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Molecular Detection of Type II Toxin *RelE* Gene and Relation with Biofilm Formation in *Escherichia Coli*

Areej Jasem Mohammad[1], and Enass Ghassan Sweedan[2*]
[1,2]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.
*Corresponding Author

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Abstract:
Escherichia coli is the principal etiological agent of urinary tract infections (UTIs) and exhibits significant antibiotic resistance, driven by various virulence factors. Fifty *E. coli* isolates were obtained from 200 urine specimens of patients, and the *relE* gene was found to be crucial for antibiotic resistance and biofilm formation. This study used cultural and biochemical tests for primary bacterial isolate diagnosis, with the VITEK 2 System for confirmation. Antibiotic susceptibility testing assessed resistance, while the Congo red technique and microtiter plate method evaluated biofilm production. Multiplex PCR was used to detect the *relE* gene. Fifty isolates were tested by using the Congo red technique; 86% formed black colonies (biofilm producers), while 14% formed pink colonies (non-biofilm producers). The study found a 72% antibiotic resistance rate to cefotaxime among isolates. Minimum inhibitory concentration (MIC) values for cefotaxime only were 512, 64, and 32 µg/mL**.** Molecular detection of toxin-antitoxin type II genes via multiplex PCR on 22 isolates revealed that 12 (54.45%) possessed the *relE* gene. The gene expression was measured using quantitative reverse transcription PCR (qRT-PCR) in the presence and absence of sub-MIC concentrations of cefotaxime. The expression of the *relE* gene showed downregulation in all isolates, and fold changes were 0.1881, 0.2044, and 0.5864, respectively. The study concluded that changes in gene expression may be induced by environmental pressure or antibiotic resistance, suggesting disruption of the regulatory systems that maintain cell stability and persistence. This phenomenon is recognized as a bacterial defense mechanism.
Keywords: *Escherichia coli*, biofilm, cefotaxime, Toxins and Antitoxins II *relE*

1. Introduction

Urinary tract infections (UTIs) are considered the most common type of infection, impacting individuals across all age groups when they are caused by bacterial pathogens that primarily inhabit the lower regions of the urinary system [1.]

Escherichia coli is a common commensal organism, but it is also responsible for urinary tract infections and other clinical disorders. The natural presence of *E. coli* is described by the presence of L-type bacteria and facultative internal variants. These bacteria are also capable of fermenting lactate and producing indole as part of their metabolic processes [2.]

Antibiotic resistance in *E. coli* poses a major public health challenge by reducing the effectiveness of standard infection treatments. [3.]

Antimicrobial drugs are significant contributors to the development of antibiotic resistance in bacteria. This is especially the case in healthcare-associated settings, where the overuse and continued use of antibiotics are key determinants of the occurrence and spread of resistant

bacterial populations. Resistance is often caused by enzymes, ESBL (extended-spectrum β-lactamases), followed by AmpC, decreased permeability, and efflux pumps [4]

Biofilm is a population of microorganisms that adhere to one another and to biotic or abiotic surfaces. The primary factor contributing to the persistence and recurrence of urinary tract infections (UTIs) is the capability of uropathogenic *Escherichia coli* (UPEC) to form biofilms. This capacity significantly enhances their resistance to antimicrobial treatments[5].

Toxin-antitoxin (TA) systems comprise a stable toxin and an unstable antitoxin. The toxins are typically proteins, while the antitoxins are RNA or proteins. Normally, the antitoxins neutralize enzymes, preventing them from acting. However, under stressful conditions, the reactive antitoxins are degraded, and stable toxins are released, which then damage cells. There are more than eight types of TA systems, classified by the principle by which the antitoxin neutralizes the toxin. A prominent example of an antitoxin is the type II toxin-antitoxin system, in which the antitoxin binds to the toxin to neutralize its activity post-translationally. [6,7]

The study aimed to assess the presence of the type II toxin-antitoxin system *relE* in uropathogenic *Escherichia coli* (UPEC) isolates. It also evaluates the possible relationship between biofilm formation in uropathogenic *Escherichia coli* and the expression of the *relE* gene, and between the expression of the *relE* gene and sub-MIC cefotaxime in UPEC. Cefotaxime may downregulate the genes. This study aids in the development of new strategies to combat antibiotic resistance.

2. Materials and methods

2.1. Bacterial collection and isolation

From 200 urine specimens collected between September and December 2024 at three hospitals in Baghdad (Child's Central Teaching Hospital, Al-Yarmouk Teaching Hospital, and Baghdad Teaching Hospital), from various ages, the mean ± SE of ages was 35.97 ± 6.011. They were cultured on MacConkey agar, Eosin Methylene Blue (EMB), and HiChrome™ UTI agar (HiMedia / India); these cultures were subsequently incubated at 37°C for 24 hours. [8.]

The identification of bacterial isolates was confirmed phenotypically via a series of biochemical tests, specifically the IMViC tests: indole, methyl red, Voges-Proskauer, and Simon citrate. (HiMedia / India) Additionally, the identification of *Escherichia coli* was validated using the Vitek-2 compact system [9,10.]

