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Quantification of *Escherichia coli* O157:H7 in organic vegetables and chickens

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Abstract

The organic foods' market is becoming one of the rapidly growing sections in agricultural economies in the world. During the last two decades, food-borne outbreaks associated with fresh produce have rapidly increased. *E. coli* O57:H7, the caustic agent of acute hemorrhagic diarrhea and abdominal cramps, is mainly associated with meat and poultry product outbreaks but frequent outbreaks linked to the consumption of vegetables have been reported. The aim of this study was to investigate prevalence of *E. coli* O157:H7 in some organic foods. A total of 230 organic food samples including four-winged bean, tomato, white radish, red cabbage, chinese cabbage, lettuce, cucumber and chicken form retailed groceries and supermarkets in Malaysia were investigated. Low prevalence of *E. coli* O157:H7 was detected in organic vegetables and chickens. The estimated quantity of *E. coli* O157:H7 in all samples ranged from <3 to >2400 MPN/g. The overall MPN/g estimate of *E. coli* O157:H7 in the samples from organic groceries was higher than supermarket with the maximum of >2400 MPN/g. Most of the samples from supermarket showed a minimum of <3 MPN/g. The specific target genes produced amplicons of 259 bp and 625 bp after PCR amplification and *E. coli* O157:H7 was detected in 5.2% of total organic samples. Prevalence of *E. coli* O157: H7 in organic foods from groceries (8.8%) was particularly higher than supermarkets (1.0%). The highest prevalence of *E. coli* O157:H7 was observed in organic chickens (40%) purchased from groceries followed by four-winged bean (10%) and white radish (3.3%).

Introduction

Modern agricultural practices have several potential hazards including the use of pesticides and fertilizers. Pesticides residues in food are associated with long-term effects on human health. Human concern about safer food in combination with the growing environmental awareness resulted in an increasing demand for organic foods. Organic foods are not genetically modified and no synthetic chemical (such as fertilizers and pesticides) is used in their production (Chen 2007). Organic foods are claimed to be more respectful to the environment (Falguera *et al*., 2012) and consumers consider organic foods to be safer and healthier (Sheng *et al*., 2009; Adl *et al*., 2011). The mentioned factors made the organic foods' market to become one of the rapidly growing sections in agricultural economies in the world (Chen 2007).

The organic market of the South-East-Asia is one of the most important in Asia but the market size is smaller than Europe and North America (OTA, 2006). Huge amounts of organic foods including vegetables (e.g. cucumbers, tomatoes and courgettes), leafy vegetables (e.g. Spinach, kale, lettuce and cabbage) and fruits (bananas) are produced in Malaysia. Malaysia set voluntary national standards for some organic products based on IFOAM regulations (ITC, 2011) but most of the consumers still show greater attention and trust in organic foods produced

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according to non-Asian standards. The health benefits and risks related to organic foods are major issues in food safety and security (Carvalho, 2006).

During the last two decades, food-borne outbreaks associated with fresh produce have rapidly increased (Warriner *et al*., 2009). Low sanitation standards were always reported in such outbreaks. The other potential hazards that increase the chance of food-borne diseases associated with fresh produce include applications of chemical fertilizers, hormones, pesticides, and their residues in food (Chen, 2007).

Minimally processed fruits and vegetables require no further treatment and are usually eaten raw. Although most of the consumers consider such minimally processed products as safe products, but their potential safety problems have been reported. Minimally processed vegetables and fruits may be contaminated with food-borne pathogens (such as *Listeria monocytogenes*, *Salmonella* and *Escherichia coli* O157:H7) (Sela *et al*., 2009; Olaimat and Holley, 2012) as they are nutritious foods and current industrial sanitising washing treatments are not capable of full elimination of food-borne pathogens. *E. coli* O157:H7 was reported to be responsible for the outbreaks linked to the consumption of fresh vegetables and fruits (such as lettuce, spinach, carrots, radish sprouts, alfalfa, unpasteurised apple cider, melon and berries) (Parish *et al*., 2003; Rangel *et al*., 2005; Abadias *et al*., 2008, 2010; Pérez Rodríguez *et al*., 2011). *E. coli* O157:H7 was responsible for 7% of fresh produce outbreaks in US (Olaimat and Olaimat, 2012).

