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Molecular detection of *Shiga toxin* genes and pathogenicity of *Escherichia coli O157: H7* isolated from salad samples in Erbil city-Iraq

Shean Ismail Saleh (MSc)[1], Lanja Jalal Mahmood (MSc)[2], Safin Tahsin Kakayi (MSc)[3], Kwestan Hassan Sdiq (MSc)[4]
[1,2,3,4] College of Health Sciences, Hawler Medical University, Iraq

Abstract

Background: *Escherichia coli* O157:H7 is one of foodborne pathogenic bacteria that causes serious infections in people around the world. Cattle are a barrage of *E. coli* O157:H7, fresh products contaminated with cattle feces are the common sources for the pathogen. *E. coli* O157:H7 has the ability to survive well in the environment. Two main virulence factor genes of *E. coli* O157 have been detected in this study, which are responsible to produce shiga toxin and increase the pathogenicity of this bacterium.

Objective: To find out the rate of fresh food contamination by *E. coli*, and detect the genes responsible for shiga toxin production and increase the pathogenicity of *E. coli* among isolates from salad samples.

Patients and Methods: Two hundred salad and vegetable samples were collected from different restaurants and small shops in Erbil city from April - September (2019). Samples were cultured on eosin methylene blue (EMB) agar plates; typical *E. coli* with a metallic shine appeared. Polymerase chain reaction technique was used for detecting (*uidA*, *rfbo157* and *stx1*, *stx2*) genes.

Results: Overall, 32% (64/200) of salad samples were contaminated by *E. coli* as identified by culturing on EMB agar. Then isolated *E. coli* was confirmed by detecting the presence of uidA gene and the rate was 88% (56/64). Pathogenicity and toxin production detection was done for all confirmed *E. coli* isolates, by detecting the presence of rfbo157 gene (479 bp), among 56 isolates only (4) isolates was positive for rfbo157 gene, and (7) isolates showed positive result for shiga toxin.

Conclusion: This study focuses on the serotype *E. coli* O157:H7, a high percentage of *E. coli* was detected in salad samples which indicated the fecal contamination. The presence of *rfbo157* gene and stx1, stx2 are the indications for pathogenicity and *shiga toxin* production respectively.

Keywords: *Escherichia coli*, *shiga toxin*, *E. coli* O157, *uidA gene*, *rfbo157 gene*

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Correspondence Address: Lanja Jalal Mahmood
College of Health Sciences, Hawler Medical University, Iraq

Email: lanja_jalal2002@yahoo.com

Website:https://djm.uodiyala.edu.iq/index.php/djm

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Introduction

Escherichia coli (E. coli) O157:H7 is one of the bacterial serotype species *E. coli* and it's also one of the shiga toxin producing types of *E. coli. Shiga toxin E. coli O157:H7* first was isolated in 1982 and it has become one of the most vital food and waterborne pathogens that cause multiple diseases such as hemorrhagic colitis, diarrhea and hemolytic uremic syndrome (HUS) in humans. *E. coli* O157:H7 is an enterohemorrhagic strain that infects the alimentary tract and stimulates abdominal cramps followed by hemorrhagic diarrhea [1]. The major way for the transmission of *E. coli O157:H7* is occurring via the fecal oral route after the ingestion of contaminated undercooked foods and liquids. Furthermore, *E. coli O157:H7* could be transmitted from person to another via fecal shedding and thus approximately an estimated 11% of infections. The production of shiga toxin is considered an essential key factor that contributes to the development of HUS [2]. On the other hand, enterohemorrhagic E. coli stimulates secondary diseases rather than its production of shiga toxin which leads to a range of gastrointestinal disease for instance watery diarrhea to hemorrhagic colitis. *E. coli* O157:H7 stimulates enterohemorrhagic diseases which might cause systemic disease by HUS, which demonstrate as acute renal failure, thrombocytopenia and hemolytic anemia. HUS can cause in both lifelong chronic diseases and acute potentially life-threatening diseases [3]. The most common signs and symptoms of *E. coli* O157:H7 infections are; acute and severe hemorrhagic diarrhea and non-hemorrhagic is also possible, a bit of fever and abdominal cramps. The diseases could be resolved between 5 to 10 days and sometimes it could be asymptomatic [4, 5].

