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Original article

Diagnosis of Shiga toxin producing *Escherichia coli* infection, contribution of genetic amplification technique

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Abstract

There has been no culture method of choice for detecting non-O157 Shiga toxin-producing *Escherichia coli* strains (STEC) because of their biochemical diversity The aim of this study was the assessment of verotoxin gene detection (*VT1*/*VT2*) within STEC PCR compared with the Vero cells cytotoxicity among O157 and non-O157 STEC serotypes.

Stool cultures were performed on Tryptic Soy Broth and sorbitol MacConkey agar with cefixitime and tellurite supplements which were identified as *Escherichia coli* (*E. coli*) by BBL crystal. Further identifications were performed including verotoxin production assessment by Vero cells cytotoxicity assay, PCR for specific *VT1*/*VT2* genotyping, and isolates were plated on blood agar and tested for enterohemolysis.

Vero cells cytotoxicity assay revealed that 58 of *E. coli* isolates (71.6%) were STEC. In PCR, 33 (56.9%) of the 58 strains were positive for the *VT*2 gene, 24 (41.4%) were positive for the *VT*1 gene and one isolate was positive for both genes. In comparison to Vero cells cytotoxicity, the sensitivity, specificity of PCR were 100%. In comparative study between verotoxin assessment by Vero cells cytotoxicity and enterohemolytic activity, concordance positive results between both were 53 (91.4%). The most common serogroups of STEC were O157 (33%) and O26 (20%).

From this study we can conclude that enterohemolysin production can be used as surrogate marker for STEC. The most rapid and promising approach for detection of STEC is by molecular method.

Keywords: *Escherichia coli*; Serotypes; Genotypes

1. Introduction

Escherichia coli (*E. coli*) is an important member of the normal intestinal microflora of humans and other mammals. However, *E. coli* is more than just harmless intestinal inhabitant; it can also be a highly pathogenic. Several different *E. coli* strains cause diverse intestinal and extraintestinal diseases by means of virulence factors that affect a wide range of cellular processes [1].

Shiga toxin producing *E. coli* (STEC) is an important emerging food borne pathogen. It has been associated with bloody and non-bloody diarrhea, hemorrhagic colitis, hemolytic uremia syndrome (HUS) and thrombotic thrombocytopenic purpura. The cattle's have been shown to be the major reservoir of STEC are foods such as ground beef and milk [2].

The term verocytoxin – producing *E. coli* was derived form the observation that these strain produce a toxin with a profound and irreversible cytopathic effect on Vero cells (African green monkey kidney) [3]. An a alternative nomenclature is Shiga toxin-producing *E. coli* STEC which reflects the fact that one of the cytotoxins produced by these organisms is essentially identical at the genetic and protein levels to Shiga toxin (stx) produced by *Shigella dysenteriae* 1 [4].

Isolation and recognition of the prominent Shiga toxin- producing strains of *Escherichia coli* serovar O157:H7 can be confirmed easily by their late fermentation of sorbitol and lack of beta glucouronidase activity. It is claimed that

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Maconkey medium containing sorbitol, tellurite and cefixime permit the growth of 94% of STEC and *Shigella soniei* but partially or completely inhibit the growth of 67% of other strains of *E. coli* [5,6].

Identification of STEC can be performed by identifying the genes coding for verotoxins or by serology with increased serum titer of specific antilipopolysaccharide antibodies [7,8].

The aim of this study was the assessment of verotoxin gene detection (*VT1*/*VT2*) within STEC by PCR compared with the vero cells cytotoxicity among O157 and non-O157 *E. coli* serotypes. In addition, phenotypic characteristic of STEC strains were studied such as production of enterohaemolysin and serotyping.

2. Patients and methods

This study included 81 *E. coli* strains isolated from 131 patients complaining of outbreak of diarrhea attending out patient clinic in Mansoura University Hospital from November 2005 to March 2006.

Stools samples were obtained from patients and transported to the laboratory in clean containers. In the laboratory, a small sample of stool was injected into 5 ml of Tryptic Soy Broth and incubated overnight at 37 °C [9]. Subculture from Tryptic Soya broth was performed on Sorbitol MacConkey Agar (SMAC) with cefixime and tellurite (CT) supplements to identify sorbitol fermenter and non-fermenter colonies. The grown strains were tested for Vero cells cytotoxicity toxicity and for *VT*-specific DNA sequences by polymerase-chain reaction (PCR) [10,11]. Subculture was performed on tryptose agar plates supplemented with sheep blood to detect enterhemolysis production (Fig. 1).

