Detection of *Escherichia coli O157:H7* in Human patients Stool and Food by Using Multiplex PCR Assays Targeting the *rfbE* and the *eaeA*Genes compared with Detection by Biochemical Test and Serological Assay

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Abstract

Escherichia coli O157:H7 is an important food borne pathogen has been linked to foods of bovine origin and fresh produce. Rapid and sensitive identification of this dangerous pathogen is important for patient management and for prompt epidemiological investigations.

This study aimed to detect *Escherichia coli* O157:H7 presence in samples from diarrhea patients, and food (imported beef and lettuce) and characterize by selective media, biochemical tests, latex agglutination test, and compared with PCR technique. during the period from September 2012 to February 2013 A total 120 samples were collected 50 from diarrhea patients and 70 food samples including (45 imported beef and 25 lettuce) All samples were screened to detect the presence of non-sorbitol fermenting colonies (NSF) on sorbitol Mac Conkey agar supplemented with Cefixime (C-SMAC). A total of 120 isolates, 22 (44%) from diarrhea patients, 18 (40%) from meat and 9 (36%) from lettuce were non-sorbitol fermenting, *E. coli* isolates were serotyped as *E. coli* O157:H7 by latex agglutination test, 10(20%) isolates of diarrhea patients, 7 (15.5%) isolates of meat and 5 (20%) isolates of lettuce, Results of the genetic diagnosis using polymerase chain reaction (PCR)with specific primers (*eaeA and rfbE genes)* by MPCR revealed that 10(20%) isolates of diarrhea patients5 (11.1%) isolate from meat and 5(20 %) of lettuce samples were positive.

PCR has become a very rapid and reliable tool for the molecular diagnosis of *E. coli* O157.

Introduction

During recent years a growing number food-borne illnesses has been associated with the consumption of fresh product [1]. *E.coli* O157:H7 and lettuce were found to be strongly associated with each other, undercooked ground beef was the most commonly dentified vehicle associated with outbreaks of *E coli* O157:H7 infection [2]. In 1982, three outbreaks of hemorrhagic colitis (HC) caused by *E. coli* serotype O157:H7 occurred in north America, at fast-food (ground beef sandwiches) prepared at restaurants in Oregon and Michigan and a nursing home in Ontario, Canada, Two common-source outbreaks probably food related in nursing homes in Canada in 1983 (31 cases) and in 1985 (73 cases) accounted for 66 cases of hemorrhagic colitis, 12 cases of hemolytic uremic syndrome (HUS), and 17 deaths [3] .Several outbreaks of *E. coli* O157:H7 infection have been reported in the United states [4], The largest of which occurred in 1993 on the west coast of the United states with over 700 individuals becoming sick, including 56 cases of HUS and 4 deaths [5].

Since recognition of this serotype, its isolation from stool samples has sharply increased, so it has been ranked as the 3rd most common bacterial pathogen of the human gut after *Salmonella* and *Campylobacter* spp. [6]. Rapid and reliable methods for the detection of *E. coliO157:H7* in different food products are needed to ensurefood safety. The combination of rapidity, good sensitivityand specificity, and ease of performance has made PCR technology an appealing alternative to culture-based and immunological-based methods for pathogen detection in foods. [7], diagnosis of the causative agent of diarrheas cannot depend only on the clinical features of the patients but requires proper diagnosis of the infectious agent in the laboratory [8].

Advantages of multiplex PCR assays, in which two or more DNA regions are co-amplified in one reaction, are lower cost and less time to obtain results. [9]. In the present study, PCR assays using two combinations of primers were designed for the detection of *E.*

coli O157:H7 in imported beef and lettuce, by detection of genes present in a limited number of serotypes. For example, the inclusion of the *E.coli*O157:H7O antigen marker *rfbEO157* limits the detection of strains to O157 sero groups [10].

The *eaeA* gene encodes a 94-kDa outer membrane protein known as Intimin that is required for intimate attachment of EPEC strains to epithelial cells [11],This study was aimed to isolate *E. coli* O157:H7 from stool patients and foods by multiplex PCR assays targeting the *eaeA* and *rfbE genes* to determine the prevalence of *E.coli* O157:H7 .

