

Detection of Escherichia coli O157:H7 Recovered from Food by multiplex PCR

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تشخيص الاشريشيا القولونية نوع ١٥٧ في الطعام باستخدام تفاعل البلمرة المتسلسل

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المستخلص

أجريت هذه الدراسة لعزل الاشريشيا القولونية نوع 157 في عينات الطعام باستخدام تفاعل البلمرة المتسلسل في محافظة واسط لفترة من ايلول 2015 الي شباط 2016. جمعت 120 عينة (40 لحم ، 40 حليب خام ، 40 لحم دجاج) من الاسواق المحلية في مدينة الكوت (العراق). كان معدل عزل جراثيم الاشريشيا القولونية نوع 157 في انواع الطعام الثلاثة كان 19. عزل (15.8%) وبواقع : 9 (22.5%) في اللحم و 7 (17.5%) في الحليب الخام و 3 (7.5%) في لحم الدجاج، باستخدام تفاعل البلمرة المتسلسل لوحظ معدل (57.9%) 11 لجين stx2 في 7 عينات لحم و 4 عينات حليب خام ، ومعدل 8 (42.1%) لجين eaeA في 2 عينات لحم و 3 عينات في كل من الحليب الخام ولحم الدجاج ، أظهرت نتائج الدراسة وجود فرق معنوي ($P < 0.05$) في انتشار البكتريا في عينات الطعام الثلاثة . كان المعدل الحسابي لجراثيم الاشريشيا القولونية في اللحم والحليب الخام ولحم الدجاج علي التوالي 5.9×10^5 CFU/m , 1.6×10^6 CFU/g , 2.4×10^3 CFU/g , وقد سجل فرق معنوي ($P < 0.05$) في عدد مستعمرات جراثيم الاشريشيا القولونية بين انواع الاغذية الثلاثة .

كما تم حساب العدد الجرثومي للبكتريا الهوائية في عينات الطعام الثلاثة ، وكان المعدل الحسابي لبكتريا في اللحم والحليب الخام ولحم الدجاج , 2.1×10^6 CFU/g , 3.1×10^6 CFU/m , 3.4×10^6 CFU/g على التوالي . لم تظهر نتائج الدراسة وجود فرق معنوي ($P > 0.05$) في العد الجرثومي للبكتريا الهوائية بين انواع الطعام الثلاثة ، كما اثبتت الدراسة أن اللحم والحليب مصدر هام للأمراض المنقولة عن طريق الغذاء التي تهدد الصحة العامة في العراق.

Abstract

A survey to detect of *E. coli* O157:H7 recovered from food by multiplex PCR was carried out in Wasit province (Iraq) during the period from September 2015 up to February 2016. (120) samples (40 beef, 40 raw milk and 40 chicken meat) were collected from local markets. The incidence of *E.coli* O157:H7 were nineteen (15.8%) : nine (22.5%) beef , seven (17.5%)raw milk and three (7.5%)chicken meat. The present study was carried out to detect the presence of stx2, and eae genes in the recovered strains by multiplex Polymerase Chain Reaction ,The amplified fragments by PCR revealed that 11 out of 19 (57.9%) *E. coli* O157:H7 isolates from (7) beef and (4) chicken meat had stx2 , while 8 out of 19 (42.1%) *E. coli* O157:H7 isolates from (2)beef, (3) chicken meat , and (3) raw milk had eaeA gene. There was statistically significant difference ($P < 0.05$) in prevalence of *E.coli* O157:H7 between the three types of samples. The median counts of *E.coli* O157:H7 was 1.6×10^6 CFU/g in beef , 5.9×10^5 CFU/ml in raw milk and 2.4×10^3 CFU/g in chicken meat. There was statistically significant difference ($P < 0.05$) in *E.coli* O157:H7 counts between the three types of sample. The median counts of aerobic plate count (APC) in beef , raw milk and chicken meat are 3.4×10^6 CFU/g, 3.1×10^6 CFU/ml 2.1×10^6 CFU/g, respectively. The results of Statistical analysis showed no significant differences($P > 0.05$) in (APC)count between the three types of food. The results of this study showed that meat and milk are a significant source for foodborn disease that concerns the public health in waist province.

