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Prevalence of Multi-Antibiotic Resistance *marA* and Quorum Sensing *luxS* Genes and Evaluation of Biofilm Formation in Uropathogenic *Escherichia coli*

[1]Asmaa A. Muhaimeed, [2]Abdulameer M. Ghareeb

[1]AL-Yarmouk Teaching Hospital
[2]Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad

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Abstract: Uropathogenic *Escherichia coli* (UPEC) are one of the main organisms that cause 80–90% of community-acquired urinary tract infections (UTIs) and catheter-associated urinary tract infections (CAUTIs). It is essential that UPEC have the ability to colonize the bladder by producing phenotypic virulence factors such flagella, pili, curli, and non-pilus adhesion. Pathogens growing on indwelling medical devices can communicate with one another using quorum sensing (QS) signals. The direction of the formation of biofilms, which result in the encasing of bacterial cells in extracellular polymeric substances (EPS), is crucially influenced by QS. The aim of this study was to evaluate the pathogenicity of the main pathogenic *E. coli* isolated from UTI patients and determine antibiotic resistance pattern and biofilm production. From December 2022 to February 2023, Out of 150 urine samples taken from UTI patients in various hospitals in Baghdad/Iraq, 25 UPEC isolates with various clinical UTI symptoms were found. These isolates were recognized by conventional and rapid techniques, and their identification was later verified by a molecular strategy based on the 16S rRNA gene. All 25 *E. coli* isolates were tested for antibiotic sensitivity using the Kirby-Bauer disk diffusion method on Muller-Hinton agar, and it was the highest resistance toward cefotaxime and amoxicillin-clavulanate, while the lowest toward meropenem, nitrofurantoin, and imipenem. Using traditional PCR techniques and specially designed primers, 25 UPEC isolates were screened for the presence of the multi-antibiotic resistance *marA* gene and the quorum sensing *luxS* gene. According to the results, both investigated genes were present in high percentages (92%). It concludes that this high percentage of *marA* is correlated with a high rate of antibiotic resistance and *luxS*, which indicate a high ability of biofilm formation; thus, the *marA* and *luxS* percent indicate the level of pathogenicity among multidrug-resistant UPEC.

Keywords: UTI; UPEC; *marA*; *luxS*.

Corresponding author: (Email: asmaa.ahmed1300a@ige.uobaghdad.edu.iq).

Introduction

Urinary tract infections (UTIs), which can occur in community and hospital, are among the most prevalent bacterial illnesses. So that, UTIs affect 150 million people worldwide each year and are one of the most pervasive infections seen in modern medical treatment, affecting people of all ages, from newborns to the elderly (1). Ninety percent of community-acquired UTIs and up to 50% of nosocomial UTIs are caused by uropathogenic *E. coli* (UPEC) strains; rates of UPEC with high resistance to antibiotics and multidrug-resistant bacteria have increased dramatically in recent years, which may make treatment challenging (2). As a result, overexpression of the *marA* gene, which is a multiple drug resistance (MDR) phenotype and an element of the *marA*/*soxS*/*rob* regulon and regulates more than 40 genes on the *E. coli* chromosome, contributes to its

own transcriptional activation. Thus, there are different levels of resistance to various antibiotics and superoxide's (3).

UPEC easily creates multicellular colonies known as biofilms on the external surfaces of catheter materials, the inner surfaces of bladder walls, and even inside bladder epithelial cells; the association between antibiotic resistance and biofilm formation has been reported many times (4). Biofilm-associated infections are a major cause for concern since they frequently lead to UTI relapses in the *E. coli* context. It is crucial to comprehend the intricacy of this process and the processes involved in biofilm production and regulation because therapeutic failure occurs in the majority of instances (5).

The biofilm-forming cells undergo cell-to-cell communication via the process of quorum sensing (QS), and the small-signal molecules, also known as auto inducers (AI), regulate gene expression based on cell density. In order for planktonic bacteria to acquire the biofilm phenotype, motility and other virulence-related activities like biofilm formation are required (6). The most extensively researched AI in *E. coli* is AI-2, which is produced by the LuxS enzyme involved in biofilm formation. This AI's production is increased, and using LSR transporters, it is quickly secreted outside. When the ideal bacterial density is reached, *luxS* is downregulated, which prevents AI-2 from being produced, the LuxS/AI-2 QS system controls virulence factors, sporulation, bacterial luminescence, motility, biofilm formation, drug resistance, and toxin generation (7). Thus, the aimed of this study to determine the prevalence of detection and identification of genes (*marA, luxS*) by molecular methods among *E. coli* collected from infections of the urinary tract.

