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ORIGINAL ARTICLE

Biofilm Mediated Antibiotic Resistance in Uropathogenic *Escherichia Coli* Isolated From Egyptian Women with Urinary Tract Infections

[1]Fatma Elzahraa Ali*, [2]Mohammed A. Elfeky, [3]Gamal H. Ali, [2]Amany Nafea, [2] Rawhia F. Abdel Hamid

[1]Department of Microbiology and Immunology, Faculty of Pharmacy, Assiut University
[2]Department of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University, Assiut, Egypt
[3]Department of Obstetrics and Gynecology, Faculty of Medicine, Assiut University, Assiut, Egypt

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Corresponding Author:
Fatma Elzahraa Ali Mohammed, Department of Microbiology and Immunology, Faculty of Pharmacy, Assiut University, Assiut, Egypt
Tel: +201099674066
Fatema.2012541@pharm.aun.edu.eg

ABSTRACT

Background*: Urinary tract infection (UTI) is a pathogenic invasion of the urothelium with resultant inflammation, encompassing a spectrum of upper and lower urinary tract disease. Biofilms provide a survival strategy to the bacteria by positioning them to effectively use the available nutrients and prevent access to antimicrobial agents, antibodies and white blood cells.* ***Objective****: This study aims to describe the profile of biofilm forming bacteria e.g. E.coli from Egyptian women with UTIs, determine the antimicrobial susceptibility patterns of isolated pathogens, Evaluate the genes responsible for biofilm formation and investigate the biofilm formation and its role in antibiotic resistance.* ***Methodology:*** *The study was conducted on female patients admitted to Naga Hammadi General Hospital from January 2022 to January 2023.Two hundred and fifty urine samples were collected from female patients. Viable count conducted on urine sample by calibrated loop technique. If the number of colonies in freshly voided urine samples is ≥ 10^5 CFU/mL, this has been usually registered as UTI. The UPEC were isolated by cultivation different culture media (MacConkey and EMB), and confirmed by biochemical confirmatory tests, TSI, Citrate and indole test.* ***Results:*** *the total studied cases were 250 participants, only 51 cases were positive for E. coli (20.4%). All the patients were female patients. Their ages varied between 16 years and 60 years including 13 single female, 38 married. The UTI rate was higher among married female patients (74.5%). Escherichia coli was the most frequent isolated bacteria 51 isolates (20.4%). All uropathogenic E.coli in this study were tested for the presence of RcsA; 46 positive for FocA, 19 positive for CsgA, 22 positive for CsgD and 13 positive for RcsC gene.* ***Conclusion****: Our study revealed that Uropathogenic E. coli (UPEC) is the most predominant causative agent of urinary tract infections. Fosfomycin is the drug of choice for treatment of Urinary tract infections (UTI) caused by UPEC. It showed a low incidence of bacterial resistance as it is taken as a single dose unit.*

INTRODUCTION

A pathogenic invasion of the urothelium that causes inflammation is known as a urinary tract infection (UTI)[1]. Based on the anatomy and comorbidities of the patient, infections are categorized as difficult or uncomplicated[2]. In women worldwide, urinary tract infections (UTIs) are the most prevalent extraintestinal infectious diseases[3].

Uropathogenic *Escherichia coli* (UPEC) is the most frequent causal agent of urinary tract infections, which are among the most common illnesses caused primarily by facultative Gram-negative bacteria[4].

The capacity to create biofilms is a prerequisite for Uropathogenic ***E. Coli*** invasion, growth, ascent, and persistence in the uroepithelium[5].

Antimicrobial resistance is created when bacteria are positioned in biofilms to take advantage of the available nutrients and obstruct the entry of antimicrobial agents, antibodies, and white blood cells. Additionally, biofilms are found to contain a high concentration of enzymes that inactivate antibiotics, such as beta-lactamases, which gives rise to an island of resistance to antibiotics[6].

Hydrophobic microorganisms generate infections that are challenging to cure and are more invasive surface features including curl cilia of type 1, flagella, and certain outer membrane adhesions may be crucial for the development of biofilms. Complex signaling

pathways, including phosphotransfer protein (*Rcsd*), signaling factor (*Rcsf*), response regulator (*Rcsb*), histidine kinase (*Rcsc*), and auxiliary activation. Numerous investigations have demonstrated that biofilms have an increased level of CA production[7]. Comparably, curli filaments are extracellular fibers that bacteria create and are essential for the production of biofilms and other community behaviors. A complex regulatory network co-regulates its synthesis, with *csgd* playing a significant role[8].

