https://doi.org/10.15407/microbiolj85.01.026

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MOLECULAR EVALUATION of *rfb*E GENE EXPRESSION CHANGES UNDER DIFFERENT CREATININE CONCENTRATIONS IN *ESCHERICHIA COLI* STRAINS VIA REAL-TIME PCR

Background and objective. *Escherichia coli (E. coli) O157: H7 as an enterohemorrhagic pathogen causes severe damage to the gastrointestinal tract and dangerous diseases in humans such as hemolytic uremic syndrome (HUS) and acute renal failure, which is associated with increased blood creatinine levels. This study aimed to evaluate antibiotic resistance of E. coli O157: H7 pathotypes to detect the virulence of gene rfbE and to study variations in its expression.* ***Methods.*** *The isolates were first inoculated on eosin methylene blue (EMB) agar and then identified using the Microgen kit and the presence of rfbE gene. Antibiotic susceptibility of the identified strains was tested by the disk diffusion technique, followed by inoculating E. coli O157: H7 strains at concentrations of 1, 3, and 6 mg dl^{-1} in BHI broth. DNA and RNA were then extracted from the bacteria, and cDNA was prepared from purified RNA. Then, the rfbE gene expression was evaluated using a real-time PCR approach, and the data were analysed with Rest software.* ***Results.*** *The research results revealed high resistance of isolated strains against some of the studied antibiotics, and variations in the expression of the rfbE gene were found to be different at different creatinine concentrations and at different time points. A significant decrease in variations in the rfbE gene expression was observed at low concentrations (1 mg dl^{-1}), but, on the contrary, a significant increase in variations in the rfbE gene expression was found at higher concentrations (3 and 6 mg dl^{-1}) ($p<0.05$).* ***Conclusions.*** *The rfbE gene is one of the factors affecting the bacterial virulence. We believe that a secondary increase in creatinine for any reason can exacerbate kidney disease and failure by affecting the rfbE gene expression while producing O antigen or bacterial endotoxin.*

Keywords: *EHEC (O157: H7), rfbE, creatinine, Real-Time PCR.*

Citation: Janbakhsh E., Mehrabi M.R. Molecular Evaluation of *rfb*E Gene Expression Changes under Different Creatinine Concentrations in *Escherichia coli* Strains Via Real-Time PCR. *Microbiological journal.* 2023 (1). P. 26—35. https://doi.org/10.15407/microbiolj85.01.026

E. coli serotype O157: H7 is a type of enterohemorrhagic *E. coli* (EHEC) pathogen, which, as a foodborne and waterborne pathogen, can infect humans: after colonization in the intestine, it leads to severe damages such as haemorrhagic colitis, purpura, idiopathic thrombocytopenia, and haemolytic uremic syndrome [1—3]. Children are the primary victims of this pathogen, and more than five million annual deaths from it are reported, particularly in children younger than five years. Haemolytic uremic syndrome (HUS) is the most common cause of acute kidney failure in children in many parts of the world. Chronic renal disease (CKD), known as a chronic renal failure, refers to the irreversible function of the kidney, which gradually progresses to the end-stage of kidney failure. As the kidney function is impaired for any reason, the creatinine level in the blood rises due to poor renal clearance of creatinine [4]. The major virulence factors of *E. coli* O157: H7, such as lipopolysaccharide (LPS), flagella, toxins, and adhesins, play a crucial role in reducing the dose of this bacterium. In *E. coli* O157: H7, the gene encoding a cluster of 12 genes encodes the antigen. Six genes are responsible for glucose-free biosynthesis, and the protein encoded by four other genes is also used to transport sugar. Two other genes also encode flippase and O antigen transporter proteins. The protein encoded by the *rfbE* gene is located in the group of proteins involved in sugar-free biosynthesis and plays a vital role in synthesizing bacterial LPS. As a biologically active and virulent part of endotoxin, LPS is one of the major and stimulating structures of the host immune system, which is located in the outer membrane of the cell wall and plays a significant role in the life and virulence of gram-negative bacteria by several mechanisms: (i) it is a barrier that is only permeable to low-weight hydrophilic molecules; (ii) it protects bacteria against the host's defence system, and (iii) it plays a vital role in pathogen-host interaction [10]. The *rfbE* gene encodes about 364 amino acids that produce the protein synthetase perosamine. This enzyme plays a vital role in forming O157 surface antigen and causes virulence of *E. coli* O157: H7 [11—14]. **This study aimed** to detect the *rfbE* gene in *E. coli* O157: H7 strains and investigate variations over time in its expression at different creatinine concentrations.