Material and methods

Collecting and detection of bacteria

One hundred fifty urine samples were collected from patients clinically diagnosed with urinary tract infections from December 2022 to February 2023 in three hospitals in Baghdad, as follows: Al-Yarmuk Teaching Hospital, Baghdad Teaching Hospital, and Al-Shahid Ghazi Hariri Hospital. These samples were taken from both genders between 5 and 78 years old and cultured on different mediums, including blood agar and MacConkey agar, and then incubated for 24 hours at 37°C. Culture on selective media (EMB) has also been used to identify and classify bacterial isolates. Afterward, API 20E was used for biochemical testing in twenty mini test tubes, and the Vitek-2 system was used to confirm the identification.

Molecular identification

The genomic DNA was extracted from all *E. coli* isolates using the Easy Pure® Bacteria Genomic DNA Kit according to the manufacturer's instructions with some modifications, as detailed in the following: 100 µl of overnight bacterial broth was centrifuged at 12,000 x g for 1 min, discarding the supernatant. Repeated many times. Then 100 µl of LB11 and 20 µl of proteinase K were added to the tube, and the bacteria were resuspended by vortexing. Then incubated overnight at room temperature. 20 µl of RNase A was added, mixed, and incubated at room temperature for 2 min. After that, 400 µl of CB11 was added and vortex for 30 sec, then transferred the entire contents to a spin column, centrifuged at 12,000 x g for 30 sec, and discarded the flow through. For cleaning and washing, 500 µl of CB11 and WB11 were added, centrifuged at 12,000 x g

for 30 sec, and the flow was discarded. This was carried out in two separate steps. To remove residual WB11 was centrifuged at 12,000 x g for 2 min. The spin column was placed into a collection tube. Then 100 µl of elution buffer was added and let stand for 2 min at room temperature. Then, centrifuged at 12,000 x g for 1 min to elute genomic DNA. Genomic DNA was stored at -20°C until use.

Detection of *E. coli* was carried out based on the 16S rRNA gene. The primer sequence for the 16S rRNA gene and expected PCR product size are shown in the Table (1), the expected amplicon size is 180 pb.

Antibiotic susceptibility test

To obtain turbidity equivalent to a 0.5 McFarland standard, *E. coli* isolates were transferred with sterile swabs to tubes of sterile saline. The surface of Mueller-Hinton agar plates was inoculated using cell suspensions and sterilized swabs. The Kirby-Bauer approach was used to determine antibiotic resistance. The sizes of the inhibition zones were measured in millimeters in accordance with the manufacturer's instructions to determine whether the inoculated plates were resistant, intermediate, or sensitive.

Biofilm production determination

Minor modifications were carried out to perform the biofilm development experiment in triplicate on a 96-well microtiter plate (MTP) (8). Briefly, each 96-well flat-bottomed sterile polystyrene microtiter plate well containing 180 µl Luria broth (LB) medium supplemented with 1% glucose was inoculated with 20 µl of a suspended bacterium of 0.5–0.7 McFarland. The plate was incubated at 37°C for 48 h under static conditions. Following incubation, phosphate buffered saline (PBS) was used to rinse the plate three times, and then 200 µl of a 0.1% crystal violet solution was added to the wells. After that, it was incubated for 15 min at 25°C. At the end of the incubation period, the MTP was washed twice with PBS. Color intensity could be used to evaluate a biofilm's capacity to form, so the plate was dried for a few hours at 60°C before being solubilized with 200 µl of 30% acetic acid to measure the biofilm formation capacity. Finally, the strains were divided into two groups based on their OD values: Group I, which produced no or weak biofilms, and Group II, which produced moderate to strong biofilms. The OD of 580 nm was measured using a microplate reader (BIO-RAD spectrophotometer). Biofilm formation received the following grades:

- Strong biofilm (S) = OD ≥ 4×ODc
- Moderate biofilm (M) = ODc ≤ OD ≥ 2×ODc
- Weak biofilm (W) = ODc ≤ OD ≤ 2×ODc
- Negative (N) = OD < ODc

