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Detection of *cnf1* and *cnf2* Genes in Clinical Isolates of *E.coli*

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ABSTRACT

Background: CNF are single-chain proteins that are created by some uropathogenic *Escherichia coli*.There are three types of CNFs detected in *E.coli*, but CNF1 represent more frequently.

Objective: The purposes of this study were to determine the prevalence of *cnf1* and *cnf2* genes in *E. coli* isolates by using specific primers.

Materials and Methods: The sensitivity test was evaluated by using different types of antibiotics in the current study, and the *cnf1 and cnf2* genes were detected by PCR in all bacterial isolates.

Results: The antibiotics sensitivity test show variable degrees of sensitivity and resistance. The high percentage of sensitivity was achieved against amikacin at a percentage of 86% and ciprofloxacin, ceftazidime, gentamicin, and tobramycin at a percentage of 80%, whereas trimethoprim and aztreonam at 64%and 60% respectively. In this study, most isolates were resistant to Amoxicillin (92%); while showing different degrees of resistance against other types of antibiotics ranging from Tetracycline (62%) to amikacin 4%. The frequency of MDR bacteria was about 64 % (32 isolates), 30 from urine, and 2 others from stool. The results revealed that the *cnf1* gene was found in Five bacterial isolates (10%), whereas the other 45 (90%) isolates don't have this gene, while *cnf2* wasn't found in any one of the bacterial isolates.

Conclusion: *E.coli* was recorded as multidrug resistance (MDR) and the *cnf1* gene was found only in uropathogenic isolates.

Keywords: Cytotoxic necrotizing factor (CNF), *E. coli*, antibiotics, genotoxins.

INTRODUCTION

Cytotoxic necrotizing factor (CNF) is a class of AB toxins generated by UPEC strains that alters the cytoskeleton and causes G2 cell cycle arrest as well as macropinocytosis of bacteria into the host cell. These behaviors encourage *E. coli* colonization and reduce epithelial turnover. Numerous cell lines that have been exposed to cnf+ E. coli sonicates exhibit multinucleation and ruffled cell borders, which is consistent with these mechanisms. Additionally, CNF1 can incite the in vitro transition of epithelial to mesenchymal tissue, which may raise the risk of cancer [(1)]. There are three CNF kinds known for *E. coli*, however only CNF1 and CNF1a are frequently seen [(2)]. CNF1 is a protein toxin that can cause multinucleation (cytotoxicity) in cultured cells and necrosis in rabbit skin. It was first identified by Caprioli and colleagues in 1983. (Necrotizing) [(3).] This toxin, which belongs to the AB class and is responsible for infant meningitis and urinary tract infections, is a 115 kDa single-chain molecule with an N-terminal receptor binding domain and a C-terminal catalytic region that contains deamidase activity [(4).]

While cnf2 is plasmid-encoded, cnf1 is chromosomally encoded 23. Additionally to harmful and disease-causing organisms, commensal strains of E. coli, a part of the natural flora in humans and warm-blooded animals' digestive systems, also develop antibiotic resistance [(5)]. The severity of UTI produced by E. coli is due to the expression of a wide spectrum of virulence factors [(6)]. As the number of *E. coli* strains resistant to many drugs has risen, treatment options have become more limited [(7,8).] Numerous research revealed that UPEC antibiotic resistance is rising annually [(9).]

E. coli infections in hospitals and the community are frequently treated with cephalosporins, fluoroquinolones, and trimethoprim-sulfamethoxazole. Resistance to these medications causes delays in the administration of the proper therapy, which increases morbidity and death [(10).] Antimicrobial resistance and virulence factors are quite prevalent in UPEC isolates, which suggests that infections brought on by these organisms require extensive healthcare monitoring and a critical need for new antimicrobials [(11).]

This study aimed to evaluate the sensitivity of *E.coli* against different types of antibiotics and also the detection of *cnf1* and *cnf2* genes in *E.coli* isolated from different sources.