According to Lund *et al*[9]**.,** transcriptional activation of the *Csg*ABC operon, *Csg*AB, and *Csg*DEFG (*Csg*, curli-specific gene) is how *CsgD* promotes curli synthesis. It was also discovered that UPEC isolates expressed type 1 pili, S, P, and F1C pili, which are all encoded by the *Foc* gene. To elevate UPEC to the bladder in the urinary tract, all of these structures are required[10].

METHODOLOGY

Ethical approval:

This research was approved by the Institutional Review of the Faculty of Medicine, Assiut University, Assiut, Egypt, The approval number is (04-2023-2003)

Two hundred and fifty urine samples were collected from female patients attended Naga Hammadi General Hospital ,Gynecology Clinics during the period of January 2022 to January 2023.The samples were rocessed in the Microbiology department, Faculty of Medicine, Qena and Assiut university.

Bacteria logical examination: For each participant, a clean-catch midstream urine sample was taken. Every specimen was shipped in an icebox to the Microbiology lab in less than one hour. Urine specimens were inspected visually, then directly examined microscially to check for pyuria. The sample was centrifuged for 5 minutes at 450 x g[11].

Viable count[12]

Viable count was conducted on urine sample by calibrated loop technique. A calibrated loop was vertically midway inserted into the well-mixed urine samples; a zigzag line was streaked on nutrient agar, then incubated overnight at 37°C. The colonies were counted and the number multiplied by 1000 as the used loop was 1µl. If the number of colonies in freshly voided urine samples is $\geq 10^5$ CFU/mL, this has been usually registered as UTI.

Isolation of *E. coli:*

Urine samples were cultured on MacConkeys agar and EMB. E .Coli was identified by the standard bacteriological methods.

Antimicrobial susceptibility test[13]

Antimicrobial susceptibility testing of the isolates was performed on Muller's Hinton agar by Kirby_bauer disk diffusion method recommended by clinical laboratory standard institution.

A range of discs were used{ Ceftazidime (CAZ)(30µg), Amoxicillin_clavulinic acid (AMC)(30µg) Ceftriaxone (CRO)(30µg), Imipenem (IPM)(10µg), Amikacin (AK)(30µg), Fosfomycin (200µg), Nalidixic acid (NA)(30µg), Ciprofloxacin (CIP)(5µg), Norfloxacin (NOR)(10µg), Ofloxacin (OFX)(5µg) and Nitrofurantoin (NIF)(300µg) }.

Biofilm formation testing of *E.coli* isolates by Congo red agar method:[14]

Congo red agar was prepared by mixing 37g brain heart infusion broth, 50g of sucrose, 0.8g Congo red dye and 10g agar. The solution was the sterilized by autoclave (121°C, 15 ibs for 15 min) and dispersed on petri dishs. Isolates were plated and incubated at 37°C for 2 hrs. The biofilm forming strains produced black colonies on Congo red plates while non-forming strains produced red colonies.

Molecular detection of Biofilm forming genes from uropathogenic *E.coli*

DNA extraction boiling method: Fresh subcultured colonies on EMB were inoculated on L.B broth .The colonies were centrifuged at 5000 xg for 3 min .the sediment was suspended in 5 milliliters molecular biology grade water mixed exposed to water bath at 100°c for 15 min and centrifuged at 5000 x g for 3 min. The supernatant was exposed to sudden cooling at ice both for 20 min. then transported to sterilized new eppendorf's tube and labeled to be used in *PCR*. The presence of *DNA* was confirmed using gel electrophoresis[15].

PCR reaction for each gene:

The *DNA* was extracted by using the boiling method, PCR was performed in a 25-µl reaction, the mixture containing 12.5 µl of PCR master mix (Thermo Fisher Scientific, United States), 1 µl each of forward and reverse primers, 2 µl of DNA template, and 8.5 µl of nuclease-free water, Initial denaturation 10 min, amplification was performed in 30 cycles as 1 minute at 94℃, 1 minute of annealing at 59℃, 1 minute at 72℃, and a final extension at 72℃ for 10 minutes[16].