Shiga toxin-producing bacteria have received substantial attention as emergent pathogens due to the dangerous toxins they produce. These exotoxins are the principal virulence factors associated with the pathogenesis of bloody diarrheal diseases, bacillary dysentery, and hemorrhagic colitis progressing to acute renal failure in infected patients, primarily in children. This phenomenon, collectively referred to as hemolytic uremic syndrome (HUS), is the main starting point for pediatric acute renal failure in many countries. Both Shiga toxins and the inflammatory innate immune cells that activated by these toxins (Stxs) contribute to the pathogenesis of Hemolytic uremic syndrome (HUS) by rendering blood vessels in the colon, kidney, and central nervous system (CNS) more sensitive to the detrimental action of Stxs [6].

The Stx family consists of two major groups, Stx1 and Stx2, sharing ca. 60% sequence identity. The Stx1 group is more homogeneous and consists of Stx1, Stx1c, and Stx1d. In contrast, the group comprised of Stx2 and related toxins is more heterogeneous. Beside Stx2, a number of variants, such as Stx2c, Stx2d, mucus-activatable Stx2d (Stx2dact), Stx2e, Stx2f, and Stx2g, have been described. Unfortunately, the Stx nomenclature is not uniform, and despite efforts to provide a uniform nomenclature, descriptions of new Stx variants are not sufficiently defined [7].

B-D-glucuronidase (GUD) is an inducible enzyme that is encoded by the uidA gene in

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E. coli cleaves the substrate 4-methylumbelliferyl-B-D-glucuronide (MUG) to release a fluorogenic radical. When the MUG compound is incorporated into conventional bacteriological media, the presence of E. coli can be easily determined by the bluish fluorescence in the medium that is observed under long-wave UV light [8].

The uidA gene has been used for detecting *E. coli* in many studies. Although, the *uidA* and *uidR* genes are present in *E. coli* and Shigella spp. ,its activity is limited to *E. coli*. A lot of studies showed that many MUG negative *E. coli* strains, including the pathogenic serotype O157:H7, were detected after PCR amplification of the uidA gene [9].

Most patients with entero-hemorrhagic *E. coli* diarrhea will recover without any treatments in 10 days other than fluid replacements. In addition to, evidence has demonstrated that antibiotics worsen the consequences as increasing the development of HUS. Severe HUS patients may benefit from hemodialysis to treat electrolyte and uremia concerns that are related to acute renal failure [10, 11].

Patients and Methods

Study protocol

This study was performed from the period between April - September (2019). Samples include salads and fresh vegetables collected from different restaurants and small shops in Erbil city. 28 places were selected to achieve such a study including 12 restaurants and 16 small shops (road shops) in Erbil city.

Sample collection: This study included 200 samples of salad and green leaves (that were collected randomly during April - September (2019) from different restaurants and small shops in Erbil city). Samples kept in sterilized plastic containers and collected inside an ice pack and transported to the microbiology laboratory at Health Sciences College /Hawler Medical University.

Culture characteristics and biochemical diagnosis: Approximately 25g of sample was transferred aseptically to (225 mL) buffered peptone water and were incubated at 37°C for 24 hr. After incubation, streaked onto eosin methylene blue (EMB) agar plates, which were further incubated at 37°C for 24 hr. Typical *E. coli* colony with a metallic shine appeared on EMB agar plates (12).

DNA Isolation: For extraction DNA from all suspected *E. coli* isolates (green metallic shine colony), 1mL of overnight brain heart infusion broth culture (approximately 108 CFU) were centrifuged at 4,000 rpm for 5 min. The pellets were washed in 1 mL of PBS (phosphate-buffered saline), then suspended in 0.5 mL of H2O, and boiled (100°C) for 15 min.

