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Prevalence and Cytotoxic Effects of Some Colibactin and *cnf* Genes among *Escherichia coli* Isolated from Urinary Tract Infections

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Colibactins (*clb*) and Cytotoxic Necrotizing Factors (*cnf*) are virulence factors that impact cell cycle through cellular differentiation, proliferation, and apoptosis. Urinary tract infections (UTIs) are the most common among type of infection among outpatients, with a lifetime incidence of about 60-65% in adult females. Here, we sought to isolate uropathogenic *Escherichia coli* (UPCE) from urine specimens and investigates the prevalence of *clb* A, B and *cnf* 1, 2 genes among these isolates. A total of 110 *E. coli* isolates were collected from patients with UTIs. All the isolates were examined for their hemolytic activity and only 46 isolates showed a halo zone of hemolysis on blood agar. The collected UPEC isolates were screened for the existence of *clb* A, B and *cnf* genes. The results revealed that out of 110 isolates, 28 harbored the *clb*A gene, 40 harbored *clb* B, and 24 isolates harboured *cnf*1. 13 isolates harbored *clb*A, *clb*B, and *cnf*1 genes, while no *cnf*2 gene was detected among isolates. The molecular detection revealed that 8 out of 28 hemolytic isolates carrying the *clb*A, 11 out of 40 were carrying *clb*B, 1 out of 24 were carrying *cnf* 1, and 5 out of 9 carrying *clb*A+*clb*B. Furthermore, 7 out of 13 isolates were hemolytic and carrying *clb*A, *clb*B, and *cnf*1 genes. Finally, we investigated the cytotoxicity of *E. coli* harboring *clb* and *cnf* genes, eukaryotic REF cells were exposed to *E. coli* producing colibactin, which induces DNA damage and leads to cell cycle arrest, senescence and death.

Keywords: **Colibactin, uropathogenic *E. coli*, cytotoxic effects**

Introduction

Escherichia coli are a group of Gram negative bacteria, facultative anaerobic. Normally found in flora of human and animal gastrointestinal tracts and symbionts participating in digestion and synthesis of vitamin K and B12, which are essential during the blood process and the formation of red blood cells [1]. However, some strains of *E. coli* can also cause intestinal and extraintestinal infections such as urinary tract infections (UTIs). These pathogenic strains carrying several virulence factors that allow them to colonize, invade and cause damage to the host [2, 3]. *E. coli* strains can be classified into the following phylogenic groups (A, B1, B2 and D). Most pathogenic strains of *E. coli* which often encode and produce virulence factors belong to groups (B2 and D). While, another strains of *E. coli* isolated from fecal belong to groups (A and B1) which lack of virulence factors [4].

A group of cyclomodulins is genotoxins and/or cell cycle modulating toxins that contribute to tumorgenesis through modulate cellular differentiation, apoptosis and

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proliferation. These cytotoxins, include Cytolethal distending toxin CTD, cytotoxic necrotizing factors *cnfs*, Colibactin and Cycle inhibiting factor (CIF) [5]. The main function of cyclomodulins is still not completely clear and understood. Reports and studies revealed that cellular modulating effects suggest that they are attributed to the bacterial colonization, persistence and the development of chronic infection [6]. *cnf*1 and Colibactin are more prevalent virulence determinants and almost exclusively closed to phylogroup B2 of *E. coli* that includes extra intestinal pathogenic *E. coli* (ExPEC) [7, 8]. The Extraintestinal pathotypic strain of *E. coli* (ExPEC) is the main causative agent of urinary tract infections and is highly frequent among neonatal meningitis and sepsis [9]. The strains of *E. coli* that produce *cnfs* are belong to the pathotype necrotoxigenic *E. coli* (NTEC), which are associated with the infections of intestinal and extraintestinal in humans and animals. Most of *cnf*s include chromosomally encoded gene *cnf*1, and plasmid-encoded gene *cnf*2 [3, 10]. The size of *cnf*1 is a 115 KDa protein toxin which activates the deamination of Rho GTPase proteins, leading to cytoskeletal alterations and some effects in the cell cycle with subsequent macropinocytosis, inducing specific effect (CPE) known as megalocytosis, induce multinucleation and enlargement.

