Gastroenterology and Hepatology From Bed to Bench.

ORIGINAL ARTICLE

Isolation of Enteropathogenic *Escherichia coli* from lettuce samples in Tehran

Somayeh Mazaheri[1], Siavosh Salmanzadeh-Ahrabi[1], Tahereh Falsafi[1], Mohammad-Mehdi Aslani[2]

[1]*Department of Biological Sciences, Alzahra University, Tehran, Iran*

[2]*Department of Microbiology, Pasteur Institute of Iran, Tehran, Iran*

ABSTRACT

Aim: The purpose of this study was to find the isolation rate of enteropathogenic *Escherichia coli* (EPEC) from lettuce samples collected in Tehran.

Background: During the last decade, the prevalence of infectious diarrheal diseases due to consumption of contaminated food especially raw vegetable has been increasingly reported. Enteropathogenic *Escherichia coli* strains are an important group of diarrheagenic *E. coli* that can cause infant diarrhea especially in the developing world.

Material and Methods: One hundred lettuce samples collected in Tehran were transported to the laboratory, homogenized by a stomacher in EC broth containing cefixime, and cultured on MacConkey agar plates. Bacterial DNA was extracted by boiling method and PCR was performed using three pairs of primers targeting stx_1, stx_2 and *eaeA* genes.

Results: Screening of 100 lettuce samples by PCR showed four samples were positive for the presence of EPEC.

Conclusion: This study suggests contamination of the lettuce by the EPEC and its possible role as the source of infection in this region.

Keywords: Enteropathogenic *Escherichia coli*, Lettuce, PCR, Tehran.

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Introduction

During the last decade, the prevalence of infectious diarrheal diseases due to consumption of contaminated food especially raw vegetable has been increasingly reported (1-2). Among the infectious disease, the acute diarrheal disease is the second cause of death in children younger than five years old worldwide (3). Although large outbreaks of infant diarrhea due to Enteropathogenic *Escherichia coli (*EPEC) have largely been disappeared from industrialized countries, this group of pathogenic *E. coli* continues to cause infant diarrhea in the developing world (4-5). One important virulence characteristic of EPEC is their 'attaching and effacing' (A/E), property, which is characterized by intimate adherence of bacteria to the intestinal epithelium (4-6). This capacity is directed by intimin, encoded by the *eae* gene which is a part of a 35-kb pathogenicity island designated as locus of enterocyte effacement (7). The intimate attachment of bacterial cells damages the intestinal epithelium, disrupts the enteric environment, and results in a severe diarrhea (6). Among the pathogenic *E. coli groups,* one group of *eae* positive strains harbors also stx_1 and/or stx_2 genes, which classify this *E. coli* group, as shiga toxin producing *E. coli (STEC)* (6-8). An important reason for the presence of pathogenic *E. coli* in raw vegetable can be the utilization of manure as fertilizer in organic agriculture. This mode

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Reprint or Correspondence: Tahereh Falsafi, PhD. Department of Biological Sciences, Alzahra University, Tehran, Iran
E-mail: falsafi.tahereh@yahoo.com

of agriculture has led to the contamination of vegetables with human pathogens such as *E. coli*, which is present in the intestinal tract of animals. Relationship between increasing numbers of food-borne disease and presence of pathogenic *E. coli* in fresh, and minimally processed or unprocessed vegetables have led to the conclusion that the majority of sporadic cases and outbreaks are due to consumption of contaminated vegetables by the pathogenic of *E. coli* (9-10). Food items have been demonstrated as a source of EPEC infection in different countries. However, little is known concerning presence of EPEC in food samples especially lettuce samples in Iran. Our objective was to investigate presence of EPEC in lettuce samples, which would be the first study in Iran to our knowledge.

Material and Methods

Sample collection and bacterial culture

One hundred lettuce samples were collected from different farmlands and markets between, 21st of June and 21st of September 2011 in Tehran. All samples were packed in separate stomacher bags and transported to the laboratory at refrigerator temperature and immediately were processed by a stomacher instrument (Bag Mixer 400 VW. Interscience. France).

