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Detection of *Escherichia coli* O157:H7 strain in diabetic foot infection patients in Basrah, Iraq

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ABSTRACT

Diabetic foot ulcers (DFUs) are defined by the typical triad of neuropathy, ischemia, and infection. The shigatoxin, Enterohemorgic *E. coli*, is considered one of the most dangerous types of toxins caused by a strain of *E. coli* O157:H7 for diabetic foot patients, which is transmitted through contamination with it, causing uremic hemolytic syndrome and renal failure for those with impaired kidney function.

To detect virulence factors, medium-high chrom was used after addition of supplements; serotyping was used; and it was revealed by the molecular method using the specific gene ECVt1.

This study showed a strain on Hi-Chrome medium, and the colonies showed a dark purple color after the addition of its supplement, Novoboicin, and Calcium Tellurite. 16(38.09%) As for serotyping, the study showed a strain after purification on Maconkey sorbitol medium 7 (16.7%). The study revealed a gene, ECVt1, of this strain, 9 (21.43%), which has a size of 637 bp.

Do not use antibiotics for this strain, as they will increase its toxicity and may lead to death.

The chromogenic substrate is broken down by *E. coli* O157:H7 and produces a dark purple to magenta-colored moiety. Samples were cultured on MacConkey sorbitol agar for the purpose of testing them serologically to ensure the appearance of *E. coli.* O157:H7, where the colonies are not sorbitol fermented Genetic detection confirmed the existence of the gene ECVt1 belonging to enterohememorragic *E. coli*, the main cause of this strain O157:H7,

Keywords: *shigatoxin, Enterohemorgic E. coli ,Hi-Chrome EC O157:H7, ECVt1*

INTRODUCTION

Diabetes foot infection (DFI), a multi-microbial infection of the soft tissues and bones in the lower extremities of diabetes patients, Several factors predispose diabetic patients to developing a DFI. (Pitocco *et al.,* 2019) including an infection, ulceration, neuropathy, vasculopathy, immunopathy, peripheral vascular illnesses, foot biomechanics, and destruction of the deep tissues linked to neurological abnormalities in the lower extremities. (Aruoah, 2021) Moderate and severe infections include those caused by facultative anaerobic Gram-negative microorganisms. The pathogens often have synergistic relationships, such as *E. coli* (Ibrahim, 2018). *E. coli* is considered the most important member of the Enterobacteriaceae family. Some strains of *E. coli* are opportunistic (Gharajalar and Sofiani, 2017; Batt, 2019; Braz *et al.*, 2020). *E. coli* are a genetically diverse group of bacteria that form part of the normal intestinal microflora of humans and animals. They are normally non-pathogenic. However, certain subgroups acquired genes that allowed them to cause extraintestinal or gastrointestinal disease (Hlad *et al.*, 2018)

.Both extra-intestinal infections that cause disease, such as urinary tract infections, as well as intestinal infections, including diarrheal disorders, are caused by pathogenic strains. Based on their traits and virulence factors, pathogenic *E. coli* strains are divided into pathotypes Shiga-Toxin Producing *E. coli* (STEC) or Enterohemorrhagic *E. coli (EHEC)*, Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), and Diffusely adhering *E. coli* (DAEC) (Naser, 2015; Yu *et al*., 2021; Neema, 2022). It is believed that Shiga toxin (Stx) is the main virulence factor of different *E. coli.* Individuals with little to no fever, severe stomach discomfort, and blood diarrhea (BD), between 5% and 15% of which evolve into a life-threatening hemolytic uremic syndrome (HUS) connected to ischemic organ damage, (Torti *et al.,* 2021; Kudva and Biernbaum, 2022). *E. coli*O157:H7 It is the most common type of infection that causes diseases, even if the disease-causing dose is small (Hlad *et al.,* 2018). *E. coli* strains are capable of causing a wide range of diseases. Shiga toxin-producing *E. coli* (STEC), one of these pathotypes, refers to strains of *E. coli* that generate at least one Shiga toxin, a kind of strong cytotoxin (Stx) (Hlad *et al.,* 2018). *E. coli* that creates STEC, often referred to as Vero toxin-producing *E. coli* (VTEC) andVTEC," is a subgroup of STX genes that are normally acquired by a bacteriophage, which results in coli that express the genes of STX. Of all the newly discovered foodborne bacteria, STEC is the most dangerous. (BD) and HUS are just a few of the serious human disorders that STEC can cause. (Torti *et al.,* 2021) Stx The characteristic thrombotic microangiop- athy triad of microangiopathic haemolytic anemia, thrombocytopenia, and renal impairment (Harkins *et al.,* 2020). Recent studies have classified pathogenic *E. coli* strains using various antibodies to recognize surface antigens associated with "183 O-groups (lipopolysaccharide) and 53 H-types (flagellar antigen)" (Younis *et al.,* 2021). The "O" and "H" antigens of *E. coli* strains and lineages are used to categorize them. The "O" (ohne) antigen is a component of the lipopolysaccharide (LPS) embedded in the outer leaflet of the bacteria's outer membrane and is defined serologically and determined by the repeating polysaccharide chains (Torti *et al.,* 2021). The O and H antigens are most frequently utilized in the serotyping of pathogenic Members of a clone of bacteria that express the same O antigen are referred to as a serogroup. *E. coli* because they have the best connection with virulence factors. The designation changes to serotype when the serogroup and the H (flagellar) antigen are specified together (Torti *et al.,* 2021). Infections can be contracted by consuming tainted food, untreated or raw water, or even water that has been contaminated with both animal and human feces. Furthermore, direct transmission from one person to another can happen when an asymptomatic carrier has been identified (Al-Wahid, 2018).

