Open Access **Baghdad Science Journal** **P-ISSN: 2078-8665**
2020, 17(3) Supplement (September):1113-1123 **E-ISSN: 2411-7986**

DOI: http://dx.doi.org/10.21123/bsj.2020.17.3(Suppl.).1113

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

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Received 30/1/2020, Accepted 29/4/2020, Published 8/9/2020

Abstract:

The members of the family of Eentrobacteriaceae harbour a gene cluster called polyketide synthase (*pks*) island. This cluster is responsible for the synthesis of the genotoxin 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 Entreobacteriaceae 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, Eentrobacteriaceae, 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 *Pseudovibiro* 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 transeferase-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-asparagine. 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 Enterobacterieaceae, 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 shaked at 180-220 rpm for 8 h at 37 ºC 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 ºC) 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, Limburing, 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 ºC for 15 min by 35 cycles at 95 ºC for 30 s, 53 ºC for 30 s, and 72 ºC for 90 s, followed by 1 cycle of 72 ºC for 10 min. PCR products were detected following the separation by electrophoresis using 1.5% agarose gel. Bacterial isolates were considered as *pks*+ after

qualitative PCR, and, for each clinical sample, *pks* prevalence was defined as the proportion of the number of *pks*+ isolates to that of isolates in that category.

Table 1. Primers used for amplification of the tested *clb* genes

clb genes	Sequences	PCR product size (bp)	Reference
clbA	*clbA* forward 5´-CTAGATTATCCGTGGCGATTC-3´ *clbA* reverse 5´-CAGATACACAGATACCATTCA-3´	1002	
clb B	*clbB* forward 5´-GATTTGGATACTGGCGATAACCG-3´ *clbB* reverse 5´-CCATTTCCCGTTTGAGCACAC-3´	550	[9]
clb N	*clbN* forward 5´-GTTTTGCTCGCCAGATAGTCATTC-3´ *clbN* reverse 5´-CAGTTCGGGTATGTGTGGAAGG-3´	700	
clbQ	*clbQ* forward 5´-CTTGTATAGTTACACAACTATTTC-3´ *clbQ* reverse 5´-TTATCCTGTTAGCTTTCGTTC-3´	821	

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 (Bioanlyse-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), Ciprofloaxacin (CIP 5 µg), Piperacillin (PRL 100 µg), Ceftazidime (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% CO_2 and atmospheric O_2 (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): -

$$\text{Cytotoxicity} = \text{A-B/A} * 100$$

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 ($p \leq 0.05$) and expressed as (Mean±SD) for triplicate measurements (21)**.**

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 Enterobaterial isolates, the other bacterial isolates include *K. pneumoniae* 31.8% (n=28), and *E. aurogenes* 10.2% (no=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.

Table 2. Different bacterial isolates obtained from different clinical specimens

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

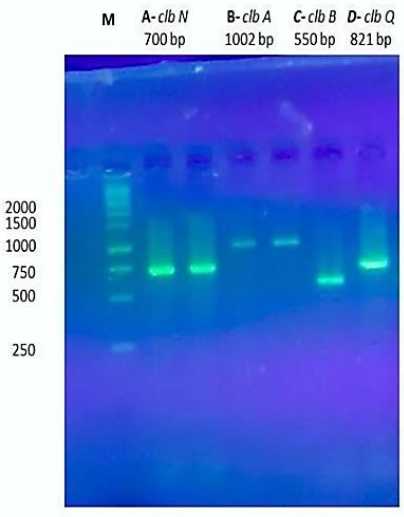

Figure 1, 5% agarose DNA gel of *clb* genes in Enterobacteria, and visualized by UV light. A: *clbN*(700bp); B: *clbA*(1002bp); C:*clbB*(550bp); and D: *clbQ*(821bp) of *E. coli* isolates *pks*+. In this case, Lane M: DNA ladder 10 Kb Molecular Marker (Bioline).

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).

Table 3. Numbers of *pks*+ and *pks*– Enterobacterial isolates according to source of isolation.

Bacteria isolates	No. of bacterial isolates		*Pks*+ prevalence %
	***pks*+**	***pks*–**	
E. coli (no=51)	7	44	8
K. Pneumoniae (no=28)	2	26	2.25
E. aurogenes (no=9)	2	7	2.25
Total (88)	**11**	**77**	**12.5**

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 ≧ 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+	*pks*–	
<=15 (no=32)	3	25	3.4
16-50 (no=39)	6	29	6.85
>=51 (no=29)	2	23	2.25
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 norofloxacin (92%). There was also a remarkable level of resistance to ceftriaxone, cefotaxime, piperacilline and tobramycin. Lower resistance level was observed against norofloxacin, ceftaxidime and nitrofurantion. Interestingly, *E. coli* showed the highest resistance level to norofloaxacin (96%) and nitrofurantion (90%), whereas, in *K. pneumoniae* the highest resistance was observed against norofloxacin (96.5 %) and neftazidime (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)
Ciprofloaxacin	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*+ Enterobactereacae 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 %).

