INTERNATIONAL JOURNAL OF ADVANCED BIOLOGICAL RESEARCH

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# COMPARATIVE STUDY BETWEEN CONVENTIONAL AND MOLECULAR ASSAYS FOR DETECTION SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN CHILDREN WITH BLOODY DIARRHEA IN KERBALA CITY

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**ABSTRACT**
Bloody diarrhea is a serious public-health problem in children especially in developing countries; it appears in approximately twenty to thirty percent of all diarrhea cases and has a higher level of mortality and morbidity. At every age and stage of life the intestinal bacterial infections are a significant cause. The epidemiology and handling of this condition are varies in the developing world, where infectious agents became predominate. This study was proceed during the period from October 2017 to December 2017 to isolate Shiga toxin-producing *Escherichia coli* (STEC) and characterize them by biochemical reactions and confirmatory diagnosis of *Escherichia coli* (*Stx*$_1$), (*Stx*$_2$) genes using conventional monoplex and multiplex polymerase chain reaction (PCR) technique. A total of 80 bloody diarrhea stool samples were achieved from hospitalized children (40 females and 40 males) under 6 years old whose suffered from acute gastroenteritis and hemorrhagic colitis with bloody diarrhea in Kerbala Pediatric Teaching Hospital in Karbala City. The overall prevalence of Shiga toxin- producing *Escherichia coli* (STEC) in stool samples were 16 /80 (20%) and 31 /80 (38.75%) by PCR and conventional bacterial cultivation techniques, respectively, distributed in a total of 11 /80 (13.75%) females and 5 /80 (6.25%) males by PCR, and 22 /80 (27.5%) females and 9 /80 (11.25%) males by cultivation techniques, with a significant variation (P≤0.05) between the two bacterial detection as says. The present study was accelerated due to endemic clinical bloody diarrhea in children for reducing the required period for laboratory diagnosis and to increase the accuracy for the detection of the specific bacterial causative agents of bloody diarrhea in children.

**KEYWORDS:** bacterial infections, *Escherichia coli*, PCR, epidemiology.

## INTRODUCTION

Acute bloody diarrhea is a medical emergency for patent of all ages, because it often signifies troubles that are life threatening and urgently, required epidemic control implications in the community. Enteric bacteria and parasites were more frequently detected in pediatric cases in developed countries than viruses and typically peak during the hottest of the temperate months. The distribution of Diarrheagenic *Escherichia coli* varies in different countries[1]. The bacterial pathogens associated with Bloody Diarrhea include mainly species of *Shigella*, *Escherichia coli* pathotypes, especially Shiga toxin-producing *Escherichia coli* "STEC" as well as enteroinvasive *E. coli* "EIEC", *Salmonella enterica*, *Campylobacter* and *Yersinia enterocolitica* [2].

*Escherichia coli*, *"E. coli*" is a Gram negative bacterium that is commonly found in the lower intestine of humans and other warm-blooded organisms. There are many strains, and sub-types of the organism. One strain is appointed as STEC O157:H7, depend on two antigens that are existent on the surface of the bacterium and flagella locomotive appendages. In contrast with other strains, the strain of *E. coli* O157:H7 is not a normal inhabitant of the human's intestinal tract. When they present in the gastrointestinal tract, by the drinking or ingestion of contaminated water and food, *E. coli* O157:H7 causes severe, even life-threatening sequelae with massive bleeding known as hemorrhagic colitis. *E. coli* O157:H7 is a serotype of enterohemorrhagic *E. coli* that was frequent isolated in Argentina in 1977[3]. The impairment of the infection results from two vigorous toxins produced by the bacterium. The toxin is known as Shiga toxin or Verotoxin. These toxins are very analogous in action and structure those produced by another bacteria cells of intestinal health concern, *Shigella dysenteriae*, which causes of dysentery in the intestinal humans. The toxins exert their effect by both partition from preventing repair of the damage and physically by damaging the host epithelial cell, because of the block of the manufacture new protein in the host cell's. The strain is later distributed in meat when, it is ground. Thorough cooking, it is necessary to kill the bacterium which buried on the ground meat. Although it is not yet clear, indications are that the uptake of as few as 10 bacterial surviving can be suitable to trigger the diseases. *E. coli* O157:H7 is also transmitted to the humans by water that have been contaminated with fecal substances[4].

The three most important phenotypic differences between *entrotoxigenic E. coli* and other *E. coli* are initially testing for sorbitol carbohydrate fermentation have been suggested as a normal means to screen for *E. coli* O157 H7, absent of ®–glucuronidase enzyme and no growth or weak at temperature more than 40°C. [5,6]. The best suitable medium for presumptive diagnosis of

*entrotoxigenic E. coli* was found to be Sorbitol Mac-Conkey Agar "SMAC", in which the colonies remain colorless (sorbitol non fermented) while other *E. coli* isolates appeared red[7]. The aim of the present study was isolation of *Shiga toxin-producing Escherichia coli* by cultivation of stool samples on SMAC medium and molecular detection of *E. coli* O157:H7 ($Stx_1$), ($Stx_2$) genes by PCR technique.