MATERIALS AND METHODS

Samples collection:

A total of 42 wounds, ulcers, and amputation swab samples were collected from patients of different ages suffering from diabetic foot infections. The samples were collected from the forefoot, midfoot, and hindfoot from November 2021 to May 2022 in Basrah Al-Sader Teaching Hospital. Wounds, ulcers, and amputations samples were collected from patients admitted to the three main hospitals in Basra and popular clinics. These are: Al-Sader Teaching Hospital, Al-Faeha General Hospital, Al-Mawana General Hospital, and Popular Clinics.

Isolation and Identification :

The primary diagnosis of the isolates appeared when cultured on the differential and selective medium MacConkey agar with crystal violet and Eosin methylene blue agar (EMB). (Naser,2015) The isolates were examined under a microscope. All samples were subjected to the classic biochemical tests (imvic tests) and confirmed by using the Vitek system for negative bacteria. The results were positive, with good, very good, and excellent rates.

Detection of E. coli O157:H7 Uesd hi-chrom E. coli O157:H7

To identify *E. coli* isolated from diabetic foot patients as belonging to the hemorrhagic O157:H7 strain, Hi-chrom *ECO*157:H7 selectivity agar was used for this strain. which appears in two colors Either a light pink mauve or a dark purple magenta Downes (2015),

TABLE 1. The table (1) shows Cultural Response

Organism	Inoculum CFU)(	Growth	Recovery	Colour of colony
E. coli ATCC 25922	50 -100	none to poor <=10%	<=10%	light pink mauve
E. coli O157:H7NCTC12900	50 -100	luxurint	>=50%	dark purple magenata

Detection of E. coli O157:H7 Uesd Serotyping of E. coli O157:H7

The serological typing of all positive strains was carried out by slide agglutination with *E. coli* O and H antisera (O157:H7). The determination of 'O' serogroups associated with the cell wall lipopolysaccharides and 'H' serogroups of the flagella has been used to identify and characterize EHEC. as seen in Figure (3).

procedure: (Use, 2020)

1. Non-sorbitol fermenting colonies (NSFC) isolated on sorbitol MacConkey agar (SMAC) are acceptable isolates for testing for *E. coli* O157 :H7

2 .A subculture of NSFC is cultured on blood agar to identify *E. coli*

For each isolate that will be evaluated, put one drop of test latex (green cap) into the test slide well.

3. Put one drop of *E. coli* Control Latex (neutral cap) onto a different test slide in the same manner

4. Using the supplied plastic stick, remove part of the NSFC from the SMAC plate, and then emulsify it in the *E. coli* O157 Test Latex on the slide.

5. Agglutination should appear after rotating the slide in three planes in a circular motion for up to a minute.

6.If the *E. coli* O157 Test Latex results in agglutination and the Control Latex is negative, proceed to step 7 by striking the isolate onto a blood agar plate and incubating it overnight.

7. if agglutination is caused by both the test latex and the control latex. Throw away the test slide after reading.

8. After a planning process on a blood agar plate for 24 hours, work to take a drop of latex with a blue cover of the virulence factor H7. Then do the agglutination test. This step does not require a latex control.