The nucleotide sequences of primers used in the study are listed in Table 1.

Table 1: The nucleotide sequences of primers used for detection of biofilm forming genes among UPEC

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	TGATCACTAGATCTTCTGCAGG	500
CsgD-R	GAACAACGAACGAGCGATCTC	

RESULTS

The study was conducted on two hundred and fifty (250) female patients admitted to Naga Hammadi General Hospital, Gynecology Clinics from January 2022 to January 2023.Total studied cases were 250 participants, only 51 cases were positive for *E. coli* (20.4%). As shown in **figure 1**

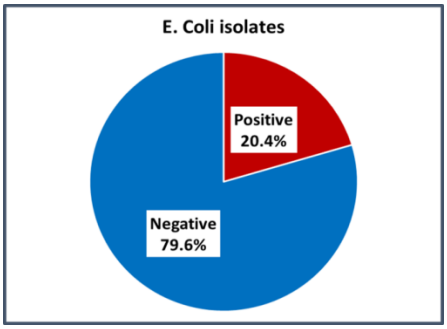

Fig. 1: Percentage of *E. coli* isolates among studied case

All patients were females. Their ages varied between 16-60 years old. Including 13 single female, 38 married, 9 pregnant and 8 were catheterized .The UTI rate is higher among married female patients (74.5%), as shown in table 2.

Table 2: Characteristics of studied patients

	N=51	%
Age (years): Mean±SD (range)	28.24 ± 8.88(16-60)	
Marital status		
▪ Single	13	25.5%
▪ Married	38	74.5%
Pregnancy	9	17.6%
Catheterized patients	8	15.7%

Data were expressed as frequency and % or mean ± SD (range).

The antibiogram of the isolated uropathogenic *E. coli* showed:

Table 3 and figure 2 showed the antibiogram of the isolates as follow showed Forty-nine isolates (96.1%) were sensitive to fosfomycin and two isolates were resistant. Four isolates (7.8%) were sensitive to amikacin and fourth seven (92.2%) isolates were resistant. Forty isolates (78.4%) were sensitive to ofloxacin eleven isolates (21.6%) were resistant. All the fifty-one (100%)isolates were resistant to imipenem. Forty(78.4%) isolates were sensitive to ciprofloxacin and eleven(21.6%) isolates were resistant. Thirty-eight isolates(74.5%) were sensitive to norfloxacin ,and thirteen isolates(25.5%) were resistant. All the isolates (100%) were resistant to ceftazidime. Twenty-four isolates (49%) were sensitive to amikacin, fourteen isolates (28.5%) showed intermediate resistance, and twelve isolates were resistant(23.5). Two isolates (3.9%) were sensitive to ceftriaxone, twenty-four isolates showed intermediate resistance, and twenty-five isolates (49%) were resistant. Eighteen isolates (35.3%) were sensitive to nalidixic acid, sixteen isolates (31.4%) showed intermediate resistance, and seventeen isolates (33.3%) were resistant. Forty isolates (78.4%) were sensitive to co meth oxazole and trimethoprim, eleven isolates (21.6%) were resistant. Forty isolates (78.4%) were sensitive to nitrofurantoin, nine isolates (17.6%) showed intermediate resistance, and two isolates (3.9%) were resistant.

Table 3: Antibiotic susceptibility pattern for isolated *E. coli*. (n=51)

	Antibiotic susceptibility pattern		
	Sensitive	**Intermediate**	**Resistant**
FF200	49 (96.1%)	0 (0.0%)	2 (3.9%)
AMC30	4 (7.8%)	0 (0.0%)	47 (92.2%)
OF5	40 (78.4%)	0 (0.0%)	11 (21.6%)
IMP10	0 (0.0%)	0 (0.0%)	51 (100.0%)
CIP5	40 (78.4%)	0 (0.0%)	11(21.6%)
NX10	38 (74.5%)	0 (0.0%)	13 (25.5%)
CAZ	0 (0.0%)	0 (0.0%)	51 (100.0%)
AK10	25 (49.0%)	14 (27.5%)	12 (23.5%)
CTR	2 (3.9%)	24 (47.1%)	25 (49.0%)
NA30	18 (35.3%)	16 (31.4%)	17 (33.3%)
COT	40 (78.4%)	0 (0.0%)	11 (21.6%)
NIT300	40 (78.4%)	9 (17.6%)	2 (3.9%)