The enrichment and the processing of the samples were carried out according to previously described protocols (11). Briefly, 25g of each sample was homogenized in bags of stomacher, which contained 225 ml *E. coli* (EC) broth medium supplemented with 0.05 mg/l cefixime. After overnight incubation at 37°C, a portion of the mixture was cultivated on MacConkey agar plate and further incubated at 37°C for overnight.

DNA extraction and Polymerase chain reaction

Detection of the EPEC isolates was performed by the polymerase chain reaction (PCR). For this purpose, the DNA was extracted from the culture plates according to the previously described protocol with some modification (12). Briefly, a loopful of growth from the first inoculation streak was suspended in 0.5 ml of distilled water and boiled for 10 minutes. After centrifugation, at 15000 rpm for 5 min, the supernatant of the lysate was used in PCR. The supernatant containing bacterial DNA was transferred to a new tube and submitted to PCR for detection of the *eaeA* gene. To exclude the group of isolates harboring stx_1 and/or stx_2 genes, they were also processed for these two genes by PCR.

The information related to the oligonucleotide primers are shown in Table 1.

Table 1. Primers and references

gene	Oligonuceotide sequence (5′-3)	size	Ref
Stx1	GAAGAGTCCGTGGGATTACG AGCGATGCAGCTATTAATAA	130bp	16
Stx2	ACCGTTTTTCAGATTTTGACACATA TACACAGGAGCAGTTTCAGACAGT	298bp	17
eaeA	GTGGCGAATACTGGCGAGACT CCCCATTCTTTTTCACCGTCG	890bp	18

The PCR was performed in a reaction volume containing 5 µl of the extracted DNA, 2.5 µl PCR buffer (10X), 0.5 µl deoxynucleoside 5-triphosphate, 0.5 µl of each primer, 0.25 µl of *Taq* DNA polymerase (5 u/µl) (Cinnagen co., Iran), and 1.5 µl $MgCl_2$ (20 mM) adjusted to the total volume of 25 µl with sterile deionized water. The reaction was carried out by an amplification thermal cycler (Peqlab, Germany). The procedure consisted of initial denaturation at 96 °C for 4 min, followed by 32 cycles of PCR. The final cycle was followed by incubation of the reaction mixture at 72 °C for 6 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel. *E. coli* ATCC 43890 (stx_1), *E. coli* ATCC 43889 (stx_2) and *E.coli* ATCC 43887 (*eaeA*) were positive

controls. The *E. coli* ATCC 11775 was included as a negative control in all assays.

Colony screening

The samples positive by PCR were submitted to colony isolation. A total number of colonies (between 20-30 colonies), located in the third and fourth area of the culture, were processed to DNA extraction and PCR. This step was repeated until isolation of pure colonies responsible for *eaeA*-positivity of PCR in the first culture. The pure bacterial cultures were stored at -20°C and -70°C. All isolates positive for *eaeA* gene were submitted to biochemical identification of *E. coli* using standard biochemical test including cultivation of the isolates on triple sugar iron agar, sulfide indole motility medium, Simon citrate agar and methyl red-Voges–Proskauer broth. The isolates positive for *eaeA* and negative for *stx* were interpreted as EPEC (6-8).

Results

The PCR protocol used in this study has permitted to detect four *eaeA* positive samples among 100 lettuce samples. To obtain the pure bacterial isolates positive for *eaeA* gene, we have had to perform multiple subcultures from the primary culture of lettuce samples. Between, 20-30 colonies located in the third and fourth area of these subcultures, were submitted to PCR reaction for detection of *eaeA* gene. However, further examination of these isolates by PCR for the detection of stx_1, and/or stx_2 genes showed the isolates did not contain stx_1 or stx_2 genes: Examination of the *eaeA* positive isolates by standard biochemical reactions confirmed that all of four isolates were *E. coli*. Agarose gel electrophoresis of PCR products for *eaeA*, stx_1, stx_2 and are shown in Figure 1 and for 3 of our isolates in figure 2.

