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Identification and Molecular Characterization of *Escherichia coli* O157:H7 Isolated from Some Iraqi Patients in Baghdad City

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Abstract: *Escherichia coli* O157:H7 is known to cause illness in animals and people. Their main criterion is to produce a potent toxin that causes damage to the intestine leading to bloody diarrhea. Outbreaks are caused by contaminated food, this strain is the prevalent one, and in severe cases, it may cause death. Forty fecal samples were collected around Baghdad from patients residing in Yarmouk and Al-Kindy Teaching Hospitals with typical symptoms of infection with *E. coli*. All 40 fecal samples (15 male, 15 female, and 10 children) were the source of the bacterium and were immediately transported to the laboratory and cultured for further processing. Among 40 samples collected from humans, only 20 were identified as *E. coli* O156:H7. Further confirmation was made by culturing on MacConkey agar, Eosin Methylene Blue (EMB) agar, to assess the development of a metallic sheen. Final bacteriological confirmation came after cultivation on HiCrome™ EC O157:H7 Selective Agar Base, modified with supplement (FD187) that gave dark purple to magenta coloration for these strains of bacteria. Molecular identification of isolated strains came from investigating two specific genes through PCR amplification, *stx*1, and *rpo*B. Both genes were amplified, sequenced, and registered in NCB with accession no. OR939814 and PP059841, respectively. Bacterial isolates studied in this research were classified to the species level and found to be STEC *Escherichia* coli O157:H7 Sakai strain. This study aimed to identify the epidemic strain of E. coli through a highly accurate approach by sequencing the *rpo*B gene, and identifying the type of Shiga toxin produced by this bacterium.

Keywords: *Escherichia coli*, O157:H7 antigene, *rpo*B, Shiga toxins, food poisoning..

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Introduction

The Enterobacteriaceae *E. coli* was the source of infection in the human diet (1). Bacterial infection may be accompanied by failure of treatment (2).Within the category of EHEC serotypes, O157:H7 is prevalent. It is characterized by flagellar (H) antigen 7 and somatic (O) antigen 157, which is responsible for significant disease outbreaks and severe morbidity, establishing this bacterium as a paramount foodborne and waterborne pathogen on a global scale (3). It may cause other serious infections that when neglected, may be life- threatening (4).

The primary strain identified in this category is O157:H7, which continues to be the dominant serotype (5). O157:H7, responsible for over one million cases of diarrhea and approximately 2000 cases of STEC-HUS worldwide, is a significant foodborne pathogen causing severe illness in humans globally (6). Contaminated food with *E. coli* O157:H7 is the primary source of

disease outbreaks in the United States (7).

Nevertheless, *E. coli* O157:H7 has been detected in a wide array of other animals, encompassing sheep, goats, heifers, birds, deer, geese, turkeys,

seabirds, dogs, cats, gulls, chickens, pigs, monkeys, reptiles, llamas, and horses(8). Enterohemorrhagic *Escherichia coli*, a subgroup of Shiga toxin-producing *E. coli* (STEC), also known as verotoxin-producing E. coli (VTEC), primarily relies on Shiga toxin (*Stx*) production as a key virulence factor within STEC. O157 VTEC strains display distinct biochemical characteristics critical for theirlaboratory identification (9).

Toxins inflict damage by impeding protein synthesis, leading to the apoptosis of endothelial cells. The regulation of these genes is subject to environmental influences such as stress and temperature (10). Moreover, these toxins, originating in the colon, can potentially induce localized harm(11). When they circulate in the bloodstream, they specifically target organs like the kidney, contributing significantly to the onset of hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) (12).

In Iraq 2022, specifically in Najaf, Baghdad, Kirkuk, and Erbil, when cases were diagnosed and confirmed, milk samples were collected and subjected to investigation using m-PCR (13).

Over the past five years (2016-2021), the United States has experienced at least one outbreak each year linked to the contamination of leafy greens by *E. coli* O157:H7, as reported by the Centers for Disease Control and Prevention (CDC) (14).

Epidemiological inquiry into the STEC strain within food and food products should become a concern by directing attention toward the food source in Iraq (15,16).

This research aims to ascertain the prevalence of *E. coli* O157 in human samples obtained from patients at both Al-Yarmouk and Al-Kindy Teaching Hospitals in Baghdad city.

Materials and methods

Sample collection

Hospitals were the main sites which 40(15 male, 15 female, 10 child) infected faecal samples were collected from patients attending Al-Yarmouk and Al-Kindy Teaching Hospitals. Patients the hospitals showed symptoms of food poisoning,heavy bloody diarrhea, and abdominal pain without fever. Samples were collected from January to June 2023.

