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Prevalence and Gene Expression Variation of Pathogenic Hemolysin Producing *E. coli* Isolated From Patients with Urinary Tract Infections and Diarrhea

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ABSTRACT

The *hlyA* gene in *Escherichia coli* (*E. coli*) bacteria codes for the protein hemolysin, which is a toxin produced by these bacteria and contributes to their ability to cause disease. Hemolysin is part of the bacteria's mechanism to increase their harmful effect by lysis red blood cells releasing hemoglobin. This toxin creates holes in the host cell membrane, leading to leakage of cell components and eventual death. The main objective of the current study is to investigate the presence and gene expression of hemolysin toxin genes in *E. coli* isolates from clinical infection cases, including samples from urinary tract infection and diarrhea from patients in Tikrit city, using real-time polymerase chain reaction (RT-PCR) technique. The study was carried out on 50 isolates, divided into 20 isolates from urinary tract infections, 20 isolates from diarrhea cases and 10 isolates as control samples (stool samples from healthy people without diarrhea). The overall prevalence of hemolysin in *E. coli* isolates was 18 out of 40 (45 %). The occurrence of hemolysin-producing *E. coli* isolates detected by RT-PCR was 10 out of 20 (50 %) in urinary tract infection samples and 8 out of 20 (40 %) in diarrhea samples. The production of the hemolysin toxin by *E. coli* isolates varies depending on the type of sample. The results also showed that the gene expression level of hemolysin gene was higher in cases of urinary tract infections compared to cases of diarrhea, with the gene expression level being (Mean folding = 5.933) in urinary tract infection cases, while it was (Mean folding = 3.712) in diarrhea cases, with significant differences at a probability level of (P value ≤ 0.05). The results showed that the prevalence of the gene responsible for the secretion of hemolysin toxins by *E. coli* is higher in cases of urinary tract infection (UTIs) than in cases of diarrhea. and the gene expression level of the *hlyA* gene showed a significant increase in cases of urinary tract infections compared to cases of diarrhea.

Keywords: Gene expression, Hemolysin, *E. coli*, Real time PCR

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مدى انتشار وتباين التعبير الجيني للإشريكية القولونية الممرضة المنتجة لسموم الهيمولايسين والمعزولة من المرضى المصابين بالتهابات المسالك البولية والإسهال

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الملخص

يقوم جين *hlyA* الموجود في بكتيريا *E. coli* بتشفير بروتين الهيمولايسين، وهو مادة سامة تنتجها هذه البكتيريا ويساهم في قدرتها على التسبب في المرض. الهيمولايسين هو جزء من آلية البكتيريا لزيادة تأثيرها الضار عن طريق تحليل خلايا الدم الحمراء وإطلاق الهيموجلوبين. يُحدث هذا السم ثقوبًا في غشاء الخلية المضيفة، مما يؤدي إلى تسرب مكونات الخلية والموت في نهاية المطاف. هدفت الدراسة الحالية الى الكشف عن مدى الانتشار والتعبير الجيني لسم الهيمولايسين في عزلات بكتيريا الإشريكية القولونية (*E. coli*) من حالات العدوى السريرية المتمثلة عينات التهاب المسالك البولية وعينات الإسهال من المرضى في مدينة تكريت، باستخدام تقنية تفاعل البلمرة المتسلسل اللحظي (Real time PCR). عزلت في الدراسة 50 عزلة *E.coli*، 20 عزلة من التهابات المسالك البولية، و20 عزلة من حالات الإسهال، و10 عينات كمجموعة سيطرة (عينات براز من اشخاص اصحاء غير مصابين بالاسهال). واظهرت النتائج ان نسبة انتشار الهيموليسين في عزلات الإشريكية القولونية الممرضة 18 من أصل 40 (45 %). وكانت نسبة وجود عزلات الإشريكية القولونية المنتجة للهيموليسين التي تم الكشف عنها بواسطة تقنية PCR في الوقت الحقيقي هي 10 من أصل 20 (50 %) في عينات التهاب المسالك البولية و8 من أصل 20 (40 %) في عينات الإسهال. كما اظهرت النتائج ان معدل التعبير الجيني لجين الهيمولايسين كان اعلى في حالة الاصابة بألتهابات المسالك البولية مما عليه في حالة الاصابة بالاسهال, حيث بلغ معدل التعبير الجيني (5.933) في حالات التهاب المسالك البولية بينما كانت (3.712) في حالات الاسهال مع وجود فروقات معنوية عند مستوى احتمالية ($P\ value \leq 0.05$). أظهرت النتائج أن نسبة انتشار الجين المسؤول عن إفراز سموم الهيمولايسن بواسطة الإشريكية القولونية أعلى في حالات التهابات المسالك البولية مقارنة بحالات الإسهال. وأظهر مستوى التعبير الجيني للجين hlyA ارتفاعاً معنويا في حالات التهابات المسالك البولية مقارنة بحالات الإسهال.

