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ORIGINAL ARTICLE

Evaluation of the frequency of *Escherichia coli* pathogroups in *Brassica oleracea* cultivars

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ABSTRACT

Background and Objectives: Pathogenic diseases resulting from microbial contamination of food have been widely distributed in many parts of the world. Among these, *Escherichia coli* is one of the most important foodborne pathogenic bacteria. Diarrhea is one of the major causes of children's death in developing countries, with approximately 2 million deaths annually. The current study aimed to determine the frequency of diarrheagenic *E. coli* pathotypes such as Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), and Shiga toxin-producing *E. coli* (STEC) in *Brassica oleracea* cultivars in order to provide information on the assessment of diarrheagenic *E. coli* pathogenesis risk.
Materials and Methods: 100 samples of vegetables were collected in Tehran, including cabbage, cauliflower, broccoli and Brussels sprouts. After homogenizing samples, enrichment was done in the EC broth medium. Five colonies of pure culture were used for DNA extraction. Pathotypes were identified by PCR using virulence genes.
Results: The results showed that the prevalence of diarrheagenic *E. coli* strains was 7%. The EPEC prevalence was 3%, All EPEC isolates were atypical. The ETEC frequency was 3%, And the EAEC prevalence was 1%.
Conclusion: These findings indicated that *Brassica oleracea* cultivars could be consideredas a source of contamination with diarrhea-causing *E. coli* strains.

Keywords: Diarrheagenic *Escherichia coli*; Virulent genes; Raw vegetables; *Brassica oleracea*

INTRODUCTION

Diarrhea disease is one of the main causes of morbidity and mortality among infants and young children in developing countries. Diarrheagenic *E. coli* strains is the most common cause of diarrhea (1). *E. coli* is commonly found in the gut as a symbiosis, but through acquiring motile genetic elements such as plasmids, transposons, bacteriophages, and pathogenicity islands, certain strains of it have acquired virulence factors (2). It has been identified that there are six different types of diarrheagenic *E. coli* pathotypes based on their specific virulence traits under the following names: enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffuse adherent *E. coli* (DAEC) (3).

Brassica oleracea vegetables are widely consumed in the daily diet due to their low calorie and bioactive

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compounds and since these vegetables are eaten raw or undercooked and, so might be a means of transmitting bacterial, parasitic, and viral pathogens (4, 5).

Transmission of diarrheagenic pathotypes takes place through the fecal-oral route (6). According to the World Health Organization (WHO) report, at least one in 10 people get infected each year due to the consumption of contaminated food which leads to 420,000 deaths (7). The current study's objectives are to investigate the frequency of pathogenic *E. coli* (EPEC, STEC, ETEC, EAEC) in *Brassica oleracea* cultivars including cabbage, cauliflower, broccoli, and Brussels sprouts in Tehran using isolation of pathogenic genes of these pathotypes.

MATERIALS AND METHODS

Sample collection and bacterial culture. From September 2018 to July 2019, a total of 100 fresh vegetable samples including cabbage (46 pieces), broccoli (16 pieces), Brussels sprouts (14 pieces), and cauliflower (24 pieces) were collected weekly from stores in the north of Tehran. The samples were transferred to the laboratory separately in sterile bags without hand touch and then deposited for further study at 4°C.

Enrichment of *E. coli* strains was performed according to the guidelines of bacteriological analysis (8). 25g of each sample was added to 225 ml of *E. coli* (EC) broth (Merck, Germany) containing 0.05 mg/l of the antibiotic cefixime in stomacher bags and then homogenized using a stomacher instrument (Bag Mixer400 VW, Inter science, France). After overnight incubation at 37°C, a loop full of culture medium containing enriched bacteria was cultured on MacConkey agar plate (Merck, Germany), then incubated for 18-24 hours at 37°C. Five colonies from each sample were selected for further assessment (9).