## MATERIALS & METHODS

### Collection of samples

Eighty stool samples were collected from children under 6 years old have been suffered from bloody diarrhea in Karbala Pediatric Teaching Hospital in Karbala city through the endemic acute gastroenteritis and colitis with bloody diarrhea during the period from the 1st of October to the 31th of December 2017. A small amount (full swab) of each stool sample was applied in two test tubes, each one was contained 5 ml of TSB-CV transport medium, stool samples were divided into two partitions, then, after 24 hours, stool samples were transported to the microbiology laboratory, one partition for conventional bacterial cultivation, biochemical identification and the other partition for traditional molecular detection by PCR technique.

### Isolation of STE

Bacterial cultivation of stool samples was performed on Sorbitol MacConkey agar (SMAC) medium which consist of one percent of sorbitol as an alternative of lactose in MacConkey agar, this medium was accomplish with Cefixime ($0.05mg/1^{-1}$), potassium tellurite ($2.5mg/1-^{1}$) and rhamnose ($5g/1^{-1}$) to become selective (SMAC-CTR) for STEC detection[8]. This medium was used to identify sorbitol fermenter *E. coli* O157HO from non-fermenter *E. coli* colonies, after overnight incubation at 37°C. sorbitol negative colonies (colorless grow colonies). For identification of STEC, colorless grown colonies were transferred to MacCokey agar plates and incubated for additional overnight to identify lactose fermentation, then, applied for conventional biochemical reactions included Indole, Methyl red Voges- proskaur, Simmon's citrate and Triple sugar iron (TSI) tests[9].

### Molecular Detection of STEC ($Stx_1$), ($Stx_2$) genes by PCR technique

### DNA Extraction

DNA extraction and purification from the grown bacteria colonies and stool samples was performed according to manufacturer instructions of (Promega Company/ USA).

### Detection of STEC ($Stx_1$), ($Stx_2$) genes by PCR technique

Detection of *E. coli* virulence genes ($Stx_1$), ($Stx_2$) was done using monoplex and multiplex PCR techniques, a new improved website to NCBI (https://www.ncbi.nlm.nih.gov) and primer design program Primer3 plus (www.bioin formatics. nl/cgi-bin/primer3plus) were used to design the primer for *Stx1 and Stx2 genes* for Shiga toxin-producing *E. coli* as demonstrated in table 1.

**TABLE 1.** DNA primers for detection *"Stx1 and Stx2"* genes of Shiga toxin-producing *E. coli*

| Primers | | Sequence of Primers Nucleotides | No. of Base pair | Tm C° | G-C contents |
|---|---|---|---|---|---|
| Sense primer | Stx1 | 5′- CAGGACGCTACTCAACCTTC –3 | 20 bp | 56 C° | 55.0% |
| Antisense primer | 455 bp | 5′- CAGAGGAAGGGCGGTTTA –3′ | 18 bp | 55 C° | 56.0% |
| Sense primer | Stx2 | 5′- CCACTCTGACACCATCCTC –3 | 19 bp | 55 C° | 55.0% |
| Antisense primer | 353 bp | 5′- GACAACGGTTTCCATGACAA –3′ | 20 bp | 55 C° | 45.0% |

**TABLE 2.** PCR thermal cycler program for detection of *E. coli Shiga toxin* genes

| Stages | Temperature C° | Time / min. | No. of cycles |
|---|---|---|---|
| Initial Denaturation | 95 | 4.5 | 1 |
| Denaturation | 95 | 1 | |
| Annealing | 55 for Stx1 and Stx2 | 1 | 35 |
| Extension | 72 | 1 | |
| Final Extension | 72 | 5 | 1 |

The PCR amplification mixture for detection of Shiga toxin genes contained 25µl of GoTaq® Green Master Mix (which contains Taq DNA polymerase, $MgCl_2$, dNTPs and reaction buffer for efficient amplification of DNA templates by PCR), 5µl of DNA, 1.5µl and 1.0µl of each forward and reverse primers for monoplex and multiplex PCR respectively, then, master mix tubes were completed to 25 µl of nuclease-free water and the PCR tubes were transferred to preheated thermal cycler system and applied the following program:-

### Agarose gel electrophoresis:

Electrophoresis for amplified DNA products was performed by directly applying of 5 µl of each DNA product sample and loaded in 1% agarose gel containing final concentration of 0.2-0.5 µg/ml Ethidium bromide and applied the electrophoresis process, then, DNA products were visualized by gel documentation system according to [10].

