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Cytotoxic Effects of Cytolethal Distending Toxin-Producing *Escherichia coli* on Normal Human Fibroblasts: Pilot Study

Yasmine Ahmed*, Ramina Khoshaba
Department of Biotechnology, College of Science, University of Baghdad

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Abstract

Cytolethal Distending Toxin (CDT) is a genotoxin produced by *Escherichia coli* and encoded by the *cdt* gene. It consists of three subunits: CDT-A, CDT-B, and CDT-C. As a virulence factor, CDT induces DNA damage in host cells, leading to cell death and potentially contributing to the development of cancer. This is a pilot study with the aims of investigating the impact of CDT-producing *E. coli* on normal cells and determining the prevalence of *cdtB* genes among clinical *E. coli* isolates. A total of 120 specimens were collected from various sources (urine, wound, blood, stool, and colorectal cancer tissue (CRC)) and biochemical tests coupled with Polymerase Chain Reaction (PCR) identified 60 samples as positive for *E. coli*. The PCR results for detecting the *cdtB* gene revealed that only 3 out of 60 isolates (one from CRC and two from stool) contained the *cdtB* gene. Moreover, Quantitative Real-Time PCR was conducted on three isolates to detect the *cdtB* gene expression. The results revealed that the expression of the isolate from stool was higher than that of CRC, followed by another stool isolate. The phenotypic effects of CDT were assessed by treating Normal Human Fibroblasts (NHFs) with *cdt+ E. coli* isolates. To evaluate the impact of CDT, an apoptosis estimation assay was employed using (propidium iodide/acridine orange), with fluorescence intensity serving as the measure of DNA damage and cell death. The results showed that NHF cells exposed to the *E. coli* CRC isolate had a significantly higher percentage of dead cells (81.46 ± 0.99%), followed by the urine isolate (74.11±1.52%), compared to the control group (4.67±1.11%). Whole genome sequencing revealed that the *E. coli* CRC isolate carries various virulence factors that may facilitate CDT-mediated DNA damage and apoptosis. These findings suggest possible DNA damage and cell death, which may reflect the genotoxic and cytotoxic potential of CDT.

Keywords: Apoptosis Estimation, CDT, *Escherichia coli*, genotoxin, NHF.

التأثيرات السامة لخلايا الإشريكية القولونية المنتجة للذيفان الخلوي في الخلايا الليفية البشرية الطبيعية :دراسة أولية

ياسمين احمد* , رامينا ميخائيل خوشابه
قسم التقنيات الاحيائية , كلية العلوم , جامعة بغداد

*Email: yasmin.abd2306@sc.uobaghdad.edu.iq

الخلاصة

الذيفان القاتل للخلايا (CDT) هو ذيفان جيني تنتجه الإشريكية القولونية ويشفر له بواسطة جين *cdt*. يتكون من ثلاث وحدات فرعية: CDT-A وCDT-B وCDT-C. كعامل ضراوة، يحفز CDT تلف الحمض النووي في الخلايا المضيفة، مما يؤدي إلى موت الخلايا ويساهم بشكل محتمل في تطور السرطان. تعد هذه دراسة أولية تهدف إلى دراسة تأثير البكتيريا القولونية المنتجة لـ CDT على الخلايا الطبيعية، بالإضافة الى تحديد معدل انتشار جين *cdtB* بين عزلات *E. coli* السريرية . تم جمع ما مجموعه ١٢٠ عينة من مصادر مختلفة (الادرار، الجروح، الدم، البراز، خزعة من القولون) شخصت 60 عينة على انها *E. coli* من خلال الاختبارات البايوكيميائية المقترنة بتفاعل البلمرة المتسلسل (PCR). كشفت نتائج تفاعل البلمرة المتسلسل أن 3 فقط من أصل 60 عزلة(عزلة واحدة من سرطان القولون والمستقيم وعزلتان من البراز) تحتوي على جين *cdt*-B. تم استخدام تفاعل البلمرة الكمي لتحديد التعبير الجيني لـ *cdtB* في العزلات الثلاثة وكشفت النتائج ان التعبير الجيني في عزلة البراز كان اعلى من تعبيره في العزلة المأخوذة من نسيج سرطان القولون والمستقيم تليها عزلة أخرى من عزلة البراز. تم تقييم التأثيرات الظاهرية لـ CDT عن طريق معاملة الخلايا الليفية البشرية الطبيعية بمصادر مختلفة من عزلات *E. coli*. لتقييم تأثير CDT، تم استخدام اختبار تقدير موت الخلايا المبرمج باستخدام (propidium iodide/Acridine orange)، مع شدة التفلور كمقياس لتلف الحمض النووي وموت الخلايا. أظهرت النتائج أن الخلايا الليفية البشرية الطبيعية المعرضة لعزلة *E. coli* المأخوذة من سرطان القولون والمستقيم أظهرت نسبة مرتفعة من الخلايا الميتة ± 81.46) (0.99%، تليها العزلة المعزولة من الادرار بنسبة (74.11±1.52%) مقارنة بمجموعة السيطرة (4.67±1.11%). كشف تسلسل الجينوم الكامل ان عزلة *E. coli* المأخوذة من سرطان القولون والمستقيم تحمل عوامل ضراوة مختلفة قد تسهل تلف الحمض النووي بوساطة CDT وموت الخلايا المبرمج. تشير هذه النتائج الى احتمال حدوث تلف في الحمض النووي وموت الخلايا، مما قد يعكس التأثيرات السمية والجينية المحتملة لذيفان CDT.