2.2. Antibiotic sensitivity assay

The study included antibiotic susceptibility testing using collected bacterial colonies resuspended in sterile saline to a 0.5 McFarland standard turbidity, as determined by the Densi-Check method. This suspension was introduced into the Antimicrobial Susceptibility Testing (AST) No.419 Vitek-2. The resulting data were processed with integrated software for accuracy. This specimen was subsequently confirmed with the VITEK 2 compact system. [11,12.] Data were analyzed according to the CLSI (2025) [13,14] criteria. Each antibiotic was identified as being resistant, intermediate, or susceptible.

2.3 Antibiotic minimum inhibitory concentrations (AMIC)

The sensitivity of bacterial isolates to cefotaxime 0.5g (Torlan /Spain) was evaluated using a microdilution broth assay to determine the minimum inhibitory concentration (MIC). Initially, bacterial cultures were standardized to a concentration of 1.5×10^8 cells/mL. This adjustment was essential for ensuring accurate and reliable findings regarding cefotaxime's effectiveness against the bacterial isolates under study. Using a sterile loop, the bacterial isolates were inoculated into Mueller-Hinton broth within microtiter plates. The plates were prepared with cefotaxime in double serial dilutions, allowing for the assessment of various concentrations. Then, the plates were inoculated and incubated at 37°C for 24 hours; after that, the MIC values were determined by identifying the first well that showed no visible growth, indicating clear media. The lowest concentration of cefotaxime that can inhibit the growth of bacteria provides critical information about the antibiotic susceptibility [15].

2.4 Biofilm Formation Assay of bacteria:

Bacterial isolates were tested for biofilm formation (BF) using qualitative and quantitative methods [16]. The Congo Red Agar (CRA) medium, prepared by adding 1% sucrose to BHI agar and mixing with Congo red solution, was used qualitatively. After streaking the organism cultures on CRA plates, incubation for 24 hours at 37C° allowed for the identification of biofilm producers: black colonies indicated strong production, grey colonies moderate, and pink colonies none [17,18] .

For the quantitative assessment, isolates from the microtiter plate method were cultured in Tryptic Soy Broth (TSB) for 24 hours. Then, it was diluted to a 1:100 ratio. A 200 µL portion was added to fresh TSB with 1% glucose and dispensed into a 96-well plate. This was repeated for each isolate alongside a negative control. After incubation, the wells were washed, fixed with methanol, stained with 0.5% crystal violet, and de-stained with 99% ethanol. According to [19], the optical density cutoff (ODc) was measured at 630 nm, with results compared to the negative control, as detailed in **Table 1.** The cutoff values for biofilm formation are also noted [20,21].

Table 1. The optical density (OD) values for biofilm formation

OD value	Biofilm formation
OD ≤ ODc	Non-biofilm former
ODc < OD ≤ 2 × ODc	weak
2ODC<ODt≤4ODC	Moderate
4 × ODc < OD	strong

2.5 Molecular Detection of Genes with PCR:

2.5.1 Bacterial DNA Extraction

The Gene Aid Presto™ Mini gDNA Bacteria Kit (Gene aid Biotech Ltd / Taiwan) was used for efficient and reliable genomic DNA extraction from bacterial cells. The quality and integrity of the isolated DNA were subsequently assessed through gel electrophoresis [22].

2.5.2 PCR Analysis

The study utilized Polymerase Chain Reaction (PCR) to amplify the toxin and antitoxin gene II in *Escherichia coli*, specifically targeting the *relE* gene. This process involved selecting specific primers (Macrogen / South Korea) [(23)] and employing a thermal cycler to maintain precise temperature control throughout the procedure. During the denaturation phase, the double-stranded DNA template was divided into two single strands. The annealing phase followed, during which the primers bound to the target DNA. Finally, in the extension phase, Taq polymerase synthesized new DNA strands [(24).] The details of this process are presented in **Table 2**.

Table2. Primers of genes used in PCR and real-time PCR

genes	Primer sequences 5' → 3'	Product size	Annealing temperature
RelE -F	5'TCTGGATTTTGACGAGCGGG3'	250 Bp	58Cº
RelE -R	5'TGTTTGCTTCAATCCGGGGT3'		

PCR amplifications were carried out in total reaction volumes of 25µl. Specifically, 1µl of each primer, 12.5µl of the premix, lyophilized powder master mix (CWbio/ Korea), 2µl DNA template, and then the volume was completed by deionized distal water, and the mixture was vortexed. The PCR reaction tubes were placed correctly into the thermocycler, where amplification was carried out according to the specifications outlined in **Table 3**.

Table 3. The program used in PCR

Cycle	Temperature	Time	Cycles
Initial Denaturation	95	3 min.	1
Denaturation	95	30 sec.	35
Annealing	58	30 sec.	
Extension	72	1 min.	
Final extension	72	3 min.	1

2.5.3 Gene expression of the *relE* gene treated with sub-MIC of Cefotaxime

PCR Product has been examined by an Electrophoresis apparatus (Cleaver / UK), agarose gel in concentration of 1.5% by combining 0.75g of agarose powder (ABIOpure/ USA) in 75ml of TBE (Bioneer/ Korea), then 4µl of Ethidium bromide dye was add (Promega /USA), and has been a look under a UV transilluminator Slimline/USA) .