Previous studies demonstrated that the cytopathic effect is a distinctive phenotypic features for the CNFs and had been differentiated from the β-hemolytic activity of uropathogenic *E. coli* [11]. *E. coli* strains which produce CNFs most notable cause meningeal and UTIs in humans, are also isolated from disease and healthy animals such as cats, dogs, pigs, and birds [3]. Colibactins are a genotoxic hybrid polyketide (non-ribosomal peptide) produced by several members of the family of *Enterobacteriaceae*. It was first discovered in 2006 from the strain of extraintestinal pathogenic *E. coli* (ExPEC) isolated from a case of neonatal meningitis [12]. Colibactins are characterized as secondary metabolites produced by encoding of *clb* A-S genes present in 54-kb pathogenicity *pks* Island. *E. coli* strains carrying colibactin genes *pks*+ demonstrated that induce enlargement of cells and nuclei without mitosis *megalocytosis*, this leads to G2 cell cycle arrest, and inducing DNA double strand breaks in eukaryotic cells [13].

Also, UPEC strains have the ability to produce hemolysin, this toxin plays an important role in the forming of pore in the cell membrane. Hemolysin is produced as either a free toxin or an outer membrane vesicle [14]. Studies showed that (50% (from bacteria that cause UPEC have the ability to produce the enzyme hemolysin [15]. One of the most important ways to detect the ability of bacteria to produce this enzyme is to hydrolyze blood agar medium, and this ability is related to the pathogenesis of *E. coli* strains, especially in acute infection [16]. In Iraq, there are many bladder and gastrointestinal cancer cases were reported. Some initial reports suggested that these cases are due to war radiation and weapons without investigation at cytotoxic and genotoxic levels of bacterial genes. To achieve the goal of our study, we hypothesized that *cnf* and *kps*$^{+}$ are the main factors to increase the cytotoxicity and genotoxicity in human cells through simulating some physiological processes such as differentiation, apoptosis, proliferation, megalocytosis, and cells enlargement.

Materials and Methods

Specimen collection

Bacterial isolates were collected from patient's urine between September 2020 and February 2021 including both sexes of different ages from different hospitals in Baghdad. A total of 485 urine samples were collected, 110 uropathogenic *E. coli* isolates (non-duplicated isolates) were obtained as grown on MacConkey and nutrient agar plates. All isolates were obtained from UTI patients (out and in patients) with their consent of hospital administration for diagnostic uses and drug of choice. Isolates were characterized and identified as *E. coli* by growth on selective media (MacConkey and EMB agar), biochemical and physiological testes, Vitek 2 system as well as EPI system.

Hemolytic assay on blood agar

The hemolytic activity of *E. coli* isolates was carried out by streaking isolates onto Blood Agar Base, Oxoide supplemented with 5% of sterile sheep blood and incubated overnight at 37℃ [17]. Plates were inspected for signs of α-hemolysis a green-hued zone around bacterial colonies, β-hemolysis halo zones around bacterial colonies, or γ-hemolysis no halo zone around bacterial colonies, and the isolates producing halo zone of the hemolysis were considered hemolytic Hly+.

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Antibiotic susceptibility testing

Antibiotic susceptibility assay was performed using the disk diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI), performed on Mueller Hinton agar (MHA) using disk dispenser (Oxoid™) [18]. 12 antibiotics were tested: Amikacin = (AK, 10 mg), Trimethoprim = (Sxt, 75 mg), Cefepime = CPM, 30 μg), Cefotaxime = (CTX, 30 μg), Ticarcillin = (TC, 75 μg), Ampicillin Sulbactam = (SAM, 10 μg), Gentamicin = CN, 10 μg), Imipenem = (IPE, 10 μg), Levofloxacin = (LEV, 5 μg), Meropenem = (MEM, 10 μg), Ciprofloxacin = (CIP, 10 mg) and Trimethoprim = (TM, 5 μg). The bacterial inoculum was standardized to 0.5 McFarland unit using spectrophotometer. The bacterial culture was streaked on the plates of MHA medium. Then, the antibiotic disks were placed on the surface of the agar medium. The plates were incubated overnight at 37℃, the clear zone of inhibition was measured. Interpretation of results was performed according to CLSI recommendations. *E. coli* isolates with intermediate resistance were considered as resistant and were added to the resistance count.

Molecular detection of colibactin *clb*A and B and cytotoxic necrotizing factors *cnf*1 and 2 Genes among *E. coli* Isolates