DNA extraction and polymerase chain reaction assay. Bacterial genomic DNA extraction was done using the boiling method (10). The presence of the following virulence genes was assessed by PCR: *E. coli* attaching and effacing gene (*eae*) encoding intimin and bundle-forming pilus gene (*bfpA*) for detection typical and atypical EPEC, heat-labile toxin gene (*elt*), and heat-stable toxin gene (*est*) for detection of ETEC strains, *pCVD432* plasmid for detection of EAEC strains, Shiga toxin 1 gene (*stx1*) and Shiga toxin 2 genes (*stx2*) with or without *eae* gene for detection STEC strains and finally the presence of rfb_{O157} and $flic_{h7}$ genes for the possible presence of O157: H7 strain (11, 12, 13). Primer sequences and PCR product sizes are shown in Table 1.

The reaction mixture contained 2.5 µL PCR buffer (10×), 0.8 µL $MgCL_2$ (50 Mm), 0.4 µLdNTP mix (10 Mm), 0.5 µL primer, 0.4 µL DNA polymerase Taq. Then PCR program are as flows: initial denaturation at 96°C for 4 minutes followed by 36 cycles for 20 seconds at 94°C for denaturation, 30 seconds from 54°C to 60°C annealing temperature of primers ([56] *est*, [55]*stx1*, [54] *elt*, *bfpA*, *pCVD432*, *eae*, [60] rfb_{O157}, $flic_{h7}$, [57] *stx2*), 50 seconds at 72°C and final cycle at 72°C for 6 Minutes.

PCR products were electrophoresed on 1.5% agarose gel. STEC ATCC 43890 (*stx1+*), STECATCC 43889 (*stx2+*), ETEC ATCC 35401 (*elt+*, *est+*), EPECATCC 43887 (*eae+*, *bfpA+*) and EAEC strain 17-2 (*pCVD432+*) were used as positive controls and *E. coli* ATCC 11775 was used as a negative control. All PCR-positive strains were subjected to biochemical tests to confirm the identification of *E. coli* strains including triple sugar iron agar, sulfide indole motility medium, Simon citrate agar, and methyl red-Voges-Proskauer broth.

RESULTS

Three EPEC isolates were isolated out of 100 vegetable samples (including cabbage, cauliflower, broccoli, and Brussels sprouts). EPEC is classified into typical (tEPEC) and atypical (aEPEC) strains based on the presence of the virulence plasmid known as EPEC adherence factor plasmid (pEAF), which is present in tEPEC (absent in aEPEC). In this study, all strains contained the *eaeA* gene and lacked the *bfpA* gene associated with the EAF plasmid, indicating that all belonged to atypical strains (*eae* positive, *bfpA* negative) (Fig. 1). Three ETEC isolates were isolated, including two strains with *elt* gene and one with *est* gene (Fig. 2). Moreover, one isolate with the *pCVD432* gene was identified as EAEC strain, and no STEC strains were not isolated from any of the samples. Table 2 represents the percentage of pathogenic *E. coli* bacteria.

Table 1. primer sequences and PCR product size.

Target Gen	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	PCR Product Size, bp	Reference
stx1	GAAGAGTCCGTGGGATTACG	AGCGATGCAGCTATTAATAA	130 bp	(11)
stx2	ACCGTTTTTCAGATTTTGACACATA	TACACAGGAGCAGTTTCAGACAGT	298 bp	(11)
$flic_{h7}$	GCGCTGTCGAGTTCTATCGAGC	CAACGGTGACTTTATCGCCATTCC	625 bp	(12)
rfb_{O157}	AAGATTGCGCTGAAGCCTTTG	CATTGGCATCGTGTGGACAG	497 bp	(13)
eae	GTGGCGAATACTGGCGAGACT	CCCCATTCTTTTTCACCGTCG	890 bp	(12)
bfpA	TTCTTGGTGCTTGCGTGTCTTTT	TTTTGTTTGTTGTATCTTTGTAA	367 bp	(11)
eltB	TCTCTATGTGCATACGGAGC	CCATACTGATTGCCGCAAT	322 bp	(11)
estA	GCTAAACAAGTAGAGGTCTTCAAAA	CCCGGTACAGAGCAGGATTACAACA	147 bp	(11)
pCVD432	CTGGCGAAAGACTGTATCAT	CAATGTATAGAAATCCGCTGTT	630 bp	(11)

Table 2. Frequency of isolates isolated from *Brassica oleracea* cultivars.

