Prevalence and Characterization of Some Colibactin Genes in Clinical Enterobacteriaceae isolates from Iraqi Patients

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Abstract:
The members of the family of Enterobacteriaceae harbour a gene cluster called polyketide synthase (pkS) island. This cluster is responsible for the synthesis of the genotoxic colibactin that might have an important role in the induction of double-strand DNA breaks, leading to promote human colorectal cancer (CRC). Eleven out of the eighty eight isolates (12.5%) were pkS+, distributed as 7 (8%) isolates of E. coli, 2 (2.25%) of K. pneumoniae and 2 (2.25%) of E. aerogenes. The cytotoxic effects of selected pkS+ isolates (E. coli and E. aerogenes) on HeLa cells were represented by decreasing cell numbers and enlarged cell nuclei in comparison to the untreated cells. Cytological changes were observed when the infected HeLa cells cultures were stained with AO/EBr and visualized under fluorescent microscope. Some changes that happened in the color of the nuclear chromatin were accompanied by DNA condensation and degradation and fragmentation of nuclei. HeLa cells with green unchanged nuclear chromatin were alive while those with orange-dark and bright red nuclei were dead. It was concluded that a proportion of the Enterobacteriaceae isolates from Iraqi patients was pkS+, which exerted cytotoxic effects upon using them to kill HeLa cells. In this study the microscopic observation of the cell morphology reveals the cellular response to the genotoxic insult, with reduced numbers, striking giant cells phenotype (megalocytosis) and fragmentation of nuclei due to the cell cycle arrest and cellular senescence.

Key words: Colibactin (pkS Island), Cytotoxicity, Enterobacteriaceae, Genotoxin, Prevalence

Introduction:
Colibactins are natural genotoxic small molecules of unknown structure, produced by human normal intestinal microbiota. These molecules are considered as secondary metabolites, and their biosynthesis is encoded by specific gene cluster which was firstly identified and characterized by Oswald with many co-workers during 2006 in an extra-intestinal pathogenic Escherichia coli strain (ExPEC) isolated from neonatal meningitis (1,2). Epidemiological studies and reports showed that colibactin can also be produced by extra-intestinal pathogenic strains of Enterobacteriaceae members including Klebsiella pneumoniae, Enterobacter aerogenes, Citrobacter koseri and was also found in Pseudovibrio spp. associated with sponges isolated from marine water (3,4). The biosynthesis of this bacterial toxin is achieved by the enzymes Polyketide synthases (PKSs) and non-ribosomal
peptide synthases (NRPSs). The major biosynthesis of these secondary metabolites is encoded by the clb A-S genes found in the 54-kb genomic pks island and any mutation in these genes except clbS results in a decreased in or loss of the genotoxic activity (5,6). The importance of this gene cluster in colibactin synthesis is represented by clbA, a phosphopantetheinyl transesterase-encoded gene (PPTase); and clbP, a D-amino peptidase; which are required for the biosynthesis and maturation of colibactin, respectively (7). The mechanisms of mode of action are poorly identified, and characterization of its structure remains partially elusive, though a previous study of Brotherton and Balskus (8) demonstrated that the assembly pathway of colibactin as a prodrug is mediated with NRPS-PKS biosynthesis machinery with an extended side chain on N-acyl-D-aspartagine. The precursor (precolibactin) is then translocated into the periplasm by clb M transporter, and the tether is removed by clbP. The production of colibactins was associated with pathogenicity and cancer. They are considered as virulence factors, immunomodulators, mutualistic factors, and antimicrobial agents, with reported anti-inflammatory and analgesic effects (9,10). This natural genotoxin induces the breakdown of double-stranded DNA, chromosome aberrations, and cell arrest in the G2/M phase in the eukaryotic host cells (2,11). Interestingly, The members of Enterobacteriaceae such as E. coli and K. pneumoniae harbouring pks were isolated from several clinical cases such as newborn meningitis (12), commensal bacteria found in human and animal intestinal tracts (13), patients with urinary tract infections (14), and haemo culture (septicemia) (7,15). In addition, E. coli harboring pks are isolated from colorectal cancer (CRC) and they could promote human CRC development (16). It was revealed that the ability of K. pneumoniae strain 1084 harbouring pks cluster to damage DNA in vitro and in vivo is significantly demolished when clbA was knocked out (12). However, it was reported that clbP can ease the harmful effect of this toxin in vitro and dramatically decreases the tumor number in vivo (17). This works aimed to investigate the prevalence of some genes responsible for the production of colibactin among the clinically isolated Enterobacteriaceae infected patients in Iraq. Additionally, determined the ability of the harbouring isolates of the pks genes to induce in vivo cytotoxic effects and genetic damage on the HeLa cell line.