The effects of the antibiotic cefotaxime (0.5g)/France gene expression in *Escherichia coli* were evaluated using the relative real-time PCR technique. The experimental procedure involved mixing 1 ml of fresh bacterial culture, grown in BHI broth, with 1 ml of a sub-minimum inhibitory concentration (sub-MIC) of cefotaxime. This mixture was then incubated at 37 degrees Celsius for 24 hours. After the incubation period, RNA extraction was performed on both the treated group and the untreated control group. RNA was isolated from the *E. coli* cells using GENEzol™ Reagent (Geneaid Biotech Ltd, Taiwan), following the manufacturer's protocol. The expression levels of toxin-antitoxin genes were quantified using the relative quantitative PCR (RT-qPCR) (Promega / USA) method, consistent with established one-step real-time PCR procedures[25]. The conditions for each cycle were programmed and run according to the protocol outlined in **Table 4**.

Table 4. (RT-PCR) program used in the expression of the gene

Steps of cycles	temperature (°C)	Time	Cycles
Reverse Transcription	55	10 minutes	1
Initial Denaturation	95	1 minute	1
Denaturation	95	10seconds	40
Extension	60	30 seconds	
Melt Curve	60-95	Different	1

The data was achieved by referencing the *GADPH* (F-ACTTACGAGCAGATCAAAGC, R-AGTTTCACGAAGTTGTCGTT) housekeeping gene. Subsequently, gene expression levels were quantified as fold changes using the ΔΔCT method elucidated by [26] .The expression of gene levels was calculated using a series of equations based on this methodology.

2.5.4 Statistical analysis

The data were systematically entered and analyzed using the Statistical Package for the Social Sciences (SPSS) version 2019 to identify the influence of various groups on the study's characteristics. T-test and LSD-Least P-value were applied to a significant comparison of means. Chi-square comparison was employed to test between percentages (0.05 and 0.01 probability) in the current study.

3. Results

3.1 Isolation and identification of bacteria

Out of 200 specimens, only 50 isolates were identified as *E.coli* through cultural, morphological, and biochemical tests. The isolates appeared as pink colonies because they were lactose fermenters when cultured on MacConkey agar and exhibited a green metallic sheen on EMB agar (**Figure 1**). This is caused by eosin Y and methylene blue dyes that interact with the acid produced by lactose fermentation, which decreases the pH and results in purple colonies on HiChrome™ UTI agar. This occurs due to the wide range of enzymes required by the bacterium for its metabolic functions, as shown in **Table 5**. Then the VITEK 2 compact system was used to confirm that the isolates belonged to *E. coli*.

Table 5. Identification of UPEC by biochemical tests:

The name of the tests	The results
MacConkey agar	Pink colonies (lactose fermenter)
EMB agar	Green metallic sheen
HiCrome™ UTI agar	purple colonies
Indole	Positive (red ring)
Methyl red (MR)	Positive(yellow)
Voges-Proskauer (VP)	Negative(red)
Simmons Citrate test	Negative(green)

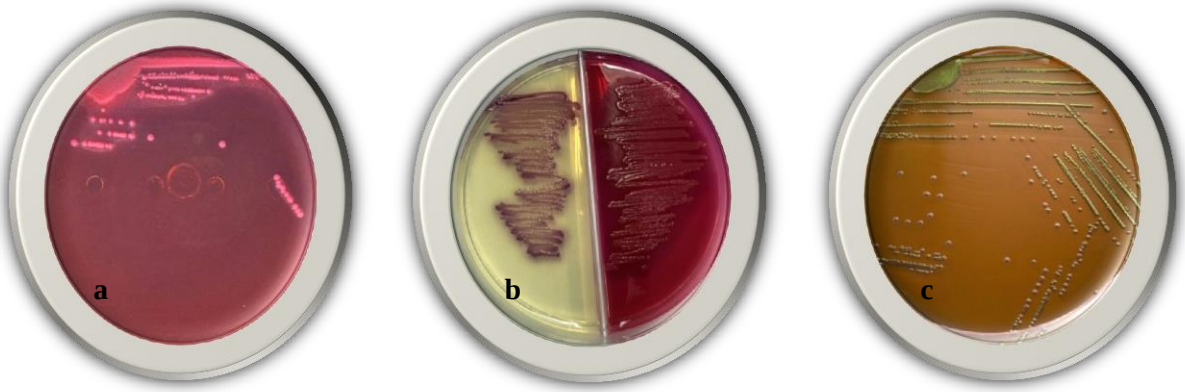

Figure 1. (a): Growth of UPEC on MacConkey agar, **(b):** Growth of UPEC on EMB agar, **(c):** Growth of UPEC on Hichrome UTI Agar