Primary isolation, purification, and identification of *Escherichia coli* O157:H7

Escherichia coli detection followed the protocol outlined in the standardfor faecal and swab samples (17). A weight of 1 g of homogenized faeces was suspended in 9 ml of modified Tryptone Soya Broth. Samples were homogenized by vortex and incubated overnight at 41°C. Following selective enrichment, 50 µl of the product was streaked onto MacConkey agar for theprimary isolation of *E. coli*, with aerobic incubation at 37°C for 24 hours.A solitary, distinct colony was chosen and subcultured on (EMB) agar. Concurrently, after colonydifferentiation, it was subjected to Gram staining and visualized using, bright-field microscopy.

Bacteriological confirmation of *Escherichia coli* O157:H7

Well-differentiated colonies were transferred to be cultivated on HiCrome EC O157:H7 with Selective Supplement (FD187), which allows the allowing this strain's growth only (18).

***Escherichia coli* O157:H7 are violet colorafter 24 h. of cultivation at 37^{0}C. Molecular identification of *Escherichia coli* O157:H7 Isolation of Deoxyribonucleic acid**

All bacterial isolates were subjected to DNA isolation. It was performed using the GenX total DNA isolation kit following company instructions. *Escherichia coli* obtained from bacterial isolates was measured for purity and concentration using Nanodrop Techne (England). Deoxyribonucleic acide samples were kept frozen until use.

Polymerase chain reaction primers used in Deoxyribonucleic acid amplification

Two primers were used to amplify both *stx*1 and *rpo*B. The sequences of primers are illustrated in Table (1).

Table (1): Primers sequences and targets.

Pathogen name	Target gene	Sequence 5'-3'	Product (bp)	Reference
E. coli O157:H7	*Stx*1	F: CAGTTAATGTCGTGGCGAAGG R: CACCAGACAATGTAACCGCTG	400bp	Cebula *et al*., (1995)
E. coli O157:H7	*rpo*B	F: CAGCCAGCTGTCTCAGTTTAT R: GGCAAGTTACCAGGTCTTCTAC	400bp	Designed in this study using reference sequence JX471606.1

Polymerase chain reaction protocol to amplify target genes

The amplification of target genes followed the same PCR program except for the annealing temperature which was different in both. Table (2) shows the PCR program used in this study.

Table (2): Polymerase chain reaction program used to amplify *stx* and *rpo*B genes.

	PCR step	Temp. / time	No. of cycles
Target gene	Initial denaturation	94^{0}C / 5 min	1 cycle
Stx1	Denaturation	95^{0}C / 3 min.	30 cycles
	Annealing	55^{0}C / 30 sec.	
	Extension	72^{0}C / 30 sec	
	Final extension	72^{0}C / 10 min	1 cycle
	Hold	4^{0}C /∞	Non
	Initial denaturation	94^{0}C / 5 min	1 cycle
***rpo*B**	Denaturation	95^{0}C / 3 min.	30 cycles
	Annealing	48^{0}C / 30 sec.	
	Extension	72^{0}C / 30 sec	
	Final extension	72^{0}C / 10 min	1 cycle
	Hold	4^{0}C /∞	Non

Resolving Polymerase chain reaction amplicons by electrophoresis

Polymerase chain reaction products were subjected to electrophoresis to resolve, identify, and measure resulting bands. Electrophoresis was performed using 2% agarose stained with 10µg/ml EtBr with a field strength of 8 v/cm for 45 min. the resulting bands were visualized and imaged using a gel documentation system with a 10-megapixelCanon camera.

Results and Discussion

Bacteriological identification of *Escherichia coli* strains

Growth on MacConkey agar

Among 40 faecal samples processed for *E. coli* isolation, only 20 samples humans were identified as *E. coli*. Initially, this was based initially on cultural conditions and the shape of the colony cultivated on MacConkey agar. Plates were examined, and colonies were distinguished by pink, indicating lactose fermentation.

Strikingly, these strains are among the *E. coli* variants lacking β-glucuronidase activity and failing to ferment sorbitol or rhamnose within 24 hours. The isolation of such strains fromfaecal specimens entails plating on mediacontaining D-sorbitol rather than lactose. Figure (1) shows the growth of *E. coli* strains on MacConkey agar.

Figure (1): *Escherichia coli* colonies cultivated on MacConkey agar. The figure shows the growth of pink colonies spread on the plate.

Growth on Eosin Methylene Blue (EMB) agar

The EMB agar may provide a distinctcriterion of *E. coli*. The growth of the bacterium gives a metallic greenish sheen after 24 h of growth under 37^0C. Figure (2) shows the growth of *E. coli* strains on EMB agar.

