

Genotypic Detection of *Enterohaemorrhagic E. coli* (EHEC) among Diarrheagenic Patients in Egypt

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Abstract: *Enterohaemorrhagic E. coli* is recognized as important food borne pathogen, responsible for sporadic cases of serious outbreaks worldwide. The morbidity and mortality associated with several recent outbreaks due to EHEC have highlighted the threat this organism poses to public health. This study was conducted to identify and characterize the virulence traits. A total of 110 samples from human were collected from 'Abou El-Reesh' Children's Hospital, Cairo, Egypt. *E. coli* isolates 91% (n = 100) were identified by conventional microbiology culture and were phenotypically characterized using biochemical and motility tests. Multiplex PCR reactions were applied for the detection of virulence genes (*stx1* and *stx2*) while uniplex PCR was applied for the detection of *eaeA* virulence gene. Among all isolated *E. coli* only one was sorbitol-nonfermenting EHEC, which also harbored *stx1*, *stx2* and *eaeA* virulence genes.

Key words: *Enterohaemorrhagic E. coli* • Shiga Toxins • PCR • Egypt

INTRODUCTION

Enterohaemorrhagic Escherichia coli is one of the five groups of *E. coli* which are recognized as aetiological agents of diarrhoea, [1]. It has been implicated in food borne, waterborne and airborne outbreaks all over the world [2]. For instance, the EHEC outbreak started in Germany in May 2011 with 3,368 cases including 36 deaths (European Center for Disease Prevention and Control; <http://www.edcde.europa.eu/en/Pages/home.aspx>).

Shiga toxins are the major virulence factors of EHEC which have cytotoxicity effects for human and animal eukaryotic cells and cause dangerous details like HUS [3]. Two major classes of Shiga toxins have been found in EHEC, Stx1 and Stx2 [4]. Both types of toxins have similar structures and mode of action. Shiga toxins possess an AB₅ structure (the holotoxin is approximately 70 kDa), composed of a pentamer of subunit B (7.7 kDa monomers) linked to a single 32 kDa A subunit [5]. In addition, the B-pentamer plays a crucial role in the binding to the

target cell, while the A subunit is responsible for the enzymatic activity of the toxin, inhibition of protein synthesis [6].

The present study was aimed at investigating the prevalence of shiga-toxin producing *E. coli* strains from clinical cases in one of the Governorates' 'Cairo' in Egypt. The virulence characteristics of isolates were also studied.

MATERIALS AND METHODS

Screening, Isolation and Identification of EHEC: Stool samples were collected from 110 patients with acute diarrhoea at early stages enteric illness before antibiotics therapy on admission to 'Abou El-Reesh' Children's Hospital, Egypt. A loopful of the stool was inoculated on air dried MacConkey (Oxoid, UK) agar plates and incubated at 37°C for 24 h. *E. coli*-like colonies that were pink to rose red colonies were selected for further examining the morphology and biochemical properties testing of growing colonies. Gram staining was evaluated

Table 1: Sequences and predicted lengths of PCR products of multiplex PCR assays for the simultaneous identification of Enterohemorrhagic *E. coli* according to Rappelli *et al.*[9]

Primer	Oligonucleotide sequence(5' to 3')	Target gene	PCR product size (bp)
Stx1	F:GAAGAGTCCGTGGGATTACG	<i>Stx1</i>	450
	R: AGCGATGCAGCTATTAATAA		
Stx2	F*: GGGTACTGTGTGCCTGTACTGG	<i>Stx2</i>	130
	R: GCTCTGGATGCATCTCGGT		
eaeA	F: TGATAAGCTGCAGTCGAATCC	<i>eaeA</i>	229
	R: CTGAACCAGATCGTAACGGC		

(* This primer was modified from GGGTACTGTGTGCCTGTACTGG according to Rappelli *et al.* [9] to be GGGTACTGTGCCTGTACTGG according to the blasting with *stx2* gene using the NCBI database and Bioinformatics tool

following the procedure described by Merchant and Packer [7] and *E. coli*-like colonies were subjected to different biochemical tests, including sugar fermentation tests, indole production test, Methyl-Red and Voges-Proskauer (IMIVC) tests and Sorbitol MacConkey (SMAC) (Oxoid,UK) agar following the standard methods described by Cowan [8].

