Iraqi Journal of Biotechnology, 2025, Vol. 24, No. 3, 255-263

Study the Toxic Effect of Colibactin Produced by Polyketide Synthase *Escherichia coli*

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Received: September 16, 2024 / Accepted: November 10, 2024 / Published: November 3, 2025

Abstract: Polyketide synthase island, also known as pks island, is a gene cluster that is present in *Escherichia coli* and this island is responsible for the creation of the genotoxin colibactin. Additionally, it has the capability to generate severe DNA distortions and damage, ultimately play a role in the progression of cancer. This study is aimed to investigate the genotoxic effect of colibactin toxin produced by pks+ *E.coli* isolates. One hundred and twenty *E. coli* isolates were obtained from various clinical sources and analyzed. A total of 63 samples tested positive for *E. coli*, representing 52.5% of the overall amount. The phenotypic and genotypic characteristics of the colibactin toxin was detected by subjecting isolates of different sources to tissue culture (Normal human fibroblast cells-NHF) and PCR assays, while utilizing a various biochemical tests. The pks+ isolates were assessed for their cytotoxicity using the (AO/PI) double staining procedure and visualized under a fluorescence microscope. The outcomes indicated that of the 63 isolates examined, only 33 were determined to be *ClbA+ E.coli*, whilst 42 out of 63 were identified as *UidA+ E.coli* by the use of traditional PCR. The NHF cells that were infected with pks+ *E.coli* sustained DNA damage due to the presence of colibactin produced by pks+ *E.coli* and resulted killing of 83.3% NHF cells.

Keywords: pks island, colibactin, NHF, genotoxic, toxin.

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Introduction

Several strains of the intestinal bacteria *Escherichia coli* carry a gene cluster that is capable of producing a secondary metabolite known as colibactin (Clb), the structure of which has been partially identified (1). Clb has been defined as a cyclomodulin produced by *E. coli*. It clings to the cells lining of the intestines and operate as a toxin. (2). In 2006, Nougayrede and his colleagues originally discovered Clb and rapidly discovered it as a toxin. Clb is a naturally-occurring genotoxic substance that is encoded, manufactured and maintained by the non-ribosomal polyketide synthases (NRPKS) island (3) and is considered one prominent virulence factor of *E.coli* (4). Studies have suggested that the pks island is present in around 34% of commensal isolates from phylogenetic group B2 in individuals who are in good health (5, 6). The human gastrointestinal microbiome is home to commensal strains of *E.coli*(7), that hold the pks island. These

strains display the capacity to endure in the colon for prolonged durations, stretching from years to even decades(8). The Clb gene cluster, responsible for amplifying DNA damage and inducing cell cycle arrest in mammalian cells, is encoded by the 54-kb genomic size of the Pks Island. (9, 10), Clb has been confirmed to induce cell cycle disruption specifically in the G2/M phase, as well as chromosomal abnormalities and DNA double strand breaks (11). The biosynthesis of Clb involves the assembly line of NRPSs and PKSs, that lead to the production of an inactive form of precolibactin (12). The *ClbQ* thioesterase modulates the synthesis of Clb and its genotoxic effects by eliminating precolibactin biosynthetic intermediates from the biosynthesis pathway. (13). The prodrug is discharged into the periplasmic region after precolibactin production is complete by the multidrug and toxic compound extrusion (MATE) transporter, *ClbM*(14). Eventually, the *ClbP* peptidase converts precolibactin into mature Clb once it reaches the periplasmic region (15). As an extra layer of defense, the pks island contains the genetic code for the *ClbS* resistance protein, which is a cyclopropane hydrolase that renders Clb inactive in the bacteria responsible for its production (16). This proves beyond a reasonable doubt that pks genomic island–carrying *E. coli* strains may promote colon carcinogenesis (17). Recent research has shown that the synthesis of Clb is at its highest in anoxic circumstances and tends to decrease as the level of oxygen in the environment increases (18). The expression of Clb is also increased by oligosaccharides, which are known to regulating the metabolism of bacteria(19).