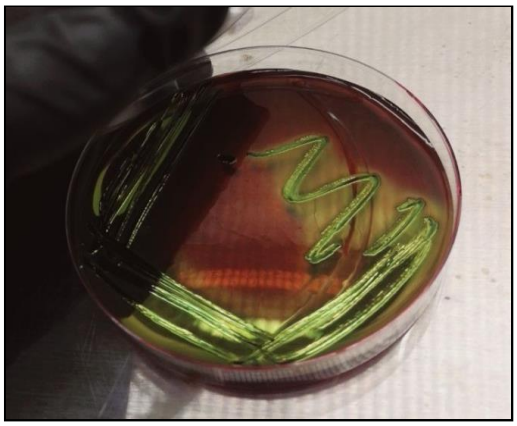

Figure (2): Growth of *Escherichia coli* on EMB agar. The figure shows colonies giving a metallic green sheen after 24 h. cultivation at 37^0C.

Confirmation of *Escherichia coli* O157:H7

The HiCrome ™ supplemented with FD187 addition is highly recommended as a selective medium forthe growth of *E. coli* O157:H7. The unique composition of this medium will allow this strain to grow well with a dark purple color which is a distinctive feature from other strains of *E. coli* that grow with a blue-green color. Figure (3) shows the growth of different strains of *E. coli* on a HiCrome medium.

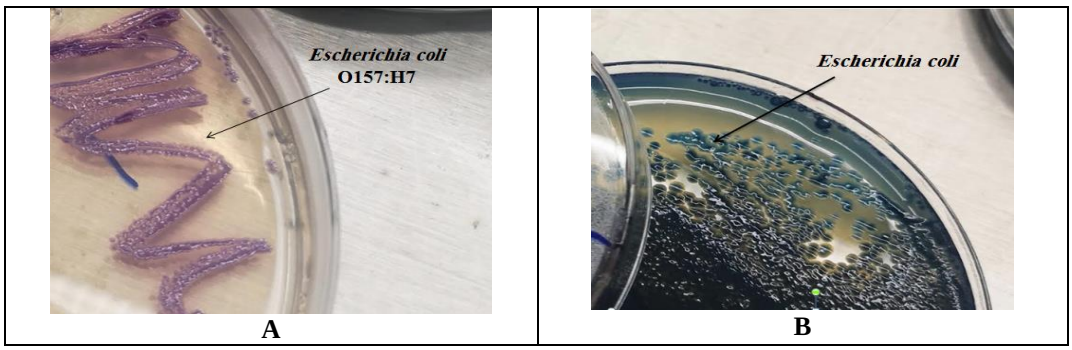

Figure (3): Growth of *Escherichia coli* O157:H7 (A) on HiCrome medium which gave a dark purple color as a distinctive feature, whereas other types of *Escherichia coli* grow with bluish green color (B).

Molecular identification of *Escherichia coli* O157:H7 Classification and strain referencing

Among bacteria, using the 16SrRNA is the main method for classification and identifying the strain of the bacteria. Hence, there is a high similarity of this gene among closely related species. The RNA polymerase gene (*rpo*B) may provide more accurate results for bacterial species identification. The DNA extracted from humans was used PCR to amplify the *rpo*B of bacterial isolates under study. Results obtained from PCR amplification were sent for sequencing and compared with NCBI-deposited data using the BLAST tool, which revealed that the strains under study were *Escherichia coli* O157:H7 strain Sakai and registered in NCBI under accession no. PP059841. Figure (4) shows *rpo*B amplification using specific primers designed for this purpose.

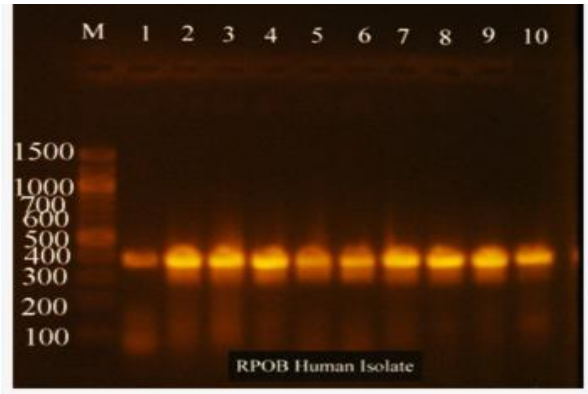

Figure (4): Polymerase chain reaction amplification of *rpo*B in *Escherichia coli* O157:H7 strain under study. The upper line show sthe gene amplified from human isolates.

Detection of *stx*1A gene

Shiga toxin production is a main feature of *E. coli* O157:H7. This toxin includes, *stx*1 and *stx*2, which cause diarrhea, nausea, stomach pain, vomiting, and fever. Thus, it becomes crucial in this study to identify the type of *stx* through gene identification. Results obtained after specific PCR amplification for the target gene showed it was sxt1A, and was registered in NCBI under accession no. OR939814. Figure (5) shows the amplification of The *stx*1 gene in *E. coli* O157:H7 strains under the study.

