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Molecular identification of some virulence related genes for *E. coli O157:H7* isolated from bloody diarrhea and UTI in Baghdad city.

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ABSTRACT

This study was carried out for detection of some virulence factors for *E coli* O157:H7 isolated from patients with bloody hemorrhagic diarrhea or urinary tract infection (UTI). A total of 200 bloody diarrhea and 150 urine samples were collected from children of both sexes between the age of 3 and 10 years, who were suffering from bloody diarrhea and urinary tract infection (UTI) in the period from September to December 2016 in, Central Children Hospital and Children Safe Hospital in Baghdad/Iraq. All samples were screened to detect the presence of non-sorbitol fermenting colonies on sorbitol MacConkey agar supplemented with Cefixime -Tellurite (CT-SMAC) also cultured on other enrichment and selective media(Hicrome and Eosin methylen blue) at 37°C for 24hrs. The isolates were identified by Vitek 2 system and they were confirmed by latex agglutination test. A total of 11 isolate, 8 (4%) from bloody diarrhea and 3 (2%) isolates from urine samples were diagnosed as *E. coli O157:H7* that appeared on CT-SMAC as small, circular and colorless colonies with smoky center whereas on Hicrome media as dark purple to magenta colored moiety colonies, positive for Vitek2 and latex agglutination. Polymerase chain reaction (PCR) was employed to detect some virulence genes of isolates ,as *hlyA* (responsible for hemolysine) *flicH7* (encoding fimbria) and *rfbO157* (encoding- lipopolysaccharide) using specific primers of 534, 625 and 259 bp for previous genes respectively . The result of PCR amplification revealed presence of *hly A* , *flic H7* and *rfb O157* genes in all isolate.

Introduction

Escherichia coli serotype *O157:H7* is a rare variety of *E. coli* that produces large quantities of one or more related, potent toxins that cause severe damage to the lining of the intestine [1] .The bacteria do not invade mucosal cells as readily as *Shigella, Yersinia* or *Aeromonas*, which are armed with fimbrae [2]; [3]. It is an emerging pathogen that causes acute human gastroenteritis and hemorrhagic colitis [4] and a major causative agent of severe UTI in children [5].

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Enterohemorrhagic Escherichia coli as a subgroup of Shiga-toxin (Stx)-producing *E. coli* (STEC) are characterized by certain serotypes that are frequently occurring in outbreaks and are associated with severe clinical illnesses such as hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) [6].

There are several virulence factors that contribute to *E. coli* pathogenicity, such as pilli, enterotoxins (LT, ST), shiga-like toxins, endotoxin (lipopolysaccharide), hemolysin, aerobactin, cytonecrotizing factor, intimin, and biofilm formation [7], [8] . This bacteria is identified by classical microbiological diagnostic procedures based on its inability to ferment sorbitol [9] which aids in the initia recognition of suspicious colonies isolated from bloody stools. The selectivity of sorbitol MacConkey

agar (SMAC) has been improved with the addition of cefiximerhamnose (CR-SMAC), cefixime-tellurite (CT-SMAC) and methylumbelliferyl-D-glucuronide (MSA-MUG) [10]. Several methods from conventional culture methods such as MacConkey agar containing sorbitol instead of lactose to serological assays are used for isolation and identification of *E. coli O157:H7* .

PCR, one of the molecular biology-based detection methods used for diagnosis of this pathogen that gives rapid, reliable results and that also has a high sensitivity and a high specificity [11].

This study aimed to diagnose *E. coli O157:H7* by different methods and detect of its virulence factors through by steps:

1-Isolation *E coli O157:H7* from children bloody diarrhea and urine on different specific media and confirm by use Vitek2 system and latex agglutination test.

2- Detection of some virulence gene *hly A* (responsible for hemolysine) , *flic H7* (encoding fimbria) and *rfbO157* (encoding lipopolysaccharid) by PCR using specific primers.

Materials and Methods

A total of 200 bloody diarrhea and 150 urine samples were collected in the period from the beginning of September to the end of December 2016 in Al-Eskan pediatrics hospital and children safe hospital from children of both sexes aged of 3 - 10 years. They were all suffered from bloody diarrhea and UTI, in Baghdad.

