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Molecular characterization of diarrheagenic *Escherichia coli* isolated from vegetables in Argentina

Juliana González, Jimena S. Cadona, Marcelo Sanz, Ana V. Bustamante, A. Mariel Sanso*

Laboratorio de Inmunoquímica y Biotecnología, Centro de Investigación Veterinaria de Tandil (CIVETAN), CONICET-CIC-UNCPBA, Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, 7000 Tandil, Argentina

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ABSTRACT

The aim of this study was to investigate the prevalence of diarrheagenic *E. coli* strains in vegetables from the humid Pampa region, Argentina, and to determine the occurrence of serotypes and virulence genes in the isolates. A total of 373 fresh vegetable samples obtained from 41 different geographical points were examined. *E. coli* was detected in 38.6% of the samples. Ten isolates could be obtained from 14 samples presumptively positive for diarrheagenic *E. coli*: 8 were identified as atypical Enteropathogenic *E. coli* (aEPEC) and 2 as Verocytotoxigenic *E. coli* (VTEC). Lettuce and beet were the vegetables most frequently contaminated with pathogenic *E. coli*. The isolates belonged to serotypes O1:H7, O28:H19, O39:H40, O86:H31, O132:H8, O139:H20, O178:H7 and O178:H19, some of which reportedly have caused human illness, and one isolate resulted non typeable. Taking into account the distribution of 16 *nle* genes, 7 profiles were detected. On the other hand, all tested isolates harbored the gene encoding for the adhesin HcpA. Other adhesion related genes were also identified: *ecpA* and *elfA* were detected in 90%, $lpfA_{O113}$ in 60%, and *ehaA* in 50% of the isolates meanwhile *ihaA* was only observed in O178:H19 isolate. This VTEC isolate harbored, also, Cdt-V toxin and megaplasmid encoding genes such as *espP*, *subA* and *epeA* and exhibited a strong cytotoxic effect. These data is the first molecular *E. coli* report that confirms the presence of *E. coli* pathotypes circulating among vegetables in Argentina. Genetic characterization showed that in addition to *eae* or *vtx* genes, isolates obtained from vegetables harbored genes encoding other toxins, adhesins, and components related to the type III secretion system that could contribute to their virulence. In conclusion, this research shows that vegetables in Argentina may be the source of VTEC and EPEC infections in the community and therefore, they should be considered as vehicles for transmission of these potentially pathogenic bacteria.

1. Introduction

Foodborne illnesses associated with consumption of vegetables and fruits have increased, and were attributed in part to higher consumption rates and to the increases in ready-to-eat (RTE) vegetable food types (Franz and van Bruggen, 2008; Sivapalasingam et al., 2004; Warriner et al., 2003) and, particularly freshly-cut leafy vegetables (Friesema et al., 2008; Herman et al., 2015; Wendel et al., 2009). Diarrheagenic *Escherichia coli* are well-known foodborne pathogens with a worldwide public health importance (Kaper et al., 2004; Nataro and Kaper, 1998). Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC) and Verocytotoxigenic *E. coli* (VTEC) are pathotypes of *E. coli* associated with gastrointestinal infections (Bettelheim, 2007).

EPEC is responsible for outbreaks and cases of prolonged diarrhea worldwide (Nguyen et al., 2006; Vieira et al., 2016). Typical EPEC strains would possess the *eae* gene (intimin) and the EPEC adherence factor (EAF) plasmid or, alternatively, the gene encoding BFP (*bfpA*), a fimbriae named “bundle-forming pilus”; atypical EPEC (aEPEC) strains, only the *eae* gene. ETEC is associated with weanling diarrhea among children in the developing world, and traveler's diarrhea. It produces two types of enterotoxins: heat-labile (LT) and/or heat-stable (ST) (Nataro and Kaper, 1998). VTEC is a zoonotic agent whose clinical spectrum includes diarrhea, hemorrhagic colitis, and the hemolytic uremic syndrome (HUS) and is defined by the presence of one or both phage-encoded verocytotoxin genes, vtx_1 and vtx_2 (Kaper et al., 2004).

