

# Distribution of CNF1 among *Escherichia coli* isolates from urinary tract infection and bladder cancer in southern of Iraq

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**Abstract**—One of the most important virulence factors produced by uropathogenic *Escherichia coli* (UPEC) is cytotoxic necrotizing factor 1 (CNF1). It plays a vital role in regulation of Ras homolog family member A (Rho), Ras-related C3 botulinum toxin substrate (Rac), and Cell division control protein 42 homolog (Cdc42) guanosine triphosphatase (GTPases) proteins, which involved in the organization of the actin cytoskeleton in eukaryotic cells. Also, CNF1 may play a role in cell proliferation, surviving and gene transcription. Therefore, detection of the gene encoding CNF1 in UPEC would be imperative in characterization of these organisms and establishing a proper management regime to prevent the poor prognosis of the associated diseases. The aim of the present study was to investigate the prevalence of the gene encoding *cnf1* in UPEC strains that have been isolated from urinary tract infection and bladder cancer patients. Out of three hundred and fifty midstream urine samples, 136 *E. coli* strains were isolated (98 urinary tract infection, 18 bladder cancer, and 20 healthy people). Detection and determination of the gene encoding CNF1 based on PCR technique and sequencing of PCR amplified target DNA. The present results showed that thirty five percent (35.34%) of the tested isolates have *cnf1* gene. All *cnf1* strains were isolated from urinary tract infection patients. This finding should bring attention to predict the prognosis of the infections caused by these organisms and follow up patients to prevent any further complications.

**Keywords**—CNF1; *Escherichia coli*; PCR; Virulence factor.

## I. INTRODUCTION

The prognosis of the urinary tract infection subjects to both the host susceptibility and the bacterial virulence. *Escherichia coli* is considered as a normal flora in human intestine. However, uropathogenic *E. coli* shows characters that make it virulent, specifically those related to specific serotypes such as O4, O6, O14, O22, O75 and O83 (Garcia *et al.* 2013) which express certain virulence factors including P-related fimbriae and haemolysin which are encoded by specific genes located in pathogenicity islands (PAIs) (Hochhut *et al.* 2006). Gene encoding CNF 1 also had

correlation with other virulence genes in PKs-island. CNF 1 is a toxic protein made by many uropathogenic *E. coli*. The glutamine residue deamidation that stimulates by CNF1 for GTP-binding protein RhoA which plays an essential role in the actin cytoskeleton organization in cells (Knust *et al.* 2010). This role represents in RhoA protein activity that reorganizes the stress fibers of actin network accompanying start membrane disrupting and multinucleation in culture cells (Fabbri *et al.* 2002).

Many studies showed that the adhesins P-related fimbriae and haemolysin play essential roles in *Escherichia coli* pathogenicity causing the further severe clinical conditions of urinary tract infections (Garcia *et al.* 2013), but the role of CNF 1 has been less understood. Previous studies showed considerable roles for CNF1 in initiation of an aggressive pro-inflammatory response to UPEC in the bladder (Garcia *et al.* 2013). Recent study demonstrated the role of CNF 1 in bladder cancer (Guo *et al.* 2017). They found that CNF1 stimulates bladder cancer cells by secreting vascular endothelial growth factor (VEGF) through the activation of Ras homolog family member C (RhoC), resulting in bladder cancer angiogenesis microenvironment. Further study found that uropathogenic *Escherichia coli* (UPEC) that CNF1-producing provokes significantly more submucosal edema and interstitial and neutrophil infiltration in the bladder than those other strains lacking CNF1 (Yang *et al.* 2018).

The aim of this study was to investigate the CNF1-producing uropathogenic *E. coli* prevalence in urinary tract infection and bladder cancer.

## II. MATERIALS AND METHODS

### A. Samples collection

Three hundred and fifty midstream urine samples were collected from patients with urinary tract infections (250) and bladder cancer (50) attended different hospitals in Basra province and 70 samples were collected from healthy people.

### B. Isolation of Bacteria

The collected samples were inoculated on MacConkey agar and eosin methyl blue agar and

incubated at 37°C for 24 hours. The culture was examined for their shape, size, color, and Gram stain reaction, for identification of *E. coli*. Then all plates were incubated at 37°C for 24 hours, after that a single pure isolated colony was transferred to Brain-Heart infusion agar medium for the preservation and to carry out biochemical tests that confirmed the identification of isolates.