Outbreaks studies showed that *E. coli* O157:H7 was able to survive and grow in different types of minimally processed vegetables and fruits (Abadias *et al*., 2012). The large nation-wide spinach outbreak in US (2006) was linked to the environmental *E. coli* O157:H7 from the field (Grant *et al*., 2006). In 2006, *E. coli* O157:H7 contamination of lettuce and spinach resulted in 81 and 199 cases (3 deaths) in US (CDC 2006; FDA 2007; Sela *et al*., 2009; CDC, 2011). In 2008, *E. coli* O157:H7 contamination of lettuce resulted in 134 cases in Canada (Warriner and Namvar, 2010). Most of the outbreaks were related to the packaged product. Products had been washed and disinfected with hypochlorite before packaging but the process were insufficient to eliminate *E. coli* O157:H7 and prevent infections (Pérez Rodríguez *et al*., 2011).

E. coli O157:H7 has the ablity to survive at refrigeration temperatures and under harsh environmental conditions. Some strains were reported to tolerate low pHs (Pérez Rodríguez *et al*., 2011). *E. coli* O157:H7 cause acute hemorrhagic diarrhea with abdominal cramps that in its severe cases result in hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Hedican *et al*., 2009; Zhu and Hill, 2013). *E. coli* O157:H7 produces potent toxins called Shiga toxins (*Stxs*) that cause severe damage to the lining of the intestine (Hedican *et al*., 2009). Young children, immunocompromised individuals and the elderly are more susceptible to hemorrhagic colitis and show more serious symptoms (Ateba and Mbewe, 2011; FDA, 2012).

Over the past two decades, consumption of fresh produce has increased for the following reasons; fresh produce is recognized as an important source of vitamins, nutrients and fibre (Olaimat and Olaimat, 2012), consumers' awareness of health aspects of vegetables and fruits have increased and consumers are more concerned about correct eating habits and staying healthy. But at the same time outbreaks of food-borne illnesses related to fresh produce have increased (Warriner *et al*., 2009).

E. coli O57:H7 is mainly associated with meat and meat product outbreaks (Abong'o and Momba, 2009) but frequent outbreaks linked to the consumption of dairy products and undercooked ground beef and chicken were reported (Belongia *et al*., 2003). According to Doyle and Schoeni (1987), 1.5% of poultry carcasses slaughtered in Madison, US were contaminated with *E. coli* O57:H7 and 9.25% of swab samples from poultry at a slaughterhouse in Slovakia were contaminated with Shiga-toxin positive *E. coli* O157 (Pilipcinec *et al*., 1999). Fode-Vaughan *et al*. 2003, used PCR technique for the detection of *E. coli* O157:H7. Organic chicken has received grate attentions as are claimed to be antibiotic and hormone free. Few studies related to *E. coli* O157:H7 contamination of organic foods has been reported in Malaysia. The objective of this study was to study the prevalence of *E. coli* O157:H7 in some organic foods marketed in Malaysia.

Materials and Methods

Sample collection

A total of 230 samples including organic chicken and six types of organic vegetables (Table 1) were purchased randomly from two supermarkets and two organic groceries in Selangor, Malaysia. Samples were collected in sterile plastic bags and analyzed immediately upon arrival to the laboratory.

Most Probable Number (MPN) method

In a sterile stomacher bag, 10 g of sample was added with 90 ml of Tryptic Soy Broth (TSB; Bacto, France) and homogenized for 60 s. The mixture was

Table 1. Organic vegetables from supermarket and groceries in Selangor, Malaysia

Local name	English name	Scientific name	Total number of samples (x)
Ulam	Four – winged bean	*Psophocarpus tetragonolobus*	30
Tomato	Tomato	*Lycopersicon esculentum*	30
Timun	Cucumber	*Cucumis sativus*	30
Lobak putih	White radish	*Raphanus sativus*	30
Lettuce	Lettuce	*Lactuca sativa*	30
Kubis cina	Chinese cabbage	*Brassica rapa*	30
Kubis merah	Red cabbage	*Brassica oleracea*	30
Total			210

Table 2. Primer sequences for the detection of *Escherichia coli* O157:H7 using a multiplex PCR