Loop full was taken for each 8 sample and enriched in modified tryptic soy broth (mTSB) supplemented with vancomycin (4mg-L) according to [12] and incubated at 37 °C for 24h. Then loop full was streaked onto CT-SMAC to seek sorbitol non-fermenting bacteria (colorless colonies) after that cultured onto Hicrome media and Eosin methylen blue (EMB) as differential Media.The bacteria were identified by VITEK2 (Biomerieux- france). The latex agglutination test (Oxoid-England) was done for confirmation the serotype.

Molecular detection:

Bacterial DNA Extraction:

DNA was extracted from 8 stool isolates and 3 urine isolates by DNA extraction Kit (promega USA) and then detected by gel Electrophoresis. The extraction protocol carried out as recommended by the manufacture data sheet. DNA Purity and concentrations of all samples were determined by using Spectrophotometer 2600 uv/vis (Unico USA).

Polymerase Chain Reaction (PCR):

Oligonucleotide primers were designed according to [13,14] (Table 1).

PCR tubes were transferred to the thermalcycler to start the amplification reaction according to a specific program for each gene (Table 2,3,4).

Table 1: Oligonucleotide primers sequences used for PCR amplification.

Primer	Sequence (5'-3')	Amplicon size(bp)	Reference
rfbO157	F: CGGACATCC ATGTGATATGG R: TTGCCTATG TACAGCTAATCC	259	Paton .and Paton (1998)
fliCH7	F: GCGCTGTCGA GTTCTATCGAGC R:CAACGGTGAC TTTATCGCCATTCC	625	Gannon et al. (1997)
HlyA	F:GCATCATCA AGCGTACGTTCC R:AATGAGCCAA GCTGGTTAAGCT	534	Paton,.and Paton (1998)

Table (2):PCR program of rfbO157:

Step	Temperature	Time	No. of cycles
Initial denaturation	94°C	4 min.	1
Denaturation	94°C	30 sec	35
Annealing	55 °C	30 sec.	
Extension	72°C	1 min.	
Final extension	72°C	4min.	1

Table (3): PCR program of flic gene:

Step	Temperature	Time	No. of cycles
Initial denaturation	94°C	30 sec.	1
Denaturation	94°C	30 sec	35
Annealing	55°C	30 sec	
Extension	72°C	1 min.	
Final extention	72°C	4min.	1

Table (4): PCR program of hlyA gene:

Step	Temperature	Time	No. of cycles
Initial denaturation	94°C	1min.	1
Denaturation	94°C	1min.	35
Annealing	55°C	2min.	
Extension	72°C	2.5 min.	
Final extention	72°C	5min.	1

Results:

The results revealed that 120 out of 200 stool samples were positive for *E. coli*. only 8 isolates were diagnosed as *E. coli O157:H7* while 87 out of 150 urine samples were positive to *E. coli*, only 3 isolates were diagnosed as *E. coli O157:H7* that appeared on SMAC as small, circular and colorless colonies with smoky center with (1-2) mm in diameter (Figure 1) and on Hicrome media that appeared dark purple to magenta colored moiety (Figure 2) (Table 5).

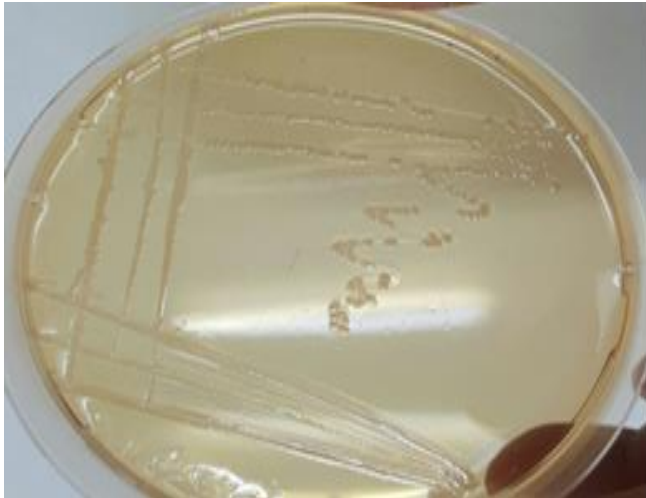

Figure (1) Colonies of *E. coli* O157:H7on CT- SMAC , after incubation for 24 hours at 37 °C.