Molecular identification of *marA* and *luxS* genes

The 25 *E. coli* isolates were examined using traditional PCR to determine whether or not the *marA* and *luxS* genes were present. The primers for the *marA* and *luxS* genes were designed using the 3pluse, V4, and University Code of Student Conduct (UCSC) programs, and they were synthesized and lyophilized by Alpha DNA Ltd. (Canada). The sequence and expected product size of these primers are listed in Table (1). The thermal cycler settings were employed with an automated thermal cycler programmed through these steps: one cycle initial denaturation stage was performed at 94°C for 5 minutes, then 35 cycles of 94°C for 30 seconds, 56°C for 40 seconds, and 72°C for 5 minutes, for

denaturation, annealing, and extension, respectively. After PCR was completed, the PCR products were analyzed on an agarose gel and visualized by a UV transilluminator.

Table (1): The primers employed in this study's oligonucleotide sequences, amplicon sizes, and references.

Primer name	Sequence (5'-3')	amplicon sizes (bp)	Annealing	References
16S rRNA-F	ACTCCTACGGGAGGCAGCAGT	180	64	(9)
16S Rrna-R	TATTACCGCGGCTGCTGGC			
***marA*-F**	ACTGGATCGAGGACAACCTG	266	56	This study
***marA*-R**	CGGCGGAACATCAAAGTAAT			
***luxS*-F**	CAATCACCGTGTTCGATCTG	380	56	
***luxS*-R**	TTCTTCGTTGCTGTTGATGC			

Statistical analysis

The Statistical Analysis System-SAS (2018) program was employed to identify the impact of various factors on study parameters (10). To statistically compare between means, the T-test Least Significant Difference –LSD test (Analysis of Variation-ANOVA) was employed. In this study, a significant comparison between percentage (0.05 and 0.01 probability) was made using the Chi-square test.

Result and discussion

Isolation and identification of *E. coli*

Phenotypic characteristics

From three hospitals in Baghdad, 150 urine samples were obtained. Urine samples that were directly cultured on MacConky agar are distinguished by their small rod shape, pink color, lack of viscosity, and lactose fermentation. *E. coli* appeared as creamy and non-hemolytic colonies on blood agar and green with metallic sheen colonies of *E. coli* on the EMB agar plate, as shown in Figure (1).

Rapid conventional identification

Analytical profile index 20 Enterobacteriaceae (API 20E) was used for biochemical testing in twenty mini-test tubes, and the Vitek-2 system was used to confirm the identification. The results showed that the full API 20E profile and Vitek-2 system indicated the presence of *E. coli* in 25 (16%) of 150 patients clinically diagnosed with urinary tract infections. (11) Suggested that it be quick, technically easy to evaluate, and reasonably priced. Although using API 20E as a partner in the combination improves the accuracy of the biotyping system.

Figure (1): Phenotypic Characteristics of *E. coli* colonies on selective media. A- Growth of pink colonies of *E. coli* on the MacConcky agar plate, B- White and non-hemolytic colonies of *E. coli* on the blood base agar plate and C- Green with metallic green shin colonies of *E. coli* on the EMB agar plate.

Molecular identification of *E. coli*

In this study, detected isolates were confirmed as *E. coli* using the 16S rRNA gene. The results of the PCR reaction on gel electrophoresis showed that 25 (100%) *E. coli* isolates were positive for the 16S rRNA gene (180 bp), as shown in Figure (2). The PCR technique was used for identifying *E. coli* bacteria by the detection of the 16S rRNA gene since it was rapid and accurate alternative to phenotypic methods of bacterial identification (12). Another study used the gene 16S rRNA as a target for detection, demonstrating the specificity of this gene in detection (13).