1. Introduction

Escherichia coli is a Gram-negative, facultatively anaerobic bacterium of the Enterobacteriaceae family [1]. Numerous *E. coli* strains are known as normal commensals that inhabit the gastrointestinal systems of humans and animals; meanwhile, certain strains are pathogenic [2, 3]. *E. coli* is associated with various extraintestinal disorders. When *E. coli* inhabits atypical environments, it can induce many infectious illnesses, including septicemia, meningitis, wound infections, urinary tract infections, and other soft tissue infections [4].

The crucial role of this pathogen arises from its capability to produce a broad spectrum of virulence factors [5, 6]. The many *E. coli* pathotypes exhibit divergent pathogenic mechanisms and unique virulence factor profiles, which are encoded by particular gene clusters. The four main classes of virulence factors in *E. coli* pathotypes are classified as colonization, fitness, toxins, and effectors, each comprising specific characteristics unique in their functions and activities [7].

The pathogenicity of *E. coli* strains depends on the presence of particular virulence factors, including toxins and adhesins. These factors facilitate evasion of host defenses and promote colonization, ultimately leading to the onset of intestinal and extraintestinal diseases [8-11].

The first identified toxins include cytotoxic necrotizing factors (CNFs) and cytolethal distending toxins (CDTs), which were initially extracted from human intestinal pathogens [12, 13]. The *cdtA, cdtB,* and *cdtC* genes, which encode the subunits of CDT toxin, are co-transcribed from a single operon [14]. The CDT family structure consists of AB2

heterotrimers, with CDT-B being the active part of the family, and CDT-A and CDT-C serving as the binding subunits. CDT-B, the active subunit, is the most conserved subunit [15, 16]. The role of CDT-A and CDT-C was primarily to bind to a receptor on the plasma membrane of host cells, facilitating the entry of CDT-B into the host cells. While CDT-B, the active subunit, carries out the catalytic activity. However, the catalytic CDT-B subunit-mediated mechanisms of eliciting these effects remain incompletely understood [16]. It has been speculated that the CDT holotoxin generates replicative stress through the CDT-B subunit. DNA replication deceleration primarily takes place during the late S phase, leading to the formation of fragile sites and significant chromosomal abnormalities. DNA defects induced by CDT treatment are accountable for the formation of anaphase bridges during mitosis and interphase DNA bridges between daughter cells in the G1 phase. Furthermore, the genotoxic potential of CDT predominantly impacts cycling human cells rather than quiescent ones. The toxin ultimately affects the proliferating cells of human colorectal organoids, leading to nuclear distension due to DNA damage, which inhibits growth [17]. The host cell effects after treatment with CDTs include cell death, cell cycle arrest, and DNA damage [16]. Timely resolution of host DNA infections is crucial to prevent host cell senescence, cancer caused by bacterial pathogens, and host cell death [18]. Researchers have identified CDT as a bacterial virulence factor that affects proliferating cells, such as human colon progenitors or stem cells, inducing replicative stress and genomic instability, which are then passed on to daughter cells, and may contribute to carcinogenesis [17].

This study aimed to detect the *cdtB* gene in *E. coli* isolates from different sources using conventional PCR, evaluate the expression of the *cdtB* gene in these isolates using Real-Time PCR, and observe the DNA damage caused by *E. coli* isolates that express the CDT toxin on NHFs using an apoptosis assay.