Introduction

Meat and milk contaminated concern the public health in both developing and the advanced countries particularly under the present concept of one world one health. In recent years some outbreaks of foodborne diseases in the United States caused by pathogenic bacteria such as *E. coli* O157:H7 and *Listeria monocytogenes*, have brought about meat and milk safety

issues to the forefront of societal concern(1). An estimated 10% of the population suffers from foodborne illnesses annually in Europe, in Iraq food borne illness in human beings due to bacterial. () pathogenesis well reported through annually report of Iraqi Ministry of health, highlighted the fact that the production, handling, sales, and

consumption of poor quality animal food products are serious public health problems in the country. The major food consumed in Iraq is beef, milk and chicken. Biological, chemical, and physical hazards are encountered in beef slaughtered and processed in the slaughterhouse. The biological hazards are mainly bacterial pathogens such as *E. coli* O157:H7, *Salmonella* and *Listeria spp.* (2). Although most strains of *E. coli* are harmless and live in the intestines of healthy humans and animals, but *E. coli* O157:H7 is an important pathogen concerning public health in the world and is now recognized as a food poisoning bacterium; this pathogen produces a powerful toxin and causes severe illness. Infection often causes severe bloody diarrhea and abdominal cramps; sometimes the infection causes non-bloody diarrhea and very severe diseases like haemorrhagic colitis (HC), haemolytic

uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). The

dangerous of *E. coli* O157 the consumers infection occurs in low doses (10-100 CFU) (3). This pathogen is especially associated with comminuted beef products such as burgers and other foods as diverse as beef jerky, bean sprouts, unpasteurised milk, apple ciders and salad vegetables such as lettuces. *E. coli* can get access to milk and products and used as marker organisms. Recovery and counting of *E. coli* is used as a reliable indicator of fecal contamination and indicates a possible presence of enteropathogenic and/or toxigenic microorganisms which constitute a public health problem. Prevalence in cattle and in sheep is generally higher than in other animals (4). This study was designed to isolate *E. coli* O157:H7 from food samples (beef, raw milk and chicken meat) and determine the prevalence of APC (Aerobic plate count) in all the (120) food samples.

Materials and Methods

Isolation *E. coli* O157 from food.

(120) samples (40 beef, 40 raw milk and 40 chicken meat) were transported in a cooler box at 4 °C. All samples were analysed immediately upon arrival at the laboratory. The samples were weighed into sterile stomacher bags Nasco Whirl-

Pak™) and homogenised for 2 min in 225 mL of Mac-Conkey broth (Difco 0020-01) (5). Each 1 mL suspension of the swabbed samples was appropriately diluted using 10-fold serial dilution; 0.1 mL of the suspension at 10⁶ dilution factor was

inoculated by spreading on MacConkey agar. Colonies growing on palatal media were identified with standard biochemical test. Biotypes were determined by API 20 kit (BioMérieux) (5). The *E. coli* O157 strain identified by *E. coli* O157 latex test reagent kit (PROLEX™), Place one drop of the Prolex™ *E. coli* O157 Latex Reagent

in a test circle on one of the test cards provided. Using a Pasteur pipette add one drop of the test suspension into the same test circle and mix using one of the mixing sticks provided. 5. Rock the card gently and examine for agglutination for up to two minutes (6).

Extraction of DNA

The DNA of the standard strains and of the other bacterial isolates yielded from bacteriological examination was extracted by DNA isolation kit (QLAamp®DNAMini Kit 50)*5 reaction

(QLAEN, Germany) according to (7) and manufacturer information with little modification. Meanwhile, the extractions of DNA from milk and meat samples were carried out (8).