INTRODUCTION

Urinary tract infection (UTI) is defined as the presence of microorganisms in urine [(1)]. The detection of microbes within the bladder signifies an infection, given that the bladder is inherently a sterile organ [(2)]. UTIs are among the most common types of community-acquired and nosocomial infections, with a significant proportion of the population and individuals of all ages affected annually, host factors such as patients age and gender may influence the prevalence of the infection [(3, 4)]. The infection can occur in any part of the urinary system and may be present with or without symptoms [(5)]. Considerably, UTIs are among the most prevalent types of infections in the body, where women are highly susceptible to this type of infections in comparison with men due to their shorter urethra, which allows bacteria to reach the bladder more swiftly. Additionally, female urethra is open and located near the anus and vagina [(6)]. Generally, *E. coli* are the primary cause of UTIs, traveling from the urinary tract to the bladder, causing cystitis, and then to the kidneys and ureters, leading to pyelonephritis [(7)]. Under health condition, urine is normally sterile and germ-free when it exits

the kidneys, however, it becomes contaminated with bacteria from the skin as it passes through the urethra, allowing bacteria to accumulate. Several factors increase the risk of UTIs, including pregnancy, diabetes, frequent sexual intercourse, prostate enlargement, repeated use of antibiotics, and the use of urinary catheters[8]. When bacteria enter the bladder and proliferate in the urine they triggrerd UTIs, the most common being cystitis, which is characterized by an inability to urinate or frequent, painful urination in the pelvic area. The severity of UTIs depends mainly on bacterial virulence factors and host susceptibility. Microscopic examination and laboratory culture of urine are essential diagnostic tests for detection of UTIs [9]. On the other hands, diarrhea is one of the most prevalent health issues globally, particularly in developing countries. It primarily affects children, especially infants, due to conditions associated with poverty, lack of health education, malnutrition, and the unavailability of safe drinking water. Diarrhea is a leading cause of child mortality, particularly among young children [10]. The word "Diarrhea" originates from the Greek words "Dia" meaning "through" and "Rhein" meaning "flow". It is a common medical condition characterized by increased frequency of bowel movements and increased stool liquidity [11, 12]. If diarrhea lasts less than 14 days, it is termed "acute diarrhea". If it persists or exceeds this duration, it is considered "persistent diarrhea", and if it lasts more than 30 days, it is referred to as "chronic diarrhea". Notably, diarrhea claims the lives of nearly 2 million children under the age of five each year. The illness can last for several days, leading to the deprivation of water and salts that the body needs to survive, with most deaths resulting from severe dehydration. Malnourished individuals, those with weakened immune systems, or those infected with the human immunodeficiency virus (HIV) are at higher risk of dying from diarrhea [13]. hemolysin gene is considered as one of the most significant virulence factors for *E. coli*. There are two types of this toxin: α-hemolysin and β-hemolysin. The latter are cytotoxins that create pores in the host cell membrane and are encoded by about (40-50 %) of Uropathogenic *E. coli* (UPEC) isolates. It has been shown that the expression of α-hemolysin increases the severity of clinical symptoms of UTIs. The activity of hemolysin is not limited against red blood cells; α-hemolysin in *E. coli* lyses lymphocytes, while β-hemolysin inhibits phagocytosis [14]. The high incidence of hemolysin-producing strains isolated from urine may indicate their importance as invasive strains [15]. Some *E. coli* isolates carry *hlyA* gene, which encodes the hemolysin toxin that secreated extracellularly from bacteria. *E. coli* produce hemolysin toxins in varying quantities, leading to different levels of effect on tissues. Additionally, the expression of *hlyA* gene differs depending on the type of isolate and other factors such as various environmental factors that trigger an increase or a reduction in the production of hemolysin. Temperature, pH, and osmolarity determine the rates and times of fluctuations in hemolysin synthesis [16, 17]. An examination of the expression of the hemolysin toxin gene in *E. coli* isolates collected from infection of the urinary tract and diarrhea cases is the main objective of this work.

