (100%) strains obtained from females with UTIs had curli fimbria (*csgA*) and type 1 fimbriae (*fimA*) genes. Curli fimbria is recognized for its role in bacterial biofilm development and the adherence of E. coli associated with human cystitis. Hemolysin produced in 8/27 of UPEC isolates and *hlyA* gene was 100% in these isolates. **Conclusion:** About 20% of the positive cases was pregnant women. All UPEC isolates were biofilm producer and all of them express (*csgA*) and (*fimA*) genes.

Keywords: UPEC, Biofilm Formation, (*csgA*), (*fimA*), (*hlyA*) Genes.

Introduction

Urinary tract infections represent a prevalent category of bacterial illnesses.[1] Enterobacteriaceae are a prevalent component of the intestinal microbiota in humans; yet, this bacterial group can also induce extraintestinal infections of the urinary tract (UTIs).[2] An infection of the urinary tract encompasses infection of the bladder (cystitis) and the kidney (pyelonephritis), potentially resulting in life-threatening sepsis. Around one quarter of women with a urinary tract infection will have a recurrence within six months of the first infection,[3] either with the identical strain or a novel organism,[4] attributed to a diverse array of microorganisms. Uropathogenic Escherichia coli (UPEC) is the primary etiological agent of urinary tract infections, encompassing both community-related and nosocomial infections.[5] Approximately 50% of women may encounter a urinary tract infection (UTI) at least one time in their lives, with recurrence being prevalent.[6] Uropathogenic Escherichia coli (UPEC) is responsible for over 90% of community-acquired (CA) urinary tract infections (UTIs) and 50% of hospital-acquired (HA) UTIs. UPEC strains possess a diverse array of virulence factors (VFs), including adhesins and toxins, to facilitate infection establishment.[7] Moreover, numerous factors facilitate the invasion of host cells by microorganisms; these factors encompass proteins associated with motility

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Address for Correspondence: Department of Microbiology, Gynecology and Pediatrics Hospital, Kirkuk Directorate of Health, Kirkuk, Iraq
Email: maral.abdul-sajjad@ntu.edu.iq

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and adhesion, enabling bacterial cells to adhere to host receptors.[8] The virulence factors of adhesion, specifically Type 1 fimbriae and curli fibers, initiate infection by facilitating surface adherence, cell aggregation, and biofilm development.[9] Extracellular matrix (biofilm) affords protection against environmental challenges, including antibiotics, dehydration, host defenses, elevated temperatures, and food deprivation.[10] *E. coli* produce hemolysin, an enzyme that significantly contributes to its pathogenicity.[11] Alpha- hemolysin is one type of these enzymes can be encoded on chromosomal pathogenicity islands or plasmids include an operon consisting of four genes one of them *hlyA* gene which encode functional toxin (HlyA).[12]

The DNA-based detection method is extensively utilized for diagnosing infectious diseases, owing to the existence of unique DNA sequences in organisms that serve as trustworthy biomarkers, complemented by amplification technologies such as (PCR).[13,14]

Materials and Methods

Research Design

A cross-sectional study was performed on females UTI patients after receiving official agreements before establishing the study.

Research Environment and Period

The research was conducted in Kirkuk city on two main hospitals (Kirkuk General Hospital, Gynecology and Pediatrics Hospital). The research was carried out from (September 2024 to December 2024).

Study Specimen and Sampling Technique

A total number of 300 midstream urine specimens were obtained from outpatients who visiting the two hospitals in Kirkuk city. Each sample was placed in a clean, clearly labeled screw-topped urine container. The patient's information included name, age, marital status, and symptoms, which were written on a questionnaire form designed for the study. Sampling done according to the following criteria:

1. Symptomatic patients (having one or more of the following; fever, frequency, burning sensation, dysuria and renal stone), asymptomatic patients were also involved.
2. Patients who have not received antibiotic treatments.
3. Pregnant and reproductive age women.

Urine sample was taken to identify the etiological agents which was responsible from the infection. In the cultures, the isolation of a singular or predominant microbe was deemed positive. The isolation was determined with the VITEK-2 GN card (BioMérieux, France)

Biofilm Detection

The characterization of biofilm by micro-titer plate method. Twenty-seven of UPEC isolates were put into Brain-Heart Infusion Broth (BHIB) supplemented with 1% glucose, incubated for 24 hours at 37°C, and subsequently diluted 1:100 with a new media. Each well in a micro-titer plate was filled with 200 µL of the diluted culture, with broth serving as a reference; the plate was incubated for 48 hours at 37°C.

