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Original Article

Investigation of the Role of Virulence Gene in Biofilm Formation of *Escherichia coli* Obtained from Clinical Specimens in Baghdad

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Abstract

Several strains of *Escherichia coli* (*E. coli*) cause many diseases, including gastrointestinal illness, urinary tract infections, pericarditis, and septicemia. The present study aimed to evaluate the prevalence of the Universal Stress Protein (*USP*) virulence gene and the level of antibiotic resistance patterns associated with biofilm formation of *E. coli* in patients with infected burns, wounds, and urinary tract infections. Cases were selected from two hospitals of Al-Yarmouk Educational Hospitals and Baghdad Medical City, Baghdad, Iraq. The clinical specimens were classified as *E. coli* according to CLSI. The frequency of the *USP* gene was determined using the PCR technique. The rate of biofilm formation and antibiotic resistance were determined using microplate and agar diffusion methods, respectively. The recorded data on the distribution of *E. coli* isolates indicated that 33 (66%) of isolates were recovered from females and 17 (34%) of them were obtained from males (P=0.02). The results of the distribution of the isolates indicated that 16 (32%) and 18 (36%) isolates were recovered from 10-20 and 21-30 and 31-40 years old participants, respectively. The recorded data revealed that the highest rate of *E. coli* isolates was obtained from urine samples while the lowest one was recovered from burn samples (P<0.0001). The frequency of *USP* gene distribution from all strains was analyzed by the PCR and gel electrophoresis techniques. The results of the PCR test identified the *USP* gene (toxin gene) at 435 bp. The *USP* gene was presented in 41 (82%) *E. coli* isolates of all samples, including 28 isolates (46%) in women and 13 isolates (26%) in men with no significant association. Concerning the distribution due to the age groups, the *USP* gene was presented in 11 isolates (22%) in the age group of 10-20 years, while 14 (28%) and 16 (32%) isolates in the age groups of (21-30) and (31-40), respectively. Concerning the distribution of samples, the *USP* gene was presented in 1 isolate (2%) from the burn, 4 isolates (8%) from the wound, and 36 isolates (72%) from the urine. The microtiter plate method was used to evaluate biofilm formation and the results showed that 7 (14%), 28 (56%), and 15 (30%) isolates were weakly, moderately, and strongly adherent, respectively. These results filled the national gap about virulence and antimicrobial resistance of *E. coli* responsible for several diseases and should be used to improve the management of patients in Baghdad.

Keywords: Antibiotic Resistance, Biofilm, *E. coli*, *USP* Gene

1. Introduction

Gram-negative infections caused by non-spore forming, facultative anaerobic *E. coli* have been identified as severe infections associated with increased mortality and significant healthcare costs. *E. coli* has been discovered in both humans and animals, as well as in healthy human intestines, urinary tract infections, pericarditis, septicemia, and other diseases (1, 2). Although pathogenicity islands of *E. coli* are two or more DNA segments that encode for the virulence factors such as the Universal Stress Protein (*USP*) gene, which is essential for bacterial survival during cellular

growth, adhesion, and motility. Six types of USP are available: A, and (C-G) which are regulated with different stresses. Although the physiological function of *USP* is unknown, an intriguing correlation is observed between the *USP*A gene and bacterial survival during cellular growth arrest. Other studies have discovered that USP has various physiological functions that reprogram the cell to defend and escape during bacterial stress (1, 3, 4). The bacteria collected from patients with pentatectis known as uropathogenic *E. coli* form a bacterial surface-dependent and involves cell surface molecules as well as various structures and is not destroyed by gentle washing, resulting in pyelonephritis (5). Furthermore, bacterial cells are tolerable substances that alter the physiology of the host immune system (6, 7). The present study aimed at phenotypically identifying *E. coli* strains by evaluating the biofilm formation and antibiotic resistance pattern, as well as, investigating the *USP* virulence gene from different sources of hospitals in Baghdad. The study concentrated on the association between the virulence factor and the antibiotic since antibiotic resistance increased.