MATERIALS AND METHODS

Fifteen *E. coli* isolates were obtained from different sources in Tikrit Teaching Hospital from August 2023 to December 2023, where 20 isolates were isolated from UTIs cases, 20 isolates from diarrhea cases and 10 isolates as control sample were obtained. The diagnosis of the bacterial isolates was confirmed by examining their diagnostic characteristics on blood agar (for identification and as a primary detection of hemolysine producer isolate), MacConkey agar and Eosine methylene blue agar, as well as through biochemical tests like Indole, Methyl red, Voges – Proskauer and Citrate utilization (IMViC) and Triple sugar iron agar (TSI) [18].

Total RNA Extraction

A total of 50 *E.coli* ribonucleic acid (RNA) samples were extracted according to the manufacturer's instructions (TransZol Up Plus RNA Kit, TransGen Biotech/China). the samples was then stored in a refrigerator at (-20°C) until use [19].

cDNA synthesis

This was performed using (5×RT PCR MasterMix, Tynzyme.co., China) [20] which contains all the reaction components (the thermo-stable M-MuLV Reverse Transcriptase, Ribonuclease (RNase) Inhibitor, Random primers, Oligo dT Primer, dNTP Mixture, Buffer). The procedure was carried out according to the manufacturer's instructions. the reaction volumes and the components used for reverse transcription as follows; Total RNA/mRNA (10 µl)\5×RT PCR MasterMix (4 µl)\ Nuclease free water (6 µl). Transforming RNA into complementary deoxyribonucleic acid (cDNA) was performed using a thermal cycling apparatus, which is illustrated within the following steps; step 1(25 °C for 10 minutes), step 2(42 °C for 15 minutes), step 3 (85 °C for 5 seconds). Subsequently, after complete the cDNA synthesis, all tubes were placed in a RT-PCR program for gene expression analysis.

The quantitative real-time PCR (qPCR) technique

Utilizing the 2-ΔΔCT Livak method, qPCR was performed to identify and measure the comparative expression of gene hemolysin mRNA Transcripting from *E. coli* isolates. A quantitative polymerase chain reaction (qPCR) was performed on a Real-Time PCR equipment from Applied Biosystem, USA. The SYBR green dye qPCR master mixture was used to identify and amplify target genes. The Recombinase A (recA) that code for the housekeeping gene was utilized to normalize the expression of gene. Table 1 presents the designed primers.

Table 1: Primers of RT-qPCR together along with their sequences

	Gene	Amplicon size	Primer	Sequence	Reference
1	*recA* Hosuskeeping	169 bp	F	GAAATCGGCGACTCTCACAT	this study
			R	CGTTACCACCGGTAGTGGTT	
2	*hlyA*	167bp	F	ACGATGTGGTTTATTCTGGA	this study
			R	CTTCACGTCACCATACATAT	

The qPCR master mix for the *hlyA* targeted gene with housekeeping gene *recA* was prepared following the instructions provided by the (Tinzyme Co., Limited ™ 2×Ultra Sybr qPCR Mix (Low Rox),China) [20], as detailed in Table 2.

Table 2: qPCR master mix preparation

Reagent Master Mix	Volume (µl)	Concentration
Forward Primer	0.5	100 pmol
×2Ultra Sybr qPCR Mix (Low Rox)	10	X1
PCR grade water	7	-
Reverse Primer	0.5	100 pmol
cDNA	2	-
Final volum	20	-

(Tinzyme Co., Limited ™ 2×Ultra Sybr qPCR Mix (Low Rox)

The components of master mix reaction qPCR were transferred in PCR Eppendorf tubes, then were vortexed for three minutes using an Exispin vortex centrifuge. The tubes were then placed in the MiniOpticon Real-Time PCR System (Applied

Biosystem, USA) under the thermos cycler conditions showed in Table 3.