In this study, however, in addition to routine culture for bacterial biochemical identification, were used for bacterial identification, CHROM Agar we used (Oxoid, USA), a new assay with simple usage, for better confirmation the identification of *Escherichia coli*. Moreover, Biochemical profile was performed by API 20E (bioMérieux, France) according to the manufacturer instructions.

Genotypic Characterization of Isolated EHEC: All isolated *E. coli* strains were subjected to molecular screening of commonly reported virulence genes. This characterization was performed through multiplex and Uniplex PCR reactions for *stx1*, *stx2* genes and *eaeA* gene respectively(Table 1). Initially, 3 pure colonies of each *E. coli* strain were picked from MacConkey agar plates and suspended in 200 μ l v/v PCR grade (mili Q) water. The bacterial suspension was then boiled for 5 min in thermostatic water bath (D3165-Kottermann, Germany) and reconstituted directly into ice bath until use. Finally, the suspension was centrifuged at 1400 g for 5 min and the supernatant was collected and used in the PCR reactions. All primers and PCR reactions were designed and conducted according to Rappelli *et al.* [9] with minor modifications. All necessary positive controls (using standard *E. coli* strains) and negative controls (without any DNA general, out of the 100 *E. coli* isolates used in the addition) were conducted within each PCR set up.

RESULTS

Screening, Isolation and Identification of *E. coli* Strains:

Among the screened collected 110 clinical specimens, 100 (91%) were verified as *E.coli* bacterial isolates (Fig. A) by showing pink to rose red colonies surrounded by a narrow zone of precipitated bile on MacConkey agar. While on EMB agar, they were showing metallic green in color. Other morphological, Gram-negative *E. coli* isolates differ in physical appearance in that some isolates were non motile rod with no flagella while other isolates were motile rod with flagella. In Addition, purple colonies on CHROM Agar (Fig.1B) and API 20E biotype was clearly established these organisms as *E. coli*.

Moreover, one of them (1%) harbored EHEC ('sorbitol-negative *E. coli*) out of 100 *E.coli* bacterial isolates in samples stool samples (Fig. 3B) by using Sorbitol MacConkey (SMAC). It was showed colourless to pale, flat and smooth, circular or serrated colonies at the edge after inoculated (SMAC) (Oxoid,UK) agar plates. However, the biochemical reactions of EHEC colonies indicate that they were similar to *E. coli* by using API 20E but they were differed in their reactions for ornithine decarboxylase and the fermentation of dulcitol, rhamnose, sucrose and melibiose in API-20E, thus colonies has 5144572 biochemical profile code(Fig.1A) .

Genotypic Characterization of Isolated EHEC: Multiplex PCR analysis of 100 *E.coli* stool samples from diarrhoeal patients were detected by a single reaction of one combination of Stx1 and Stx2 primers. It gave distinct and adequate amplification of their respective targets: *stx1*, *stx2* which showed PCR products 130 bp and 510 bp respectively (Fig.2).Moreover, Uniplex PCR analysis of primers for amplification of *eaeA* which showed PCR products 229 bp (Fig.2). While no products were obtained from the EHEC negative control.

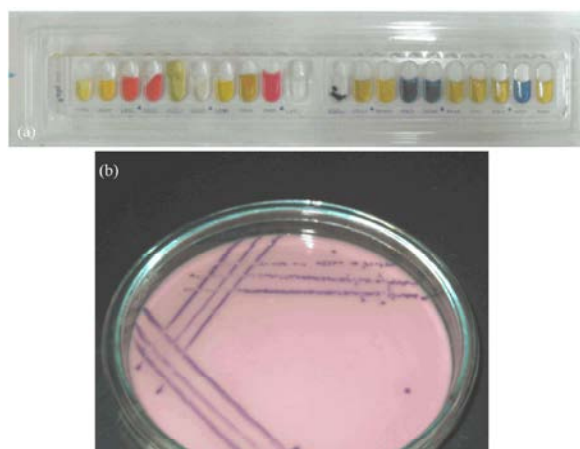


Fig. 1: Photographic picture showing the biochemical reactions profiles of EHEC clinical isolate using API 20 kit system (A) and the growth of the isolated *E. coli* strains on CHROM Agar (B).

