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RESEARCH ARTICLE

Genetic Characterization of *Escherichia coli* O157 Isolated From Human Stool Specimens in Wassit Province of Iraq

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Abstract

The goal of this study was to assess the severity of diarrhea caused by Shiga toxins produced by *Escherichia coli* (STEC) isolated from human infections in Wassit province of Iraq. Stool specimens from (161) sporadic cases of diarrheal Patients with the mean age of (20 years), and range from 1 month to 75 years, were collected in AL-Karamma Teaching Hospital between March to July 2019. Then they were processed by culture, microscopic tests and VITEK which were used for the identification as *E. coli*. PCR was performed for detecting shiga toxin genes in *E. coli* isolates, and *rfb* gene (encoding O-antigene) of STEC- O157. DNA sequencing was done on some positive isolates. The results of PCR detected *stx* genes in 19 (12 %) culture isolates of *E. coli* isolated from human diarrheal specimens. While 9 cases were positive for *stx* genes and have *rfb* gene. DNA sequences that depend on the sequence of the *vtx2* gene have shown a highly homologous sequencing identity with NCBI-Blast *Escherichia coli* strain(O157:H7) isolates from humans and animals. The phylogenetic study revealed a clear genetic relationship between human and animal *E. coli* isolates and then gene sequencing was deposited with accession number into NCBI-Genbank (0MN944014.1). In conclusion, prevalence of *E. coli O157* in humans remains undiagnosed because there are no traditional O157 detection methods in all our hospitals. Our study showed that the use of molecular methods in the detection can be used to detect STECs which cannot be detected using routine methods.

Keywords: Diarrhea, Shiga toxin-producing *Escherichia coli* (STEC), *E. coli O157*.

1. Introduction

Escherichia coli are gram-negative bacteria which are non-sporulating, flagellated and rod-shaped, with a agrowth potential (in aerobic and anaerobic conditions) in various types of food [1]. Shiga toxin or (verotoxin) producing by E. coli Is a division of an important virulent bacterial group referred to as enterohemorrhagic Escherichia coli (EHEC) [2,3]. The majority of human infections caused by STEC originate from the ingestion of contaminated food, particularly bovine ones such as (undercooked ground beef, unpasteurized cows' milk) and by contact from person to person. Within the (STEC family) Some strains, such as O157:H7, which is the primary serotype of Enterohemorrhagic E. coli seem to be more virulence to humans. These strains were classified as major foodborne pathogens [4,5]. STEC causes diseases of the human digestive system with different clinical spectra, ranging from watery diarrhea, bloody diarrhea , hemorrhagic colitis , and in some cases develope to hemolytic uremic syndrome (HUS) [6].

The expression of Shiga toxins and other virulence factors is characterized in STEC O157: H7 [7]. Shiga toxins are composed of two main groups : shiga toxin 1 (Stx1) and shiga toxin 2 (Stx2) which have been encoded to the prophage [8,9]. The (shiga toxin genes) can be transmitted horizontally to E. coli [10,11], allowing to convert non-producing shiga toxin strains into toxin producing strains. This study aimed at isolating and identifying local STEC O157 strains obtained from humans with acute diarrhea to describe these strains using molecular methods.

2. Materials and methods

2.1 Specimens collection

One hundred and sixty-one stool specimens were collected from patients suffering from diarrhea in different locations in the Iraqi province of Wasit. These specimens were placed in tryptic soya broth (TSB) as enrichment media and transported to the bacteriology Laboratory of AL-Karamma teaching hospital.

2.2 Isolation and identification of *E. coli* by using Traditional methods

E. coli was isolated from stool specimens by overnight culture of tryptic soya broth (TSB) at 37 ° C. Then bacterial growth was cultivated in MacConkey agar, Eosin methylene blue agar (EMB), Sorbitol MacConkey agar, and O157-CHROM agar overnight to make pure *E. coli* isolates. Biochemical determination tests were performed by the VITEK @ 2 system using GN-ID cards [12,13].

