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Molecular Detection of *Stx1* and *Stx2* Genes in *E. coli* O 157:H7 Isolated from Soft White Cheese

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Abstract

Foodborne diseases are increasingly reported to be caused by *Escherichia coli* O157:H7. Therefore, the current study was conducted to determine the incidence of this bacterium in locally produced soft white cheese. A total of 36 samples were collected from local markets in different places of Baghdad city. The process of bacterial isolation was performed by culturing onto different media, such as MacConkey Agar, Eosin Methylene Blue Agar and Sorbitol MacConkey Agar (SMAC). The identification of bacteria was achieved by biochemical tests and confirmed by a molecular technique. Multiplex polymerase chain reaction (m-PCR) was used through the detection of *rfb* O157 and *flic* H7 genes. Some genes of virulence factors, such as Shiga-toxin genes (*Stx1*and *Stx2*), were also investigated by PCR. In this study, the results showed that the total number of *E. coli* isolates was 6/36 (16.7%) and only 1/36(2.8%) of the isolates were of the *E. coli* O157:H7 strain. The identified isolate of *E. coli* O157:H7 was positive for the *Stx2* gene; 1/6 (16.7%) of *E.coli* isolates, while it was negative for *Stx1;* 0/6 (0%). In terms of occurrence, this bacterium was less found than those reported by previous researches in Iraq. This study demonstrated that soft white cheese made locally in Baghdad City has a significant probability of containing the Shiga-Toxin *E. Coli* (STEC).

Keywords: *E. coli* O157: H7, **S*tx1*and S*tx2*,** multiplex PCR, soft white cheese

التشخيص الجزيئي لبكتيريا *E. coli* O 157 في الجبن الابيض الطري

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الخلاصة

تعد جراثيم الإشريكية القولونية O157: H7 واحدة من اهم المسببات الرئيسية للأمراض في الانسان التي تنتقل عن طريق الأغذية. أجريت الدراسة الحالية لتحديد نسبة وجود هذه الجراثيم في الجبن الأبيض الطري المنتج محليًا . تم جمع اجمالي 36 عينة من اسواق محلية ومن مناطق مختلفة في مدينة بغداد. تم إجراء عملية العزل بطريقة الزراعة على وسط محدد مثل(Sorbitol MacConkey Agar (SMAC. كما تم تأكيد التعرف على هذه الجراثيم بتقنية الجزيئية باستخدام طريقة تفاعل البلمرة المتسلسل المتعدد (m–PCR) وذلك من خلال الكشف عن جينات *rfb O157* و *flic H7*. كما تم الكشف عن بعض جينات عوامل الضراوة مثل جينات سموم الشيغا (*Stx1* و *Stx2*) حيث تم فحصها ايضا بواسطة طريقة تفاعل البلمرة المتسلسل PCR. في هذه الدراسة, أظهرت النتائج أن العدد الإجمالي لعزلات الإشريكية القولونية هو 6/36 (16.7٪) وان هناك

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عزلة واحدة فقط 1/36 (2.8٪) من *E. coli* O157: H7. حيث كانت العزلة المحددة للإشريكية القولونية O157: H7 موجبة فقط لجين *Stx2* 1/6: (16.7 ٪) من عزلات الإشريكية القولونية, كما ان نسبة حدوث هذه الجراثيم أقل من تلك المذكورة في البحوث السابقة في العراق.