2. Materials and Methods

2.1. Determination of Phenotype

2.1.1. Bacterial Sources

Samples were collected from men and women in sterile tubes with different sources (urine, burn, and wound) from two hospitals of Al-Yarmouk Teaching Hospital and Baghdad Medical City, Baghdad, Iraq. While burning, samples were taken from the location of the infection using a smear from January to March 2021. All samples were isolated after cultured on brain heart infusion (BHI), blood, and MacConky agars. The biochemical tests (Oxidase, Indol, Methyl-Red, Catalase, and Voges-Proskauer) were used to identify the *E. coli* isolates.

2.1.2. The Sensitivity Test for *E. coli* Isolates

Seven types of antibiotics were used to perform a local sensitivity analysis for all *E.coli* isolates after being cultured by the agar diffusion method, then, the zone region of media was measured in which bacteria fail to grow (8).

2.1.3. Biofilm Forming Assay (Microtiter Plate)

All 50 isolates grown in tryptic soy (TS) broth supplemented with 20% glucose were evaluated for biofilm formation using the 96-well microtiter plate. Each isolate was grown overnight at 37° C, then cultures were diluted by 0.5 McFarland, 1.5×10^8 cell/ml. Later, 180 µL aliquots of these cultures and 20 µL of TS broth were added to the microtiter plate. Some wells only contained sterile TS broth supplemented with 20% glucose to serve as controls. Incubation of the plates was done in aerobic condition incubation at 37 ℃ for 24 h. Then the plates were washed three times using 200 µL phosphate saline (PBS) to get rid of other non-adherent cells. Then the plate was dried. A 1% solution of 180 µl purple crystal was used and the wells were stained for 15 min, the unbound crystal violet was done before washing with 200 µl of PBS. Fixing dye released by adding 200 µL of 96% ethanol. A quantitative examination of biofilm production was then performed. The absorbance of each well was measured at 630 wavelengths. This experiment was performed based on the adsorption values calculated in three replications: if the absorbance of samples is less than or equal to the absorbance of the control, it means that no biofilm is produced; however, if the absorbance of the control is less than the absorbance of the samples or equal for 2× absorbance the control, that means weak biofilm is produced, and if 2×absorbance of control is less than absorbance of samples or equal the 4×absorbance of the control that means moderate biofilm is produced, while if the 4×absorbance of control less than the absorbance of the samples that means strong biofilm production.

2.2. Determination of Genotype

2.2.1. Molecular Assay

The genomic DNA of all *E. coli* isolates extracted by AccuPrep ® Genomic DNA Extraction Bioneer/Korea) Kit. To detect the distribution of *USP* (toxin gene) from *E. coli*, by the forward primer 5′-ACATTCACGGCAAGCCTCAG-3, and the reveres primer 5′AGCGAGTTCCTGGTGAAAGC-3′, the expected amplification product was 435 base pears. The reaction was performed at a total volume of 25µl

for polymerase chain reaction (PCR), which contained 12µl (2X) of Green Master (GoTaq®) Mix, 1µl of primers and 3µl of DNA template, then 7.5 µl of water (nuclease-free). The USP (toxin gene) response amplification program was expressed at 94◦C for 5 min of initial denaturation, then 30 s at 58◦C and 30 s for 10 min on 72◦C, and then adjusted for 30 cycles. The amplification products of PCR were detected by gel electrophoresis in agarose gel (1.5%) for 75 min. DNA ladder 100 bp (Solarbio, China) was used. The ethidium bromide dye was used for staining, then visualized by UV-transilluminator (9).

2.3. Statistical Analysis

Data were analyzed using SAS Software 2010. The differences among proportions were assessed by the Chi-squared test. A *P*-value less than 0.05 is statically significant.

3. Results

3.1. Identification of Sample

A total of 125 samples, including 61 urine, 40 burn, and 24 wound samples were collected from patients in two hospitals of AL-Yarmouk Teaching Hospital, and Baghdad Medicine City, Baghdad from January to March 2021. Out of 125 samples, 110 samples were cultured; however, only 50 isolates (45.45%) were identified as *E. coli* based on morphological and biochemical characteristics as shown in figure 1.

