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Research Article

HlyA Gene in Hemolysin-Producing Uropathogenic and Enteric *Escherichia coli* Isolated from Iraqi Patients and the Effect of Gamma Rays on It

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About Article

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ABSTRACT

Escherichia coli is among the most common causes of bacterial infections. Haemolytic *E. coli* poses a significant threat to public health worldwide. This study aimed to identify haemolytic *E. coli* isolates, detect the *hlyA* gene, and evaluate the effects of gamma rays on it. A total of 400 urine and diarrhea samples were collected from patients with urinary and diarrheal diseases in Al-Diwaniya Province, Iraq, between November 2016 and April 2017. Haemolytic *E. coli* was confirmed using phenotypic and genotypic methods. The isolates were then exposed to mutagenic gamma rays for 10 and 15 minutes. The nucleotide and amino acid sequences of the *hlyA* gene were analyzed using the BLAST program and compared with the sequence of a standard isolate from the NCBI database. Out of 345 samples, 156 (45.2%) isolates tested positive for *E. coli*, including 80 (51.2%) from diarrhea cases and 76 (48.7%) from urine samples. The higher prevalence of diarrhea among males was statistically significant ($p < 0.01$), while the higher rate of urinary tract infections (UTIs) among females was also statistically significant ($p < 0.01$). Phenotypically, hemolysin enzyme production was observed in 40 *E. coli* isolates. Genotypically, the *hlyA* gene was detected in 15 isolates. DNA sequencing was used to determine the nucleotide and amino acid sequences of the hemolysin gene before and after exposure to gamma rays (60 cobalt). The results demonstrated substitution mutations, including both transitions and transversions, in the *hlyA* gene. According to the NCBI BLAST analysis, these mutations altered the protein translation by changing the amino acid asparagine to glutamine. This may reduce the expression level of the *hlyA* gene, potentially impacting the virulence of *E. coli*. These findings suggest that haemolytic-producing *E. coli* are widely circulating among populations affected by enteric and urinary tract infections in central Iraq.

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1. INTRODUCTION

Escherichia coli (*E. coli*) is a gram-negative bacillus and a natural part of the normal intestinal microbiota. However, some strains of *E. coli* can cause disease in humans, other mammals, and birds, ranging from intestinal infections like dysentery and diarrhea to extraintestinal infections such as urinary tract infections, respiratory tract infections, sepsis, and CNS infections like meningitis in humans. Pathogenic *E. coli* has a significant impact on public health, resulting in an annual economic cost of several billion dollars worldwide (Pokharel *et al.*, 2023). *E. coli* strains produce various virulence factors, including alpha-hemolysin (*HlyA*), which promotes invasion and infection. The presence of the *hlyA* gene in clinical isolates has been linked to the severity of disease (Sánchez-Magraner *et al.*, 2006). Hemolysin helps bacteria invade tissues and lyses red blood cells (RBCs), releasing hemoglobin. Hemolysins are pore-forming proteins produced by various bacteria, including *E. coli* (Chester & Kyung, 2000). *E. coli* hemolysins are classified into three types: α-hemolysin (*HlyA*), and hemolysin E (HlyE) (Burgos & Beutin, 2010). enterohemolysin (EhxA), EhxA and HlyE are found in enterohemorrhagic *E. coli* (EHEC). EhxA is highly similar to *HlyA*, which is a key virulence factor encoded on the chromosome and plasmids of pathogenic *E. coli* strains (Murase *et al.*, 2012). It belongs to the RTX family of proteins and is encoded on virulence plasmids of typical EHEC strains, such as O157:H7 (Welch, 2005). EhxA is a potential virulence factor that strongly correlates with severe diseases like hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) (Bielaszewska *et al.*, 2013). However, EhxA generally shows weaker hemolytic activity than *HlyA* (Beutin *et al.*, 1986). Ionizing radiation types such as gamma rays, X-rays, and higher-energy UV rays are commonly used to induce mutagenesis. Ionizing radiation excites and ionizes molecules by removing electrons, causing direct and indirect effects on bacterial DNA, which can lead to mutations. Excessive DNA damage results in cell death. An indirect effect involves reactive oxygen species—free radicals like hydroxyl (•OH) and superoxide anion (O2-•)—generated by water radiolysis, which can cause oxidative stress in bacterial cells and molecules (Hashemabad *et al.*, 2018). *E. coli* is an important model organism in scientific research due to its rapid growth, short generation time, and ease of cultivation, making it valuable for studying genetic and metabolic traits. Consequently, bacterial cells are used as biosensors to assess the effects of gamma radiation on *E. coli* in the human intestine, and how radiation impacts living organisms indirectly or directly through molecules like DNA and organelles such as the cytoplasmic membrane (Rohde, 2018). This study aimed to identify hemolytic *E. coli* causing UTIs and GIT infections, perform phenotypic detection of hemolysin enzyme on human and sheep blood agar, and evaluate its ability to lyse human blood groups. The study also aimed to assess the effect of gamma radiation on the potency of hemolysin, its ability to lyse human and sheep red blood cells, and its impact on the structure of the *hlyA* gene before and after exposure to gamma rays.