3.2 Antibiotic susceptibility assay

The present study examined the antibiotic susceptibility of fifty UPEC isolates by using the VITEK 2 compact system. This finding revealed a significant level of antibiotic resistance among the isolates, as shown in **Table 6**. Six (12%) isolates were classified as multidrug-resistant (MDR) to antibiotics, including cefotaxime, as indicated by the AST VITEK 2 system. 72% were resistant to cefotaxime, while intermediate resistance to ciprofloxacin was 52%, and ceftazidime was 38%. However, 94% of them were sensitive to Ceftazidime-Avibactam, Ceftalozane-Tazobactam, Imipenem, Meropenem, Amikacin, and Tigecycline. As well, 92% of them were sensitive to piperacillin–tazobactam, cefepime, gentamicin, and colistin, while ampicillin was 80%, and trimethoprim-sulfamethoxazole was 84%. The findings concluded that the majority of UBEC isolates were multidrug resistant. The results of antibiotic sensitivity were a highly significant P-value (0.001) for all antibiotics except Ceftazidime.

Table 6. The results of antibiotic sensitivity of *E. coli*

Antibiotics	**R**	**I**	**S**	***P-value***
Ampicillin	5(10%)	5(10%)	40(80%)	0.001 **
piperacillin –Tazobactam	4(8%)	0(0%)	46(92%)	0.001 **
Cefotaxime	36(72%)	0(0%)	14(28%)	0.001 **
Ceftazidime	13(26%)	19(38%)	18(36%)	0.269 NS
Ceftazidime- Avibactam	3(6%)	0(0%)	47(94%)	0.001 **
Ceftolozane-Tazobactam	3(6%)	0(0%)	47(94%)	0.001 **
Cefepime	4(8%)	0(0%)	46(92%)	0.001 **
Imipenem	3(6%)	0(0%)	47(94%)	0.001 **
Meropenem	3(6%)	0(0%)	47(94%)	0.001 **
Amikacin	3(6%)	0(0%)	47(94%)	0.001 **
Gentamicin	4(8%)	0(0%)	46(92%)	0.001 **
Ciprofloxacin	9(18%)	26(52%)	15(30%)	0.0048 **
Tigecycline	3(6%)	0(0%)	47(94%)	0.001 **
Colistin	4(8%)	0(0%)	46(92%)	0.001 **
Trimethoprim-Sulfamethoxazole	8(16%)	0(0%)	42(84%)	0.001 **
P-value	*0.001* **	*0.001* **	*0.001* **	---
** (*P≤0.01).*				

R: resistant, I: Intermediate, S: Sensitive

3.3 Antibiotics Minimum Inhibitory Concentrations

The MIC results for cefotaxime in 3 isolates of *E. coli* were (512, 64, 32) µg/ml by using a microdilution broth assay. Also, results of sub-MIC for 3 isolates as results were shown in **Table 7**.

Table 7. Antibiotic minimum inhibitory concentrations of Cefotaxime

Isolate number	**1**	**23**	**24**
Cefotaxime (MIC) µg/ml	512	64	32
Sub-MIC	256	32	16

3.4 Biofilm formation

According to the Congo Red technique's results, 43 isolates (86%) produced black colonies on the medium (producing biofilm), while only seven isolates (14%) produced pink growth on the medium (non-biofilm-producing), as shown in **Figure 2**. This phenomenon can be elucidated by the capacity of biofilm-forming bacteria to release significant quantities of extracellular polysaccharides. These polysaccharides undergo chemical interactions with the Congo red dye, leading to the formation of dark pigment complexes. This process is attributed to the enhanced pigment absorption exhibited by biofilm-forming bacteria, in contrast to strains that do not form biofilms. The microtiter plate (MTP) method was applied for quantitative assessments of biofilm production among the isolates. The findings indicated that the isolates were capable of producing biofilms. Specifically, 32 isolates, representing 64%, were identified as biofilm producers. Among these, 13 (26%) were classified as moderate biofilm producers, whereas 19 (38%) were categorized as weak biofilm producers. The remaining 18 isolates, comprising 36%, did not produce any biofilm. These results, along with the final digital values quantifying biofilm production by the bacterial suspensions in the wells, are illustrated in **Figure 3** and summarized in **Table 8.**