Primers	Primer sequence (5' to 3')	Target gene	Amplicon size	Reference
flicH7-F	GCG CTG TCG AGT TCT ATC GAG	*flic H7*	625 bp	Sarimehtoglu *et al.*,2009
flicH7-R	CAA CGG TGA CTT TAT CGC CAT TCC			
rfb O157-F	CGG ACA TCC ATG TGA TAT GG	*rfb O157*	259 bp	Jamshidi *et al.*, 2011
rfb O157-R	TTG CCT ATG TAC AGC TAA TCC			

incubated at 37ºC for 24 h. To perform the three-tube MPN, 100 fold and 1000 fold dilutions of the stomacher fluids were prepared. One ml of the aliquot from each dilution was transferred into triplicate MPN tubes, and then incubated at 37ºC for 24 h. The positive MPN tubes were then subjected to PCR for the detection of *rfbO157* and *flicH*$_7$ genes specific for *E. coli* O157:H7.

DNA extraction and PCR amplification

MPN tubes showing visible turbidity were used for DNA extraction using a modified boil cell method (Tunung *et al*., 2010). Briefly, 1 ml of aliquot from each MPN tube was centrifuged at 12,000 x g for 3 min. The pellet was resuspended with 500 µL of TE buffer and after mixing was boiled for 10 min. Boiled mixture was cooled at -20˚C for 10 min and centrifuged at 12,000 x g for 5 min. Final supernatant was used as template for PCR amplification. *rfbO157* and *flicH*$_7$ genes were detected by multiplex PCR. The sequence of the two primer pairs is shown in Table 2. *rfbO157* primers are specific for somatic antigen (O157) and *flicH*$_7$ primers are specific for the flagellar antigen (H7).

PCR amplification was performed in a 25µl reaction mixture consisting of 5 µl of 5 x PCR buffer, 10 mM of deoxynucleoside triphosphate, 25 mM $MgCl_2$, 5U/µl *Taq* polymerase, 2 µl of DNA template and 0.5 µM of each primer. The thermal cycling started with pre denaturation at 94^{o}C for 2 min, followed by 35 cycles of 1 min denaturation at 94^{o}C, 1 min primer annealing at 55oC, 1 min elongation at 72^{o}C, and the final extension at 72^{o}C for 10 min. Amplified products were electrophoresed in 1.0% agarose gel at 100 V for 30 min. PCR products were visualized under UV light after staining by ethidium bromide. The expected sizes of amplicons for *rfbO157* and *flicH*$_7$ genes were 259 bp and 625 bp respectively.

Results and Discussion

Results of prevalence and MPN-PCR for detection of *E.coli* O157:H7 in organic vegetables and chicken is summarized in Table 3 and 4. The results showed higher frequency of *E. coli* O157:H7 in samples from groceries compared to the sample from supermarkets (Table 3). Results showed low level of contamination in Malaysian organic vegetables. As mentioned before *E. coli* O157:H7 was reported in vegetables. In a recent study Oliveira *et al*., reported that *E. coli* O157:H7 was present in 53.1% of minimally processed leafy vegetable samples in Brazil (Oliveira *et al*., 2011) which is quite high.

The estimated quantity of *E. coli* O157:H7 in all samples ranged from <3 to >2400 MPN/g. The overall MPN/g estimate of *E. coli* O157:H7 in the samples from organic groceries was higher than supermarket with the maximum of >2400 MPN/g. Most of the samples from supermarket showed a minimum of <3 MPN/g and the maximum of 19MPN/g.

The target genes specific to *E. coli* O157:H7 produced amplicons of 259 bp and 625 bp (Figure 1). *E. coli* O157:H7 was detected in 5.2% of total organic samples (Table 3). Prevalence of *E. coli* O157: H7 in organic foods purchased from groceries were particularly higher (8.8%) than supermarkets (1.0%) (Table 3). Among organic vegetables four-winged bean (10%) and white radish (3.3%) were contaminated with *E. coli* O157:H7.

The highest prevalence of *E. coli* O157:H7 was observed in organic chickens (40%) purchase from the groceries. In a recent study conducted by Ateba and Mbewe in South Africa, the prevalence of *E. coli* O157:H7 in pork meat, cattle and beef, and water samples was reported to be 67.7%, 27.7% and 2.3%, respectively (Ateba and Mbewe, 2011).

