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Full Length Research Paper

Isolation of *Escherichia coli* O157:H7 from manure fertilized farms and raw vegetables grown on it, in Tabriz city in Iran

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Concern for pathogens in vegetables has risen because of increasing number of outbreaks of foodborne illnesses caused by consumption of fresh and minimally processed vegetables. Using bovine feces as fertilizer, which is the common organic fertilizer on farmlands in Iran, can pose risk of spreading and transmission of enteric pathogens such as *Escherichia coli* O157:H7 both by direct contact and consumption of produces grown on such farms. We obtained a total of 282 samples of soil and vegetables (lettuce, cabbage, carrot and radish sprout), from manure fertilized agriculture farms in Tabriz city. Phenotypic/biochemical characteristics followed by biochemical tests were performed. Multiplex PCR technique was used to confirm *E. coli* O157:H7 isolates. The data indicated that 5 samples of soils (1.77 %) and one sample of vegetable (0.35 %) of total samples were contaminated with *E. coli O157:H7*. Organic fertilizer used in farm lands in this study poses a supposed low risk in vegetables.

Key words: Foodborne, bovine feces, *Escherichia coli* O157:H7, raw vegetables, multiplex PCR.

INTRODUCTION

Increased consumption of fresh and raw vegetables has increased the focus on how the microenvironment of these kinds of food affects produce safety. Concern for pathogens in vegetable has risen because of increasing number of outbreaks of foodborne illnesses caused by consumption of fresh and minimally processed vegetables (Tauxe et al., 1997). There is a great variation in the number of microorganisms on vegetables, and factors like nutritional substances, microbial competition, structural damages on plant (wounds) and the potential in internalization of pathogens, affect the proliferation of pathogens on vegetables. However, when infective dose of pathogens is low, the persistence of microorganisms is as important as proliferation of these pathogens (Aruscavage et al., 2006).

In organic agriculture, manure is widely used as fertilizer and use of manure has led to concern for the potential of contamination of minimally processed vegetables such as salad vegetables (e.g. lettuce, cabbage, cucumber and carrot) with enteric pathogens (Ingham et al., 2004). In Iran, bovine manure is commonly used as fertilizer and it is well documented that cattle is the predominant reservoir of shiga-toxin producing *Escherichia coli* (STEC). All STEC produce cytotoxic factors to African green monkey kidney (Vero) cells and hence has been named verotoxin (VT) or shigatoxin (stx) because of similarity with shigatoxin produced by Shigella dysenteriae type1 (Obrien et al., 1992). In the UK, Advisory Committee on dangerous pathogens (ACDP) classify STEC into hazard group 3, because of the low infective dose, the potential severity of the infection and the possibility of laboratory-acquired infections (Pimbley, 1999).

There are at least 100 serotypes of STEC and of this group, *E. coli O157:H7* first recognized as foodborne pathogen in 1982, is the most well known microorganism. Clinical symptoms of the disease caused by these bacteria range from mild diarrhea and hemorrhagic colitis (HC) to complicated infections such as hemolytic uremic

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syndrome (HUS) and thrombocytopaenic purpura. (Meng et al., 2007; Willshaw et al., 2001; Law, 2000). The main transmission route of *E. coli O157:H7* outbreaks in the US is foodborne and raw vegetables, particularly lettuce, alfalfa and vegetable sprouts have been implicated in several outbreaks of *E. coli O157:H7* infections. (Meng et al., 2007). The present study was undertaken in order to investigate the distribution of *E. coli O157:H7* in the soil and vegetables obtained from manure fertilized agricultural farms in Tabriz. We used phenotypic and biochemical characteristics to detect this microorganism and finally, isolates were confirmed by multiplex PCR (m-PCR) technique.

MATERIALS AND METHODS

Sample collection

This study was carried out on agriculture farms fertilized with cow manure in Tabriz city between 23rd July and 22nd October 2008. A total of 282 samples of soils and vegetables were collected from 27 farm producers. Vegetables included in this study were lettuce, cabbage, carrot and radish sprout that could be consumed without cooking. All samples were packed in separate sterile plastic bags together with ice in cardboard boxes and transported to the laboratory immediately after sampling and were analyzed after arrival in the laboratory.

Enrichment

Twenty five grams of each sample (vegetable or soil) were added to 225 ml of buffered peptone water and incubated at 36°C for 12 - 18 h, after homogenization for one minute.

