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Assessment the role of *sfa* and *afa* Genes in the Antibiotics Resistance of Uropathogenic *Escherichia coli*

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Abstract

Background. Urinary tract infections (UTIs) are a prevalent bacterial infectious illness. *Escherichia coli* is the main cause of urinary tract infections (UTIs). Uropathogenic *Escherichia coli* (UPEC) strains possess various distinct virulence characteristics that can exacerbate (UTIs). **Aim.** The aim of this study was to identify the *sfa* and *afa* genes found in UPEC isolates and evaluation the effect of both genes in biofilm formation of uropathogenic *Escherichia coli* and relation with antibiotic resistance. **Methods.** Between October and December of 2023, 140 sample were collected. It was found that 21 isolates were UPEC detected by culturing, biochemical tests, and VITEK-2 system. Using the disk diffusion method, the susceptibility to fourteen different antibiotic types were examined; and ability of formation biofilm was tested. **Results.** The findings showed that the *E. coli* isolates were resistance to, imipenem (4.76%), amikacin (9.52%), gentamicin (33.3%), ciprofloxacin (76.19%), azithromycin (28.57%), tetracycline (80.95%), cefotaxime (90.48%), ceftriaxone (85.71%), co-trimoxazole (52.38%), amoxiclav (amoxicillin- clavulanic acid) (9.52%), aztreonam (33.3%), ampicillin (90.48%), chloramphenicol (9.52%), while all the isolates were sensitive to the nitrofurantoin. For biofilm formation of isolates the findings indicated that 1 (4.76%) isolate produced a weak biofilm, 13 (61.90%) isolates formed a moderate biofilm, and 7 (33.33%) isolates formed a strong biofilm. While the frequency of presence of biofilm formation genes was 17 (80.95%) for *sfa* and 3(14.28%) for *afa.* **Conclusion.** The results of present study concluded that the *sfa* and *afa* genes were had important role in the adhesion and biofilm formation of UPEC as well as in the antibiotic resistance of these isolates.

Keywords: *afa*, antibiotics, UPEC, UTIs, *sfa*.

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Introduction

Humans regularly have urinary tract infections (UTIs). Pyelonephritis and cystitis are two UTI etiologies that are substantially linked to the UPEC (1). UPEC possesses numerous virulence characteristics that facilitate its ability to colonize, invade, and persist within the urinary system of the host (2,3,4). In UPEC strains linked to severe UTI, the most significant virulence genes are S fimbriae adhesion (*sfa*) and afimbrial adhesin I (*afa*I) (5). These virulence factors aid the pathogen in establishing itself on host surfaces, evading or manipulating the host's defense mechanisms, causing harm or invading host cells and tissues, and triggering a harmful inflammatory response, ultimately resulting in a clinical disease (6). Bacteria that form biofilms generate a matrix consisting of proteins, extracellular DNA, and polysaccharides. This matrix offers various advantages to the bacterial communities, such as defense against

immune cells, adhesion (aided by bacterial adhesins), and structural integrity (7). The ability of bacteria to have resistance genes that imitate the potential antibiotics' inhibitory effects and allow them to survive is known as antibiotic resistance (8). The generation of genetic material can encompass two distinct mechanisms: intrinsic processes, which involve the natural recombination and integration of genetic material into the bacterial genome, and the acquisition of genetic material from external sources through horizontal gene mutation events such as conjugation, transformation, and transduction (9). The main processes leading to the development of bacterial resistance are the blocking of the porin channel, altering the targets of antibiotics, and using enzymatic action to neutralize the effectiveness of antibiotics (10). Multiple research investigations have provided evidence of antimicrobial resistance in (UPEC), with a noticeable rise in resistance to routinely employed antibiotics, including ciprofloxacin and trimethoprim-sulfamethoxazole, among others (11,12). The patterns of antimicrobial resistance exhibit variations across different geographical regions (13).

Materials and methods

Samples collection

Between October and December of 2023, patients at Ghazi Al-Hariri Hospital for Surgical Specialties and Al-Yarmouk Teaching Hospital provided 140 distinct specimens, including midstream urine and urinary catheter swabs were collected. Following the cultivation of all samples on MacConkey agar and EMB agar, the isolates were kept at 37ºC for 24 hours. Different biochemical techniques were used to characterize the isolates of *E. coli*. Colonies were identification by VITEK-2 system.

