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MULTIPLEX PCR RAPID AND SENSITIVE SCREENING METHOD FOR DETECTION OF LOCAL STRAINS OF *ESCHERICHIA COLI* O157 : H7 IN HILLA CITY

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ABSTRACT : This study was performedto investigate molecular characterization of Shiga toxin-producing *E. coli* associated with acute diarrhea (bloody , watery or both) that collected from Children under 5 years old in Babylon province.For the detection of the pathogenic *E. coli* O157:H7 virulence genes (*stx1, stx2, eaeA, hlyA*) in a multiplex PCR protocol using fourspecific primer pairs. The target genes produced species-specific amplicons at (614bp), (779bp), (890bp) and (165bp), respectively. A total of 500 stool samples were collected, cultured on theSorbitol MacConkey agar and *E. coli* 0157: H7 Chromogenic Agar Base with cefixime tellurite supplement. Out of the 500 samples, 223 (44.6%) were non-sorbitol fermenting (NSF). *E. coli* isolates were serotyped as *E. coli* O157:H7 11(2.2%), byserological detection of O157 somatic antigen by using slide agglutination of heat-treated organisms and Vitek2 system. All latex agglutination positive isolates were positive to mPCR test. The results obtained show that the established PCR protocol is suitable for a rapid and specific analysis of the pathogenic *E. coli* O157:H7 in clinical samples for the assessment of microbiological risks.

Key words : *Escherichia coli* O157:H7, Shiga-like toxins, Multiplex PCR.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) strains represent one of the most important groups of food-borne pathogens that can cause several human diseases such as hemorrhagic colitis (HC) and hemolytic – uremic syndrome (HUS) worldwide (Januszkiewicz and Rastawicki, 2016). Hemorrhagic colitis occasionally progresses to hemolytic uremic syndrome (HUS), an important cause of acute renal failure in children and morbidity and mortality in adults (Adams *et al*, 2016). Enterohemorrhagic *E. coli* O157:H7 (EHEC O157:H7) has been known to cause these syndromes since the 1980s, but clinical cases and outbreaks caused by members of other EHEC serogroups are increasingly recognized (Centre for Food Security and Public Health, 2016). The ability of STEC strains to cause disease is associated with the presence of wide range of virulence factors including those encoding Shiga toxin (Mayer *et al*, 2012). The ability of *E. coli* O157:H7 to cause severediseases in humans is related to their capacity to secreteshiga toxins (Stx1 and Stx2) and variants of these toxins, which associated with hemolytic uremic syndrome (HUS). Stx is essential in the pathogenesis of HUS, which has been mostly related to Stx2-producing isolates (Guirro *et al*, 2014). Another virulence associated factors of most STEC isolates associated with severe disease are intimin and haemolysin (Karger and Homayoon, 2015).

MATERIALS AND METHODS

Sampling

A total of 500 stool samples were collected from children under 5 years old with diarrhea from both sexes, who attended to Babylon Maternity and Pediatrics Hospital in Hilla city, 208 females and 292 males.

Stool samples were collected from children, who hospitalized with acute diarrhea (bloody, watery or both) and persistent diarrhea symptoms during the period from July 2016 to February 2017. A detailed information of the patients were obtained, including age, sex, source of drinking water, type of feeding and antibiotics usage.

Isolation of *E. coli* O157:H7

The fecal swabs were collected by using sterile disposable wooden swabs, they were inoculated into MacConkey agar and incubated aerobically at 37ºC for 24hrs. Rose pink coloured colonies with typical appearance of *E. coli* were re-streaked on Eosin Methylene Blue agar, then incubated for 24hrs. at 37°C. The green metallic sheen colonies were considered as *E. coli*.

Morphological identification completed by Gram's stain technique according to Paresh *et al* (2013). Each colony suspected *E. coli* was further diagnosed by using biochemical tests catalase, oxidase and IMViC tests.

Sorbitol MacConkey agar – SMACwas used as selective medium for *E. coli* 0157:H7, in which *E. coli* were inoculated, then incubated at 37°C for 24hrs., pale pink colonies were appeared (sorbitol non-fermenting colonies).