Isolation by selective plating

Each enrichment culture was serially diluted 10-fold up to 10^{-5} in 0.1% peptone water and 100 µl volumes of the 10^{-4} and 10^{-5} dilutions were spread onto Sorbitol–MacConkey agar supplemented with 0.05 mg/l Cefixime and 2.5 mg/l Potassium-tellurite, (CT-SMAC agar). The plates were incubated at 36°C for 18 to 24 h (Chapman et al., 1991; Zadik et al., 1993).

Confirmation tests

Sorbitol- non fermenting colonies that were colorless, (up to 10 colonies per sample) were selected for verification. These colonies were tested for catalase and oxidase activity and catalase positive and oxidase negative colonies were confirmed to be *E. coli*, using Indole test with SIM medium (Merk, Germany) and citrate utilization test with Simmons citrate agar (Merk, Germany) and the Methyl red and Voges-proskaure tests using MR-VP medium (Merk, Germany) (Harrigan,1998).

PCR test

Presumptive colonies and *E. coli O157:H7* that were used as positive control (ATCC 25922) were grown in 5 ml of Brain Heart Infusion (BHI) broth for 12 h at 36°C and then the DNA was extracted according to the protocol below:

a) Centrifuge 1.5 ml of prepared broth in eppendrof tube at 10000 rpm for 5 min and discard the supernatant.
b) Add 800 µl of lyses buffer (containing 5 mol NaCl, 100 mmol Tris-base, 20 mmol EDTA-$Na_{2\ and}$ CTAB 20%) and homogenize the sample with shaker.
c) Incubate the tube at 65°C for 10 to 20 min and centrifuge again at 12000 rpm for 10 min.
d) Add equal volume of chloroform-isoamylalcohol (24:1) to the supernatant.
e) Centrifuge the tube at 12000 rpm for 10 min and transfer the supernatant to a new tube.
f) Add equal volume of -20°C isopropanol and keep the sample at -20°C for 30 min.
g) Centrifuge the tube at 12000 rpm for 10 min and discard the supernatant.
h) Add 250 µl of 4°C ethanol (70%) and centrifuge at 10000 rpm for 5 min.
i) Discard the supernatant and let the pellet to be dry at room temperature.
j) Dissolve the dried pellet in 50 µl of sterile distilled water.

The oligonucleotide primers (MWG, Germany) used in this study are listed in Table 1. PCR assay was performed in 25 µl volume containing 50 ng of extracted DNA, 2.5 µl PCR buffer mix, 0.1 µl (each) 2-deoxy nucleoside 5-triphosphate, 0.1 µl of eaeA O_{157} and fliC h_7 and 0.2 µl of $stx1_1$ and stx2, 0.5 µl of Taq DNA polymerase and 1.1 µl $mgcl_2$. The reaction was carried out with amplification thermal cycler (Eppendrof model 22331, Germany). The procedure consisted of initial denaturation at 94°C for 4 min, followed by 35 cycles of templet denaturation at 94°C for 30 s, primer annealing at 62°C for 30 s and extention at 72°C for 75 s. The final cycle was followed by incubation of the reaction mixture for 10 min at 72°C.

$$\left(\frac{}{wt\ vol}\right)$$

PCR products were analyzed by electrophoresis with 1.5% agarose gels containing 0.5 mg of ethidium bromide per ml. These were visualized with UV illumination and photographed. DNA standard molecular weight size marker (ladder) was included in each agarose gel electrophoresis run.

RESULTS

The phenotypic and biochemical characteristics of isolates are shown in Table 2. Presumptive *E. coli* O157:H7 was detected in 17 samples (including 8 samples of soil and 9 samples of vegetables) of total 282 samples. Of 17 presumptive *E. coli* O157:H7, 4 isolates obtained from farm number 13 (3 samples of soil and one sample of lettuce grown on it) and 2 samples of farm number 19 (2 samples of soil of cabbage producing farms) were confirmed as *E. coli* O157:H7 detecting the genes encoding intimine (*eaeA* O_{157}), flagellar H7 (*fliC* h_7) and shigatoxins (*stx*)1+2 by multiplex PCR technique (Figure 1) . Also, we used simple PCR assay to detect the presence of each stx1 and stx2 genes in 6 positive isolates and as shown in Figure 2, all six samples contained both stx1 and stx2 genes.