Subsequent to incubation, the plate was meticulously rinsed three times with phosphate-buffered saline (PBS) to remove non-adherent cells. Subsequently, 200µL of 0.1% crystal violet was added and incubated for fifteen minutes at laboratory temperature. The stain was then removed from the plate by washing it three times with distilled water, and the plate was inverted for several hours to facilitate drying. Two hundred microliters of a 95% ethanol solution were added to re-solubilizing the colorant. The absorbance measured by Enzyme Linked Immunosorbent reader, at 630nm.[15,16] These absorbance readings are interpreted as:

ODi ≤ ODc = Non biofilm generator
ODc < OD ≤ 2 ODc = Weak biofilm generator
2 ODc < OD ≤ 4 ODc = Moderate biofilm generator
4 ODc < OD = Strong biofilm generator

Molecular Characterization of Biofilm Formation

Genomic DNA has been obtained from fresh colonies.as manufacturer's instructions. To identify curli fimbriae and type 1 fimbriae genes including distinct PCR reactions were conducted as outlined in Table 1 below:

Table 1: Primer Sequence for Investigated Genes of UPEC Isolates.

Genes	Primer Sequence	Size bps	References
csgA	F 5′-GCAGCAATCGTATTCTCCGG-3′ R 5′-CGGTCGCGTTGTTACCAAAG-3′	415 bps	designed for this study
fimA	F 5′-GGCAATCGTTGTTCTGTCGG-3′ R 5′-CCGCATTAGCAGCACCCGGG-3′	503 bps	
hlyA	F 5′-CAGTCTGCAAAGCAATCCGC-3′ R 5′-CGCTTCTGCCATCTCAGAAG-3′	510 bps	

A commercially available PCR Master Mix was used for the PCR (Promega, USA) according to the guidelines provided by the manufacturer. Briefly, 13.5 µL of Master Mix, 1 µL of forward and reverse primer (10 pmoles/µL), and 4.5 µL DNase-free distilled water and finally, 5 µL DNA template was added in a final volume of 25 µL. An initial denaturation phase was conducted at 95°C for 3 minutes prior to the PCR amplification of genes, 30 cycles of denaturation at 95°C for 20 s, annealing at primer-specific temperatures for 25 s, and extension at 72°C for 50 s are then. Following electrophoresis in a 1% agarose gel using 1x TBE (Tris/Boris/EDTA) buffer, the PCR products were dyed with a safe stain load dye (Promega, USA) and examined under UV light.

Analysis of Statistics

A statistical analysis was conducted utilizing SPSS Statistics program, version 26.1. Comparative studies across groups were performed using suitable statistical methods, chiefly the Chi-square test, to assess significance

via generated p-values. Beyond 0.05 was deemed statistically non-significant, whilst below 0.01 was classified as highly significant.

Ethical Considerations

Ethical consent for conducting the study was secured by Kirkuk Health Department (approval No.714 2024-9-23).

Results

In this study, out of the collected samples, 49/300 (16.33%) were identified as causative agents of UTI in females. Depending on cultural and biochemical characteristics, with a higher infection rate in Non-Pregnant than pregnant women (69% vs 31%). The results of the marital status among the infected females are shown in Table (2). The highest rate, 206/300 (68.67%), was reported among the married patients, while 94/300 (31.33%) were among the singles. From the patients, 93/300 (31%) were pregnant and 207/300 (69%) were non-pregnant. Furthermore, the infection rate in married females was found to be higher than in single females (19.90% vs. 8.51%) respectively. Statistically, a significant difference was found (P=0.021).

Table 2: UTI Culture Positivity According to Demographic and Clinical Characteristics.

Category	Symptomatic Presentation		Marital Status		Pregnancy Status		Total
	Present	Absent	Married	Single	Pregnant	Non-pregnant	
Positive samples No.%	40 (19.42%)	9 (9.57%)	41 (19.90%)	8 (8.51%)	10 (10.75%)	39 (18.84%)	49
Total of examined samples No.	206 (68.67%)	94 (31.33%)	206 (68.67%)	94 (31.33%)	93 (31.00%)	207 (69.00%)	300
P-value	0.0487		0.021		0.113		

In this research ten different genus of bacteria was identified, with high frequency to *Escherichia coli* and *Klebsiella pneumoniae* (55%, 10%), respectively. The predominant isolates with their frequency demonstrated in (Figure 1)