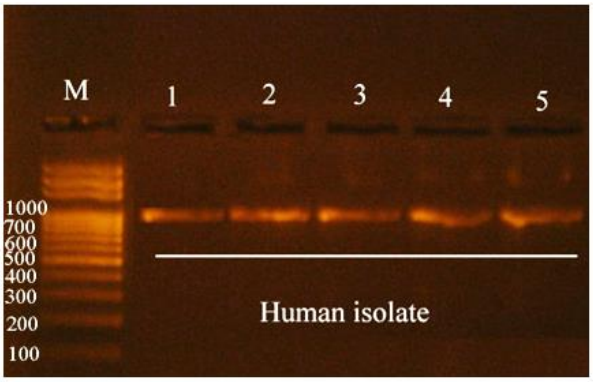

Figure (5): PCR amplification of *stx*1 gene in *E. coli* O157:H7 strains obtained from humans.

Escherichia coli is a group of Gram-negative bacteria that includes highly similar species making differentiation. The enterohaemorrhagic *E. coli*, like serotype *E. coli* O157:H7, may be considered a leading cause of food- associated outbreaks. The primary source of infection is contaminated food with any remains from these animals(20). Initially, it was believed that these foods were contaminated with faecal material, but reports suggested that *E. coli* O157:H7, regardless of food contamination, may find a suitable environment to grow and propagate through them (21).

However, detecting *E. coli* O157:H7 on routine enteric media may be time-consuming and costly. Since this strain is not a sorbitol fermenter, previous reports suggested the use of MacConkey agar as a selective medium for this strain, as the sorbitol is a useful marker to aid the detection of *E. coli* O157:H7 from stool (21). Eosin methylene blue agar, on the other hand, provides a rapid and accurate method to differentiate *E. coli* from other Gram-negative pathogens by observing the greenish sheen on the surface of the colony, which is pH sensitive and does not appear due to alkalinity interfering with the acid requirement of EMB agar to produce metallic sheen (23). Thus, using HiCrome agar proved highly recommended in identifying *E. coli* O157:H7 from the other types and species of the same genus (24).

This medium is based on three differential components: lysin decarboxylase, which is positive for *E. coli* O157:H7, sorbitol fermentation, and beta- glucuronidase (25). Selectivity is achieved through the use of monensin, which inhibits Gram-

positive bacteria.

Molecular analysis of any organism under study provides powerful, accurate, and reliable results since it's built on the DNA of the organism studied. In this research, amplifying and sequencing of *rpo*B was the approach for identifying *E. coli* O157:H7 and classifying the isolates obtained. The β subunit of RNA pol is encoded by the *rpo*B gene, which is a highly conserved sequence and was established as a tool for bacterial species and sub-species identification and considered more robust than 16SrRNA (26). Resolving *rpo*B sequencing enabled accurate DNA-DNA hybridization research and enabled scientists to reach better results. Thus, our work used such information to conduct accurate results during theidentifying and classifying of bacterial isolates under study. They were found to be Sakai sub-species with similarity reaching 99%, which facilitated the way of registering them in NCBI under accession no. PP059841. The importance of studying this gene comes from its characteristic of providing *E. coli* O157:H7 with the ability to resist adverse conditions that encounter their growth by expressing various multiple response genes that preserve their survival (27).

Moreover, STEC, are a major health concern even in developed countries (28). Shiga toxin *E. coli* (STEC) drew the focus of scientific studies for its clinical importance. It is encoded by *stx*1 and *stx*2 genes of temperate, lambdoid bacteriophage that is permanently integrated in the *E. coli* genome (29). Shiga toxins are the major virulence factors of STEC that belong to the AB_5 family of proteins with active A and B moieties responsible for binding to the host cell receptor (30). The importance of studying *stx*1 A in this study came from its role in cleaving the N-glycosidic bond in the 28SrRNA of the 60SRNA subunit which subsequently leads to cytotoxicity. Our results from sequencing the *stx*1A gene howeds a similarity of 99% with the Sakai, strain and the results were deposited in the NCBI database under accession OR939814.

Finally, performing molecular studies on virulence factors such as *stx* may provide the future conception in research regarding antibiotic usage against STEC since previous reports indicated that β-lactams, quinolines, andtrimethoprim could induce Shiga toxin production after DNA damage inflected by these antibiotics (31).

Conclusion

Shiga toxin-producing *E. coli* were found widely spread as food contaminant and the causative agent of food poisoning. Contaminated food isthe source of this type of bacteria and is mainly found to be O157:H7. Cultural conditions can provide preliminary identification of *E. coli* O157:H7 and the type can be conffiemed when the bacterium is cultivated on HiCrome medium since it providesdifferent growth characteristics and colors. Identification and classification of bacteria using the *rpo* gene is highly recommended and provides accurate results of more than 16SrRNA. Molecular and evolutionary studies concerning the *stx* gene are an important aspect of scientific research for itsclinical importance.

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