Detecting Stx1 and Stx2 Genes from *Escherichia coli* O157:H7 Isolated from Soft Cheese Samples Collected from Different District in Baghdad City/Iraq

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Abstract

Escherichia coli O157:H7 colonies have been obtained from eighty produced local Buffalo's and Cow's soft cheese specimens that have been collected randomly from different retail markets in various districts of Baghdad city. The results showed that the presence of Escherichia coli O157:H7 as a contamination indicator in the soft cheeses. Six isolates were detected on the differential and selective media MacConkey agar, sorbitol such as MacConkey agar, chromogenic media and routine biochemical tests were used to diagnose and identify the bacteria. Results of *Escherichia coli* O157:H7 diagnosis have been confirmed using PCR technique which exhibited that, 6 culturing strains serotype *Escherichia coli* O157:H7 were positive using PCR assay and 5 of these strains expressed gene of Stx1 while1strain expressed gene of Stx2.

Keywords: Detection, Genes, Cheese, PCR and *E. coli*

التحري عن Stx1 وStx2 في بكتريا القولون O157: H7 المعزولة من عينات الجبن الطري المجموعة من مناطق مختلفة من بغداد/العراق

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الخلاصـــة

عزلت مستعمرات بكتريا القولون O157: H7 من 80 عينة من الجبن الطري المنتج محليا، مصدره حليب الابقار والجاموس والتي جمعت بشكل عشوائي من مختلف الأسواق في مواقع مختلفة من مدينة بغداد. أظهرت النتائج وجود الاشريشيا القولونية O157: H كمؤشر للتلوث بهذه الأجبان الطرية. تم الكشف عن ستة عزلات *Escherichia coli* O157:H7 على الاوساط التفاضلية والانتقائية: وسط الماكونكي والسوربيتول ماكونكي اكار والكروموجينيك واستخدمت الاختبارات الكيميائية الحيوية الروتينية لتشخيص وتحديد هذا النوع من البكتريا المعوية. اكدت النتائج باستخدام تقنية ال PCR التي اظهرت عزل ستة أنواع من العزلات المصلية بكتريا القولونO157:H7 خمسة من هذه العزلات تعبر عن جين Stx1 وعزلة واحدة تعبر عن جين Stx2.

الكلمات المفتاحية: تحري والجينات والجبن والتفاعل الانزيمي المتسلسل وبكتريا القولون

Introduction

Escherichia coli O157:H7 has become a major pathogen in products of dairy and food since its identification in 1982 as a pathogen of human (Fratamico and Smith, 2006), because of its capability for causing hard illness especially, thrombotic thrombocytopenic purpura hemorrhagic colitis, and hemolytic uremic syndrome (Maher, *et al*., 2001; Govaris, *et al*., 2001). Commonly, this bacterium is the most recognized STEC in the US, nevertheless, several other STEC sero- groups involving O103, O26, O145, and O111, were related with sporadic cases of HUS and HC and outbreaks globally (Paton, *et al*., 1996).This bacterium is a member in the pathogenic strains group of *E.coli*, Shiga toxigenic *E.coli* (STEC), entro-haemorrhagic *E.coli* (EHEC), and Verotoxin producing *E.coli* (VTEC), which was obtained from individuals who modified severe abdominal crampand bloody diarrhea (Yoon and Hovde, 2008). One of the largest *E. coli* O157:H7 (One of the EHEC Serotypes) outbreaks related with consumption of food taken place in Japan in 1996 in City of Sakai, (Nobuyasu, *et al*., 2003). Most of outbreaks of the food borne of *E. coli* O157:H7 were related with the food consumption of the originated from cattle, particularly the contaminated food by feces of cattle, because *E. coli* O157 was found regularly in healthy cattle feces, as aasymptomatic carrier (OKsus. *et al*., 2004). Over 11% of the total number of the recorded cases of *Escherichia coli* O157:H7 infections in Wales and England in 1999 were because of the products of dairy (Vernozy - Rozand, *et al*., 2005). Due to results, the contamination of the environment and carcasses using *E. coli* O157:H7 and *E. coli* O157 from contents the cattle intestine during slaughter is one of the most important risk agents intrans mission to the human (Phillips, 1999; Mead and Griffin, 1998). The made cheese from unpasteurized milk is the potential method to the present bacterium transmission to consumers. In Iraq, domestic cheese is until too popular that generally manufactured from the raw milk with the imperfect hygienic practice. The huge amount of traditional cheese is made from the raw milk and used up after ripening in saline solutions freshly (Najm and Amer, 2017). The pathogenicity of *E. coli* O157:H7and *E. coli* O157, involving STEC, are related with many agents of virulence. The major agent contributing to its pathogenicity is its capacity for producing 2 potent phages encoded cytotoxins known Shiga-toxins (Stx2 and Stx1) (Garrido, *et al*., 2006). The toxins production, another virulence-related agent expressed using STEC is the protein known Intimin, which is encoded using the gene of EAE and responsible for the intimate STEC attachment to cells of the intestinal epithelium (Law, 2000). Over the last many years, detection approaches of STEC in food were significantly modified from culture-based approaches into immune and DNA-based tests with each approach having its weaknesses and strengths (Derzelle, *et al*., 2011).