Introduction

One of the most common food-borne pathogens that have received more attention recently is *Escherichia coli* (*E.coli*) O157:H7 [1]. It is a grave concern for public health, since it causes a number of illnesses that span in severity from mild to fatal [2]. There are several well-known pathotypes of *E. coli* that can cause intestinal diseases, including enterotoxigenic *E. coli* (ETEC) and enterohaemorrhagic *E. coli* (EHEC) [3]. ETEC is the most prevalent, especially in developing nations [4]. The most typical serotype of EHEC linked to foodborne outbreaks is *E. coli* O157:H7 [5]. This bacterium can cause extremely severe diarrhea as well as potentially fatal post-diarrheal conditions such as hemorrhagic colitis and hemolytic uremic syndrome [6].The moderately invasive EHEC can use bacterial fimbriae for attaching (*E. coli* common pilus, ECP), and it has a phage-encoded shiga toxin that causes a significant inflammatory reaction [7]. It is generally known that the following four EHEC virulence factors were identified: Shiga toxins 1 and 2 (*Stx1* and *Stx2*), intimin (*eae*), and EHEC-hemolysin (EHEC-*hlyA*) [8].

E. coli serotype O157:H7 has been linked to a number of epidemics [9]. Because *E. coli* O157 is frequently present in healthy bovine feces, the majority of *E. coli* O157:H7 outbreaks have been linked to the consumption of foods derived from cattle, particularly those contaminated with cattle feces [10**]**. EHEC colonization in adult ruminants, however, is asymptomatic [11]. The absence of *stx* receptors on enterocytes was thought to be the root of this organism's asymptomatic illness in cattle [12]. Cattle are a major source of toxigenic *E. coli* and have been linked to the transmission of *E. coli* disease to humans [13]. Dairy products were to blame for more than 11% of all E. coli O157:H7 illnesses that were recorded in England and Wales in 1999 [14]. In accordance with other research, ingestion of dairy and milk items was linked to 1-5 % of foodborne infections, contaminated cheese was the source of 53% of cases, and EPEC was responsible for 18.33% of these infections [15]. In recent foodborne STEC outbreaks, unprocessed dairy products that are contaminated, like raw milk and raw-milk cheese, have been implicated [16]. It has been shown that the pathogen can survive in raw (soft) cheeses, unpasteurized milk cheddar cheese [17], feta cheese, and yoghurt [18].

Numerous works have been created based on polymerase chain reaction (PCR) for quick detection of *E. coli* O157:H7 because molecular tests are more time-efficient than conventional analysis for identifying E. coli O157:H7 isolate [19]. The primary objective of this study was to use multiplex PCR to identify this bacterium and its virulence components (shiga-toxin1 and shiga-toxin2) in order to highlight the probable presence of STEC in samples of cheeses made locally which were collected from various parts of Baghdad city.

Materials and Methods

Sampling

In the current study, to look into the possibility that there is *E. coli* O157: H7 in locally produced (homemade) soft white cheese made of bovine milk, 36 samples were obtained from local markets in different places in Baghdad city, including Al-Jehad, Al – Amerria, Al-Bayaa, and Abu Ghraib, during the period from March to May 2023. These samples were transferred to the department of microbiology at the College of Veterinary Medicine /University of Baghdad for further specific microbiological analysis. Each sample of fresh cheese (250 grams) was placed into a sterile bag made of plastic, and then the contents were

mashed manually and homogenized. After that, 10 grams were taken from each sample and initially cultured in 90 ml of Trypton Soya Broth which contains sodium citrate (2%) in order to dissolve the small cheese lobules [20-22], then incubated at 37°C for 24 hours as initial activation. The implanted samples were plated with MacConkey agar (the colonies of *E. coli* appeared red in color) as well as with Eosin methylene blue (EMB) agar (the colonies were with greenish metallic sheen color) for identification of *E. coli*. *E. coli* was sub-cultured to obtain pure cultures which were cultured onto nutrient agar slants and preserved inside a refrigerator for further examinations [23]. The suspected *E. coli* isolates were examined by some biochemical tests, such as Indole and Methyl Red tests which both were positive, but the Voges proskauer and Citrate utilization tests were negative [24]. The identified bacteria were cultured onto a specific medium, namely Sorbitol MacConkey Agar (SMAC) [25]. *E. coli* O157: H7 colonies appeared light brown in color (non-sorbitol fermenters) [26].

