# Using of PCR for Identification and Toxins Typing of Enterotoxigenic Strains of *E. coli* Isolated from Dairy and Meat Products

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## Abstract

**This study was done to determine the enterotoxin of *E.coli* using polymerase chain reaction technique (PCR). One hundred and twenty samples (20 raw milk, 15 zabady, 15 ice cream , 10 kariesh cheese ,10 soft cheese, 15 sausage,15 luncheon and 20 faecal calves diarrhea) were collected from different localities in Kaluobia Governorate and examined for the occurrence of pathogenic and toxigenic *E.coli*. *E. coli* was isolated at a higher percentage from kariesh cheese (66.3%) followed by faecal samples and zabady (50%), raw milk (41.6%) sausage (40%), ice cream (36.3%), luncheon (25%) and at a lower percentage from soft cheese (20%). *E. coli* could be isolated onto Sorbitol MacConkey agar media (SMA) and Eosin methylene blue agar media (EMB) as method for recovery of the pathogenic *E.coli* which were isolated in percentages of 44.4% and 48.8% respectively. Serological typing of the isolated *E.coli* strains (12 isolates randomly selected) using specific (O) antisera, revealed the identification of different (11) serotypes (O111, O86A, O124, O146, O158, O8, 078, O18, O25, O114, O148). By using polymerase chain reaction technique (PCR) for detecting enterotoxins encoding genes for shiga like toxin (STx) and effacing attaching primer (eae), the results revealed that toxigenic *E.coli* encoding eae gene was detected in 6 out of 8 randomly chosed *E.coli* isolates, while shiga like toxin was found in 4 out of the same 8 randomly *E.coli* isolates.**

## Introduction

Enterotoxigenic bacteria are characterized by production of enterotoxins in food that may cause many diseases such as: food- born illness and food intoxication which results from bacterial growth in food as, milk, meat and their products (9). One of the major sources of contamination of the environment is the stools of infected humans and animal reservoirs as well as untreated water and food resources (1). Milk, meat, other food constituents are subjected to different sources of contamination by food poisoning pathogens either from endogenous origin or exogenous origin.

Milk, meat and their products have been associated with food borne illness which is caused by various pathogens such as *E. coli* including dangerous *E. coli O157, Staphylococcus aureus* and many other microorganisms (**26**). Enteropthogenic *E. coli* has been incriminated as a potential food poisoning agent and it was usually associated with infant diarrhea and gastroenteritis in adults (**34**). Infection by enterotoxins producing *E.coli* (ETEC) resulting diarrheic secretions due to the actions of one or more enterotoxins, and can lead to dehydration and death. These bacteria may produce thermo-labile (LT-1 and LT-2) and thermo-stable (ST a and ST b) enterotoxins (**10**). Shiga-toxin is considered as an important virulence factor produced by *E.coli* strains (STEC) and is similar to the shiga –toxin produced by *Shigella dysenteriae* type 1. *E.coli* strains producing ST x1 and or 2, cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans. Most cases of these diseases are caused by ingestion of food and drinks contaminated with animal faeces (**36**). Although *E. coli* O157:H7 serovar is the dominant STEC in many parts of the world, it is recognized that STEC strains were belonged to a very broad rang of O: H serovars (**38**). Since non *E. coli* O157 (STEC) is more prevalent in animals and as contaminants of foods, humans are probably more exposed to these strains, some of which have been associated with sever illness (**7**). Recent investigations indicated that *E. coli* O157:H7 is a cause of food borne illness and that young dairy cattle are reservoir for it. A particularly dangerous type of infection with was caused EHEC strain which associated with food borne outbreak traced to milk, dairy products and meat products lead to hemorrhagic colitis and hemorrhagic uremic syndrome in human (**1 and 42**). The majority of strains the *E. coli* produce STx2 and some produce STx1 and STx2 and a few produce STx1 only (**31**). So diseases caused by STEC had become a major public health problem, which may cause outbreaks (**44**). PCR is a genotypic technique which could be used extensively in diagnosis bacterial samples in dairy and meat products specially multiplex PCR with higher specificity and sensitivity which could reach a level of 99% accuracy **(6 and 20).**