PCR design and amplification condition

PCR design and amplification according to (9) All the extracted DNA of the standard strains and of the recovered *E. coli* O157:H7 isolates by bacteriological examination were examined using multiplex-PCR for molecular typing of the toxic and virulence genes (shiga toxin type 2 (stx2) and intimin gene (eaeA) using specific oligo nucleotide primers. The sequence of the primers and the size of the amplified fragments are listed in (Table 1). The reaction mixture consisted of 1 µl (200 µg) of the extracted DNA from the

bacterial isolates or from the standard strains, 5 µl of 10× PCR buffer (BIOTOOLS) (75 mM Tris base-HCl, pH 9.0, 2 mM MgCl₂, 50 mM KCl, 20 mM (NH₄)₂ SO₄, 1 µl dNTPs (40 µM) (BIOTOOLS), 1 µl (1 U AmpliTaq DNA polymerase) (Qiagen), 1 µl (50 pmol) of the forward and reverse primers and the final volume made up to 50 µl using deionized distilled water (DDW). 40 µl paraffin oil was added and after denaturation firstly occurs for 5 min at 96 °C, followed by 35 PCR cycles that consist of denaturation at 95 °C/for 3 min, annealing at 55 °C/45 s, extension at

72 °C/45 s (10^{-1}), and final extension at 72 °C/7 min. Agarose gel electrophoresis was carried out according to (11) to evaluate the amplified fragments using standard PCR markers and 100 bp ladder.

Aerobic Plate Count (APC)

The aerobic plate count (APC) was evaluated from several naturally contaminated meat and milk samples that were held at 4 °C for 24 hours from time the collection . the dilutions were made

from each sample (10^1 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6) . APC of the samples was measured by plating a 1-ml aliquot of each dilution onto Nutrient agar (3M™ Healthcare, St Paul, MN, USA). The agar was incubated at 37°C for 18-20 h, APC count evaluated using colony counter (12).

Statistical Analysis

the Statistical Analysis performed with statistical package for social sciences (SPSS) 19.0 and Microsoft Excel 2010.

Table (1) PCR primer used for multiplex PCR

Specificity	Oligonucleotides sequence (5'–3')	Amplicon size
Intimin gene (eaeA)	F-GTGGCGAATACTGGCGAGACT R-CCCCATTCTTTTTCACCGTCG	602-620
Shiga toxin type 2 (Stx2)	F-ATCAGTCGTCCTCACTGGT R-CTGCTGCTGTACAGTGACAAA	346-350

Results and Discussion

Prevalence of *E.coli* in Food Samples

The *E.coli*0157 ;H7 is important pathogen and is now recognized as a foodborne bacterium of concern in many countries (13). This pathogen is especially associated with comminuted beef products such as burgers in the USA and other foods as diverse as beef jerky beansprouts, unpasteurised milk,

apple ciders and salad vegetables such as lettuces. Prevalence in cattle and in sheep is generally higher than in other animals (14). Out of (120) food sample(40 beef , 40 raw milk and 40 chicken meat). The incidence of *E.coli* 0157:H7 were nineteen (15.8%) : nine (22.5%) beef , seven (17.5%)raw milk

and three (7.5%) chicken meat (Fig.1)(Table.2).

The present study was carried out to detect the presence of *stx2*, and *eae* genes in the recovered strains by multiplex Polymerase Chain Reaction. The amplified fragments by PCR revealed that 11 out of 19 (57.9%) *E. coli* serotype O157:H7 isolates from (7) beef and (4) chicken meat had *stx2*, while 8 out of 19 (42.1%) *E. coli* O157:H7 isolates from (2) beef, (3) chicken meat, and (3) raw milk had *eaeA* gene (Fig. 2) (Table. 1). The results of Statistical analysis showed significant differences ($P < 0.05$) between the three types of food. Our result confirms the conclusion of Onzian (15) who mentioned that Serotyping of *E. coli* O157:H7 isolates yielded from bacteriological examination of milk samples were 26 (4.81%) and from raw

meat samples 6 (4%) by multiplex-PCR. The findings of present study are agreement with (16) in Iraq that reported the prevalence of *E. coli* in local minced meat and imported minced meat and chicken meat were (80%, 65%, 56%) respectively, and with (3) who reported the prevalence of *E. coli* in raw milk was 12% and Buffalo meat was 22%. In beef carcass processing, *E. coli* associated with cattle carcasses can increase or decrease during processing depending on factors such as the levels of contamination of live cattle, efficiency of evisceration and hygienic practice in the Slaughter house, Slaughter plants have also been required to test carcasses for generic *E. coli* as an indicator of the adequacy of the plant's ability to control fecal contamination, The prevalence *E. coli* in raw milk is a strong indication of animal fecal contamination (17).