The prevalence of *clb* A and B and *cnf* 1, 2 genes among *E. coli* isolates was screened by Polymerase chain reaction (PCR), For DNA extraction, each *E. coli* isolates was grown in Luria-Bertani (LB) broth medium at 37 ± 2℃ for 24 h. Genomic DNA was extracted using genomic DNA Extraction Kit supplemented by (Promega, USA), according to the manufacturer's instructions. Purity concentration, and integrity of extracted genomic DNA was measured using Nano drop photometer (IMPLEN, Germany). The extracted DNA was stored at -20℃ until used for gene amplification. Nucleotides of *clb* A and B; *cnf* 1 and 2 genes in the *E. coli* were amplified by using specific primers. All primers for target genes used in the current study were listed in the original studies of [19]. The sequences primers were listed in Table 1. PCR amplifications were performed in a DNA thermal cycler (Applied Biosystem, Germany) according to protocol published by [17]. PCR programmed with cycling conditions: Initial denaturation at 95℃ for 10 min followed by 35 cycles of amplification. Each cycle consisted of a denaturation step at 94℃ for 1 min, annealing at the specified temperature Table 1, for 40 s, and elongation at 72℃ for 1 min. Final extension step was done at 72℃ for 10 min. For visualization of the amplicons, agarose gel electrophoresis was performed and compared with appropriate DNA Ladder: GeneRuler 100 base pair or Gene Ruler 100 bp plus (Thermo Fisher Scientific™, UK).

Cell culture of cytotoxicity assays for colibactin

The cytotoxicity assay was used to investigate the genotoxicity of *E. coli* bacteria bearing *clb* and *cnf* genes by a short infection of cultured mammalian cells such as Rat Embryonic Fibroblast cells with *E. coli*. This assay was performed with some modifications according to [3, 20]. The REF cells were attained from the Biotechnology Research Center, Al-Nahrain University, and these cells were maintained in RPMI-1640 complemented with 10 % fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml Streptomycin at 37℃ with 5% CO_2. The 1×10^5 cells were seeded onto 96-well tissue culture plates and incubated at 37℃ with 5% CO_2 for 24 h. After incubation, cells have been washed 3 times with PBS.

Table 1. Primers used for amplification of the *clb* and *cnf* genes and their sequences.

Primer name	Sequence	Annealing temperature (℃)	Amplicon size (bp)	Reference
*cnf*1-F	5'-GGGGGAAGTACAGAAGAATTA-3'	55℃	1111	[17]
cnf 1-R	5'-TTGCCGTCCACTCTCTCACCAGT-3'			
cnf 2-F	5'-TATCATACGGCAGGAGGAAGCACC-3'	55℃	1240	
cnf 2-R	5'-GTCACAATAGACAATAATTTTCCG-3'			
*clb*A-F	5'-CTAGATTATCCGTGGCGATTC-3'	54℃	1002	[19]
*clb*A-R	5'-CAGATACACAGATACCATTCA-3'			
*clb*B-F	5'-GATTTGGATACTGGCGATAACCG-3'	54℃	575	
*clb*B-R	5'-CCATTTCCCGTTTGAGCACAC-3'			

After that, a new medium of RPMI-1640 (100 µl) was replaced. Bacterial inoculum was prepared under an aseptic condition with turbidity equivalent to that of a 0.5 McFarland standard, the turbidity was measured by using Biomerieux Densi CHEK plus turbidity meter. The multiplicity of infection (MOI) is done by making serial dilutions 100, 50, 25, 12.5, and 6. The cells infected have been re-incubated for 4 h. The cells have been washed 3 times with PBS and the 100 micro of fresh RPMI complete medium was added to each well. The plates were then re-incubated under the same conditions for 48 h [21, 22]. After that, the treated REF cells were washed twice by PBS fixed with absolute methanol for 5–7 min. Then the methanol was discarded and the cells were washed again with PBS [23]. Staining with 100 µl/well of methylene blue staining solution for 1 h. Rinsing it several times with methylene blue wash buffer, tapping the plate between each wash on a paper towel to remove all the liquid. Repeat the procedure until no more blue stain is visible on the paper towel. Air drying it a few hours at 37℃ [21, 23]. Observing the cells is conducted with an inverted microscope. Controlling cells should be confluent with normal cellular morphology. In contrast, cells infected with high MOI of *E. coli* producing *clb* and *cnf* MOI around 100 should exhibit a cytopathic phenotype characterized by low numbers of cells, megalocytosis, and signs of cell death. At lower MOI around MOI 25, the phenotype is less marked, and islets of normal cells that we're able to repair the moderate DNA damage and resumed proliferation should be visible. At MOI 6 the cells were similar to control cells [3].

Statistical analysis

The Statistical Analysis System-SAS (2012) program was used to detect the effect of difference factors in study parameters. Least significant difference -LSD test Analysis of Variation-ANOVA was used to significant compare [24]. Three replicates were used to each treatment.

