The Egyptian Journal of Hospital Medicine (October 2023) Vol. 93, Page 7803-7809

Detection of *cdt* and *clb* Genes in *pathogenic E.coli*

Zainab Zamel Khalaf and May Talib Flayyih
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.
***Corresponding author:** Zainab Zamel Khalaf, **E-mail**: zainab.alnaji@sc.uobaghdad.edu.iq, **Mobile:** +9647707802415

ABSTRACT

Background: Many diseases create the cytolethal-distending toxin (CDT), which is representative of an emerging family of recently identified bacterial compounds.

O**bjective**: This study was performed to detect the antibiotic resistance properties and distribution of *cdtB* and *clbA* in *E. coli* strains isolated from urine and stool samples.

Materials and Methods: The sensitivity test was evaluated by using different types of antibiotics in the current study, and the *cdtB and clb* 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 showed that the *clbA* gene was found in 6 bacterial isolates (12%), whereas the other 44 (88%) isolates don't have this gene, while the *cdtB* gene wasn't found in any one of the bacterial isolates.

Conclusions: *E.coli* was recorded as multidrug resistance (MDR) and the *clb* gene was found only in 6 bacterial isolates but it was not the *cdtB* gene detected in all isolates.

Keywords: *E.coli*,*cdt*,*clb*, antibiotic sensitivity, genotoxins.

INTRODUCTION

Escherichia coli is an opportunistic pathogen that is frequently found in the intestinal flora of both humans and animals. Depending on where it dwells, it can be classified as commensal, intestinal, or extraintestinal *E. coli* [(1)]. The expression of a broad spectrum of virulence factors contributes to the severity of UTI caused by *E. coli* [(2)] The members of the family of Enterobacteriaceae harbor a gene cluster called polyketide synthase (pks) island. This cluster is responsible for the synthesis of the genotoxin colibactin which might have an important role in the induction of double-strand DNA breaks [(3)].

One class of cyclomodulins contains genotoxins and/or cell cycle-regulating toxins that influence cellular differentiation, death, and proliferation, all of which promote tumorigenesis. These cytotoxins include Colibactin, Cytolethal Distending Toxin (CTD), and Cytotoxic Necrotizing Factors (cnfs) (CIF) [(4)]. Intestinal and extraintestinal infections caused by *E. coli* strains are correlated with virulence factors like CDT and CNF [(5)].

In mouse sepsis and meningitis models, the role of colibactins in the virulence of E. coli and *K. pneumoniae* has been established. Isogenic mutants with defective colibactin production show diminished virulence in comparison to wild-type pks-positive (pks_) strains. [(6)]. The 54-kb pathogenicity pks Island contains the clb A-S genes, which code for the secondary metabolite known as colibactins [(7)]. Numerous Enterobacteriaceae strains, including particular strains of gut commensal and extraintestinal pathogenic E. coli (ExPEC) and Klebsiella pneumoniae, among others, include the clb locus [(8)]. The ability of *E. coli* strains from phylogenetic group B2 to establish prolonged colonization of the host gut is likewise correlated with the presence of the pks island [(9)].

The first CDT, a novel form of toxin activity produced by pathogenic strains of E. coli, was reported in 1987 [(10)]. Being able to cause DNA double-strand breaks in both proliferating and nonproliferating cells makes CDT unique among bacterial toxins and results in the irreversible cell cycle arrest or death of the targeted cells [(11)]. In addition to harmful and disease-causing organisms, commensal strains like Escherichia coli (*E. coli*), a component of the natural flora in the digestive system of people and warm-blooded animals, also develop antibiotic resistance [(12)].

Although there are many contributing factors, the development of antibiotic resistance in *E. coli* and other bacteria has coincided with the addition of these medications to the treatment toolkit in both human and veterinary medicine [(13)]. This study was designed to isolate *E. coli* from urine and stool samples, study the antibiotics susceptibility tests for ten types of antibiotics against *E.coli*, screen for genotoxins-producing *E. coli* from these samples, and detect the *clbA* and *cdt* genes as genotoxins in *E. coli* using PCR technique. This study was limited by the paucity of molecular studies on genotoxic *E. coli* and multidrug resistance of bacteria.