Table 3: Real-Time PCR thermoscycler conditions

The Repeat cycle	The Time	The Temperature	qPCR step
1	10 min	95 ℃	Pre-denaturation
40	15 s	95 ℃	Denaturation
	1 min	60 ℃	Annealing/Extension
1	15 s	95 ℃	Melting curve analysis
	1 min	60 ℃	
	15 s	95 ℃	
	15 s	60 ℃	

(Tinzyme Co., Limited ™ 2×Ultra Sybr qPCR Mix (Low Rox)

Gene expression analysis

The gene expression value was measured using the Livak method [21] with the following equation:

$\Delta CT(target) = CT_{(target\ gene)} - CT_{(reference\ gene)}$

$\Delta CT(control) = CT_{(control\ gene)} - CT_{(reference\ gene)}$

$\Delta\Delta CT = \Delta CT(target) - \Delta CT(control)$

Fold change = $2^{-\Delta\Delta CT}$

Statistical Analysis

The T-test for two different samples, SPSS, and GraphPad Prism 9 software were used for statistical analysis at a probability level of (P value ≤ 0.05).

RESULTS

Detection of hemolysin gene using Real-time PCR

As shown in Table 4, the results revealed that only 18 *E. coli* strains were capable of producing hemolysin:

Table 4: Number and percentage of hemolysin production isolates

The source of isolates	Total number of analysed isolates	Analysis of positively isolates with perception %
Urinary tract infection	20	10/20 (50 %)
Diarrhea	20	8/20 (40 %)

Relative gene expression analysis

Table 5 and Table 6 show the levels and average gene expression of the HlyA gene in cases of urinary tract infections and diarrhea

Table 5: Relatively expression of genes analysis for *E. coli* isolates producing gene of hemolysin by utilizing the Livak technique.

Source of isolates	CT (*hlyA*)	CT (*recA*)	ΔCT	Expression for folding Change
UTIs	28.38588	12.30142	16.084459	4.054547
UTIs	29.99131	13.93607	16.055246	4.137485
UTIs	27.66153	12.38249	15.27904	7.085943
UTIs	25.441	9.638002	15.802998	4.928
UTIs	27.41491	11.45567	15.95924	4.422187
UTIs	30.66298	14.83401	15.82897	4.840077
UTIs	30.01022	14.85871	15.151501	7.740887
UTIs	28.66094	13.32844	15.332498	6.828184
UTIs	25.22449	10.18046	15.044036	8.339519
UTIs	29.55656	14.25206	15.304496	6.962011
Diarrhea	30.86153	13.72	17.141531	1.948642
Diarrhea	28.551	12.58172	15.9692797	4.39152
Diarrhea	28.41491	12.03257	16.3823462	3.298143
Diarrhea	29.88298	14.23251	15.6504733	5.477535
Diarrhea	28.10022	12.17912	15.9210932	4.540675
Diarrhea	35.99094	11.50263	16.1943155	3.757269
Diarrhea	30.38449	13.93806	16.4464331	3.15484
Diarrhea	29.00156	12.54538	16.4561763	3.133606

Table 6: The expression level of the *hlyA* gene and the standard deviation in patients with (UTIs) and diarrhea

Gene	Case	Number of isolates	Folding($2^{-\Delta\Delta ct}$) Mean ± SD	P-Value
hlyA	UTIs	10	5.933 ± 1.615	0.004
	Diarrhea	8	3.712 ± 1.081	

(Probability Value of 0.05)

Figures 1 and 2 display the amplification curve and the melting curve of the hemolysin gene in *E.coli*, and Figure 3 illustrate the average gene expression and the standard deviation of the HlyA gene in both cases (UTIs and diarrhea).

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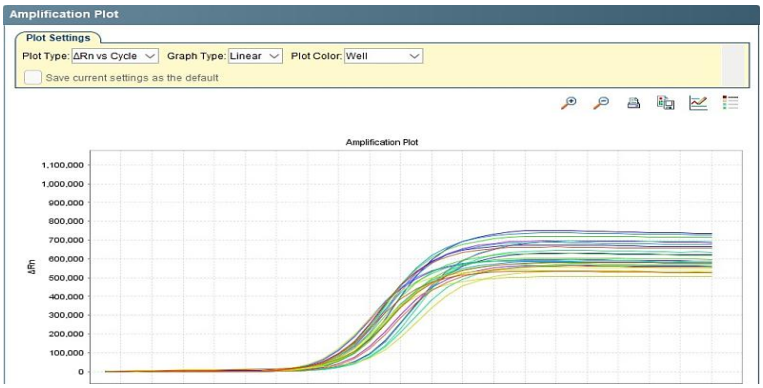

Fig. 1: Application of SYBER green in amplifying real-time of PCR plots for the identification of hemolysin gene generating by *E. coli* isolates.