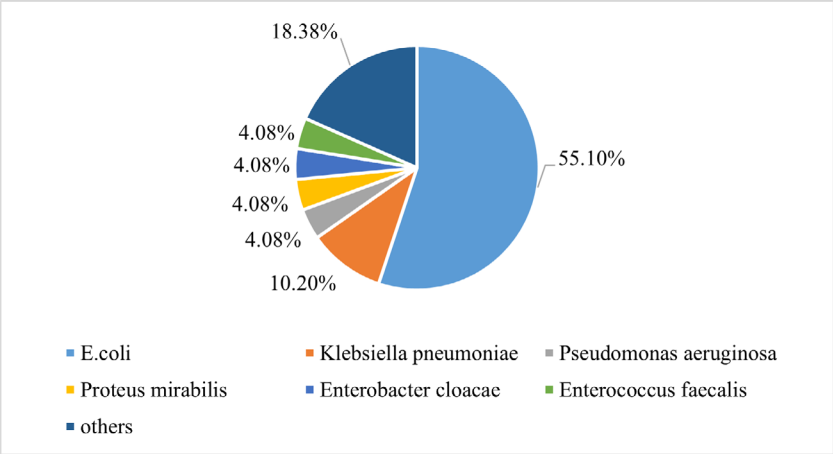

Figure 1: Distribution of Isolates in UTI Infections.

E. coli isolates was 27/49 (55.10%). All of them diagnosed by chemical tests and confirmed by VITEK2 compact system as demonstrated in (Figure 2) and (Table 3).

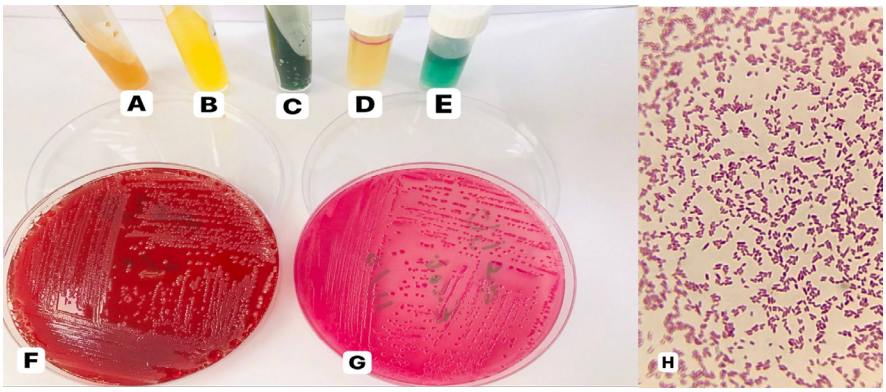

Figure 2: *E. coli* on A: Kligler Iron Agar (A\A with gas). B: Christensen´s Urea Agar (-ve). C: Simmon´s Citrate Agar (-ve). D: Peptone Water (indole test +ve). E. Mannitol Salt Semi-solid Agar (motility test +ve). F: Blood Agar (β hemolysis). G: MacConkey Agar (lactose-fermenter). H: Gram Stain (gram negative).

Hemolysin Production

The detection of hemolysin enzymes was performed by cultivating the identified bacteria on 5% blood agar, revealing two forms of hemolysis: beta and gamma. Eight isolates exhibited beta-hemolysis, whereas the remaining isolate had gamma-hemolysis. The beta-hemolysis shown in [figure 3]:

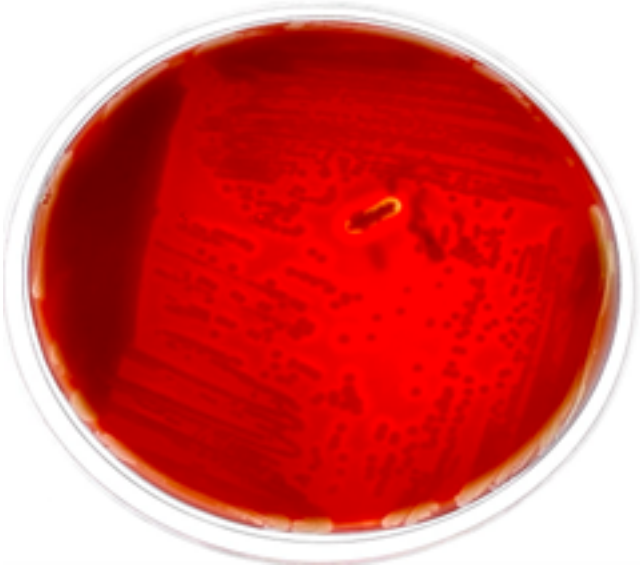

Figure 3: Beta Hemolysis of UPEC on 5% Blood Agar Plate.