Materials and Methods

Sample collection:

A total of ١٢٠ samples were collected during the period from September 2012 to February 2013 including fifty samples were collected from patients with diarrhea from both sexes ranged between the age of one and five years who attend to Al-yarmok, aben albalady, central children's hospitals in Baghdad, severity food samples (45 imported beef and 25 lettuce) were collected from supermarkets of Baghdad, included the new Baghdad, al-Shaab district, Baya, Adhamiya, and sayedia, Samples were collected in sterile plastic bags and saved in Refrigerated container until transported to the laboratory.

Isolation of *E. coli* O157:H7

1-Loopfull of each Stool samples of humans was enriched in modified tryptic soy broth (mTSB) supplemented with vancomysin (4 mg/L) according to Sanderson [12]and incubated in 37°C for 24h. Enrichment aliquots of 100 µl were plated onto sorbitol MacConkey agar (SMA, Oxoid) to test for non-fermenting bacteria (colorless colonies).

2- Each food sample (25 g) of meat and lettuce was combined with 225 ml of modified tryptic soy broth (mTSB) supplemented with vancomysin (4 mg/l) according to Sanderson [12] then incubated with Agitation (120 r.p.m.) for 24 hs at 37 °C .After 24 hs enrichment aliquots of 100 µl were plated onto sorbitol MacConkey agar (SMA, Oxoid) supplemented with cefixim to test for sorbitol non-fermenting bacteria (colorless colonies).

Confirmation tests

Sorbitol-non fermenting colonies that were colorless, (up to 10 colonies per sample) were selected for verification. These colonies were tested for oxidase activity, oxidase negative colonies were confirmed to be *E. coli*, using Indole test and citrate utilization test with Simmons citrate agar (Oxoid, England) and the Methyl red and Voges-proskaure tests using MR-VP medium (Biolife) then KCN . Latex agglutination kit (Oxoid *E.coli* O157:H7/USA) was used for conformity identification of *E. coli* O157:H7 to detect the somatic antigen O157, Biochemical identification was performed by the API 20E (Biomerieux).

Bacterial genomic DNA extraction and purification:

This procedure was done by using commercially available DNA extraction and Purification kit (Geneaid/ Korea).

Detection of the Whole DNA

The purified DNA was detected by electrophoresis (80 V) in 1% agarose gel with ethedium bromide. Methelen blue (loading dye) stain added to the DNA sample and visualizes the DNA by the UV light.

Detection of virulence factor genes by polymerase chain reaction (PCR) analysis:

The oligonucleotide primers (*rfb O157*and *eaeA*) were chosen to have adjacent annealing temperatures and minimal interactions and resulted in different-sized products distinguishable in agarose gels [13]. Target primers for amplifying segments of genes are listed in Table 1. MPCR was performed with a reaction mixture 1µl of each set of primers, 5µl of extracted DNA in concentration (50µg/mL), frozen dry mastermix kit (BIONEER/Korea) and completed with distilled water to the final volume of 20 µl The reactions were carried out with amplification thermal cycler (Verti 96 wells DNA thermal cycler (AB: applied Biosystem Company, USA). The procedure consisted of initial denaturation at 94°C for 4 min, followed by 35 cycles of template denaturation at 94°C for 30 s, primer annealing at 58°C for 1 min and extension at 72°C for 1 min. The final cycle

was followed by incubation of the reaction mixture for 4 min at 72°C. Reaction products were separated by agarose gel electrophoresis loading 10µl on to a 1.5% agarose gel (Difco) The buffer in the electrophoresis chamber and in the agarose gel was TBE(1X). 80 volts were applied across the gel. Finally the gel was visualized by exposing the gel to UV light and was photographed. DNA standard molecular weight size marker (100 bp/Promega / USA) was included in each agarose gel electrophoresis run.

Table (1)
Oligonucleotide primers sequences used for PCR amplification.