There are few available papers discriminating prevalence by *E. coli* pathotype in vegetables. Feng and Reddy (2013) pointed out that prevalence for VTEC strains isolated from vegetables has been scarcely reported and determined that this pathotype was present in 0.5–0.6% of the spinach and 0.04–0.2% of the lettuce samples from USA. Rúgeles et al. (2010) identified one VTEC among 12 strains (isolated from 38 leaf samples) from Bogotá. Ozpınar et al. (2013) found average VTEC

* Corresponding author.
E-mail address: msanso@vet.unicen.edu.ar (A.M. Sanso).

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prevalence in vegetables from Istambul of 7.2%, although in parsley and lettuce VTEC was detected in 20% and 10%, respectively. In relation to ETEC, in USA, Feng and Reddy (2014) estimated prevalence rate in these products around 0.2%, and, in Mexico, ETEC was found in 2 and 12% of the jalapeno and Serrano peppers, respectively (Cema-Cortes et al., 2012).

E. coli strains belonging to a particular pathotype carry specific virulence determinants involved in the diseases. However, hybrid types of intestinal pathogenic *E coli*, VTEC/enteroaggregative *E. coli* (EAggEC), such as the German outbreak strain O104:H4 (Bielaszewska et al., 2011) and O111:H21 (Dallman et al., 2012) have appeared. EAggEC harbor a set of plasmid virulence-associated genes regulated by a transcription factor encoded by the *aggR* gene (Dudley et al., 2006). Therefore, monitoring of virulence gene profile of *E. coli* is important to detect newly emerging pathogenic *E. coli*. Several other virulence factors are also involved in *E. coli* pathogenesis including adhesions, host cell surface-modifying factors, invasins, toxins and secretion systems, which are ideal targets for determination of pathogenic potentials of any given *E. coli* isolate (Kaper et al., 2004).

The increase in consumption of fruit and vegetables is a worldwide trend due to healthy lifestyle recommendations (Callejón et al., 2015). In Argentina, there is no information on *E. coli* and diarrheagenic *E. coli* contamination in these fresh products. The aim of this study was to investigate the prevalence of diarrheagenic *E. coli* strains in vegetables from the central humid Pampa region of Argentina and to determine the occurrence of serotypes and virulence genes in the isolates.

2. Materials and methods

2.1. Sample collection

A total of 373 vegetables samples were obtained from 41 different geographical points, including retail shops, markets, farms and fairs in the central humid Pampa region of Argentina. Samples were collected between November 2013 and March 2015 from lettuce, beets, chard, spinach, parsley, arugula and ready-to-eat salads. Vegetables collected were fresh and none of them were precooked or frozen. The samples were placed in separate sterile plastic bags and then immediately transported to the laboratory and processed within 24 h of sampling.

2.2. Sample preparation and detection of virulence genes

Twenty-five grams of vegetable sample were placed in 225 ml Luria-Bertani (LB) broth and incubated overnight with shaking at 37 °C. An aliquot of bacterial cultures was boiled for 10 min and the crude genomic DNA used as DNA template for PCR reactions. In a first screening, *uspA* (universal stress protein gene) was amplified for the detection of presumptive contamination with *E. coli* (Chen and Griffiths, 1998). Virulence genes *vtx*$_1$ (verocytotoxin 1), *vtx*$_2$ (verocytotoxin 2), *eae*, *estIa* (heat-stable enterotoxin a), *estIb* (heat-stable enterotoxin b), *elt* (heat-labile enterotoxin) were detected by different PCR reactions (Blanco et al., 1997; Paton and Paton, 2002; Woodward et al., 1992). Samples *eae*-positive but *vtx*-negative were considered as presumptively contaminated with EPEC, *vtx*-positive but *eae*-negative as presumptively contaminated with VTEC. Samples *eae*-positive and *vtx*-positive could be presumptively contaminated with EPEC and/or VTEC. Samples *estIa*, *estIb* and/or *elt*–positive were considered as presumptively contaminated with ETEC. ETEC reference strains LD1 (*estIa*), LD22 (*estIb*, *elt*) and *E. coli* O157:H7 strain EDL933 (*eae*, *vtx*$_1$, *vtx*$_2$) were used as positive controls (Parma et al., 2000).