E. coli O157:H7 contamination of organic foods is usually originated from environment, human or animal sources (WHO/FAO, 2008). Contamination of organic products can occur at harvesting or processing stages. Food handlers act as another source of contamination and the surroundings area of stalls or grocery shops is usually less hygienic. In Malaysia, farmers usually send their organic vegetables directly to the grocery shops early in the morning. Unlike supermarkets that have dedicated sections for each organic product, organic vegetables in groceries are in close contact with other vegetables and are kept together in the same plastic polystyrene.

As shown in Table 3, supermarket samples were less contaminated. Organic products in the studied

Table 3. Prevalence of *E. coli* O157:H7 in organic vegetables and chickens

Organic foods	Supermarkets		Organic groceries		Total	
	No.[a]	%[b]	No.[a]	%[b]	No.[a]	%[b]
Four- winged bean	1/14	7.1	2/16	12.5	3/30	10.0
Tomato	0/17	0.0	0/13	0.0	0/30	0.0
White radish	0/15	0.0	1/15	6.7	1/30	3.3
Red cabbage	0/15	0.0	0/15	0.0	0/30	0.0
Chinese cabbage	0/15	0.0	0/15	0.0	0/30	0.0
Lettuce	0/16	0.0	0/14	0.0	0/30	0.0
Cucumber	0/13	0.0	0/17	0.0	0/30	0.0
Chicken	-	-	8/20	40.0	8/20	40.0
TOTAL	1/105	1.0	11/125	8.8	12/230	5.2

[a]Number of positive samples/number of samples examined.
[b]Frequency (in %) of positive samples among the samples examined.

Table 4. Densities (MPN/g) of *E. coli* O157:H7 in organic food samples

Organic foods	Supermarkets			Organic groceries		
	[a]Min	[b]Med	[c]Max	[a]Min	[b]Med	[c]Max
Four- winged bean	<3	<3	19	<3	3	>2400
Tomato	<3	<3	<3	<3	<3	<3
White radish	<3	<3	<3	<3	<3	9.1
Red cabbage	<3	<3	<3	<3	<3	<3
Chinese cabbage	<3	<3	<3	<3	<3	<3
Lettuce	<3	<3	<3	<3	<3	<3
Cucumber	<3	<3	<3	<3	<3	<3
Chicken	[d]_	[d]_	[d]_	<3	9.1	1100
Min/Med/Max	<3	<3	<3	<3	<3	>2400

[a]Min= Minimum MPN/g value
[b]Med= Median MPN/g value
[c]Max= Maximun MPN/g value
[d]= Sample not available

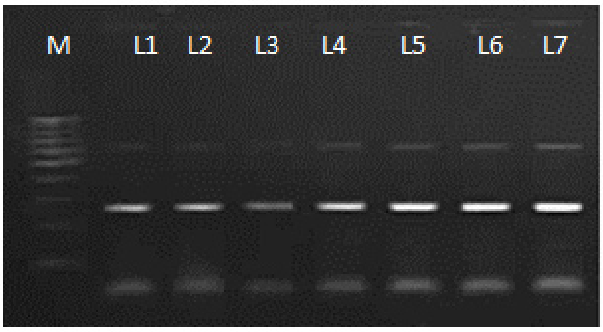

Figure 1. Results of the PCR assay, amplifying 259 base pair segment of *rfb* O157 and 625 base pair of *flicH*$_7$ gene of *E. coli* O157:H7. Lane M: 100 bp DNA marker. Lane 1- 6: representative positive samples. Lane 7: positive control

supermarkets are mostly provided by one of the leading food trade companies in Malaysia which offers a wide range of organic products in the dedicated counters (OTA, 2006). Although less prevalence was observed in the mentioned supermarkets comparing to the groceries, low contamination with *E. coli* O157:H7 was found in Four- winged bean from supermarkets. Possible ways of contamination are cross–contamination during handling, contaminated food handlers, instruments and utensils. Previous studies revealed that the bacterium has the ability to attach to the different surfaces (glass, rubber, hydroxyapatite, polystyrene and stainless steel surfaces) (Young *et al*., 2012).