Primer	*Sequence (5'-3')*	*Ampliconsize (bp)*	*Reference*
RfbE	**F :5-CGGACATCCATGTGATATGG-3 R: 5-TTGCCTATGTACAGCTAATCC-3**	**259 bp**	**13**
eaeA	**F:5-AAGCGACTGAGGTCACT-3 R:5-ACGCTGCTCACTAGATGT-3**	**473 bp**	**30**

Results and Discussion

The frequency of NSF *E. coli* was 44% in human stool samples, 40% in meat Samples and 50% in lettuce samples, that show in (Table (2)). The frequency of NSF isolates in human fecal samples (44%) was higher than the rate reported by Issa who reported that NSF isolates from patient children was (28.57%)[14], also higher than Naahma who showed isolation rate (27.3%)[15], but less than rate obtaind by jassim Out of 125 samples 58 (46.4%) of stools diarrheic patients positive result [16]. In addition atotal of 31 samples of imported minced meat the samples were collected from different areas of Baghdad city who reported lower NSF isolates rate (1.14%) compared with the rate in the present study [17]. The differences in isolation rate between the present study and the other studies may be related to the selective enrichment procedure used in this study which revives the stressed and injured cells. On the other hand, the selective medium (SMAC supplemented with cefixcim) that inhibits other enteric organisms which compete with and over grow the targeted organism in the present study.

Table (2)
Frequency of NSF, biochemically and Api 20 tests to confirm E. coli in human stool, meat and lettuce.

Isolates source	*Total samples*	*Non sorbitol fermenting*	*No. of positive isolates with biochemical Test*	*No. of positive isolates for Api20*
Human stool	**50**	**22 (44%)**	**19 (38%)**	**20 (40%)**
meat	**45**	**18 (40%)**	**16 (35.5%)**	**15 (33.3%)**
lettuce	**25**	**13 (50%)**	**8 (32%)**	**7 (28%)**
total	**120**	**49**	**43**	**42**

Serological identification of *E. coli* O157:H7

Quality detection of somatic O157 antigen was done by latex agglutination test, The results revealed that 20, 15.5 and 20% of *E. coli* isolates from human, meat and lettuce respectively were positive for O157 somatic antigen Table (3) .Latex agglutination test is sensitive and specific in identification of *E. coli* O157:H7. Also it is simple and easy to use. The use of this method in the present

study was to reduce the time and effort use in isolation, also to eliminate other serotypes of pathogenic *E. coli* which have the same cultural and biochemical characteristics. The initial diagnosis methods which relied on phenotypic traits and biochemical tests is not sufficient to determine the serotype in a final and definite so was resorting to the use of latex agglutination kit. The positive rate of recovery *E. coli* O157:H7 in the present study to latex agglutination test from diarrhea patients 10 (%٢٠) was higher than that recovered by Temelli [18] which were 5.55%, the high rate of recovery in the present study can be explained by the area from which patients came from, The direct contact with animals, material contaminated with animal feces or contaminated buildings where animal are confined, this will increase the chances of infection. Three *E. coli* O157:H7 isolate (2%) were detected from 100 samples of beef meat products Confirmated by biochemical and serological tests and this lower than rate in present study [19]. These result was higher than serological identification by the rapid latex agglutination test for both O157 and H7 because of the uses of latex agglutination kit (Oxoid, USA) that identify O157 antigen only Fig. (1).

Table (3)
Frequency of latex agglutination positive E. coli O157:H7.

Isolates source	*Isolates NO.*	*O157 positive*
Human stool	**50**	**10 (20%)**
meat	**45**	**7 (15.5%)**
lettuce	**25**	**5 (20%)**

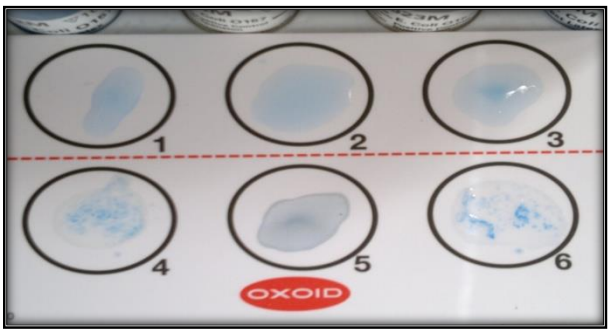

Fig.(1) Latex agglutination test for somatic O157 antigen No.1 negative control; No.4 positive control; No.6 Positive sample (show visible agglutination).