2.3. Isolation of diarrheagenic Escherichia coli

The overnight culture of the enrichment broth of each presumptive sample EPEC, VTEC or ETEC was inoculated into MacConkey agar. The plates were incubated at 37 °C for 24 h. After incubation, presumptive colonies of each sample were analyzed by PCR for the presence of *vtx*$_1$, *vtx*$_2$, *eae*, *saa* (STEC autoagglutinating adhesin), *ehxA* (enterohemolysin), *estIa*, *estIb* and *elt* (Blanco et al., 1997; Paton and Paton, 2002; Woodward et al., 1992). Isolates were confirmed as *E. coli* by PCR amplification of *uspA* (Chen and Griffiths, 1998).

2.4. Determination of bfpA for EPEC strains

All EPEC strains were tested for the presence of *bfpA* by a monoplex PCR (Gunzburg et al., 1995). An *E. coli* O157:H45 *bfpA*-positive strain (kindly supplied by Dr. A. Bentancor, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Argentina) was used as positive control, and double distilled water as negative control.

2.5. Serotyping

Identification of somatic (O) and flagellum (H) antigens for the strains was performed by the microagglutination technique with specific antisera, as described by Blanco et al. (1992).

2.6. Detection of additional virulence factors

The isolates were also characterized by the presence of plasmid-encoded genes, non-LEE effector (*nle*) genes, genes encoding putative adhesion factors and genes encoding putative virulence determinants (described in the TW14359 O157 strain associated with the raw spinach outbreak in the U.S. in 2006). The additional virulence genes (35) amplified in this study are summarized in Table 1.

2.7. Data analysis

Taking into account the combinations of the genes detected/

Table 1
Additional putative virulence genes amplified in this study and references.

Target gene	Encoded protein	References
espP	Extracellular serine protease	Bustamante et al., 2011
katP	Periplasmic catalase peroxidase	
stcE	Zinc metalloprotease	
subA	Subtilase cytotoxin	
nleB2, *nleC*, *nleD*, *nleH1-1*	OI-36 non-LEE effectors	Coombes et al., 2008
nleG2-3, *nleG5-2*, *nleG6-2*	OI-57 non-LEE effectors	
nlA, *nleF*, *nleG*, *nleG2-1*, *nleG9*, *nleH1-2*	OI-71 non-LEE effectors	
ent/esp L2, *nleB*, *nleE*	OI-122 non-LEE effectors	
ecpA	*E. coli* common pilus	Hernandes et al., 2011
elfA	*E. coli* laminin-binding fimbriae	
hcpA	Pilin subunit hemorrhagic coli pilus	Xicohtencatl-Cortes et al., 2007
ihaA	IrgA homologue adhesin	Tarr et al., 2000
ehaA	EHEC autotransporter	Wu et al., 2010
lpfA$_{O113}$	Long polar fimbriae	
epeA	Serine protease autotransporter	Leyton et al., 2003
cdt-V	Cytolethal distending toxin	Cergolle-Novella et al., 2007
sfpA	Sorbitol-fermenting EHEC O157 fimbriae plasmid-encoded	Brunder et al., 2001
ECSP_0242	Virulence factor that facilitates protein-protein interactions	Kulasekara et al., 2009
ECSP_1773	Protein that interferes with innate immunity	
ECSP_2687	Protein that reduces the cytokine expression	
ECSP_2870/72	Gene regulating adaptation to plant host	
ECSP_3286	Outer membrane protein	
ECSP_3620	Nitric oxide reductase NorV	

undetected in isolates analyzed in the present study, a cluster analysis of virulence-associated genes was generated using the BioNumerics v.6.6 software.

2.8. Cytotoxicity assay

The cytotoxicity of the O178:H19 VTEC isolate was evaluated by an assay on Vero cell monolayers (Fernández et al., 2013). EDL933 was used as a positive control strain. The isolates were cultured in 5 ml of LB broth for 18 h at 37 °C with shaking. After an overnight growth, the cultures were centrifuged (10 min, 10,000 × *g*, 4 °C) and the supernatants were stored at − 20 °C.