Molecular detection of E.coli O157:H7 strain:

TABLE 2. Primers used in the study

Primer Name	Sequence (5 to 3)	Product size (bp)	Reference
EC-vt1-2-F	CGT CTT TAC TGA TGA TTG ATA GTG GC	637	(Oh *et al.*, 2014)
EC-vt1-2-R	CGC GAT GCA TGA TGA TGA C	637	

To investigate the gene responsible for the secretion of shiga toxin, polymerase chain reaction (PCR) using ECVt1 gene-specific primer sets was used for the detection of some virulence factors. primers mentioned in Table 2 were used for the detection of some virulence factors. as seen in Figure 5.

Reagent

TABLE 3. Reagent(50µl) of PCR for amplifying gene *ECVt1*Pioneer (master mix)

No	Reagent	Volume
1	Genomic DNA	5 µl
2	Fowered primer	2 µl
3	Revears primer	2 µl
4	master mix	25 µl
5	N.F.W	16 µl
6	Total volume	50

TABLE 4. PCR condition of ***ECVt1*** gene amplification

Step	Tim	Temprature	No. of cycle
Inatial denaturation	95.c	7min	1
denaturation	95.c	30sec	35
Annealing	58.c	1min	35
Extenation	72.c	1min	35
Final extenation	72.c	10min	1

Antibiotic sensitivity:

Clinically, the use of antibiotics is not advised due to the activation of the lytic cycle, which might result in the release of toxins. Studies have shown that fluoroquinolones increase the synthesis of Stx2 in STEC O157:H7. **(Torti *et al.*, 2021).**

RESULTS

Identification of E. coli

The results of the culture indicated pink-dry colonies on MacConkey agar, with bile salt precipitating around the colonies concerning *E. coli*. While cultured on EMB selective media, the cells revealed a green color when examined under a microscope. The bacteria cells appear as short, red, and not spore-forming rods. Test results on the Vitek®2 System revealed the diagnosis of a strain of *E. coli* O157

Detection of bacterial E.coli O157:H7

The strain *E. coli* O157:H7 was isolated from patients with diabetic feet using Hi-Chrome *EC*O157:H7 agar, which contains sorbitol and a chromogenic combination. The colonies are light pink mauve and dark purple magenta. The turquoise color indicates the presence of *E. coli* only and not the strain O157:H7. Figure (2) shows the color of the pure and mixed colonies Figure (1).

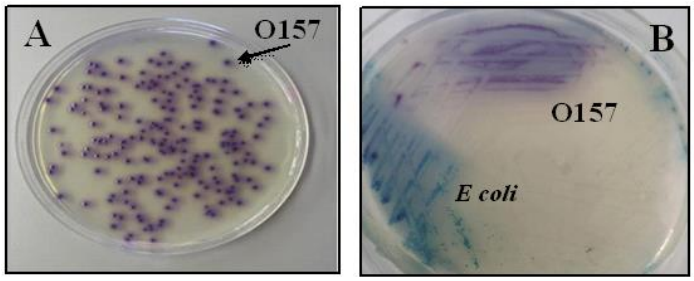

FIGURE 1. A Shown *E. coli* O157:H7 on Hi-Chromo *EC*O157:H 7 agar and B *E.coli* MixedO157:H 7 and NO O157:H 7 on Hi-Chromo *ECO*157:H 7

serotyping E.coli O157:H7

After confirming the pathological strain that appeared on the medium of the chromogenic agar, it was cultivated on the selective culture medium MacConkey with sorbitol agar, and the appearance of the non-ferment sorbitol colonies (NFSC) was confirmed. **Figure (2)** shows sorbitol ferment colonies (SFC) and (NFSC) .

A positive response is shown by an apparent agglutination in a clear fluid, whereas a negative response is indicated by a homogeneous, milky turbidity. Seven isolates out of 16 isolates grown on HiChrome medium gave a positive result for serotyping 7 (43.75%). The **figure (4)** illustrates this agglutination (O157:H7).

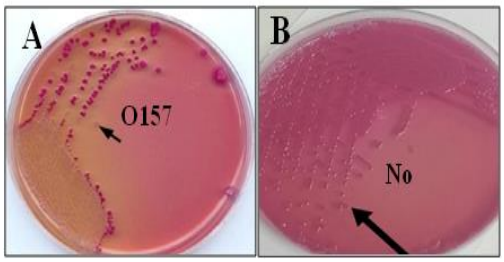

FIGURE 2. shown A. *E.coli* O157:H7 (NSFC) and B. *E.coli* No O157:H7 (SFMAC) .