MATERIALS AND METHODS

Specimen collection

Bacterial isolates were collected from the patient's urine and stool between January 2021 to March 2021. A total of 120 urine and stool samples were collected, 35 uropathogenic E. coli and 15 enteropathogenic isolates were obtained as grown on MacConkey and EMB agar plates. By growing on specific media including MacConkey, EMB agar, and Hichrom agar (Himedia India), as well as through biochemical tests and the use of the Vitek 2 system as a confirmation test, isolates were described and recognized as *E. coli*.

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Antibiotic susceptibility testing

The isolates on Muller-Hinton agar (Himedia India) were tested for antibiotic susceptibility using the disk diffusion method. Using a panel containing the following antibiotics, each isolate was tested for antibiotic susceptibility: amoxicillin (A) 10 µg, Tobramycin (TOB) 30 µg, gentamicin (GEN) 10 µg, Tetracyclin (TE) 30 µg, ciprofloxacin (CIP) 5 µg, aztreonam (ATM) 30 µg, ceftazidime (CAZ) 30 µg, amikacin (AK) 30 µg, piperacillin (PRL) 30 µg, and trimethoprim (TMP) µg (Himedia\India). The inhibitory zones' sizes were determined following a 24-hour incubation period at 37°C. Results were interpreted following Clinical Laboratory Standard Institute (CLSI) guidelines (2020).

Screening the genotoxins-producing isolates:

Local isolates of *E.coli* were screened to select the efficient isolates in DNase production. The ability of these isolates in DNase production was assayed after culturing at 37 C in DNase agar with toluidine blue, so the DNA hydrolyzing effect was detected by converting the color from blue-purple to pink [(12).] The uninoculated medium turns blue as a result of toluidine blue O and DNA complexing. DNA depolymerizing organisms produce a compound of a dye, oligonucleotide, and mononucleotide.

Detection of *cnf1* and *cnf2* gene by PCR:

DNA extraction

In this study, 50 E. coli isolates—35 from urine and 15 from diarrheal stool—were chosen for cnf1 and cnf2 gene identification. A commercial Wizard genomic DNA purification kit from Promega in the United States was used to extract the genomic DNA from these isolates, and a Quantus Fluorometer was used to measure DNA concentration and purity**.**

PCR amplification

The sequence of the particular pair of primers was used, as per a prior study [(13)].

To find bacteria that have the USP gene, PCR was utilized. The green master mix (Promega, USA), 1 µl of each primer (10 Pmol), and 2 µl of DNA template were used in the 25 µl volume PCR reactions. It took 25 µl of deionized distilled water to complete the reaction volume. The thermocycler's cycling parameters were one cycle of 94 oC denaturation for 2 min, one cycle of annealing for 1 min, one cycle of 72 oC elongation, and 30 cycles of 94 oC denaturation for 1 min. The PCR reaction products were either separated right away on 2% agarose gels or kept at -20 C for future use.

Ethical approval

This study was approved by The Research Ethics Committee, College of Science Baghdad University. This work has been carried out following the "Guide for the care and use of Laboratory Animals" for the use and welfare of experimental animals, published by the US National Institutes of Health (NIH publication No. 85–23, 1996).

RESULTS

Isolation and Identification

Fifty bacterial isolates from *E.coli* were achieved after culturing it on different culture media such as MaCconkey agar, and EMB agar (Himedia/India). Hichrom UTI agar (Himedia/India), was also used, by which uropathogenic *E.coli* were confirmed. The uropathogenic *E.coli* represent 35 isolates (70%), while the other 15 isolates (30%) were isolated from stool and represent enteropathogenic *E.coli.* The current study's findings showed that the majority of *E. coli* isolates were from urine samples (70%) compared to stool (30%) since it is the primary causal pathogen in women's recurrent UTI, which accounts for 80% of all infection episodes [(14)].

Antibiotic sensitivity test:

Antibiotic susceptibility test was done according to the Kirby-Bauer method by using ten types of different antibiotic discs with different concentrations. The results showed variation in the susceptibility of isolates to different types of antibiotics depending on the antibiotic type and the source of bacterial isolate.