Sorbitol non-fermenting colonies were re-cultured on MacConkey agar medium to eliminate non lactose fermenting bacterial species, incubated at 37°C for 24hrs., then colonies transferred on *E. coli* 0157:H7 Chromogenic Agar base with cefixime tellurite supplement, for first screening for *E. coli* 0157:H7 incubated at 37ºC for 24hr. Amber color colonies were appeared. Specific biochemical tests for *E. coli* 0157:H7 (Cellobiose fermentation test, Potassium Cyanide growth test and Enterohemolysin production) were performed on the sorbitol nonfermenting colonies (Sanderson *et al*, 1995; Stapp *et al*, 2000). Serological detection of O157 somatic antigen by using Slide agglutination of heat-treated organisms and Vitek2 system were usedfor further identification of *E. coli* O157:H7.

Detection of O157 somatic antigen

This test was used for more specific identification of *E. coli* O157:H7 by using slide agglutination of heat-treated organisms to detect the somatic antigen O157.

Table 1 : Oligonucleotide primers sequences used for mPCR amplification.

Primer	Sequence (5'-3')	Primer length	Amplicon size (bp)
Stx1 F Stx1 R	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	20 20	614
Stx2 F Stx2 R	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	21 23	779
eaeA F eaeA R	GTGGCGAATACTGGCGAGACT CCCCATTCTTTTTCACCGTCG	21 21	890
hlyA F hlyA R	ACGATGTGGTTTATTCTGGA CTTCACGTGACCATACATAT	20 20	165

Table 2 : PCR conditions of *stx1*, *stx2*, *eaeA* and *hlyA* genes.

Stage	Step	Temperature	Time	No. of cycles
1.	Initial denaturation	95°C	3 min.	1
2.	Denaturation	95°C	20 s.	35
	Annealing	58°C	40 s.	
	Extension	72°C	1.5 min.	
3.	Final extension	72°C	5 min.	1

Identification process with Vitek 2 system

The suspected *E. coli* 0157:H7 strains were subjected to Vitek2- GN system for more accurate identification.

Genotypic identification using Multiplex PCR amplification

The genotyping of the studied groups were performed using Multiplex PCR technique after genomic deoxyribonucleic acid (DNA) extracted from bacterial samples. Genomic DNA was extracted and purified using extraction and purification Kit (Geneaid Company, UK) according to manufacturer's instructions. The concentration of DNA and purity were measured using the NanoDrop spectrophotometer. The extracted DNA was kept at ™20°C in aliquots.

Amplification of *stx1*, *stx2*, *eaeA* and *hlyA* genes by multiplex PCR

Genotyping detection by multiplex PCR for the presence of *stx1*, *stx2*, *eaeA* and *hlyA* genes by using four pairs of specific primers (Table 1). *stx1*, *stx2*, *eaeA* and *hlyA* genes are encoding for the Shiga – like toxin1, Shiga – like toxin2, Intimine, and Hemolysin. The amplification mixture contain 12.5 ìl of Gold multiplex PCR premix (*Taq* DNA polymerase, dNTPs, MgCl2 and reaction buffer at optimal concentration for efficient amplification of DNA templates by PCR), 4 ìl of purified DNA, 2.5 ìl of each forward and reverse primers), then volume completed to 50 ìl by deionized water. All tubes were centrifuged in microcentrifuge for 10 seconds. PCR tubes were transferred to the thermo-cycler to start the amplification reaction according to a specific program (Table 2) as described previously by Kim *et al* (2005). Amplified DNA fragments were resolved by gel electrophoresis using 1.5% agarose gels. Gels were stained with 0.5 µL of ethidium bromide per mL, visualized and photographed under UV illumination.

RESULTS

DNA was extracted from*E. coli* O157:H7 that isolated fromstool samples (Fig. 1), the results were detected by electrophoresis on 1% agarose and exposed to U.V light in which the DNA appears as compact bands.

All the isolates, which were positive forserological detection of O157 somatic antigen by using slide agglutination of heat-treated organismsand Vitek2 system, were amplified to detect *stx1*, *stx2*, *eaeA* and *hlyA* genes. The results of PCR amplification were confirmed by electrophoresis analysis.

Out of 500 stool samples 223 were non-sorbitol fermenting isolates. 2.2% (11 of 223) of the isolates

were suspected *E. coli* O157:H7, by using multiplex PCR, we examined the presence of virulence gene markers (*stx*1, *stx*2, *eaeA*, *hlyA*). Out of 11 isolates, 5 isolates were positive for all four genes (*stx1, stx2, eaeA, hlyA*). 3 isolates were positive for3 genes,in the following sequence:- (*stx1*, *eaeA*, *hlyA*for first isolte) *;* (*stx2, eaeA, hlyA* for second isolates); (*stx1*, *stx2*, *eaeA* for third isolate). 3 isolates were positive for 2 genes, in the following sequence: (*stx1*, *eaeA* for first isolate); (*stx2*, *eaeA* for second); (*stx1*, *hlyA* for third isolate).