2.3 Molecular methods

2.3.1 Extraction of DNA from bacterial genome:

DNA was extracted from activated pure culture of *E. coli* bacteria using DNA bacteria kit (Geneaid. USA). Detection of DNA bands was done using agarose gelelectrophoresis (1%). The DNA concentration was also checked with a nanodrop (spectrometer) and kept in the freezer at -20 ° C [14,15]. The primers used in this study are shown in Table-1.

Table -1: The sequence forward and reverse of primers *vtx1*,*vtx2*, and *rfb* genes.

Genes	Primer Sequence (5' to 3')	Product size (bp)	References
Shiga-toxin 1 *(vtx1)*	F: AAATCGCCATTCGTTGACTACTTCT R: TGCCATTCTGGCAACTCGCGATGCA	750 bp	[16]
Shiga-toxin 2 (*vtx2*)	F: CTT CGG TAT CCT ATT CCC GG R: CTG CTG TGA CAG TGA CAA AAC GC	366 bp	Design in NCBI
rfb	F: CGGACATCCATGTGATATGG R: TTGCCTATGTACAGCTAATCC	259 bp	[16]

2.3.2 Detection of virulence genes by PCR

In PCR study , different primers of the virulence genes were used to identify STEC as shown in table-1. In addition, the reaction mixture contained Green Master Mix (Promega Company), which is pre-prepared to use solution containing (Taq DNA polymerase dNTP, MgCl2), and the reaction mixtures were formulated in 0.2 ml eppendorf tube with 25 µl reaction volumes PCR according to Promega protocol.

The reaction was followed by adding 3.0 µl of purified genomic DNA, 1.0 µl of 10pmole from the (F primer), 1.0 µl of 10 pmole from the (R primer) to a premix PCR tube, and volume completion to a total volume of 25 µl by PCR deionized water according to the group's instructions. In short, it is mixed with vortex and rotation. PCR thermocycler apparatus (Agilent Technologies, USA) used to produce a reaction. There are 35 cycles in this reaction. The reaction consists of four Phases including the first Phase being DNA denaturation at (95 ° C for 45 seconds), the second Phase being primary annealing at (58 ° C for 45 seconds), and the third Phase being primary extension at (72 ° C for 45 seconds). The last Phase was a 7 minute extension stage for *vtx2* gene at 72 ° C. Thus in the *vtx1* gene (95 ° C was for 4 minutes) in the initial denaturation, the next 35 cycles were for (30 seconds at 95 ° C) as denaturation, for (45 seconds at 58 ° C) as annealing, and for (60 seconds at 72 ° C) as first extension and finally extension for 8 minutes at 72 ° C. The last step was the electrophoresis used to test the PCR product band at (1.5 % agarose gel electrophoresis at 70 volt for 60 min), then stained with ethidium bromide stain and monitored under UV light [16].

2.3.3 Identification of STEC serogroup O157

The identified O157 isolates by conventional methods were examined for the detection of STEC serogroup O157 by Uniplex PCR assay with the use of specific primer sets which targeted *rfb* gene specific to *E. coli* O157 producing an amplicon of 259 base pairs. The PCR were performed with PCR system (Agilent Technologies, USA) at 95° C for 3 minutes for one cycle followed by 30 cycles of 94°C for 1 minute , 55°C for 1 minute and 72°C for 1 min. with final extension at 72°C for 10 minutes . The amplified PCR products were detected by (1.5 % agarose gel electrophoresis at 70 volt for 60 min), and visualized by staining with ethidium bromide through using gel documentation system (SCIE-PLAS, UK) [16].

2.3.4 Sequencing and Sequence Alignment

On 2 % agarose gel electrophoresis PCR products have been separated and visualized by exposure to ultra violate light (302 nm) after staining with ethidium bromide. Sequencing of genes was performed by MACROGEN KOREA, (biotechnology lab) machine DNA sequencer 3730XL, Applied Biosystem), Homology check was performed using (Basic Local Alignment Search Tool ,BLAST) program which is available at the (National Center Biotechnology Information ,NCBI) online at (http:// www.ncbi.nlm.nih.gov) and BioEdit program.
Phylogenetic analysis for sequenced genes has been conducted using ClustalW in MEGA 6, a neighbor-binding tool to recognize genetic similarities and create a phylogenetic tree for O157 STEC isolates from specimens.