Materials and methods. Isolation and identification. Urine sampling of patients admitted to the Imam Khomeini Hospital in Boroujerd, Iran, was performed by a nurse. The samples were then transferred to the laboratory of the relevant hospital for culture. The primary culture was performed on eosin methylene blue (EMB) agar media. The samples were then transferred to the Microbiology Department of the Islamic Azad University, the Boroujerd branch, for identification.

To identify the strains, the heat-staining procedure, biochemical tests of catalase, oxidase, and motility were conducted. Then the bacteria identification was performed using a Microgen kit according to the manufacturer's instructions.

Antibiotic susceptibility testing. Antibiotic susceptibility of isolates was tested according to the standard Kirby-Bauer disk diffusion method based on the Clinical Laboratory Standards Institute (CLSI). Antibiotic discs used for this experiment were: ceftriaxone (5 μg), cefixime (30 μg), co-amoxiclav (30 μg), streptomycin (10 μg), gentamicin (10 μg), enrofloxacin (5 μg), neomycin (30 μg), tetracycline (30 μg), sulfamethoxazole (100 μg), and fluorophenicol (30 μg), prepared from UK Mast.

Polymerase chain reaction (PCR) to detect the rfbE gene. According to the kit instructions (Sinaclon, Iran), a DNA extraction kit was used to extract genomic DNA after culturing the identified *E. coli* O157:H7 isolates in BHI broth. Each PCR requires a final volume of 25 μL, containing 3 μL of DNA extracted as a template, 1 μL of forwarding primer, 1 μL of reverse primer, 12.5 μL of Master Mix (AMPLIQON), and 7.5 μL of double-distilled water (Table 1). Then, PCR was

performed for the 16S rRNA gene using Table 2 primers and for the *rfbE* gene using the forward (5' CGTGGTATAGCTACTGTCACC 3') and reversed (5' CGCTGAATGTCATTCGCTCTG 3') primers according to the temperature and schedule (Table 2), positive control; well #2: 16S rRNA gene. Electrophoresis of PCR products was performed by 1% agarose gel. The Gel Doc system then photographed the bands. A 100 bp marker prepared from GenedireX, a joint production of Taiwan and the USA, was used to identify PCR products. Distilled water and DNA of ATCC25922 standard bacteria were used as negative and positive controls, respectively.

Bacterial culture at different creatinine concentrations. EHEC (O157: H7) isolates containing the studied gene were tested at different creatinine concentrations. Isolates were cultured at concentrations of 1, 3, and 6 mg dL^{-1} in BHB broth and incubated for 24, 48, and 72 h, respectively.

RNA extraction. RNA extraction was performed using the Trizol extraction kit (Invitrogen Life Technologies) according to the instructions. The quantity and quality were evaluated by UV spectrophotometric methods, using light absorption at 260 nm and 280 nm and agarose gel electrophoresis for the presence of the S16 and S23 bands.

The cDNA synthesis and real-time PCR. According to the manufacturer's instructions, RNAs extracted from the kit (Sinaclon, Iran) were used for cDNA synthesis. To that end, RNA, a random hexamer, and RNase-free water were mixed and kept at 70° C for 5 min. The enzyme and the other reaction components were then added to the mixture and placed in a Bio-Rad thermocycler (USA) at 42° C for 60 min (for the reaction) and at 70° C for 10 min (for the RT enzyme inactivation). Following cDNA synthesis, the real-time PCR was performed in the volume of 12 μL consisting of 6.25 μL of Master Mix containing SYBR® Green, 1.5 μL of cDNA, 0.5 μL of specific primers for each gene, and 3.75 μL of nuclease-free water in

Table 1. **List of materials used for PCR of *rfbE* gene**

Material Mix PCR:	Amount of material for a sample, μL
Master mix	12/5
Forward Primer	1
Reverse Primer	1
DNA template	3
Distilled water	7/5
Total volume	25

Table 2. **Temperature and time conditions of PCR for *rfbE* gene**

Gene *rfb*E	Primary denaturation	Secondary denaturation	Annealing	Polymerization	Final polymerization	Cycles
Temperature, (°C)	94	94	50	72	72	35
Time	5 min	45 sec	30 sec	90 sec	10 min	