The high sensitivity ratio was achieved by amikacin, ciprofloxacin, tobramycin, and gentamicin in percentages of 86% for amikacin and 80% for each one of the other antibiotics respectively. Aztreonam and trimethoprim show a sensitivity ratio in percentages of 60% and 64% respectively. In this study, most isolates were resistant to amoxicillin (92%); furthermore, the isolates showed resistance to other antibiotics involving tetracycline (62%), ceftazidime (52%), and piperacillin (50%). Whereas only 10 (20%) isolates were resistant to ciprofloxacin, 11 isolates (22%) to gentamicin, 7 isolates (14%) to tobramycin, 13 isolates (26%) to aztreonam, 18 isolates (36%) to trimethoprim and 2 isolates only (4%) to amikacin (**Figure 1**).

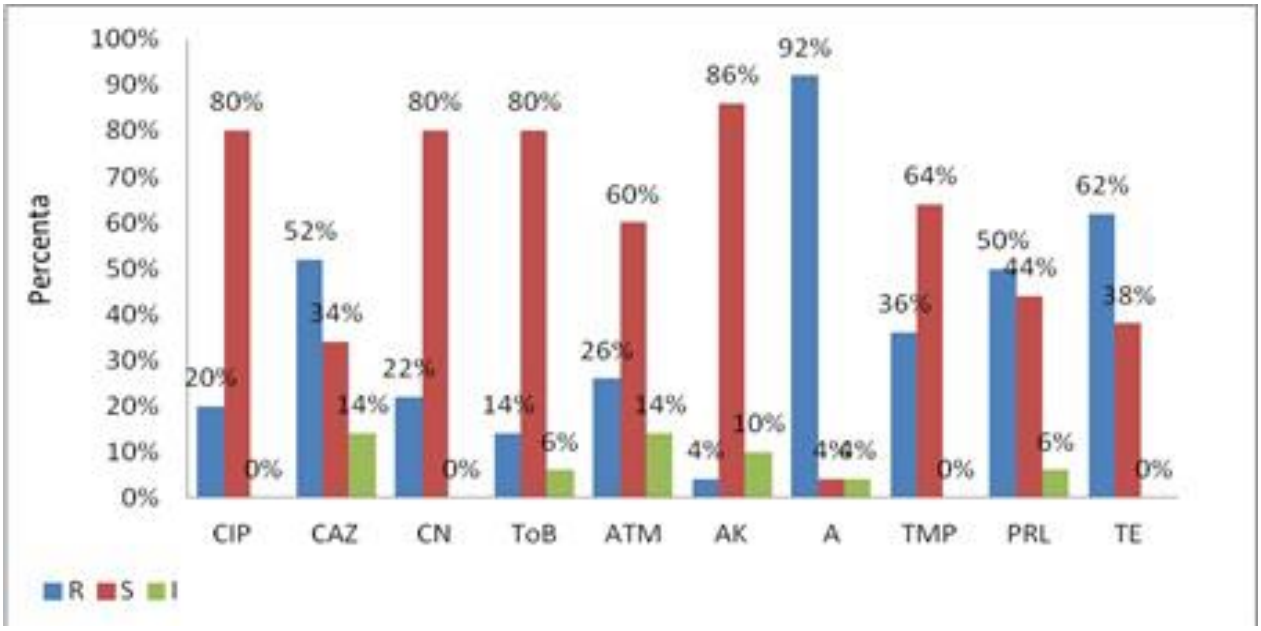

Figure (1): The percentage of susceptibility pattern for *Escherichia coli* isolates against antibiotics

In the current study, the percentage of MDR bacteria represents (64%), from 35 isolates taken from urine 30 isolates were recorded as MDR. It was observed in this study that only 2 isolates from stool show MDR from 15 isolates taken from this source.