3. Results

This study included 161 Iraqi patients, all of them from the province of Wassit suffering from diarrhea, whose ages ranged from one month to seventy-five years.. STEC was found in 19 (12 %) patients. The highest rate 63% (12/19) were in the age group (15 – 65 years). The lowest rate 10.5% (2/19) in the age group more than 65 years.

3.1 Bacteriological results

Cultural characterization of the *E. coli* growth appeared on MacConkey agar as pink colonies while delivering a metallic green sheen on Eosin-methylene blue agar (Figure -1).

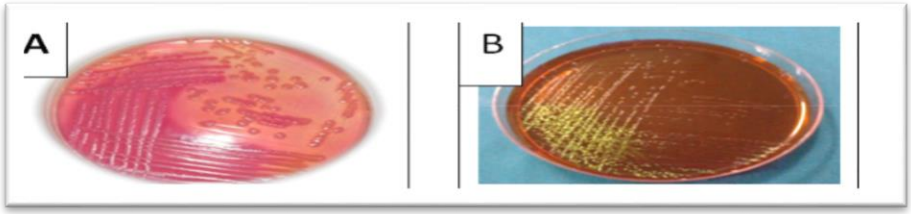

Figure- 1: (A) Pink colonies on MacConkey agar, (B) Metallic green sheen on Eosin-methylene blue agar

Culture results of Sorbitol MacConkey agar showed colorless colonies called (NSF), representing about 14 (8.7%) isolates, while sorbitol fermenting isolates (SF) of *E. coli* appeared with pink color colonies representing about 147 (91.3%) isolates. (Figure -2)

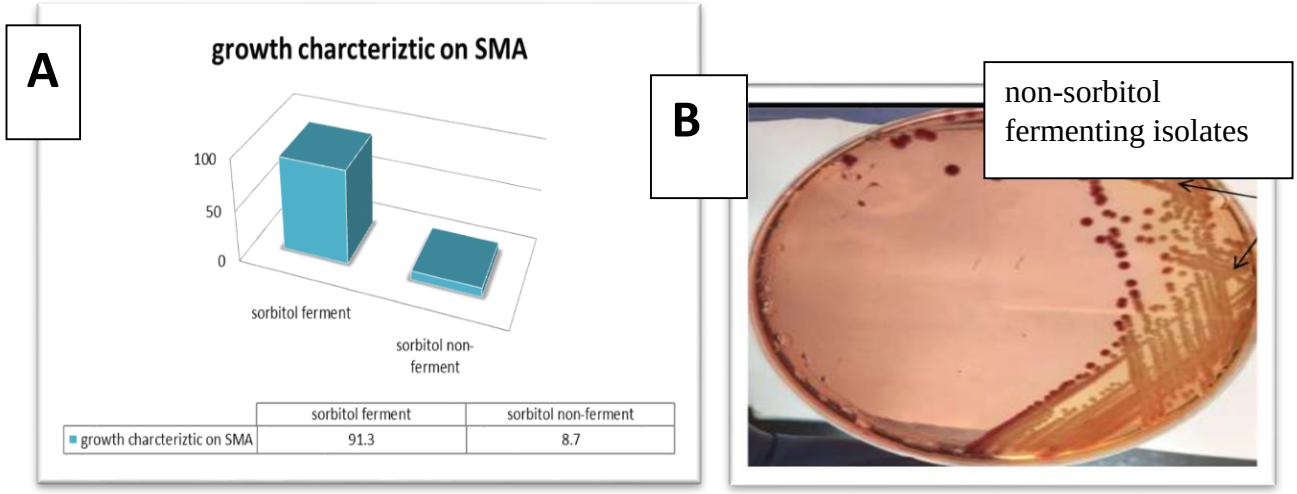

Figure -2: (A) Prevalence of non-sorbitol *E. coli*, (B) Appearance of *E. coli* colonies on such medium.

All colonies (positive or negative for sorbitol on sorbitol MacConkey agar) were grown on O157 CHROMagar in which O157 STEC provided mauve color colonies, while non –O157 and other *E. coli* that either gave or inhibited blue or white colonies. The results of growth on O157 CHROMagar medium with cefixime tellurite supplement were exhibited 16 (9.93%) isolates as mauve color colonies, 11 (6.83%) colonies appeared pale pink to faint red color colonies and 134 (83.22%) were blue color colonies. (Figure-3).