Table 3. **Specifications of primers used in this study**

Gene	Primer sequence	Length (bp)	Reference
16S rRNA	F: CATTGACGTTACCCGCAGAA R: CGCTTTACGCCCAGTAATTCC	100	(16)
rfbE	F: CGGACATCCATGTGATATGG R: TTGCCTATGTACAGCTAATCC	259	(17)

an ABI Step One device (USA) according to the program as a single-step of initial denaturation at 95°C for 30 s and 45 cycles of amplification reaction including denaturation at 95 °C for 15 s and binding of primers at 50 °C for 30 s and the temperature conditions of the melting curve formation stage including 95 °C for 15 s, 50 °C for 30 s and 95 °C for 15 s on the *rfbE* gene. The sequence of studied primers is listed in Table 3. The16S rRNA gene was used as a housekeeping gene. All experiments were performed in at least three replicates, and then the obtained data were processed by REST software.

Statistical analysis. T-test was used to analyze the results of Real-Time PCR using SPSS-23 software to compare the differences between the two groups, and $p < 0.05$ was considered significant.

Results. Isolate colonization. Twenty isolates of *E. coli* O157: H7 were identified after removing duplicate and clinically unrelated isolates from the Imam Khomeini Hospital in Boroujerd. The studied samples contained 16S rRNA and *rfbE* genes specific to this species (Figs. 1 and 2).

Antibiotic susceptibility. *E. coli* O157: H7 isolates exhibited resistance to most antibiotics under study. The lowest resistance was observed to cefixime, co-amoxiclav, and enrofloxacin, and the highest resistance was to sulfamethoxazole, tetracycline, and neomycin (Fig. 3).

RNA extraction. The quantity and quality of the extracted RNAs were evaluated by spectrophotometry and horizontal electrophoresis. The presence of the 16S and 23S bands in rRNA indicates that the RNA is safe, and the absence of additional bands indicates its purity (Fig. 4). Moreover, the absorption ratio of the extracted samples at A260 to A280 wavelength was within 1.8—1.9, which shows the appropriate and desirable quality of the extracted RNAs.

Variations in rfbE gene expression under 1 mg dL^{-1} creatinine at different time points. Analysis of the *rfbE* gene expression variations at different time points under 1 mg dL^{-1} creatinine showed that the *rfbE* gene expression decreased compared to the housekeeping gene 16S rRNA at 24, 48, and 72 h after creatinine treatment, with a significant decrease at 24 and 48 h ($p<0.05$) (Fig. 5).

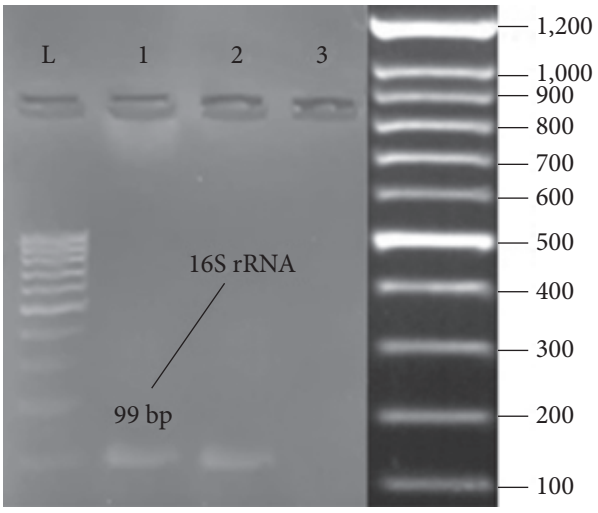


Fig. 1. Electrophoresis results of PCR products for 16S rRNA gene, Ladder 50 bp: L
Well #1: Positive control; well #2: 16S rRNA gene with 99 bp; and well #3: Negative control (distilled water)

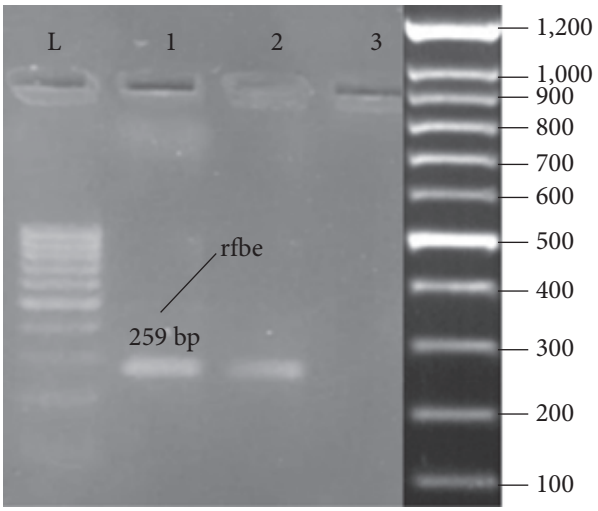


Fig. 2. Electrophoresis results of PCR products for *rfbE* gene, Ladder 100 bp: L
Well #1: Positive control; well #2: *rfbE* gene with 259 bp; and well #3: Negative control (distilled water)