## Material and Methods

**I – Materials:**

1: **Samples**: One hundred and twenty samples (20 raw milk ,15 zabady ,15 ice cram, 10 kariesh cheese,10 soft cheese,15 sausage,15 luncheon and 20 faecal calves diarrhea) were randomly collected from different localities in Kaluobia Governorate. The collected samples were transferred to the laboratory in an insulated ice box with minimum delay and immediately examined for the occurrence of *E. coli*

**2- Media:**

**2:1: Media for isolation and identification of *E.coli*:** MacConkey agar, Eosin Methylene Blue (EMB) and Sorbitol MacConkey agar (SMA) **(37)**

**2:2: Media for biochemical identification of *E .coli*: (37)** and **(5).**

**3: *E.coli* antisera (13)** were used for determination of *E. coli* and were kindly obtained from Animal Research Institute, Dokki, Egypt. (Polyvalent sera, pigs and monovalent, rabbits.

4: **Polymerase chain reaction (PCR):**

**4:1: primers**: Four primers for *E. coli* isolates were used (Genotec Company)

2 STX primers

STX2F-------GGCACTGTCTGAAACTGCTCC.

STX2R------- TCGCCAGTTATCTACATTCTG

Specificity at ------ 603- 857 of a subunit coding region of ST2 including ST2 variants

Amplicon size (bp) ------- 255

2 Eae primers

Eae F --------- GACCCGGCACAAGCATAAGC

Eae R -------- CCACCTGCAGCAACAAGAGG

Specificity ----27- 410 of eae this conserved between EPEC and STEC.

Amplicon size (bp) ---------384

**4:2:Deoxynucleosides Triphosphate Amersham Pharmacia Biotech, 27-203501)**

**4:3: Mastermix PCR (Bioron, 101605, 2007)**

**II: Methods:**

**1: Collection and preparation of samples**: All collected samples of each product were sent to the laboratory to be prepared according to the American Public Health Association (4).

**2: Microbial examination:**

**2:1: Isolation and identification of *E.coli*:**

**2:1:1: Onto MacConkey and EMB agar media:** The samples were cultured and incubated at 37 C for 24 hours according to **(19 and 28).**

**2:1:2: Onto Sorbitol MacConky agar media**: The samples were cultured and incubated at 37 C for 24 hours. The plates were examined for fermentation *E. coli* to sorbitol sugar in the media and up to 5 suspected colonies were picked up and cultured onto agar slants and incubated for 24 hours at 37C and these tubes were used for further studies.

**2:2: Methods of identification**: According to **(11-and 40).**

**3: Serological identification of isolated entero-pathogenic *E. coli* (EPEC) strains**: According to **(13)** using slide agglutination method for determination of the O-antigen

**4: Identification of *E. coli* isolates using multiplex PCR:**

**4:1: Genomic DNA extraction:** Distilled water (one ml) was added to *E. coli* growth on slope agar then shaken well. The bacterial suspension was centrifuged and the pellet was suspended in distilled water by using vortex. The genomic DNA was extracted by boiling of the suspension for one minute in water bath to ensure lysis of cells and complete DNA denaturation.

**4:2: Oligonuclutide primers**: Primers were dissolved in nuclease free water to obtain 50-1000 pmol concentration **(41)**. 5ul of the up stream and 5ul of down stream primers were used in the PCR mixture.

## Results

**Table (1): Results of samples grown onto MacConkey agar media.**

| Number of examined samples | No. of positive isolates | Percentages % |
|---|---|---|
| 120 | 85 | 70.1 |

The percentage were calculated according to number of total samples (120)

**Table (2): Distribution of positive isolates in different samples grown onto MacConkey agar media (lactose fermenting bacteria):**

| Types of samples | No. of samples | Results | | | |
|---|---|---|---|---|---|
| | | Positive | % | Negative | % |
| Raw Milk | 20 | 12 | 60 | 8 | 40 |
| Zabady | 15 | 10 | 66.6 | 5 | 33.3 |
| Ice cream | 15 | 11 | 73.3 | 4 | 26.6 |
| Kariesh cheese | 10 | 7 | 70 | 3 | 30 |
| Soft cheese | 10 | 5 | 50 | 5 | 50 |
| Sausage | 15 | 10 | 66.6 | 5 | 33.3 |
| Luncheon | 15 | 12 | 80 | 3 | 20 |
| Fecal samples | 20 | 18 | 90 | 2 | 10 |
| Total | 120 | 85 | 70.1 | 35 | 29.2 |