Materials and Methods:

Bacterial isolation and Identification

For the isolation of Enterobacteriaceae, various clinical specimens (stool, urine, blood and wound swabs) were collected from different patients to avoid duplication. All isolates were collected during eight-month period from April 2018 to January 2019, from selected medical centers and hospitals that were (Ibn Al-Baladi Hospital for Children and Women, Educational Laboratories/ Medical City and Central public Health Laboratory), in Baghdad/Iraq. The collected Enterobacteriaceae isolates were further identified and characterized to species level using several approaches like morphology and colony characters. Several types of culture media were used for isolating and purifying which include (Nutrient agar, MacConkey agar, Eosin-Methylene blue agar and Blood agar Simmon citrate agar). All bacterial isolates undergone biochemical tests, physiological characters, API 20E identification system (BioMérieux, La Balme-les-Grottes,France) and were finally confirmed by VITEK-2 system.

DNA Extraction and manipulation

Total genomic DNA for all Enterobacteriaceae isolates was extracted and prepared using Wizard genomic DNA purification Kit (Promega, USA). Next, 2-4 colonies of each isolate was picked up and incubated in 1 ml Luria-Bertani broth (LB) medium and shaken at 180-220 rpm for 8 h at 37 °C according to the manufacturer’s recommendation for Gram-negative bacteria, with the exception that DNA was rehydrated with 10 mM Tris-HCl (pH 8.0). The quality and quantity of the extracted DNA were assessed by gel electrophoresis and spectrophotometer (NanoDrop; Thermo Fisher) respectively. The extracted DNA was stored at freezing (−20 °C) and used as a template for subsequent amplifications and detection of pks genes. The presence of colibactin genes (pks) among the collected clinical Entrobacterial isolates was determined using Polymerase Chain Reaction (PCR) technique. The primers used for the amplification of the tested clbA, B, N and Q genes are listed in Table 1. PCRs were performed using a Hot Start Taq Kit (Qiagen, Limubing, Netherlands) in a 25-µl reaction volume containing 1 pmol µl-1 of FWD and R primers, 1 X buffer, 1 X Hot Start Taq buffer mix, 1 µl DNA, and distilled water (dH2O). PCR conditions were 95 °C for 15 min by 35 cycles at 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 90 s, followed by 1 cycle of 72 °C for 10 min. PCR products were detected following the separation by electrophoresis using 1.5% agarose gel. Bacterial isolates were considered as pks+ after
Table 1. Primers used for amplification of the tested clb genes

| clb  | Sequences                      | PCR product size (bp) | Reference |
|------|--------------------------------|-----------------------|-----------|
| clbA | clba forward 5′-CTAGATTATCCGTCGGCGATTC-3′ | 1002                  |           |
|      | clba reverse 5′-CAGATTACAGATACCATTCA-3′ |                       |           |
| clbB | clbB forward 5′-GATTGAGAAGCAGCAGATCCAC-3′ | 550                   | [9]       |
|      | clbB reverse 5′-CAATCCTTCTGGTTAGCAGAC-3′ |                       |           |
| clbN | clbN forward 5′-GTTCGTCCTCGAGATGATATCC-3′ | 700                   |           |
|      | clbN reverse 5′-GATTTGGATACTGGCGATAACCG-3′ |                       |           |
| clbQ | clbQ forward 5′-CTTGTATAGTACACAACATTTC-3′ | 821                   |           |
|      | clbQ reverse 5′-TTATCCGTTAGCTTTCGTC-3′  |                       |           |