2. LITERATURE REVIEW

E. coli is a bacterium that exists as a beneficial symbiont in the digestive systems of humans and various other animals. It is therefore crucial in aiding digestion and synthesising specific vitamins. However, it can also cause bacterial infections, making it a significant public health concern. Furthermore, the emergence of drug-resistant strains of *E. coli* is a serious global issue (Naqid *et al.*, 2020). *E. coli* is a key pathogen that causes gastrointestinal and urinary tract infections. Diarrheagenic *E. coli* (DEC) and uropathogenic *E. coli* (UPEC) are two distinct pathotypes with specific clinical associations. This study, therefore, aimed to compare DEC and UPEC isolates in terms of their distribution, antimicrobial resistance, serotypes, resistance, and virulence gene profiles (Abdelkhalig *et al.*, 2025). This resistance enables the bacterium to survive and multiply even when treated with antibiotics that are generally effective against bacteria. The production of certain chemicals, including hemolysin, is considered an essential factor in the pathogenicity of *E. coli* (Schindel *et al.*, 2001). Hemolysins facilitate bacterial invasion and cause haemolysis of red blood cells (RBCs), resulting in the release of haemoglobin. Hemolysins are proteins known for their ability to form pores and are produced by various bacteria, including *E. coli* (Provoda & Lee, 2000). This process results in the release of haemoglobin, a vital protein involved in transporting oxygen in the blood. Several types of hemolysins have been identified in *E. coli*, including α-hemolysin (*HlyA*), hemolysin E (HlyE) and enterohemolysin (EhxA) (Murase *et al.*, 2012). These proteins help the bacteria to colonise and invade host tissues, causing significant damage to the host organism and making *E. coli* a key focus in the fight against bacterial infections. A subtype of the broader *E. coli* species, enterohemorrhagic *E. coli* (EHEC), is known to produce two specific hemolysins: EhxA and HlyE. Burgos and Beutin (Burgos & Beutin, 2010). A significant similarity was discovered between EhxA and *HlyA*. The latter is a major virulence factor present in the chromosomes and plasmids of pathogenic *E. coli* strains. EhxA is a potent virulence factor associated with severe illnesses such as haemolytic uraemic syndrome (HUS) and haemorrhagic colitis (HC) (Bielaszewska *et al.*, 2013). This highlights the importance of such factors in the development of life-threatening diseases. Interestingly, the *HlyA* secretion mechanism in uropathogenic *E. coli* — a strain that is known to cause urinary tract infections (UTIs) — was the first example of a type 1 secretion system (T1SS) to be identified (Kanonenberg *et al.*, 2018). The prognosis for patients with a UTI who also secrete alpha-hemolysin is generally poor (Caetano *et al.*, 2022). This is primarily due to the fact that uropathogenic *E. coli* (UPEC) strains are frequently linked to more severe infections. Common EHEC strains can be identified by their high levels of EhxA, which makes it a useful phenotypic marker (Caetano *et al.*, 2022). This extracellular hemolysin, produced by *E. coli*, is a notable virulence factor (Bielaszewska *et al.*, 2013).

Gamma rays can importantly affect the production of the hemolysin protein in *E. coli* bacteria, reducing or eliminating their ability to lyse human and sheep red blood cells. Mutagenesis using gamma rays has been shown to cause genetic alterations that can inhibit hemolysin production. Additionally, gamma rays can trigger a generalised stress response in haemolytic *E. coli* bacteria, affecting various metabolic pathways, including those involved in virulence and DNA repair. This can indirectly affect hemolysin activity.

3. METHODOLOGY

3.1. Ethical declaration

The present study was conducted in accordance with the 1975 Declaration of Helsinki, as revised in 2013. All procedures performed in this study adhered to national and international guidelines. Written informed consent was obtained from each patient before the study commenced, and the researchers anonymised all patient data. Ethical approval was not sought for this in vitro study. Samples were obtained using standard diagnostic and therapeutic protocols for managing gastrointestinal infections. All of the authors can confirm that this study complies with the Health Insurance Portability and Accountability Act (HIPAA) of 1996. The researchers followed all mandatory health and safety procedures.

3.2. Study Design and collection of samples

A total of 400 urine and diarrhea samples from patients were collected at AL-Diwaniya General Teaching Hospital to identify hemolytic *E. coli* isolates during the period from November 2016 to April 2017. The samples were gathered from patients of various age groups, ranging from under 10 years to over 52 years, including those suffering from UTIs and diarrhea. Specimens were collected in the morning using sterile, leak-proof containers, and it was confirmed that patients had not taken any medication for three days prior to sample collection. Additionally, information on the patients' gender and age was recorded.

3.3. Bacteriological assay

Each urine and diarrheal sample was cultured directly on MacConkey agar and Eosin Methylene Blue agar (EMB) (Oxoid, Basingstoke, Hampshire, UK). The plates were then incubated for 24 hours at 37 °C. The agar plates were examined and those showing no growth after 48 hours were recorded as negative cultures. Any bacteria exhibiting growth were identified using standard techniques based on their morphological, cultural and biochemical characteristics (Hernández-Chiñas *et al.*, 2021).

3.4. Hemolytic activity

Hemolytic activity was detected in human blood agar containing 5% washed erythrocytes in PBS (phosphate-buffered saline, pH 7.4) and in sheep blood agar supplemented with 10 mM $CaCl_2$ (Magalhães *et al.*, 2011). The bacterial strains were cultured in tryptic soy broth overnight. Two microlitres of the homogenised liquid culture were streaked onto blood agar plates. After 24 hours' incubation at 37 °C, hemolysis surrounding the bacterial culture was visually examined.