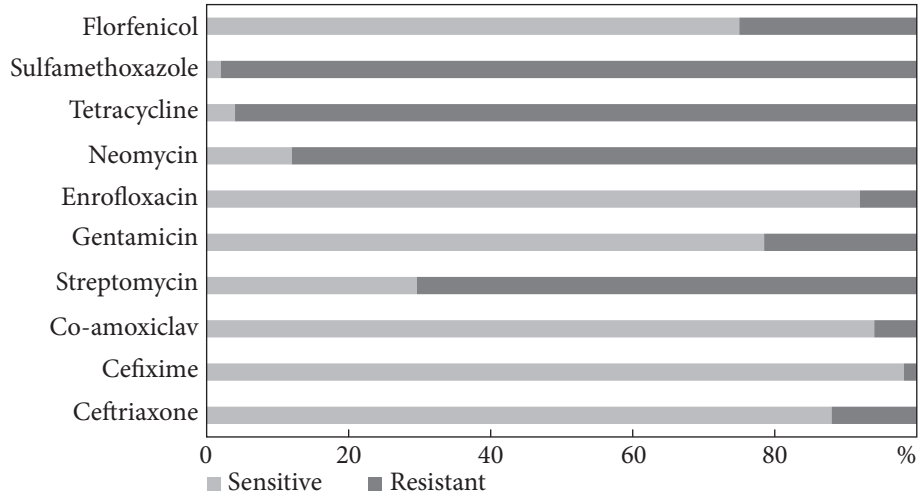
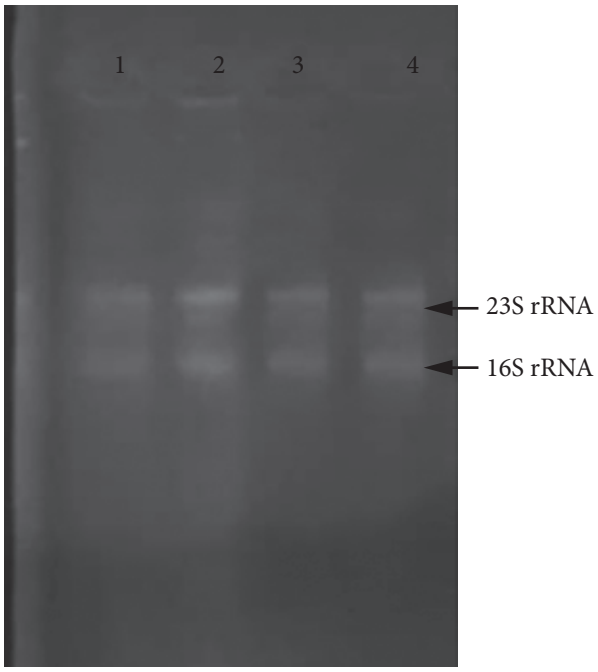


Fig. 3. Resistance pattern of *E. coli* O157:H7 isolates against different antibiotics



◄ ***Fig. 4.*** Quality of RNAs extracted from samples treated with 0, 1, 3, and 6 mg dL^{-1} creatinine concentrations in columns 1—4, respectively

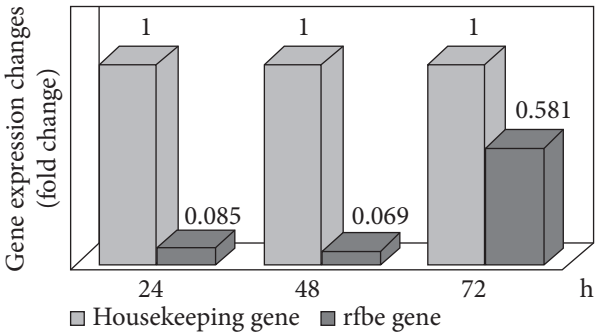


Fig. 5. Variations in relative *rfbE* gene expression under creatinine concentration of 1 mg dL^{-1} at 24, 48, and 72 h, showing its significant decrease at 24 and 48 h