DNA extraction

The diagnosis of *E. coli* O157: H7 was confirmed by using the multiplex PCR (m-PCR) technique. PCR was applied to identify *E. coli* O157: H7 through the detection of the *O157* and *H7* genes (*rfb* O157 and *flic* H7) in *E. coli* isolates. Some virulence factors' genes such as Shiga-toxins genes (*Stx1*and *Stx2*) were also detected using the PCR technique. The primers for these genes were supplied in lyophilized form by Macrogen Company. Lyophilized primers were dissolved in nuclease free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at -20 C) to 90 µl of nuclease free water to obtain a working primer solution of 10 pmol/µl. The primers' sequences and the PCR products with their expected sizes are shown in Table 1. This study's primers, amplification conditions, and reagents were all based on those reported by Obaid *et al*., 2022 [27].

Table 1: The primers' sequences of *E. coli* O157: H7 and the expected size of PCR products.

Gene	Primer	Sequence (5- 3)	Amplicon Size [bp]	Reference
Rfb	rfb O157-F	5-CGGACATCCATGTGATATGG-3	259	[27]
	rfb O157-R	5-TTGCCTATGTACAGCTAATCC-3		
Flic	flic H7-F	5-GCGCTGTCGAGTTCTATCGAG-3	625	[27]
	flic H7-R	5-CAACGGTGACTTTATCGCCATTCC-3		
Stx1	Stx1-1	5-ACACTGGATGATCTCAGTGG-3	602	[27]
	Stx1-2	5-CTGAATCCCCCTCCATTATG-3		
Stx2	Stx2-1	5-CCATGACAACGGACAGCAGTT-3	780	[27]
	Stx2-2	5-CCTGTCAACTGAGCACTTTG-3		

Genomic DNA was extracted from bacterial culture using the ABIOpure - Extraction Protocol (ABIOpureTM Total DNA Kit, ABIOpure, USA). DNA concentration ranged from 54 to 81 ng/µl as detected by Quantus Fluorometer (Promega, USA) in order to be sure about the goodness of the samples for downstream applications. The PCR component calculations for the primers are shown in Tables 2 and 3.

Table 2: PCR Component Calculation for *rfb O157*(primer 1) and *flic H7*(primer 2).

Master mix components	stock	unit	final	unit	volume (1 sample)
Master mix	2	X	1	X	10
Forward primer1	10	µM	0.5	µM	1
Reverse primer1	10	µM	0.5	µM	1
Forward primer2	10	µM	0.5	µM	1
Reverse primer2	10	µM	0.5	µM	1
Nuclease Free Water					4
DNA		ng/µl		ng/µl	2
Total volume					20
Aliquot per single rxn	18 µl of master mix* and 2 µl of template per tube				

Master mix* (GoTag Green Master Mix, Promega, USA)

Table 3: PCR Component Calculation for each primer of *Stx1* and *Stx2* genes.

Master mix components	**stock**	**unit**	**final**	**unit**	**volume (1 sample)**
Master mix	2	X	1	X	10
Forward primer	10	µM	0.5	µM	1
Reverse primer	10	µM	0.5	µM	1
Nuclease Free Water					6
DNA		ng/µl		ng/µl	2
Total volume					20
Aliquot per single rxn	18 µl of master mix and 2 µl of template per tube				

The program of PCR (Thermal Cycler, Thermo Fisher Scientific, USA) used the following conditions: Initial denaturation was done by 1 cycle at 95°C for 5 minutes, followed by 30 cycles for each denaturation at 95 °C for 30 seconds. Annealing was done at 52 °C or 60 °C for 30 seconds, while extension was done at 72 °C for 30 seconds. Then 1 cycle was performed for each of the final extensions (at 72 °C for 7 minutes) and held at 10 °C for 10 minutes. The program of multiplex PCR is shown in Table 4.

Table 4: Program of multiplex PCR.