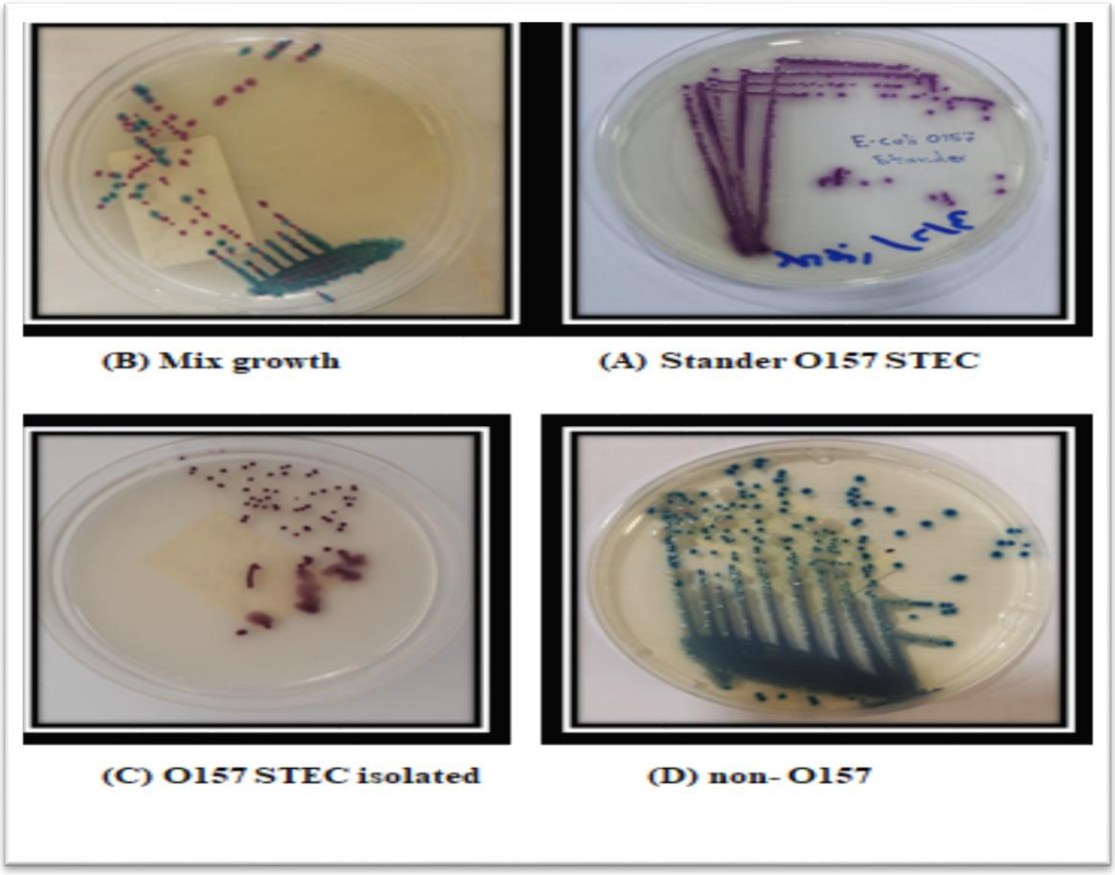

Figure -3: Cultural characterization of STEC on O157 CHROMagar where the STEC O157 appeared mauve color while non O157 STEC and other species appeared as blue color **(A)** Stander O157 STEC, **(B)** Mix growth, **(C)** O157 STEC and **(D)** non- O157.

According to the data revealed in table (1), the most important items in this table were age and gender because its relatedness to STEC Infections. Of the 19 cases with STEC, O157 were detected in nine cases (47%) and Non-O157 detected in 10 cases (53%). STEC present with seven cases in age group > 15-65 years (78%) of O157 serotype, and two cases in children aged 0-5 years, with no cases in other age groups. Fifty percent of Non-O157 were present in age group > 15-65, three cases in children and two cases in old age people. Male to female ratio was 1.25:1 in O157, while gender ratio was 0.6:1 in Non-O157 serotype, demographic characteristics of patient confirmed with STEC explained in table (2).