Variations in rfbE gene expression under 3 mg dL^{-1} creatinine at different time points. Analysis of *rfbE* gene expression variations under 3 mg dL^{-1} creatinine at different time points showed that *rfbE* gene expression did not alter significantly compared to the housekeeping gene (16S rRNA) at 48 and 72 h after creatinine treatment. Still, the *rfbE* gene expression increased particularly compared to the housekeeping gene (16S rRNA) within 24 h after creatinine treatment ($p < 0.05$) (Fig. 6).

Variations in rfbE gene expression in the presence of 6 mg dL^{-1} creatinine at different time points. Analysis of variations in the rfbE gene expression at different time points showed that at 24 and 48 h after 6 mg dL^{-1} creatinine

treatment, the rfbE gene expression did not alter significantly compared to the housekeeping gene (16S rRNA). Still, the rfbE gene expression increased significantly at 72 h compared to the housekeeping gene (16S rRNA) ($p < 0.05$) (Fig. 7).

Discussion. Since the discovery of bacteria, humans have been searching for an effective medicine to treat infections caused by them. Treatment of infections caused by various microorganisms with the emergence of antibiotic resistance today faces many challenges and difficulties.

E. coli is one of the most frequent causes of several common bacterial infections in humans. Multi-drug resistance of this bacterium is critical in hospitalized patients. This bacterium is one of the most essential causes of the urinary tract and nosocomial infections, such as sepsis, wound infections, gastroenteritis, and neonatal meningitis. Also, *E. coli* is known to be one of the opportunistic nosocomial pathogens that has become resistant to β-lactam antibiotics due to the acquisition of plasmid-encoded broad-spectrum β-lactamases. Consequently, the treatment of infections caused by *E. coli* has become difficult. We are today witnessing the global expansion of the resistance to this bacterium, particularly in developing countries. According to the results of the study of the antibiotic resistance of *E. coli* O157:H7 isolates, the highest resistance was observed against sulfamethoxazole (98%), tetracycline (96%), neomycin (88%), streptomycin (69%), and cefixime (65%). In comparison, the lowest levels of resistance were revealed with co-amoxiclav (6%), enrofloxacin (8%), ceftriaxone (12%), gentamicin (20%), and florfenicol (25%). Furthermore, the resistance of this bacterium is increasing day by day, and the best options for the treatment of infection caused by this bacterium are co-amoxiclav and enrofloxacin. Sanchez et al. (2011) in the USA evaluated the antibiotic resistance of *E. coli* from 2002 to 2010 and found that the resistance rate to ciprofloxacin increased from 3% to 17%, while that to cotrimoxazole increased from 17% to 24%. The highest resistance to cefotaxime and ceftazidime and the lowest resistance to gentamicin were reported in a study by Kermanshah in 2011, whose results are consistent with our research findings showing the increasing resistance of this pathogen.

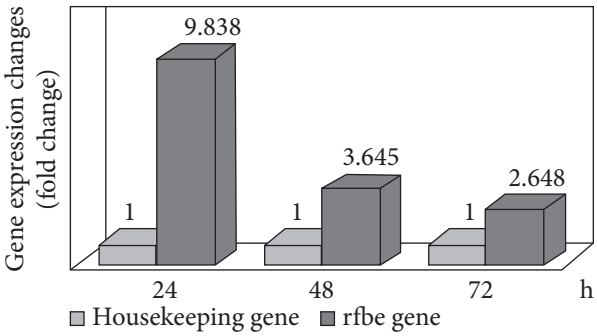


Fig. 6. Variations in *rfbE* gene expression under 3 mg dL^{-1} creatinine at 24, 48, and 72 h

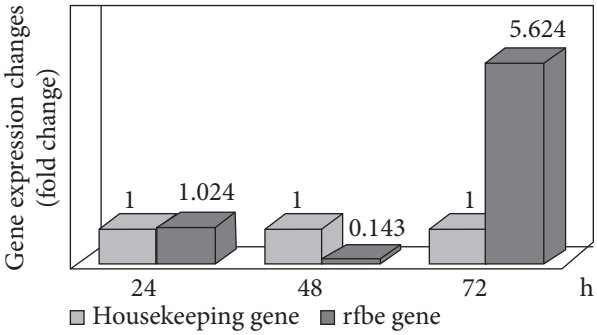


Fig. 7. Variations in the *rfbE* gene expression under 6 mg dL^{-1} creatinine at 24, 48, and 72 h