2.1. Vero cell cytotoxicity assay

Colony sweeps of the isolates were grown overnight at 37 °C in Penassay broth (antibiotic medium no. 3; Difco Laboratories, Detroit, Mich.) containing mitomycin (0.2 mg/liter).

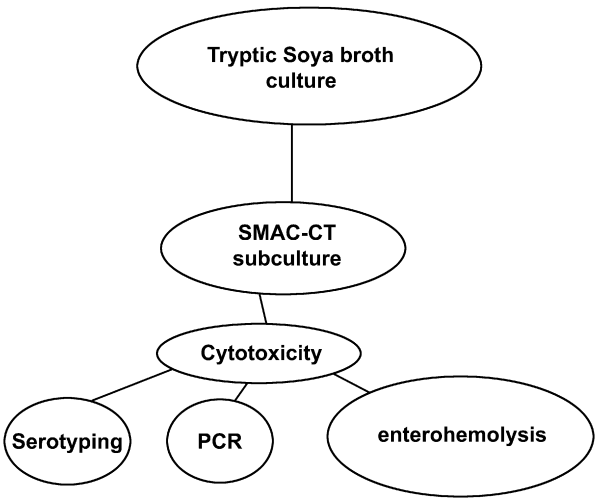

Fig. 1. Schematic representation for steps of stool culture.

Supernatants obtained by centrifuging the fluid cultures at 10,000 × *g* for 10 min were filtered through 0.2-μm-pore-size membrane filters. Volumes (50 μl) of serial twofold dilutions of the filtrates were applied to confluent Vero cell monolayers (in 96-wells trays)and were evaluated for cytopathic effect after 48 to 72 h of incubation (Rounding of 50% or more of the cells) [12].

2.2. PCR amplification methods

For *VT*1 forward primer was (5′ ACCCTGTAACGAAGTT TGAC 3′) and the reverse primer was 5′ ATCTCATGCGAC TCTTGAC 3′).

For amplification, 1.5 μl of boiled bacterial supernatant was used as template and 0.5 μl of *AmpliTaq* Gold (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NJ) was used as the polymerase. The steps from denaturation to elongation were repeated 35 times.

For VT2 forward primer was 5′ TTAACCACACCCACGG CAGT 3′ and the reverse primer was 5′ GCTCTGGATGCAT CTCTGGT 3′.

The PCR amplification mixture for the detection of *VT*2 contained 26.2 μl of sterile water, 3.5 μl of 10× PCR buffer solution (Finnzymes, Espoo, Finland), 0.98 μl of 4 dNTP mix (containing 5 mM dATP, 5 mM dCTP, 5 mM dTTP, and 5 mM dGTP), 1.0 μl each of forward and reverse primers (10.0 μM), and 0.7 μl of DNA polymerase (Dyn5′ azyme II; Finnzymes). Predenaturation was carried out for 4.5 min at 95 °C, and annealing was carried out for 1 min at 62 °C [13]. Five microlitre of the PCR reaction product run on a 3% high resolution agarose gel and stained with ethidium bromide. The *VT1* and/or *VT2* product of gave bands at 135 and 346 bp, respectively. Using PCR markers greatly aids in the interpretation of the results [9,10].

2.3. Detection of Enterohemolysis activity (EHEC)

Enterohemolytic activity of the strains was detected on tryptose agar plates (Difco Laboratories, Detroit, Mich.) supplemented with 10 mM $CaCl_2$ and 5% defibrinated sheep blood washed three times in phosphate-buffered saline (pH 7.2), as described previously [14]. The inoculated plates were observed for hemolysis after 3 h of incubation (for detection of alpha-hemolysis) and after overnight incubation in ambient air at 37 °C (for detection of enterohemolysis or non-hemolysis).

2.4. Serotype identification

The *E. coli* strains were serotyped for their O (lipopolysaccharide) previously described [15]. Colony of bacterial growth from the SMAC-CT agar plate was then tested in latex reagents for the O antigen. The latex agglutination assays were performed according to their manufacturers' instructions (Oxoid). A positive result with the O latex reagents was interpreted as large clumps of agglutinated latex and bacteria with partial or complete clearing of the background latex within 1 to 2 min.