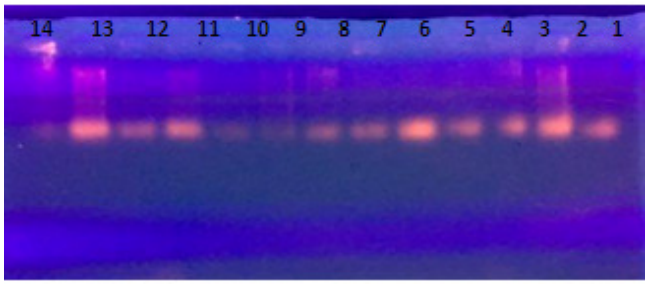
82% were positive for *stx1*, 64% were positive for *stx2*, 91% were positive for *eaeA*, 73% were positive for *hlyA* and (*stx1*+*stx2*) were 54.5%. The *eaeA* gene was the most commonly found of the four target genes. Fig. 2 showed the agarose gel electrophoresis photographed of multiplex polymerase chain reaction for *stx1* and /or *stx2*, the *E. coli* attaching and effacing *eaeA* and *hlyA* genes, amplification products which up to (614bp), (779bp), (890bp) and (165bp) fragment size, respectively.

DISCUSSION

Shiga toxin-producing *Escherichia coli* O157:H7 strains are food borne infectious agents that cause a number of life-threatening diseases, including hemorrhagic colitis and hemolytic uremic syndrome equally in male and female children (Fujii *et al*, 2015), its pathogenicity is usually linked to a Shiga toxins, EHEC producing two phage-encoded cytotoxins called shiga toxins (encoded by *stx1* and *stx2*), shiga-like toxin 1 and shiga-like toxin 2 or a combination of both are responsible for most clinical symptoms of these diseases (Giau *et al*, 2016). In addition to toxin production, *E. coli* O157:H7 possesses other virulence factors such as intimin (*eae*) and haemolysin (*hlyA*) (Fakih *et al*, 2016).

The results of this study were exhibited presence of *stx1* (82%), *stx2*(64%), *eaeA*(91%), (*stx1*+*stx2*) 54.5% genes mainly in most experimented isolates, while *hlyA*(73%) were associated with some isolates by multiplex PCR, with notably high percentage of intimin (*eaeA*) gene, occurrence of these genes gave an indicator and conformity for *E. coli* O157:H7 identification.

These results were compatible with the study of Bai *et al* (2010), they found that all human *E. coli* O157:H7 strains (57 strains) possessed *eaeA* and *hlyA* genes and differed in the *stx1* and *stx2* occurrence,of the 57 human strains, 22 had both stx1 and stx2, 34 had stx2 and only one had stx1 alone. These results were agreed with our results in the occurrence of *eaeA* in most of strains, (*stx1*+*stx2*) and *stx2*, which appear in relative ratios, but differed in the presence of *stx1* gene that appear in one strain from 57 and *hlyA* gene, which appear in all strains.



Lane 1 - 14 refers to extracted DNA from bacterial samples; Electrophoresis conditions, 1% agarose, 75 V, 20 mA for 1h (10 µl in each well), U. V. 260 - 280.

Fig. 1 : The electrophoresis pattern of DNA extracted from bacteria.

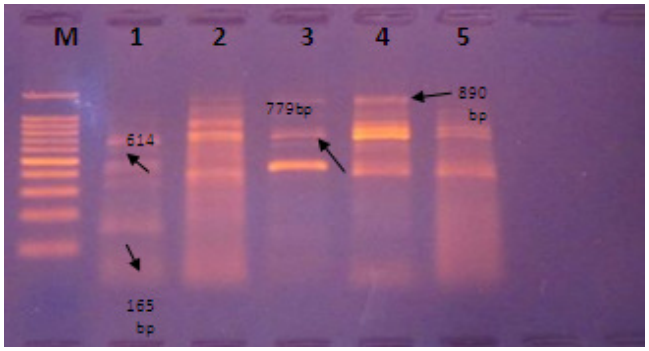


Fig. 2 : Agarose gel electrophoresis of Multiplex-PCR. Amplification products which up to 614bp, 779bp, 890bp and 165bp fragment size.Electrophoresis conditions: agarose concentration 1%, power applied: 75 V, 20 mA for 120 min. U. V. 260 - 280. Staining method; precast ethidium bromide.