Data were expressed as frequency and %

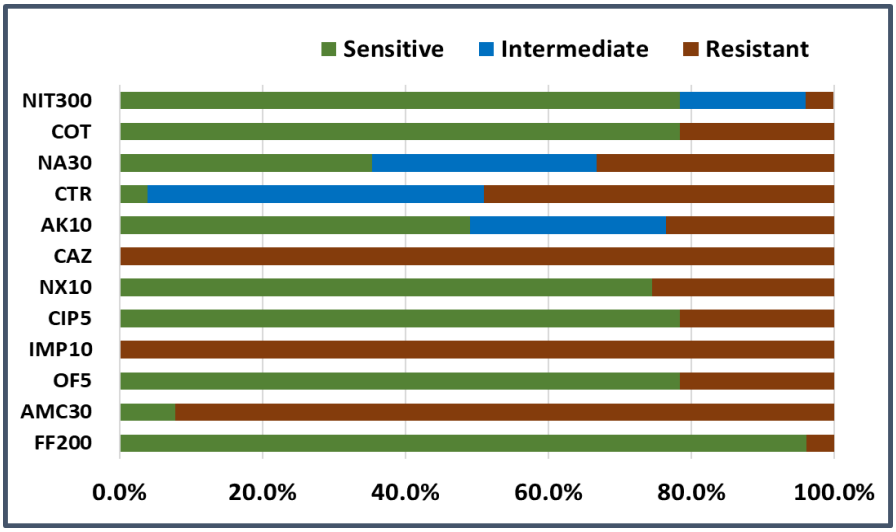

Fig. 2: Antibiotic susceptibility pattern for isolated *E. coli*.

Table 4: Association between biofilm formation and antibiotic resistance for *E. coli.*

Resistance to:	**Biofilm formation**			**P-Value***
	Weak	**Moderate**	**Strong**	
FF200 (n=2)	1 (50.0%)	0 (0.0%)	1 (50.0%)	0.999
AMC30 (47)	4 (8.5%)	34 (72.3%)	9 (19.2%)	**<0.001**
OF5 (n=11)	1 (9.1%)	9 (81.8%)	1 (9.1%)	**0.003**
IMP10 (n=51)	4 (7.8%)	37 (72.5%)	10 (19.6%)	**<0.001**
CIP5 (n=11)	1 (9.1%)	9 (81.8%)	1 (9.1%)	**0.003**
NX10 (n=13)	1 (7.7%)	10 (76.9%)	2 (15.4%)	**0.004**
CAZ (n=51)	4 (7.8%)	37 (72.5%)	10 (19.6%)	**<0.001**
AK10 (n=12)	2 (16.7%)	9 (75.0%)	1 (8.3%)	**0.009**
CTR (n=25)	2 (8.0%)	18 (72.0%)	5 (20.0%)	**<0.001**
NA30 (n=17)	2 (11.8%)	13 (76.5%)	2 (11.8%)	**0.001**
COT (n=11)	1 (9.1%)	9 (81.8%)	1 (9.1%)	**0.003**
NIT300 (n=2)	1 (50.0%)	1 (50.0%)	0 (0.0%)	0.999

Data were expressed as frequency and %
*Chi square test was used to compare proportion between groups.
All isolates were 100% drug resistant.

Biofilm formation among isolated *UPEC*

As shown in table (4) the ability of uropathogenic *E .coli* for biofilm formation was determined by Congo red media. All the isolates were biofilm formers 72.5 % (37 isolates) were moderate biofilm formers, while 19.6 % (10) were strong biofilm formers and 7.8% (4 isolates) were weak biofilm formers.