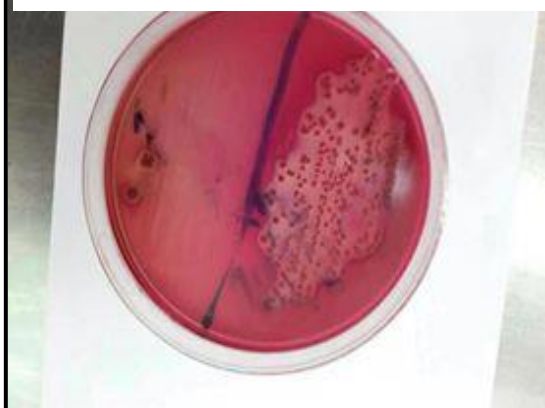


Fig. 1. *E. coli* 0175 on MacConkey agar

Table (2) Characterization of the recovered *E. coli* O157:H7 by multiplex PCR from food samples

Food types	Number of sample	E. coli O157:H7	Multiplex PCR of E. coli O157:H7	
			stx2gen	eaeA gen
Beef	40	9 (22.5%)	7	2
Raw milk	40	7 (17.5%)	4	3
Chicken meat	40	3 (7.5%)	0	3
Total	120	19 (15.8%)	11(57.9%)	8(42.1%)

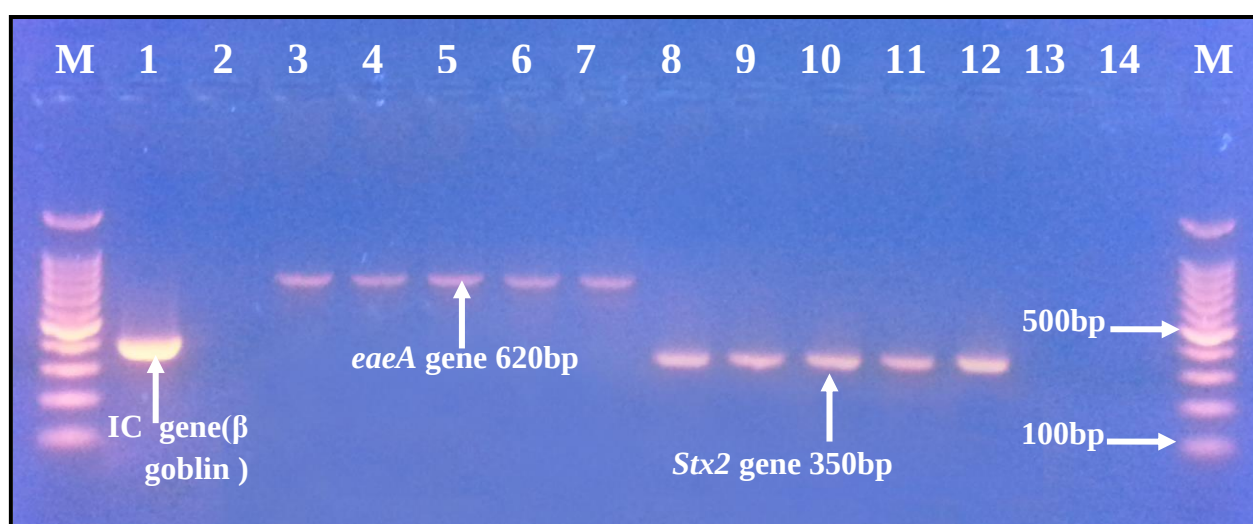


Fig. 2. PCR products, M (MW): One hundred base pairs DNA ladder; lane1: Internal control(β goblin gen 402bp); lane2: Negative control; lane(3-7): Positive samples for *E.coli* H7 (eaeA gene 620bp); lane (8-12): Positive samples for *E.coli* H7 (Stx2 gene 350bp); lane(13-14): Negative samples