3.5. Irradiation

The radioisotope cobalt-60, with a dose of 1 Gy, was used as a source of gamma rays. A test tube containing 5 ml of nutrient broth was inoculated with a single colony of *E. coli* and incubated at 37°C for 24 hours in a shaking incubator at 200 cycles per minute, then centrifuged at 800 rpm for 15 minutes. The cells were suspended in a saline solution, and the tube was transferred to a spectrophotometer to adjust the optical density to obtain a cell concentration of 1x107/ml (Trampuz *et al.*, 2004). Then, 1 ml of bacterial suspension was added to three test tubes: one exposed to gamma rays for 10 minutes, another for 15 minutes, and the third served as a control. After irradiation, the tubes were coated with aluminum foil, transported to the laboratory, and kept in the dark until mutations in the *hlyA* gene were detected (Xiao *et al.*, 2006).

3.6. PCR assays

3.6.1. DNA preparation:

DNA was extracted from *E. coli* using a Wizard Genomic Extraction Kit (Promega, USA), following the manufacturer's instructions. The DNA was then stored at −20°C. This chromosomal DNA was then used as a template for all PCR experiments.

3.6.2. *HlyA* gene amplification:

The *hlyA* gene of *E. coli* was amplified using the primers shown in Table 1. PCR mixtures were prepared in a total volume of 20 µl according to the kit instructions (AccuPower® PCR PreMix kit), by adding 5 µl of genomic DNA, 1.5 µl of forward primer and 1.5 µl of reverse primer to a PCR premix tube, and then completing the volume to 20 µl with deionised PCR water. The PCR products were visualised using 1.5% agarose gel electrophoresis at 100 V for 30 minutes.

Table 1. The primer sequence and PCR conditions optimal conditions for the *hlyA* gene

Gene name	Primer sequence (5´- 3´)	Size	Conditions	References
hlyA	F GGAGTTAGTGCAGCCTCCAG	360bp	Step1:95°C, 5 min.	Ojaimi and Al-Nashe (2019)
	R ACCACTCTGACTGCGATCAG		Step2: 95°C, 30 sec.	
			Step3:58°C, 30 sec.	
			Step4:72°C, 2min.	
			Step5: 72°C,10 min.	

F: Forward; *R: Reverse*

3.6.3. Sequenced analysis of *hlyA* gene

The *E. coli* PCR amplicons were sequenced at Macrogen in South Korea. The *hlyA* gene sequences were compared with those in the NCBI bacterial database to identify the bacteria. The *E. coli* sequences were also aligned using the BlastN program to detect mutations in the *hlyA* gene. A neighbour-joining phylogenetic tree was constructed based on the *hlyA* gene sequences of *E. coli* and related bacterial genera using Molecular Evolutionary Genetics Analysis version 11 (MEGA11) software. The newly generated *hlyA* gene sequences were deposited in GenBank (Ali

et al., 2019).

3.7. Data analysis

The data study's analysis was done using 'SPSS software version 23 (IBM SPSS Statistic). The significance test was calculated using a chi-square test, and a P value < 0.05 was taken as a minimal significance level

4. RESULTS AND DISCUSSION

The results revealed that 185 out of 200 diarrhea samples tested positive for growth. Only 80 isolates were identified as *E. coli*, comprising 33 males (58.75%) and 47 females (41.25%). Additionally, 160 out of 200 urine samples showed positive growth, with 76 isolates identified as *E. coli*, comprising 55 females (72.36%) and 21 males (27.63%). *E. coli* colonies appeared on MacConkey agar as small, circular, pink colonies (1-5 mm in diameter) and on Eosin methylene blue agar as a green metallic sheen Table 2. Under the microscope, they were Gram-negative bacilli.

Table 2. Distribution of *E. coli* isolated from various clinical samples based on sex.

Clinical Source	Sex		*E. coli* No	X2	P value
	Female	Male			
Diarrhea	33 (41.25%)	47 (58.75%)	80 (43.24%)	4.9*	0.027
Urine	55 (72.36%)	21 (27.63%)	76 (47.5%)	30.42*	0
Total	**88 (56.41%)**	**68 (43.85%)**	**156 (100%)**	**29.53***	**0**

4.1. Hemolysin production

Hemolytic activity was assessed for all *E. coli* strains. Each strain showed hemolytic activity to different extents, from a small, diffuse zone around the bacteria to a large, clear, well-defined hemolysis area. Our results indicated that some strains exhibited β-hemolytic activity on human blood agar, while others showed α-hemolytic activity on sheep blood agar. Out of the 80 *E. coli* GIT strains isolated, 10 only displayed β-hemolytic activity on blood agar and α-hemolytic activity on sheep blood agar. Conversely, 70 strains showed no hemolytic activity on either human or sheep blood agar. In comparison, 30 of the 76 *E. coli* strains from UTI samples exhibited β-hemolytic activity on blood agar and α-hemolytic activity on sheep blood agar. The remaining 46 uropathogenic *E. coli* strains showed no hemolytic activity, as summarized in Table 3.

The results showed a clear effect of gamma rays on the effectiveness of the hemolysin enzyme in lysis human and sheep red blood cells. This is evidenced by the smaller lysis area surrounding the bacterial colony on culture media. This suggests that the enzyme's lytic activity has decreased, which has a negative impact on the bacteria's virulence and its potential to cause intestinal and urinary infections.

Table 3. Hemolytic activity of clinical *E. coli* strains.