Results and Discussion

Isolation and identification of UPEC

In this study, during the period from September 2020 to February 2021, 485 urine samples were collected from different hospitals in Baghdad governorate. Patient's age ranged from one month to 51 ≥ years old. Altogether, a total of 110 non-duplicated isolates were identified as *E. coli* based on microscopic observation, typical and cultural characteristics appeared on the selective media such as MacConkey and EMB agar. Identification of *E. coli* isolates was confirmed by Vitek 2 system and EPI 20E system. Out of the 110 uropathogenic *E. coli* isolates were recovered from different ages, including 27 isolates 24.4% were obtained from patients with age below one year, 33 isolates 29.9% from the age ranged between 1–14 years, 11 isolates 9.9% obtained from patients with age ranged 15–30 years. Also, 14 isolates 12.6% were collected from patients with age ranged between 31–50 years. Finally, 25 isolates 22.6% were isolated from patients with age ≥50 years, number of isolates obtained from male patients was 53 (48.1%), while, 57 isolates 51.8% were obtained from female as shown in Table 2.

Table 2. The percentage of UPEC isolates according to the age of patients and gender.

Age	Male	Percentage	Female	Percentage
(1-12)m	19	(17.2)%	8	(7.2)%
(1-14)Y	13	(11.8)%	20	(18.1)%
(15-30)Y	5	(4.5)%	6	(5.4)%
(31-50)Y	6	(5.4)%	8	(7.2)%
(51≥)Y	10	(9.0)%	15	(13.6)%
Total	53	(48.1)%	57	(51.8)%

Antibiotics sensitivity of UPEC tested isolates

The antimicrobial sensitivity results showed that a clear variation in response among *E. coli* isolates to antibiotics used in this study. The highest percentage of resistance among the *E. coli* isolates was recorded against Ticarcillin 96%, Cefepime 94% and Cefotaxime 93% followed by Trimethoprim 82%, Levofloxacin 81%. Moderate resistant was appeared against some antibiotics Sulbatam 79%, Ciprofloxacin 69%, Amikacin 68% and Gentamycin 54%. While a relatively small proportion of the isolates were resistance to Meropenem 5%, while none of them exhibit any resistance to Impenem, Table 3.

UTIs are among the most common infections worldwide and caused predominantly by uropathogenic

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Table 3. Antimicrobial sensitivity pattern of the UPEC tested isolates against 11 antibiotics.

Antibiotics	Symbol	Number of bacterial isolates and their percentage		
		R	I	S
Amikacin	AK	75 (68%)	21 (19 %)	14 (13%)
Sulbactam	SAM	79 (71%)	26 (23%)	5 (4%)
Cefepime	CPM	104 (94%)	-	6 (6%)
Ciprofloxacin	CLIP	76 (69%)	10 (10%)	24 (21%)
Cefotaxime	CTX	103 (93%)	4 (4%)	3 (3%)
Gentamycin	CN	60 (54%)	12(11%)	38 (35%)
Imipenem	IPM	-	-	110 (100%)
Levofloxacin	LEV	81 (73%)	6 (6%)	23 (21%)
Meropenem	MEM	6 (5%)	-	104 (94%)
Trimethoprim	TM	82 (74%)	-	28 (26%)
Ticarcillin	TC	106 (96%)	-	4 (4%)

E. coli, which lead to recurrence, renal damage, sepsis, or even death. Thus, early and appropriate empirical antibiotic therapy is of great importance. UTIs are a significant cause of morbidity in infant boys, older men and females [25]. The resistance rates of antibiotics among UPEC strains encoded for virulence factors genes was demonstrated against quinolones and Aminoglycosides. Thus, these drugs could be used for empirical treatment of UTIs. The resistance of antibiotics between members of *E. coli* isolated from urine was associated with lower prevalence of virulence factors and pathogenicity [26, 27]. As reported by [23] that the antibiotic susceptibility patterns for the cytotoxic genes carrying isolates, it became evident that there is a cost paid by the *cnf*1^+, *clb*A^+ and *clb*Q^+ isolates on the expense of their antimicrobial resistance since high antimicrobial susceptibility was detected among these isolates. According to [28] that uropathogenic *E. coli* PAIs including PAI II_{536} which encodes the *cnf* 1, hemolysin and *pks* island are characterized by their genetic flexibility and horizontal gene transfer abilities which rendered them mutation prone region, highly unstable and labile to external stimuli including the exposure to antibiotics. Hence, the disappearance of the UPEC PAIs among the resistance UPEC strains supported of these island.

In the last decades, as revealed by [26, 29, 30] that an increase in the level of resistance to antimicrobial drugs such as quinolones in these microorganisms has been reported worldwide, possibly due to the fact they are considered the treatment of choice for UTIs. Reports among UPEC causing disease have been performed to establish the pattern of resistance, virulence, and possibility of determine a relationship between these features [23]. In our study, *E. coli* isolates encoding colibactin and *cnf* genes were isolated from Iraqi patients with UTIs showed a high resistance against some antibiotics like Cefepime, Cefotaxime and Ticarcillin, in contrast to reported acclamation of virulence associated traits, antibiotic resistance was not prominent for the *E. coli* strains infected prostatitis in the men aged between 20–40 year, this results was in consistence with [30] who suggested that the low levels of antimicrobial usage in our healthy outpatient population and with the observation that these patients hadn't previous UTI, sustainable contact with the health care system and urological risk factors.