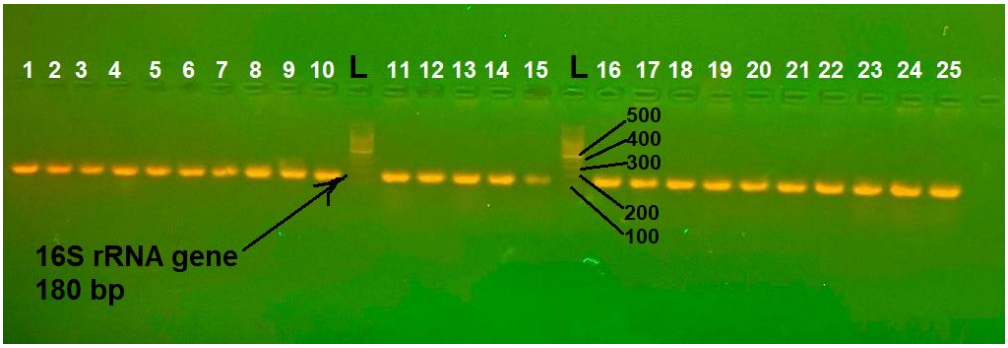

Figure (2): Agarose gel electrophoresis of conventional PCR amplification products of 16S rRNA gene in *E. coli* isolates. L: marker (100 bp ladder); lanes 1-25: PCR amplicons of target genes. (Expected size 180 bp; 1.5% agarose, 50 Vol / 1.30 hour).

Distribution of *E. coli* by gender factor

In this study, out of a total of 25 *E. coli* isolates, 19 (76%) were from females, whereas only 6 (24%) were from men. Patients' ages for the study were between 5 and 78 for both genders. This research shows that female patients with UTI have a greater incidence of *E. coli* isolates than male patients, as shown in Figure (3). This finding was similar to Iraqi study by (14), which found that females were more likely than males to get an infection, with percentages of 71.2% and 28.8%, respectively, of 200 samples taken from UTI patients in Baghdad.

A woman's short urethra and proximity to the anus allow germs to enter the bladder and cause illness, whereas a man's longer urethra makes it more difficult for bacteria to infection the bladder (15).

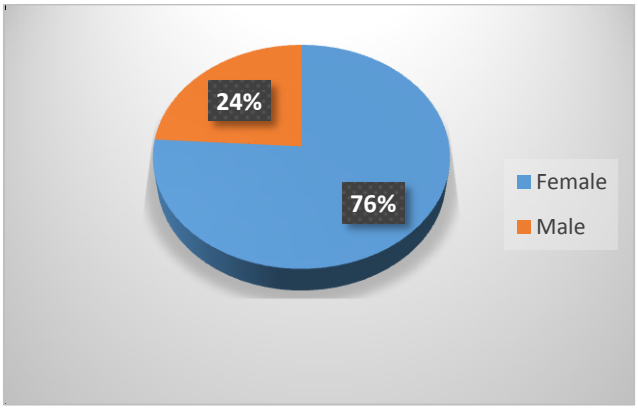

Figure (3): Percentage of females and males suffering from UTI

Antibiotic susceptibility test.

The susceptibility of *E. coli* isolates to certain concentrations of some antibiotics was performed using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar. Resistance of *E .coli* isolates were tested against several types of antibiotic including: Imipenem (IMI); Meropenem (MEM); Cefotaxime (CTX); Ciprofloxacin (CIP); amoxicillin-clavulanate (AUG); piperacillin-tazobactam (TZP); Aztreonam (ATM); Trimethoprim-Sulfamethoxazole (SXT); Levofloxacin (LEV); Amikacin (AK); Gentamycin (CN); Naldixic acid (NA); and Nitrofurantoin (F) as shown in table (2).

In the present study all *E. coli* isolates have shown highest resistance to Cefotaxime was recorded (80%) the resistance rate was similar with other study reported by (16,17), who discovered that (74.4% and 79%), respectively. However, this result was slightly lower than the rate of cefotaxime resistance reported in other research by (18), who recorded 92.45%. The results of the present study showed resistance to levofloxacin (60%), which was in agreement with the Iraqi study by (19,20), which recorded a resistance percentage of 60.1% and 61.2%, respectively. In this study, the dominant isolate *E. coli* was sensitive to meropenem and nitrofurantoin 96%. The results presented here agree with those of (21), who recorded an 89.8% sensitivity for nitrofurantoin, and the Iraqi study by (22), who recorded a 95.6% sensitivity for meropenem. Also, this result is in agreement with (23), who recorded (96.9% and 99.9%) resistance to nitrofurantoin and meropenem, respectively.

The *E. coli* isolates have a variety of ways to resist antibiotics, including creating biofilms. The outer membrane that encloses the cell wall of gram-negative bacteria has channels called porins that block the entry of antibiotic molecules into the bacterial cell. In addition, *E. coli* can produce a broadspectrum B-lactamase enzymes, which are very important mechanisms in the fight against B-lactam antibiotics in the family of Enterobacteriaceae (24).

Table (2): Percentages of antibiotic susceptibility rate of 25 *E. coli* isolate against 13 antibiotic agents.