Clinical source	*E. coli*	hemolytic *E. coli*	Hemolysin type	Blood type	
				Human	Sheep
Urine	76	30 (39.47%)	β-hemolysis	30 (39.47%)	-
			α-hemolysis	-	30 (39.47%)
			No-hemolysis	46 (60.52%)	46 (60.52%)
Diarrhea	80	10 (12.5%)	β-hemolysis	10 (12.5%)	-
			α-hemolysis	-	10 (12.5%)
			No-hemolysis	70 (87.5%)	70 (87.5%)
X2			8.855*		
P value			0.003		

Hemolytic activity was tested for all *E. coli* strains for blood groups four main blood types: A, B, AB, and O. All uropathogenic *E. coli* isolates exhibited hemolysis activity for AB group cells (40%), followed by A, B, and O groups with incidences of 33.3% and 20%, respectively. The fewest urinary isolates showed hemolysis activity with human O group cells (6.6%). A similar pattern was observed with enteric *E. coli* isolates, which showed nearly the same range of hemolysis activity, from 20% to 50%, against AB, A, and B cells. However, only 10% of isolates exhibited hemolysis activity against human O cells, as shown in Table 4. The results showed that blood group AB had the highest rate of hemolysis, while blood group O was the least affected by *E. coli*.

Table 4. Haemolytic activity of clinical *E. coli* strains for human blood groups.

Blood groups	Hemolytic uropathogenic *E. coli*		Hemolytic enteric *E. coli*	
	No	**%**	**No**	**%**
A group	12	40	5	50
B group	10	33.3	2	20
AB group	6	20	2	20
O group	2	6.6	1	10
Total	**30**	**100**	**10**	**100**
X2	10.48*		4.8	
P value	0.015		4.186	

4.2. *hlyA* gene-PCR result

The PCR results of the current study showed that 10 out of 30 isolates of hemolytic uropathogenic *E. coli* possess the *hlyA* gene both before and after exposure to gamma rays at 10 and 15 minutes, which is 33.33%. Meanwhile, 20 isolates lack the gene, accounting for 66.66%, with a molecular weight of 360 bp, as shown in Figure 1A. Additionally, the study found that 50% of hemolytic enteric *E. coli* isolates carry the *hlyA* gene, both before and after exposure to gamma rays at 10 and 15 minutes, with a molecular weight of 360 bp, as depicted in Figure 1B.

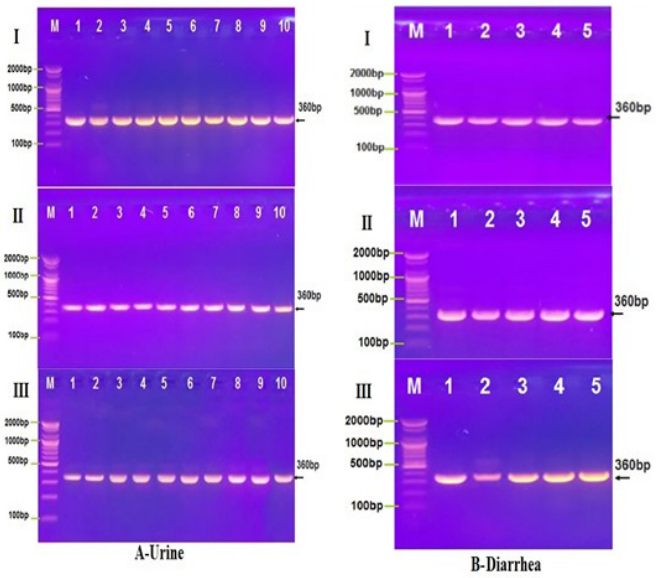
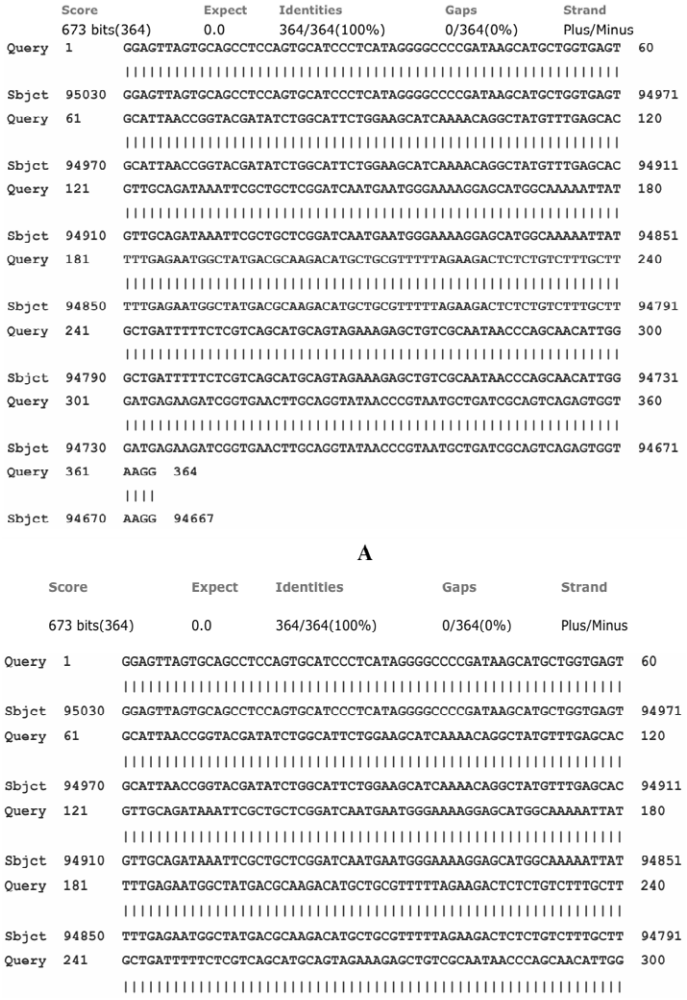


Figure 1. Electrophoresis of the PCR amplicon for the *hlyA* gene in hemolytic *E. coli* isolated from A- urine and B- diarrhea:
I. E. coli not exposed to gamma rays.
II. E. coli exposed to gamma rays for 10 minutes.
III. E. coli exposed to gamma rays for 15 minutes.