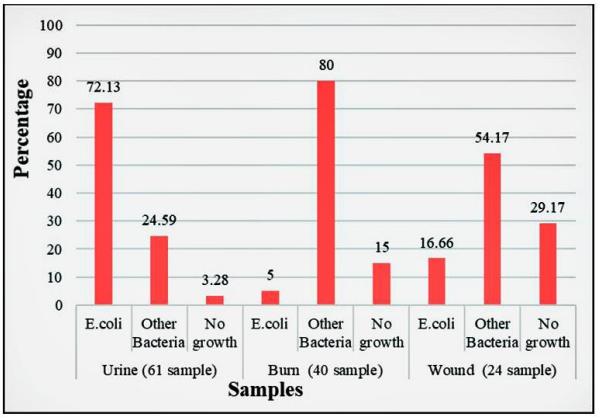

Figure 1. *E. coli* and other bacterial distribution in clinical samples

3.2. The Isolates of *E. coli*

The recorded data on the distribution of *E. coli* isolates showed that 33 (66%) of isolates were recovered from females and 17 (34%) of isolates were obtained from males (Table 1) (*P*=0.02). The results of the distribution of the isolates indicated that 16 (32%) of isolates were recovered from 10-20 and 21-30 years old participants, and 18 (36%) were recovered from 31-40 years old participants (Table 1). The recorded data revealed that the highest rate of *E. coli* isolates was obtained from urine samples while the lowest rate was recovered from the burning sample (Table 1) (*P*<0.0001).

3.3. Detections of *USP* Virulence Gene

The frequency of *USP* gene distribution from all strains was analyzed by the PCR method and the gel electrophoresis. PCR assay results identified the *USP* gene (toxin gene) at 435 bp as shown in figure 2. The *USP* gene was presented in 41 (82%) *E. coli* isolates of all the samples, including 28 isolates (46%) in women and 13 isolates (26%) in men with a non-significant association. Concerning the distribution due to the age groups, the *USP* gene was presented in 11 isolates (22%), 14 (28%) and 16 (32%) isolates in the age groups of 10-20, 21-30, and 31-40, respectively. According to the distribution due to the type of samples the *USP* gene was presented in 1 isolate (2%) from the burns, 4 isolates (8%) from the wounds, and 36 ones (72%) from the urine.

3.4. Investigation of Antibiotic Susceptibility

The results of susceptibility to seven types of antibiotics are shown in table 2. All *E. coli* isolates showed high resistance to Aztreonam, Ceftriaxone, Ciprofloxacin, and Ampicillin at 86%, 84%, 80%, and 72%, respectively. The high sensitivity to each of the Imipenem, and Amikacin was 98%, and 68%, respectively, while the same susceptibility was shown for resistance and sensitivity to Chloramphenicol.

Table 1. Distribution of isolates (*E. coli*) according to gender, age, and sample type

	No. of Isolates (%)		Chi-Square	*P*-value
Gender	Women	33 (66%)	5.12	0.02 *
	Men	17 (34%)		
Age (year)	10-20	16 (32%)	0.15	0.92 NS
	21-30	16 (32%)		
	31-40	18 (36%)		
Sample Type	Urine	44 (88%)	52.1	< 0.0001 **
	Burn	2 (4%)		
	Wound	4 (8%)		

Data were presented as a Chi-square goodness of fit test
NS: Non-significant
**P*-value less than 0.05 (Significant)
** *P*-value less than 0.01(Highly Significant)