The cell debris was separated by centrifugation at 12,000 rpm for 5 min, the released DNA was accumulated in the supernatant, then transferred into a sterilized eppendorf tube and stored under - 20˚C (13).

Confirmation identification of *E. coli*: All green metallic shine colonies of *E. coli* on Eosin methylene blue agar isolates were identified by detecting specific uidA gene by PCR Table (1).

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Table (1): Sequencing of primers that are used to amplified uidA gene

Gene name	Sequence (5′……..3’)	Size	PCR condition	Reference
uidA	F: 5′ TGGTAATTACCGACGAAAACGG R: 5′ ACGCGTGGTTACAGTCTTGCG 3	162 bp	*uidA* gene program cycle PCR program: Initial Denaturation 94 c for 5 min (one cycle) Denaturation. 94 for 30 second Annealing 55 For 50 second Extention 72 for 1 min Final extention 72 for 5 min(one cycle) Cycle: 30 cycle	**(14)**

Detecting of pathogenicity and toxin production

Studying of shiga toxin producing *E. coli* was determined by two genes (*Stx1and Stx2)* while pathogenicity of *E. coli* was determined by molecular detection of *rfbo157* gene Table (2).

Table (2): Sequencing of primers that used to amplified Stx1, Stx2, and rfbo157

Gene names	Sequences (5′……..3’)	Size	PCR condition	Reference
toxin 1 (*Stx1*)	Stx1f AAATCGCCATTCGTTGACTACTTCT Stx1r TGCCATTCTGGCAACTCGCGATGCA	366 bp	*Stx1* gene program cycle PCR program: Initial Dena 94 c for 8 min (one cycle) Denaturation. 94 for 50 sec Annealing 60 For 50 sec Extention 72 for 1 min Final extention 72 for 10(one cycle) min Cycle: 30 cycle	**(15)**
Shiga toxin 2 (*Stx2*)	Stx2f CGATCGTCACTCACTGGTTTCATCA Stx2r GGATATTCTCCCCACTCTGACACC	282 bp	*Stx2* gene program cycle Pcr program Initial Dena 94 c for 8 min (one cycle) Denaturation. 94 for 50 second Annealing 60 For 50 second Extention 72 for 1 min Final extention 72 for 10 min (one cycle) Cycle: 30 cycle	**(15)**

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rfbo157	rfbo157f AAGATTGCGCTGAAGCCTTTG rfbo157r CATTGGCATCGTGTGGACAG	479 bp	*Rfbo157* gene program cycle PCR program Initial Dena 94 c for 10 min (one cycle) Denaturation. 94 for 45 second Annealing 57 For 40 second Extention 72 for 1 min Final extention 72 for 5 min (one cycle) Cycle: 35 cycle	**(16)**

Results

Isolation and identification of *Escherichia coli*: Among 200 salad samples, 64 samples samples (32%) were contaminated with *E. coli* as detected by cultivation on EMB agar. Pre-enrichment samples in buffered peptone water, after incubation for 24 hours at 37°C, were subcultured on eosin methylene blue (EMB) agar. *E. coli* colonies appeared dark and blue-black with a metallic green sheen, as shown in Figure (1).

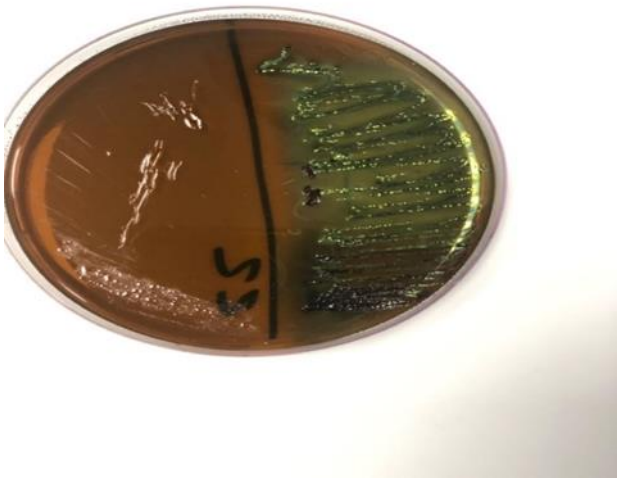

Figure (1): The appearance of *E. coli* on EMB agar

Confirmation of *E. coli* isolates

All *E. coli* isolates on EMB agar were confirmed by detection of a specific diagnostic gene (uidA gene) using Polymerase Chain Reaction (PCR) technique. Among 64 isolates of *E. coli* (56) isolates, about (88%) were positive for uidA gene as determined by the presence of 162 bp amplified product Figure (2).