Detection of *cnf1, cnf2, clb*A and *clb*B genes among the UPEC tested isolates

All the collected UPEC isolates were screened for existence of *cnf*1*, cnf*2*, clb* A and *clb* B genes. The detection of target genes was achieved using PCR techniques, the results revealed that 28 isolates out of 110 (25.4%) harboured the *clb* A gene with a size of 1002 bp, 40 isolates out of 110 (36.3%) harboured *clb B* gene with a size of 575 bp, 24 isolates out of 110 (21.8) % harboured *cnf*1 gene with a size of 1111 bp. In this study, 13 isolates out of 110 (11.8%) were harboured *clb*A*, clb*B and *cnf*1 genes, while no *cnf*2 gene was detected among the all obtained isolates as shown in Table 4 and Fig. 1.

All the obtained isolates were subjected to examined their ability to produce hemolysin, out of 110 UPCE

Table 4. Prevalence of Colibactin and *cnf* genes among UPEC isolates.

Genes	Number of positive isolates (%)	Number of isolates with (β-hemolytic) positive
clb A	28/110 (25.4%)	8/28 (28.5%)
clb B	40/110 (36.3%)	11/40 (27.5%)
*cnf*1	24/110 (21.8%)	1/24 (4.1%)
*cnf*2	/	/
clbA+clbB	9/110 (8.1%)	5/9 (55.5%)
clbA+clbB+cnf1	13/110 (11.8%)	7/13 (53.8%)

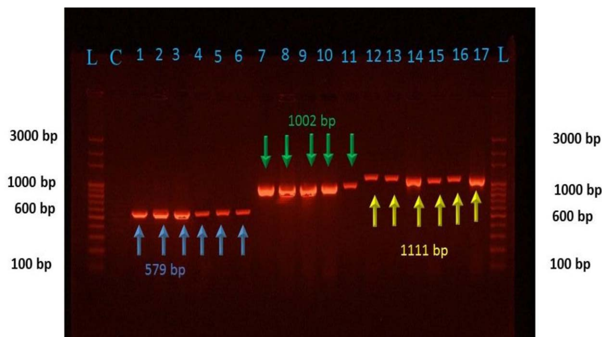

Fig. 1. PCR amplification of *cnf*1, *cnf*2, *clb*A and *clb*B genes in representative uropathogenic *E. coli* isolates. Lane C, Control. Lane (1-6), the isolates harboured *clb*B, Lane (7-11), the isolates harboured *clb*A, and Lane (12-17), the isolates harboured *cnf*1 gene. Lane L: DNA ladder 3000 pb. Electrophoresis was done in agarose gel (1.5%) at (5 V/cm) for 65 min.

isolates, 46 (41.7%) showed a halo zone on blood agar supplemented with 5% sheep blood (β hemolysis phenotype) as shown in the Table 4. According to these results the isolates were considered as *Hly*$^{+}$, the molecular detection revealed that 8 out of 28 (28.5%) were hemolytic isolates and carrying *clb* A gene, 11out of 40 (27.5%) carrying *clb* B gene, 1out of 24 (4.1%) carrying *cnf*1, 5 out of 9 (55.5%) carrying *clb* A and B. Finally, 7 out of 13 (53.8%) isolates were hemolytic and have a cytotoxicity effect against REF cells, only 32 out of 46 isolates exhibited a β-hemolytic activity on blood agar were carrying cytotoxic genes as shown in the Table 4. Previous studies and reports have shown an association between the production of both CNF and colibactin toxins and the production production of hemolysin enzyme [17, 30, 31]. Our results are consistent with the previous reports referred that, there is a cytotoxic effect as a result of existence the *cfn* and hemolysin genes among UPEC strains. CNF and colibactin are a cell active toxin produced by certain hemolytic UPEC strains, it's play an important role in the activation of Rho proteins and induce multinucleation in mammals cells culture [3]. Furthermore, in separated study was revealed that 96% of the *E. coli* strains carrying *cnf* gene obtained from marmosets were non-hemolytic isolates. Also, isolates of *E. coli* with a similar deactivating insertion in the *HylA* gene were previously isolated from laboratory rats from a specific vendor [33].