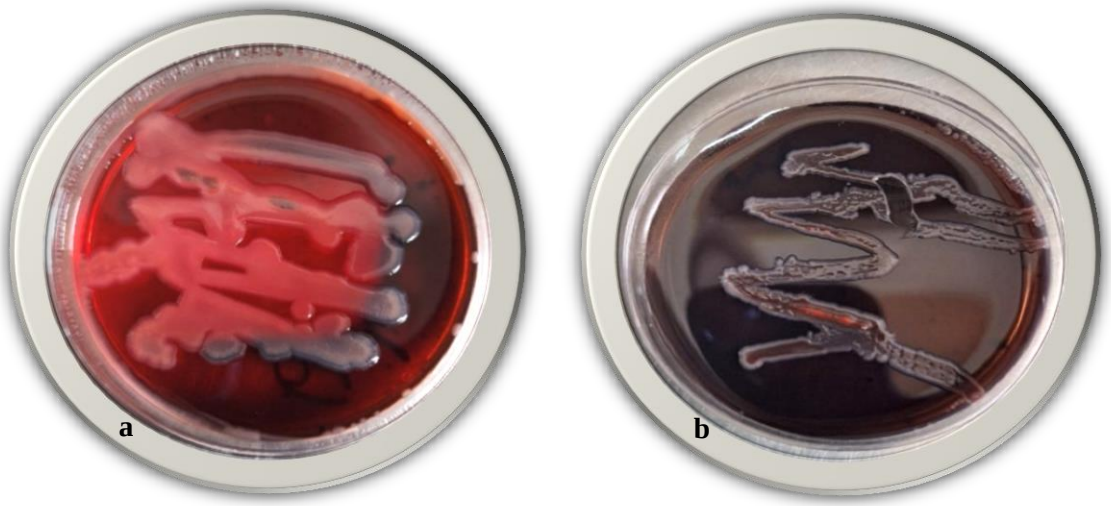

Figure 2. Growth of UPEC isolates on Congo red medium, a: Black colonies (biofilm production), b: Pink colonies (non-biofilm producing)

Table 8. Biofilm results of *Uropathogenic E. coli* by the MTP method

Biofilm formation	**Number &Percentage%**
Non-producer	18 (36.00%)
Moderate	13 (26.00%)
Weak	19 (38.00%)
Total number of biofilm producers	32 (64.00%)
Total of isolates	50(100%)
P-value	0.378 NS
NS: Non-Significant.	

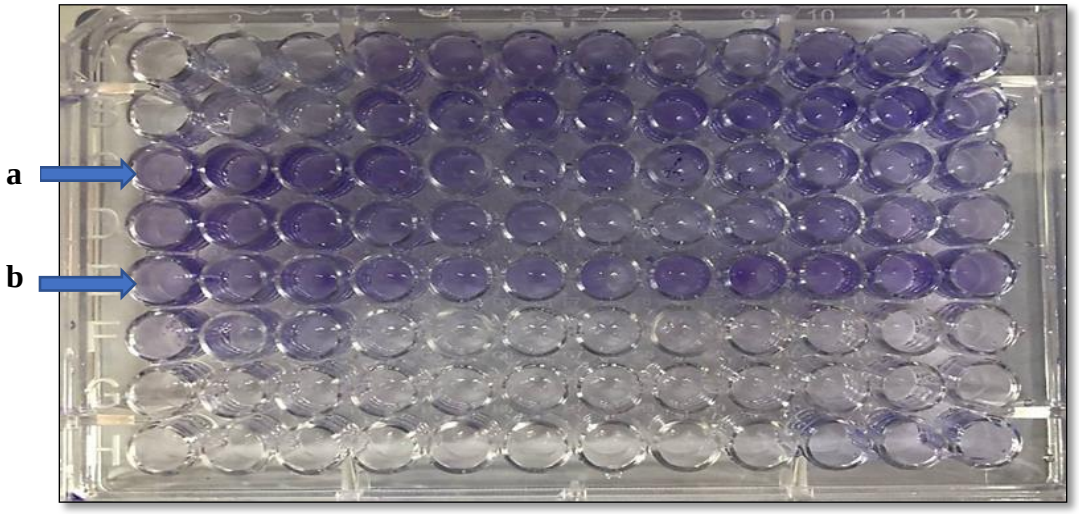

Figure 3. The microtiter plate, **a.** dark purple biofilm producer, **b.** light purple non-biofilm producer

3.5 Detection of type II toxin *relE* gene with PCR:

The current study included 22 UPEC isolates diagnosed based on phenotypic characteristics and antibiotic resistance, particularly to cefotaxime. The molecular detection was carried out by PCR for the *relE* gene, then electrophoresis; the product size was 250bp. For the *relE* gene to detect the presence or absence of this gene in the UPEC isolates, as shown in **Figure 4**, the percentage of the frequency of genes in isolates was 54.45% positive for the *relE* gene.