4.3. Analysis of the *hlyA* gene sequencing

DNA sequencing was used to determine mutations in the nucleotide and amino acid sequences in the hemolysin (*hlyA*) gene before and after exposure to (60 cobalt) gamma rays. Three *E. coli* isolates were selected: the first was not exposed to gamma rays, the second was exposed for 10 minutes, and the third for 15 minutes. The nucleotide and amino acid sequences of the *hlyA* gene were analysed using the BLAST program and compared with the gene sequence of the standard isolate in the NCBI database. BLAST analysis reported that the nucleotide and amino acid sequences of the *hlyA* gene in *E. coli* isolates from urine and diarrhea that were not exposed to gamma rays were 100% similar to the standard isolate gene with the accession number (CP009107.1), and there was no change in the sequence of nitrogen bases (Figure. 2).



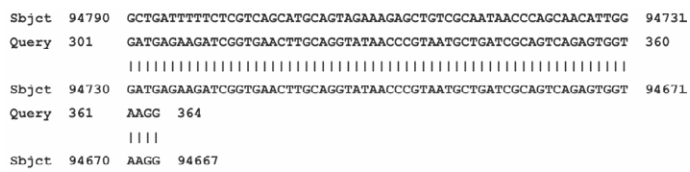


Figure 2. A comparison of the sequencing analysis results of the *hlyA* gene in hemolytic *E. coli* with the isolate standard (CP009107.1).
A: Uropathogenic E. coli not exposed to gamma rays (MF983702.1).
B: Enteric E. coli not exposed to gamma rays (MF983705.1).

In contrast, in urine and diarrhea isolates exposed to gamma rays, the results showed substitution mutations in both types of Transition and Transversion in the *hlyA* gene. The number of mutations in the uropathogenic *E. coli* exposed to the gamma ray at 10 and 15 minutes were 7 mutations, the ratio of gene match with the standard isolate gene bears accession number (CP009107.1)., was 99% Figure. 3, The number of mutations in the enteric *E. coli* exposed to the gamma ray at 10 and 15 minutes were 4 mutations, the ratio of gene match with the standard isolate gene bears accession number (CP009107.1)., was 99% as show in Figure 4.