MATERIALS AND METHODS

Specimen collection

Between January 2021 and March 2021, bacterial isolates from urine and stool samples were collected. One hundred and twenty samples of urine and stool were taken in total, and 35 isolates of enteropathogenic *E. coli* and 15 isolates of uropathogenic *E. coli* were acquired and grown on MacConkey and EMB agar

Received: 20/08/2022
Accepted: 02/12/2022

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*.

Antibiotic susceptibility testing

The isolates were tested on Muller-Hinton agar (Himedia India) by using the disk diffusion method for antibiotic susceptibility. Each isolate was tested for antibiotic susceptibility using a panel comprising the following drugs: amoxicillin (A), tobramycin (TOB), gentamicin (GEN), tetracycline (TE), ciprofloxacin (CIP), aztreonam (ATM), ceftazidime (CAZ), amikacin (AK), piperacillin (PRL), and trimethoprim (TMP) (Himedia-India). After 24 hours of incubation at 37°C, the plates were examined for inhibitory zone diameters. Clinical Laboratory Standard Institute (CLSI) guidelines (2020) were followed for the interpretation of the results.

Screening the genotoxins-producing isolates

To find the most effective isolates for producing DNase, local *E. coli* isolates were examined. After incubating these isolates at 37 °C in DNase agar with toluidine blue, the ability to produce DNase was tested.

The DNA hydrolyzing effect was identified by changing the color from blue-purple to pink [(14)]. 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 *cdt* and *clbA* 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

According to a previous study, the specific pair of primers' sequence was employed see (**Table 1).** PCR reaction was used to detect bacteria that possess the *usp* gene. The PCR reactions were conducted in a 25 µl volume and contained 2 µl of DNA template, 1 µl of each primer (10 Pmol), and 12.5 µl of green master mix (Promega, USA). The reaction volume was increased to 25 µl using deionized distilled water. The thermocycler was cycled through 30 times of denaturation at 94 ºC for 1 minute after 1 cycle of denaturation for 2 minutes, annealing for 1 minute, and elongation for a minute at 72 °C. To preserve the PCR reaction products, they were either separated right away on 2% agarose gels or stored at -20 °C.

Table (1): Primers used for *cdtB* and *clb* genes detection

Primer Name	Seq.	Annealing Temp. (°C)	Product size (bp)
cdtB-F	5`-GCCGTAGATATTTTAGCTGTGC-3`	60	175
cdtB-R	5`-CACCAAAGGCATCAACAGCAG-3`		
clbA-F	5`-CAGATACACAGATACCATTCA-3`	54	1002
clbA-R	5`-CTAGATTATCCGTGGCGATTC-3`		

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, 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%) are isolated from stool and represent enteropathogenic *E.coli.* The majority of *E. coli* isolates were found in urine samples (70%) compared to stool samples (30%).