This work aims to identify *Escherichia coli*: O157: H7 from the locally produced home-made soft cheese specimens using PCR Genotyping for detecting virulence factors (Stx2 and Stx1) of isolates of this bacterium.

Materials and Methods

Cheese Sampling

A total of local eighty produced cow's soft cheese specimens have been collected (250 gm for Each Sample) in the sterile 500ml polyethylene bag randomly from the local market at Baghdad city, after transferring them to Food Contamination Center, samples

were analyzed and identified depending on the type of sample.

E. coli Isolation

About 25 gm of samples of cheese has been taken and homogenized with 225 mL of the buffered peptone water 0.10% in the stomacher for 1 minute, then the homogenate has been diluted serially and 0.10 ml of each dilution have been plated on 3 media, MacConkey agar and Sorbitol MacConkey agar (SMAC) supplemented with potassium tellurite (2.5 mg/L) and cefixime (0.05 mg/L) were used to detect *E. coli* and EHEC colonies. McA and SMAC agar after culturing with bacterial cultures, incubated for 24 h at 37 °C. Seven lactose fermenting colonies from MacConkey agar and non-sorbitol fermenting (Colorless) colonies from SMAC agar were transferred individually to fresh nutrient broth and incubated at 37 °C for 18 h, then streaked on CHROM agar O157 and on plates containing EMB agar (Eosinmethylen Blue) then incubated at 37 °C for 24 h. These isolates, with typical *E. coli* metallic sheen on the EMB medium, were described using the bio- chemical analyses, involving conventional gram staining, indole production, methyl red, vogesproskauer, citrate utilization and urease and hydrogen sulphide was used to confirm the *E. coli* species.

Molecular Detection of Genes of Verotoxine (Stx2 and Stx1) by PCR Technique

DNA Extraction

The commercial kits of Genomic DNA and DNA extraction of isolates of *Escherichia coli* O157:H7 have been extracted using (USA, Kit Promega, Wizard Genomic™ Mini g DNA Bacteria) as in the kit of purification (USA, Geneaid). However, the purified DNA has been checked using electrophoresis in 1.5% agarose gel with addition of ethedium bromide. Stain of bromophenol blue was added to samples of DNA and visualized the DNA using light UV.

PCR Gene Reaction

One protocol of Pollared, *et al*., (1990) has been employed for studying genes of Stx1. This has been achieved by the customize primers exhibited above.

Stx1:f

ATAAATCGCCATTCGTTGACTACS
tx1:rAGAACGCCCACTGAGATCAT
C

Stx2:f

GGCACTGTCTGAAACTGCTCC.Stx
2:r TCGCCAGTTATCTGACATTCTG

The reaction mixture of PCR contains five micro liters of purified bacterial DNA, five microliters of green master mix, and two microliters of each primer for reverse and forward primers, then the volume has been completed to twenty-five microliters using deionized water. Then, tubes of PCR have been centrifuged for 10 sec. and placed in the thermal cycler for starting the reaction of amplification for each gene depending on particular programs, cycling condition for amplification included 94ºC for 3 min, for annealing 1 min and 72 ºC for 1min followed by 72 ºC for 10 min. PCR results have been achieved in post amplification process. About 10 µl of the amplified specimen has been immediately loaded in the 1.5% agarose gel containing 0.5 µl/25ml ethidium bromide with the addition of DNA size and loading buffer. The gel and marker as standard in electrophoresis has been run at 100 Volts for 30 min, then products have been visualized using transilluminator of UV (Sambrook, *et al*., 1989).

Results and Discussion

This research involves detecting the STEC producing the bacterium of *E. coli* O157:H7 in specimens which collected from local soft cheeses after the isolation of this bacterium on the selective medium like (EMB, McA, SMCA), and chromogenic media, bio-chemical tests, and using technique of duplex PCR as shown in Figure (1).