Table 3: Outcomes of Biochemical Characteristic Details of E. coli by VITEK2 Compact System.

Laboratory Report

bioMérieux Custormer:
System #:
Patient Name: najma ali. Printed by: Labadrnin
Isolate: 305-1 (Approved) Patient ID: 305
Card Type: GN Bar Code: 2412780403018656 Testing Instrument: 000B4B8115 (VITEK2C)
Setup Technologist: Laboratory Administrator(Labadmin)
Bionumber: 0405610550426610
Organism Quantity: Selected Organism: Escherichia coli

Comrnents:

Identification Inforrnation	Card :GN Status: Final	Lot Number: 241 2780403 Analysis Time: 4.08 hours	Expires: Jul 7, 2025 13:00 CDT Comleted: Nov 13, 2024 20:46 CST
Organism Origin	VITEK 2		
Selected Organism	99%0 Probability Bionumber: 0405610550426610		Escherichia coli Confidence: Excellent identification

Analysis Organisrns and Tests to Separate:
Analysis Messages:
Contraindicating Typical Biopattern(s)
Biochernical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTP	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAIaP	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	VINT	-	39	SKG	-
40	ILATk	-	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	Gl A	-	47	ODC	+	48	LDC	+	53	IHISa	-	56	CMT	+	57	BGUR	+
58	0129R	+	59	GGAA	-	61	IMLTa	-	62	ELL-M	-	64	1 LATa	-			

Biofilm Formation

All *E. coli* isolates shown the potential to build biofilms. Most of them was weak biofilm forming bacteria, about 17 (62.96%), then 9 (33.33%) was moderate biofilm former, whereas 1 (3.70%) was strong biofilm former. No evidence of non-biofilm forming was observed. All results demonstrated in (Table 4) and in (figure 4) the microtiter plate demonstrate all 27 *E. coli* isolates was biofilm producer.

Table 4: Quantitative Assessment of Biofilm Generation in *E. coli*.

Biofilm Production Level	No. of Isolates	Percentage %
Strong	1	3.70
moderate	9	33.33
Weak	17	62.96
Negative	0	0
Total	27	100

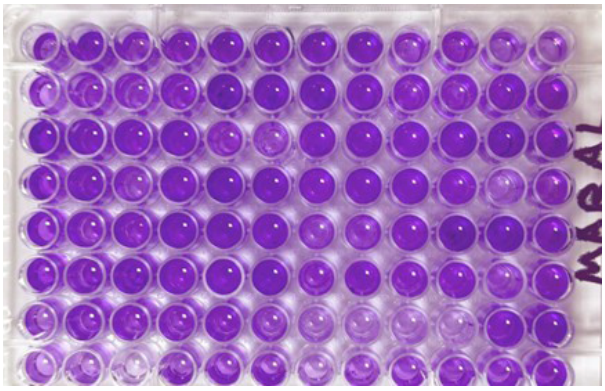

Figure 4: Detection of Biofilm Forming Capability of E. coli on Micro Titer Plate.

Molecular Detection

The PCR approach was used on 27 E. coli isolates to identify genes associated with type 1 and curli fimbriae (curli-specific gene). The molecular detection results indicated that all 27 clinical isolates possess the *csgA* gene, as illustrated in [Figure 5].

Molecular analysis of the *fimA* gene showed that each of the 27 isolates of E. coli possess *fimA* gene, as illustrated in [Figure 6].

The molecular analysis demonstrated that all isolates contained both the *csgA* and *fimA* genes. Furthermore, 8 out of 27 isolates generate β-hemolysin on 5% blood agar, and all 8 (100%) of these isolates exhibit the *hlyA* gene. Two samples utilized as controls are prominently displayed in [Figure 7]. The rates are illustrated in Table 5.

Table 5: Genotypic Expression of UPEC Virulence Factors.

No.	Genes	Positive n (%)	Negative n (%)	Total
1.	*csgA*	27 (100%)	0	27
2.	*fimA*	27 (100%)	0	27
3.	*hlyA*	8 (100%)	0	8

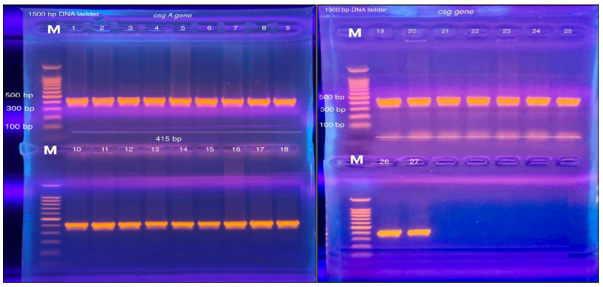

Figure 5: Detection using Agarose Gel Electrophoresis of *csgA* Gene in UPEC (415 bp). Lane M: 1500 bp DNA ladder, lane (1-27): Amplicons of the *csgA* Gene.