2. Materials and Methods

Sample Collection and Isolation

A total of one hundred twenty specimens were obtained from various sources (urine, wound, blood, stool, and colorectal cancer tissue) during the period from October 2023 to February 2024, with the approval of the College of Science Research Ethics Committee (CSEC/0624/0046), University of Baghdad. All samples were provided by Medical City Hospital, Al-Kindi Teaching Hospital, Al-Yarmouk Hospital, and Al-Kadhimiya Teaching Hospital. Wound and stool specimens were obtained directly from patients utilizing sterile transport medium swabs and swiftly transported to the laboratory under aseptic conditions for diagnostic analysis and isolation. Urine specimens were obtained from patients in sterile containers containing boric acid for preservation and were promptly transported to the laboratory for analysis. Blood samples were collected in blood culture bottles and thereafter transported to the laboratory for additional processing. The CRC biopsies were placed in tubes with normal saline, transported to the laboratory, and gently homogenized with sterile normal saline. An aliquot of the tissue homogenate was collected for culture. The specimens were cultured on MacConkey agar and Eosin Methylene Blue (EMB) agar (Himedia, India), and then incubated the culture for 24 hours at 37°C. Biochemical examinations (indole test, urease, and Simmons citrate test) from Himedia, India, were also performed [19-21].

Molecular Detection of E. coli 16s rRNA

DNA Extraction

The genomic DNA was extracted using a Bacterial DNA Extraction kit (Elk, China) from a single colony taken from the culture medium and introduced into Brain Heart Infusion

broth (BHI), Himedia, India. After incubation for 24 hours at 37°C, the extraction procedure was performed according to the manufacturer's recommendations.

PCR amplification for the detection of 16S rRNA and cdt gene

Polymerase Chain Reaction has been done to check the presence of the *16S rRNA* and *cdtB* genes. *16s rRNA* is the most molecular marker that is used to identify *E. coli* [22]. The amplification was performed utilizing the PCR Kit (2.5X Master Mix Synthol) (Synthol, Russian). The Primers sequence that was used were supplied by Macrogen Company in a lyophilized form, as shown in Table 1. The PCR program for each gene is mentioned in Table 2. A 2% agarose gel electrophoresis was performed to monitor the amplification of these genes at 50 V for 40 min.

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Table 1: Primer sequences and amplicon size of *16S rRNA* and *cdt* gene

Primer Name	Primer Sequence F 5′→3′	Primer Sequence R 5′→3′	Tm.	Product size (bp)	Reference
16S rRNA	ATGGCTGAGATTGAACCCTGG	TTATCCCCCTCCATCAGGCAG	63	134	This study
Cdt-Bcomu	TAAATGGAATATACATGTCCG	TTTCCAGCTACTGCATAATC	53	588	[23]

Table 2: The program of PCR analysis for the detection of *cdtB* and *16s rRNA* genes

Steps	Temperature °C	Period m : s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:15	40X
Annealing	53*,63*	00:15	
Extension	72	00:15	
Final Extension	72	10:00	1

*53 annealing temperature of *cdtB* gene, *63 *annealing* temperature of *16s rRNA* gene

Real-Time PCR of the cdtB gene among isolates

RNA Extraction

RNA was extracted from three isolates by following the guidelines of TRIzol™ Reagent from ELK (Biotechnology, China).

Formation of complementary DNA from mRNA

The preparation of cDNA derived from mRNA was performed according to the protocol of the company OT-1 Reagent Kits (Reverse Transcription kits, Synthol, Russia). The component of the reaction mixture (10 µL) was 1 µL enzyme MMLV-RT, 1 µL primer oligo, 2 µL extracted RNA, and 6 µL nuclease-free water. The program for the thermal cycler to create cDNA is shown in Table 3.

Table 3: Thermal cycler program for cDNA formation

Steps	°C	min: sce	Cycle
Reverse transcription	37	30:00	1
Heat inactivation	92	05:00	
Hold	4	10:00	

Assessment of the gene expression of the cdtB gene

The experiment was conducted using Luna® Universal qPCR Master Mix Protocol (M3003) (New England Biolabs, USA). The primers used in this experiment were the same as those in conventional PCR, with different temperatures, 60°C for each primer (Table 1). The housekeeping gene used in this experiment was *16s rRNA*. The procedure was conducted according to the company's instructions. The first step was the preparation of the Master Mix. The total volume consists of 10µL of Luna® Universal qPCR, 0.5µl of each primer forward and reverse, 7µL of nuclease-free water, and 2µL of cDNA. Following that, the Real-Time PCR protocol is divided into three steps: initial denaturation for 1 min at 95°C, denaturation for 15 sec at 95°C, and annealing for 30 sec at 60°C. Repeat the last step 45 times. The results were displayed as Cycling Threshold (Ct), which was calculated using software used by a real-time cycler. The analysis of expression data for the targeted gene was normalized compared to housekeeping genes. The outcomes were presented as fold change according to the Livak equation [24]. The ΔΔCt method, as described by Livak, calculates the difference in Ct values obtained from real-time PCR for each required gene and housekeeping gene. The gene expression data obtained were analyzed using one-way ANOVA to compare the relative expression levels of the *cdtB* gene among the three groups, and the results are expressed as mean ± standard deviation (SD). Statistical significance was determined by considering a P-value < 0.05.