Biofilm Assay

O'Toole using the Crystal Violet Binding Assay, which he had modified, to investigate the biofilm production of various strains of *E. coli* (14). The UPEC isolates were subjected to subculture using this approach for a duration of 24 hours at a temperature of 37°C in the Brain Heart Infusion Broth. After the incubation period, the cultures were transferred to 96-well polystyrene plates in a 1:100 ratio and subjected to a 24-hour incubation period at 37°C. Subsequently, a 1% crystal violet solution was employed to stain the wells. Subsequently, 96% ethanol was introduced into each well to dissolve the crystal violet that was attached. Finally, the absorbance of solubilized crystal violet was quantified at a specific wavelength of 595 nm for each well. The experiment was performed in triplicate. The biofilm forms of *E. coli* strains were used to categorize them into four groups that shown in (Table 1).

Table (1): The biofilm groups (15).

Mean OD_{595}	Biofilm intensity
OD ≤ ODc	None Biofilm Former
ODc < OD ≤ 2 × ODc	weak
2 × ODc < OD ≤ 4 × ODc	moderate
ODc > 4 × ODc	strong

Antibiotic susceptibility test

The antibiotic susceptibility test was done for all UPEC isolates by using disk diffusion method against 14 antibiotics chosen according CLSI including imipenem, amikacin, gentamicin, ciprofloxacin, azithromycin, tetracycline, cefotaxime, ceftriaxone, co-trimoxazole, amoxiclav (amoxicillin- clavulanic acid), aztreonam, ampicillin, chloramphenicol, and nitrofurantoin. The plates were subjected to incubation at a temperature of 37°C for a period of 24 hours. after incubation the dimeter of inhibition zones developed clear area around the disk were measured by millimeter (mm) using metric ruler according to (CLSI, 2023) (16).

DNA extraction

UPEC DNA was extracted using Presto mini gDNA bacteria kit (Geneaid, Taiwan). Nanodrop device was utilized to quantify the concentration and purity of the extracted DNA, which was later assessed using gel electrophoresis.

Molecular detection of *sfa* and *afa* genes by single plex PCR

In this study, *sfa* and *afa* species- specific primers for UPUC was used. The primers were supplied by Macrogen as lyophilized form then dissolved in nuclease free water to prepare the stock solution then diluted to obtained working. Then single plex PCR was used for the detection of these genes in UPEC isolates, which were isolated from patients with urinary tract infections. Primers used to detect *sfa* gene were *sfa*1 ('5-GAGTCAGCCCTCCGTTTTCA-3') and *sfa*2 ('5-TCTGGGTGAATGTCAAGGCC-3'), *afa* gene were *afa*1 ('5-TTACCECCACCTITCAGCAT-3') and *afa*2 ('5- AAGCAGTTTGAGGCAGAGCT-3') were designed by the primer 3 program through the following steps: 1. Downloaded reference sequence from ncbi. 2. Input sequence into generous prime software. 3. Choose primer with best specs, Tm (melting temperature), GC (guanine and cytosine) content and has no self-dimer or hairpin loop. *sfa* product size was 176bp and it's amplified a portion of this gene, while product size for *afa* gene was 215bp and it's amplified a portion of *afa* gene.

The PCR amplification program for *sfa* gene includes the following steps: 1. Initial Denaturation 95°C for 3 min, one cycle. 2. Denaturation 95°C for 30 sec. 3. Annealing 55°C for 30 sec. 4. Extension 68°C for 1 min. (last 3 steps includes 35 cycles) 5. Final Extension 68°C for 5 mins, one cycle. The PCR amplification program for *afa* gene includes the following steps: 1. Initial Denaturation 95°C for 3 min, one cycle. 2. Denaturation 95°C for 30 sec. 3. Annealing 52°C for 30 sec. 4. Extension 68°C for 1 min. (last 3 steps includes 35 cycles) 5. Final Extension 68°C for 5 mins, one cycle.