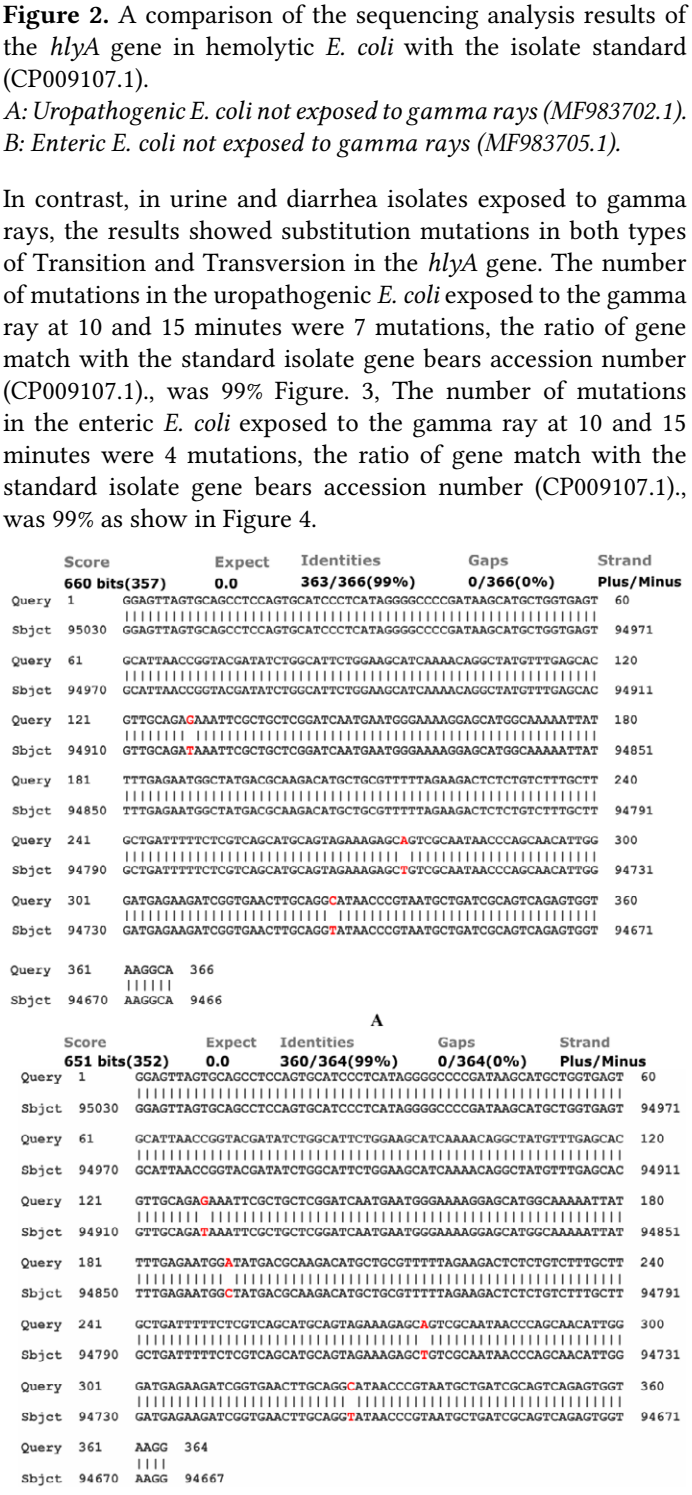


Figure 3. Results of the analysis of *hlyA* gene sequencing in uropathogenic *E. coli* exposed to gamma rays for A-10 and B-15 minutes, compared with the isolate standard (CP009107.1).

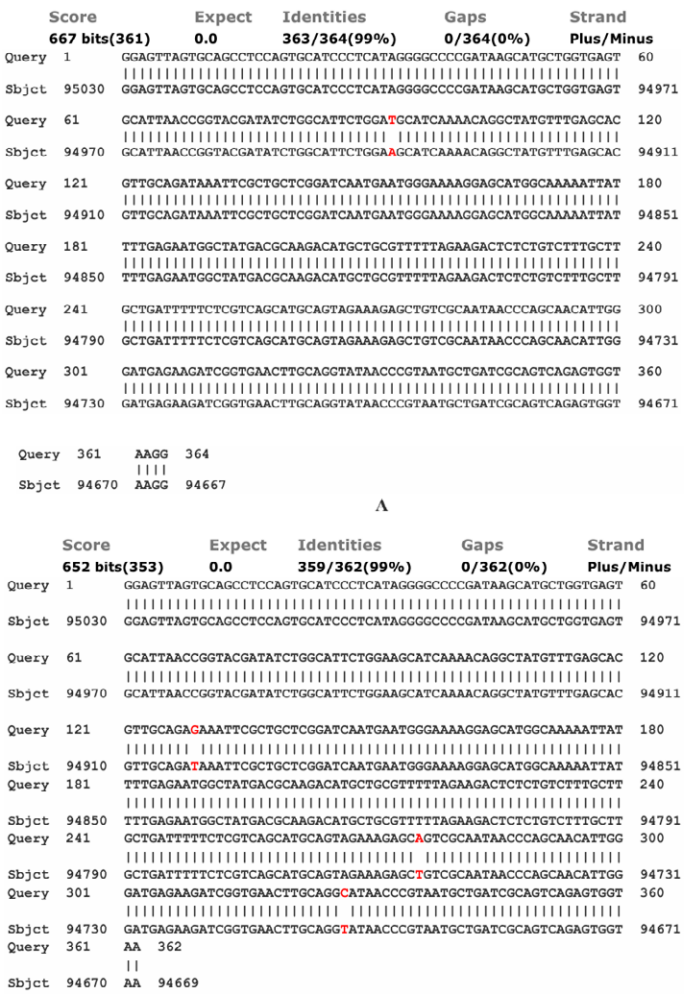


Figure 4. Results of the analysis of *hlyA* gene sequencing in enteric *E. coli* exposed to gamma rays for A-10 and B-15 minutes, compared with the isolate standard (CP009107.1).

On the other hand, when comparing the results of amino acid translation of the *hlyA* gene of *E. coli* isolated from urine and diarrhea, non-exposed to gamma rays, has 100% similarity with amino acid translation of the standard isolate gene, bearing accession number (AKN56576.1), as shown in (Figure 5). But after exposure to gamma rays at 10 and 15 minutes, the results of amino acid translation of the *hlyA* gene of uropathogenic *E. coli* and enteric *E. coli* show with the results of amino acid translation of the standard isolate with the accession number (AKN56576.1), the effect of mutations on the translation of amino acids was observed by altering the protein translation pathway and thus converted the amino acid Asparagine to Glutamine in different location of as shown in (Figures 6, 7).

Figure 5. A comparison of the results of the amino acid sequence analysis of the *hlyA* gene in haemolytic *E. coli* that were not exposed to gamma rays and were isolated from A- urine and B- diarrhea, with the isolate standard (AKN56576.1).

Figure 6. Results of the analysis of amino acid sequencing of the *hlyA* gene in uropathogenic *E. coli* exposed to gamma rays for A-10 and B-15 minutes, compared with the isolate standard (AKN56576.1).

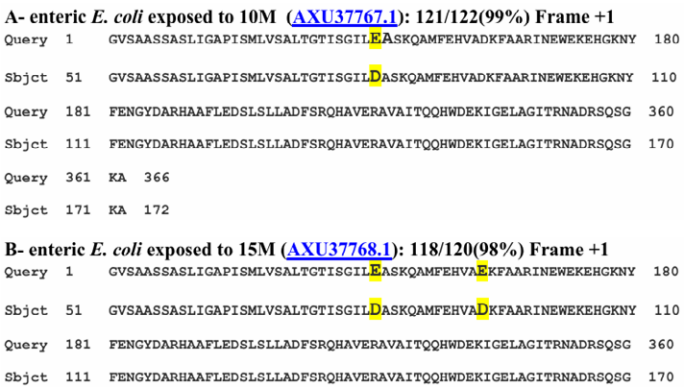


Figure 7. Results of the analysis of amino acid sequencing of the *hlyA* gene in enteric *E. coli* exposed to gamma rays for A-10 and B-15 minutes, compared with the isolate standard (AKN56576.1).