Screening the genotoxins-producing isolates

The results of the current study revealed that all *E.coli* isolates can produce DNase enzymes and cause DNA damage, so all bacterial isolates may have genotoxic activity **(Figure (1).**

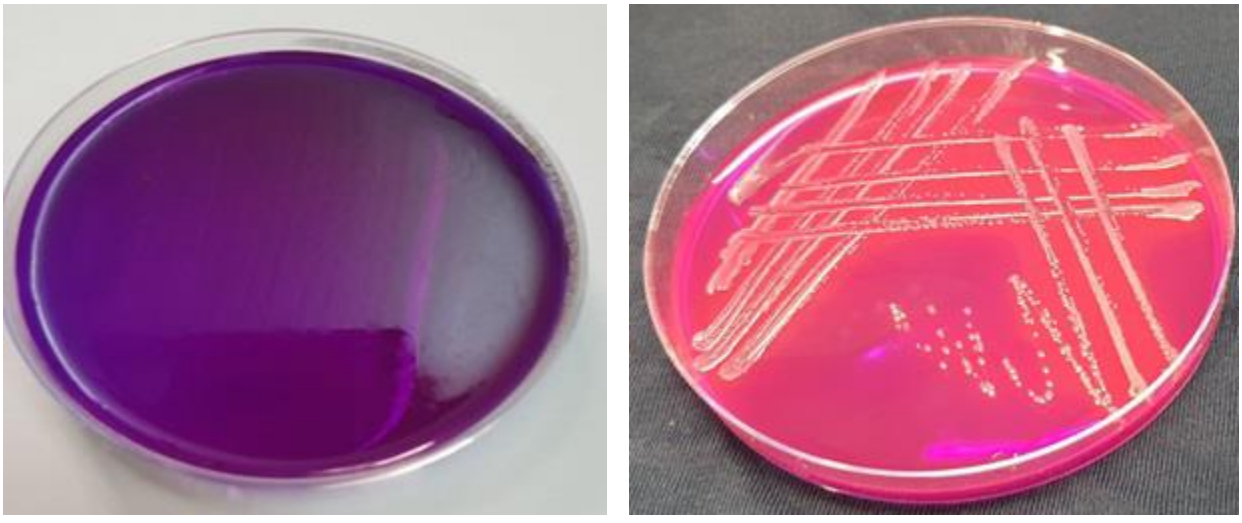

Figure (2): *E.coli* on DNase agar.

This approach made use of a semi-synthetic medium that also contained a nucleic acid solution. Placing a flood of 1 N hydrochloric acid on the plate allows for the detection of enzyme activity (HCl). A clean zone around growth indicates a positive reaction.

Detection of *cnf1* and *cnf2* genes by PCR:

This research was done to detect *cnf1* and *cnf2* genes in all *E.coli isolates* by PCR technique. Specific primers for these genes were used for the detection of the presence of it. The results showed that 5 isolates (10%), only were positive for the *cnf1* gene, whereas 45 isolates (90%) don't have this gene (Table (1)). All these isolates were taken from urine except one from stool. Our finding showed that the *cnf2* gene wasn't detected in any one of the bacterial isolates see **Table (1).** The product of PCR detected by using gel electrophoresis is shown in **Figures (3, 4, and 5**) for the *cnf1 gene* and **Figures** (**6,7,8**) for the *cnf2 gene*.

Table (1): Frequency of *cnf1* and *cnf2* genes in *E. coli* isolated from urine and stool.