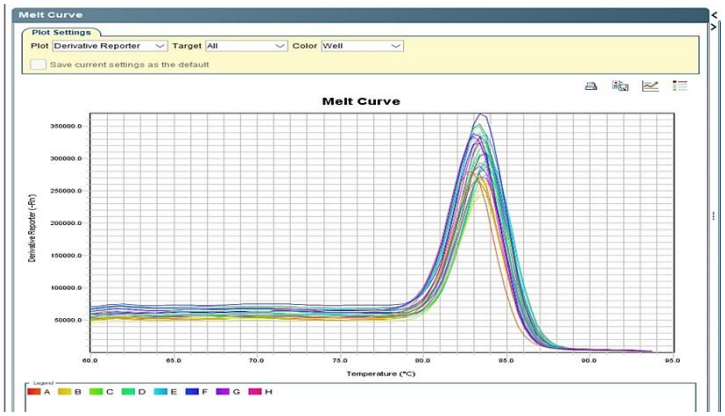

Fig. 2: Multing Curve of *hlyA* gene

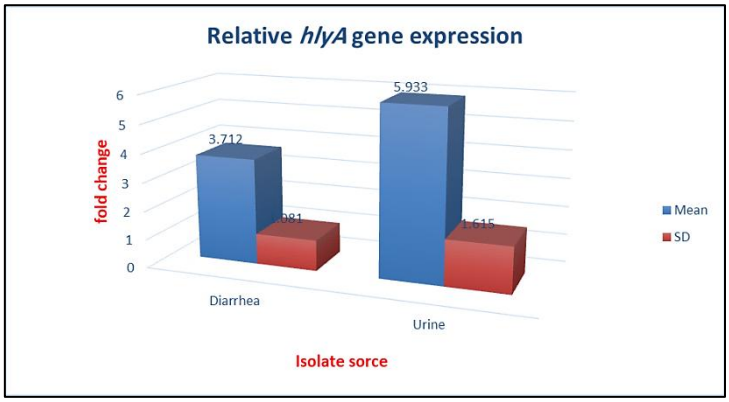

Fig. 3: Analysis the levels of *hlyA* mRNA gene expression. These values have been standardized to represent expression of *recA*m RNA. The bars represent the average and the variability (SD) of two separate controlled experiments, statistically significant at a significance level of P-Value < 0.05.

DISCUSSION

Factors related to virulence enable *E. coli* to establish itself on the mucosal surface of specific tissues and organs, such as the urinary tract and the intestines. These genetic elements (virulence factors) enhance the pathogenicity and immunological resistance of *E. coli's* capacity to generate multiple virulence factors greatly enhances its ability to cause disease [22, 23]. The findings of this study indicate that (45 %) of the hemolysin produced by isolates of *E. coli* was quantified utilizing real-time PCR, which closely aligns with the results of [24] that found the percentage was (45.3 %) and [25] which found it was (44 %), while