Vero cells were grown in 96-well plates, at 37 °C in Eagle™ minimal essential medium (MEM) supplemented with 10% (vol/vol) fetal calf serum, 100 mg/l penicillin, 200 mg/l streptomycin, and 2.2 g/l $NaHCO_3$ in an atmosphere of 5% CO_2. Two-fold serial dilutions of the bacterial supernatants were added to each well and incubated at 37 °C under 5% CO_2 in air. Morphological changes of the cells were examined microscopically after 72 h of incubation. The cell monolayers were fixed and stained with 10% (vol/vol) formaldehyde and 0.2% (wt/vol) crystal violet in phosphate-buffered saline solution. Wells having partial or total cell destruction were considered positive.

3. Results

3.1. Presence of E. coli and diarrheagenic E. coli

The presumptive presence of *E. coli* and diarrheagenic *E. coli* pathotypes in the set of 373 vegetable samples, detected by specific virulence genes amplification on the enrichment broths, is shown in Table 2. *E. coli* was detected in 144 samples (38.6%). Lettuce and beet were the vegetables most frequently contaminated with *E. coli*. However, it should be noted that the number of samples of lettuce taken was much higher than the others because of the plant's commercial significance and because of repeated outbreaks linked to lettuce consumption. Fourteen samples positive for *vtx*$_1$, *vtx*$_2$, *eae*, *estIa* or, *estIb* were detected by screening of the enrichment by PCR.

3.2. Strain characterization and virulence genes

Ten isolates could be obtained from fourteen samples presumptively positive for diarrheagenic *E. coli* inoculated into MacConkey agar (Table 3). Eight strains were identified as aEPEC (*eae*-positive, *bfpA*–negative) and two strains were identified as VTEC (Rel5 and Rel3).

It was not possible obtain positive isolates on agar of two samples from lettuce that were found to be *vtx*$_2$-positive/*eae*-positive. Two samples were detected as ETEC positive, one from beet was STa (*estIa*) and the other one, from lettuce was STb (*estIb*). However, positive isolates could not be obtained.

In relation to the serotyping, nine isolates belonged to eight different serotypes and one isolate was non typeable. Two isolates, from lettuce and chard, were serotyped as O28:H19.

Table 3
Serotypes, pathotype, source, date and location sampling of VTEC and EPEC strains isolated from vegetables in Argentina.

N°	Isolate	Serotype	Pathotype	Source	Date	Sampling location
1	C5	O1:H7	aEPEC	Lettuce	Nov. 2013	Retail shop 1
2	A2V1	O28:H19	aEPEC	Chard	Dec. 2013	Retail shop 5
3	L45V1	O28:H19	aEPEC	Lettuce	Apr. 2014	Retail shop 11
4	L2O1	O39:H40	aEPEC	Lettuce	Dec. 2013	Organic farm 1
5	L66VE1	O86:H31	aEPEC	Lettuce	May 2014	Retail shop 11
6	L1683	O132:H8	aEPEC	Lettuce	Nov. 2014	Retail shop 34
7	L1581	O139:H20	aEPEC	Lettuce	Nov. 2014	Retail shop 24
8	L2051	O178:H7	aEPEC	Lettuce	Dec. 2014	Retail shop 6
9	Re15	O178:H19	VTEC	Beet	Jun. 2014	Retail shop 6
10	Re13	NT:NM	VTEC	Beet	Jun. 2014	Retail shop 6

Fig. 1 shows the distribution of virulence-associated genes in the 10 *E. coli* isolates. Both O28:H19 isolates shared the same virulence profile and were recovered from the same sampling site, but they were not temporally related. VTEC isolates were *vtx*$_2$-positive or *vtx*$_1$-*vtx*$_2$-positive and only one of them (O178:H19) harbored the *ehxA* gene, encoding an enterohemolysin.

Taking into account the distribution of *nle* genes, 7 profiles were detected. The isolate C5 (O1:H7) and both VTEC isolates, Re15 (O178:H19) and Re13 (NT:NM), were negative for all *nle* genes. On the other hand, according to the *nle* profile, the O132:H8 isolate showed complete most of the pathogenicity islands (PAIs) (OI:57, OI:71 and OI:122) and partially complete OI:36.