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Studying of pathogenicity and toxins production: All confirmed *E.coli* isolates were examined for the presence of rfbo157 gene (479 bp), only (4) isolates (7%) among (56) *E. coli* isolates were positive for this gene depending on DNA ladder of 1000 bp as illustrated in Figure (2). All confirmed *E. coli* isolates were also examined for the presence of Stx1 gene (366 bp) and Stx2 gene (282 bp); in the present study only 7 isolates (13%) of the samples showed positive results for these genes, as shown in Figure (2).

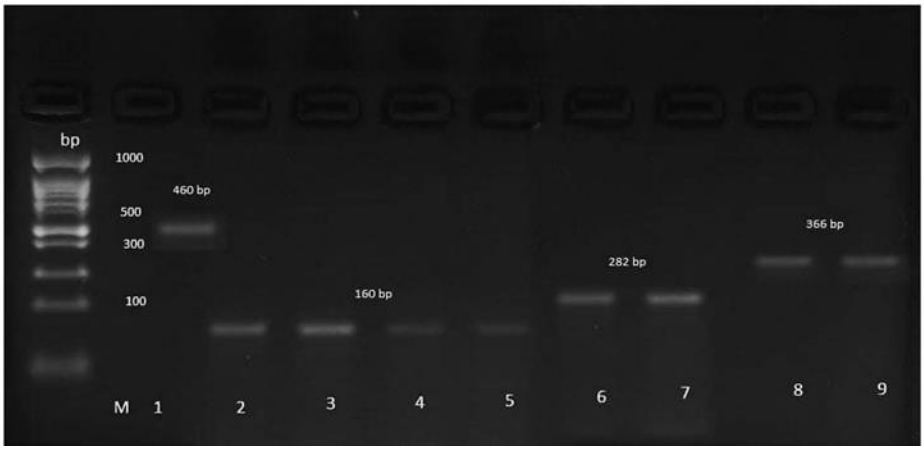

Figure (2): Agarose gel electrophoresis of PCR amplified lane 1 rfbo157 (460 bp) sequence from *E. coli* isolates ; lane 2-5 of uidA gene (160 bp); lane 6 &7 of Stx2 gene (282 bp); lane 8 & 9 of Stx1 gene (366 bp) and Lane M:1000 bp DNA ladder, the used voltage was 90 volt and running time 45 minutes

Distribution of *E. coli* isolates among different kind of salads: After collecting 200 samples of salads and vegetables around the city of Erbil in different locations for isolating *E. coli* strains and then detecting pathogenic strains of *E. coli* as in Table (3), our results showed that in 200 samples only (32%) were positive indicated the presence of *E. coli* depending on culture identification and (68%) were negative.

Table (3): The isolation of pathogenic *E. coli* in vegetables and salads

Type of salad	Number of samples	Number of positive *E. coli*	positive *E. coli* %	Number of negative *E. coli*	negative *E. coli* %
Tomato+cucumber	30	10	5%	20	10%
Tabola	20	10	5%	10	5%
Cabbage	24	4	2%	20	10%
Carrot	18	6	3%	12	6%
Tomato	16	4	2%	12	6%
Tomato + Lettuce	12	4	2%	8	4%
Lettuce	12	2	1%	10	5%
Tomato + Cabbage	12	6	3%	6	3%
Cucumber	8	2	1%	6	3%
Pepper	6	2	1%	4	2%