Table 5. Biofilm formation among isolated *UPEC.*

	N=51	%
Biofilm formation		
▪ Weak	4	7.8
▪ Moderate	37	72.5
▪ Strong	10	19.6

Data were expressed as frequency and Percentage

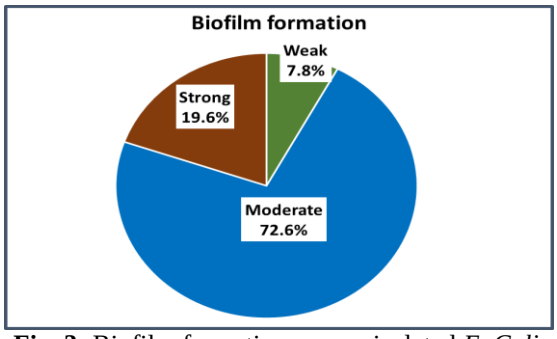

Fig. 3: Biofilm formation among isolated *E. Coli.*

Gene amplification and detection:

Gel electrophoresis: The desired amplicon of 587, 706, 450, 400 and 500 was visualized under UV illumination after staining by ethidium bromide as follow in fig 4 & 5.

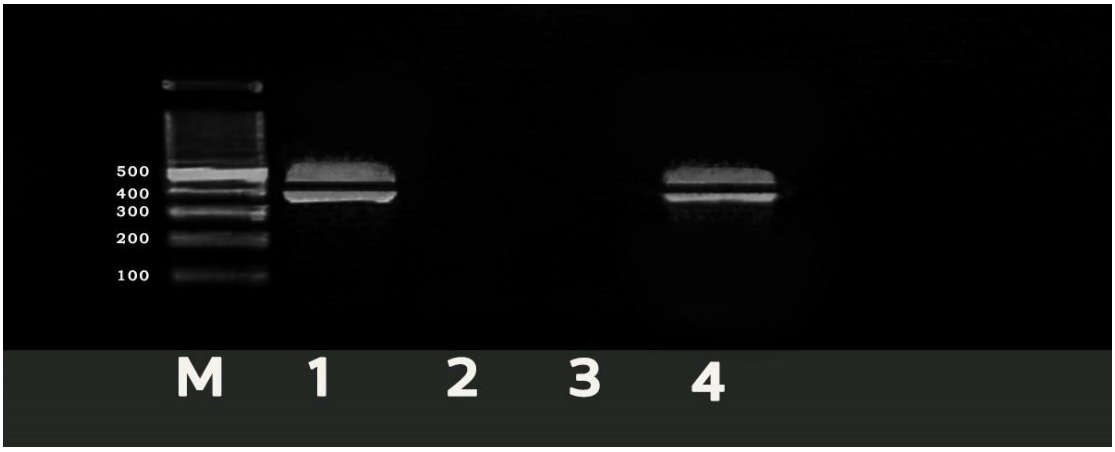

Fig. 4: Representative gel picture of PCR amplification of genes *CsgA* and *CsgD***.** showed samples 1 and 4 were positive for both genes.

Fig. 5: Representative gel picture of PCR amplification of genes *Foc-A* and *RcsC* showed samples From 1 to 14 were positive for *Foc-A*. Only sample five was negative for *RcsC.*

Bio film producing genes as shown in table 5, All uropathogenic *E.coli* in this study were tested for the presence of *RcsA*, 46 positive for *FocC*, 22 positive for CsgD, 19 positive for *CsgA* and 13 positive for *Rcsc* gene.

Table 6: Biofilm producing genes among isolated *E.coli* organisms

Gene	N=51	%
***RcsA* gene**		
▪ Positive	46	90.2%
▪ Negative	5	9.8%
***FocC* gene**		
▪ Positive	46	90.2%
▪ Negative	5	9.8%
***CsgD* gene**		
▪ Positive	22	43.1%
▪ Negative	29	56.9%
***CsgA* gene**		
▪ Positive	19	37.3%
▪ Negative	32	62.7%
***RcsC* gene**		
▪ Positive	13	25.5%
▪ Negative	38	74.5%

By comparing

Three isolates were positive for *rcsA* gene (75%), One isolate was positive for *focC* (25%) and One isolate was positive for *csgD* (25%). Thirty-three isolates were positive for *rcsA* gene (89%),Thirty-five isolates were positive for *foc* gene (94.6%),Eleven isolates were positive for *csgA* (29.7%),Eleven isolates were positive for *csgD* (29.7%) and Five isolates were positive for *RcsA* (13.5%). ten isolates were positive for *rcsA* gene (100%).Ten isolates were positive for *foc* gene (100%).Eight isolates were positive for *csgA* gene (80%).Ten isolates were positive for *csgD* gene (100%).Eight isolates were positive for *rcsA* gene (80%).