Detection of *rfbO157* and *eaeA* genes

The bacteria was *Ecoli O157* when *rfbO157* gene (259bp) was amplified. *E. coli* O157 existed in the patiants stool tested 20%, 15.5% meat and 20% lettuce .Multiplex PCR were considered positive for *E. coli* O157:H7 when *rfbE* was amplified with the *eaeA* gene (473bp) as standard strain and the result was 10 (20%), 5(11.1%), 5(20%) for human stool, meat and lettuce respectively Fig.(2) shows amplification of the target genesegments in *E. coli* O157 H7-positive samples.

Table (4)
Frequency of rfb_{O157} and eae Agenes in the samples.

Isolates source	*Total*	*E. coli isolates*	*rfb_{O157} gene*	*eaeAgene*
Human stool	**50**	**19**	**10 (20%)**	**10(20%)**
meat	**45**	**16**	**7 (15.5%)**	**5(11.1%)**
lettuce	**25**	**8**	**5 (20%)**	**5(20%)**

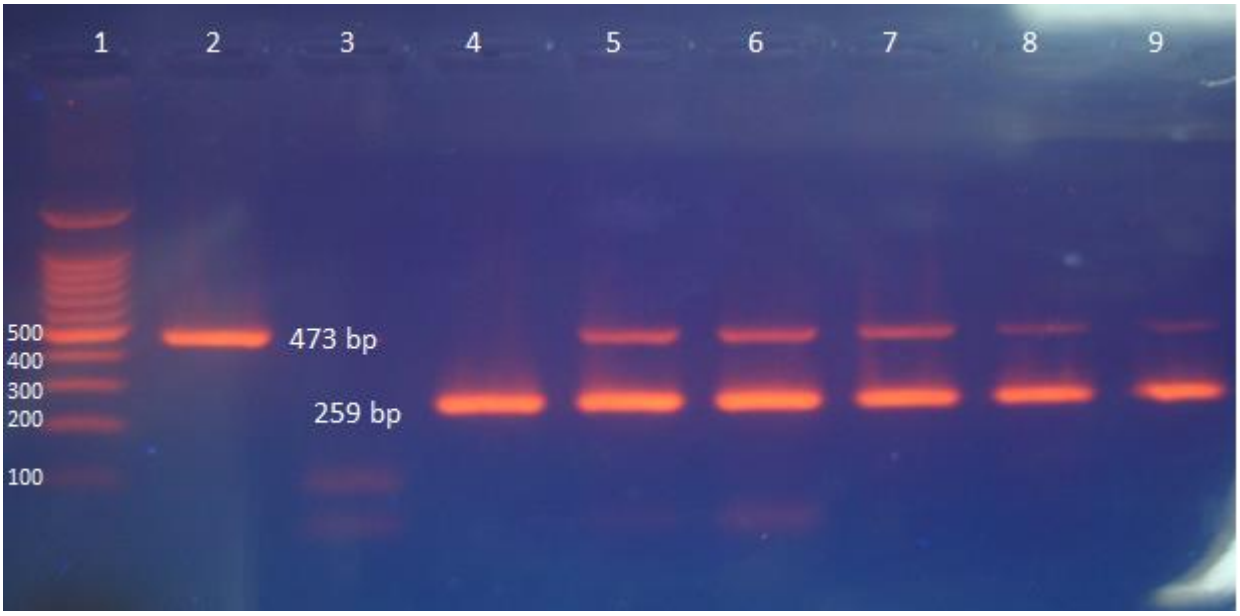

Fig.(2) Lane's 1: 100 bp (DNA size marker,), Lanes 2:eaeA positive, Lane 3: PCR assay control (no template DNA). Lanes 4:rfbO157 positive .Lanes 5 to 9: Multiplex PCR assay with rfb O157and eaeA.

The rate of PCR positive for *E. coli* O157:H7 isolates from human samples (20%) and that was higher than results obtained by Blanco [20] (3%), the frequencies of contamination found for ground beef by pathogen was detected in 5 (١١.1%) beef samples were similar to those reported by Abdul-Raouf, that stated there was a need to handle raw meats separately from other foods to prevent cross-contamination and perhaps illness [21]. In addition these results disagreed with Ahmed when a total of 74 raw uncooked minced beef meat from 3 different areas in Baghdad 1.4% positive for multiplex PCR[22].