**Table (3): Results of growth and action of lactose fermented bacteria onto Eosin Methylene Blue (EMB) and Sorbitol MacConkey agar (SMA) and biochemical tests:**

| Types of samples | No. of samples | Onto EMB agar | | Onto SMA agar | | *Indol test | |
|---|---|---|---|---|---|---|---|
| | | No. of samples | % | No. of samples | % | No. of samples | % |
| Raw Milk | 12 | 5 | 41.6 | 4 | 33.3 | 5 | 41.6 |
| Zabady | 10 | 3 | 30 | 6 | 60 | 5 | 50 |
| Ice cream | 11 | 6 | 54.5 | 5 | 45.4 | 4 | 36.3 |
| Kariesh cheese | 7 | 4 | 57.1 | 5 | 71.4 | 4 | 57.1 |
| Soft cheese | 5 | 2 | 40 | 2 | 40 | 1 | 20 |
| Sausage | 10 | 5 | 50 | 4 | 40 | 4 | 40 |
| Luncheon | 12 | 4 | 33.3 | 3 | 25 | 3 | 25 |
| Fecal samples | 18 | 12 | 66.6 | 8 | 44.4 | 9 | 50 |
| Total | 85 | 41 | 48.2 | 37 | 43.5 | 35 | 41.1 |

*The results of indol test which is the most accurate test used for diagnosis in combination with other biochemical and culturing onto specific media as EMB and SMA

**Table (4): Results for testing the growth of *E. coli* of feacal origin (growth at 45C) using Eijkeman test:**

| Types of samples | NO. of samples | Results | | | |
|---|---|---|---|---|---|
| | | Positive | % | Negative | % |
| Faecal samples | 18 | 13 | 72.2 | 5 | 27.7 |
| Kariesh cheese | 7 | 4 | 57.1 | 3 | 42.9 |
| Soft cheese | 5 | 2 | 40 | 3 | 60 |
| Total | 30 | 19 | 63.3 | 11 | 36.6 |

**Table (5): Serotyping of the isolated *E.coli* using polyvalent and monovalent (O) antisera (randomly selected 12 isolates).**

| The reference number of isolated *E. coli* | Polyvalent (O) antisera | Monovalant (O) antisera |
|---|---|---|
| 42 | 3 | O18 |
| 55 | 1 | O86a |
| 60 | 1 | O86a |
| 6 | 1 | O111 |
| 14 | 7 | O124 |
| 57 | 3 | O158 |
| 41 | 3 | O114 |
| 37 | 5 | O25 |
| 8 | 4 | O148 |
| 3 | 6 | O8 |
| 15 | 4 | O78 |
| 18 | 2 | O146 |
| Total of *E .coli* | 40 | 11 |

**Table (6): Using multiplex PCR reaction using mastermix and 2 primers for identification of *E. coli* isolates.**

| No of randomly E.coli isolates | Results | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Effacing attaching primer | | | | Shiga like toxin | | | |
| | positive | | negative | | positive | | negative | |
| | No | % | No | % | No | % | No | % |
| 8 isolates | 6 | 75 | 2 | 25 | 4 | 50 | 4 | 50 |