Enumeration of *E.coli* O157 in Food Samples

The median counts for the pathogen load estimates of *E. coli* O157 from Beef, raw

milk and chicken meat are 1.6×10^6 CFU/g, 5.9×10^5 CFU/ml 2.4×10^3 CFU/g

respectively(Fig.3). The results of Statistical analysis showed significant differences($P<0.05$) in *E.coli* count between the three types of food. Total of (19) isolations *E. coli* 0175 counts in (5) food samples(3 Beef, 2 raw milk) were $<10^5$ CFU/g,ml. And count on(4) food samples (2 Beef, 1raw milk,1chiken meat) were $<10^4$ CFU/g,ml . Only (5)samples(1 beef, 3 raw milk ,1chiken meat) had *E. coli* counts of $<10^2$ CFU/g,ml and (5) sample(3 Beef , 1 raw milk,1 chicken meat) had >10 CFU/g,ml. This results agree with (18) who showed the counts of *E.coli* in minced meat were 3.3×10^2 CFU/g. (19)reported the counts of *E.coli* in beef were 3×10^2 CFU/g. the poor hygienic culture of labor in supermarket of meat effect on the level of meat contamination and Cattle's faeces and hides are considered to be sources of *E.coli* contamination of carcasses during slaughter and it can occur during removal of the hide or the gastrointestinal tract(20). The variability in contamination and cross-contamination may be originated in factors such as plant size design, age, equipment, automation, speed of slaughter, and animal holding facilities; geographic location; season of the year; type, lot and origin of

animals; labor shift ; and personnel training and turnover. As the hide is separated for removal, contamination may be introduced onto the carcass surface. A single source (one animal or the plant environment and equipment) may contaminate carcasses not only during dehiding but also during later steps, Some operations such as skinning and evisceration are more likely than others to result in carcass contamination, and some carcass areas are more prone than to exposure to potential contamination or cross-contamination. Contamination of meat others with *E.coli* during slaughter is the principal route by which these pathogens enter at the meat supply chain (21). The counts of *E.coli* in Chicken meat which found in this work is quite different from previous studies reporting mainly *E. coli* counts in chicken meat in the United Kingdom were 10 CFU/g (22) . In Turkey (23) reported an occurrence of 10 CFU/g of *E. coli* on chicken meat contamination of chicken occur during removal the digestive system because the *E.coli* present in intestine of Chicken.