Table 7: Correlation between biofilm formation and the type of isolated gene

	Biofilm formation			P-Value*
	Weak (n=4)	Moderate (n=37)	Strong (n=10)	
Positive rcsA gene	3 (75.0%)	33 (89.2%)	10 (100.0%)	0.337
Positive foc gene	**1 (25.0%)**	**35 (94.6%)**	**10 (100.0%)**	**<0.001**
Positive csgA gene	**0 (0.0%)**	**11 (29.7%)**	**8 (80.0%)**	**0.004**
Positive csgD gene	**1 (25.0%)**	**11 (29.7%)**	**10 (100.0%)**	**<0.001**
Positive RcsC gene	**0 (0.0%)**	**5 (13.5%)**	**8 (80.0%)**	**<0.001**

Data were expressed as frequency and %

*Chi square test was used to compare proportion between groups

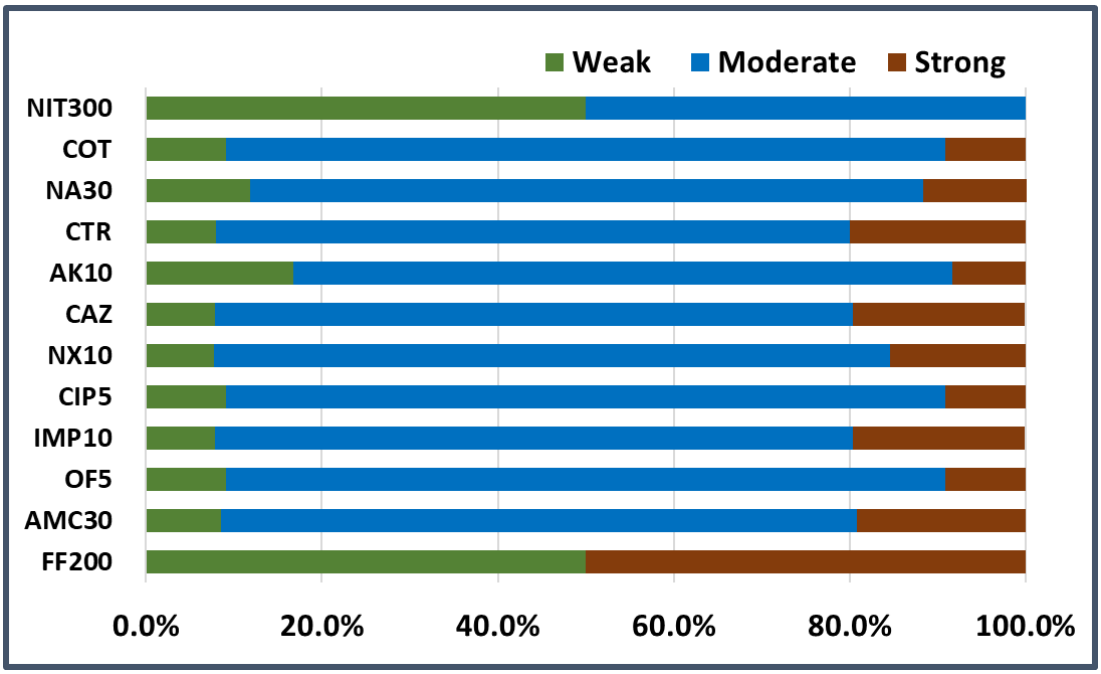

Fig. 6: Biofilm formation and antibiotic resistance for isolated *E. coli.*

DISCUSSION

One of the most prevalent bacterial illnesses acquired in community hospitals and worldwide is urinary tract infection (UTI). *Escherichia coli* is the predominant cause of urinary tract infections (UTIs), responsible for 80-85% of the estimated 150 million cases identified annually[17].

Among the most often isolated causal agents of urinary tract infections (UTIs), *E. coli* is accounting for 80% of uncomplicated UTIs, 95% of community-acquired infections, and 50% of hospital-acquired infections[18].