The water used for washing is another possible source of cross-contamination. Vegetables are usually washed and immersed together before packaging process. According to Wang and Doyle reported that *E. coli* O157:H7 is able to survive in water for a long period (especially at cold temperatures) (Wang and Doyle, 1998). In such conditions the chance of *E. coli* O157:H7 contamination will increase.

In 2011, Pérez Rodríguez *et al*. conducted a modelling study for *E. coli* O157:H7 cross-contamination of lettuce during processing. They spiked lettuce samples with *E. coli* at 0.01, 1 and 100 cfu/g, and reported that the initial concentration in the contaminated batch did not influence significantly the *E. coli* O157:H7 levels in final bags. The highest prevalence level of 13.39% was observed for 100 cfu/g contamination level. They evaluated the impact of some interventions such as cleaning/disinfection procedures, irradiation and chlorination on *E. coli* prevalence and reported that irradiation (0.5 KGy) was the most effective decontamination method.

Storage temperature is one the important factors that affects the growth and distribution of *E. coli* O157:H7 (McKellar *et al*., 2012). Compared to the refrigerated foods at the supermarket, contaminations of organic foods from organic groceries were more prevalent (Table 3). As observed, the temperature in organic groceries was higher compared to the supermarkets. High temperature promotes the growth and survival of *E. coli* O157:H7. Organic foods in groceries are kept for a long period and such longer holding time increase the chance of contamination. However, *E. coli* O157:H7 was reported to grow at low temperature on fresh produce. Knudsen *et al* reported *E. coli* O157:H7 in frozen strawberries kept for more than 1 month (Knudsen *et al*., 2001).

Some environmental factors at pre-harvest stage play significant role in *E. coli* O157:H7 contamination (Steinmuller *et al*., 2006). At pre-harvest activities fresh produce are more likely to be contaminated through irrigation water containing untreated sewage and improperly composted manure (FDA, 2012). In some cases water is used as a vector of transmission of animals within the herds (McGee *et al*., 2002). If such water is used for irrigation, the vegetables will be contaminated with microorganisms from animals. To reduce the risk of infection from sewage-borne pathogens in environmental waters, *E. coli* is used as fecal indicator bacteria (Liang *et al*., 2011). Total fecal coliforms (*E. coli*) and enterococci are indicators for the microbiological safety of water resources. A research conducted by Okeke *et al*. (2011) on water samples from treatment plants in Alabama, revealed the total coliform most probable number (MPN) was between 385.6 - 847.5 MPN/100 ml (Okeke *et al*., 2011). By monitoring the indicator microorganisms, health risks posed by microbial contamination of water resources can be reduced. As there is a close association between food-borne disease and water quality, farmers, food producers and food handlers should pay special attention to the quality of water to maximize consumer's health protection (Kirby *et al*., 2003).

Soil is a potential source of *E. coli* O157:H7 infection for fruit and vegetables, especially if the soil contains slaughterhouse waste or untreated sewage sludge (Avery *et al*., 2005). In organic agriculture, manure is used as soil conditioner and fertilizer. Nutrients in the manure maintain and increase soil fertility. Using manure promotes the growth of beneficial organisms, increase water holding capacity, improve soil tilt and aeration (Doyle *et al*., 2005). Although the original source of contamination in fresh vegetables has not been identified yet, the leading vehicle for transmission of *E. coli* O157:H7 is suspected to be manure from farm animals.

Malaysia set national standards for organic farming, however there is no enforcement. The voluntary nature of standards allows some producers to present their product in organic market even if they are not produced according to related standards. The non-labeled organic products in organic groceries are such examples.

Conclusion

MPN-PCR method was used for detection of *E. coli* O157:H7 in organic foods. Comparing to the traditional confirmation techniques, MPN-PCR technique had advantages of rapid detection, less workload and less material consumption. The combination of MPN-PCR method resulted in the identification of *E. coli* O157:H7 within two days whereas the MPN-plating method needs 4 to 5 days to complete the identification. According to the results of the present study organic foods presented at local groceries showed higher contamination frequency. Contamination of organic foods with *E. coli* O157:H7 can pose possible health risks to the consumers. Results of the current study can be used as baseline data in risk estimation for the prediction of human illness associated with organic foods and provide useful information for the microbial risk assessment of *E. coli* O157:H7.

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