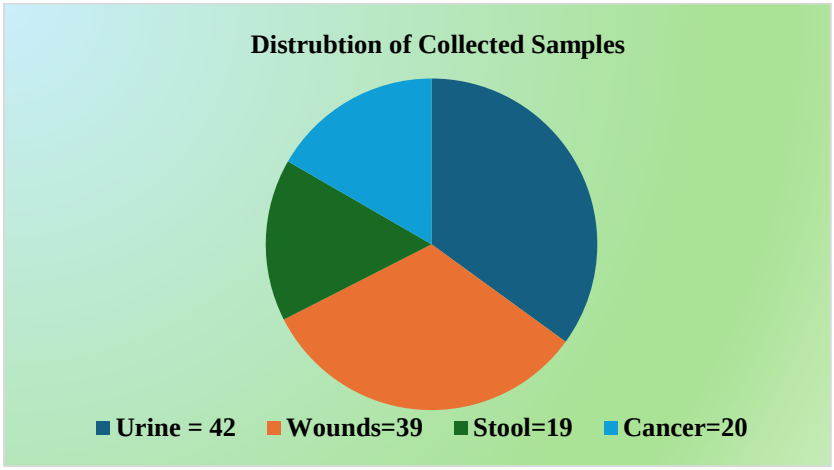

Figure (1): Distribution of collected samples from different sources. Urine, Wounds, Stool and patients with Colorectal Cancer

Additionally, the *UidA* gene is commonly utilized to verify the existence of *E. coli* isolates, as it encodes the enzyme β-glucuronidase (GUS), a distinctive marker unique to the majority of *E. coli* strains. Such prevalence is uncommon in other species, hence offering exceptional specificity for *E. coli* (20).

The purpose of this study was to examine the effect of Clb genotoxin on chromosomal abnormalities of human cells.

Materials and Methods

Ethical Approval

This study was approved by the ethical committee of the Biotechnology Department college of Science/

University Of Baghdad according to the Ref number (CSEC/0224/0020).

Sample Collection

A total of 120 samples were gathered from various sources, which included wounds, urine, stool and individuals diagnosed with colorectal cancer. The distribution of the clinical samples is shown in (Figure 1), representing data collected from the Medical City and Yarmouk Hospital between December 2023 and April 2024. The samples were obtained by the process of cultivating in BHI broth. By utilizing selective synthetic media, specifically MacConkey Agar and Eosin Methylene blue (EMB). Moreover, chemical testing methods including Indole, Urease, and Simmon Citrate were applied for identification of the samples. In addition, further validation was accomplished by a set of specific primers for the *ClbA* and *UidA* genes through the deployment of polymerase chain reaction (PCR) methods.

Molecular Detection of *E.coli ClbA* and *UidA* genes

DNA Extraction

The bacterium's genomic DNA was obtained using the Bacterial DNA Extraction kit (Elk, China) according to the manufacturer's instructions without any alterations. The DNA was isolated, refined, and stored at a temperature of -20°C for further use.

Conventional PCR Technique

By employing conventional PCR, the *ClbA* and *UidA* genes were detected. The primer sequence used in the present study is shown in (Table 1). The 20 µl reaction mixture included 10 µl of Master Mix, 1 µl of forward primer (10 pmol), 1 µl of reverse primer (10 pmol), 2 µl of DNA template (30 ng), and 6 µl of Nuclease-free water. The Thermal Block was the tool used to conduct the PCR. The PCR technique for gene identification first started with a single cycle lasting 5 minutes at a temperature of 95 °C, with an initial denaturation step. In turn, the respective genes (*ClbA* and *UidA*) were subjected to 40 denaturation cycles at 95°C for 30 sec, followed by annealing at 58°C for 30 sec. Then, a 30-second extension period was implemented at a temperature of 72°C. Finally, a single cycle of extension was performed at a temperature of 72°C for a duration of 7 minutes. The PCR results was then displayed on agarose gel of 1.5% concentration, that contained ethidium bromide and was exposed to ultraviolet light (UV).

Table (1): The primers that were used this study for the detection of *ClbA* and *UidA* genes through conventional PCR.