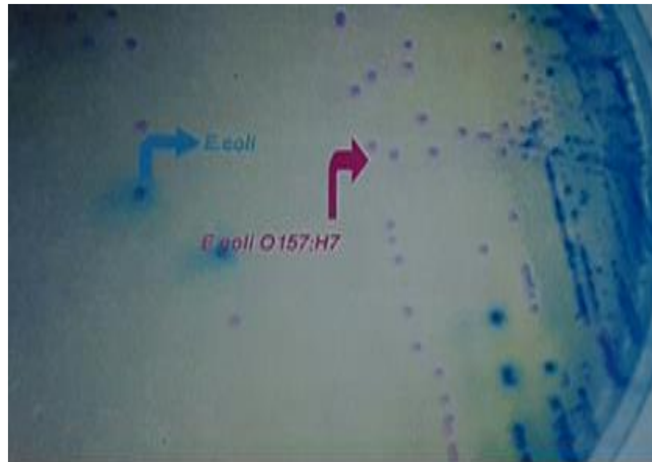

Figure (2) colonies of E .coli O157:H7 on Hicrome media (purple color) and other E. coli (blue color) after incubation for 24 hours at 37°C.

Table (5): Frequency of Non sorbitol fermenting (NSF) and CT-SMAC confirmed E. coli in Human stool and urine.

Isolates Source	Total samples	Non sorbitol fermenting	No. of E coli O157 positive isolates for CT-SMAC
Human stool	200	120(60%)	8(4%)
Human urine	150	87(58%)	3(2%)
Total	350	207	11(6%)

Diagnosis of bacteria by Vitek2:

Vitek2 was conducted for identification, of *E. coli O157:H7* the results clarified that 8 isolates from stool samples and 3 from urine samples were identified as *E. coli O157:H7.*

Serological identification of *E. coli* O157:H7:

All of the *E. coli* isolates were confirmed by latex agglutination test for the somatic *O157* antigen (Figure 3).

Molecular detection of virulence factors of isolates:

All isolates (11) were investigated for some virulence factors that responsible for pathogenicity of this pathogen and results found that all the isolates have these genes as demonstrated in Table (6) and Figures (4,5,6,7,8,9).

Figure (3): Latex agglutination test for somatic O157 antigen. No.1 positive sample, No.2 positive sample(agglutination) and No.3 control (noagglutination).

Table (6): Frequency of hly A, flicH7 and rfbO157 genes of isolates in the samples:

Isolates source	No.of positive isolates with SMAC media	hly A gene	flic H7 gene	rfbO157 gene
Human stool	8	8(100%)	8(100%)	8(100%)
Human urine	3	3 (100%)	3(100%)	3(100%)
Total	11	11	11	11

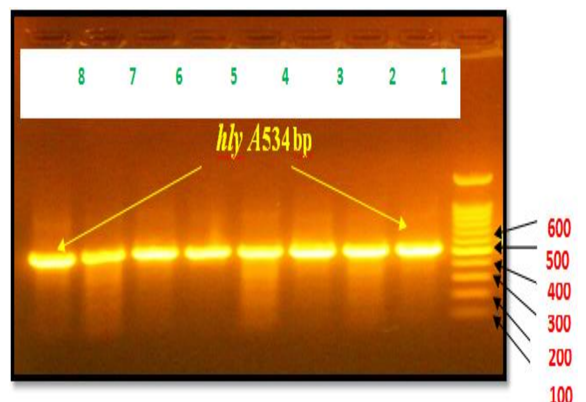

Figure (4): Gel electrophoresis of human stool (1,2,3,4,5,6,7,8) ,the amplified of *hly A* gene (534 bp)

was electrophoresed in 2% agarose gel at 75 volt/cm2 for 60 min.