Table -2: Demographic characteristics of patients confirmed with STEC.

Demographics ▼		Shiga toxin-producing Escherichia coli (STEC)	
		O157 №=9	Non-O157 №=10
Age groups (years)	0-5	2 (22%)	3 (30%)
	> 5-15	-	-
	> 15-65	7 (78%)	5 (50%)
	> 65	0 (0%)	2 (20%)
Gender	male (9)	5 (56%)	4 (40%)
	female (10)	4 (44%)	6 (60%0

3.2 Molecular results

The results of the PCR assay relied on the isolated bacterial genomic DNA (Geneaid. United States) which used (Nucleic acid purification based on spin column) to rapidly purify nucleic acids through its solid phase extraction process. This technique is based on the fact that nucleic acid under certain conditions attaches to the solid phase of silica. Consequently, this kit appeared rapidly in 1h of extraction and easy method for purifying bacterial genomic DNA of (*Escherichia coli*) from overnight bacterial growth on the 37 ° C brain heart infusion broth media.

3.2.1 Detection of virulence genes: STEC strains examination by PCR showed that of the 161 *E. coli* isolates, 19 of which ensured genes of the shiga toxins (*stx1* and *stx2*). Of the 19 isolates, 14 isolates (73.68 %) were positive for the *stx1* gene, 16 isolates (84.21 %) were positive for the *stx2* gene. However, only 11 (57.89 %) of these isolates were found to contain both *stx1* and *stx2* genes, as shown in figures (4 and 5).

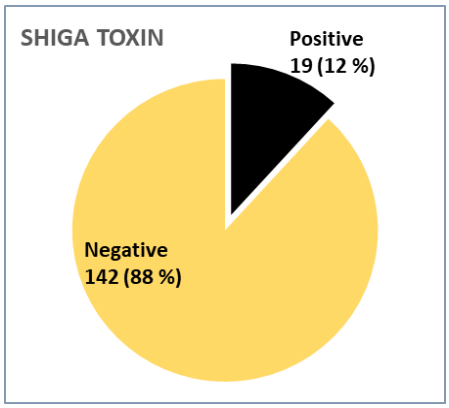

Figure -4: Shiga- toxins distributions among *E. coli* isolates.

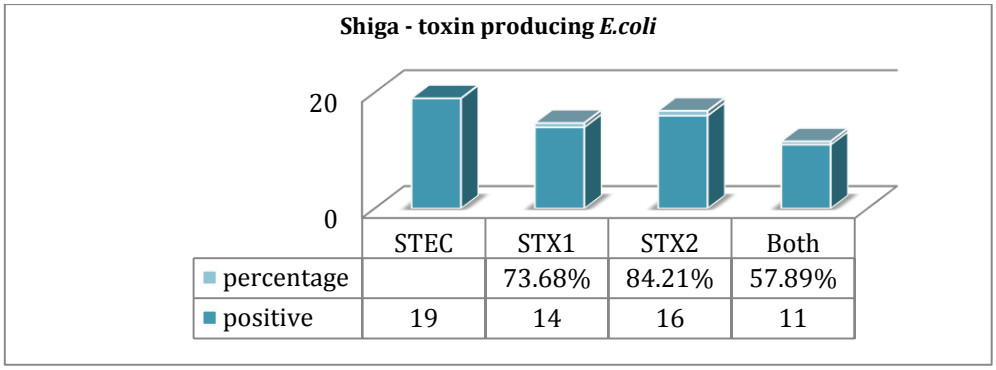

Figure -5: Distributions of (*stx1*, *stx2* or both) among positive STEC isolates.