### C. Identification of Isolates

*Escherichia coli* isolates were identified depending on morphological features on culture medium (MacConkey agar and eosin methylene blue agar), biochemical tests including indole, methyl red, Voges Proskauer, and citrate test (IMViC tests) and Gram stain according to the classification of Bergey's manual (Whitman *et al.* 2015).

### D. Molecular detection and determination of *cnf1* gene.

#### DNA Extraction

In order to extract the genomic DNA, the bacterial isolates were cultured overnight in 10ml of broth at 37°C. Then the extraction was carried out by using (Promega kit).

#### PCR Protocol

The (PCR) reaction mix used a final volume (25 µl) containing (2 µl) of DNA, (1 µl) of each primer, (12.5 µl) master mix and (8.5 µl) nuclease free water.

The (PCR) amplification was done under the following conditions: initial denaturation at 94°C for 4 minutes (1 cycle), then 30 cycles were performed: denaturation 94°C for 30 seconds, annealing temperature 65°C for 30 seconds, followed by extension of 72°C for 1 minute, then final extension of 72°C for 4 minutes.

The primer used to detect *cnf1* were as follows:

F-AAGATGGAGTTTCCTATGCAGGAG

R-CATTCAGAGTCCTGCCCTCATTATT

PCR product size 498 bp. (Chapman *et al.* 2006).

#### DNA sequencing

For DNA sequencing, 20 µl of PCR products of selected *E. coli* isolates were sent to Macrogen Company (Seoul, South Korea). The above forward primer was used for DNA sequencing (Chapman *et al.* 2006). All the obtained sequences were analyzed and aligned to each other and to the reference gene recorded in the National Center for Biotechnology using the Bio Edit program (Hall 1999).

### III. RESULTS AND DISCUSSION

The present results showed that out of 350 urine samples, 136 (38.7%) *E. coli* isolates were identified of which, 98/250 (39.2%) were found in patients infected with urinary tract infection, 18/50 (36%) were found in bladder cancer and 20/70 (28.6%) were isolated from healthy people. *E. coli* grew on eosin methylene blue which was used as a differential and selective medium for genus *E. coli* (Leininger *et al.* 2001). The colonies showed green sheen. This confirmed in principle that isolates

belonged to genus *E. coli*. Isolates have the ability to ferment lactose & production of strong acids and form large green colonies on the EMB agar by quick reduction in the pH of the EMB was observed on the medium.

MacConkey agar also was used as a selective medium for genus *E. coli*. All *E. coli* isolate showed pink colonies (Cundel *et al.* 2019).

The isolates and the cells appeared as Gram-negative bacilli rod shape after staining by Gram stain under microscope.

However for further identification, the oxidase test was performed and all isolates gave negative results. IMViC tests were performed to differentiate and identify *E. coli* from other *Enterobacteriaceae*.

*E. coli* has been considered as one of the most common pathogens associated with UTI in many other countries (Gajdác *et al.* 2019). The results showed that the prevalence of *E. coli* among UTI patients was slightly low compared with other international studies which reported that the prevalence of *E. coli* was 50 to 57.5% (Kabugo *et al.* 2016, Mwaka *et al.* 2011) and much higher than the prevalence rate found in Kurdistan Region in Iraq (21.1%) (Mahde *et al.* 2022). The variation of the prevalence rate could be due to the differences in the sample size, different population, or the improvement in management of urinary tract infections.

Detection of *cnf1* gene was carried out by using PCR technique using specific primers. *CNF1* encoding gene was detected in 41 (35.3%) strains (table 1), all of them were isolated from patients with urinary tract infections (table 1, figure 1). None of the bladder cancer samples showed positive results for *cnf1* gene.

Alignment of *cnf1* gene sequence with reference gene recorded in the National Center for Biotechnology Information showed 98 to 100% similarity (figure 2).