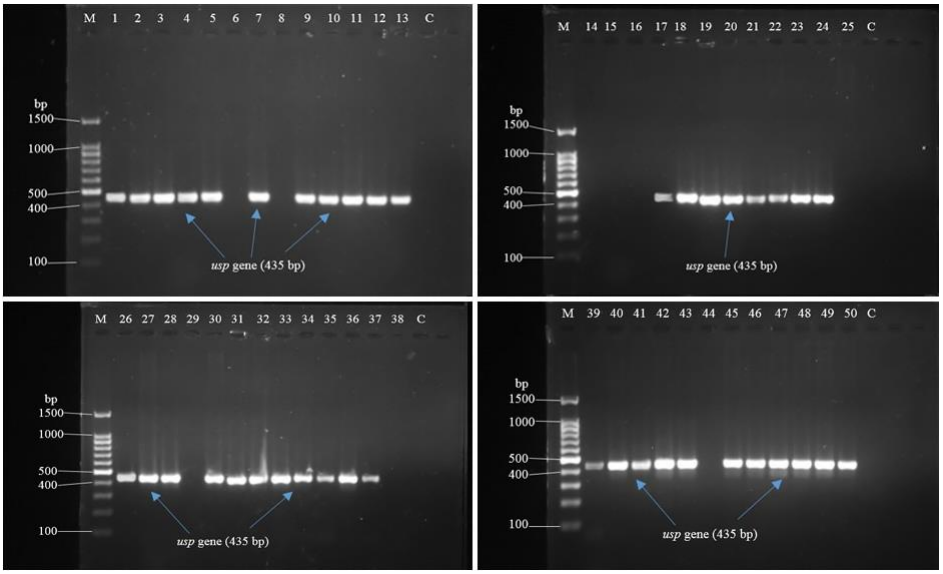

Figure 2. The *USP* gene in *E. coli* gel electrophoresis after PCR, *M: Lader (DNA marker 100bp), C: negative control (contain all PCR reaction except DNA templet). The lines (1-50): DNA product from isolates, the agarose gel concentration: 1.5%, Voltage: 5/cm,1: 15h.

Table 2. Properties of antibiotic susceptibility in *E. coli* isolated from clinical samples

Antibiotics	Resistance No. (%)	Sensitive No. (%)	*P*-value
Amikacin (AK)	16 (32%)	34 (68%)	0.011 *
Ampicillin (AM)	36 (72%)	14 (28%)	0.002 **
Aztreonam (ATM)	43 (86%)	7 (14%)	<0.0001 **
Ceftriaxone (CRO)	42 (84%)	8 (16%)	<0.0001 **
Chloramphenicol (C)	25 (50%)	25 (50%)	1 NS
Ciprofloxacin (CIP)	40 (80%)	10 (20%)	<0.0001 **
Imipenem (IMI)	1 (2%)	49 (98)	<0.0001 **

3.5. Biofilm Formation Assay

The microtiter plate method was used to evaluate biofilm formation and the results indicated that 7 (14%), 28 (56%), and 15 (30%) isolates were weakly, moderately, and strongly adherent, respectively. The differences between the adherent rates were highly significant (P<0.01). The distribution of the *USP* gene with biofilm is shown in table 3. The antibiotic resistance and the biofilm formation are presented in table 4. Aztreonam and Ceftriaxone resistance was higher in the biofilm-forming isolates (86.7 %), followed by Ciprofloxacin (73.3 %). Weak and strong biofilm formation was highly resistant to Aztreonam, Ceftriaxone, and Ciprofloxacin. Sensitivity in biofilm formation against Amikacin (57.1% and 80%), Ampicillin (0% and 53.3%), and Chloramphenicol (14.3% and 100%) were found to be weak and strong.

Table 3. Relationship between biofilm formation and USP gene distribution

			Biofilm Formation			Total	Chi-Square Test	*P*-value
			Weakly Adherent	Moderately Adherent	Strongly Adherent			
USP Gene	Absent	Count (%total)	1 (2%)	3 (6%)	5 (10%)	9 (18%)	3.462	0.177 **NS**
	Present	Count (%total)	6 (12%)	25 (50%)	10 (20%)	41 (82%)		
Total		Count (%total)	7 (14%)	28 (56%)	15 (30%)	50 (100%)		