### Statistical analysis

All produced results were analyzed by Chi square statistic at the level of significant when p- value < 0.05. the statistical analysis was performed using SPSS program[11].

## RESULTS & DISCUSSION

The conventional assays for identification of Shiga toxin-producing *Escherichia coli* involve the growth of *E. coli*

colonies on Sorbitol MacConkey agar culture "SMAC" media, following by screened and reviewing by serological test by using H7 and O157 specific antisera[12]. Bacterial culture alone may be unconcerned and insensitive, particularly for identification of small cells number of Shiga toxin-producing *E. coli* and it is unable to identify non Shiga toxin-producing *E. coli* [13]. Modern studies have been appeared that enzyme immunoassay (EIA) may be more sensitive and precise than classical culture assays because the apparatus have able to visualize the private antigen serotypes from stool specimens[12,14]. The present study have authenticated the use of PCR for specific detection of Shiga toxin-producing *E. coli* strains that are not instantly detected by classical culture and have documented by its ability to provide time consumption results (figure 1).The grown colonies were transferred directly from the primary enrichment media into selective medium SMAC and analyzed by PCR technique, the results demonstrated that molecular technique have been reduced the required time to achieve an expected result in few hours (5-6 hours) instead of (5-6 days) consumed for bacterial phenotypic detection by the traditional cultivation on selective media and determination by biochemical activity techniques (15).

Multiplex and monoplex PCR were speculate positive for *E. coli* when Shiga toxin (*Stx1 and Stx2*) genes was amplified according to molecular weight as 455 and 353 base pairs respectively and the results compared with DNA marker ladder of 100 base pair as demonstrated in (Figure 2). The identification of Shiga toxin genes might signalize just in the *E. coli* serotypes, which is another technique not be identified by traditional culture (16, 17).

The overall prevalence of STEC in a total of 80 bloody diarrhea stool samples of children under 6 years old (40 males and 40 females) hospitalized in Karbala Pediatric Teaching Hospital were 16 (20 %) and 31(38.75 %) by PCR and bacterial cultivation techniques, respectively, distributed in 11 (13.75 %) females and 5 (6.25 %) males by PCR technique and 22(27.5%) females and 9(11.25%) males by bacterial cultivation.

The present study was accelerated in Karbala Pediatric Teaching Hospital due to an endemic and elevation of the clinical acute gastroenteritis and hemorrhagic colitis with bloody diarrhea in children under 6 years old in kerbala city, these results become a clear benefit when demonstrate that a significant differences ($P \leq 0.05$) between two bacterial detection assays as demonstrated in table 3.

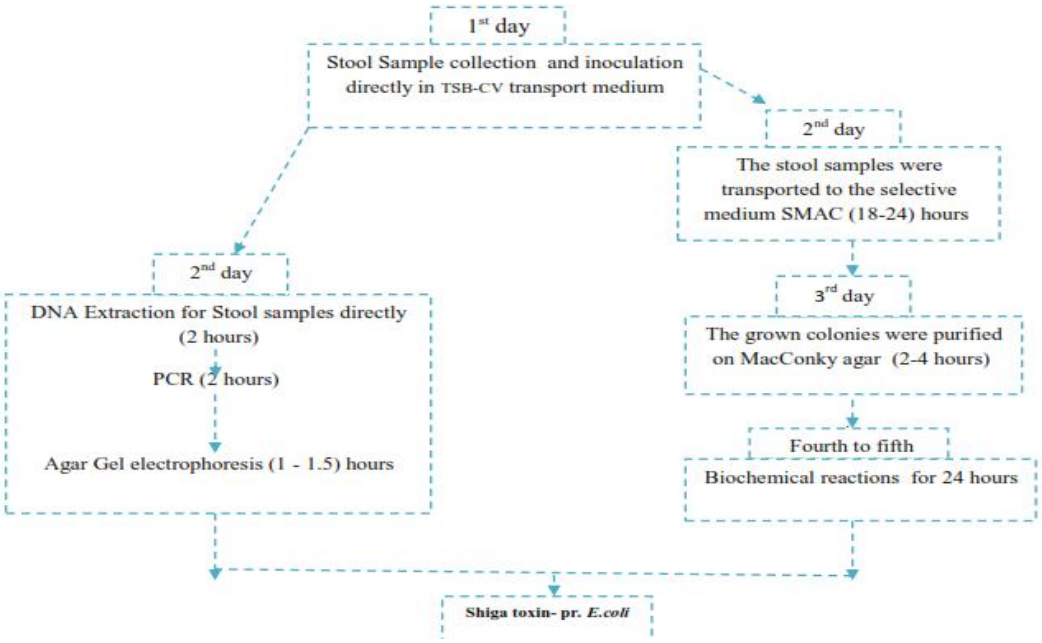


**FIGURE 1:** demonstrated the difference in required time for bacterial detection and identification of *E. coli O157 H7* strains between conventional and molecular assays