In comparison to other countries, the prevalence reported in this study was higher than values reported in studies conducted in France (0.12%) [23]. A study in Argentina (8.3%) [24], and the outbreak of diarrhea in northern Palestine when examined 250 stool samples and reported 124 positive cases of *E. coli* O157:H7 (about 50%) [6]. The reasons that can be suggested for this high frequency of recovery of the serotype were the timing of sample collection since it was during an outbreak of diarrhea and overcrowding, insanitary conditions and poverty in the area .It was lower than prevalence of *E. coli* O157:H7 in cattle and beef was reported to be 27.7% [25]. Such high values suggest point source contamination during primary meat production and processing, and/or subsequent temperature abuse of the meat and meat products within the production/retail chain.

As mentioned before the results of *E. coli* O157:H7 prevalence in vegetables by PCR in organic vegetables 20% showed higher frequency of *E. coli* O157:H7 compared with Results showed low level of contamination in Malaysian organic vegetables [26].

In the study of Oliveira *et al*., [27] they reported that *E. coli* O157:H7 was present in 53.1% of minimally processed leafy vegetable samples in Brazil which is quite high.

Contamination of organic foods with *E. coli* O157:H7 was usually originated from

environment, human or animal sources, Contamination of organic products can occur at harvesting or processing stages. Food handlers' act as another source of contamination and the surroundings area of stalls or grocery shops was usually less hygienic. Possible ways of contamination were instruments and utensils. Previous studies revealed that the bacterium has the ability to attach to the different surfaces (glass, rubber, hydroxyapatite, polystyrene and stainless steel surfaces) [28] Storage temperature is one the important factors that affects the growth and distribution of *E. coli* O157:H7 [29].

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الخلاصة

Ecoli O157: H7 من اهم الممرضات التي تصيب الانسان وتنتقل عن طريق الاغذية ذات المنشأ البقري والفواكه والخضروات الطازجة. وان سرعة الكشف عن هذا الممرض الخطر مهم لمعالجة المرضى وإجراء تحليلات سريعة خلال حدوث الاوبئة، هدفت هذه الدراسة الى الكشف عن بكتريا *Ecoli O157:H7* المتواجدة في عينات من خروج المرضى والاغذية (لحوم البقر المستوردة والخس) وتمييزها بواسطة الاوساط الانتقائية، الاختبارات الكيموحيوية، اختبار التلازن بحبيبات اللاتكس ومقارنتها مع تقنية الـ PCR.

خلال الفترة من سبتمبر ٢٠١٢ وحتى فبراير ٢٠١٣ تم جمع ١٢٠ عينة ٥٠ من مرضى بالإسهال و ٧٠ عينة من المواد الغذائية بما في ذلك (٤٥ عينة من اللحوم المستوردة و ٢٥ عينة خس) كل العينات فحصت للكشف عن وجود المستعمرات غير المخمرة لسكر السوربيتول (NSF) على اكار سوربيتول ماكونكي المضاف اليه بالسفيكسيم C-SMAC وكان من مجموع ١٢٠ عينة، ٢٢ (٤٤٪) عينة موجبة من مرضى الإسهال، ١٨ (٤٠٪) من اللحوم و٩ (36٪) من الخس غير مخمرة لسكر السوربيتول، كانت نتائج عزلات *Ecoli* التي وصفت على انها*Ecoli O157 H7* بالفحص السيرولوجي بواسطة اختبار تراص اللاتكس، 10(20٪) من مرضى الإسهال، 7 (15.5٪) عزلة من اللحوم و 5 (20٪) عزلة من الخس. اما نتائج التشخيص الوراثي بالـ (polymerase chain reaction) PCR من خلال التحري عن البكتريا باستخدام بادئات محددة لجينات (*rfbE ،eaeA*) من خلال تفاعل MPCR كانت النتائج 10 (20٪)، 5(11.1٪) و 5 (20٪) من عينات الخروج، اللحم و الخس على التوالي ايجابية.