	Primer sequence 5′→3′		Product size (base pair)	Temp.	Reference
ClbA	**F**	ATCATGGCAATTTAGTACGTGCAA	250 bp	58	This study
	R	TTGACCTTCATAACGAGGAAGTG			
UidA	**F**	AAATCGGCGAAATTCCATACC	333 bp	58	This study
	R	CGTCAATGTAATGTTCTGCGA			

Maintaining and cultivating cell cultures

Normal human Fibroblast (NHF) cells are a cell line derived from a biopsy sample of human adipose tissue and were acquired from the Iraqi Biotech Cell Bank Unit in Baghdad, Iraq. The cells were cultured in Minimum Essential Medium (MEM) because it provides a balanced mix of essential amino acids, vitamins, and minerals that are necessary for the growth and maintenance of fibroblasts. Enriched with 10% fetal bovine serum, 100

units/mL penicillin, and 100 µg/mL streptomycin in addition to amphotericin B (2.5 µg/ml) as a fungicidal agent. The cells were propagated using Trypsin-EDTA and replanted when they reached 80% confluence twice a week. The cells were then cultured at a temperature of 37 °C, with a CO2 concentration of 5% and atmospheric oxygen levels (21).

Apoptosis Estimation (propidium iodide (PI) / (AO) Acridine orange staining assay)

The Acridine orange / propidium iodide (PI/AO) double staining method was implemented to evaluate the capacity of Clb, produced by pks+ bacterial isolates of *E. coli*, to promote cytotoxicity and fragmentation of double-stranded DNA (DSBs) In healthy NHF cells. The apoptotic levels in both infected and control cell lines were quantified using the AO/PI staining method. The plate was first seeded with 5000 cells per well, which were then infected with (+pks bacterial isolates of *E. coli*) for a duration of 24 hrs in a 37 °C incubator. Following the incubation period, the cells were rinsed with a solution of 1X phosphate-buffered saline (PBS). For the conventional method of using two different dyes to stain a sample, the wells that were examined were given precisely 50µl of the AO/PI stain combination (At room temperature of 25°C) for a duration of 30 sec. Subsequently, the stain was eradicated. The cells that were infected with pks+bacterial isolates were used as a positive control, whereas the uninfected cells served as a negative control. The photos were captured with a Leica DM 5000B epifluorescence imaging system microscope. fluorescence intensity is quantified by means of fluorescence microscopy and analyzed utilizing image J software.

Statistical Analysis

The obtained data were statically analyzed using an unpaired t-test with GraphPad Prism 6. The values were presented as the mean ± SD of triplicate measurements.

Results and Discussion

Bacterial Samples

A total of 120 clinical samples were obtained and identified using the MacConkey agar medium. The results indicated that among the 120 samples. A total of 63 samples tested positive for *E.coli*. The samples that were detected showed a positive growth of *E. coli*, which displayed very noticeable pink colonies on MacConkey agar. Afterwards, the culturing process was extended with EMB. *E. coli* displayed green colonies with a rich green metallic sheen. After that, additional biochemical assays were performed on the isolates, which included urease, indole and Simmon citrate. The results for indole were positive for *E. coli*, but the results for simmon citrate and urease were negative.

Polymerase chain reaction (PCR) assay detection of *UidA* and *ClbA* genes

The presence of the *UidA* gene in all detected *E. coli* samples was verified using the PCR method. The results revealed that *UidA* gene was detected in 42 isolates, accounting for 66.6% of the total. These isolates had a product band with a size of 333 base pairs (bp) as depicted in (Figure 2). Furthermore, the same *E.coli* isolates were examined for the presence of the *ClbA* gene by the use of Conventional PCR amplification. The investigation found that the *ClbA* gene was detected in 33 isolates, representing 52% of the total samples. These isolates showed a product band with a size of 250 bp as shown in (Figure 3).