Then a non-fermented lactose colony is taken and cultured on the blood agar in order to test it serologically to ensure the presence of the virulence factor H7 **Figure(3)**

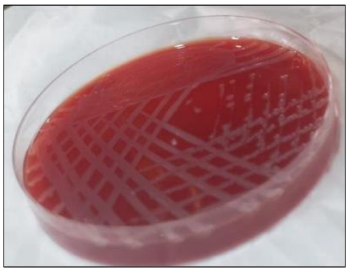

FIGURE 3. Shown *E.coli* O157:H7 on the blood agar

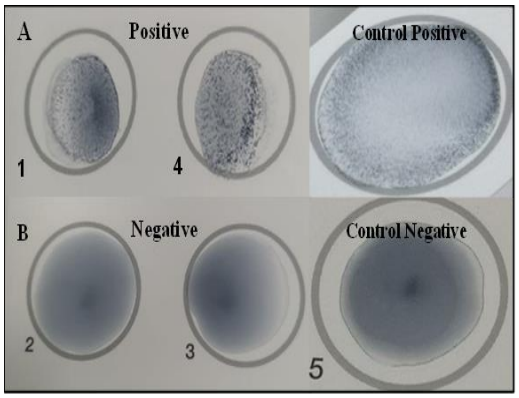

FIGURE 4. shown Slide Agglutination *E.coli* O157 H7A. positive and B. negative

The PCR technique was used to amplify the gene EHEC ECVt1 that detects the virulence factor of *E. coli*. Through genetic detection The ECVT1 responsible for Shiga toxin secretion was

investigated by the PCR technique of EHEC, the pathological strain of diabetic foot patients was identified by Hi-Chrom ECO157:H7 agar, and the O157:H7 strain was confirmed by serotyping. Figure6 The table (5) shows the detection of nine isolates of EHEC producing Shiga toxin out of 42 isolates of *E. coli* **9 (21.43%).** Then came the detection of 16 isolates of the strain O157:H7 on Hi-Chrome media out of 42 isolates of *E. coli* **16 (38.09%).** These isolates were confirmed by serotyping seven isolates of the O157:H7 strain out of the 16 isolates of the O157:H7 strain tested on Hi-Chrome medium **7 (16.7%).**

TABLE 5. Isolation of EHEC from the samples of the diabetic foot ulcer patients

EHEC No. (%)			**Total No. of sample tested**
EHEC *ECVt1*	**Hi-Chrom *ECO*157:H7 agar**	**serotyping *E.coli* O157:H7**	
9(21.43 %)	16 (38.09%)	7 (16.7%)	42

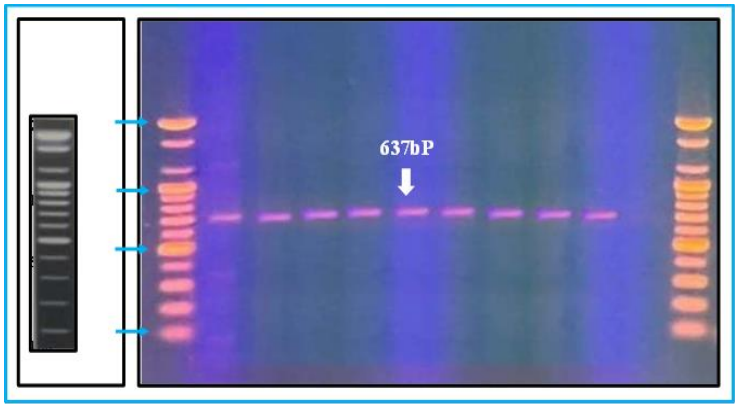

FIGURE 5. shows PCR-amplified EHEC *ECVt1* of 1% agarose gel electrophoresis, *E. coli* Approximately the size . (637bp) In comparison to a DNA lader, L1-L2 100-2000 bp isolate No1-9*Ecoli* Temperatures 58ºC, 70V, and 80 min

DISCUSSION

Ecoli was diagnosed based on the differential medium, MacConkey agar. Which contains crystal violet dye and yellow salts that inhibit the growth of gram-positive bacteria. Identical results with Albadri (2021), while EMB appeared green with a metallic sheen. Identical outcomes were found in Naser *et al.* (2015) and Antony *et al.* (2016).