All tested isolates harbored the gene encoding for the adhesin HcpA. Other adhesion related genes were also identified. The genes *ecpA* and *elfA* were detected in 90%, *lpfA*$_{O113}$ in 60%, and *ehaA* in 50% of the isolates. The *ihaA* gene was only detected in the O178:H19 isolate meanwhile *saa* and *sfpA* genes were not found. The presence of cytolethal distending toxin gene *cdt-V* was demonstrated in two isolates belonging to O1:H7 and O178:H19 serotypes, whereas megaplasmid encoding genes such as *espP*, *subA* and *epeA* were found only in this last VTEC isolate but *katP* and *stcE* were not detected. The gene ECSP_3286 (gene encoding an outer membrane protein that facilitate heme complex extracellular transportation) was present in all the isolates, ECSP_2687 (gene encoding a protein that reduces the expression of cytokines), only in one and, the remaining putative virulence determinants were not detected.

The only VTEC typeable isolate was cytotoxically evaluated by an assay on Vero cells. This O178:H19 isolate (Re15) exhibited a strong cytotoxic effect on agar plates, even more pronounced than that showed by EDL933 strain (O157:H7).

4. Discussion

Escherichia coli is an important indicator microorganism of fecal contamination in food. *E. coli* prevalence was of around 40% in

Table 2
Occurrence of presumptive *E. coli* and diarrheagenic *E. coli* detected by specific virulence genes amplification (PCR) in vegetable samples from Argentina.

Commodity	No. of samples	*E. coli* (*uspA*)	EPEC (*eae*)	EPEC/VTEC (*vtx*$_1$ or *vtx*$_2$ and *eae*)	VTEC (*vtx*$_1$ or *vtx*$_2$)	ETEC (*estIa, estIb or elt*)
Lettuce	267	110 (41)	7 (2,6)	3 (1,1)	n.d.	1 (0,4)
Beet leaves	48	21 (44)	n.d.	n.d.	1 (2,1)	1 (2,1)
Spinach	19	2 (10,5)	n.d.	n.d.	n.d.	n.d.
Parsley	16	4 (25)	n.d.	n.d.	n.d.	n.d.
Chard	13	5 (38,5)	1 (7,7)	n.d.	n.d.	n.d.
Arugula	5	2 (40)	n.d.	n.d.	n.d.	n.d.
Ready-to-eat salads	5	n.d.	n.d.	n.d.	n.d.	n.d.
Total	373	144 (38,6)	8 (2,1)	3 (0,8)	1 (0,3)	2 (0,5)

The numbers in parenthesis represent percent values.
E. coli, *Escherichia coli*; EPEC, *Enteropathogenic E. coli*; VTEC, *Verocytotoxigenic E. coli*; ETEC, *Enterotoxigenic E. coli*; n.d., not detected.

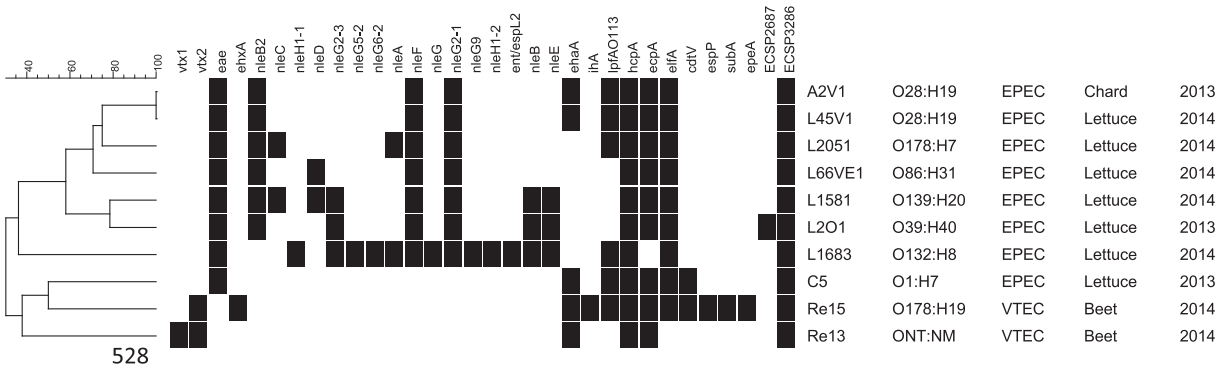

Fig. 1. Cluster analysis of virulence-associated genes in *Escherichia coli* isolated from vegetables in Argentina. The dendrogram was generated using the BioNumerics v.6.6 software. The presence (black) or absence (blank) of genes, isolate denomination, serotype, *E. coli* pathotype, source and isolation year are shown.
Genes not found in any of the studied strains: *saa*, *sfpA*, *katP*, *stcE*, ECSP_0242, ECSP_1773, ECSP_2870/72, and ECSP_3620.