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).**

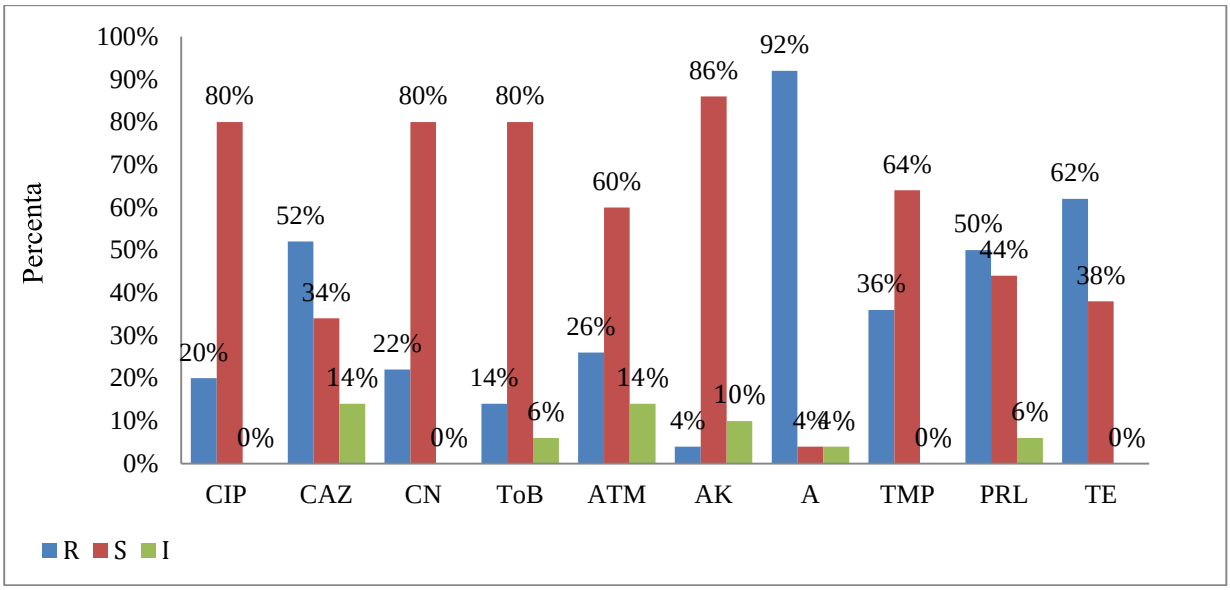

Figure 1: The percentage of susceptibility pattern for *Escherichia coli* isolates against antibiotics.

In the current study, the percentage of MDR bacteria represent (64%), of 35 isolates taken from urine and 30 isolates recorded as MDR. It was observed in this study that only 2 isolates from stool show MDR from 15 isolates taken from this source. Over the past few decades, multidrug-resistant *E. coli* has emerged in many nations.

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 2**). 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.

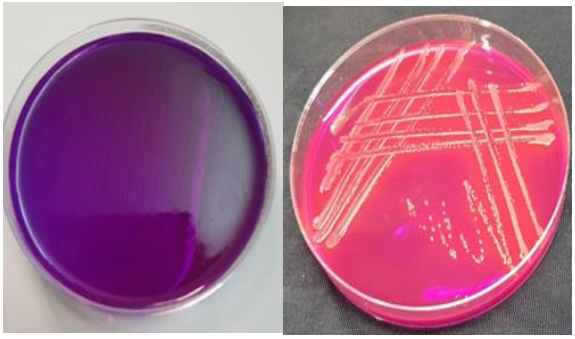

Figure 2: *E.coli* on DNase agar.

Detection of *cdtB and clb* genes by PCR:

This study was carried out to detect *cdt* and *clbA* genes in all 50 *E.coli isolates* by PCR technique. Specific primers for this gene were used for the detection of its presence of it, these genes are responsible for the presence of genotoxins such as cytolethal distending toxin and colibactin. The results showed that 6 isolates were positive for the *clbA* gene, whereas 44 isolates don't have this gene (**Table 2**).

On the other hand, the *cdt* gene was not found in any one of the bacterial isolates. All these isolates were taken from urine except one from the stool. The product of PCR detects by using gel electrophoresis as shown in (**Figures 3, 4, and 5**). No *cdt* gene was detected in any of the bacterial isolates tested in this investigation, which was designed to find and identify various cdts genes among *E. coli* strains isolated from urine and feces, see (**Figure 6).**

Table (2): Frequency of *cdt* and *clb* gene in *E. coli* isolated from urine and stool samples.

Isolates	source	*cdt*	*clbA*	Isolates	source	*cdt*	*clbA*
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	-	-