The Gram-negative identification card was utilized in the current study to identify Gram-negative bacteria, and the Vitek®2 System test was employed to support and corroborate the results of the biochemical identification test in addition to diagnosing a strain of *E. coli* O157:H7. This study indicates excellent results. Hano (2019), Identical with Neema (2022).

colonies appeared in a turquoise color for *E. coli* without adding any supplements Due to the bacteria's production of the enzyme glucuronidase, *E. coli* absorbs the chromogenic substrate p-nitrophenyl-p-D-glucopyranosiduronic acid (PNPG) from the medium. The intracellular glucuronidase enzyme then breaks the bond between the chromophore and the glucuronide, releasing the chromophore, and resulting in the formation of a colony that is blue or green in color. chromogenic combination, which are used in the medium in place of lactose and indicator dyes. So, in particular and selectively, the chromaginous substrate was cleaved from the chromogenic substrate, *E. coli* O157:H7, and produced a dark purple to magenta-. Colonies of *E. coli* can range in color from pale pink to purple. This study is supported by a study Abdul Razzaq *et al.*, 2021). There are other reasons for the efficiency of the medium. Downes (2015), (1) The medium becomes selective when HiCrome *EC* O157:H7 Selective Supplement (FD187) is added (2). Aeromonas and Providencia species are specifically inhibited by potassium tellurite. Gram-positive bacteria are prevented from growing by novobiocin. diagnosis of *E. coli* O157:H7. Hichrom agar is considered a suitable medium for isolation and identification of *E. coli* O157:H7, as confirmed in a study by Jenkins *et al.* (2020) compared to MacConkey and EMB agar, as Hichrom medium contains sorbitol and a chromogenic mixture instead of lactose and indicator dyes, respectively. The chromogenic agent X-glucuronide used in this medium helped in the

detection of the glucuronidase activity of *E. coli* cells that absorb the X-glucuronide. The released chromophore resulted in light pink to mauve-colored colonies. recorded serotype results: 7 out of 42 isolates (16.7%) (O157:H7) While in another Al-Shatty (2016) study. recorded results for the same serotype (O157:H7) but isolated from fresh beef contaminated with *E. coli*, an estimated 5% out of 540 samples. length of his search in the city of Basra.

In the present test, samples were cultured on MacConkey sorbitol agar for the purpose of testing them serologically to ensure the appearance of *E. coli.* O157:H7, whereas in the study Gambush *et al.,* (2022), To detect O157:H7 and increase *E. coli* selectivity, tellurite potassium, cefixime, and vancomycin were also added to the SMAC agar. As a result of mutations, some O157:H7 strains lost their ability to ferment sorbitol, including their capacity to produce -glucuronidase. However, a subgroup of O157:H7 that had the ability to ferment sorbitol lost its ability to move, creating an O157: H-lineage. The technique most commonly used in clinical laboratories when samples identified as O157:H7 are examined is O-antigen determination, which was done in the current study by latex agglutination. When mixed with bacterial growth, these latex granules are coated with antibodies against the O157 antigen, and the O157 STEC bacteria adhere to the latex particle to produce visible agglutination, which indicates a positive result. This was confirmed by the previous study by Mohseni (2022).

Serotyping was one of the fundamental methods used in the 1930s to distinguish between various species and subspecies of bacteria based on antigen-antibody responses. Even more sensitive phage typing techniques were later established in the 1950s. These methods have always been used to determine the origin of infections (Uelze *et al.,* 2020).

Genetic detection of the virulence factor The ECVT1 is responsible for Shiga toxin secretion by the PCR technique, which is considered one of the most dangerous and life-threatening strains of hemorrhagic *E. coli* (in addition to the previous methods that were mentioned for the strain O157:H7, whereas another study found that EHEC O157:H7 released the Shiga toxin after taking antibiotics but not subtype O104:H4 (Corogeanu *et al.,* 2012) and another study by Zhang *et al.,* 2021). The death rate in the study (2.38%) resulting from uremic hemolytic syndrome after renal failure occurred in the end stage of the disease, and the treatment did not work to reduce the severity of the disease but increased its complexity. This is consistent with the study by Harkins et al. (2020) (3%). The death rate increases as the treatment increases toxicity in the blood due to this strain. This is consistent with the study (Mody *et al.,* 2015; Harkins *et al.,* 2020). Rapid detection, identification, and distinction of foodborne pathogens have all been accomplished using polymerase chain reaction techniques. They have been applied to the detection and diagnosis of infectious diseases as well as the diagnosis of hereditary and infectious disorders, genetic fingerprint identification, and DNA cloning. In order to identify common bacterial strains that occur in viable but uncultivable conditions and are frequently missed by the traditional method (Adzitey *et al.,* 2013). Since phenotypic methods are not sufficient for identifying bacterial species, determining the phenotype of these bacterial species may be difficult or impossible in microbiology laboratories (Clark *et al.,* 2013; Hanan, 2022).

CONCLUSIONS

This is a summary of the results of a study:

1- appearance of ECVT1, belonging to the bacterial species enter hemorrhagic *E. coli*, in patients with diabetic foot infection, which appeared in Basra hospitals and some popular clinics.

2- The emergence of a strain O157:H7

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