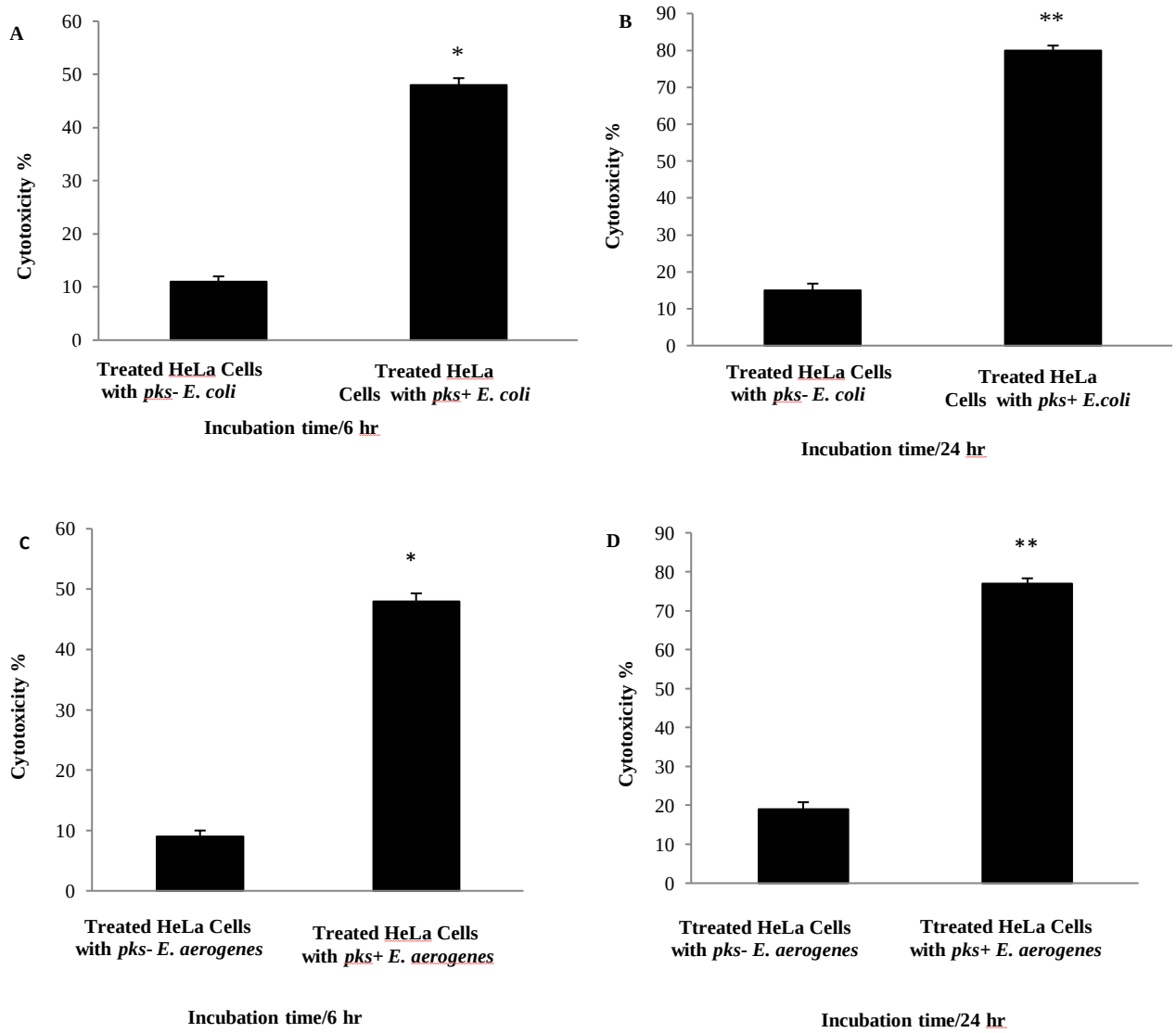

Figure 2. Cytotoxic activity of clb in Enterobactereacea.

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).

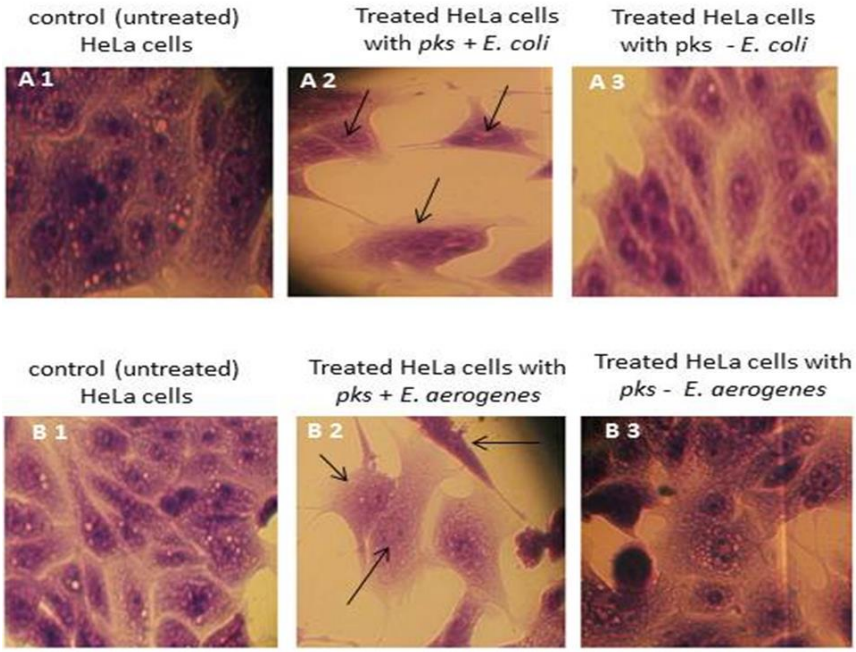

Figure 3. Cell culture assay for cytotoxicity.