**Antimicrobial susceptibility test**

All bacterial isolates pks+ were assayed by Kirby-Bauer disk diffusion method for antimicrobial susceptibility to the following commercially available antibiotic disk (14 antibiotics) obtained from (Bioanalyse-Turkey), Amikacin (AK 15 µg), Cefotaxime (CTX 30 µg), Gentamicin (GN 10 µg), Ceftriaxone (CRO 30 µg), Imipenem (IMP 10 µg), Meropenem (MEM 10 µg), Ciprofloxacain (CIP 5 µg), Pipericillin (PRL 100 µg), Cefazidime (CAZ 30 µg), Norfloxacin (NOR 30 µg), Chloramphenicol (C 30 µg), Nitrofurantion (NI 30 µg), Cefepime (CPM 30 µg) and Tobramycin (TOB 10 µg). The susceptibility test was done using Mueller Hinton agar (MHA) according to Clinical laboratory Standard Institute (CLSI) guidelines (18). Interpretive criteria applied for classification of the clinical isolates were categorized as Susceptible (S), Intermediate (I) and (R) for Resistant group.

**Cytotoxic activity of Bacterial isolates encoding colibactin (pks*) against HeLa cell line (ex vivo)**

**Cell cultures and Maintenance**

HeLa cells were obtained from the Iraqi Biotech Cell Bank Unit, Baghdad, Iraq. These cells were maintained in the culture medium (RPMI-1640) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. The cells were passaged using Trypsin-EDTA and reseeded at 80% confluence twice a week and incubated at 37 °C, 5% CO₂ and atmospheric O₂ (19).

**Genotoxicity Assay**

Genotoxic effect of pks+ bacterial isolates was accomplished according to Bossuet-Greifet al., [20] with some modifications. Bacterial cultures of E. coli and E. aerogenes pks+ and pks-negative (pks−) were activated on LB agar plates, and grown overnight (16–24 h) at 37 °C with shaking 240 rpm. Then bacterial cultures were inoculated into 9.5 ml pre-warmed (37°C) Dulbecco’s modified Eagle medium (DMEM) with 25 mM HEPES in a tube with 0.5 ml of LB medium containing bacterial overnight culture. The culture was grown till OD600 =0.4 to 0.5. The cell viability assay was done using 24 well plates. Cell lines were seeded at 1 × 105 cells/well. After 24 hr or until a confluent monolayer was achieved, the cells were infected with pks+ bacterial isolates of E. coli and E. aerogenes at a multiplicity of infection (MOI) [The number of bacteria per cell at the onset of infection] of 50 for 6 and 24hr (20). The cells were washed three times with 1X PBS. Then, they were incubated with RPMI medium containing 10% FCS and 100 µg/ml gentamycin. Following 48 hr incubation, they were washed with 1X PBS then stained with crystal violet for 15 minutes. The absorbency and dye binding were determined on a microplate reader by measuring the optical density (OD) at 600 nm. The cells infected with pks− bacterial isolates were used as a positive control, and uninfected cells were used as a negative control. The assay was performed in triplicate. The control and treated HeLa cells were observed under an inverted microscope. The Hela cells used as a positive and negative controls were confluent with a normal cells in the number and morphology.

- The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated according to the following equation (19): -

\[
\text{Cytotoxicity} = \frac{A-B}{A} * 100
\]

Where A and B are the optical densities of the control and the tested groups, respectively.