Cell culture assays were performed to investigate cytotoxicity of selected UPEC isolates which harboring tested genes *clb* A, B and *cnf*1 to REF cells, these cells were either exposed to harboring isolates and/or non-harboring isolates during 4 h with bacteria under optimal conditions at different MOI. The results in Fig. 2A–E show the cytotoxic effect of the isolates on the REF cell culture. Notably, only 3–23% of cells were killed after incubation time 48 h with non-harboring UPEC isolates positive control at MOI ranged from 100 to 6.25 compared to the negative control which does not show any effect, whereas the cytotoxicity percentage of the same cells was increased remarkably 69% when they were exposed to isolates harboring *clb* A, B, and raised up to 84% when the cells were incubated with UPEC isolates harboring *clb* A, B and *cnf*1 genes at 100 MOI compared to the positive and negative control as shown in Fig. 2. The cytotoxicity of all observed treatments were decreased with the gradual increase of MOI, whereby the lowest cytotoxic effect was at 6.25 MOI for all treatments. The bacterial isolates that harbouring *clb* A and B severally showed moderate cytotoxic effects especially at the MOI 100 and the percentage of cytotoxicity was 60, 76% for *clb* A and B receptively, and the percentages are increased gradually with the increasing of the MOI rates as shown in Figs. 2A–E. Statistical analysis showed that there was a significant difference ($p \leq 0.05$) between the effects of isolates at MOI ranging from 100 to 6.25 compared to the treatment of negative control, which did not show any significant difference.

In this study, the cell culture assay was performed to investigate the cytotoxic effect of six selected representative UPEC isolates against REF cells. Based on the microscopic observations, UPEC isolates which harboring *clb* A, B, and *cnf*1 induce megalocytosis striking giant cells phenotype. As well as, the cytotoxicity in REF cells that treated by different isolates harboring tested genes *clb*A, B and *cnf*1 in a manner corresponding to MOI was ranged from 100 to 6.25. The obtained results shown in Fig. 3 revealed that the REF cells treated with high concentrations of bacteria have a significant effect and causes enlargement, elongation, and decreasing in the number of living cells, in comparison with the treatment of negative control (isolate non-harboring targeted genes).

Furthermore, the treated REF cells with 100 MOI of UPEC isolates carrying genes *pks*+ and *cnf*1 exhibit a