Steps	°C	m:s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	52	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

Agarose gel electrophoresis (OWL Electrophoresis System, Thermo, USA) was done to confirm the presence of amplification. All the solutions used, such as 1X TAE buffer, DNA ladder marker, ethidium bromide (10 mg/ml), and agarose originated from Promega, USA. After preparation of agarose (1.5 %) and casting of the horizontal agarose gel, the PCR product (5µl) was directly loaded into the well. Electrical power was turned on at 100v/m Amp for 60 min. DNA moves from the cathode to the plus anode poles. The ethidium bromide-stained bands in gel were demonstrated utilizing a gel imaging system (Major Science, Taiwan).

Results

During this study, 6/36 (16.7%) isolates of *E. coli* were recovered from cheese samples, and only one 1/36(2.8%) isolate was *E. coli* O157:H7, which was cultured onto the specific Sorbitol MacConkey Agar (SMAC) medium and appeared light brown or golden in shading, as shown in Figure-1. The results of culturing were in agreement with the results of the multiplex PCR technique. The results of the amplification of the *rfb-O157* and *flic-H7* genes of *E. coli* samples are shown in Figure 2. In addition, the results of this study revealed that the only isolate of *E. coli* O157:H7, i.e. 1/6 (16.7 %) of *E.coli* isolates, possessed the *Stx2* gene, as shown in Figure 3.

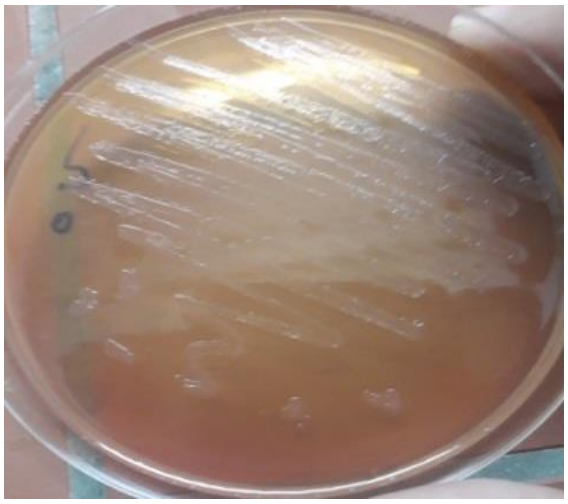

Figure-1: Pale brown colonies of *E. coli* O157: H7 on SMAC agar.

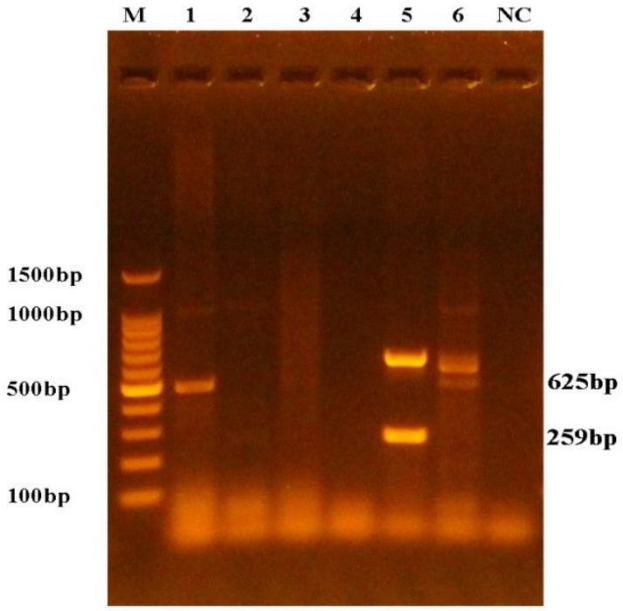

Figure 2 : Results of the amplification of *rfb-O157* and *flic-H7* genes of *E. coli*. Samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lane 5 resembles 625, 259 bp PCR products.