Name of vegetables	No of sample	EPEC (%)	ETEC (%)	EAggEC (%)	STEC (%)
cauliflower	24	1	1	n.d	n.d
cabbage	46	2	1	n.d	n.d
broccoli	16	n.d	1	1	n.d
Brussels sprouts	14	n.d	n.d	n.d	n.d
Total	100	3	3	1	n.d

EPEC, Enteropathogenic *E. coli*; ETEC, Enterotoxigenic *E. coli*; EAggEC, Enteroaggregative *E. coli*; STEC, Shigatoxigenic *E. coli*; n.d., not detected.

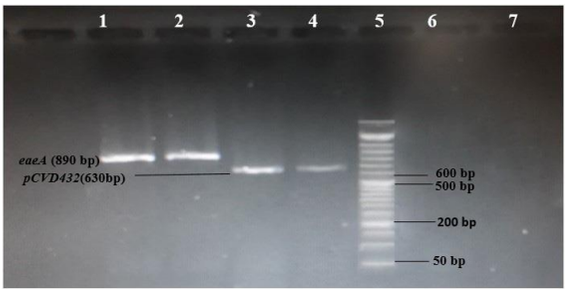

Fig. 1. PCR products of the *eae, pCVD432* genes on 1.5% agarose gel.
Lane 1 and 2, sample and positive control for *eae* gene (890 bp), lane 3 and 4, sample and positive control for *pCVD432* gene (630 bp), lane 5, a molecular marker of DNA, lane 6 and 7, negative control for *eaeA* gene and *pCVD432* gene, respectively.

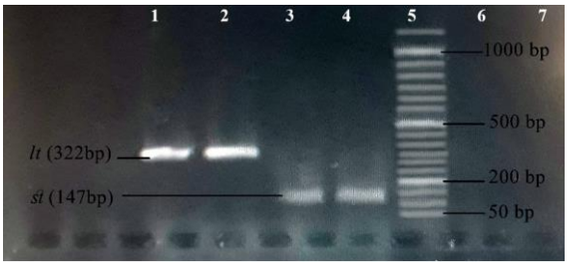

Fig. 2. PCR products of the *elt, est* genes on 1.5% agarose gel.
Lane 1 and 2, respectively, sample and positive control for *elt* gene (322 bp), lane 3 and 4 respectively, sample and positive control for *est* gene with (147 bp), lane 5, a molecular marker of DNA, lane 6 and 7 negative control for *elt* gene and *est* gene, respectively.

DISCUSSION

The incidence of leafy vegetable-associated outbreaks which was reported to the Centers for Disease Control and Prevention (CDC) (1973 to 2012) was higher than that other food types associated outbreaks (14). Due to the importance of these vegetables in the transmission of infection. The frequency of infection and genotypic distribution of pathogenic *E. coli* strains associated with diarrhea in *Brassica oleracea* cultivars was investigated in this study. As far as we know from the reported information, this is the first study of the prevalence of pathogenic *E.*

coli in cultivars of *Brassica oleracea* in Iran using molecular methods.

From the 100 samples of Brassica vegetables gathered in the present investigation, the prevalence of pathogenic *E. coli* strains, including EPEC, ETEC, STEC, EAEC was 7%. The frequency of EPEC acquired in this study was 3%. In different studies on other vegetables, the rate of isolation of this pathotype are as follows: Iran (4%), Argentina (2.1%), and Korea (0.8%) (9, 10, 15). Our findings are approximately consistent with those studies. However, these figures were higher in the case of EPEC studies on vegetables in Jakarta (15.79%) and the Czech Republic (11.1%) (16, 17).

The *bfpA* gene was not observed in any of the samples, indicating that vegetables were contaminated with atypical EPEC. The results of studies conducted in Iran and other countries show that atypical strains are likewise ready to colonize the intestine and cause diarrhea. In some studies, atypical EPEC strains have been isolated from children with diarrhea. For instance, a study conducted in Brazil indicates an increase in the prevalence of atypical EPEC strains isolated from patients of diarrhea (18). Furthermore, studies in Peru, Japan, and Melbourne suggest a rise in diarrhea due to atypical EPEC. These show that the incidence of diarrhea with atypical EPEC is rising in developing countries as well as industrialized countries (19-21).