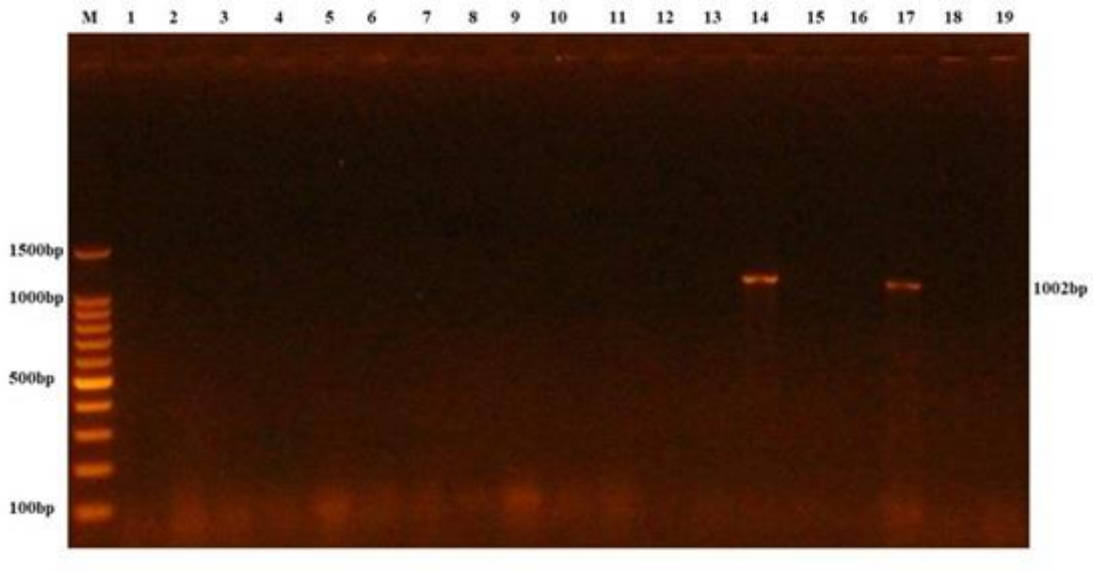

Figure 3: Results of the amplification of the ClbA *gene* of *Escherichia coli* samples were fractionated on 1.5% agarose gel electrophoresis stained with Ethidium Bromide M: 100bp ladder marker. Lanes 1-19 resemble 1002bp PCR products.

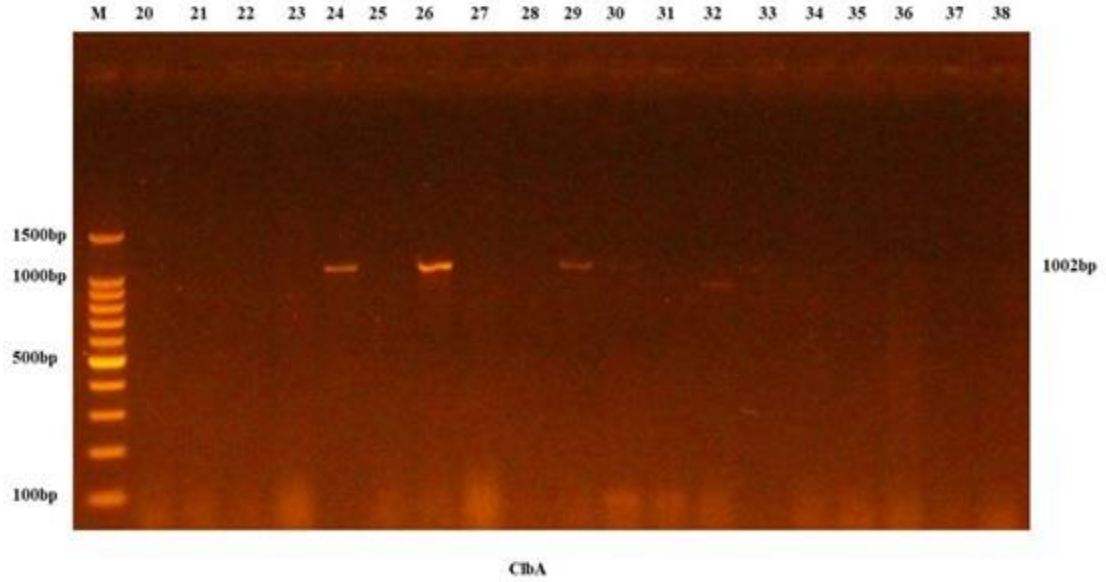

Figure 4: Results of the amplification of the ClbA *gene* of *Escherichia coli* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 20-38 resemble 1002bp PCR products.