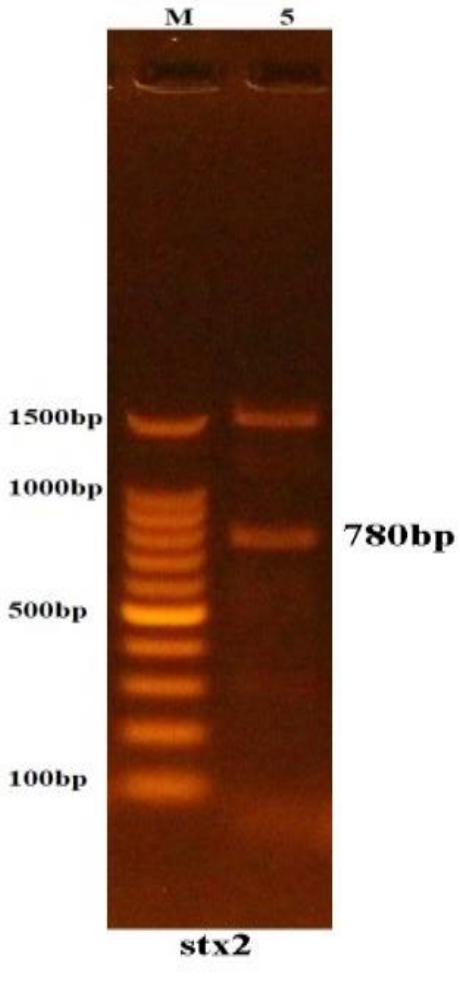

Figure 3: Results of the amplification of stx2 gene of *E. coli O157:H7* isolate. Sample number 5 was fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lane 5 resembles 780 bp PCR products.

Discussion

It has been approved that *E. coli* O157:H7, which has been a linked to several cases of infection and fatalities globally, ranks among the most hazardous foodborne pathogenic bacteria. It is commonly known that *E. coli* O157:H7 is regarded as the primary pathogen responsible for human gastroenteritis in underdeveloped countries [27].

Information on the frequency of *E. coli* O157 in dairy products in Iraq is limited. Therefore, this work was prepared to assess the prevalence of *E. coli* O157:H7 in locally made white soft cheese collected from various locations throughout Baghdad city. The isolation process was performed by the culturing method using specific media, including Sorbitol MacConkey Agar (SMAC) [25]. The total number of *E. coli* isolates was 6/36 (16.7%); 5 isolates were sorbitol fermenters and only 1/36(2.8%) isolate was non- sorbitol fermenter. The latter might be the bacteria of *E. coli* O157: H7, which is distinguished from the other species of *E. coli* by its incapacity to ferment sorbitol; it was found to be light brown or golden in shading, whereas the fermented sorbitol was purple [28].

Numerous factors, including the poor condition of the raw supplies utilized throughout the production process, their high microorganism content, the ineffectiveness of the thermal procedures applied during the manufacturing process, and the unsanitary practices used throughout the handling and preservation procedure, may be to blame for the presence of these large numbers of *E. coli* isolates.

In the present work, the detection of a suspected isolate of *E. coli* O157 : H7 was effectively verified by the *flic H7* and *rfb O157* genes, utilizing the m-PCR technique. According to these genes, a PCR-based technique for identifying *E. coli* O157:H7 strains appears to be appropriate, which is in line with [29]. In one previous study, the *Stx* target gene was also used for the detection of this bacterium [30]. During this study, the genes of some virulence factors, like the Shiga- toxins genes (*Stx1*and *Stx2*), were also investigated to point out attention to any possible Shiga-Toxin *E. coli* (STEC) presence in the cheese samples. The identified isolate of *E. coli* O157:H7 was toxigenic; it had positive expression of *Stx2* gene; 1/6 (16.7 %) of *E.coli* isolates. The Shiga toxin 2 (*Stx2*) and adhesion intimin (*eae*) are *E. coli* O157's two main virulence factors [31]. Both toxins (*Stx1* and *Stx2*) have numerous subtypes, and some subtypes of *Stx2* seem to be connected to disease in humans [32]. Improved health outcomes and lower healthcare costs would result from the prevention of Shiga toxin infections.