Estimation of Apoptosis (Propidium Iodide/Acridine Orange Assay)

Measuring apoptosis in cell lines (infected and control) using acridine orange/propidium iodide(AO/PI). The effect of cytotoxic *E. coli* on NHFs was assessed using three samples. The first sample (A) was not infected with *E. coli* and acted as a control, showing normal live cells. The second sample (B) was treated with *E. coli* isolated from a urine specimen. The third sample (C) was treated with *E. coli* collected from the tissue of colorectal cancer. The use of AO/PI stains to indicate cell apoptosis occurs when the AO is attached to a single strand of DNA, which generates red fluorescence, indicating that DNA damage has occurred. In contrast, when it attaches to double-stranded DNA, it generates green fluorescence, which means the DNA is intact [25]. The other dye, PI, is an alternative dye that binds to DNA and only enters dead bacteria, generating red fluorescence [26]. NHFs were used in this experiment. This is an Iraqi cell line established by Prof. Dr. Ahmed Majeed Al-Shammari, derived from a human adipose tissue biopsy. These NHFs were grown in minimal essential medium (MEM), which contains fetal bovine serum (FBS) and antibiotic/antimyotic, and incubated for 24hours at 37°C in a humidified environment with 5% CO_2. After that, the cells were washed with phosphate buffer solution (PBS) to remove the antibiotic and FBS. The trypsin EDTA was added, and the cells were incubated for 2-5 minutes at 37°C to detach the

NHFs cells from the tissue culture flasks. Neutralized the trypsin EDTA with complete MEM medium, and 5000 cells/well were taken and seeded in 8-well plate. At this time, the *E. coli* was cultured in BHI broth for approximately one night. The sample was subsequently centrifuged, and the supernatant was discarded. Rinse the bacterial pellet with PBS and resuspend it in MEM. After the preparation of both NHF cells and *E. coli* isolates, the NHF cells were infected with *E. coli* at a 50 Multiplicity of Infection (MOI) and incubated for 24hours at 37°C. After incubation, the cells were washed with a 1X PBS solution. For standard dual staining, the wells used for analysis received precisely 50µl of the AO/PI staining mixture (at room temperature) for 30 seconds. Then, the stain was washed out with PBS. The images were captured using the Leica fluorescence microscope [27, 28]. Fluorescence intensity was assessed through fluorescence microscopy and analyzed using ImageJ software [29].

Whole-genome sequencing

Genomic DNA extracted from the CRC isolate was subjected to whole-genome sequencing using the Illumina NovaSeq platform, employing next-generation sequencing (NGS) technology (Macrogen, Korea). The Quantity of DNA was assessed by the QuantiFluor® dsDNA System on a Victor Nivo Multimode Microplate Reader. DNA integrity was assessed using the 2100 Bioanalyzer, while the actual fragment size was evaluated using the Femto Pulse System. For the purity of DNA, the N120 Nanophotometer has been used. After that, library preparation was conducted using the TruSeq Nano DNA Library Preparation Kit, and paired-end 151 bp reads were generated. The raw sequencing data were obtained in FASTQ format and then assessed for quality, length, and assembly using the FastQC tool. Then, adapter sequences were trimmed from the reads using the Trimmomatic system. The reference genome that has been used and aligned to the reading results for downstream analysis was (GCF_000005845.2).

3. Results and Discussion

Sample Collection and Isolation

A total of one hundred twenty specimens were collected from various sources, 60 of which were identified as *E. coli* (30 urine isolates (50%), 15 wound (25%), 10 stool (16.6%), and 5 CRC (8.3%). No *E. coli* from blood samples was detected. These isolates exhibited pinkish colonies on MacConkey agar and green metallic sheen colonies on EMB agar. Furthermore, showed positive results for the indole test and negative results for urease and Simmon citrate. In a study by Anwer *et al.,* only 41 *E. coli* isolates were obtained from 144 samples collected from patients with urinary tract infection [30]. Additionally, Mahdavi *et al.,* showed that only 140 isolates were isolated from 250 samples of *E. coli,* which is a close rate to our study [29]. The presence of the *16S rRNA* gene in all 60 samples verified these results, as shown in Figure 1