DISCUSSION

The data presented in this study have shown that 5 samples of soils (1.77 %) and one sample of vegetable

Table 1. Primer sequences and predicted length of PCR amplification products.

Primer	Direction	Primer sequence (5′-3′)	Size	Reference
stx$_1$	Forward Reverse	CATTGTCTGGTGACAGTAGCT CCCGTAATTTGCGCACTGAG	732	Gannon et al., 1997
stx$_2$	Forward Reverse	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCACTTTG	779	Gannon et al., 1997
eaeA O$_{157}$	Forward Reverse	CAGGTCGTCGTGTCTGCTAAA TCAGCGTGGTTGGATCAACCT	1087	Gannon et al., 1997
fliC h$_7$	Forward Reverse	GCGCTGTCGAGTTCTATCGAGC CAACGGTGACTTTATCGCCATTCC	625	Gannon et al., 1997

Table 2. Phenotypic/biochemical characteristics of soil and vegetable samples.

Samples		No. of samples	No. of samples contain colonies that were		
			Sorbitol Negative	Oxidase negative and catalase positive	Indole and MR positive and VP and citrate negative
Lettuce	Soil	41	32	12	4
	Vegetable	41	34	9	4
Cabbage	Soil	38	30	8	3
	Vegetable	38	27	9	4
Carrot	Soil	27	21	5	0
	Vegetable	27	21	6	0
Radish sprout	Soil	35	30	11	1
	Vegetable	35	30	15	1
Total		282	225	75	17

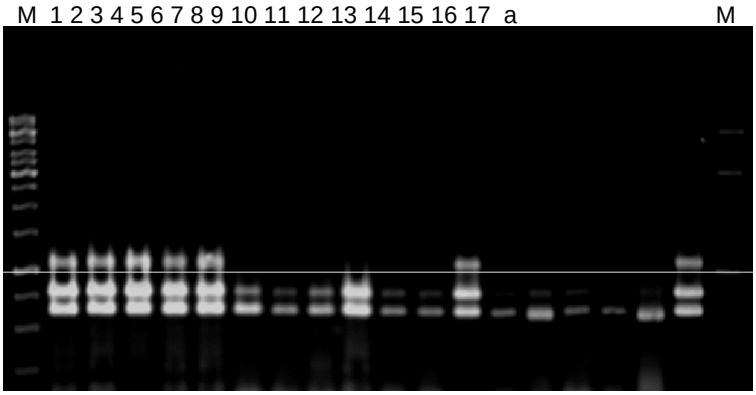

Figure 1. Multiplex PCR assay with stx$_{1+2}$, eaeO$_{157}$ and fliCh$_7$ oligonucleotide primer pairs. Lanes M; molecular size marker, Lane a; positive control, Lanes 1 to 17; 17 presumptive *E. coli O*$_{157}$*: H*$_7$ isolates.

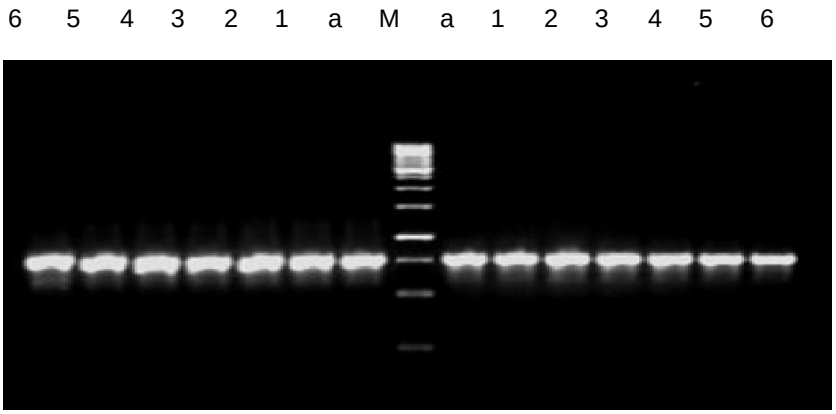

Figure 2. PCR assays with stx_1 and stx_2 oligonucleotide primer pairs. (Side A) Agarose gel of stx_1; (side B) Agarose gel of stx_2. Lane M: molecular size markers, Lanes a: positive controls, Lanes 1 to 6: 6 positve samples.