**TABLE 3: p**resence of *E. coli* isolates in bloody diarrhea stool samples of children by both PCR and Cultivation techniques

| NO. | PCR | | | | Cultivation | | | |
|---|---|---|---|---|---|---|---|---|
| | + ve | % | - ve | % | + ve | % | -ve | % |
| 80 | 16 | 20% | 64 | 80% | 31 | 38.75 % | 49 | 61.25 % |
| Statistical analysis | $X^2 = 3.72$, $P < 0.05$ significant differences | | | | | | | |

Furthermore, the results of the present study was clearly showed that the conventional culture assays represent a challenge for the detection of Shiga toxin-producing *E.coli*, especially in stool with high levels of residents flora as appeared in similar study (16) demonstrated that conventional bacterial cultivation of stool samples of children with bloody diarrhea on a relatively selective medium was utilized to determine *E.coli* O157 H7, but the time that needed to reach the phenotypic characterization of bacteria takes about 3-4 days.

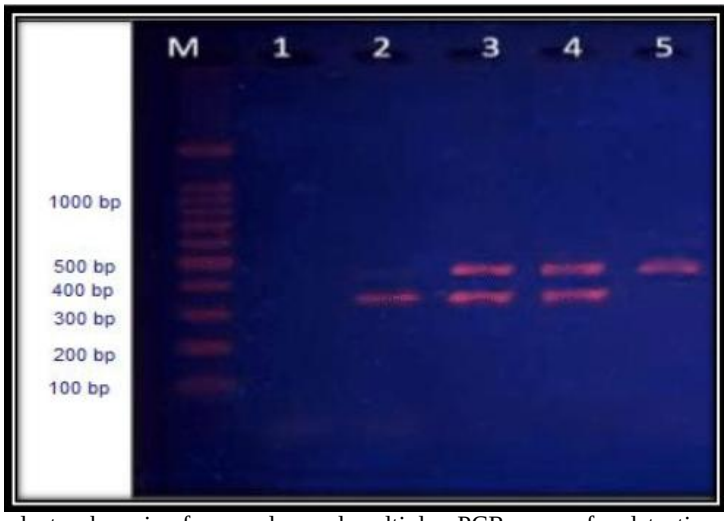


**FIGURE 2:** Agarose gel electrophoresis of monoplex and multiplex PCR assays for detection of Shiga toxin-producing *E.coli* : M lane= DNA ladder marker100 bp.; lane 1= negative control, lane 2=( *Stx2*) 353 bp. (monoplex PCR); lans 3, 4=( *Stx1* and *Stx2*) 353 bp. and 455 bp. (multiplex PCR); and lane 5=( *Stx2*) 455 bp. (monoplex PCR).

**TABLE 4:** presence of *E. coli* isolates in bloody diarrhea stool samples of children by PCR and Cultivation techniques according to gender

| Gender | PCR | | | | Culture | | | |
|---|---|---|---|---|---|---|---|---|
| | + ve | % | - ve | % | + ve | % | -ve | % |
| Male | 5 | 6.25% | 75 | 93.75% | 9 | 11.25 % | 71 | 88.75 % |
| Female | 11 | 13.75% | 69 | 86.25% | 22 | 27.5% | 58 | 72.55 |
| Statistical analysis | $X^2$= 2.5, P > 0.05 non significant differences | | | | $X^2$= 6.76, P < 0.05 highly significant differences | | | |

Table.4 demonstrated that Shiga toxin-producing *E. coli*, in females were higher percentage than in males of the studied children with bloody diarrhea with significant differences (P<0.05), these results were different from another previous studies (18) reported that the Shiga toxin-producing *E.coli* was in males encountered 55.81% more than in females 44.19%, and (19) who was documented that from all 72 cases positive samples for "*Stx1 or Stx2*" genes, there were18 samples positive in males more than 9 in females but There were no significant variations in gender frequency of STEC positive isolates in that study, although some studies found that Shiga toxin-producing *E.coli* was higher in younger and female patients (20).

Conclusion and Recommendations Acute bloody diarrhea is a challenging diagnostic and therapeutic problem in children, and It is essential to recognize patients with infectious causes simultaneously, infectious hemorrhagic colitis due to enteric Shiga toxin-producing *E.coli* is so rapid in children and have the priorities in bacterial evaluations thorough microbiology. Monoplex and multiplex PCR technique for rapid and correct diagnosis of bloody diarrhea causative agent in stool samples of children minimizes risk and delay.

For patients at different ages the acute bloody diarrhea by serotype of Shigatoxogenic *E.coli* is a medical emergency that delegate serious medical inspection. Affected peoples appropriately must be cured with antimicrobial agents and so that infection surveillance must be put in place.

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