**Acridine orange/Ethidium bromide staining assay**

To examine the capacity of Colibactin produced by pks+ bacterial isolates to induce damage and breaking of ds DNA of HeLa cells, the acridine orange/ethidium promide (AO/EBr) double-staining assay was used. Briefly, HeLa cells were
grown and placed in 96-well plates followed by infection with pks+ bacterial isolates at MOI 50 for 6 and 24 hr. After incubation time, the cells were washed with IX PBS then stained with AO/EBr (10 μg mL⁻¹) and measured within 5 minutes by a BX51 UV fluorescent microscope. This experiment was carried out in triplicate [20].

**Statistical analysis**

The obtained data were statistically analyzed using an unpaired t-test with GraphPad Prism 6. Significant differences between means were analyzed at (p ≤ 0.05) and expressed as (Mean±SD) for triplicate measurements (21).

### Table 2. Different bacterial isolates obtained from different clinical specimens

| Types of bacterial isolates | Types of clinical specimen | Total of bacterial isolates No. | % |
|----------------------------|---------------------------|---------------------------------|---|
| E. coli                    | Stool: 38 Urine: 9 Blood: 1 Wound swab: 3 | 51 | 58 |
| K. pneumonia               | Stool: 4 Urine: 15 Blood: 6 Wound swab: 3 | 28 | 31.8 |
| E. aurogenes               | Stool: 7 Urine: 2 Blood: 0 Wound swab: 0 | 9 | 10.2 |
| Total                      | Stool: 49 Urine: 26 Blood: 7 Wound swab: 6 | 88 | 88 |

**Detection of pks encoding genes among the Enterobacterial isolates**

Existence of clb A, B, N and Q genes in the isolated bacteria was determined. The gene of interest was PCR-amplified using specific primer pairs, and the resulted product for each gene was electrophoresed on 1.5% agarose gel. The DNA gel revealed that clbA was around 1002bp, clbB 550bp, clbN 700bp and clbQ 821bp in length (Fig. 1).

**Results:**

**Occurrence of Enterobacteriaceae in different clinical specimens**

A total of 88 bacterial isolates of Enterobacteriaceae were collected from 285 clinical samples of hospitalized patients having clinically-evident infection (patients with urinary tract infection, diarrhea, and infection of different wounds and blood with bacteraemia and septicemia) in various hospital wards. The results showed that the prevalence of E. coli was 58% (n=51) among the total Enterobacterial isolates, the other bacterial isolates include K. pneumonia 31.8% (n=28), and E. aurogenes 10.2% (n=9), as indicated in Table 2, the highest percentage of bacterial isolates was obtained from stool and urine samples (49 and 26%) respectively. Followed by 6% obtained from wound swab and 7% from blood samples.

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![Figure 1](image_url) 5% agarose DNA gel of clb genes in Enterobacteria, and visualized by UV light. A: clbN(700bp); B: clbA(1002bp); C:clbB(550bp); and D: clbQ(821bp) of E. coli isolates pks+. In this case, Lane M: DNA ladder 10 Kb Molecular Marker (Bioline).

**The prevalence of pks genes among Enterobacteriaceae species**

Based on the results of PCR analyses, it was noticed that a total of 11 (12.5%) out of 88 tested isolates carried all the screened genes for pks+ island (colibactin-positive) (clb A, B, N and Q). The distribution of pks+ among the collected clinical isolates showed that 7 isolates belonged to E. coli, 2 isolates to K. pneumoniae and 2 to E. aurogenes (Table 3).

| Bacteria isolates | No. of bacterial isolates | Pks+ prevalence |
|-------------------|--------------------------|-----------------|
| E. coli (n=51)    | 7                        | 44              | 8               |
| K. Pneumoniae (n=28) | 2                        | 26              | 2.25            |
| E. aurogenes (n=9) | 2                        | 7               | 2.25            |
| Total (88)        | 11                       | 77              | 12.5            |

**Table 3. Numbers of pks+ and pks− Enterobacterial isolates according to source of isolation.**
Table 4 illustrates the numbers of \( pks^+ \) and \( pks^- \) bacterial isolates according to the age of the patients. The highest percentage of \( pks^+ \) isolates was found in the age between 16 to 50 years. The results also showed that the young people and children (age below 15 years) can be infected with bacteria harbouring \( clb \) genes (3 isolates were \( pks^+ \)), while 2 isolates were isolated from patients aged \( \geq 51 \) years.