Since *E. coli* plays a major role in gastrointestinal infections, it is vital to study the virulence factors assisting bacteria in invading the host. To that end, the effect of different concentrations of creatinine was investigated in this study as a simulated model for patients with renal failure to assess for the first time one of the most important genes in bacterial virulence. The *rfbE* gene was further studied because all isolates that express this antigen have severe clinical symptoms. Verotoxigenic *E. coli* (VTEC) or Shigatoxin producing *E. coli* (STEC) isolates are among

the most important pathogenic *E. coli* isolates that cause diarrhea and hemorrhagic colitis in humans. Hemorrhagic colitis occasionally progresses to hemolytic uremic syndrome (HUS), a significant cause of acute renal failure in children and adults. In the present study, the effect of creatinine concentrations of 1, 3, and 6 mg dL^{-1} on the *rfbE* gene expression in *E. coli* O157:H7 has been investigated. A survey of the *rfbE* gene expression variations at different time points under 1 mg dL^{-1} creatinine showed that the *rfbE* gene expression decreased compared to the housekeeping gene (16S rRNA) at 24, 48, and 72 h after creatinine treatment, a significant decrease was observed at 24 and 48 h ($p < 0.05$). In the study of variations in the *rfbE* gene expression at different time points at a creatinine concentration of 3 mg dL^{-1}, there was no significant variation in the *rfbE* gene expression at 24 and 48 h after creatinine treatment compared to the housekeeping gene (16S rRNA). Still, at 72 h, the *rfbE* gene expression increased significantly compared to the housekeeping gene (16S rRNA) ($p < 0.05$).

Moreover, variations in the *rfbE* gene expression at different time points at a concentration of 6 mg dL^{-1} showed that at 24 and 48 h after creatinine treatment, the *rfbE* gene expression did not change significantly compared to the housekeeping gene (16S rRNA). Still, at 72 h, the *rfbE* gene expression significantly increased ($p<0.05$). The present study showed for the first time that the *rfbE* gene expression is different at different creatinine concentrations and different time points. Further studies are required to evaluate the expression of this gene at different creatinine concentrations and determine its role and function.

Bakhshi et al. [27] argued that the protein-encoding *rfbE* gene plays an essential role in the group of proteins involved in glucose-free biosynthesis and bacterial LPS synthesis. It is likely that the *rfbE* gene is involved in activating antibodies associated with *E. coli* antigen diseases and increases bacterial activity in the presence of sugar and LPS [29]. Since most of the specific enzymes involved in the biosynthesis of O polysaccharides are encoded by genes collected in the *rfb* region (O antigens), located in region 42 in *E. coli* and *Salmonella enterisa*, these enzymes complement biosynthesis and transport nucleotide sugar precursors to a lipid carrier to complement O duplicated oligosaccharide units [30]. The other components of the *rfb* gene decode the product present in the specific polymerization and O polysaccharide assembly [31]. As shown in literature, *rfbEcO7* is a gene involved in antigen biosynthesis, and it is more conserved in various intestinal and non-intestinal bacteria than other similar genes [32].

According to the knowledge obtained on the *rfb* gene function and its main activity as O antigens in *E. coli* through this study, creatinine, as a nutrient with a high level of nitrogen, can enhance the activities of O antigen and *rfbE* gene effectively. Its desired level can increase the expression of the *rfbE* gene, which requires further investigation with the direct presence and effect of the nitrogen extracted from creatinine.

The study by Stevenson et al. [33] showed that O antigen is removed during in vitro culture that effectively reduces the expression of *rfb* cluster genes [33]. Still, in the present study, the expression of *rfbE* gene was enhanced as one of the 11 *rfb* genes, which indicates the importance of creatinine amplification compared to the genes encoding O polysaccharide in this bacterium. The temperature changes lead to the expression of the *rfbE* gene. Therefore, the O-region chain is produced *in two degrees 25 and 37*, as shown in the study [34], which indicates that the temperature factor at the culture time can also affect the *rfbE* gene expression.

In the study by Feng et al. [35], the effect of anti-O157 kits was analyzed, and the results showed that the presence of *rfbE* is required for the expression of O157 and leads to amplification of virulence factors of O157:H7. By [36], *rfbE* is the most critical diagnostic factor to identify E. coli O157:H7.