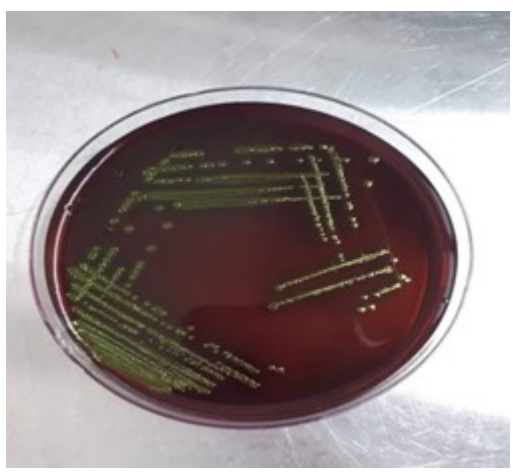

Figure (7) *E. coli* in EMB Media

Results showed that from 80 cheese samples, 22 isolates were *E. coli*. These isolates are confirmed as *E. coli* by biochemical tests. From 22 positive results on MacConkey agar and SMCA, 12 isolates were confirmed on HiCrome EC O157:H7 selective agar as *E. coli* 0157:H7, morphologically, formed dark purple to magenta colored moiety on HiCrome medium. Six isolates were confirmed by PCR gene reaction as illustrated in Table (1). Five isolates sorbitol negative carrying Stx1 was detected by PCR to identify as *E. coli* O157:H7 and another isolate which sorbitol positive also carried Stx2 as illustrated in Table (2). Furthermore, the results showed that six isolate of *E. coli* O157:H7 was detected from 80 cheese samples. Therefore, the fact that the occurrence of *E. coli* O157:H7 was low, it should be taken into consideration that the cultural approaches employed in the current work may contribute to the low isolation average. The exact contamination rate may be at least two times higher than stated here due to the low isolation average of culture approaches compared with other genetically and immunological approaches (D'Boer and Heuvelin, 2000). Similar study carried out in Iraq, from 100 samples of meat only two isolates and from 98 dairy product samples were detected as *E. coli* O157:H7 (Dhaher, *et al*., 2010) and one isolate of *E. coli* O157:H7 was detected from 61 food samples (Zahraa, *et al*., 2016). In Iran, another study proved that, from 130 bulk tanks of milk just one isolated was *E. coli* O157:H7 (Brenjchi, *et al*., 2011). While, in another study, from 125 sample of soft cheese prepared from raw milk, found 5 isolates of *Escherichia coli* O157:H7 (Najand and Khalilli, 2007). Out of 50 ground beef samples 7 strains of this bacterium were detected, while no isolate of this bacterium isolated from chicken drumsticks in Turkey (Fatma and Murat, 2000). PCR assay was used to confirm the non-sorbitol fermenting colonies as *Escherichia coli* O157:H7.

Table (1) *Escherichia coli* O157:H7 Occurrence in Specimens of Soft Cheese

Source of Cheese Samples	No. of Samples	Chromo Genic Media	Selective Media	PCR Positive
Buffaloes	20	2	8	-
Cows	60	10	14	6
Total	80	12	22	6

Table (2) The Prevalence of Genes of Stx2 and Stx1 in *Escherichia coli* O157:H7 that Isolated from Samples of Soft Cheese

No. of Isolates	Culturing Positive	PCR Positive	Stx1	Stx2
6	6	6	5	1

Many studies showed that multiplex PCR as a reliable identification approach for this bacterium (Gannon, *et al*., 1997). The particular genes were chosen for the reason that the simultaneous targeting of sequences of STX1 and STX2 was significant in detecting the contaminated food with the virulent STEC.

In the multiplex-PCR assay the presence of the main virulence genes (STX1 and STX2), which have been widely used by other researchers (Keen and Elder 2002; Holland, *et al*., 2000). According to results, STX1 and STX2 was detected in isolated *E. coli* O157:H7. It has been reported that the STX1 gene was more common than the STX2 as represented in Figure (2). This agreed with research achieved in the European countries, Japan, and USA (Johnsen, *et al*., 2001; Holland, *et al*., 2000; Zhao, *et al*., 1995).

It pointed out that samples contaminated with strains of *Escherichia coli* in such level could produce potential hazard to the consumer. Next to that, further exploration of other virulent strains such as entero pathogenic *E. coli* (EPEC), entero invasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC) are required for knowing the specific situation of Escherichia coli contaminated food at the local markets. It can be concluded from current study that the level of presence of this bacterium in soft cheese samples are low.

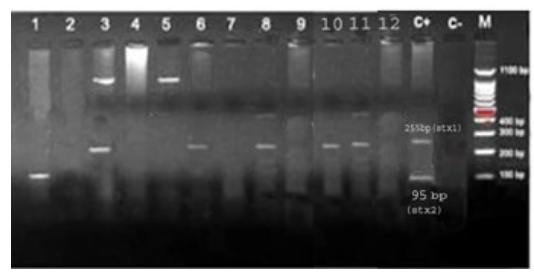

Figure (2) Agarose Gel Electrophoresis of PCR Products to Detect Stx1 and Stx2. M (DNA Marker), C- (Negative Control), C+ (Positive Control)

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