Results

Isolation and characterization of *Escherichia coli*

After culturing of specimens on MacConkey agar and EMB agar, biochemical tests and identification by VITEK-2 system it seemed that out of 140 specimens, 21(15%) were *E. coli*, 30 (21.42%) were gram negative bacteria, 30 (21.42%) were gram positive bacteria and 59 (42.14%) specimens showed no growth.

Biofilm formation by *E. coli*

By using micro-titer plate method to discover the ability of 21 *E. coli* isolates to produce biofilm, the results showed that 1 (4.76%) isolate was weak biofilm producer, while 13 (61.90%) isolates were moderate biofilm producer, and 7 (33.33%) isolates produce a strong biofilm.

Antibiotic susceptibility test

This study involved doing antibiotic susceptibility testing on all isolates of UPEC. The isolates capable of generating biofilms

exhibited the highest level of resistance to Ampicillin and Cefotaxime followed by Ceftriaxone (85.71%). Also, most resistance was recorded for Tetracycline (80.95%), Ciprofloxacin (76.19%), Co-Trimoxazole (52.38%), Gentamicin and Aztreonam (33.33%). The current study demonstrated that UPEC isolates possessed a low - level resistance against Azithromycin (28.57%), Amoxicillin- Clavulanic acid, Amikacin and Chloramphenicol (9.52%), Imipenem (4.76%), and Nitrofurantoin was no resistance as (Figure 1) shows.

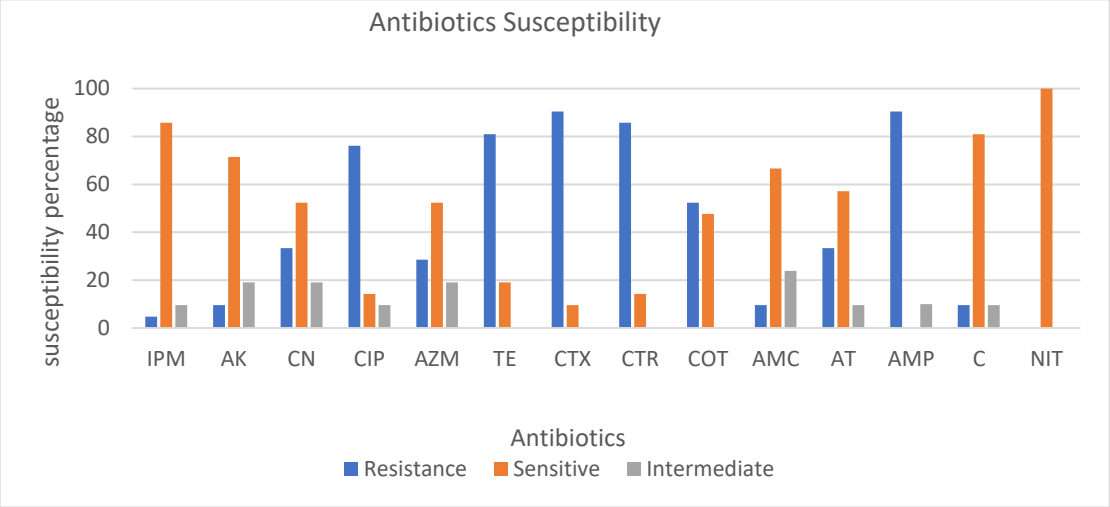

Figure (1): Antibiotics susceptibility of UPEC isolates, Imipenem (IPM), Amikacin (AK), Gentamicin (CN), Ciprofloxacin (CIP), Azithromycin (AZM), Tetracycline (TE), Cefotaxime (CTX), Ceftriaxone (CTR), Co-Trimoxazole (COT), Amoxicillin- Clavulanic acid (AMC), Aztreonam (AT), Ampicillin (AMP), Chloramphenicol (C) and Nitrofurantoin (NIT).

Detection of *sfa* gene by PCR technique

The results of the study showed that detect *sfa* gene in 17 (80.95%) isolates of UPEC by analyzing the bands on gel electrophoresis shown in (figure 2).