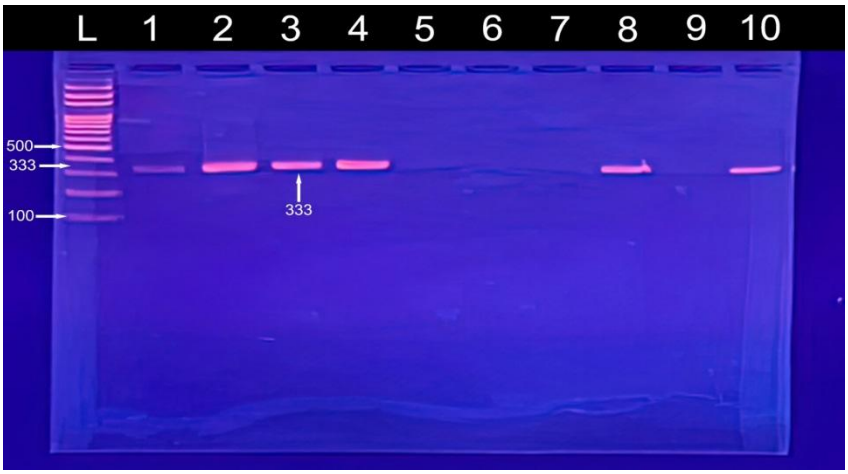

Figure (2): *E. coli UidA* gene PCR products on Agarose Gel Electrophoresis (333 bp). UV-Light 1.5% Agarose gel electrophoresis stained with Ethidium Bromide. 60 min at 70V. 100-bp ladder marker.

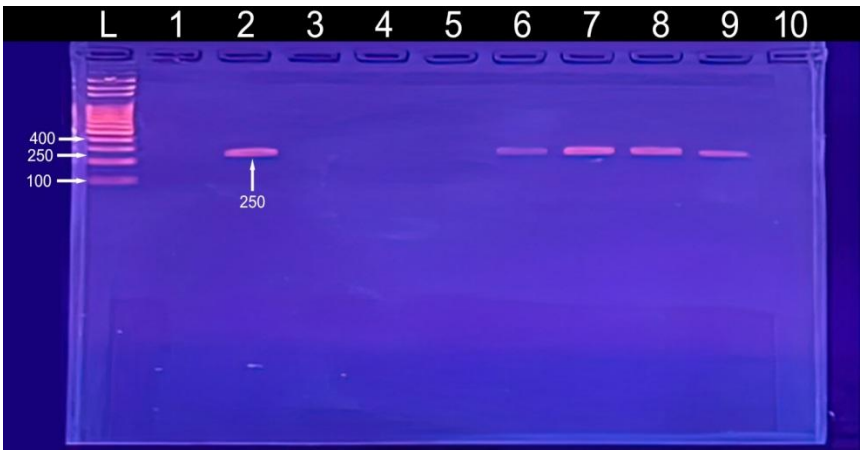

Figure (3): *E.coli ClbA* gene (250 bp) PCR amplified products on Agarose Gel Electrophoresis. UV-Light. 1.5% Agarose gel electrophoresis stained with Ethidium Bromide. 60 min at 70V. 100-bp ladder marker.

The cytotoxic effects of pks+ *Escherichia coli* on normal human fibroblast (NHF) cells

The infection of the NHF cell line was conducted using three samples. Sample A: was an untreated sample which served as a control to demonstrate the healthy cell state, sample B was performed using *E. coli* isolate obtained from wounds, whereas sample C was performed using *E. coli* isolate obtained from colon cancer. Both sample B and C went through testing to assess the possible cytopathic effects of Clb. The results showed in Figure 4-A, displayed healthy untreated NHF cells with a vibrant green color, indicating a high concentration of AO and PI stains. As none of the cells exhibit DNA damage. Next, Figure 4-B indicated that NHF cells had an average level of viable cell count, which was less stable and showed greater damage compared to the situation depicted in figure 4-A. The yellowish/greenish coloring indicates a somewhat negative effect of Clb, while yet preserving a slightly viable state. Lastly, Figure 4-C clearly depicts the

NHF cells in a severely damaged condition, showing a significant reduction in the number of viable cells. (PI), a dye that only enters cells with damaged membranes, serves as a definitive indicator of the existence of dead or advanced apoptotic cells. The NHF cells suffered damage by Clb, When PI is added to DNA, it causes the DNA to emit a reddish to orange/yellowish fluorescence. This was observed in this particular case with the presence of Acridine Orange dye. Within the RNA interaction system, AO emits an orange fluorescence, whereas in the case of DNA, AO fluoresces in green color. AO is able to permeabilize the outer cytoplasmic membrane and gets lodged within areas of the cells characterized by acidity, which causes intense orange coloration of the damaged structures when viewed under a blue light.