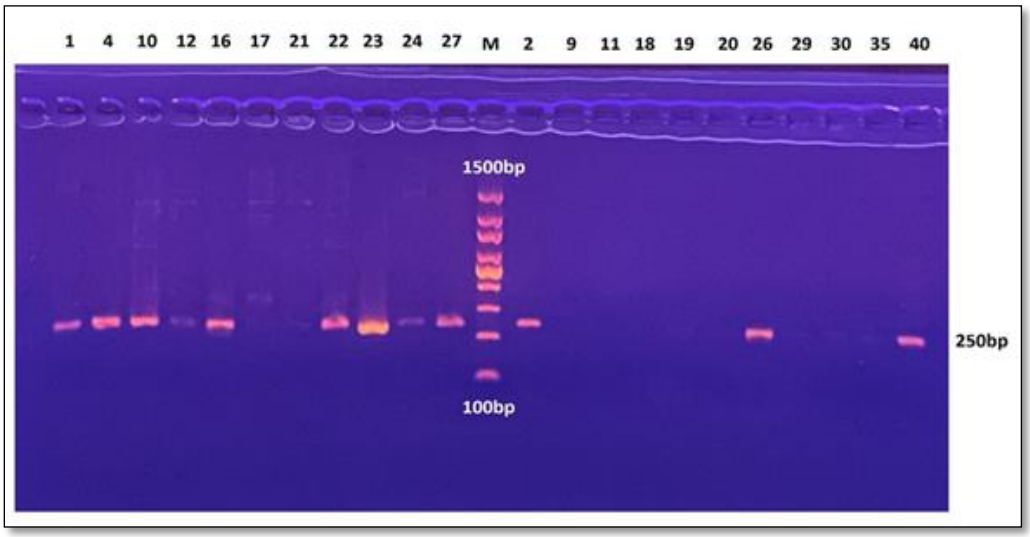

Figure 4. Gel electrophoresis of the PCR: the product size 250 bp. Electrophoresis was performed in a 1.5% agarose gel at 70 volts for 1 h. Representative results are shown; lane M: DNA ladder (100-1500 bp); 1, 4, 10, 12, 16, 22, 23, 24, 27, 2, 26, and 40 were positive *for relE*, and 17, 21, 9, 11, 18, 19, 20, 29, 30, and 35 were negative.

3.6 Relationship between *relE* and biofilm formation

As shown in **Figure 5**, the *relE* gene was out of 22 isolates; 12 were positive. The comparison of biofilm-forming isolates and non-forming isolates reveals the presence or absence of these genes. In this finding, the non-forming isolates recorded the highest value (59.2%), which were positive for *relE*, followed by moderate, which recorded 27.2% of them, and then the weak ones recorded 13.6%. The comparison among the groups indicates non-significant differences ($P \leq 0.0266$) for the *relE* gene. The scarcity of experimental work in this field makes the interpretation of results not easy.

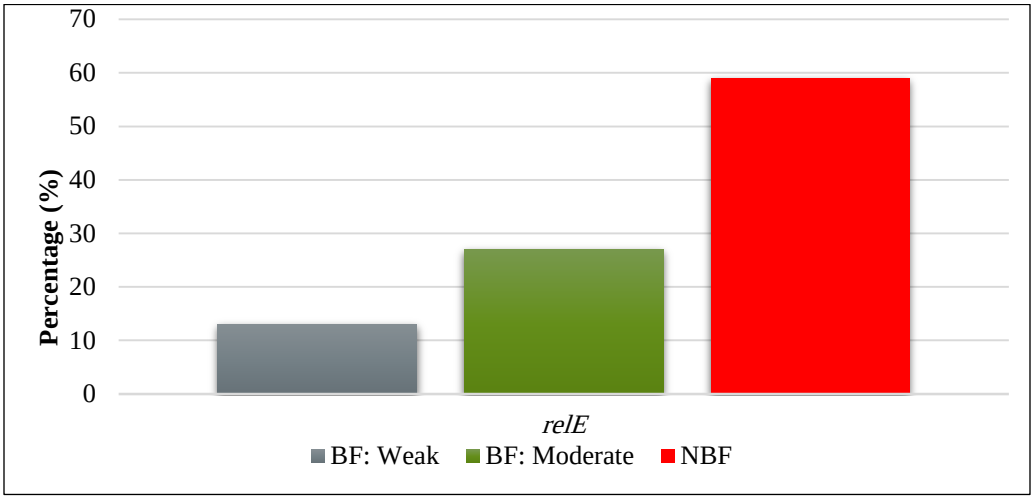

Figure 5. Results of the relationship between *relE* with biofilm formation in *E. coli*

3.7 Gene expression of the *relE gene* without and with sub-MIC of Cefotaxime

Gene expression of three genes was identified for three positive isolates for these genes, their numbers (1, 23, and 24); they were multidrug resistant to antibiotics and especially to cefotaxime 0.5g (Torlan /Spain).

The current finding indicates that there is no significant difference in the expression of the *relE* gene, which recorded 0.1881, 0.2044, and 0.5864-fold change to control, as shown in **Table 9** and **Figure 6.**

Table 9. Fold change of *relE genes* in *Uropathogenic E. coli* in the presence of sub-MICs of Cefotaxime and control

Isolate code	Control			Sub-MIC Cefotaxime			ΔΔCt	Fold change	Result
	hkg	*relE*	ΔCt	*hkg*	*relA*	ΔCt			
1	12.77	27.74	14.97	10.49	27.87	17.38	2.41	0.1881 ±0.02	Decrease
23	11.78	11.16	-0.62	14.41	16.08	1.67	2.29	0.2044 ±0.05	Decrease
24	11.7	25.02	13.32	12.48	26.57	14.09	0.77	0.5864 ±0.08	Decrease
L.S.D. (P-value)	-	-	-	-	-	-	-	*0.419 NS (0.0889)*	-
*** (P≤0.01).*									