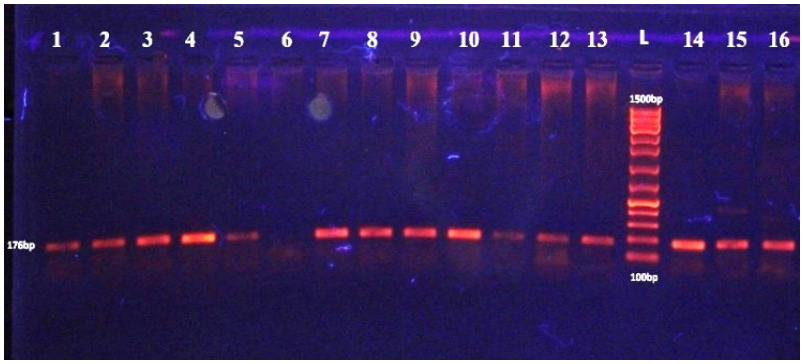

Figure (2): **PCR amplification of *sfa* gene in *E. coli* isolates using gel electrophoresis run on agarose gel at 1%, voltage 150 for 30 minutes stained with Ethidium bromide M: DNA Marker (ladder) (100-1500 bp).**

Detection of *afa* gene by PCR technique

The results raveled that 3 (14.28%) of UPEC isolates carried *afa* gene by analyzing the bands on gel electrophoresis as shown in (figure 3).

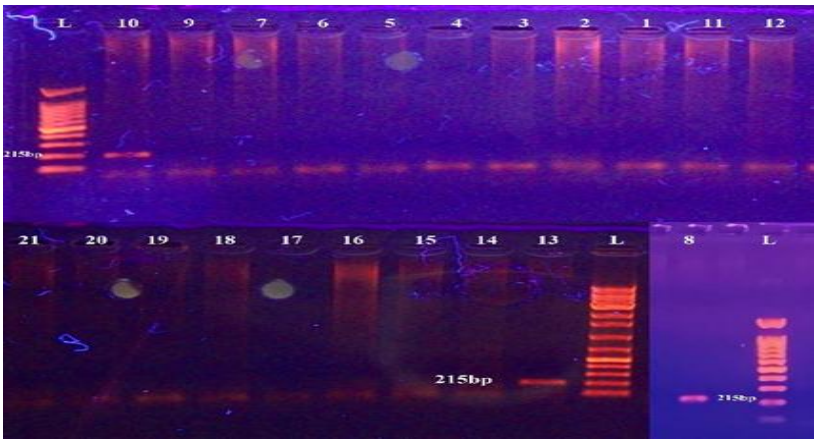

Figure (3): **PCR amplification of *afa* gene in *E. coli* isolates using gel electrophoresis run on agarose gel at 1%, voltage 150 for 30 minutes stained with Ethidium bromide M: DNA Marker (ladder) (100-1500 bp).**

Discussion

Isolation and characterization of *Escherichia coli*

Based on the morphological characteristics observed on MacConkey agar *E. coli* appears in pink/red colonies which ferments lactose the result agree with (17,18) and on Eosin methylene blue agar (EMB) culture media the green metallic sheen colonies indicates the presence of UPEC and its agree with (19) result, as well as biochemical tests, out of a total of 140 specimens, 21 (15%) were identified as *E. coli*, 30 (21.42%) were determined to be gram negative bacteria, 30 (21.42%) were identified as positive bacteria, and 59 (42.14%) specimens showed no growth. The results of biochemical tests revealed positive for indole, negative for citrate, negative for oxidase, and positive for catalase were used to detect UPEC and (20) was reported same tests.

Biofilm formation by *E. coli*

pathogens that produce biofilms are frequently responsible for recurring and complex urinary tract infections, typically linked with multidrug-resistant pathogens (21). Comprehending the process by which biofilms form and the factors linked to them is crucial for the advancement of novel treatments (22). Among the 21 UPEC isolates, all of them were biofilm-forming with different intensities. There is one (4.76%) isolate that produces a weak biofilm, while 13 (61.90%) isolates indicate a moderate biofilm, and 7 (33.33%) isolates produce a strong biofilm. These percentages are shown in (Table 2). Also, biofilm formation ability shows a (P-value = 0.0055) which is ($P \leq 0.01$) and Chi-square test was done to test it.