### Table 4. Numbers of \( pks^+ \) and \( pks^- \) bacterial isolates according the age of the patients

| Age  | No. of Bacterial isolates | \( pks^+ \) prevalence | \( pks^- \) prevalence |
|------|---------------------------|-------------------------|------------------------|
| <=15 | 3                         | 25                      | 3.4                    |
| (no=32) |                      |                         |                        |
| 16-50| 6                         | 29                      | 6.85                   |
| (no=39) |                      |                         |                        |
| >51  | 2                         | 23                      | 2.25                   |
| (no=29) |                      |                         |                        |
| Total| 11                        | 77                      | 12.5%                  |

**Antibiotic sensitivity profile of \( pks^+ \) isolates**

All of the \( pks^+ \) bacterial isolates were examined for susceptibility against 14 antibiotics as shown in Table 5. In total, the highest sensitive level was recorded for imipenem (100%) in all these isolates. Among the 7 \( pks^+ \) of \( E.\ coli \) isolates, 6 isolates were susceptible to nitrofurantion (94%) and norfloxacin (92%). There was also a remarkable level of resistance to ceftriaxone, ceftaxime, piperacilline and tobramycin. Lower resistance level was observed against norfloxacin, ceftriaxone and nitrofurantion. Interestingly, \( E.\ coli \) showed the highest resistance level to norfloxacaxin (96%) and nitrofurantion (90%), whereas, in \( K.\ pneumoniae \) the highest resistance was observed against norfloxacin (96.5%) and netfazidim (93%), and thus it was also observed that \( E.\ aerogenes \) carrying the \( pks \) colibactin gene cluster are highly associated with low antimicrobial resistance.

### Table 5. The antibiotic sensitivity patterns of all \( pks^+ \) isolates

| Antibiotics | \( E.\ coli \) (n=7) | \( K.\ pneumonia \) (n=2) | \( E.\ aerogenes \) (n=2) |
|-------------|----------------------|-------------------------|-------------------------|
|             | Sensitive (%) | Resistant (%)   | Sensitive (%) | Resistant (%) | Sensitive (%) | Resistant (%)   |
| Amikacin    | 4 (57)         | 3 (43)          | 1 (50)         | 1 (50)          | 1 (50)          | 1 (50)          |
| Cefotaxime  | 3 (43)         | 4 (57)          | 2 (100)        | 0 (0.0)         | 1 (50)          | 1 (50)          |
| Gentamicin  | 1 (14)         | 6 (86)          | 0 (0.0)        | 2 (100)         | 1 (50)          | 1 (50)          |
| Ceftriaxone | 3 (43)         | 4 (57)          | 1 (50)         | 1 (50)          | 1 (50)          | 1 (50)          |
| Imipenem    | 7 (100)        | 0 (0.0)         | 2 (100)        | 0 (0.0)         | 2 (100)         | 0 (0.0)         |
| Meropenem   | 1 (14)         | 6 (86)          | 2 (100)        | 0 (0.0)         | 2 (100)         | 0 (0.0)         |
| Ciprofloxac | 2 (28.5)       | 5 (71.5)        | 0 (0.0)        | 2 (100)         | 0 (0.0)         | 2 (100)         |
| Piperacillin| 3 (43)         | 4 (57)          | 1 (50)         | 1 (50)          | 1 (50)          | 1 (50)          |
| Ceftazidime | 2 (28.5)       | 5 (71.5)        | 1 (50)         | 1 (50)          | 1 (50)          | 1 (50)          |
| Norfloxacin | 4 (57)         | 3 (43)          | 1 (50)         | 1 (50)          | 1 (50)          | 1 (50)          |
| Chloramphenicol | 2 (28.5)       | 5 (71.5)        | 0 (0.0)        | 2 (100)         | 1 (50)          | 1 (50)          |
| Nitrofurantion | 3 (43)       | 4 (57)          | 2 (100)        | 0 (0.0)         | 1 (50)          | 1 (50)          |
| Cefepime    | 4 (57)         | 3 (43)          | 2 (100)        | 0 (0.0)         | 1 (50)          | 1 (50)          |
| Tobramycin  | 2 (28.5)       | 5 (71.5)        | 1 (50)         | 1 (50)          | 2 (100)         | 0 (0.0)         |

