

Quantitative detection and characterization of Shiga toxin-producing *Escherichia coli* O157 and non-O157 in raw vegetables by MPN-PCR in Malaysia

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Abstract

Foodborne diseases are mainly caused by bacterial contamination which can lead to severe diarrhea. This study aimed to detect the presence of Shiga toxin-Producing *Escherichia coli* O157, *Escherichia coli* non-O157 and virulence gene in raw vegetables. The samples were purchased from wet market and hypermarket in Selangor. The detections were carried out by using the combination methods of Most Probable Number-Polymerase Chain Reaction (MPN-PCR). A total of 37(18.5%) samples were found to be contaminated by STEC. Out of these 37 isolates, four (10.8%) of the isolates were *E. coli* O157 while 33(89.2%) were *E. coli* non-O157. However, there was no *E. coli* O157:H7 detected in all the samples. The occurrence of Shiga toxin-Producing *E. coli* in edible raw vegetables samples suggests the importance of this pathogen in vegetables. Therefore, more studies are required to remove this pathogen from vegetables.

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Introduction

Foodborne diseases are caused by consuming the food contaminated by bacteria, viruses or chemicals. However, bacterial contamination in foods remain the most important source of food related diseases (Su *et al.*, 2005). Outbreaks occur due to the consumption of contaminated food, direct contact with the infected animal or consumption of foods which cross-contaminated by infected workers. Foodborne diseases can cause severe diarrhea, especially for those who has weak immune system such as the elderly, infants, children and immunocompromised patients (Kennedy *et al.*, 2004).

Shiga-toxin producing *Escherichia coli* (STEC) are one of the important emerging pathogens which cause foodborne infection. STEC which encoded shiga-toxin genes (*stx1* and *stx2*) are able to adhere to the epithelial cell of the gastrointestinal tract and cause bloody diarrhea. The severe diarrhea may develop into hemolytic uraemic syndrome (HUS) (Mead and Griffin, 1998).

Fresh vegetables are known as one of the important sources of vitamins, nutrients and fiber. The demand of fresh vegetables has increased as people nowadays are more concerned to healthy lifestyle

and balance diet (Warriner *et al.*, 2009; Olaimat and Holley, 2012). Usually the primary source for STEC is linked to cattle (Chapman *et al.*, 1997); however, raw vegetables have become one of the vehicle of transmission for this foodborne pathogen. This is supported by Rangel *et al.* (2005) who reported that in United State, there are 21% of STEC O157 outbreak are linked to fresh product from 1982 to 2002; while from 2000 to 2004, fresh products have become the second most important vehicle of transmission associated to STEC O157 outbreak (Rangel *et al.*, 2005; Aruscavage *et al.*, 2006; Olaimat and Holley, 2012). Besides that, Delaquis *et al.* (2007) also reported that many outbreaks of foodborne diseases occurred after consuming raw leafy green vegetables which contaminated by STEC.

There are many methods in identification and detection of *E. coli* such as polyclonal and monoclonal antibodies technique (De Boer and Heuvelink, 2000). However, in this study, multiplex-PCR was used in the detection of virulence genes of STEC. The Most-Probable-Number (MPN) was applied together with PCR which known as MPN-PCR. This is a simple method in quantification and estimation the number of bacteria in foods (Hara-Kudo *et al.*, 2001). The objective of this study was to determine the

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prevalence of Shiga-Toxin Producing *E. coli* in raw vegetables.

Materials and Methods

Sampling

A total of 200 samples were purchased randomly from wet market and hypermarket in Selangor. The samples used in this research including Japanese Parsley (*Oenanthe javanica*), cucumbers (*Cucumis sativus*), tomatoes (*Solanum lycopersicum*), carrots (*Daucus carota*) and Romaine lettuce (*Lactuca Sativa*). The samples were collected in between June 2011 and October 2011. All the samples were transported to laboratory immediately for analysis.

Most-probable-number (MPN)

Ten gram of the samples was placed in the stomacher bag. The samples were then added with 90 ml of Tryptic Soy Broth (TSB; Merck, Germany) and stomached for 60 s using stomacher (Interscience, France). The homogenized samples were incubated at 37°C for 18-24 h. After the incubation, 10⁹ fold dilutions to 10¹¹ fold dilutions of the pre-enrichment samples were carried out for the three tube MPN analysis. After the dilution, 1 mL from each dilution was transferred into three centrifuge tubes, followed by incubation at 37°C for 18-24 h.

Genomic DNA extraction

The DNA was extracted from the turbid MPN tubes using boil cell method as described by Tunung *et al.* (2011) and Ubong *et al.* (2011). The MPN tubes were centrifuged at 13,400 x g for 1 minute. The supernatant were discarded and pellets were re-suspended with 500 µl of Tris-EDTA buffer (TE buffer). The boiling of the mixture at 100°C was done for 10 min and cooled at -20°C for another 10 min. The mixture was then centrifuged at 13,400 x g for 2 min and the supernatant was kept at -20°C for further use.