3.3.2 Detection of O157 serogroup: All *E. coli* isolates that carry shiga-toxin genes (*stx1*, *stx2*, or both), were subjected to PCR for the investigation of the presence of (*rfb* 0157) gene size (259 bp) which was estimated depending on DNA marker (100bp DNA ladder). Depending on the (*rfb* O157) gene patients with positive isolates for STEC infection were classified into two groups (STEC O157 and STEC Non-O157). The PCR amplification of virulence genes (*stx1* and *stx2*) at 366 and 750bp respectively, and (*rfb* O157) gene at 259 bp on agarose gel electrophoresis. (Figure -6)

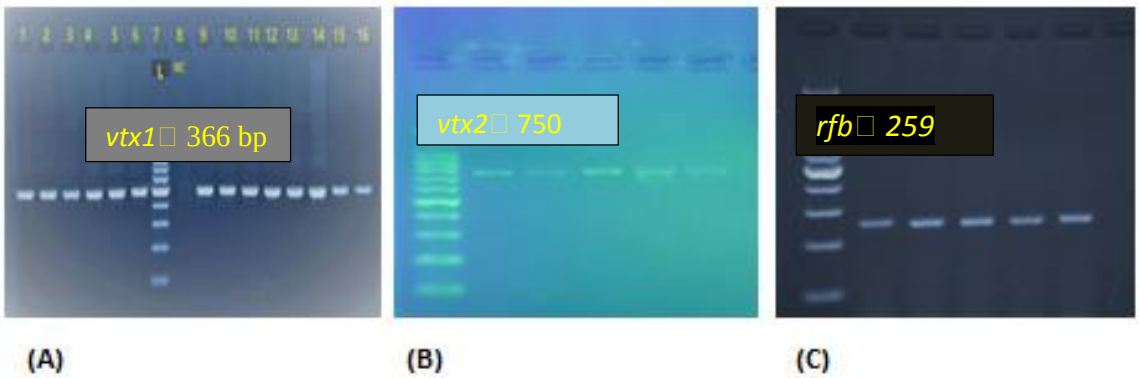

Figure -6: PCR detection of *vtx1* (A), *vtx2* (B) *rfb* O157 (C) genes using molecular size marker (100 bp Ladder); *vtx*1 at 366 bp, *vtx2* at 750 bp and *rfb* o157 at 259 bp.

DNA Sequencing: Three isolates of STEC-O157 were randomly selected and sent for sequencing and then submitted to the NCBI-GenBank database to obtain accession code (0 MN944014.1). The DNA sequencing method has been performed on *vtx2* detection, and phylogenetic analysis for phylogenetic confirmation of O157. (Figure -7).

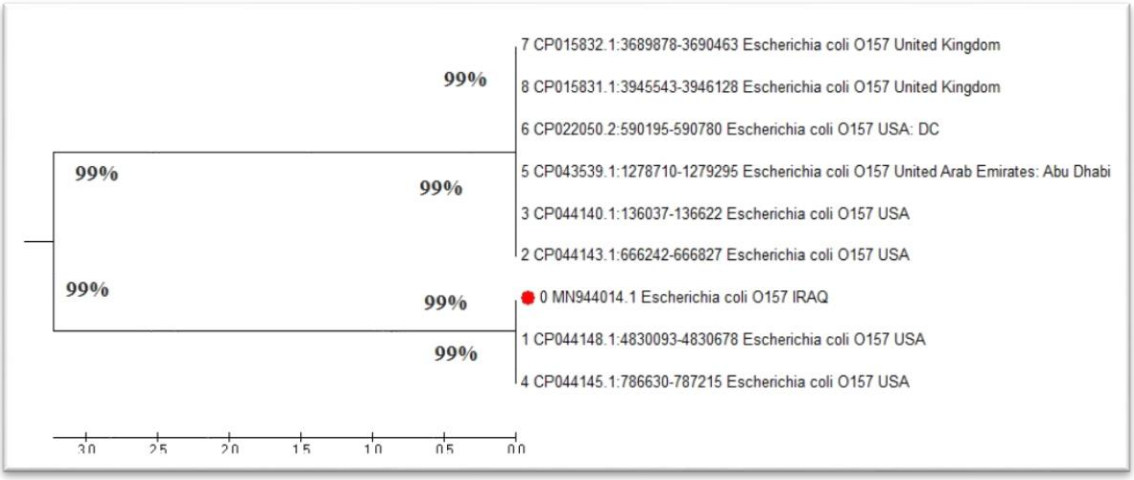

Figure -7: Unrooted phylogenetic tree of STEC O157 isolated from stool of human.