**Cytotoxicity of \( pks^+ \) Enterobactereae towards Hela cells**

In order to determine the functionality of \( pks \) cluster in \( E.\ coli \) and \( E.\ aerogenes \) in killing HeLa cells, these cells were either exposed to negative \( pks \) isolates and/or positive \( pks \) isolates for two time intervals (6 and 24hr ) under optimal conditions at MOI 50. The results in Fig. 2 show the cytotoxic effect of all isolates on the normal HeLa cell cultures. Notably, only 10-15 % of normal cells were killed after incubation with \( pks^- \) \( E.\ coli \) strain under different times intervals, whereas the percentage of the same cells was increased remarkably (50%) when they were exposed to \( pks^+ \) \( E.\ coli \) strains, and raised up to 80% after 24 hr exposure time (Fig. 2 A and B).

Similarly, \( E.\ aerogenes \) exhibited the same killing pattern towards HeLa cells when they harboured \( pks \) cluster genes (Fig.2 C and D). When normal cells were treated with \( pks^- \) \( Enterobacter \) for 6 hr, they conferred viability of around 90% , however, they cannot get longer when they were exposed for 24 hr with \( pks^+ \) \( E.\ aerogenes \) isolates (killing activity around 75%).
Normal HeLa cell cultures were exposed to 2 isolates of *Enterobactereacea* at MOI 50 for 6 and 24 hr under optimal conditions. A: HeLa cells with *pks*– *E. coli*; B: Hela cells with *pks*+ *E. coli*; C: HeLa cells with *pks*– *E. aerogenes*; and D: HeLa cells with *pks*+ *Enterobacter*. Data expressed as mean±SD. *P*<0.05, **P**<0.01.

**Induced megalocytosis in response to *E. coli* and *E. aerogenes* infections**

In the current study, the efficiency and cytotoxicity of *pks*+ bacterial isolates encoding *clb* genes were examined on HeLa cell cultures at MOI 50. The cells were treated with both *E. coli* and *E. aerogenes* harbouring *clb* genes for 6 and 24 hr. From the microscopic observation of the cellular morphological phenotypes, in this study, the treated HeLa cells revealed some responses to the colibactin; the cytopathic phenotype of this genotoxin was represented by decreasing cell numbers and enlargement of cell nucleus, which could be due to DNA fragmentation and cell cycle arrest, in comparison to the untreated cells (control) (Fig. 3 A and B).
Figure 3. Cell culture assay for cytotoxicity.

(A1, B1) HeLa cells were untreated (negative control), (A2, B2) HeLa cells treated with pks+ E. coli and E. aerogenes (A3, B3) HeLa cells treated with pks– E. coli and E. aerogenes at MOI of 50 for 6 hr incubation in Gentamicin-containing medium. The black arrows in (A2, B2) indicated the morphological changes in treated HeLa cells with pks+ isolates such as enlargement and multinucleation (displayed megalocytosis). Images were taken at 40x magnification using Inverted microscopic.

The results of fluorescence microscopy of HeLa cells treated with bacterial isolates (pks+ and pks–) with AO/EB staining showed different staining patterns of the nuclei. After the treatment of HeLa cells with pks– bacterial isolate E. coli and E. aerogenes (living cells) (Fig. 4 A and D), the nuclear chromatin appeared in different forms of condensation and degradation; the nuclei were green-yellow, yellow-orange to yellow in color. While, in the dead HeLa cells treated with pks+ E. coli and E. aerogenes, the nuclei appeared orange, dark and bright red in color. Following the treatment of control (living cells), the nuclei appeared in green color and without any condensation, degradation or fragmentation (Fig. 4 C and F untreated cells).