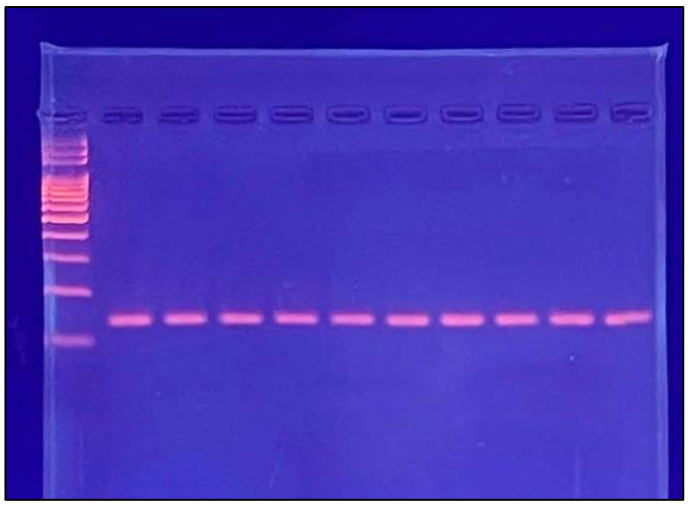

Figure 1: Gel electrophoresis of the PCR product of the 16S rRNA gene. 2% agarose gel at 50V for 40min. L: Ladder marker 100 bp. From 1 to 10, the DNA bands appear with a size of 134bp

Prevalence of the cdtB gene among E. coli isolates

Molecular detection was performed using conventional PCR to identify the presence of the *cdtB* gene. A specific primer for the B subunit, which is the active subunit. The outcomes showed the presence of the gene in only three isolates (5%) out of 60. The sources of these isolates were one CRC tissue specimen and two stool samples. The findings of this study are supported by Mahdavi *et al.,* and Onlen *et al.*, who also reported a low frequency of the *cdt* gene in *E. coli* isolates [31,32]. As shown in Figure 2, only three bands had been observed in gel electrophoresis with a size of 588bp. A study revealed that mucosa-associated CDT-producing *E. coli* is more common in tumor tissues than in healthy colonic tissues [33]. This association suggests that these bacteria may have a part in the development of colorectal cancer. Several studies have found that stool samples from both diarrheal and non-diarrheal people contain. *E. coli* that produces CDT, for example, a Brazilian study discovered that the *cdt*-I gene, which produces CDT, was present in a tiny proportion of pediatric fecal *E. coli* isolates. This discovery suggests that the gut microbiome of healthy people may contain *E. coli* that produces CDT [34].

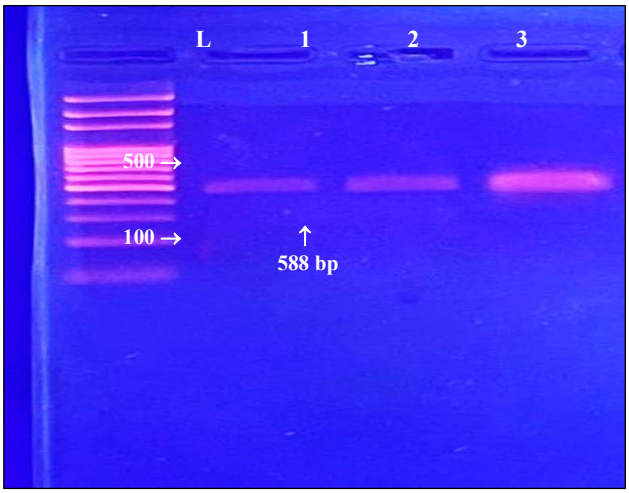

Figure 2: Gel electrophoresis of the PCR product of the *cdtB* gene. 2 % agarose gel at 50V for 40min. L: ladder marker 100 bp. Lanes: 1 to 3 represent the bacterial isolates.

Gene expression of the cdtB gene

The results of real-time PCR for the samples that exhibited the presence of the *cdtB* gene showed a high gene expression rate of the B subunit of that gene. SYBR Green dye was used in this experiment, which is an intercalating fluorescence dye that binds to double-stranded DNA. This study utilized the 16S *rRNA* housekeeping gene as a reference gene. The melting curve confirmed the absence of other compounds in the reaction, such as primer dimer, as shown in Figure 3.