(Siegfried), [26-29] recorded that the percentage was (68.45 %), (68.5 %), (62.5 %) and (75%) respectively. Seasonal and geographical variables significantly influence the abundance of genes associated with virulence among isolates of *E. coli*. There is evidence to suggest that these genes exhibit higher frequencies throughout the summer season rather than winter along with other distinct seasons [30]. Isolates of *E. coli* carrying *hlyA* gene were detected using real-time PCR, showing the following percentages of occurrence: (50 %) (10/20) in UTIs cases and (40 %) (8/20) in diarrhea cases. These results was near to results of [31] that revealed about (45 %) of the isolates were capable of producing hemolysin toxin onto blood agar and [32] who found the percentages was (41.36 %). On the other hands, our results are disagreed with [33] and [34] who found that the percentage was (23.7 %), (21 %) and (0 %) respectively. While [35] recorded a high percentage of (93.7 %), which is higher than our study, furthermore, in Spain [36] recorded a rate of (56 %). According to our results, *E. coli* isolates producing hemolysin exhibited higher toxin production (greater activity and effectiveness) in UTIs samples, followed by diarrhea samples. This can be explained by the presence of a large amount of blood in urinary tract due to the destruction of blood vessels. In contrast diarrheic feces contain little or no blood. The presence of blood stimulates *E. coli* isolates to increase the expression levels of the *hlyA* gene, the higher blood levels leading to increased hemolysin secretion[37]. Several studies, including one conducted by [25] in Egypt, have demonstrated that the prevalence of this gene is higher in cases of UTIs compared to diarrhea cases. This study found that the *hlyA* gene was present in (44 %) of UTI cases, whereas it was found in only (10 %) of diarrhea cases. Many studies have confirmed that Uropathogenic *E. Coli* (UPEC) secrete high concentrations of alpha-hemolysin (*hlyA*), which integrates into the host cell membrane in a calcium-dependent manner, forming pores in umbrella cells and promoting their lysis. This facilitates bacterial access to iron and nutrients and stimulates exfoliation, exposing deeper layers of the urothelium for colonization and aiding in the spread of bacteria to adjacent cells. Additionally, the production of hemolysin toxins by *hlyA* gene is highly expressed in intracellular bacterial communities (IBCs). In UPEC, these intracellular bacterial communities play a significant role in certain infections, particularly UTIs, where hemolysin production in these communities is deemed essential at this stage of infection [38]. Other studies have also confirmed that hemolysin gene enhances invasion and infection in UTIs, with the presence of the *hlyA* gene in the genome of clinical isolates being associated with the severity of infection. Studies have shown that *hlyA* genes are present in (31- 48 %) of *E. coli* strains isolated from cases of uncomplicated UTIs, while their prevalence in isolates from pyelonephritis or urosepsis ranges from (50 to 78 %) [39]. Several factors exhibit a substantial impact on the expression of the hemolysin gene in E. coli isolates. Specific isolates demonstrate decreased synthesis of hemolysin when exposed to streptomycin, sodium cyanide, rifampin, and nalidixic acid, among other substances. Additionally, hemolysin gene expression reaches its maximum at a pH range of (7-8) [40]. Calcium is one of an essential ion for the enhanced production of hemolysin genes. Furthermore, the duration of colony expansion is another crucial determinant in augmenting hemolysin synthesis. Microbial culture in a controlled environment at (37 °C) during (16 hours), a temperature analogous to that of the human body, show increased hemolysin production. Oxygen, glucose, and Iron additives are crucial for promoting the regulating of the *hlyA* gene in isolates of *E. coli*. Hemolytic *E. coli* exhibits increased activity in a liquid medium containing minerals, salts, and hemoglobin. Energy metabolism inhibitors like sodium azide, potassium cyanide, and dinitrophenol inhibit the production and release of hemolysin toxin. likewise, neuroactive

pharmaceuticals and procaine reduce protein synthesis, have a comparable effect. Furthermore, certain drugs, such as nalidixic acid, rifampin, streptomycin, and chloramphenicol, affect gene expression in *E. coli* isolates. Every one of these elements are crucial in regulating the gene expression that produces hemolysin toxin in isolates of *E. coli*, explaining the variation in expression levels among different samples [27]. *hlyA* produced by UPEC causes hemolysis of human erythrocytes without the need for toxin interaction with membrane proteins, The high doses of *hlyA* can lyse cells without relying on a receptor, likely because they form pores that disrupt colloidal osmotic pressure. These pores, described as proteolipidic, have their size and stability influenced by the cell membrane composition. Moreover, factors like temperature, exposure time, and toxin concentration have been shown to affect the size of these pores in red blood cells (RBCs) [39]. Numerous studies have shown that hemolytic activity, mannose-resistant haemagglutination are more commonly found in UTIs isolates compared to other extra-intestinal and fecal isolates [41, 42].

CONCLUSION

The current study's results showed that the prevalence of the gene responsible for the secretion of hemolysin toxins by *E. coli* is higher in cases of UTIs than in cases of diarrhea. The study also demonstrated that the gene expression of *hlyA* was more pronounced in UTIs. *hlyA* gene expression increased in *E. coli* in response to the presence of blood in the surrounding environment. To summarize, the release of the toxin of hemolysin from *E. coli* is influenced by various both inside and outside environmental conditions that dictate the level of gene expression.

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Author contribution: Authors contributed equally in the study.

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