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Apple + Carrot	10	4	2%	6	3%
Beet	4	2	1%	2	1%
Babaghnoge	8	2	1%	6	3%
Arugula	10	4	2%	6	3%
Cucumber + Carrot	10	2	1%	8	4%
Total	200	64	32%	136	68%

Discussion

E. coli can be identified by using eosin methylene blue (EMB) agar on the basis of a green-metallic sheen appearance on the surface of bacterial colonies. Eosin methylene blue agar used as a rapid and accurate method for identification *E. coli* and distinguish it from other gram-negative bacteria, The dyes in EMB agar, eosin Y and methylene blue, are inhibitors of gram-positive bacteria and pH indicators, that at an acid pH combine to produce a green-metallic precipitate (sheen) [17, 18].

In this study, it was tried to combine cultural and molecular techniques to shorten the time needed for identification.

Gene uidA is one of the particular housekeeping genes in *E. coli* that code for beta-di-glucuronidase and is determined as one of the *E. coli's* virulence markers. Many studies depended on this gene to identify *E. coli*; also PCR techniques based on the detection of this gene have been used to diagnose *E. coli* [19, 20].

The results of uidA gene were similar to Kingsley Ehi Ebomah and Anthony Ifeanyi Okoh [21] which were (81%) of isolated *E. coli* positive for this gene, also in another study (83%) of isolated *E. coli* from gallstones in Iraq were positive for this gene [22]. In a study conducted by [23], they found that all the isolates confirmed as *E. coli* by biochemical tests were also found positive for uidA gene [24]. Only (4) isolate (6%) among (64) *E. coli* isolates was positive for rfbo157 gene (479 bp), this percentage is similar to the study done by [25] in which (7.3%) of pathogenic *E. coli* isolated from the same type of samples in Nigeria.

The results of the current study were higher than [26] in Dhok city in which no isolate (0%) of their sample were positive for rfbo157. *E. coli* O157 is the most studied strains among all other pathogenic strains.

Among all other strains of *E. coli*, the most studied strain is *E. coli* O157, because it has been identified as one of the world's main sources of human food-borne infections, with severe complications such as hemolytic uremic syndrome, which leads to renal failure. Humans can be infected with this strain through direct and indirect methods such as contaminated beef ground meat, vegetables, fruit juice and water. Vegetables have long been suspected of being key vehicles for the transmission of *E. coli* to humans[25].

In the present study only (11%) of the samples showed positive results for shiga toxin; this result close to the result of study by [26] in which 37 (18.5%) of the raw vegetable samples were contaminated by shiga toxin producing *E. coli.* Foodborne infections like Shiga-toxin-producing E. coli (STEC) are well known for their ability to trigger numerous outbreaks. STEC can cause hemolytic uremic syndrome (HUS),

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hemorrhagic colitis (CS) and bloody diarrhea [27].

In the restaurants and hypermarkets, vegetables and fresh product contamination occurred during the holding period and packing process. One of the causes for the high incidence of STEC in raw vegetables could be cross contamination from the handler, cutting instruments, and washing water.Cross-contamination is frequently caused by poor hygiene practices and the working environment [28].

Cross-contamination can be exacerbated by the handling and processing way in both the hypermarkets and restaurants [29]. Shiga toxin producing *E. coli* are able to survive under hard and pressured environments [30]. During the preharvest process, soil is one of the contamination sources. The pathogens' ability to survive in the soil is dependent on a variety of factors such as temperature, moisture level, and soil type [31]. Animal fertilizer, chemical insecticides, irrigation water, excrement and dust are further pre-harvest contamination sources. In both fresh and processed freshcut products, microorganisms are naturally occurring contaminants. After harvesting, fresh products are more susceptible to being contaminated by microorganisms [27]. This is due to the fact that the fresh product's microbiological safety is affected by harvesting and preparation processing like slicing, cutting, and peeling. The release of nutrients from fresh products will enhance microbial multiplication and growth [31].

Recently, PCR-based detection technique have become very important for identification of bacteria. The fundamental reason for this is that DNA from a single bacterial can be amplified in about one hour, which is much faster than other methods [32].