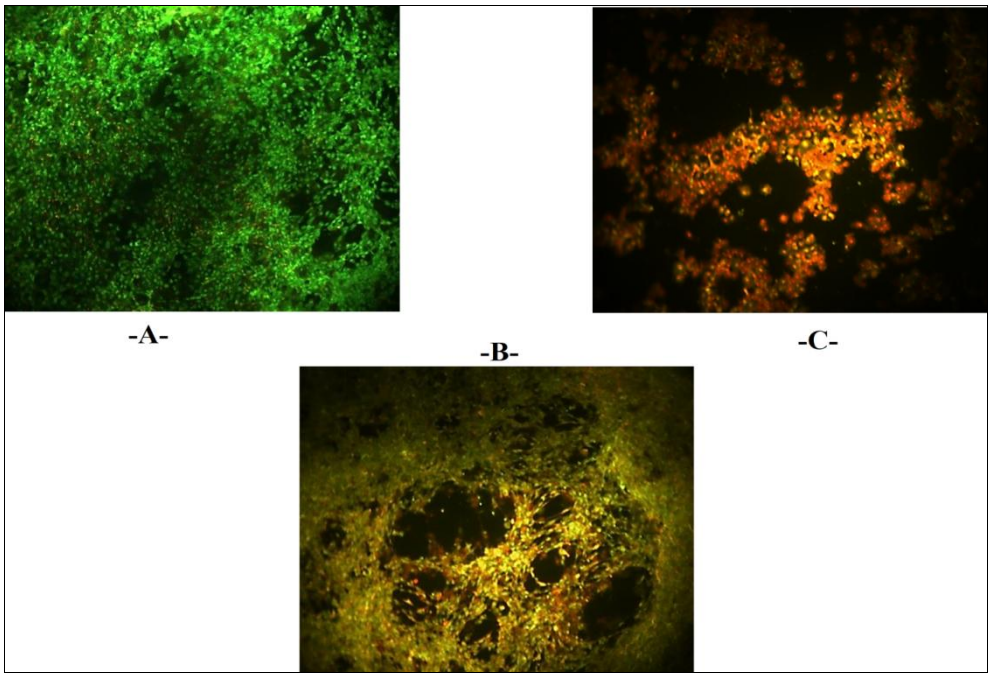

Figure (4): Showed several NHF cells exhibiting varying degrees of DNA damage caused by pks+ *E.coli* isolates. (Sample A): Served as control which was untreated. (Sample B): infected with pks+ *E.coli* derived from wounds. Lastly, (Sample C): infected with pks+ *E.coli* derived from colon cancer.

Apoptotic Assay on NHF cells, infected with pks+ *E. coli*

NHF cell cultures were subjected to a 24-hour exposure to two isolates of *E.coli* under optimal conditions, the fluorescence intensity of AO/PI was measured in (Figure 5). The findings indicated that the wound sample had a modest cytotoxic impact on the NHF cells while still preserving a large number of viable cells. The sample of colon cancer had a significant cytotoxic impact, resulting in substantial damage and a decrease in viable cells, ultimately causing their death, with a relatively low count of viable cells remaining. Finally, the control sample was used to demonstrate the typical condition of the viable cells in contrast to the impact of our cytotoxic isolates.