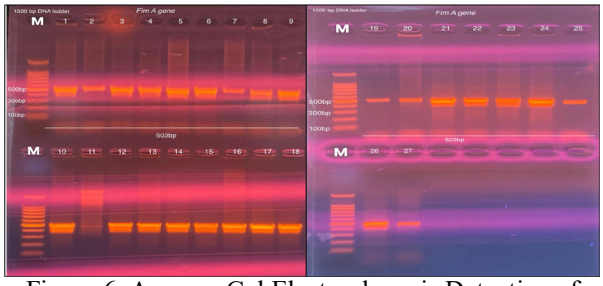

Figure 6: Agarose Gel Electrophoresis Detection of *fimA* Gene in UPEC (503 bp). Lane M: 1500 bp DNA Ladder, Lane (1-27): Amplicons of the *fimA* Gene.

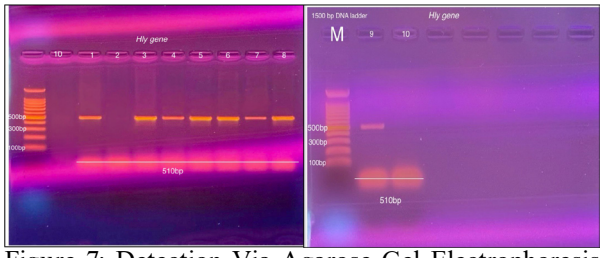

Figure 7: Detection Via Agarose Gel Electrophoresis of *hlyA* Gene in UPEC (510 bp). Lane M: 1500 bp DNA Ladder, Lane (1-10): Amplicons of the *hlyA* Gene

Discussion

Urinary tract infections, like other preventable diseases, severely impact the lives of women in resource-limited countries. The problem becomes more serious by the presence of pregnancy and resistance to the standard treatment regime.[17]

In this study, the included symptomatic cases were more than asymptomatic, (68.67%, 31.33%) respectively. This consistent with a study in Iraq that (82.1%) were symptomatic, and (17.9%) were asymptomatic.[18]

However, (16.33%) of the individuals that took part had a positive culture. These results are in consistent with a study in Riyadh, SAU, they receive 44,884 urine samples for culture. Within these urine cultures (19%) were positive for uropathogens.[19] Though some studies in Iraq reported a higher rate of culture-positive in UTIs as in Najaf, they received 194 urine samples from females, 93 samples (48%) tested positive for bacterial growth at $\geq 10^4$ CFU/mL.[20] This could be because the study's significant growth is interpreted as a high-burden bacterial count (10^5 CFU/mL). The inequality between researches could be caused by variations in the type and the size of the sample in the study population, methodology, drug intake, definition of bacteriuria and the study period length. Another cause may be that an overactive bladder and interstitial cystitis/bladder pain syndrome provide a clinical picture akin to a urinary tract infection (discomfort, urgency, pressure or pain), thus leading to a clinical diagnosis of UTI despite a negative culture result.[17,21]

The highest rate of infection according marital status was (19.90%) reported among the married patients, while (8.51%) were not-married. These results are consistent with another study in Iraq, that the incidence of bacteriuria was (14.7%)

in married women, and (8%) in not-married women, this attributed to sexual activity, that facilitates the transmission of pathogens to the urinary system.[22] A significant relation existing between marital status and UTIs, p-value was 0.021. Because pregnancy causes physiological and hormonal changes, it is often regarded as one of the risk factors for UTI. In the current study only (10.75%) of pregnant women had positive urine culture. The comorbidities, generally lacks a substantial correlation with positive culture outcomes, p-value=0.113. This outcome aligns with the findings given in Saudi Arabia in (2024), which indicated a rate of 5%.[23] This finding is in contrast to other studies in Iraq, in which the rate of infections in pregnant women was (86%).[24] These disparities might be attributed to economic, hydration, environmental, social and regular antenatal visits and continuous monitoring of laboratory tests in pregnant women can significantly reduce the incidence of urinary tract infections (UTIs). Gram-negative bacilli are frequently documented in UTI patients, the spectrum of pathogens may vary according to time of the study, area and population. Out of forty-nine isolates in this study, (83.67%) were gram-negative bacteria, similar results were noted in the (2022) survey conducted in Iraq, which yielded a result of 67.03%,[25] opposed to the study of (2024) in the same country, which was reported (51.4%) for gram positive.[26] *E. coli* was the prevalent pathogen, it was about (55.10%), this result is in line with the findings of the study in the (2024) in Iraq, which was reported (67%) for this bacteria.[27] Although it contradicts the findings revealed in the 2024 study in Iraq, which indicated a prevalence of 29.7% for the same bacteria.[26]