Table 4. Susceptibility of the antibiotic pattern of *E. coli* biofilm formation

Antibiotic	Biofilm (weakly adherent		Biofilm moderately adherent		Biofilm strongly adherent		*P*-value
	Resistance No. (%)	Sensitive No. (%)	Resistance No. (%)	Sensitive No. (%)	Resistance No. (%)	Sensitive No. (%)	
Amikacin	3 (42.9%)	4 (57.1%)	10 (35.7%)	18 (64.3%)	3 (20%)	12 (80%)	0.46 NS
Ampicillin	7 (100%)	0 (0%)	22 (78.6%)	6 (21.4%)	7 (46.7%)	8 (53.3%)	0.017 *
Aztreonam	5 (71.4%)	2 (28.6%)	25 (89.3%)	3 (10.7%)	13 (86.7%)	2 (13.3%)	0.47 NS
Ceftriaxone	5 (71.4%)	2 (28.6%)	24 (85.7%)	4 (14.3%)	13 (86.7%)	2 (13.3%)	0.62 NS
Chloramphenicol	6 (85.7%)	1 (14.3%)	19 (67.9%)	9 (32.1%)	0 (0%)	15 (100%)	<0.0001 **
Ciprofloxacin	7 (100%)	0 (0%)	22 (78.6%)	6 (21.4%)	11 (73.3%)	4 (26.7%)	0.33 NS
Imipenem	0 (0%)	7 (100%)	0 (0%)	28 (100%)	1 (6.7%)	15 (93.3%)	0.33 NS

4. Discussion

In the present study, isolates of *E. coli* from urine were found to be more prevalent than other infections. E.coli cells attach to uroepithelium, increasing infection due to their virulence factors and components (10). The results of *E. coli* distribution indicated a higher prevalence in women than in men, and similar findings had been previously reported (11). According to urinary tract infections and individual differences, shorter urethra and position close to the urethral meatus to the anus, the anatomical features of the female genital tract predispose to the growth of bacteria in the urinary tract (12). Urinary tract infection by E. coli in women is associated with fecal perineum which occurs at least once in about 50% of women (13). Sexual activity is another cause of transmitted infections for UPEC isolates (14), while other causes such as wounds and burns in men are related to blacksmithing, carpentry, and women to household chores, especially in the kitchen. The genotypic assay used in this study to detect the *USP* virulence gene using PCR confirmed it for all isolates from various clinical samples. Another study discovered that the *USP* gene contributes to the development of urinary tract infections (15). The other

study investigated that *USP* as a new agent is responsible for the DNA damage from *E.coli* that affects mammalian cells (5). Our results of *E.coli* positive isolates for the *USP* gene (82%) are consistent with those of another study from Mexico which was (87.1%) (16), as well as the same study from Japan which was (71.7%) (15). Furthermore, antibiotic resistance in *Enterobacter* and other bacteria become a public health concern (7, 17). This study indicated the *E. coli* resistance properties for different antibiotics. The highest resistance is for Ampicillin and Aztreonam which agrees with another study from Iraq which found a higher resistance for both the Aztreonam and Ampicillin reaching 100 %. Also, Ceftriaxone had a lower resistance (52 %) (18), so antibiotic resistance is likely to rapidly increase. Antibiotic tolerance in this study is greatly increased in bacteria with biofilms formation. The results of the latest research confirm the findings of previous studies (19) in which a difference was observed in the resistance of *E.coli* to form biofilms under various conditions. This finding confirms previous studies that pathogenic bacterial cells are more resistant than planktonic ones (11, 20). In conclusion, the results of the present study indicated that resistance mechanisms are associated with the production of *E. coli* biofilms.

5. Conclusion

In general, we found that infecting *E. coli* with plasmids containing various antibiotic resistance genes had a significant impact on biofilm formation. Throughout the study, all clinical isolates of *E. coli* developed excessive biofilms, including those with the lowest levels of antibiotic resistance; however, *USP* virulent gene was found in all isolates, which increases bacterial pathogens. Therefore, collecting scientific data is significantly important to improve healthcare and prevent or minimize nosocomial infections.

Authors' Contribution

Study concept and design: R. K. M.
Acquisition of data: R. K. M.
Analysis and interpretation of data: R. K. M.
Drafting of the manuscript: R. K. M.
Critical revision of the manuscript for important intellectual content: R. K. M.
Statistical analysis: R. K. M.
Administrative, technical, and material support: R. K. M.

Ethics

The human study was approved by the University of Baghdad, Baghdad, Iraq ethics committee.

Conflict of Interest

The authors declare that they have no conflict of interest.

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