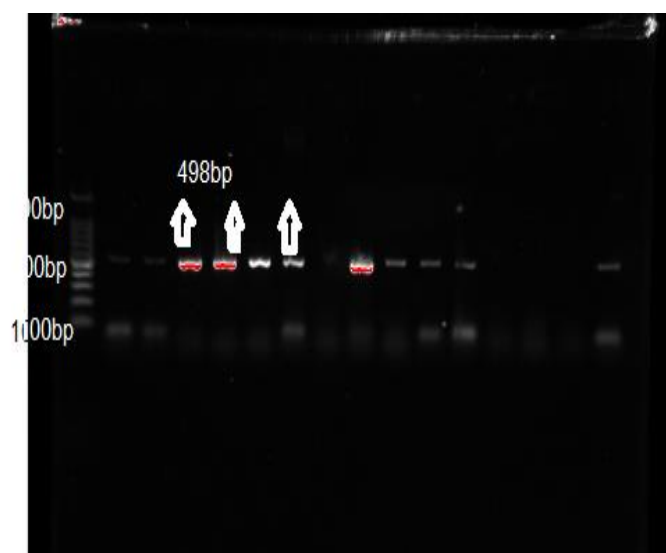


Figure 1. Gel electrophoresis of PCR products of Cytotoxic Necrotizing Factor 1 (*cnf1*) gene (498bp) for *E. coli* strains.

TABLE 1. DISTRIBUTION OF CYTOTOXIC NECROTIZING FACTOR 1(CNF1) GENE AMONG *E. COLI* ISOLATES

| Sample source           | Number of isolates | cnf1 geneN (%) |
|-------------------------|--------------------|----------------|
| Healthy people          | 20                 | 0              |
| Urinary tract infection | 98                 | 41 (41.83%)    |
| Bladder cancer          | 18                 | 0              |
| Total                   | 136                | 41 (35.3%)     |

collected from patients with bladder cancer and healthy people were *cnf1* negative.

In conclusion findings indicated that *cnf1* positive strains were markedly associated with UTI (41.83%). This virulence gene (*cnf1*) may help these isolates to persist even with proper treatment and could be responsible for frequent persistent infections. Despite the importance of findings, there are certain limitations such as limited patients' information regarding their disease prognosis and responding to chemotherapy. Further study is required for longer time to follow up patients and to determine more phenotypical and genotypical characters of the infecting *E. coli* strains.

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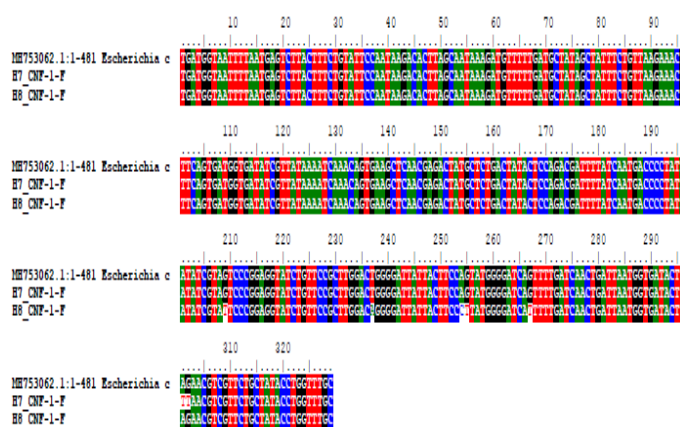


Figure 2. Alignment of the sequence of *cnf1* gene of representative tested isolates with reference *Escherichia coli* strain W010 cytotoxic necrotizing factor CNF-1 (CNF-1) gene, Sequence ID: MH753062.1.

The roles that cytotoxic necrotizing factor 1 (CNF1) play in the pathogenicity of UPEC has been broadly studied. It is one of the most important UPEC toxins. Several studies reported that about 30% of the UPEC isolated from pyelonephritis cases have the *cnf1* gene, suggesting its effect in kidney infection (Bien *et al.* 2012). Other studies reported that CNF1 positive strains induce other inflammation in prostate, kidney and bladder (Guo *et al.* 2020, Smith *et al.* 2008). It has been reported that CNF1 causes bladder cells apoptosis that may lead to bladder cell exfoliation providing bacterial access to underlying tissues (Mills *et al.* 2000). Other recent study found that CNF1 persuades the migration and invasion of prostate cancer cells encouraging prostate cancer progression through activation of the Cdc42-PAK1 axis (Guo *et al.* 2017). Furthermore, many reports showed that CNF1 markedly involves in activating Rho GTPases inducing cell motility and invasion by uropathogenic *E. coli* (Carlini *et al.* 2021, Doye *et al.* 2002, Diabate *et al.* 2015). The present study showed that the prevalence rate of CNF1-positive strains (41.83%) was higher than that reported in Europe (34%), Iran (28%) and India (29%) (Landraud *et al.* 2000, Shabani *et al.* 2018, Arindam *et al.* 2017). The present results showed that all the *cnf1* positive strains were associated with urinary tract infection and all *E. coli* strains isolated from urine

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