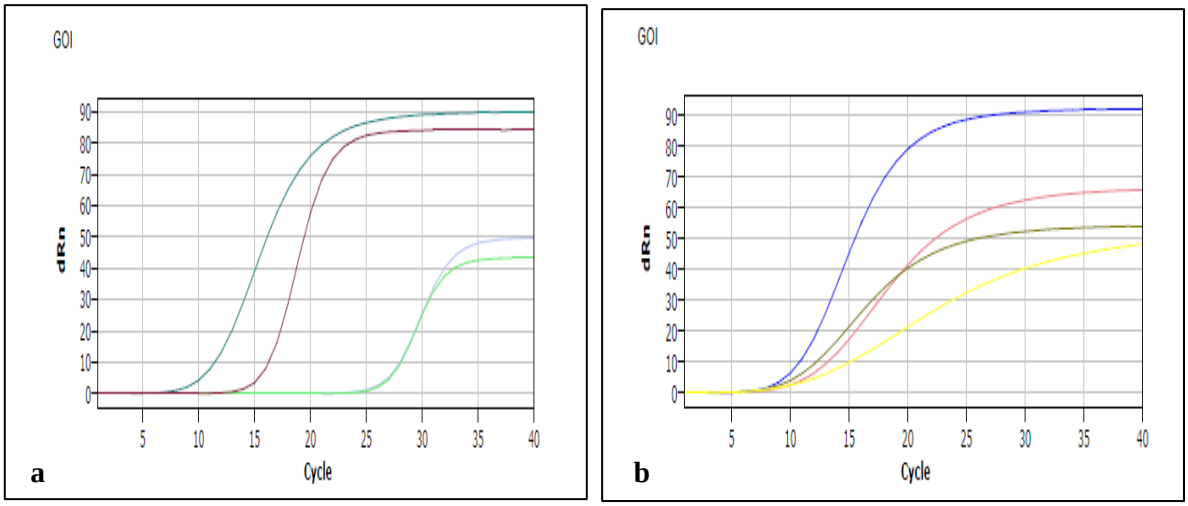

Figure 6. The Real-time PCR quantitation analysis for cycling: (a) *relE* gene, (b) *GAPA* housekeeping gene.

4. Discussion

This study's findings show that only 50 isolates were identified as *E. coli* through cultural, morphological, and biochemical tests. These results agree with [27], who identified 57 *E. coli* isolates from urine samples. The differences in results are attributed to variations in sterilization conditions, temperature, incubation period, inoculation methods, environment, and human factors. This was subsequently confirmed with the VITEK 2 compact system. It concluded from these findings that UPEC bacteria are highly resistant to cefotaxime, followed by ceftazidime and then ciprofloxacin. This bacterium resists more than two antibiotics from different groups; therefore, it is considered an MDR strain. The observed phenomenon may be attributed to the overutilization of broad-spectrum antibiotics within both healthcare facilities and the broader community. This increases the survival and growth of resistant strains, further complicating the treatment strategies. These results agree with [28], who reported that the prevalence of 3rd G Cephalosporins Cefotaxime antibiotic resistance was higher in UPEC isolates than in the commensal strains, which recorded 78.7% of total isolates, and [29], who found a high prevalence of resistance to Cefotaxime (91.73%), Rifampin (97.8%), Neomycin (97.4%), Ceftazidime (96.8%), Piperacillin (92.09%), Ceftriaxone (90.32%), and Ciprofloxacin (89%). The results of antibiotic sensitivity showed a highly significant P value (0.0001) for all antibiotics except Ceftazidime. These findings are in accordance with the research conducted by [30], which indicated significant resistance rates in phenotypic testing, specifically 87% for penicillin and 61% for fluoroquinolones. This finding is also consistent with [(31),] who reported that (52.5%) of isolates were resistant to ciprofloxacin. Resistance was highest to Ampicillin (85%) and Cefazolin (82.5%), while zero resistance was seen to Amikacin and Tigecycline. In the current study, it was found that isolates are more resistant to Cefotaxime; therefore, three isolates of *E. coli* were selected for further study. The variation in antibiotic susceptibility test results for the same bacteria across studies can be attributed to technical, biological, and methodological factors. The results agree with [32], who reported a value increase in cefotaxime-induced MIC [33]. reported that sub-MIC of cefotaxime can promote horizontal transfer of antibiotic resistance genes. Biofilm formation of isolates was detected. And the results agree with other research. The Cutoff value was calculated according to [19]. It concluded that determining the MIC is essential for choosing the appropriate antibiotic for treatment; high concentrations indicate the development of resistance in these bacteria, which is often caused by enzymes ESBL (extended-spectrum β-lactamases), followed by AmpC, decreased permeability, and efflux pumps. Previous research has indicated that biofilms are microbial communities surrounded by a layer of extracellular polymeric substances. This matrix enables them to adhere to various surfaces. Recent studies have shown that the production of biofilms in *E. coli* is mediated by the expression of curli and cellulose. This production helps uropathogenic *E. coli* (UPEC) survive for extended periods in the urethra by enveloping entire communities of bacteria in a hydrophobic layer. [34,35.]