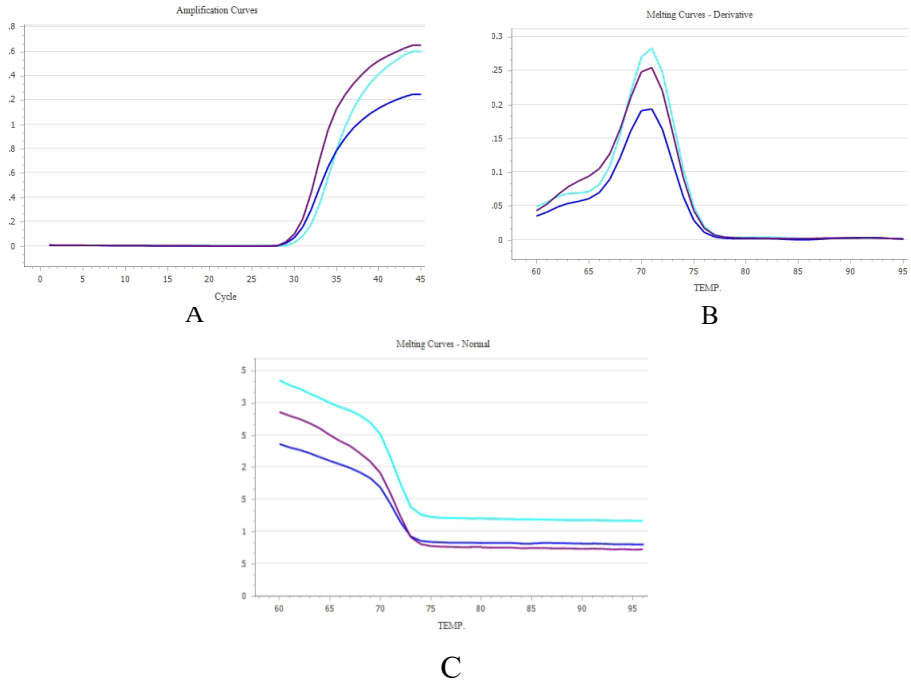

Figure 3:A-Amplification curve, B- Derivative melting curve, and C- Normalized melting curve of the *cdtB* gene by using PCR. Light blue represents colorectal cancer isolate, dark blue and purple represent stool isolates.

The highest folding change in *cdtB* gene expression was observed in the stool sample, with a value of 1.29 ± 0.016. In comparison, the colorectal cancer tissue and the second stool sample showed lower fold changes of 0.82±0.014 and 0.78 ±0.015, respectively. A statistically significant difference was observed among the three samples, as shown in Figure 4. The study results are consistent with another study, which indicates a low occurrence of the *cdt* gene among different sources of *E. coli*, suggesting that the *cdt* gene is present in 9% of the total samples [35].

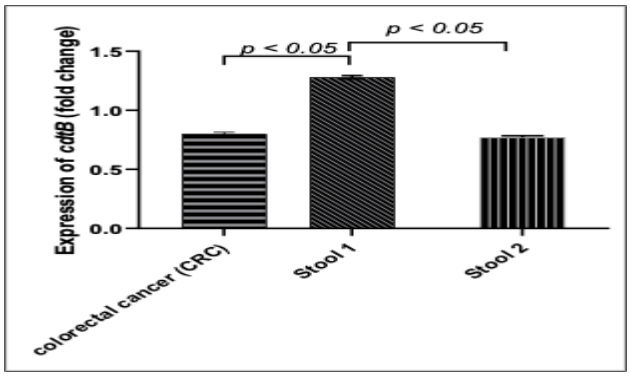

Figure 4: Comparative expression analysis of the *cdtB* gene in colorectal cancer stool-derived *E. coli* isolates.

The Apoptotic Effects of cdt⁺ E. coli on NHF cells

Both samples B and C showed positive results for DNA damage in the NHF cells, leading to cell death. As shown in Figure 5A , the green fluorescence indicates viable, healthy, untreated NHF cells with high cell viability. In contrast, Figure 5B represents the NHF cells treated with *E. coli* isolated from a urine sample, demonstrating a significant reduction in cell viability, a high average number of dead cells, and substantial DNA damage. Finally, Figure 5C shows that cells treated with *E. coli* isolated from CRC tissue exhibited even lower viability and more dead cells compared to urine group, suggesting higher DNA damage. The AO/PI fluorescent intensity was assessed using Image J and analyzed with GraphPad Prism 6, as shown in Figure 6. AO/PI staining revealed that the NHF population treated with the urine-derived isolate had a large percentage of non-viable (PI-positive) cells, which means that more cells died than in the control group. Additionally, the colorectal cancer isolates exhibited a larger percentage of NHFs dead cells, with only a small percentage of viable cells. Lastly, the control sample was used to demonstrate how the cytotoxic isolates affected the viability of cells compared to their normal state.