Taking the above results together, this indicate that the colibactin-producing E. coli and E. aerogenes were able to decrease the number of infected mammalian cells and generate morphological changes.

Figure 4. Fluorescent micrographs of AO/EBr-double-stained.
(A) HeLa cells treated with pks− E. coli, (B) HeLa cells treated with pks+ E. coli, (C) Control (untreated) HeLa cells, (D) HeLa cells treated with pks− E. aerogenes, (E) HeLa cells treated with pks+ E. aerogenes, (F) Control (untreated) HeLa cells, at MOI 50 as indicated. All treated cells were incubated for 24 hr at 37°C. Scale bar 10μm.

**Discussion:**

The pks gene cluster encodes a number of multi domain enzymes that are responsible for the synthesis of the genotoxin colibactin that typically synthesize secondary metabolites in several types of microbial species. This genotoxin was shown to play an important role in the induction of the damage and breaks in the DNA of host cells. Colibactin may contribute in causing different disease entities, in addition to cell damages and increased virulence in bacteria. A member of the family Enterobacteriaceae is found widely in the gastrointestinal tract of many mammals, including almost all humans. The presence of pks gene in some of pathogenic and non-pathogenic E. coli has been implicated in the induction of the formation of colorectal tumors in animals like mice. Some studies reported that the pks gene was more highly represented in the intestinal mucosa in CRC patients than in non-CRC controls. The presences of pks genes vary according to clinical samples; these genes were found in 20% of the stool samples investigated by (22) and found in 32% of E. coli strains isolated from feces of hospitalized veterans (5). In this study, a total of 88 clinical isolates were obtained from stool, urine and blood, which then be diagnosed as an E. coli, K. pneumoniae and E. aerogenes. The prevalence of pks genes was found in all these isolates. The distribution of colibactin-producing genes is not restricted to such strains; K. pneumoniae strain1084 was shown to promote severe damage to the DNA of liver parancymal cells in BALB/c mice, and the molecular detection revealed that it harbors clbA gene, compared to clbA deletion mutant strain which was unable to persist the infection. In addition, the predominance of pks genes in K. pneumoniae strain1084 is around 25.6 % in Taiwan (3). Moreover, K1 CC23 K. pneumoniae is considered as a hyper virulence strain, due to the presence of pks genes, particularly clbA, which participated in the development of meningitis in BALB/c mice, in comparison to the mutant strain where the production of colibactine is attenuated [8]. In Iran, it was found that the occurrence of clb genes is varied, with 23.23 % is for clbB and 20% for clbN and 13.33 % for both genes in 30 samples of K. pneumoniae isolated from CRC patients (24). Interestingly, it was found that clbB+ E. coli isolated from colonic mucosa of patients with familial adenomatous polyposis (FAP) is able to increase the level of IL-17 in colon and induce DNA damage in colonic epithelium. This suggests the relatedness of colibactin-producing genes to increase and induce tumor in the host cell (25). The indispensable role of clbB and clbN in colibactin synthesis is at the early step of producing pre-drug (N-acetyl-D-Asparagine), which then undergoes protease activity to produce a mature colibactin (15). It was stated that both cob and clone are related to bacteremia, with prevalence percentage of 58 % from blood samples and 23 % from feces (5). This is not consistency with our findings of the occurrence of clb B and clone not only in blood samples, but also in urine and stool specimens. Our results are in agreement with (24), where both genes were isolated from E. coli, K. pneumoniae, E. aerogenes and C. koseri. All of the pks+ bacterial isolates were significantly more susceptible to 10 of the 14 antibiotics tested (Table 5). The present results showed that amongst antibiotics, imipenem was the strongest antibiotic inhibitor for the growth of all pks+ bacterial isolates, also including fluoroquinolons, β-lactam and glycosides. It has been also observed that theses isolates show susceptibility to nitrofurantoin with a percentage of inhibitory effects of 94%. On the other side, ciprofloxacin was the only antibiotic to which there was no significant difference in rates of susceptibility between the pks+ and pks− isolates. There are several studies which reported that the bacterial isolates particularly K. pneumoniae harbouring the pks colibactin gene cluster are considered as highly associated with low levels of antimicrobial resistance, since most of the isolated bacteria belong to K1 and K2 capsular types, as isolates of these capsular types recovered from KLA cases are usually less resistance to other antimicrobial agent. On the other hand, (26, 27) reported that the emergence of multidrug resistance, combined with genotoxicity in hypervirulent K. pneumoniae strains, is worrisome and that there might be more strains of Enterobacteriaceae that will be discovered with the resistance of antibiotics phenotypes. Careful monitoring of isolates with genotoxic colibactin pks gene cluster for acquired antimicrobial resistance is warranted (28, 29). To mimic the genotoxic efficiency of colibactin-producing isolates in vitro, HeLa cell was used. The histological changes of infected HeLa cells were associated with reduction in the number of cells, and enlargement of cells size, compared to the normal cells. In addition, the cytotoxic level of pks+ isolates was determined and observed. The ability of pks+ harboring strains to induce DNA damage was
reported in the studies of (30-33). The HeLa cell culture was exposed to colibactine-producing *E. coli* for 4 hours and stained with a marker for DNA damage. The DNA inter-strand cross link was clearly obvious, and thus those data are in agreement with our results. The cytotoxicity of genotoxin (colibactin) requires live bacteria and direct attachment between the bacteria harbouring *clb* genes and mammalian cells (1, 34). This part supports the hypothesis that the bacteria which have the ability to produce the genotoxin colibactin can play an important role in inducing tumorigensis and development of colorectal cancer.