In the current study, the frequency of ETEC was determined at 3%; two isolates had *elt* gene and one isolate had *est* gene. In a comparative report in China, 559 ready-to-eat food samples were tested for *E. coli* contamination, of which 6% ETEC was reported from vegetable samples (22). In another study in Korea, out of 416 samples from vegetables and fruits, the frequency of ETEC was 0% (9). In another study in Mexico, out of 100 samples of beet juice were collected, 2% ETEC was found, which shows that the figures obtained in these studies are almost identical to the present study's statistics (23). Some studies have also evaluated the prevalence of ETEC in other foods, including the study in Korea where the rate of meat contamination with ETEC was 43% (24). In another study in Italy milk contamination was assessed. Out of 149 *E. coli* isolates, 35.6% of the isolates were classified as pathogenic, of which 54.7% belonged to the ETEC pathotype (25). Therefore, the prevalence of ETEC in fresh vegetables seems to be relatively lower than in other foods.

The variety and geographical distribution of bacterial strains is the main issue in dealing with ETEC disease. Targeting enterotoxins and CFs, which play an important role in the pathogenicity of ETEC, are the most effective approaches to the ETEC vaccine advancement strategy. Consequently, the determination of enterotoxin types and CF types in various geographical regions is critical to battle ETEC-induced disease (26).

In this study, the frequency of enteroaggregative *E. coli* was also explored, and just one isolate was isolated from broccoli. EAEC causes acute and chronic diarrhea in children, adults, and individuals living with HIV in both developing and developed countries. However, EAEC infections are often self-restricting and must be managed individually (27).

No STEC pathotypes were observed in this study. Some studies in Brazil, the USA, and South Korea also reported the prevalence of STEC in vegetables as 0% (9, 28, 29). However, in some examinations conducted in Iran, the prevalence of STEC strains on vegetables was reported 8%, 16.66%, and 33.33% (30- 32). In Istanbul, out of 186 vegetable samples, 13 cases (11.9%) of STEC were reported (33).

Lack of STEC isolation and low contamination of other pathogenic *E. coli* pathotypes in *Brassica oleracea* specimens can be correlated to several factors, including differences in the type and number of studied samples, the geographical location of sampling, seasonal changes, and water quality used in agricultural lands (34). Studies have shown that since rising temperatures can increase the proliferation and survival of bacteria in the environment, the high prevalence of pathogenic *E. coli* occurs during the warmer seasons (35). This result was inconsistent with a study on *E. coli* in Bolivia, in which the causative agent of diarrhea was isolated from the fecal samples of children with and without diarrhea. They concluded that ETEC and EPEC infections are more common in warm seasons (36).

In another study out of a total of 140 *E. coli* isolates from children with diarrhea and 110 isolates from children without diarrhea, most of the diarrhea patients infected with EPEC were referred to hospitals during the summer (37). In our investigation, the frequency of pathogenic strains in summer was not examined, so this could be one of the potential reasons for the low frequency of it in this group of vegetables.

CONCLUSION

In general, the present study results revealed the possible role of *Brassica oleracea* specimens as a source of EPEC, ETEC, and EAEC infections. This way, they can be considered one of *E. coli* strains' transmission factors, causing the disease. Contamination of raw vegetables by pathogenic microorganisms may occur at all stages before and after harvesting through factors such as irrigation water, soil, manure, animals, washing water, processing, and distribution (7). The results indicated that the *Brassica oleracea* specimens had inappropriate microbial quality and represented a potential risk for customers. Knowing the existence and frequency of infection may lead to close monitoring of these vegetables to find the source of contamination. The examined epidemiological information (Epidemiological surveillance data) about *E. coli* pathotypes will help to provide useful solutions to prevent and control diarrheal diseases related to *E. coli*.

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