Contrary to the results of the present study, it was reported by McDonald et al. [37] that creatinine phosphate or CRN-HCI reduced single- and multiple-drug resistance of *E. coli* and *Staphylococcus aureus* and prevented bacterial growth in media as well as had a potential to prevent the progression of skin infections. Still, no research has been found to investigate the direct effect of creatinine on the expression of the *rfbE* gene and other O antigens. On the other hand, in the study of Teal et al. [38], inconsistent with the results of the present study, it was observed that the blood creatinine level in patients with renal failure containing Shigatoxin producing *E. coli* was very high, which could be a symptom of Shigatoxin having *E. coli* infection.

In addition to the *rfbE* gene, EHEC contains the *STX*, *eae*, *H7*, *wzx*, and *wbd1* genes, each of which is involved in producing factors affecting bacterial virulence. The combination of these factors can affect the kidney function, cause HUS, and increase blood creatinine levels. The secondary increase in creatinine by affecting the *rfbE* gene expression producing the O antigen or bacterial endotoxin boosts the coagulation system's activity by activating coagulation factor XII and triggering canine and complement systems exacerbate kidney disease and failure.

Conclusions. The results of this study indicate a significant effect of concentration of3 mg dL^{-1} creatinine within the first 24 h on the gene expression and adverse effects on kidneys, which was not like this at higher concentrations and more extended periods. Therefore, controlling the gene expression within the first 24 h may be necessary for reducing kidney damage. On the other hand,, the increased creatinine concentration and extended period cannot have the initial effect (the first 24 h) on the gene expression. Probably, the most damage caused by the gene expression occurs within the first 24 h.

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Received 26.01.2022

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МОЛЕКУЛЯРНА ОЦІНКА ЗМІНИ ЕКСПРЕСІЇ ГЕНА *rfbE* ПІД ВПЛИВОМ РІЗНИХ КОНЦЕНТРАЦІЙ КРЕАТИНІНУ У ШТАМАХ *ESCHERICHIA COLI* ЗА ДОПОМОГОЮ ПЛР У РЕАЛЬНОМУ ЧАСІ

Escherichia coli (*E. coli*) O157: H7 як кишково-геморагічний збудник викликає важкі ураження шлунково-кишкового тракту та небезпечні захворювання у людей, такі як гемолітико-уремічний синдром (HUS) і гостра ниркова недостатність, яка пов'язана з підвищенням рівня креатиніну в крові. **Мета роботи** оцінити резистентність *E. coli* O157: H7 до антибіотиків, щоб виявити ген вірулентності *rfbE* та вивчити варіації в експресії гена. **Методи.** Ізоляти спочатку висівали на еозиновий метиленовий синій (EMB) агар, а потім ідентифікували за допомогою набору Microgen і наявності гена *rfbE.* Чутливість ідентифікованих штамів до антибіотиків перевіряли методом дискової дифузії з подальшим посівом штамів *E. coli* O157: H7 в концентраціях 1, 3 та 6 мг дл$^{-1}$ у бульйон BHI (бульйон із серцево-мозковим екстрактом). Потім ДНК і РНК екстрагували з бактерій у вище зазначених концентраціях, а кДНК готували з очищеної РНК. Потім експресію гена *rfbE* оцінювали за допомогою ПЛР у реальному часі, а дані аналізували за допомогою програмного забезпечення Rest. **Результати.** В результаті проведених досліджень виявлено високу резистентність виділених штамів проти деяких досліджуваних антибіотиків, а варіації експресії гена *rfbE* виявилися різними при різних концентраціях креатиніну в інші моменти часу. Значне зниження варіацій експресії гена *rfbE* спостерігалося при низьких концентраціях (1 мг дл$^{-1}$), але, навпаки, значне збільшення варіацій експресії гена *rfbE* було виявлено при більш високих концентраціях (3 і 6 мг дл$^{-1}$) ($p<0,05$). **Висновки.** Ген *rfbE* є одним з факторів, що впливають на вірулентність бактерій. Ми вважаємо, що вторинне підвищення рівня креатиніну з будь-якої причини може загострити захворювання нирок і недостатність, впливаючи на експресію гена *rfbE* при продукуванні антигену О або бактеріального ендотоксину.

Ключові слова: *ЕНЕС (O157: H7), rfbE, креатинін, ПЛР в реальному часі.*