Antibiotics	Sensitive	Intermediate	Resistance	P-value
Imipenem	13 (44%)	10 (40%)	2 (8%)	0.0198 *
Meropenem	24 (96%)	1 (4%)	0 (0%)	0.0001 **
Cefotaxime	4 (16%)	1 (4%)	20 (80%)	0.0001 **
Ciprofloxacin	7 (28%)	2 (8%)	16 (64%)	0.0001 **
amoxicillin-clavulanate	0 (0%)	6 (24%)	19 (76%)	0.0001 **
piperacillin-tazobactam	11 (44%)	7 (28%)	7 (28%)	0.523 NS
Aztreonam	6 (24%)	3 (12%)	16 (64%)	0.0001 **
Trimethoprim-Sulfamethoxazole	9 (36%)	0 (0%)	16 (64%)	0.0001 **
levofloxacin	9 (36%)	1 (4%)	15 (60%)	0.0001 **
Amikacin	22 (88%)	2 (8%)	1 (4%)	0.0001 **
Gentamycin	17 (68%)	0 (0%)	8 (32%)	0.0001 **
Naldixic acid	7 (28%)	2 (8%)	16 (64%)	0.0001 **
Nitrofurantoin	24 (96%)	1 (4%)	0 (0%)	0.0001 **
P-value	0.0001 **	0.0037 **	0.0001 **	------
* ($P \leq 0.05$), ** ($P \leq 0.01$)				

Biofilm formation of *E. coli*

The microtiter plate method (MTP) revealed that 25 isolates (100%) had the capacity to adhere and produce a thin layer with significantly different degrees of thickness (strong, moderate, and weak), where the variation in biofilm thickness may be due to differences in the isolates ability to produce biofilm. Results showed that 7 (28%) isolates produced strong biofilm, 10 (40%) isolates produced moderate biofilm, and 8 (32%) isolates produced weak biofilm.These results are in agreement with a study in Baghdad/Iraq, by Singh (28), who determined that all isolates of *E. coli* isolated from patients with UTI were 100% biofilm-producing.

While other studies done in Iraq have shown results when using MTP to investigate biofilm production, as in the study by (25) and (26) where they found that 83.3% and 80%, respectively, of isolates were biofilm producers.

Another study using MTP in southern Italy by Maione (23) found the rate of biofilm production in multidrug-resistant (MDR) *E. coli* isolates from urine samples to be 80%, with 30% strong biofilm. Bacteria create biofilms to shield themselves from potential injury, which makes them more resistant to drugs. Numerous components of the biofilm contribute via various antibiotic resistance pathways (27). The transformation of the colony from an exponential to a sluggish or without growth/persisters phenomenon supports antibiotic resistance. The glycocalyx matrix inactivates antimicrobial substances and safeguards the biofilm's periphery through the efflux system and enzymes. It's interesting to note that cells in a biofilm's middle location lack a certain nutrient, which slows their growth (28).

Molecular detection of *marA* and *luxS* genes

Each of the 25 DNA samples from *E. coli* isolates was subjected to PCR amplification for molecular identification and used designed primers to determine the prevalence of the multi-antibiotic resistance and quorum sensing genes *marA* and *luxS*, respectively. Figures (4,5) exhibit the results, which revealed that 23 of the isolates (92%) produced positive results for sharp amplified bands for both genes with approximate molecular sizes of 266 bp and 380 bp, respectively.

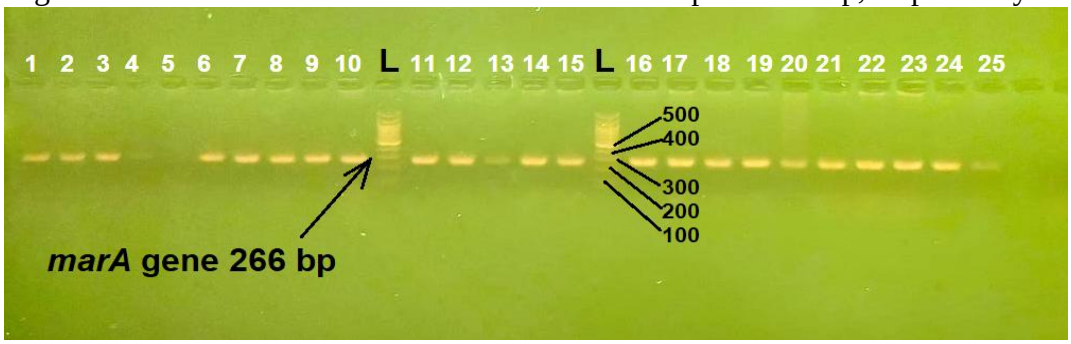

Figure (4): Agarose gel electrophoresis of conventional PCR amplification products of *marA* gene in *E. coli* isolates. L: marker (100 bp ladder); lanes 1-25: PCR amplicons of target genes. (Expected size 266 bp; 1.5% agarose, 50 Vol / 1.30 hour).