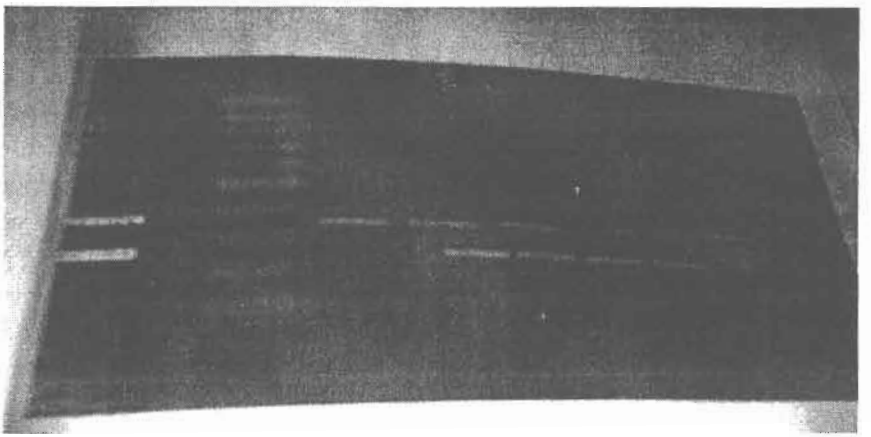

**Photo (1): Showing PCR results of STx and Eea, band of (6) isolates where positive with Eae coding gene primer at 348 bp, and bands of (4) isolates where positive with STx coding gene primer at 225bp. On agarose gel**

## Discussion

*E.coli* and other coliform microorganisms are used as indicators to serve as a measurement of feacal contamination and thus, of the presence of enteric pathogens in food. Although some of these pathogens are found in intestinal tract of man, most are found throughout the environment and have little sanitary significance **(21)**. The results illustrated in table (1) and (2) revealed the presence of lactose fermenting microorganism on MacConkey agar media (70%), feacal contamination is suggested to be the main source infection as shown by the percentage of isolation from calves with diarrhea, luncheon, ice cream, kariesh cheese, zabady, sausage, raw milk and soft were 90%, 80%, 73.3%, 66.6% 66.6%, 60% and 50% respectively. These findings simulate those reported by **(24 and 12).**

*E.coli* is the traditional indicator of possible faecal contamination and also reflects the sanitary measures adapted in the food preparation. The presence

of pathogens in food is supposed to indicate unsanitary conditions or poor practices during production, processing and / or storage **(1)**. Some strains of *E. coli* have been shown to be enteropathogenic, which means that they can produce a form of food infection resulting in severe diarrhea, but it would be almost impossible to eliminate all forms of coliforms because they are easily killed by heat **(27)**.

The Results in table (3) revealed to the percentage of *E. coli* isolated from different types of samples from dairy and meat products using specific cultures and biochemical tests, the percentage of isolation in kariesh cheese, faecal sample, zabady, raw milk, sausage, ice cream, luncheon, and soft cheese were 66.6%, 50%, 50%, 41.6%, 40%, 36.3%, 25%, and 20%, respectively. These results found confirmation in those reported earlier by **(23, 17, 28 and 1)**.

Results obtained in table (4) show the effect of high incubating temperature (45C) on the growth of *E. coli* of faecal origin and those isolated from contaminated food samples. Positive recovery was recorded from calf faeces, Kariesh cheese and soft cheese (72.2%, 66.6% and 40%, respectively).These findings goes hand in hand with those detected by **(29, 25 and 3)** who detected shiga toxin producing *E. coli* in examined samples of diarrheic calves which were the main reservoirs of shiga toxigenic *E. coli*. **Soomro et al. (43)** reported that faecal contamination of food during preparation was the common cause of enteropathogenic *E. coli* causing outbreaks.

Results of serological typing recorded in table (5) of twelve randomly selected *E. coli* isolates of different samples revealed the identification of 11 different monovalent serotypes. The predominant serotypes were O124, O86a , O8, O25 , O158, O148, O111, O18, O146, O78 and O114. These results agreed with the earlier findings **(18, 2 and 33)**. STEC from different sources and geographical areas belong to many different (O) serogroups. However, most of the documented outbreaks incriminated only few serotypes as O26, O111 and O157 which have been designated as enterohemorrhagic *E.coli* (EHEC) **(32)**. Recently, increased concern about

a new group of pathogenic *E. coli* implicated in hemorrhagic colitis and diarrhea in dairy cattle termed shigatoxigenic *E. coli* (STEC) **(22).**

Results of detection for toxins of *E. coli* isolated using PCR technique are shown in table (6), six isolates of *E. coli* out of 8 under testing was positive with effacing attaching primer (eae) and 4 isolates was positive with shiga toxin primer (STx). The results indicating that most of isolated *E. coli* was toxigenic, PCR is genotyping technique which is a highly sensitive, rapid and specific method used for identification and diagnosis of *E. coli* in food samples and other suspected samples **(6).**