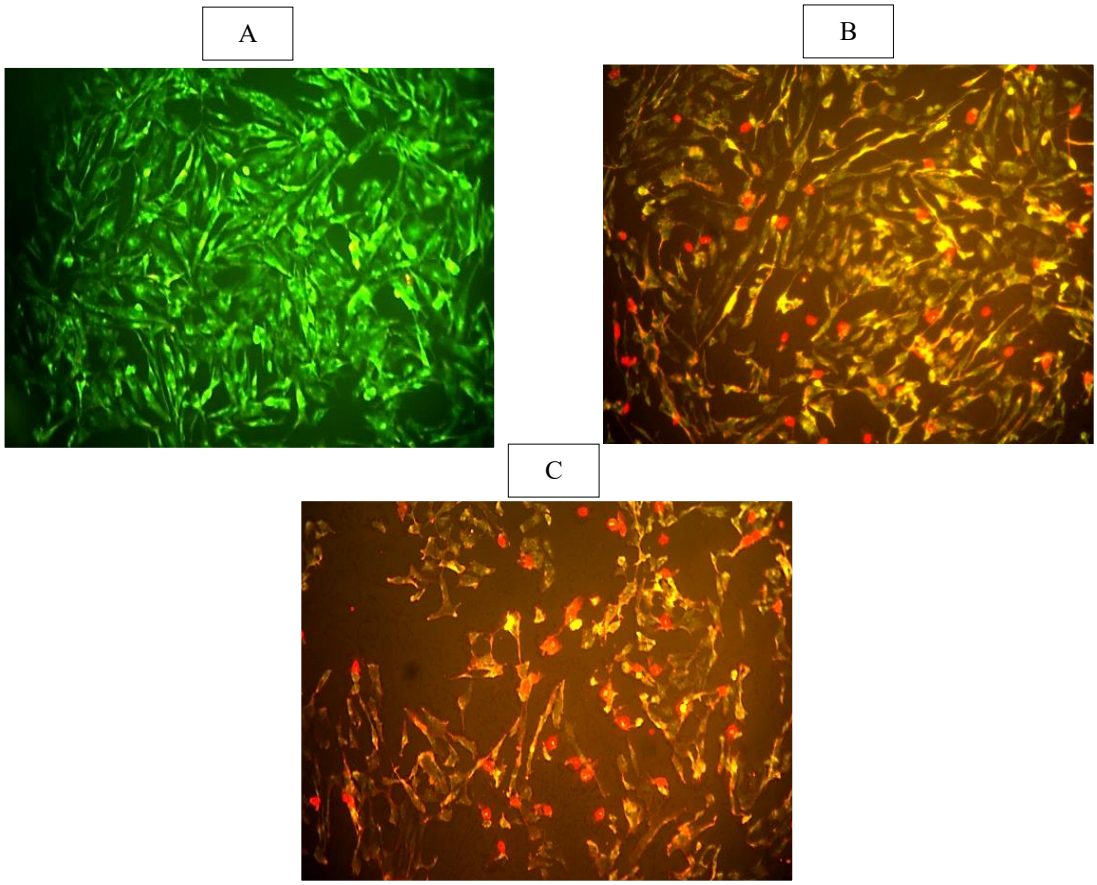

Figure 5: The apoptotic effect on NHF cells was caused by *cdt+ E. coli*. Sample (A) Control cells that were non-treated with *E. coli* isolate. Sample (B) cells were treated with *E. coli* isolate from urine. Sample (C) Cells treated with *E. coli* isolate from colon cancer tissue. The images were captured using a Leica fluorescence microscope at 10X magnification after 24 hours of cell exposure**.**

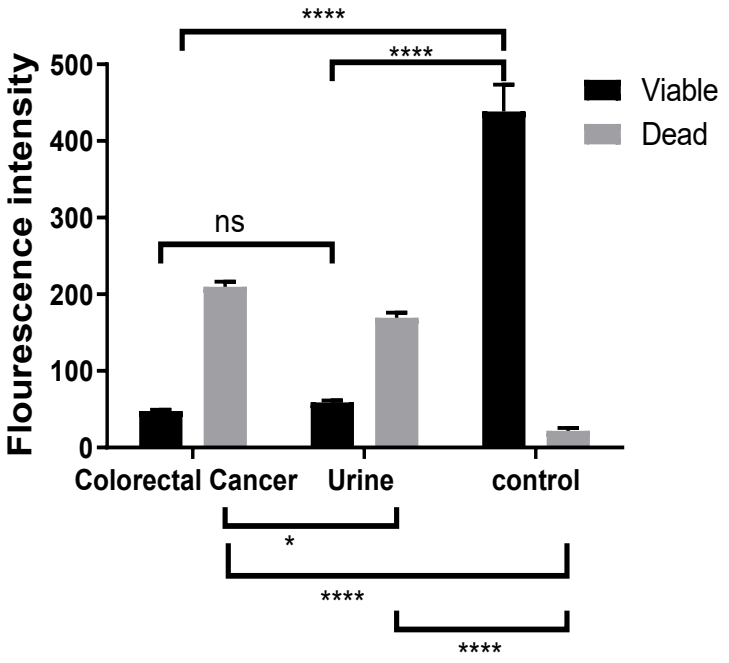

Figure 6: Fluorescence intensity of NHF cells infected with a CRC and urine isolates compared with the control, untreated sample.