**Conclusion:**

In the present study, the occurrence and prevalence of colibactin-encoding genes among various clinical bacterial specimens, including those from stool, urine, and blood was confirmed using a PCR technique. The presence of *pk* polyketide clusters in the isolated bacteria spots the light on how these microbes could cause damage to the host cell. One of the most efficient ways to disrupt host cell is by triggering endotoxins, exotoxins and genotoxins. Overall, this pilot study indicates that the *pk* Enterobacteriaceae isolates have the ability to produce genotoxin which may induce damage in the DNA and make a histological transformation. Colibactin is polyketide molecules that is released from pathogenic and non-pathogenic bacteria, and from bacteria related to CRC in particular. There are four genes detected to be involved in the production of colibactin; *clbA, clbB, clbN* and *clbQ* and to play an important role in the biosynthesis of genotoxin. Moreover, our finding indicate that the ability of *pk* members of the family Enterobacteriaceae to persist and colonize the human gut and infect the organs, and their cytotoxicity towards mammalian cells.

**Authors' declaration:**

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

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الخلاصة:

افراد العائلة المعوية تكون مجموعة من الجينات تدعى Polysynthetic polyketide synthase (pks) التي يطلق عليها Colibactin عن تصنيع الذيفان الذي يطلق عليه Colibactin والتي لها تأثير مضر. هذه الجينات تقع ضمن عائلة الـ E. coli E. aerogenes K. pneumoniae وتعود عزلة E. coli E. aerogenes K. pneumoniae في الدراسة. تم اختبار تأثير السم المخلوط في عائلة E. coli E. aerogenes K. pneumoniae التي تشير إلى كسر النواة وانفصال العصريا يشير إلى كسر النواة وانفصال العصريا. استُهلكت النتائج باستخدام ضعف المجهر الفلورسيني. بعض هذه التغيرات تم ملاحظتها في عزلات البكتيريا المعوية المعزولة من مرضى عراقيين يمكنها أن تفرز مواد سامة (ذيفان الكولبكتين) يمكنها أن تكسر كرات الدنا والخلايا، وتسبب تغييرات نسيجية في الخلايا بسبب تغير في العناصر، والتي تشير إلى تأثيرات سامة على الخلايا.