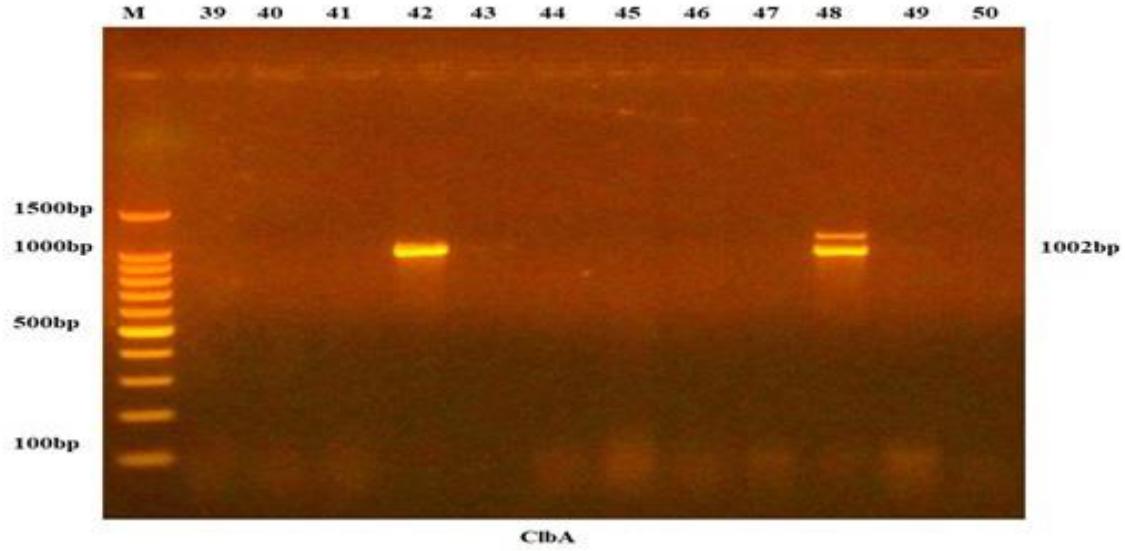

Figure (5): Results of the amplification of the *ClbA gene* of *Escherichia coli* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes 39-50 resemble 1002bp PCR products.

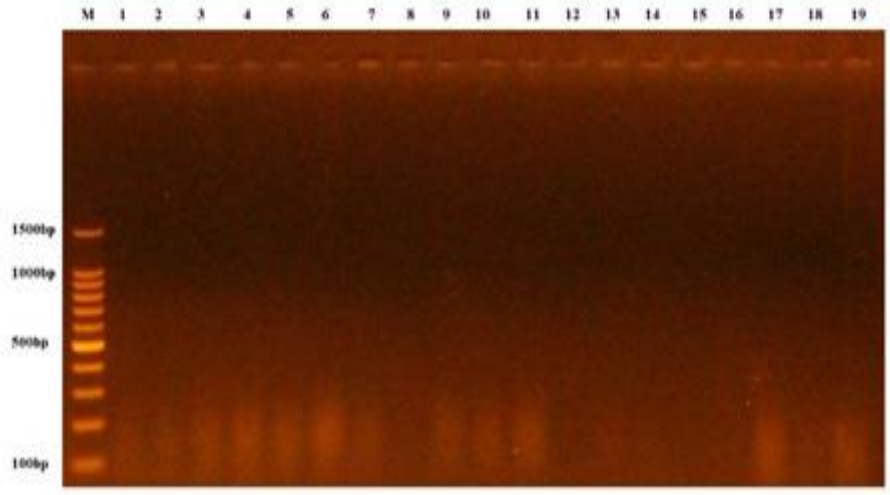

Figure (6): Results of the amplification of the *CdtB gene* of *Escherichia coli* samples were fractionated on 1.5% agarose gel electrophoresis stained with Ethidium Bromide M: 100bp ladder marker. Lanes 1-19 resemble PCR products.