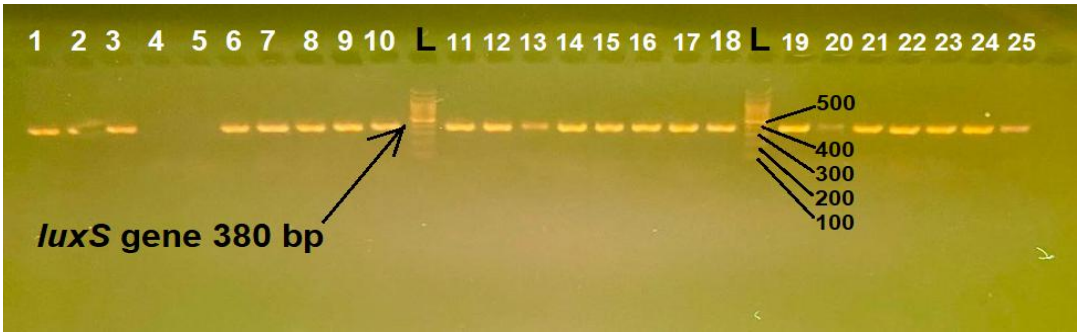

Figure (5): Agarose gel electrophoresis of conventional PCR amplification products of *luxS* gene in *E. coli* isolates. L: marker (100 bp ladder); lanes 1-25: PCR amplicons of target genes. (Expected size 380 bp; 1.5% agarose, 50 Vol / 1.30 hour).

Gel electrophoresis results in figures (3,4) show that the *marA* and *luxS* genes are present in all isolates except isolates no. 4 and 5.

The *luxS* gene, which is highly conserved among bacterial species, encodes LuxS, a crucial enzyme in this set of events. Recent studies have focused heavily on the intricate process through which QS affects the development of biofilms; bacteria communicate with one another within and between species using chemical signals and related receptors (6). These chemicals bind to their receptors when an extracellular threshold concentration is achieved, activating the QS system; the first potential interspecies QS system was found with the identification of autoinducer-2 (AI-2) and its associated synthase, both gram-positive and gram-negative bacteria contain the synthase LuxS, and research suggests that the LuxS/AI-2 QS system regulates the production of virulence factors, sporulation, bacterial luminescence, motility, biofilms, drug resistance, and toxins (7). Antibiotic resistance could increase by 10–1000 times as a result of biofilm formation, complicating therapeutics; during biofilm development, those cells can interact with one another and control their physiology through quorum sensing (QS). On the other hand, the main resistance mechanism is reduced drug accumulation brought on by activation of efflux mechanisms and down-regulation of outer membrane porins; the *marA* encourages antibiotic outflux and inhibits their influx, resulting in a variety of antibiotic-resistant phenotypes; the *mar* operon, one of the primary controllers of drug resistance in *E. coli*, is the multiple antibiotic resistance (29). Genetic studies revealed that the *mar* locus was made up of two divergently transcribed units: the *marC,* which encodes for a putative integral membrane protein, and the *marRAB* operons, which encode genes for three transcription proteins (MarR, MarA, and MarB) (30). Nevertheless, the presumed integral inner membrane protein MarC does not lead to the MDR phenotype, whereas MarA and MarR are transcription factors, MarB's function wasn't fully understood until 2013, when it was discovered to suppress expression from the *marRAB* operon by slowing the rate of *marA* transcription through an unidentified method (31). As a result, , *marA* encourages antibiotic outflux and inhibits their influx, resulting in a variety of antibiotic-resistant phenotypes (32).

Conclusion

It is concluded that this high percentage of *marA* is connected with a high rate of antibiotic resistance and *luxS*, which represents a high capacity

for biofilm formation; hence, the *marA* and *luxS* percent reflect the amount of pathogenicity among multidrug-resistant UPEC.

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