The results revealed that substitution mutations of the type transversion were more prevalent than transition mutations. The most frequent substitution mutations in the nitrogenous base sequence of the *hlyA* gene of *E. coli* isolates from urine and diarrhea were the substitution of thymine with guanine and the substitution of thymine with adenine. This may be due to the effect of gamma rays, as shown in Table 5. The results of the phylogenetic tree analysis showed that the E.coli isolates that were not exposed to gamma rays (the control group) were more genetically similar to each other than the bacterial isolates exposed to gamma rays at times 10 and 15, as illustrated in the Figure 8.

4.4. Discussion

Due to the diseases caused by *E. coli* that affect humans, particularly urinary and intestinal tract infections, which are caused by important virulence factors such as the production of the hemolysin enzyme (especially the beta type), resulting in harmful effects for patients such as the destruction of red blood

Table 5. Mutations types and sites in the *hlyA* gene sequencing

Clinical source	Accession number	Time of exposed to gamma rays	Mutate type	Mutate site	Mutation
Urine	MF983702.1	Control	-	-	-
	MF983703.1	10 minute	Transversion	948902	T-G
			Transversion	94756	T-A
			Transtion	94704	T-C
	MF983704.1	15 minute	Transversion	94902	T-G
			Transtion	94839	C-A
			Transversion	94756	T-A
			Transtion	94704	T-C
Diarrhea	MF983705.1	Control	-	-	-
	MF983706.1	10 minute	Transversion	94939	A-T
	MF983707.1	15 minute	Transversion	94902	T-G
			Transversion	94787	T-A
			Transtion	94704	T-C

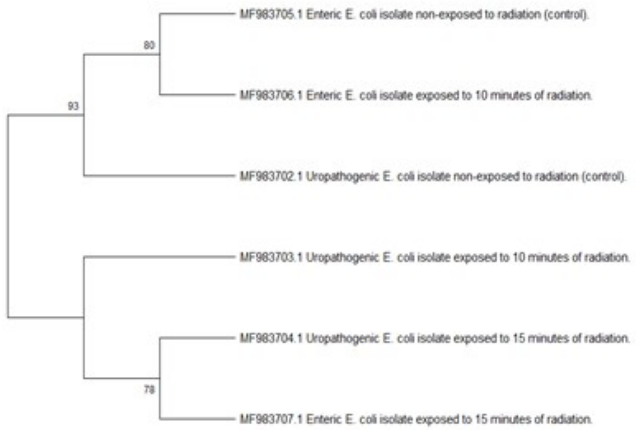


Figure 8. Phylogenetic tree analysis of *E. coli* before and after exposure to gamma rays.

cells, necrosis and cell lysis, we conducted this study to reduce the harm caused by this enzyme through the use of gamma rays.

Pathogenic *Escherichia coli* poses a significant economic threat worldwide due to its high mortality rate (Garcia *et al.*, 2022). There are two main types of *E. coli*: intestinal pathogenic *E. coli* (IPEC) and extraintestinal pathogenic *E. coli* (ExPEC). These are key pathogens responsible for urinary tract infections, such as cystitis and pyelonephritis, as well as gastrointestinal tract infections and bacteremia. They can pose a threat to human and animal health (Sarowska *et al.*, 2019). ExPEC is commonly found in the animal industry, where it causes economic losses and poses a risk to human health through foodborne transmission or cross-infection (Li *et al.*, 2024). Its virulence factors include adhesins, toxins such as hemolysin, lipopolysaccharides, iron acquisition factors, polysaccharide capsules, and invasion proteins. These are usually found on pathogenicity islands, plasmids, and other mobile genetic elements, helping the bacterium to survive in adverse conditions (Sarowska *et al.*, 2019). Our study found that the prevalence of *E. coli* isolates was 45.2% (43.2% enteric *E. coli* and 47.5% uropathogenic *E. coli*). The prevalence of *E. coli* clinical isolates in our study was higher than the figures of 0.22% and 28.44% reported in the Republic of Zambia (Kasanga *et al.*, 2024). The prevalence of *E. coli* isolates from clinical sources in our study was also higher than that reported for *E. coli* isolated from urine (38.2%), but lower than that reported for *E. coli* isolated from diarrhea (52.5%) in Iran (Alipour & Poursina, 2021). Also, the prevalence of *E. coli* isolated from clinical sources in our study was higher than that reported in Saudi Arabia and Iraqi, where the prevalence of *E. coli* was 20.5% from urine and 28% from diarrhea sources (Abdelkhalig *et al.*, 2025; Raid & Al-Nashi, 2019). The frequency of uropathogenic *E. coli* isolates was higher in females than in males. This finding aligns with other studies reporting a higher prevalence of *E. coli* in UTIs among females (Cag *et al.*, 2021; Naqid *et al.*, 2020). The high incidence of UTIs in women is due to several factors. One is that they have more type 1 fimbria (fimH) receptors than men, which are thought to be a virulence factor of UPEC (Getaneh *et al.*, 2021). Furthermore, women's urethras are shorter and located closer to the anus, facilitating the passage of UPEC to the bladder and causing infection. In contrast, the male urethra is longer, making it more difficult for bacteria to reach the bladder (Fazly Bazzaz *et al.*, 2021). Hormonal changes, such as a decrease in oestrogen during and after pregnancy, also lead to an increased risk of UTIs (Mouhammed & Gdoura, 2024). Regarding diarrhea samples, clinical isolates of *E. coli* were more prevalent in males than in females. our study similar results to a study conducted in Ahvaz, Iran, which also found a higher incidence of *E. coli* isolates in male diarrheal samples than in female ones (Sirous *et al.*, 2020). This variation is difficult to explain, and further research with larger sample sizes is needed to determine its cause.