DISCUSSION

According to some studies' findings, *E. coli* is the primary causative bacteria in recurrent UTI in women, which accounts for around 80% of all episodes of infection [15]. About 90% of urinary tract infections are caused by uropathogenic *E. coli*; the bacteria invade from feces or the perineum and ascend the urinary tract to the bladder [16]. 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 [17, 18].

A local study by **Shakur** [19] reported that *E.coli* isolates formed (42, 85%) of urine specimens. **Momoh *et al*.** [20] the University Of Benin Teaching Hospital (UBTH), Benin, Edo State, Nigeria, collected a total of 407 urine samples from patients with suspected UTI both on-site and off-site. Male and female samples yielded 162 (39.8%) and 245 (60.2%) *E. coli* isolates, respectively. A local study by **Sweedan *et al*.** [21] 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). All isolates were considered multidrug-resistant if they show resistance to at least three antibiotics from three separate classes (MDR) [22].

A local study by **Abdul-Ghaffar and Abu-Risha** [23] also reported *E.coli* as multidrug-resistant bacteria and this is in agreement with the present study. Concern over how to treat *E. coli* sickness is growing due to the growth in cephalosporin resistance, particularly given the parallel rise in the prevalence of multidrug-resistant *E. coli* [24]. Multidrug-resistant *E. coli* has been seen more frequently over the past few decades. The developing cephalosporin resistance, notably the accompanying rise in the prevalence of multidrug-resistant *E. coli*, is raising concerns about the treatment of *E. coli* disease [25]. Multidrug-resistant *E. coli* strains have become more common, which has limited treatment options [26, 27]. Antibiotic resistance in UPEC is rising annually, according to several researchers [28]. For the treatment of bacterial infections, antibiotic therapy is crucial. Gram-negative and Gram-positive bacterial infections are commonly treated with the second-generation fluoroquinolone antibiotic ciprofloxacin (CIP). The percentage of MDR *E. coli* isolates was 82.1% in a different study on UTIs in Iran [29]. Major repercussions of MDR include the empirical treatment of *E. coli* infections and a potential co-selection of antibiotic resistance, which is mediated by MDR plasmids [29]. It is advised for the detection of DNase in gram-negative microbes by using DNase agar containing toluidine blue because toluidine blue may be inhibitory to some gram-positive species [30]. This method represents a quick agar plate method for demonstrating the DNase activity of microorganisms was published by **Jeffries *et al.*** in 1957 [31]. 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 clear zone around growth denotes a favorable response. 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 [17]. The results of [32] revealed that out of 110 isolates, 28 harbored the *clbA* gene, 40 harbored *clb B,* and 13 isolates harbored *clbA, clbB,* and *cnf1* genes. The results of **Hussein *et al.*** [3] showed that the distribution of pks+ among the collected clinical isolates showed that 7 isolates belonged to *E. coli*. The findings by **McCarthy *et al.*** [33] indicate that the *pks* island responsible for colibactin production is widely distributed among neonatal *E. coli* K1 isolates, suggesting that this virulence factor has an important role to play in invasive disease in susceptible neonates. Numerous studies have revealed that intestinal and extraintestinal pathogenic microorganisms can produce cdt [34]. These studies were in agreement with the present study. Both diarrhoeagenic and uropathogenic *E. coli* strains, as well as those that do not belong to any known pathotype, have CDT-encoding genes found [35]. A study by **Hinenoya *et al.*** [36] reported that all four cdt-types genes were identified among their strains; most of them (60 %) carried the cdt-I type which was also the predominant type in Japan. These findings were in agreement with the present study.

CONCLUSIONS

The findings of this study show that multi-drug resistance (MDR) is highly spreading among uropathogenic *E.coli*. Our findings showed that most. *E.coli* isolates were resistant to amoxicillin while highly sensitive to amikacin, ciprofloxacin, tobramycin, and gentamicin. On the other hand. the current study revealed that the colibactin (*clb*) gene was found in 6 bacterial isolates, while the other 44 isolates don't have this gene. Cytolethal distending toxin B gene (*cdtB*) was not recorded in any one of the bacterial isolates.

Financial support and sponsorship: Nil

Conflict of interest: None, the authors declare that they have no conflict of interest.

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