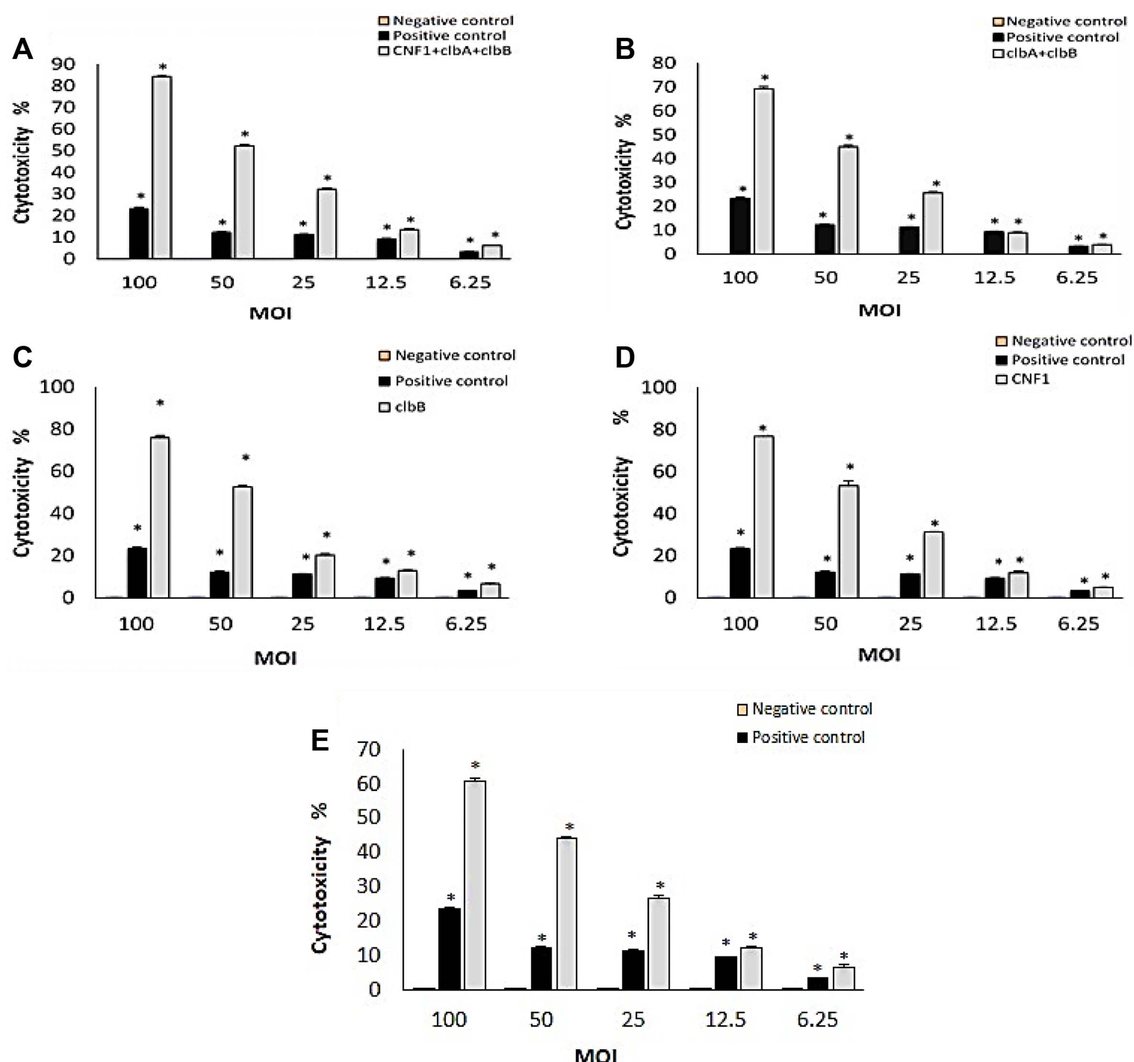

Fig. 2. Cytotoxic effects of UPEC isolates against REF cell line: (A) Normal cultures were exposed to isolate harbouring *clb* A, B and *cnf*1. (B) REF cells were exposed to isolate harbouring *clb* A, B. (C) REF cells were exposed to isolate harbouring *clb* B. (D) REF cells were exposed to isolate harbouring *cnf*1. (E) REF cells were exposed to isolate harbouring *clb* A. All treatments compared with treatment of control positive (non-harbouring bacteria) and control negative (only REF cell line). Data were presented as mean ± SD. *$p < 0.05$, **$p < 0.01$.

cytopathic phenotype which characterized by decreasing the number of cells. Microscopic observations demonstrated the presence of giant cells Megalocytosis, and some dead REF cells (cell debris, apoptotic bodies). From the results shown in Fig. 3 the isolate harboring *clb* A, B, and *cnf*1 gene at the MOI 100 and 50 was more cytotoxic against REF cells, this was noticed from the reduction in the number of cells, enlargement, and the presence of high number of giant cells. In contrast, no cell changing was observed at lower MOI 25, 12.5, and 6.25 of UPEC harboring tested genes.

Recently, some reports demonstrated that *E. coli* strains carrying *clb* genes colonize the intestinal of human and animals may play a role in the etiology and pathogenesis of colorectal cancer, the strains of *E. coli* strains from human and animal's gut which are classified according to serotype groups B2 are often the same responsible for UTIs [3, 23]. In a published study [34] reported that during UTIs, *clb* and *cnf*1 genes may play a role in induction of profound changes in cellular signal-