Table (2): The percentage of Biofilm formation in UPEC isolates.

Biofilm	UPEC isolates	Percentage (%)
Strong	7	33.33
Moderate	13	61.90
Weak	1	4.76
Total	21	100%
Chi-square test $-\chi^2$	---	10.391 **
P-value	---	0.0055
** ($P \leq 0.01$)		

These results were closed to the results of (23) who reported that *E. coli* isolates were categorized under four groups and accordingly, 41 (31%) isolates were strong biofilm producers, 31(23.5%) were moderate, and 31(23.5%) were weak biofilm producers whilst 29 (22%) were unable to produce biofilm. Another study done by (24) shown the percentage of biofilm formation was 31%, 29%, 32%, and 8% of the isolates were strong, moderate, weak, and non-biofilm producers, respectively. According to (25) among the biofilm-producing strains, eleven and one strain formed strong and moderate biofilms, respectively. While (26) results showed that the combined rate of biofilm formation in UPEC isolates was 87.9%. Also, 26.3%, 26%, and 47.1% of UPEC isolates were able to create strong, moderate and weak biofilms, respectively.

Antibiotic susceptibility test

UPEC isolates resistance to Cefotaxime and Ampicillin was concordant with (27) who referred that resistance of *E. coli* to these antibiotics reached 100% and 97.8%, respectively. Also, the increasing of UPEC resistance to Ceftriaxone was agreed with (28) and (29) who reported in their study (91%) and (90.57%) resistance to Ceftriaxone. While Tetracycline in this study shows 80.95% resistance, which agrees with (30) findings. On the other hand, the percentage of resistance to Ciprofloxacin was (76.19%) which is slightly higher than the percentage mentioned in (31) research. For Co-Trimoxazole, the result didn't agree with the study by (32) which reported that resistance to this antibiotic was (100%) while in this study it was (52.38%). Also, in current study, the UPEC isolates show (33.33%) resistance to Gentamicin and Aztreonam, which agrees with (33) study on resistance to Gentamicin and disagree with their study on Aztreonam resistance. Who reported that resistance to Gentamicin was (42%) while resistance to Aztreonam was (87%). Furthermore, the resistance to Azithromycin in this study was (28.57%) and the result was slightly matched with (34) who found that the resistance of UPEC to this antibiotic was (30.3%). As well, the resistance of UPEC isolates to Amoxicillin- Clavulanic acid, Amikacin and Chloramphenicol was (9.52%), for Amoxicillin- Clavulanic acid the result agree with (35) which found (6%) resistance isolates to this antibiotic and disagree with (36) who found resistance to this antibiotic was (100%), for Amikacin its clearly disagree with (37) and (38) findings which found (90%) and (80%) resistance to Amikacin while the result in this study agree with (34) , and for Chloramphenicol the resistance was (9.52%) which disagree with (39) and (40) results who found (73%) and

(28%) resistance to this antibiotic. however, (37) reported 93% resistance to Imipenem while in our study was (4.76%) which is clearly disagree with their findings and it agree with (41) result who reported (7.69%) resistance to this antibiotic. The isolates under study showed no resistance to Nitrofurantoin compared to (33) and (42) which reported that resistance to Nitrofurantoin was (9%) (5.37%) respectively.

Detection of *sfa* gene by PCR technique

The results of the study showed that detect *sfa* gene in 17 (80.95%) isolates of UPEC by analyzing the bands on gel electrophoresis. The result was agreed with (43) who found that *sfa* presence was (81%) in 100 *E. coli* isolates. Also, the finding of this study agreed with (44) findings who reported that *sfa* found in E. coli isolates in (21%). Another finding by (45) who reported the presence of *sfa* was *sfa* (39.1%) in 23 UPEC isolates.

Detection of *afa* gene by PCR technique

The results raveled that 3 (14.28%) of UPEC isolates carried *afa* gene by analyzing the bands on gel electrophoresis. By comparing the results with other studies, (43) who study the prevalence of urovirulence genes in 100 UPEC isolates obtained from patients with UTI, rates were (12%) for *afa* gene. In other study by (45) who study 23 UPEC isolates, to identify genes associated with UTIs in patients, the results showed *afa* was found in (39.1%) in UPEC isolates.