In the study performed by [33] found that more than half of food samples were contaminated by *E. coli*, this is higher than results of our study in which only (32%) were positive indicated the presence of *E. coli*. There have been several reports of food contamination by *E. coli* from different countries.

Nonpathogenic *E. coli* may acquire virulence genes by horizontal gene transfer, resulting in the establishment of virulent strains.

Conclusions

This study focuses on the serotype *E. coli* O157:H7, a high percentage of *E. coli* was detected in salad samples which indicated the fecal contamination. The presence of rfbo157, stx1and stx2 genes are the indications for pathogenicity and shiga toxin production respectively.

Recommendations

The present study suggests that checking of restaurants and road shops should be carried out at organized periods and the products should be tested for the presence of pathogenic microorganisms, also the water supply should be tested since it is one of the most common source for fecal contamination and then lead to salads and fresh vegetable contamination through washing.

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Ethical clearance: The present study suggests that checking of restaurants and road shops should be carried out at organized

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Conflict of interest: Nill

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التحري عن عزلات الممرضة (O157: H7) والمنتجة للسموم لبكتريا (*Escherichia coli*) المعزولة من السلطة في مدينة اربيل-عراق

شيان اسماعيل صالح[١] ، له نجه جلال محمود[٢]، سفين تحسين كاكه يى[٣]، كويستان حسن صديق[4]

الملخص

خلفية الدراسة: بكتريا (*Escherichia coli* O157:H7) واحدة من اهم مسببات تسمم الغذهئي في العالم حيث تسبب اعراض خطيرة للانسان. تعد المواشي نواقل لهذه بكتريا، حيث تعد تلوث منتجات الغذائية بمخلفات هذة الحيوانات واحدة من المصادر الرئسية لانتقال المرض، كما ان بكتريا (E. *coli*) تاقلمت بصورة مع الظروف البيئية المختلفة.
(*E. coli* O157) تمتلك العديد من عوامل الضراوة من اهمها انتاج السموم و جين (*rfbo157*) .
اهداف الدراسة: هدفت الدراسة الحالية الى تحديد نسبة انتشار العزلات الممرضة و منتجة للسموم لبكتريا (*coli.E*) المعزولة من الخضراوات و سلاطات التى تباع في مطاعم و المحلات الصغيرة.
المرضى والطرائق: تضمنت الدراسة جمع (٢٠٠) عينة من سلطة و الخضراوات من المطاعم و المحلات الصغيرة في اسواق مدينة اربيل من شهر ايلول الى نيسان ٢٠١٩ . زرعت العينات على وسط (EMB). حيث نمت مستعمرات *E. coli* بلون اخضر معدني بعد ذلك تم تاكيد التشخيص بالتحري عن *uidA gene* باستخدام تقنية PCR وكذلك تحديد نسبة البكتريا الممرضة و منتجة للسموم بدراسة جيني *rfbo157* و *stx1, stx2*على التوالي.
النتائج: كانت نسبة العزل (64/200) 32% (*E. coli*) من اجمالي العينات التى تم تشخيصها باستخدام وسط EMB . اما نسبة العزل بالاعتماد على جين *uidA* فكانت ٨٨% (٥٦|٦٤).الامراضية و انتاج السموم ل ٥٤ بكتريا من *E. coli* فكانت ٤ عزلات موجبة لجين *rfbo157* المسؤل عن الامراضية و ٧ عزلات موجبة لجيني المسؤلين عن انتاج السموم .
الاستنتاجات: كانت نسبة التلوث عينات السلطة و الخضراوات ببكتريا *E. coli* عالية بنسبة ٣٢% باستخدام وسط الزرعي الانتقائي EMB و اقل باستخدام جين *uidA gene* اما النسبة العزلات الممرضة فهي قليلة ٧% اما ١٢ % من العزلات كانت منتجة للسموم.

الكلمات المفتاحية: scherichia coli, shiga toxin, *E. coli* O157, uidA gene

البريد الالكتروني: lanja_jalal2002@yahoo.com

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