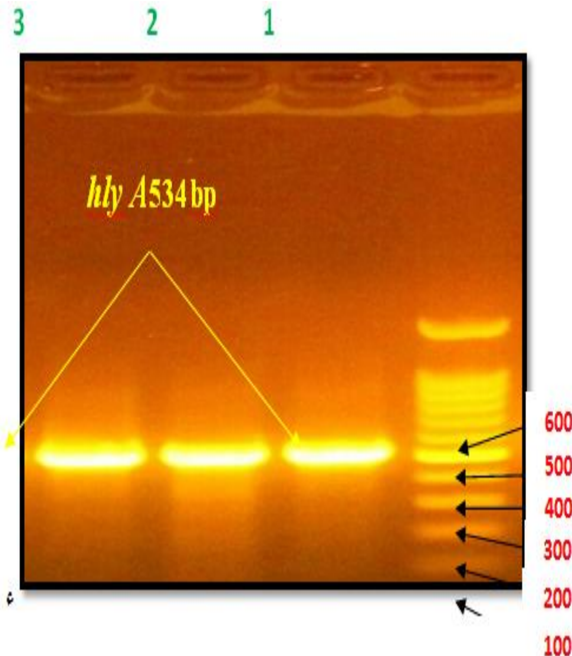

Figure (5): Gel electrophoresis of human urine (1,2,3), the amplified Of *hly A* gene (534 bp) was electrophoresed in 2% agarose gel at 75 volt/cm2 for 60 min.

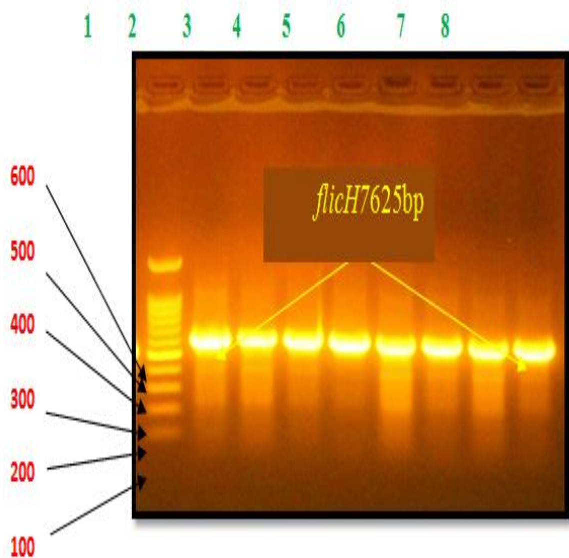

Figure (6): Gel electrophoresis of isolates from human stool (1,2,3,4,5,6,7,8) , the amplified of *flic* gene (625 bp) was electrophoresed in 2% agarose gel at 75 volt/cm2 for 60 min.

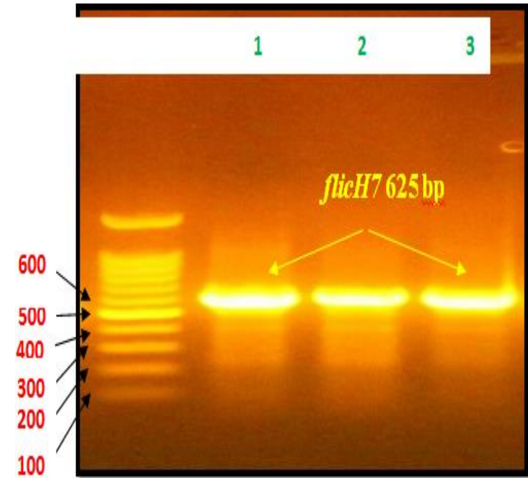

Figure (7): Gel electrophoresis of isolates from human urine (1,2,3) , the amplified of flic gene (625 bp) was electrophoresed in 2% agarose gel at 75 volt/cm2 for 60 min.

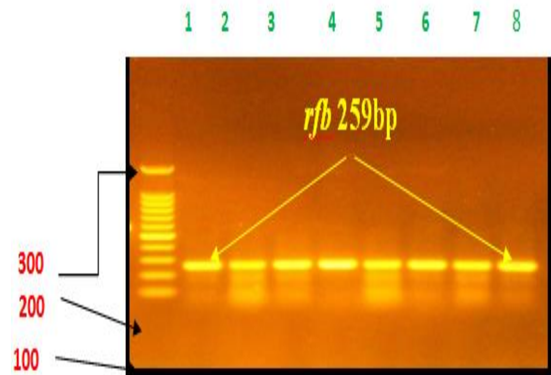

Figure (8): Gel electrophoresis of isolates from human stool (1,2,3,4,5,6,7,8) ,the amplified Of rfb gene (259 bp) electrophoresed in 2 electrophoresed % agarose gel at 75 volt/cm2 for 60 min.