4. Discussion

Escherichia coli O157 is considered one of the most harmful causes of gastrointestinal diseases, as well as the cause of most food poisoning cases worldwide [17,18]. PCR is an effective molecular technology that is used not only to determine the target DNA in different clinical samples and to identify many forms of pathogens but also for rapid and accurate analysis of stool specimens, it can help to distinguish shiga toxin- producing *E. coli* from other strains.

Recently, PCR-based methods targeting the virulence genes (*stx1* and *stx2*) have been used to diagnose and confirm STEC infections, leading to rapid and improved detection rates [19]. The current results were similar to the study at Al-kufa Hospital by Aljanaby [20], between October 2015 to April 2016. Out of 50 *E. coli* isolates from severe diarrhea, these results showed that (*stx1* and *stx2*) genes were present only in diarrheal specimens, two isolates (11.1 %) and three isolates (16.6 %) respectively. And in the same line with Mohsin [21], a total of 200 samples of patients with diarrhea were tested for stx genes along with other virulence factors. In April to September 2005, Allied Hospital Faisalabad, Pakistan found that one or both *stx* genes were detectable in 22 (11 %) patients. On a global level, this study was completely identical to the study conducted by Loconsole [22], in southern Italy for patients from one month to 15 years old where the STEC incidence was (12.1 %). The present study was in conflict with the results of Abdul-hussein *et al.*, [23], who found that all the pathotypes of diarrheagenic *E. coli*. Either nether (*stx1 or stx2*) genes are produced by all 19 study isolates.

Also in comparison to Al-Dulaimi *et al*., [24], who also performed the study in Dhi Qar city, where the research focused on the molecular diagnosis of DEC. The findings were as follows: EAEC (64.73%), ETEC (19.5%), EPEC (10.5%) and STEC (5.27%) diarrhea . This variation in findings was the cause of several factors such as diet, age, stress and seasonal changes influencing the excretion of Shiga toxin producing *Escherichia coli*. The present study is relevant to a study conducted by Baba *et al.*, [25], when whole genome sequencing analysis demonstrated the importance of observing the genomic properties of STEC isolates from asymptomatic food handlers as well as symptomatic patients in Miyagi Prefecture, Japan. The results of patients showed O157 was 50% (19/38).

In the analysis of the phylogenetic-tree *vtx2*, for *E. coli* isolated from human. High identity of human isolates in the phylogenetic tree is identical to O157: H7 which is the strain of virulence and animal impotence. The strain coded 0MN944014.1 and reported as 99% high resemblance to O157 with CP014314.1 O157H7 in the US isolated from camels meaning zoonotic significance and high virulence in transmission of disease [26]. In the phylogenetic-tree analysis and dependent on a gene (*vtx2*), the study isolate was recorded in the gene bank where it was recorded with this code (0MN944014.1). The percentage of congruence was 99% with 8 isolates in one in the United Arab Emirates/ Abu Dhabi which bear the code (CP043539.1: 1278710-1279295 *Escherichia coli O157*) Isolated from camel, which means zoonotic importance and high virulence in transmission of disease [27] , and two isolates in United Kingdom under the code (CP015832.1:3689878 and CP015831.1:3945543-3946128) the investigation examines the genomic variations in STEC O157: H7 correlated which caused two outbreaks at the same restaurant, eight weeks apart. Isolates clustered not with local strains but with international travel to the Middle East / North Africa. The second outbreak was markedly higher and several secondary cases were found that suggested that human-to - human transmission was effective [28], and five isolates in USA under the code (CP022050.2:590195-90780, CP044140.1:136037-136622, CP044143.1:666242-666827, CP044148.1:4830093-4830678, and CP044145.1:786630-787215) [29].

Conclusions

PCR is a fast detection technique for the virulence (stx) gene. Sigha toxin-Produced by *E. coli* have a *stx* gene that is an essential element of virulence anywhere isolated from humans. The molecular analysis of the local isolate (0 MN944014.1) obtained from patients with diarrhea confirmed that it was *E. coli* O157. Furthermore, the local O157 isolate is also reported to be homologous to the nucleotide sequence of 8 different E. coli O157:H7 in the database of GenBank.

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