The hemolytic activity of *E. coli* is considered one of the fundamental virulence factors influencing its pathogenicity. This is an important virulence trait, as it enhances the severity of infections caused. The production of cytolysin has been linked to septicaemia and a fivefold increase in the risk of acute, terminal outcomes in patients. In this study, 25.6% of isolates (19.23% uropathogenic and 6.41% enteric *E. coli*) exhibited β-hemolytic activity on human blood agar and α-hemolytic activity on sheep blood agar. Additionally, uropathogenic and enteric *E. coli* isolates demonstrated similar characteristics. coli isolates showed the ability to exhibit hemolytic activity against blood groups. Comparable to our findings, a Romanian and Iraqi study reported that 40.2% and 7.23% of *E. coli* isolates from diarrhoeal and urine samples were haemolysin-producing (Mare *et al.*, 2020; Juda & Khalaf, 2024). Furthermore, genetic analysis of these *E. coli* strains revealed the presence of a hemolytic activity-associated gene (*hlyA*), which was present at a higher proportion (33.33%) in the UPEC strains. Conversely, a study found that 50% of *E. coli* strains carrying the *hlyA* gene were responsible for diarrhoeagenic cases. These findings are consistent with those of (Yazdanpour *et al.*, 2020). who found that 26% of *E. coli* isolates from urine samples carried the *hlyA* gene. According to (Shahbazi *et al.*, 2018; Moeinianzadeh & Shaheli, 2021), the *hlyA* gene was prevalent in urine-derived *E. coli* isolates at a rate of 41.7%. However, disagreement with this study another study found that the *hlyA* gene was present in 18.2% of enteric *E. coli* isolates. The presence of the *hlyA* gene in the genome of clinical isolates has been associated with more severe infections. Our investigation showed that bacterial cells are sensitive to a dose of 1 Gy, which can be classified as either sensitive or sublethal. Notably, the hemolysin enzyme became vulnerable after irradiation. In our study, gamma rays were found to impact the *hlyA* gene by causing substitution mutations in nucleotide sequences and altering amino acids. Exposure to gamma rays can decrease *hlyA* gene expression levels. This reduction affects the virulence of *E. coli* and its ability to cause urinary and gastrointestinal tract infections. determining patterns and frequencies of substitution mutations in genomes is important for studying molecular evolution and understanding the molecular basis of these mutations. Substitution mutations are mutations in a gene that lead to changes in structure, function, and gene expression. These changes can affect the amino acid composition of a protein. However, replacing amino acids in a particular area of a protein does not always affect its function, as the genetic code consists

of three nitrogen bases, and changes can occur in just one of these bases (Takahashi *et al.*, 2013). The genetic variation observed between bacterial isolates may be caused by mutations in the nucleotide sequence within genes. One type of mutation is a substitution mutation, also known as a point mutation, which causes a single nucleotide base to change in the DNA sequence. These changes are replicated during the replication process, resulting in a permanent alteration to the order of nitrogen bases in the genome (Shaker *et al.*, 2018). Point mutations include translation mutations, in which a purine base is replaced by another purine base, or a pyrimidine base is replaced by another pyrimidine base. A transversion mutation occurs when the purine base is replaced with the pyrimidine base, or vice versa. There are two types of mutation. Transition mutations are more common than transversion mutations because replacing one loop structure with another is more likely than replacing a double-loop structure (pyrimidine) with a single-loop structure (purine) (Ohno *et al.*, 2014). Identifying the functional domains of *HlyA* remains an active area of research. The repeat regions of *HlyA* first bind to calcium, and then to cell membranes, resulting in cytolytic activity (Boehm *et al.*, 1990). Studies of mutagenesis have revealed the important regions of the protein that interact with red blood cells (RBCs). The amino acids 9–37 have a dampening effect on haemolytic activity. Mutants lacking this region are 2.5 times more haemolytic than the wild type and are able to form more stable pores (Ludwig *et al.*, 1991). The results of the genetic analysis showed that the highest rate of mutations when comparing local and global isolates was due to the transformation of thymine into guanine, thymine into adenine and thymine into cytosine. This is because it is easier to replace nitrogen bases within the same chemical category than to replace them with bases from a different category.

5. CONCLUSION

To address the growing prevalence of *E. coli* virulence factor genes, new strategies and approaches must be developed. Our study explores how a given dose of radiation can restrict the dissemination of virulence factor genes in a healthy environment. It also highlights the effectiveness of modern gamma ray applications in current practices, such as treating blood components and sterilising medical equipment. To understand the direct and precise impact, we also recommend studying the effect of gamma radiation on the gene expression of hemolysin before and after exposure to gamma rays.

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