Maldonado *et al* (2005) were found that by using multiplex PCR the food and clinical *E. coli* isolates, 84% of *E. coli* isolates were positive for all four genes (*stx1*, *stx*2, *eaeA* and *hlyA*). Most food and clinical isolates from outbreaks carried three or four of the virulence-associated genes and in general induced higher levels of cytotoxicity. The presence of virulent strains of *E. coli* in the environment may be a potential source of contamination of food and the water supply. Moreover, these strains may comprise a potential reservoir of virulence genes acquired from different sources (bacteriophages and plasmids). *E. coli* is a very dynamic organism; it has a capacity for horizontal gene transfer to increase genetic diversity, and under certain circumstances this can lead to the emergence of new pathogenic strains implicated in human gastrointestinal disease and other serious complications (Eaton *et al*, 2017).

Gerrish *et al* (2007) found that the 3 chromosomal genes (*stx*1, *stx*2 and *eaeA*) were detected with (100%) efficiency by both PCR and Southern blot hybridization to detect 9 virulence factors among 40 EHEC type-strains. (75%) of the pathogenic *E. coli* strains tested contained at least 1 *stx* gene (23%) were positive for both (*stx*1 and *stx*2). The most common gene detected was *eaeA*, which was positive by both PCR and Southern blot hybridization

in 93% of the strains and *hlyA* (96%).While *eaeA* gene is strongly correlated with Shiga toxin, the adherence phenotype conveyed may be sufficient to cause a pathogenic state because 4 of the clinical isolates investigated contained only the *eaeA* gene.

The results of study of Naseer *et al* (2017) were showed that presence of an *stx2*a and *eae*-positive STEC in children Aged (d™5 years) have been identified as risk factors for thedevelopment of HUS, results showed that the distribution and combination of virulence genes were clustered within distinct serotypes, with cases of HUS dispersed among the differentvirulent gene clusters. The largest accumulation and combination of virulence genes was seen among isolates of serotype O157:H-/H7, genes sequenced as a following *stx1* (64%), *stx2* (34%), *eaeA*(74.5%), *stx1*+*stx2* (43%).

Variation in virulence factor targets and use of different PCR primers contribute to variable results in detecting the most common virulence factors: *stx1*, *stx2*, *eaeA*, and *hlyA*. Variation in amplification success is likelyto increase because more virulence factor variants are certain to emerge as more Shiga toxin–producing *E. coli* strains are identified.

In this study, 3 isolates have 2 genes arranging as (*stx1*, *eaeA*; *stx2*, *eaeA*; *stx1*, *hlyA*), Ethelberg *et al* (2004) showed in their study that the combined presence of the *eaeA* and *stx2* genes is an important predictor of HUS which caused by O157:H7. Kargar and Homayoon (2015) exhibited that the isolates harbored *stx1* and *eaeA* genes that causes infections in south of Iran and non of the isolates had *stx2* and *hlyA* genes.

In a multiplexing assay, more than one target sequence, can be amplified by using multiple primer pairs in a reaction mixture. Almost all of the *E. coli* O157:H7 contains *stx1* or/and *stx2* gene, which are a virulence genes encoding a family of related toxins called Shiga. The *stx1* or/and *stx2* gene have been proven to be *E. coli* O157:H7 (Gyles, 2007; Orth *et al*, 2007).

The identification of *E. coli* O157:H7 serotype using stx as a target was carried out. According to Toma *et al* (2003), the primer stx was specific identification of *E. coli* O157:H7, which detects both *stx1* or/and *stx2* genes, and this has also been applied in different studies (Toma *et al*, 2003; Tobias and Vutukuru, 2012), while the *hlyA* gene codes for the action of Hemolysin protein (*hlyA*), this was considered as the target gene for PCR detection (Kaur *et al*, 2007; Dharmendra *et al*, 2013).

CONCLUSION

Multiplex PCR is a specific and reliable tool, which allows low cost-effective detection for *stx1* and/or *stx2*, *eaeA* and *hlyA* genes bacterial pathogens in one reaction tube.

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