Isolates	source	*Cnf1*	*Cnf2*	Isolates	source	*Cnf1*	*Cnf2*
E1	Stool	-	-	E26	Urine	+	-
E2	Stool	-	-	E27	Urine	-	-
E3	Stool	-	-	E28	Urine	-	-
E4	Stool	-	-	E29	Urine	-	-
E5	Stool	-	-	E30	Urine	-	-
E6	Stool	-	-	E31	Urine	-	-
E7	Stool	-	-	E32	Urine	-	-
E8	Stool	-	-	E33	Urine	-	-
E9	Stool	-	-	E34	Urine	-	-
E10	Stool	-	-	E35	Urine	-	-
E11	Stool	-	-	E36	Urine	-	-
E12	Stool	-	-	E37	Urine	-	-
E13	Stool	-	-	E38	Urine	-	-
E14	Stool	+	-	E39	Urine	-	-
E15	Stool	-	-	E40	Urine	-	-
E16	Urine	-	-	E41	Urine	-	-
E17	Urine	-	-	E42	Urine	+	-
E18	Urine	-	-	E43	Urine	-	-
E19	Urine	-	-	E44	Urine	-	-
E20	Urine	-	-	E45	Urine	-	-
E21	Urine	-	-	E46	Urine	-	-
E22	Urine	-	-	E47	Urine	-	-
E23	Urine	-	-	E48	Urine	+	-
E24	Urine	+	-	E49	Urine	-	-
E25	Urine	-	-	E50	Urine	-	-

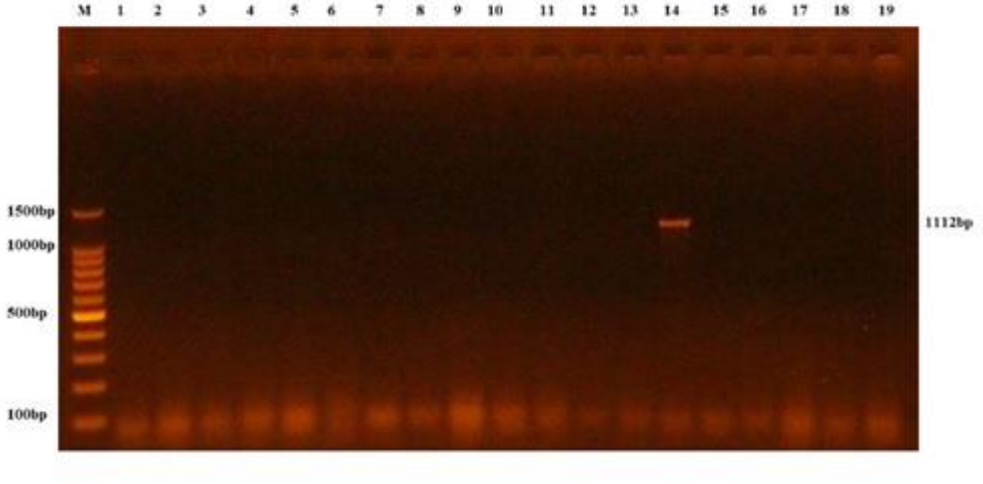

Figure (3): Results of the amplification of the CNF1 *gene* of *Escherichia coli* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 1-19 resemble 1112bp PCR products.

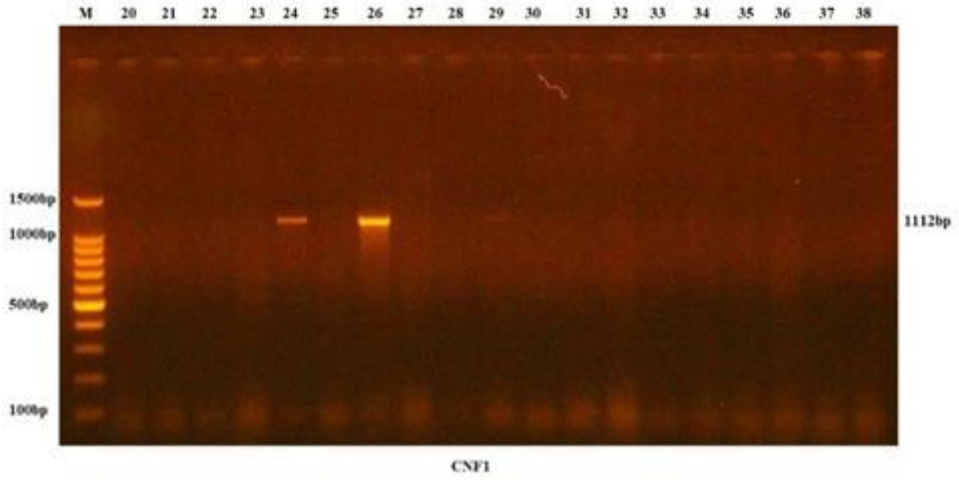

Figure (4): Results of the amplification of the CNF1 *gene* of *Escherichia coli* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 20-38 resemble 1112bp PCR products.