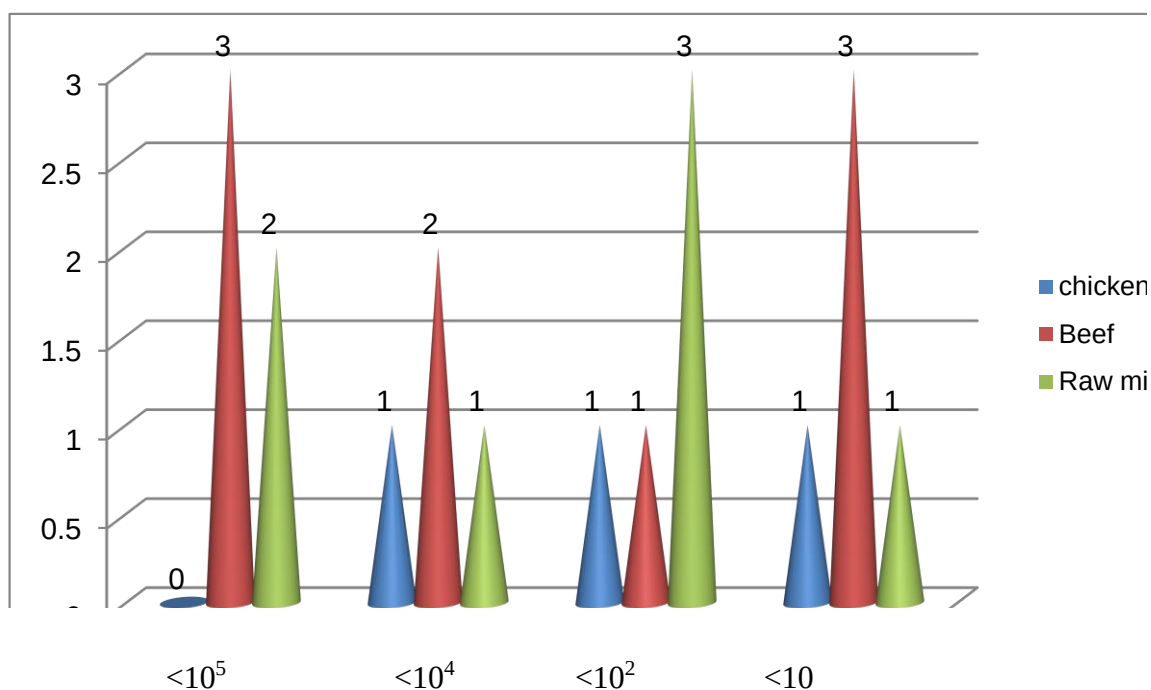


Fig.3. Enumeration of *E.coli*0157 in Food Samples

Evaluation of food background flora grow

The evaluation of food background flora growth was done through counting the aerobic plate count (APC) in meat samples that stored at 4 °C after 24 hours from collection. The median counts of APC in Beef, milk and chicken meat are 3.4×10^6 CFU/g, 3.1×10^6 CFU/ml, 2.1×10^6 CFU/g, respectively (Fig.4). The results of Statistical analysis showed no significant differences ($P > 0.05$) in (APC) count between the three types of food. the growth natural flora occurred during

marketing, the finding of (24) were similar to those of the present study. the high number of bacteria may be transmitted from fleece of animals to the carcass surface during hide remove (25). This study agree with (26) they report APC in raw milk in Kuwait were 9×10^5 CFU/g, High incidence of APC in raw milk is indicated poor hygienic standard being observed during milk production and handling.

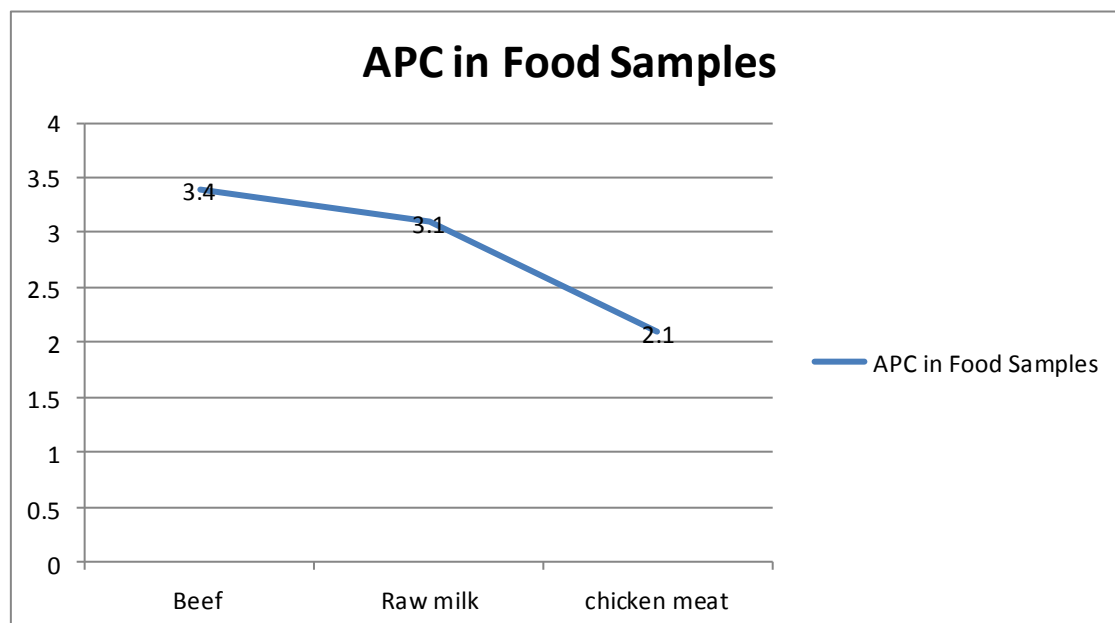


Fig.4. APC in Food Samples in Log₁₀⁶

Conclusions

1. The results of this study revealed the efficiency PCR reaction for detection of *E. coli* 0175:H7 isolates recovered from food of animal origin.
2. These results show an increase in the counts of *E. coli* 0157:H7 in the food, This

situation represents an increased risk for the consumers and a challenge for those working in the food sanitary control service.

3. The level of (APC) were high in the three types of food.

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