Multiplex PCR was used to detect presence of genes encoding shiga toxin and eae in *E .coli* isolates, the shiga toxin producing isolated were tested for production of shiga toxin **(35).** Due to the increasing incidence of *E. coli* infection allover the world, the need for improved epidemiological surveillance and development of simple and rapid detecting method seemed to be urgent. PCR technique is rapid, highly specific and sensitive **(39).**

In conclusion, the results of the present study revealed that *E. coli* was the most common bacterial pathogen isolated from calves with diarrhea. The main *E. coli* serovars which were also associated with food poisoning cases in human beings and diarrhea in calves were O111, O86A, O124, O146, O158, O8, 078, O18, O25, O114, O148. PCR technique proved that enterotoxigenic *E. coli* more common than shiga toxigenic *E. coli* in examined samples.

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# استخدام اختبار البلمرة المتسلسل فى معرفة وتحديد عترات الايشريشيا كولاى المفرزة للانترو توكسين والمعزولة من منتجات الالبان و اللحوم

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## الملخص العربى

تعتبر الاغذية بجميع مشتملاتها وبخاصة الالبان واللحوم ومنتجاتها من المواد الغذائية الهامة بالنسبة للانسان نظرا لما تحتوية من عناصر غذائية اساسية . ولما كانت هذة المنتجات عرضة للتلوث من مصادر مختلفة اثناء صناعتها وتداولها مما يودى الى فساد المنتج فضلا مما قد تودى الى اضرار صحية الى المستخدم وتعتبر الايشريشيا كولاى من الميكروبات المهمة فى حدوث التسمم الغذائى على مستوى العالم ولذلك اجريت هذة الدراسة للتعرف على ميكروب الايشريشيا كولاى المفرزة للانتروتوكسين المسبب فى التسمم الغذائى وقد اجريت هذة الدراسة على مائة وعشرون عينة من منتجات الالبان واللحوم (٢٠ عينة من اللبن الخام و١٥عينة من الزبادى و١٥ عينة من الايس كريم و١٠ عينة من الجبن القريش ١٠ عينة من الجبن الابيض و ١٥عينة من السجق و ١٥عينة من اللانشون و٢٠ عينة من عجول مصابة بالاسهال). وقد تم عزل ميكروب الايشريشيا كولاى من اللبن الخام ومن الزبادى ومن الايس كريم ومن الجبن القريش و من الجبن الابيض و من السجق و من اللانشون ومن عجول مصابة بالاسهال بنسبة ( ٤١,٦%, ٥٠%, ٣٦,٦%, ٦٦,٦%, ٢٠%, ٤٠%, ٢٥%, ٥٠%) على التوالى. وتم عزل ميكروب الايشريشيا كولاى من براز العجول المصابة بالاسهال وانواع الجبن الملوثة بفضلات الحيوانات المريضة مثل الجبن القريشى والجبن الابيض وكانت النسبة (٧٢,٢% ,٦٦,٦% , ٤٠%) على التوالى. وتم عمل الفحص السيرولوجى لعدد ١٢ عينة أخذت عشوائيا بعد ان ثبت تطابقها لميكروب الايشريشيا كولاى بالطرق المرفولوجيوة والبيوكيمائية ووجد ان العترات التى تم فحصها تنتمى الى ١١ عترة مختلفة هى

( O146, O78, O8, O148, O25, O114, O158, O124, O111, O86a, O18)

وتم عمل اختبار البلمرة المتسلسل على ٨ عينات عشوانيا من الذين تم تصنيفها سيرولوجيا لتحديد وجود الجينات المسئولة عن السم المماثل لذيفان بكتيريا الشيجلا ٢ والجين المسئول عن الالتصاق وتبين وجود ٦ عينات ايجابية لجين الالتصاق ووجود ٤ عينات ايجابية للجين المسئول عن السم المماثل لذيفان بكتيريا الشيجلا ٢. ولذلك يعتبر اختبار البلمرة المتسلسل من الاختبارات الحديثة, السريعة والدقيقة فى معرفة وتحديد العترات المفرزة للانتروتوكسين من ميكروب الايشريشيا كولاى.