In this work, our findings of occurrence frequency were lower than those reported by previous studies in Iraqi regions [33-38, 22]. These rates were different because of the source of milk used for dairy products and its being collected from infected cows. In addition, bacterial contamination may be reduced during the processing of dairy products, especially when increasing hygienic measures in such procedures. In Iran, similar to our findings, a study conducted by Al-Khyat [39] revealed that 0.5% of the samples were *E. coli* O157:H7 isolates. The frequency of *E. coli* varies in different countries due to similarities and variances in temperature and hygienic conditions across the globe.

Globally, the documented isolation rate was very high as pointed out by Mora *et al*., [40], who isolated the bacteria from 7.6 % of soft cheeses manufactured from raw cow's milk in Canada, along with 7.8 % of soft cheeses made from cow's milk in Peru [16] and 4 % in Turkey[10]. According to [41], STEC appeared more frequently in summer and less frequently in winter. The bulk of human cases of *E. coli* O157:H7 outbreaks occurred between June and September [42].

Investigations into the presence of *E. coli* O157:H7 in dairy products have taken place in many regions of the globe [43]. In Ireland, the presence of this bacterium was confirmed in fresh white cheese [44], and this is a sign of poor milk quality, subpar manufacturing procedures, or contamination during processing [45]. Mastitic udders and fecal contamination are both viewed as significant entry points for *E. coli* O157:H7 into the milk supply [46]. The source of the disease has been determined to be feces-contaminated ground in 48 out of 196 *E. coli O157: H7* outbreaks reported in USA between 1982 to 1998 [47]. Additionally, it has been discovered that flies can spread *E. coli* O157:H7 in farms [48, 49].

The most significant methods for isolating and characterizing *E. coli* O157:H7 in samples taken from dietary and environmental sources include conventional culture and serological and molecular testing. Techniques for detecting *E. coli* O157:H7 in food have advanced dramatically over the past few years, moving from culture-based techniques to DNA-based and immunological tests [50]. Multiplex PCR assays offer the advantages of reduced costs and quicker findings [51, 52]. The disadvantage is that the need for specialized laboratory equipment required by DNA-based approaches could be expensive, especially in underdeveloped countries. To ensure food safety, quick and accurate methods for detecting *E. coli* O157:H7 in various food items are required. In comparison to cultural-based and immunological-based approaches for pathogen identification in food, PCR technology has increased in popularity as a result of its quickness, great sensitivity, and specificity [53]. As noted by [54,55], PCR assays have been demonstrated to be specific and sensitive for finding microbial infections like *E. coli* O157:H7 and, for the diagnosis of *E. coli* O157:H7, gene-based methods like the PCR technique were more accurate than both the biochemical and the serological examinations. The capacity to identify isolates with a masked O antigen was the primary benefit of the used PCR technology [56].

A significant milk product made in practically every region of the world is cheese. Identifying *E. coli* O157 is frequently used as a gauge for determining the safety of fresh white cheese. Transmission happens when raw dairy products are consumed [57].

Conclusions

In this work, despite the extremely low incidence of *E. coli* O157:H7, the detection of this pathogen in cheese samples has an important value, particularly in Iraq, due to the strong inclination of Iraqi inhabitants to consume such dairy products. Similar to some other countries, domestic cheeses made from raw milk with inadequate hygiene requirements are still quite popular in Iraq. This study has demonstrated that soft white cheese made locally in Baghdad city has a significant probability of containing the Shiga-toxin *E. coli* (STEC). Cheese prepared from milk that has not been pasteurized could be a potential means to acquire bacteria such as *E. coli* O157:H7 by the consumer. Further studies of larger number of samples might be necessary to clarify the incidence of such bacteria in dairy products.

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Conflict of Interest: The authors declare that they have no conflict of interest**.**

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