The difference between the two methods could be a result of the two different media used in each method (brain heart infusion broth for the CRA method and tryptic soy broth for MTP), as well as the difference in bacterial concentration and incubation period, which could also affect the results. These results agree with [36]. Prior studies have shown a similar result when using MTP to investigate biofilm production, as was found by [37], when they found 19 (40.4 %) out of 47 *E. coli* isolates were biofilm producers, while the remaining 28 (59.6 %) isolates were non-biofilm producers. Out of the 19 biofilm producers' isolates, only one isolate was a strong biofilm producer, and 18 isolates were moderate biofilm producers. Twenty extremely drug-resistant UPEC were screened using Congo-red agar (CRA) to see if they could form biofilm. All these (100%) were biofilm producers. *E. coli* biofilm formation was classified into three groups. The first group was strong, 7(35%) isolates, the second was moderate, 13 (65%), and the third was weak, 0(0%) [38]

It concluded that biofilm-producing isolates displayed higher levels of antibiotic resistance than

non-biofilm producers, which cause Prostatitis, Biliary tract infections, and urinary catheter cystitis, among other major health issues that can result from biofilms formed by clinical *E. coli* strains [39].

These results showed non-significant results for the *relE* gene. The presence of the *relE* gene in only 12 isolates may be due to the occurrence of mutations, genetic diversity, or horizontal gene transfer within bacterial populations. UPEC strains enable the acquisition or loss of genetic elements, such as pathogenicity islands, plasmids, transposons, and other mobile genetic elements. These elements often carry toxin-antitoxin (TA) systems, including the *relBE* module, which prevents the association of the *relE* primer at its appropriate position and prevents amplification of this gene; therefore, this gene was not detected in all isolates. The results of this study also agree with [40], who noticed the prevalence of TA type II in *K. pneumoniae*, particularly *RelEB* genes. The researcher and his colleagues[41] reported that genes encoding a toxin-antitoxin (TA) system significantly reduced biofilm biomass in the absence of antibiotic stress. Similarly,[42] suggested that the TA system is linked to biofilm formation. However, another study indicated that there is a relationship between biofilm formation and the type II toxin-antitoxin system as well.

It was concluded from this study that these genes have an indirect effect on biofilm formation; the presence or absence of these genes may depend on environmental or regulatory factors that must be included in this study to maintain biofilm formation. The relation of *relE* with biofilm formation is not clear, but some studies have found no significant direct relationship between the presence of TA systems like *relE* and biofilm formation, while others suggest indirect links through stress response. The expression was changed by exposing the bacterium to sub-MIC of Cefotaxime after incubation for 24 hours in broth media. GAPDH is crucial for glycolysis, a fundamental metabolic pathway, making it a prime candidate for a housekeeping gene in prokaryotes and eukaryotes, especially found in *E.coli*[43].

The *relE* gene is responsible for the production of a protein toxin that inhibits protein synthesis through inhibiting the translation. This gene in three isolates was shown to have downregulation in expression genes in treated isolates with sub-MIC of cefotaxime, which refers to Ct values of genes in contrast to the control reduce the activity of this toxin and antitoxin II system in these isolates that are associated with increasing antibiotic resistance and persistence, which could reducing the rate of growth arrest or the formation of persistent cells. When the toxin level gets elevated again, the structures within the toxin-antitoxin II (TA) complex are changed, forming the three-dimensional resistance at the binding sites, inducing the TA system transcription. Repression conditions of high concentrations of the *RelE* toxin block the interaction between adjacent *RelB* dimers and prevent *RelB* from binding to the DNA to further activate transcription [7.] Interestingly, *relE* gene expression was down-regulated relative to the *infB1* control gene, suggesting that the bacterial cells might continue at sub-MIC of antibiotics (gentamicin and doxycycline) rather than grow.

The researcher and his colleagues[23] indicated that the *relB* gene is responsible for conferring resistance of *K. pneumoniae* to antibiotic stress by the action of the type II TA system. Furthermore, such a pathway is directly associated with the generation of persistent cells- a specialized bacterium that is particularly tolerant to antibiotics and environmental signals. This tolerance is mostly directed to a phenotypic change to a latent state, which supports persistence during disadvantageous conditions.

5. Conclusion

This study found that the *relE* gene, which is part of the type II toxin-antitoxin system, is downregulated. This reduction may be associated with environmental stress or antibiotic resistance, indicating that a disrupted regulatory mechanism could impact bacterial stability and persistence. It has been proposed that cefotaxime influences the expression of toxin-antitoxin (TA) genes under sub-minimum inhibitory concentration (sub-MIC) stress. Although we did not

observe a significant impact of *relE* inhibition on biofilm formation after exposure to cefotaxime, further experiments are needed to investigate this relationship under various conditions, including different concentrations and exposure times.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Ethical Clearance

The study was conducted following the receipt of participant consent and ethical approval from the Ethics Committee of the Biology Department at the University of Baghdad's College of Sciences (CSEC/0922/0117) on December 30, 2024. This procedure is in line with the guidelines set forth by the Iraqi Ministry of Health and Environment.

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