The second predominant pathogen was *K. pneumonia* (10.20%), this result is comparable to those in the (2022), who reported a rate of (8%) for this bacteria.[25] In contrast with the result reported in (2025) in Kirkuk which was reported (33.4%) for this bacteria.[28]

Other pathogens included *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterobacter cloacae*, and *Enterococcus faecalis*, each exhibiting an equivalent prevalence of approximately (4.08%). This outcome parallels a (2022) study conducted in Iraq, which reported a prevalence of (4.76%) for *Pseudomonas aeruginosa* and *Proteus mirabilis*. Furthermore, *Enterobacter cloacae* and *Enterococcus faecalis* were identified at rates of (6.35%) and (12.70%), respectively; these findings align with the results of our investigation.[29]

The prevalence of Enterobacteriaceae, particularly *E. coli*, in urinary tract infections is due to their role as constituents of the normal flora in the gastrointestinal tract. The anatomical closeness of the urethra to the anus enables the transfer of these bacteria from the gastrointestinal tract to the urinary tract, which possess distinct virulence factors that promote colonization and infection, alongside various human behaviors and medical practices that aid their introduction and perseverance inside the urinary system.[6] Their capacity to adhere and invade uroepithelial cells, form biofilms, and evade immune responses makes them particularly effective at causing UTIs.[30]

In a study of 27 UPEC isolates for biofilm production assessed via the micro-titer plate method, (3.70%) were identified as strong biofilm producers, (33.33%) as moderate producers, and (62.96%) as weak producers. The p-value was 0.001, signifying a substantial association between bacteriuria and biofilm production. These results contrast with a (2024) study conducted in Iraq, which reported biofilm-forming bacteria at rates of (33.33%, 61.90%, and 4.76%) for strong, moderate, and weak classifications, respectively.[31] Accordingly, a PCR program was adopted to detect the presence of *csgA* and *fimA* genes, which are thought to be responsible for biofilm development.

The gel electrophoresis data indicate that both genes (*csgA* and *fimA*) were present in (100%) of all isolates in our study. This outcome aligns with the findings reported in (2016 and 2017), when the result for *csgA* gene was also (100%) in these studies.[32,33] Furthermore, a study conducted in (2024) in Iraq, reported a (100%) prevalence of the *fimA* gene.[34] In contrast to our findings, a study conducted in (2022) reported a prevalence of (73.3%) for the *csgA* gene[35] and (76%) for *fimA* gene in this study.[36] *Escherichia coli* is characterized by production of various hemolysin enzymes, the most important of which is alpha-hemolysin. Which plays a significant role in its pathogenicity. UPEC strains that express hemolysin are linked to severe advanced renal diseases, including cystitis and pyelonephritis.[11,37] In this study 8/27 isolates were beta-hemolysis caused complete hemolysis for red blood cells around the colonies on blood agar. These hemolysin produce isolates submitted to hemolysin alpha (*hlyA*) gene detection, consequently (100%) of the isolates expressed this gene. Similar results were observed in the study conducted in (2025) in Iraq, which found that the *hlyA* virulence gene was the most prevalent gene among the isolates, occurring in (86%) of cases.[38] In contrast, a study conducted in Iraq in (2020) revealed that the *hlyA* gene prevalence was (26.32%).[39]

Conclusion

1. Urinary tract infection was predominant in married females and the prevalence of causative agents were *E. coli*.
2. The *csgA* and *fimA* gene which encodes for curli and type 1fimbriae was observed in 100 % of UPEC strains isolated from females with urinary tract infections, which indicate its role as an important virulence factor used to adhesion and biofilm formation in extra-intestinal pathogenic *E. coli* infections.
3. Study have shown that pregnant women who consistently attend prenatal check-ups are less likely to develop UTIs compared to those women with irregular follow-up.

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