The present investigation revealed a greater incidence of infection among married female patients, estimated at 74.5%.Comparable findings have been reported by Enas *et al*., which revealed a rise in the occurrence of urinary tract infections (UTIs) among individuals aged 26 to 45 years. This age group, which includes the most sexually active individuals, accounted for 71.99% of all UTI-related cases[19].

The study revealed that 17.60% of the 51 female patients were diagnosed with urinary tract infections (UTIs) during pregnancy. The study conducted by Noura *et al*., revealed a proportion of 35.9%. The variability in UTI rates among different research may be attributed to the environmental, cultural, social, and religious elements that impact sexual behaviors in diverse groups [20].

Escherichia coli was the predominant bacterium isolated in our investigation, accounting for 20.4% of the 51 isolates.

Noura *et al*., found that the UPEC was the most predominant isolated microorganism, accounting for 37.4% of the samples [20].

UPEC is the predominant microorganism found in the vaginal area. The identical observation was also identified by Moshera *et al*., [21].

In contrast to our study, Ahmed *et al*., found that *S. saprophticus* was the most common microorganism, accounting for 35%. This discrepancy may be attributed to inaccuracies in collecting midstream samples [22].

High resistance to Amoxicillin-Clavulinic acid, Imipenem, and Ceftazidime was observed in the antibiotic susceptibility pattern of the isolated *E. coli* cultures. Furthermore, our *E. coli* isolates exhibited a significant susceptibility to Fosfomycin, which were consistent with the results reported by Noura *et al*., in their study conducted at Assiut University hospitals in 2023 [20].

The isolates isolated by Noura *et al*., exhibited a high sensitivity to fosfomycin, making it a potential first choice for treating MDR-UPEC. The presence of MDR may be attributed to the improper usage of antibiotics. Development of resistance to Fosfomycin is unlikely due to its administration as a prescribed dosage [20].

A bacterial biofilm leads to persistent infections by exhibiting heightened resistance to antibiotics and disinfectant agents, as well as evading phagocytosis and other elements of the host immune system [21].

The biofilm development in our work was quantified using the Congo Red Agar technique. Nearly all of the isolates exhibited biofilm formation, with 72.5% classified as medium biofilm formers, 19.5% as strong biofilm formers, and only four as weak biofilm formers. These observations corroborated the findings made by Jahromy in Iran. Who identified 404 of the isolated UPEC strains as capable of initiating biofilm formation[23].

In contrast to the study conducted by Moshera *et al*., at Suez Canal University hospitals, our findings indicate that 63.8% of UPEC isolates were biofilm formers, which is lower than our observed results. This may be due to the different methodology employed in the study[21].

Molecular identification of *CsgD* genes showed that the prevalence of each gene was 37.3% and 43.1%, respectively. These results were comparable to Jahromy results carried out in Iran, which were 33% and 35% for *CsgA* and *CsgD* genes, respectively. Our findings for the prevalence of *RCsA*, *FocA*, and *RcsC*genes were 90.2% for both *RCsA* and *FocA* genes and 25.5% for *rcsc* genes, which are higher than those of Jahromy, which was 35%, 29%, and 16% for *RcsA*, *FocA*, and *RcsC* genes, respectively. This variance may be related to different geographical locations [23].

Our analysis revealed a significant correlation between the isolated genes and biofilm development. All the isolates that formed biofilms were found to express the *RcsA*, *CsgD*, and *FocA* genes (p.value > 0.001), and 80% of the isolates expressed the *CsgA* and *CsgD* genes. These findings coincided with those of Jarhomy [23].

CONCLUSION

Our study revealed that UPEC (Uropathogenic ***E. coli***) is the most predominant causative agent of urinary tract infections.

Urinary tract infections are more common in female patients due to anatomical reasons. The short urethra of females is considered to be a very important predisposing factor.

Biofilm formation has a particular role in UPEC drug resistance. As the biofilm former bacteria requires a higher concentration of antibiotics to cure infection. UPEC showed multi-drug resistance (MDR) patterns due to the misuse of antibiotics and the biofilm formation.

Fosfomycin is the drug of choice for treatment of urinary tract infections (UTI) caused by UPEC. It

showed a low incidence of bacterial resistance as it is taken as a single dose unit.

Conflict of Interest: The authors report no conflicts of interest in this work.

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