(0.35%) of total 282 samples obtained from manure fertilized agriculture lands in Tabriz were contaminated with *E. coli* O157:H7. The results of this study may conclude that the distribution of this microorganism is rather low. One possible reason could be that in our region, farmers generally use the manure on lands, in late winter and there is a rather long period of time between incorporation of manure into soil and harvesting the vegetables, so the pathogen number can be reduced. An isolation plating medium adapted for use in processes for the presumptive detection of *E. coli* O157:H7, generally contain ingredients that inhibit the growth of strains of other microorganisms under incubation (Szabo et al., 1986; Vernozy-Rozand, 1997). But it is clear that the presence of interfering bacteria, hinders the isolation of *E. coli* O157:H7 from sources with high bacterial counts such as soil samples. For example aerobic bacteria such as *Pseudomonas* spp., are widely distributed in nature and in plants that hinder isolation of *E. coli* O157:H7. In addition, presence of sorbitol negative, gram negative facultative anaerobic rods other than *E. coli* O157:H7 have been isolated in plants that produce colorless colonies in CT-SMAC agar medium and can hamper the isolation of *E. coli* O157:H7 (Fujisawa et al., 2002).

Therefore, it is possible that *E. coli* O157:H7 colonies were missed and that the actual prevalence is higher than the one reflected by the isolates obtained in this study. However, it can also be possible that the prevalence of *E. coli* O157:H7 in this study was indeed low. As shown in Table 2 , of total 282 samples, 225 samples contained sorbitol negative colonies and just 75 samples were confirmed by determining catalase and oxidase activity. So these tests seem to be efficient as screening test for isolation of *E. coli* O157:H7 from vegetables and soils samples. Also, it should be noticed that using some detection methods pose some difficulties to efficient and economical diagnosis of *E. coli* O157:H7. For example, using ingredients such as MUG (4-methyl umbelliferyl -D-glucoronide) will be useful for differentiation of *E. coli* O157:H7 from other *E. coli* stains (Typically 90 - 95% of the investigated *E. coli* strains have glucoronidase activity and produce 4-methyl umbelliferone from hydrolysis of MUG which is detectable because it shows blue fluorescence when illuminated with UV light (Frampton et al., 1993), but STEC O_{157} are glucoronidase negative and cannot hydrolysis MUG and do not produce blue illumination). But a major consideration when using MUG is that, when many glucoronidase positive colonies are grown on a single agar plate, it is necessary to separate colonies and restreak them on the other CT–SMAC agar medium to detect glucoronidase negative colonies, because this fluorogenic substance is water soluble and diffuse through the agar and is present in entire plate, therefore high number of MUG-positive flora will prevent the recognition of MUG- negative colonies after overnight incubation (Fujisawa, 2002). Other considerations when using some culture media such as rainbow agar or chromagar O_{157} that contain chromogenic, water insoluble substances that can be observed in normal daylight, is the cost of media because these media are expensive for the routine analyses of foods.

Cattle are thought to be the main source of STEC, and perhaps important way of spreading of these groups of pathogens on farmland is bovine manure/slurries (Duffy, 2003; Johannessen et al., 2004).Studies on prevalence of *E. coli* O157:H7 in cattle feces in different countries show a wide variety of positive cases from 0.1 to even 62% (Duffy, 2003). On the other hand, these groups of pathogens have been isolated from different animals and wild life such as ruminants, dog, cat wild birds and

rodents. Therefore, further studies are necessary to determine the prevalence of *E. coli* O157:H7 in cattle herds in our region to have accurate judgment about the possibility and range of transmission of this pathogen to agricultural farms from the origin of cow manure. In the farmlands examined in this study, *E. coli* O157:H7 was found to be able to persist for a long time in the soil and could contaminate crops such as raw vegetables. Long term persistence of this organism that poses a risk of transmission of pathogens both by direct contact or ingestion of produce grown in contaminated farmlands has been demonstrated previously (Avery et al., 2005; Fremaux et al., 2007; Fukushima et al., 1999; Lau and Ingham, 2001)

Finally, alternative strategies such as developing practical and economical treatment like composting, heat drying or digestion to reduce/eliminate pathogens from animal manure is needed to reduce the prevalence of STEC in farm environment and farmers should be prohibited from using raw manure on agriculture lands within at lest 3 months of harvest or are required to use treated manure to reduce pathogens.

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