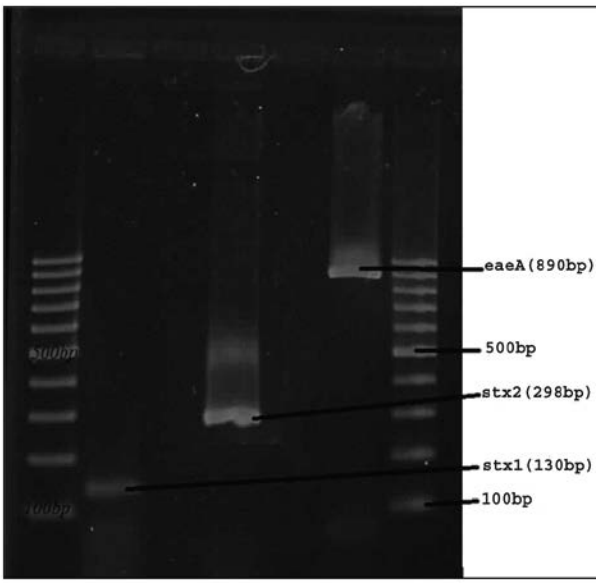

Figure 1. Agarose gel electrophoresis of *eaeA*, stx_1 and stx_2, gene products of control strains.

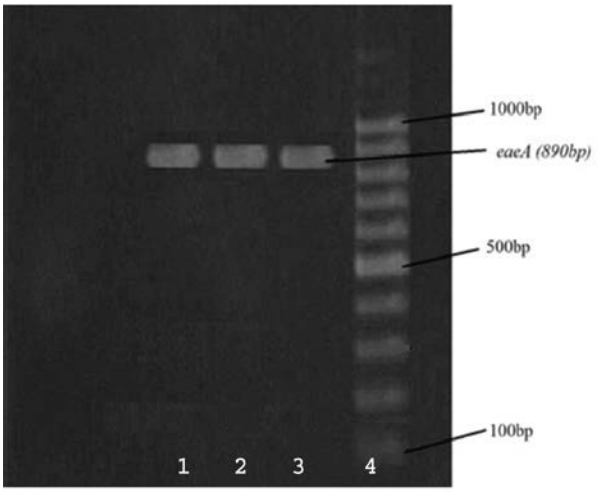

Figure 2. PCR assay for the detection of *eaeA* gene (890 bp) in the isolates. 1, 2 and 3: positive samples 4:ladder (100 bp).

Discussion

The samples were put into an *E. coli* broth incorporated with cefixime for proliferation of the pertinent bacteria. This enrichment is critical, as the bacteria feel stressed under sampling and transformation conditions. Under selective conditions due to presence of cefixime in EC broth, the growth of gram-negative such as *E. coli* is favored.

The advantage of the PCR screening method performed in this study is its remarkable potential for detection of the EPEC strains in a complex mixture since it is a rapid, sensitive and reliable method for detection of *eaeA, stx_1 and stx_2* genes.

To our knowledge, there is no report in Iran concerning presence of EPEC in raw vegetables such as lettuce. Using a PCR protocol, we assessed 100 lettuce samples collected in Tehran. The results have indicated that 4% of the samples were contaminated with EPEC strains.

This is the first study providing an evidence for the contamination of vegetable samples by the EPEC strains in Tehran. However, the isolation rate of EPEC strains in the lettuce samples in the present study has been lower than other studies. This low isolation rate may be attributed to geographical area, variation in sampling, variation in types of samples evaluated, and differences in detection methods (13).

Some investigators in Iran have studied the presence of EPEC strains in human fecal samples and in milk samples (12, 13). The isolation rate of this pathogen in milk samples in Kermanshah has been 22.1% (13).

EPEC strains have been detected in 25% of milk samples and in feces of 14% of children with diarrhea in Brazil (14, 15). Jafari et al (3) studied the prevalence of EPEC strains among 1087 children under five years old with acute diarrhea in Tehran and found that 12.6% were positive for EPEC strains.

As the mode of agriculture may be responsible for contamination of vegetables with human pathogens such as EPEC strains, official health protection policies is required in this area for suitable control and monitoring the utilization of contaminated wastewaters as well as. Replacing non-contaminated fertilizers with those obtained from contaminated animals. Consistent with this necessity, we have noted that our positive samples were originated from the farmlands where a wide range of bovine manure was employed for soil nourishment and wastewaters was used for watering in some cases.

As a conclusion, the results of this investigation indicated the possible role of lettuce samples in the transmission of the EPEC strains and the necessity of preventative measures to control contamination of lettuce samples by EPEC strains.

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