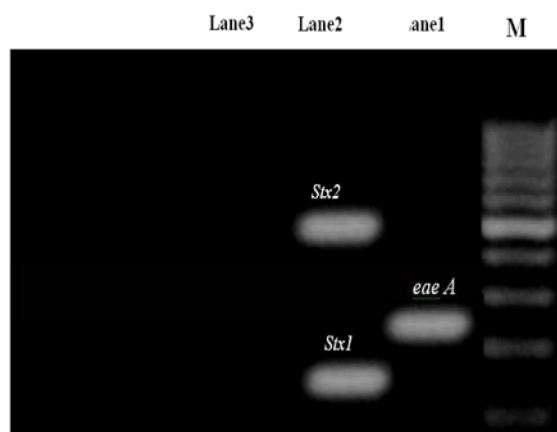


Fig. 2: The PCR products, which have been amplified by the sets of multiplex PCR reactions using DNA from isolated EHEC strain. Lane M is 100-bp ladder, lane 1 *eaeA* gene, lane 2 *stx1* gene and *stx2* gene and Lane 3 (negative control).

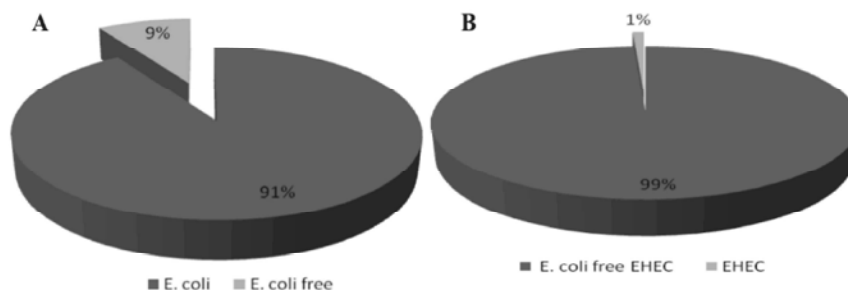


Fig. 3: The recorded prevalence (91%) of the isolated *E. coli* strains compared to the *E. coli* free samples collected from 110 clinical samples (A) and the recorded prevalence (1%) of the isolated EHEC strain out of 100 of the clinical *E. coli* samples (B).

The distributions of EHEC infection with various virulence traits were as follows: (100%) One patients harboured *E. coli* containing both *stx1* and *stx2* genes and were also 100% positive for Intimin gene.

DISCUSSION

The isolated bacterial isolates showed typical culture and biochemical characteristics to members belonging to genus *E. coli* [8]. Indeed, EHEC is associated with a broad spectrum of illnesses in humans including non-bloody and bloody diarrhoea, haemorrhagic colitis (HC) and the often deadly haemorrhagic uremic syndrome (HUS) [10]. However, some studies have suggested that there is an interesting phenomenon in developing countries in which EHEC is much less frequently isolated than other diarrheagenic *E. coli* as reported by Akbar, [11] in Kalar

town in Iraq and other countries, as by Alikhani *et al.* [12], by Güney *et al.* [13] in Turkey, by Ghenghesh *et al.* [14] in Libya and by Raji *et al.* [15] in East Africa, This was simply confirmed in the present study where EHEC had low prevalence (1%).

Although, EHEC and enteropathogenic *E. coli* EPEC share *eaeA* (the intimin structural gene), but the major virulence factors defining the characteristics of EHEC are *Stx1* and *Stx2*. In the present study only 1 (1%) isolates was identified to be EHEC had *eae* gene and both *stx1* and *stx2* genes.

The present study, most frequently observed of both *stx1* and *stx2* genes were detected together in 1 (100%) isolate out of 100 *E. coli*. This result was in contrast to the studying of Shiga toxin-producing *Escherichia coli* strains by Wieler *et al.* [16], *stx1* genes alone in 107 strains (61%) included was the higher prevalence.

Our result was similar to the study of prevalence of enterohemorrhagic *Escherichia coli* O157 in cattle feces during slaughter by Elder *et al.* [17], 58.4% of total EHEC isolates included for both *stx1* and *stx2* genes together was higher prevalence. This difference in results subject that excretion of Shiga toxin-producing *Escherichia coli* is affected by several factors like food, age, stress and seasonal changes

CONCLUSION

Although around 100 *E.coli* isolates have been collected, only one was genotypically characterized as EHEC. This isolate harbored both genes of Stx proving potential risks, therefore, genotypic characterization is crucial to show any possible sequestered risks.

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