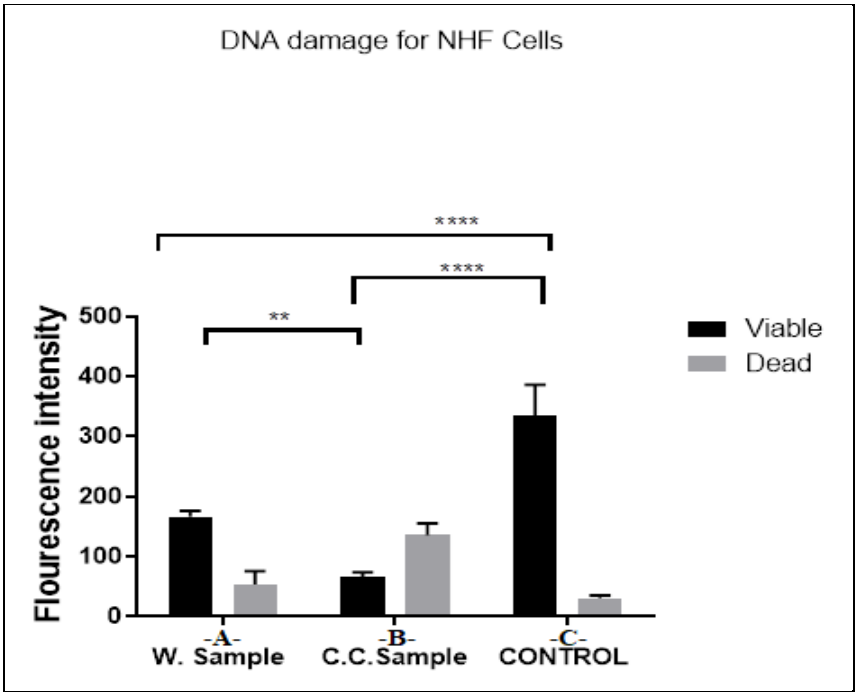

Figure (5): DNA damage for NHF Cells, infected with wound sample, cancer sample and a control sample.

Figure (5) Shows the varying impact of different samples on NHF cells measured by the intensity of the fluorescence of AO/PI stains specifically. Depending on the levels of fluorescent intensity of AO/PI stains inside the infected NHF tissue, these results can be interpreted as; Clb's ability to cause genetic changes and DNA damage as a result of destroying the cellular consistency of wound and cancer infected NHF cells. The harmful impact of Clb was demonstrated by comparing AO/PI staining fluorescence patterns of infected and control (uninfected) NHF cells, when AO is attached to double-stranded DNA, it produces green fluorescence (22). Alternatively, when it is attached to single-stranded DNA or compromised tissue it causes them to emit a red fluorescence (23). On the other hand, PI is a dye that cannot enter cells with intact membranes and only enters cells with damaged membranes and produces a red fluorescence (24). Thus, by comparing the fluorescence patterns of infected and uninfected NHF cells, the findings indicated that the existence of the genotoxin led to harmful effects on the infected cells. It alkylated DNA, leading to the formation of DNA cross-links and strand breaks. This damage triggered a DNA damage response in the infected cells, resulting in various biological effects that included cell cycle arrest and programmed cell death (apoptosis). Thus, Sample "A" obtained from wounds demonstrated a moderate level of DNA damage, affecting 27.78% of NHF cells as shown by the fluorescence intensity data. The sample labeled "B" obtained from a patient with colonic cancer showed significant DNA damage in 83.3% of the NHF cells. Finally, Sample C, which consists of untreated NHF cells, has a normal cellular condition. The study of (25) showed significant damage of Clb produced by different pks+ *E.coli* strains on different cell lines which agrees to our results. While another recent study on mice reported that Clb producing *E.coli* strains were able to generate

tumors on variable levels (26). The capacity of Clb-containing *E. coli* strains to induce in vitro infection in cultured human cells and mice models caused the development of DSBs leading to cell cycle arrest and disruption of intestinal epithelial homeostasis which promotes tumor progression.

Table (2): Comparison of Viable Cell Counts Across Different Sample Groups Using Sidak's Multiple Comparisons Test.

Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary
W. Sample vs. C.C.Sample	101.4	38.14 to 164.7	Yes	**
W. Sample vs. CONTROL	-169.1	-232.4 to -105.9	Yes	****
C.C.Sample vs. CONTROL	-270.6	-333.9 to -207.3	Yes	****

Conclusions

This study identified the presence of the Clb genotoxin and its impact by exposing NHF cells, which were labeled with AO and PI , by applying *E.coli* isolates retrieved from cancer and wounds. The fluorescence intensity served as an indicator of the condition of the NHF cells, distinguishing between healthy and damaged tissue. This allowed for the identification of Clb that was responsible for causing DNA damage and DSBs in the tissue.

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