(**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 multi-nucleation (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.

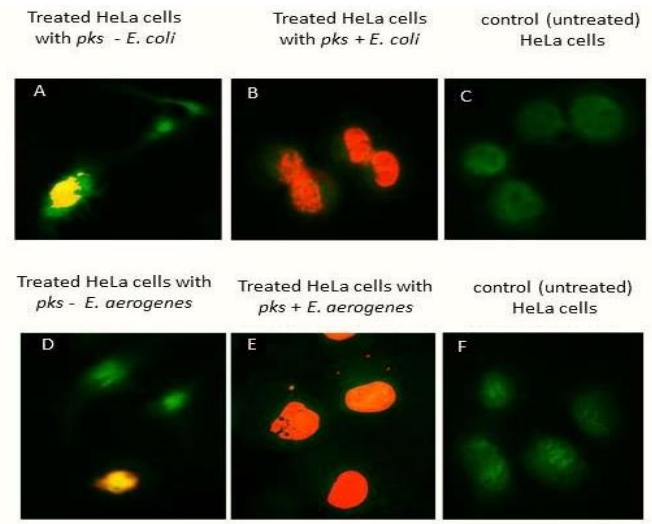

Figure 4. Fluorescent micrographs of AO/EBr-double-stained.

(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. pnuemonia* 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. pnuemoniae strain*1084 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. pnuemoniae* 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-acyl-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 consistence 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, ß-lactam and glycosides. It has been also observed that theses isolates show susceptibility to nitrofurantion 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 Enterobacterial 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 colibactine-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 *pks*-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 *pks*+ 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 *pks*+ 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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Open Access **Baghdad Science Journal** **P-ISSN: 2078-8665**
2020, 17(3) Supplement (September):1113-1123 **E-ISSN: 2411-7986**

انتشار و تشخيص بعض جينات الكولبكتين في العزلات الممرضة في العائلة المعوية المعزولة من مرضى عراقيين

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الخلاصة:

افراد العائلة المعوية تمتلك مجموعة من الجينات تدعى (*pks*) Polyketide synthase. هذه المجموعة من الجينات تكون مسؤولة عن تصنيع الذيفان الذي يطلق عليه Colibactin والذي له دور مهم في استحثاث تكسر اشرطة الدنا المزدوجة DNA والذي يؤدي الى استحثاث ورم او ما يعرف بسرطان القولون، احد عشر من اصل ثمانية وثمانين عزلة بكتيرية وتمثل (12.5%) كانت قد توزعت 7(8%) عزلة تعود لبكتريا *E. coli*، 2(2.25%) عزلة تعود لبكتريا *K. pneumonia* و 2(2.25%) تعود لبكتريا *E. aerogenes* كانت حاملة لجينات الكولبكتين قيد الدراسة. تم اختبار لتأثير السمي الخلوي لعزلتين كانت موجبة للجينات قيد الدراسة وهي *E. coli* and *E. aerogenes* تجاه خط الخلايا السرطاني المعروف بـ HeLa بينت النائج انخفاض عدد الخلايا وحصول استطالة في انوية الخلايامقارنة بالخلايا الغير معاملة. اظهرت النتائج حصول تغيرات نسيجية في الخلايا بأستخدام صبغة AO/EBr تم ملاحظتها بأستخدام المجهر الفلورسيني: بعض هذه التغيرات تم ملاحظتها في لون كروماتين النواة ومصحوب بتكثف الدنا النووي وكذلك حصول تكسر في النوية، خلايا الـ HeLaالتي ظهرت بلون اخضر ولم تحصل فيها اي تغيرات في لون المادة الكروماتينية هي خلايا حية ولم تتم معاملتها مع البكتريا الحاملة للجينات قيد الدراسة، بينما الخلايا المعاملة مع خلايا بكتيرية حاملة للجينات ظهرت انويتها بلون برتقالي داكن وهي خلايا ميتة. يستنتج من ذلك ان عزلات البكتريا المعوية المعزولة من مرضى عراقيين يمكنها ان تفرز مواد سامة (ذيفان الكولبكتين) يمكنها قتل قتل الخلايا السرطانية نوع HeLa وهذا ناتج عن تغيرات حصلت في انوية الخلايا المعرضة للبكتريا وكان واضح في كثافة وتكسر المادة الوراثية للخلايا قيد الدراسة.

الكلمات المفتاحية: الكولبكتين، السمية الخلوية، العائلة المعوية، الذيفانات الجينية، انتشار