Figure 6 illustrates the impact of DNA damage caused by the cytotoxic effect on NHF cells, as measured by the fluorescence intensity of AO/PI. The data obtained by AO/PI may indicate that the treatment is inducing DNA damage and leading to cell death. The effect of CDT can be demonstrated by comparing the normal control sample and the infected sample using AO/PI staining. Consequently, comparing the data obtained, the DNA damage that occurred and led to cell death may indicate the effect of genotoxin on NHF cells. Therefore, the control sample, which consists of uninfected cells, displays a healthy state of cells with a low percentage of cell death (4.67±1.11%). Sample B, which was taken from a urine isolate, exhibits a significantly high cell death effect on NHFs (74.11±1.52%). Finally, Sample C, obtained from colorectal cancer, shows significantly higher cell death in NHF cells (81.46 ± 0.99%) compared to the control group. According to another study, the *cdt+ E. coli* had shown significant DNA damage in different cell lines, which confirms the current findings [36]. Additionally, a recent study reported the vital role of CDT-producing *E. coli* in causing senescence or apoptosis of target cells when the damage is beyond repair [33]. Furthermore, this investigation demonstrated the effect of CDT toxin to cause cell apoptosis [37]. Data were analyzed using Sidak's multiple comparisons test to determine statistical significance among the groups: CRC, urine, and control, as illustrated in Table 4. The viable cell counts of NHFs in the urine sample and CRC sample exhibit no significant difference, with a *P*-value of 0.9350. The mean difference observed between the control, untreated NHF cells and both urine and CRC exhibited highly substantial disparities with *P*-value of <0.0001. Conversely, comparison between CRC and urine isolates regarding dead cells showed a significant difference with *P*-value of <0.0001. This indicates that the apoptosis induced by exposure to *E. coli* from cancer isolates was greater than that resulting from *E. coli* derived from urine isolates. Furthermore, the analysis of dead cell levels between control NHF uninfected cells and isolates from urine or cancer reveals markedly significant differences. Consequently, the level of DNA damage and the resulting cell death induced by *E. coli* associated with colorectal cancer exceeded that observed in urine samples. The results of Sidak's test revealed

that both *E. coli* strains from different sources (colorectal cancer and urine) significantly reduce cell viability and increase cell death, which may confirm the cytotoxic effect of CDT. Due to the limited number of CDT+ *E. coli* isolates, additional assays, such as the comet assay and caspase activation assay are required to confirm the DNA damage.

Table 4: Sidak's multiple comparisons test for comparing cell counts in different sample groups.

Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff	Significant	Summary	Adjusted P Value
Viable (Colorectal cancer vs. Urine)	-11.40	-49.51 to 26.71	No	ns	0.9350
Viable (Colorectal cancer vs. Control)	-391.0	-429.1 to -352.9	Yes	****	<0.0001
Viable (Urine vs. Control)	-379.6	-417.7 to -341.4	Yes	****	<0.0001
Dead (Colorectal cancer vs. Urine)	40.40	2.292 to 78.52	Yes	*	0.0354
Dead (Colorectal cancer vs. Control)	188.1	150.0 to 226.2	Yes	****	<0.0001
Dead (Urine vs. Control)	147.7	109.6 to 185.8	Yes	****	<0.0001

Whole-genome sequencing of the CRC isolate

Sequencing results of the CRC isolate reveal several virulence genes in *E. coli* that could potentially act synergistically with CDT in inducing DNA damage in a normal cell line, leading to apoptosis of host cells. These virulence factors were *fimH, chuA, hlyE, gad, kpsMII_K5, traT, irp2,* and *iutA.* The effect of CDT toxin on the DNA requires other virulence factors for it to mediate the entry of CDT into host cells and thereby evade the immune system response [17, 38]. These virulence factors are: Adhesion factors, such as *fimH*, help secure the bacteria on epithelial surfaces, which is necessary for efficient toxin delivery, and iron acquisition systems (*chuA, irp2, iutA*) sustain bacterial survival and replication, indirectly enhancing CDT's impact on host cells.

4. Conclusion

This study highlights the potential cytotoxic and genotoxic effects of CDT-producing *E. coli* on normal cells. The detection of the CDT gene in *E. coli* isolated from stool and colorectal cancer samples suggests an association between these bacteria and colorectal carcinogenesis. CDT+ *E. coli* isolates show their effect on NHF by staining with AO/PI. The fluorescent intensity demonstrates cell viability, suggesting that the CDT plays a role in membrane integrity and general cell viability, which may be caused by DNA damage, depending on the presence of other virulence factors that promote adhesion, iron uptake, and immune evasion. As mentioned previously, this is a pilot study. Further research is necessary using a larger number of CDT+ *E. coli* isolates to explore the molecular mechanisms underlying CDT-mediated genotoxicity and its implications in human health. This should include experiments involving different normal cell lines and assays such as the comet assay to assess DNA damage more precisely.

Conflict of Interest

There are no conflicts of interest between authors.

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