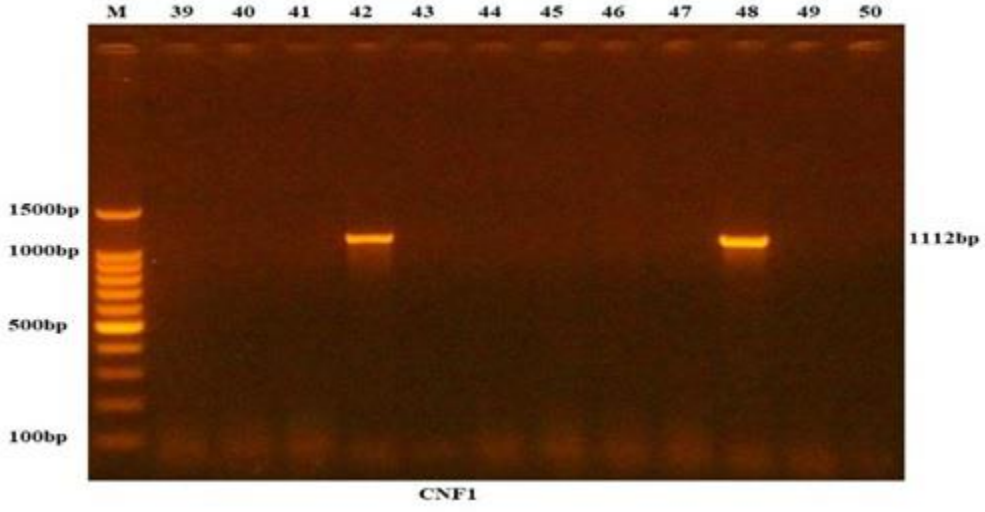

Figure (5): Results of the amplification of the CNF1 *gene* of *Escherichia coli* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 39-50 resemble 1112bp PCR products.

DISCUSSION

Uropathogenic *E. coli* is responsible for 90% of urinary tract infections; the bacteria enter through the perineum or feces and travel up the urinary tract to the bladder [15]. Specific virulence traits that are strongly associated with bacterial colonization and persistence in the urinary system are used to identify UPEC strains. These elements consist of poisons, siderophore systems, and adhesins or fimbriae [16, 17].

According to a local investigation by Shukur [18], 42 urine samples contained *E. coli* isolates or 85% of the total. A total of 407 urine samples were taken from patients with suspected UTIs at the University of Benin Teaching Hospital (UBTH), Benin, Edo State, Nigeria, according to Momoh *et al*. [19]. Male and female samples yielded 162 (39.8%) and 245 (60.2%) E. coli isolates, respectively. The local study by **Sweedan *et al.*** [20] reported that uropathogenic *E.coli* resist all antibiotics under study such as Ceftazidime (30µg), Cefotaxime(10µg), Amikacin (10µg), Amoxicillin (10µg), Ciprofloxacin(5µg), Trimethoprim (30µg), Kanamycin(25µg), Norfloxacillin(10 µg), and Cefalexine (10µg), Tetracycline(5 µg), Doxycillin (5µg). Aminoglycoside antibiotics are used to treat the bulk of illnesses brought on by aerobic Gram-negative bacteria like *E. coli* and *Klebsiella pneumoniae* as well as non-