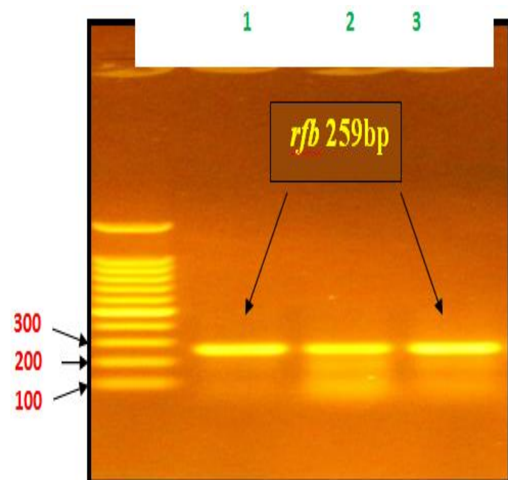

Figure (9): Gel electrophoresis of isolates from human urine (1,2,3), the amplified of rfb gene (259 b electrophoresed p) in 2% agarose gel at 75 volt/cm2 for 60 min.

Discussion:

Escherichia coli O157:H7 is well documented Shiga toxin–producing (STEC) serotype, it is one of hundreds of *E. coli* strains. Although the most strains are harmless and live in the intestine of healthy human and animals, STEC strain produces a powerful toxin and can cause severe illness [16,17].

In the present study, the virulence of *E. coli O157:H7* was assessed in bloody diarrhea and urine samples using three genes including: *fli*CH7, *rfbO157* and *hly*A.

Patients selection was restricted to those children suffered from bloody diarrhea and UTI because *E. coli O157:H7* is mostly associated with this clinical features. Resent study showed that 8 (4%) of Non Sorbitol Fermenting (NSF) revealed from bloody diarrhea samples which is similar to the study conducted by [18] who diagnosed *E .coli O157:H7* serotype as a cause of bloody diarrhea in children in Nasiriya city with ratio of 4% of patients.

E. coli O157:H7 isolated from 3(2%) samples of urine , and came in accordance with [5] who isolated this bacteria from 2.3 % of Urinary Tract Infections in an Iranian Children Hospital, while less than [8] who showed that 36 (14%) from 259 of children with UTIs in USA.

In North America, serotype *O157:H7* alone is responsible for a number of *E. coli* outbreaks involving 75,000 human infections per year [19].

The use of selective enrichment procedure in this study was due to this microorganism is fastidious and need revives the stressed and injured cells. Non sorbitol fermenting *E. coli* was isolated on selective enrichment Tryptic Soy Broth (TSB)supplemented with vancomycin as described by [20]. Also selective medium (CT-SMAC) was used to inhibit other enteric organisms that compete with and over grow the targeted organism. Then identification of *E. coli O157:H7* depend on a combination of biochemical and serological tests was done.

The confirmation of *E. coli O157:H7* was done by latex agglutination which confirmed identification of 8 isolated from stool and 3 from urine. [21] who evaluated agglutination test as a rapid presumptive detection of *E. coli* serotype *O157:H7* when isolated *E coli O157:H7* from both sex, males and females in Pediatric Hospital of Karbala governorate - Iraq. Latex agglutination test is sensitive and specific in identification of *E. coli O157:H7* but is expensive compared to ordinary conventional agglutination tests[15] . The use of this method in the present study was to eliminate other serotypes of pathogenic *E. coli*. Also it is simple and easy to use [22].

E. coli O157:H7 can be biochemically distinguished from common *E. coli* isolates based on enrichment step, a screen for sorbitol fermentation, and a final serological confirmation of *E. coli O157:H7* [23].