vegetable samples from Argentina. Previous studies carried out in different countries showed heterogeneous *E. coli* prevalence data in vegetables. In Brazil, Oliveira et al. (2012) detected *E. coli* in 53% of the leafy vegetables; in Istambul, Turkey, Ozpınar et al. (2013) showed that 60% of vegetable samples were positive for *E. coli*; in Iran, around 50% vegetables were contaminated with *E. coli* and from these, 87% were VTEC (Shakerian et al., 2016) and, in Alexandria, Egypt, presumptive *E. coli* O157 isolates were detected in 71% of leafy greens samples (Khalil et al., 2015). On the contrary, in some other countries, lower prevalence was reported. In Finland, *E. coli* was isolated from 15% of packaged fresh leafy vegetables (Nousiainen et al., 2016). A similar percentage was detected in lettuce from Canada (Wood et al., 2015) and in Switzerland it was only found in 2% of lettuce (Althaus et al., 2012).

In this study, EPEC predominated over VTEC contamination. The *bfpA* gene was not detected in any of the samples, indicating that *eae*-positive vegetables were contaminated with aEPEC, an emerging diarrheagenic pathogen in developing and industrialized countries (Contreras et al., 2010; Estrada-Garcia et al., 2009). In Argentina, Alonso et al. (2012) detected contamination with aEPEC in 20% from chicken and their products and VTEC/aEPEC in 2.4% of them. Silveyra et al. (2015) described the isolation of an aEPEC O157:H16 from a bloody diarrhea case in a child occurred in La Pampa.

The isolates were primarily of serotypes that have been seldom associated with human diseases in Argentina. However, some of them belonged to serotypes and pathotypes associated with patients in other countries. Moreno et al. (2010) reported an O39:H40 aEPEC isolated from a child with diarrhea in Brazil and, several strains O178:H7, both EPEC and VTEC, have been isolated from humans in Australia and Germany (Miko et al., 2014; Prager et al., 2009; Staples et al., 2013). In relation to O178:H19 strains, they have been previously identified among patients from Germany (Bielaszewska et al., 2006; Werber et al., 2008) and Brazil (De Toni et al., 2009) and from HUS cases in Belgium (Buvens et al., 2010) and Argentina (Miko et al., 2014), as well as from cattle and meat and chicken-derived food in the Central Eastern area of Argentina, the region considered in this study (Alonso et al., 2016; Blanco et al., 2004; Galli et al., 2010; Kruger et al., 2011; Sanz et al., 2007).

Genetic characterization showed that in addition to *eae* or *vtx* genes, isolates harbored genes encoding other toxins, adhesins, and components related to the type III secretion system that could contribute to their virulence. The analysis based on the presence/absence of genes associated with virulence identified two main clusters, one containing VTEC plus an EPEC isolate (C5) which lacked *nle* genes and another one with the remaining EPEC isolates. The VTEC isolates were *eae*-negative so that was an expected result, but C5 is an *eae*-positive strain that noticeably lack of any *nle*. The gene ECSP_3286 found in all the isolates was postulated, together with other six, as a putative virulence factor that could be responsible for the high virulence of a spinach strain responsible for an outbreak occurred in USA (Kulasekara et al., 2009).

This research shows that vegetables in Argentina may be a source of VTEC and EPEC infections in the community and therefore, they should be considered as vehicles for transmission of these potentially pathogenic bacteria. Contamination of vegetables can occur in different steps of the food production. Pre-harvest contamination through VTEC-excreting animals, soil, water and application of manure as fertilizer on fields is regarded as most important (Franz and van Bruggen, 2008). However, all pathogenic (EPEC/VTEC) isolates obtained came from retail shops, not from farms. Therefore, vegetables, mostly, could be contaminated or cross-contaminate during transport or by food-handler. These strains are potential foodborne pathogens and their presence in fresh vegetables points to the need to keep a careful watch over these foods and to emphasize for more educational efforts on producer, handler, seller and consumer.

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