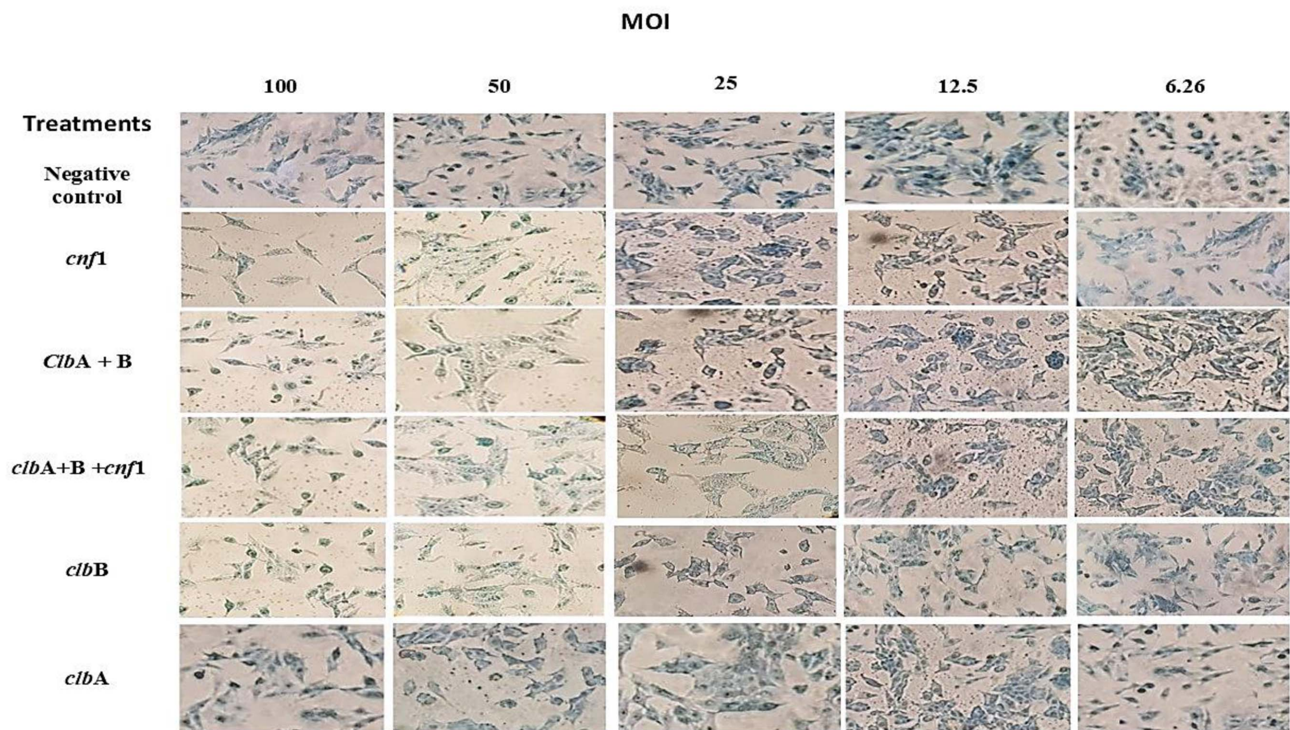

Fig. 3. Megalocytosis and enlargement phenotype observed two days after a 4 h exposure of REF cells to a UPEC isolates harbouring tested genes at a multiplicity of infection (MOI) of 100 to 6.25 bacteria per cell. The REF cells were stained with methylene blue and photographed with inverted microscope (40x).

ing pathways, also this study revealed that the colibactin induces DNA double-strand breakdown. On the other hands, *cnf*1 modulates a high number of cellular functions by hijacking of Rho GTPase [35].

By affecting the immune response, *cnf*1 could lengthen the brief time window between the establishment of bacteriuria and activation of a host defense during UTIs. Consequently, enhancing UPEC survival and allowing invasion of the parenchyma and bacteremia [36]. Colibactin and probably *cnf* may also favor host colonization, since their encoding genes have been found together in group B2 strains responsible for asymptomatic bacteriuria [36]. The study was achieved by [23] suggested that the existence of these virulence factors render the bacteria more virulent and aggravates the severity of the infection. Furthermore, the hyperplastic changes in the bladder transitional epithelium, proliferation and thickening of the Bowman's capsule exhibited by isolates of hemolytic *E. coli* harbouring the colibactin genes. In this study, the obtained results of the prevalence and distribution of *clb* A, B and *cnf*1 genes was in consistence with results obtained by [23], who referred that the percentage of hemolytic *E. coli* among the 125 isolates was only 21 (16.8%), among these β-hemolytic isolates, there were 10 isolates (47.62%) harbored the toxins encoding genes *cnf1*$^{+}$, *clb* A^{+} and *clb* Q^{+}.

The culture of REF cells was infected with live *E. coli* isolates harbouring *clb* and *cnf*1 genes showed megalocytosis and enlargement, a cytopathic phenotype consistent with colibatin and CNF toxins production, and depending on the MOI, Multiplicity of Infection. The cytotoxic effect of *E. coli* isolates requires direct contact with cells culture, perhaps due to the instability of produced colibactin by bacteria, which is matured upon secretion [31, 34]. The obtained results in the current study are in agreement with the results from previous reports and studies showing cytotoxic effects of *E. coli* harbouring *clb* and *cnf* genes against cell lines *In vitro* [3, 31, 33, 34]. The genomic data support the hypothesis which refers to the strains of *E. coli* harbouring virulence and antibiotic resistance genes that may have the cytopathogenic effect to influence clinical and subclinical

disease [3].

In this study, PCR *E. coli* isolates encoding colibactin and CNF were isolated and identified in patients with UTIs, All isolates were subjected to molecular investigation to detect the prevalence of *clb* A, B and *cnf* 1, 2 genes. Among the 110 isolates, 13 isolates out of 110 (11.8%) were positive for *clb* A, B and *cnf*1 genes, while no *cnf*2 gene was detected among the all obtained isolates. Among the obtained isolates only 46 isolate (41.7%) were positive for hemolysin production on blood agar supplemented with 5% sheep blood. *E. coli* isolates harbouring tested genes appeared cytotoxic effects against REF cells and this effect increased with the increasing of MOI during the period of incubation.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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