fermenters like *P. aeruginosa* (Amikacin, Gentamicin, Tobramycin, Kanamycin, and Netilmicin) [21,22]. For the treatment of UTIs and other infections, fluoroquinolones and extended-spectrum cephalosporins are widely used in Mongolia. Because of the widespread use of these antibiotics, the rates of AMP, CEF, and CIP resistance may be higher than usual [23]. The high prevalence of E. coli isolates resistant to ampicillin, amoxicillin/clavulanic acid, ceftriaxone, and sulphamethoxazole/trimethoprim and the low cost of these drugs cannot be ruled out, which makes them widely accessible and affordable to the general public. Resulting in abuse and misuse. According to the study's findings, imipenem, ciprofloxacin, ceftazidime, gentamycin, and cefotaxime were all effective against isolated *E. coli* bacteria. The cost of the medicines and the environment may contribute to the susceptibility of E. coli isolates [24]. A second-generation fluoroquinolone antibiotic called ciprofloxacin (CIP) is frequently used to treat both Gram-negative and Gram-positive bacterial infections. The study by Al-Hasnawy *et al.*[25] concluded a high prevalence of uropathogenic *E. coli* (UPEC) with Multidrug-resistant (MDR) isolated from urinary tract infection in Babylon province, Iraq. Another study on UTIs in Iran found that 82.1% of the isolates were MDR *E. coli* [26]. Since most isolates have substantial resistance to many kinds of antimicrobial drugs, particularly penicillins, tetracyclines, cephalosporins, fluoroquinolones, aminoglycosides, and macrolides, the advent of antimicrobial resistance has increased the relevance of UPEC [27].

As a result, the majority of therapeutic choices are unsuccessful, increasing hospital stays and the expense of care. All isolates were considered multidrug-resistant if they show resistance to at least three antibiotics from three separate classes (MDR) [28]. As a result of the rise in MDR, the antibiotic resistance crisis has become one of the most urgent observed issues in global public health [29].

Antibiotic-resistant microorganisms have emerged, posing a global health hazard. This has prompted efforts worldwide to create new, more effective antimicrobial chemicals and new delivery and targeting techniques [29].

Treatment of E. coli infections and a potential co-selection of antibiotic resistance are two of the major impacts of MDR, which are mediated by MDR plasmids [30].

A metachromatic dye is present in DNase Test Agar with Toluidine Blue to do away with the need to add reagents to the agar after incubation. Toluidine blue should only be used with Enterobacteriaceae since it may be harmful to some gram-positive cocci [12]. As a result of the indicator's metachromatic qualities, the area around the organisms that have the DNase enzyme appears to have a vivid rose-pink color. It is advised for the detection of DNase in gram-negative microbes because toluidine blue may be inhibitory to some gram-positive species [31].

In conjunction with the current findings, a local study by **Hassan *et al*.** [32] found that only 4 E. coli isolates possess the cnf1 gene. In addition to being isolated from sick and healthy animals like cats, dogs, pigs, and birds, *E. coli* strains that produce CNFs are also known to cause meningitis and UTIs in people [33]. A study by **Alhadidi *et al***. [34] found that no cnf2 gene was found in any of the collected isolates, but 13 isolates out of 110 (11.8%) had the *clbA*, *clbB*, and *cnf1* genes. These findings are in agreement with the current study.

The pathotype necrotoxigenic *E. coli* (NTEC), which is linked to extraintestinal and intestinal infections in both humans and animals, includes the *E. coli* strains that cause cnfs. The majority of cnfs contain the plasmid- and chromosomally-encoded genes *cnf1* and *cnf2*, respectively [33, 35]. The cnf1 virulence factor is thought to work by releasing iron from red blood cells, disrupting phagocytic cells, and damaging renal tissue [36]. The activation of cnf1 is necessary for the spread and persistence of UPEC [36].

CONCLUSIONS

The results of the current study suggested that *E.coli* isolates resist more than 3 types of antibiotics so recorded as multidrug-resistant (MDR). All bacterial isolates were positive for the DNase test, so they have genotoxic activity. The *Cnf1* gene was found in uropathogenic E.coli only, while enteropathogenic *E.coli* don't have this gene. All *E.coli* isolates in this study show negative results for the *cnf2* gene.

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