Table (3): Correlation between biofilm formation and antibiotics resistance in UPEC isolates.

Spearman r	Biofilm vs. IPM	Biofilm vs. AK	Biofilm vs. CN	Biofilm vs. CIP	Biofilm vs. AZM	Biofilm vs. TE	Biofilm vs. CTX	Biofilm vs. CTR	Biofilm vs. COT	Biofilm vs. AMC	Biofilm vs. AT	Biofilm vs. AMP	Biofilm vs. C	Biofilm vs. NIT
R	-0.1882	0.2995	-0.2403	-0.5161	0.05858	0.04697	-0.4398	-0.1054	0.1846	-0.05588	0.2271	0.1885	-0.02446	-0.1882
95% confidence interval	-0.5824 to 0.2776	-0.1651 to 0.6553	-0.6174 to 0.2265	-0.7805 to -0.09504	-0.3944 to 0.4886	-0.4042 to 0.4797	-0.7387 to -0.003646	-0.5237 to 0.3538	-0.2811 to 0.5800	-0.4866 to 0.3967	-0.2397 to 0.6087	-0.2774 to 0.5826	-0.4622 to 0.4228	-0.5824 to 0.2776
P (two-tailed)	0.4138	0.1872	0.2940	0.0166	0.8009	0.8398	0.0460	0.6493	0.4230	0.8099	0.3221	0.4132	0.9162	0.4138
P value summary	ns	ns	ns	*	ns	ns	*	ns	ns	ns	ns	ns	ns	ns
Significant? (alpha = 0.05)	No	No	No	Yes	No	No	Yes	No	No	No	No	No	No	No

Table (4): Correlation between presence of *sfa* and *afa* genes according to biofilm formation.

Spearman r	*sfa* vs. Biofilm formation	*afa* vs. Biofilm formation
r	-0.1879	0.5534
95% confidence interval	-0.5822 to 0.2780	0.1466 to 0.8001
P (two-tailed)	0.4148	0.0093
P value summary	ns	**
Exact or approximate P value?	Approximate	Approximate
Significant? (alpha = 0.05)	No	Yes

Correlation between biofilm formation and antibiotics resistance in UPEC isolates.

The correlation between biofilm formation and antibiotics resistance was detected by using (two-tailed) test, for Cefotaxime and Ciprofloxacin there is a significant difference between these antibiotics and the ability to produce biofilm with p value (p= 0.0460) for Cefotaxime (CTX) and (p= 0.0166) for Ciprofloxacin (CIP). For other antibiotics there is no significant difference and the results was shown in (Table 3). Our research suggests that biofilm formation and antibiotic resistance were associated for XDR-*E. coli* isolates, and it would provide new information on how to prevent and treat infections caused by *E. coli*. On the other hand, the correlation between biofilm formation and presence of *sfa* and *afa* genes was study. There is a non- significant for *sfa* gene while *afa* gene show a significant difference with biofilm formation and it was (r= 0.5534, p= 0.0093). As (Table 4) illustrate the results.

The result of this study shows *sfa* gene were more frequent than *afa* gene which means means *sfa* gene are more important than *afa* gene in biofilm formation and have correlation with antibiotic resistance. The importance of biofilm detected in these UPEC isolates reflects antibiotic resistance and also reflects the importance of biofilm formation for antibiotic resistance. The significance of biofilm formation genes lies in their role as virulence factors, antibiotic resistance, and capacity to enhance the specificity of pathogenicity in a wide range of pathogenic bacteria. Consistent with our findings, prior research investigating the prevalence of the *sfa* and *afa* gene combination among UPEC isolates has indicated that this gene combination is either nonexistent or has received little documentation (45,46).

Conclusion

The study reveals varying biofilm-forming capacities in clinical *E. coli* isolates. Increasing infection risk and antibiotic resistance. Future research should focus on anti-biofilm coatings and biofilm active therapeutics. In addition, it was found that the *sfa* gene was more strongly associated with strong biofilm formation in UPEC.

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