The differences between the current result and other studies depending on site of collection, season, number of hospitals surveyed and medication especially exposure to antibiotics, in addition to the use of significant parameters and methods to detect these pathogens. High percentage results in some studies may be due to the life style, rural individuals were more contact with source of infections such as carrier animals and their dairy or meat products [24]. The primary reservoir of this strain is the cattle. Cattle feces are a major source of contamination of beef, other food products, and water [25]. The direct contact

with animals and material contaminated with animal feces, will increase the chances of infection. Many studies done about this bacteria in Iraq and worldwide , that isolated it from different sources such as [25] who isolated *E coli O157* from Tigris River and children stool, also [26] who detected this bacteria in food and patients in Baghdad, while [27] isolated of *Escherichia coli O157:H7* from beef hamburgers in Khuzestan Province, Iran.

The molecular study was done by PCR for detection of 3 genes (*hlyA* – *rfb*O157 – *flic* H7) responsible for some virulence factors, and the result revealed that all isolates (11) were harbored these genes through amplification the specific fragments of these genes. The *hly A* was chosen in this study as enterohemolysin protein that cause lysis of red blood cells by destroying their cell membrane that lead to cause bloody diarrhea when *rfb* O157 gene choose as lippolysaccharide the O antigen and *flic* H7 gene (encoding fimbria) that help bacteria to attachment. These results confirmed that this pathogen considered as an important causes of gastrointestinal tract impairment and urinary tract infection in human.

PCR is a powerful molecular biology technique for the detection of target DNA in various clinical specimens and for the diagnosis of many kinds of pathogens. It is not only highly sensitive and specific, but it also provides rapid and reliable results for stool samples, it can help to distinguish diarrheagenic *E. coli* from those of the normal flora [28].

The serotype *O157:H7* positive for gene (*hly* A) isolated from urine , may indicated that this pathogen play crucial role in urinary tract infection in the humans and cause renal failure in the infected individuals. [29] identified 2 *E coli O157:H7* isolates positive for *hly A* from patients with UTI, other research by [30] reported 8 isolates from urine have *hly* genein the urinary tract infections of humans in Baghdad.

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الكشف الجزيئي لبعض الجينات ذات الصلة بعوامل الضراوة للاشيريشيا القولونية O157:H7 المعزولة من الاسهال الدموي والتهاب المجاري البولية في مدينة بغداد

محمد بكر الجبوري امنه نعمه الثويني ليث مصلح نجيب

الخلاصة:

هذه الدراسة نفذت للكشف عن بعض عوامل الضراوة لبكتريا *E coli* O157:H7 المعزولة من المرضى بالاسهال الدموي والتهاب المجاري البولية . جمعت 200 حالة اسهال دموي و150 عينة ادرار من الاطفال من كلا الجنسين بين عمر 3 الى10 سنوات والذين يعانون من الاسهال الدموي والتهاب المجاري البولية في الفترة من شهر ايلول الى شهر كانون الاول 2016 في مستشفى الطفل المركزي ومستشفى حماية الطفل في بغداد/العراق. جميع العينات فحصت للكشف عن وجود المستعمرات الغير مخمره للسوربيتول على وسط سوربيتول ماكونكي الاكار المدعم بالسفكسيم وكذلك زرعت على اوساط اخرى غنيه وانتخابيه (هايكروم , وايوسين مثيلين الازرق) على درجة حرارة 37 درجه مئويه ولمدة 24 ساعه .العزلات تم الكشف عنها عن طريق جهاز الفايتك وتم تاكيدها باستخدام فحص التلازن. من مجموع 11 عزلة, 8 (4%) من المرضى المصابين بالاسهال الدموي و3 (2%) عزلت من الادرار شخصت كبكتريا *E coli O157:H7* والتي ظهرت على وسط سوربيتول ماكونكي اكار صغيره , دائرية , عديمة اللون مع مركز داخن وعلى وسط الهايكروم ظهرت المستعمرات بلون ارجواني مظلم الى محمر, موجبة لفحص الفايتك وفحص التلازن. تفاعل البلمرة المتسلسل استخدم لكشف بعض جينات الضراوة للعزلات مثل *hly A* (المسؤول عن تحلل كريات الدم الحمراء),*flicH7* المسؤول عن الالتصاق) و *rfbo157* (المسؤول عن عامل الضراوه lipopolysaccharide) من خلال استخدام بادئات خاصه 534, 625 و259 لكل قاعده للجينات السابقه على التوالي. نتيجة تضاعف تفاعل البلمره المتسلسل اظهرت وجود الجينات *hly A, flicH7, rfb O157* في جميع العزلات.