Multiplex-PCR

The detection of the Shiga-toxin gene (*stx1* and *stx2*), *fliCh7* gene and *rfbO157* was carried out using multiplex-PCR assay. The specific forward and reverse primer sequences used for the detection of shiga toxin gene (*stx* gene) are *stx1-F* (5'-ATA AAT CGC CAT TCG TTG ACT AC-3'), *stx1-R* (5'-AGA ACG CCC ACT GAG ATC ATC-3') and *stx2-F* (5'-GGC ACT GTC TGA AAC TGC TCC-3'), *stx2-R* (5'-TCG CCA GTT ATC TGA CAT TCT G-3') (Çadİrcİ *et al.*, 2010). A specific forward (*rfbO157* -5'-CGG ACA TCC ATG TGA TAT GG-3') and

reverse (*rfbO157* - 5'-TTG CCT ATG TAC AGC TAA TCC-3') and forward (*fliCh7* -5'-GCG CTG TCG AGT TCT ATC GAG -3') and reverse (*fliCh7* - 5'-CAA CGG TGA CTT TAT CGC CAT TCC-3') were used to detect the O antigen and flagellar antigen in *Escherichia coli* O157:H7 (Hashemi *et al.*, 2010). A total of 25 µl reaction mixtures which containing of 5x PCR buffer, 0.5 mM l-1 deoxynucleoside triphosphate (dNTP) mix, 1.5 mM l-1 MgCl₂, 0.5 µM l-1 of each primer, 1 U of *Taq* polymerase and 2 µl of DNA were used in performing multiplex-PCR.

For the detection of *fliCh7* gene and *rfbO157* gene, the amplification condition was started with initial denaturation at 94°C for 5 min, then following by 35 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 30 s, and elongation at 72°C for 60 s. Finally, a final extension at 72°C for 10 min was carried out at the end of the process. For the detection of Shiga-toxin gene, amplification condition as follow: initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension for 72 °C for 45 s and followed by final extension at 72°C for 7 min.

The PCR products were loaded onto a 1.5% agarose gel and undergo electrophoresis at 100V for 28 min in Tris-Borate-EDTA buffer (TBE). The separation of the products were stained by ethidium bromide staining and visualized under UV-transilluminator using gel documentation apparatus (Gel Documentation System, SynGene, UK).

Results

The amplicon size obtained from MPN-PCR for shiga-toxin like gene (*stx 1* and *stx 2*) was shown at 180bp and 255bp respectively (Figure 1) while the amplicon size for *rfbO157* and *fliCh7* genes was shown at 259bp and 625bp respectively (Figure 2).

The prevalence of the Shiga-toxin Producing *Escherichia coli* O157 (STEC O157) and *Escherichia coli* non-O157 (STEC non- O157) are shown in Table 1. *Escherichia coli* O157:H7 was not detected in all the samples. A total of 37 (18.5%) samples were found to be contaminated by STEC. The prevalence of STEC O157 and non-O157 in 200 samples were 10.8% and 89.2% respectively. Romaine lettuce showed the highest prevalence (6.0%) among the raw vegetables tested. Tomatoes showed the second highest prevalence (5.0%), followed by carrots (4.0%) and Japanese parsley (3.0%). There was no detection of STEC was found in the cucumbers. The overall result showed that the prevalence of STEC in raw vegetables from hypermarket (13.5%) were higher than the prevalence in raw vegetables from

Table 1. Prevalence of Shiga-Toxin producing *E. coli* O157 and *E. coli* non-O157 in raw vegetables using MPN-PCR

Vegetable Types	Wet Market						Hypermarket					
	<i>E. coli</i> O157			<i>E. coli</i> non-O157			<i>E. coli</i> O157			<i>E. coli</i> non-O157		
	PCR+	n	%	PCR+	n	%	PCR+	n	%	PCR+	n	%
Japanese Parsley	0	20	0.0	0	20	0.0	0	20	0.0	7	20	35.0
Cucumber	0	20	0.0	0	20	0.0	0	20	0.0	0	20	0.0
Carrot	0	20	0.0	2	20	10.0	0	20	0.0	6	20	30.0
Tomato	0	20	0.0	4	20	20.0	0	20	0.0	6	20	30.0
Romaine Lettuce	4	20	20.0	0	20	0.0	0	20	0.0	8	20	40.0
Total	4	100	4.0	6	100	6.0	0	100	0.0	27	100	27.0

n = Number of samples
% = Percentage of the positive samples

Table 2. Microbial load of Shiga-toxin producing *E. coli* O157 and *E. coli* non-O157 (MPN/g) in raw vegetables from wet market and hypermarket

Vegetable types	Wet Market			Hypermarket		
	Min (MPN/g)	Med (MPN/g)	Max (MPN/g)	Min (MPN/g)	Med (MPN/g)	Max (MPN/g)
Japanese Parsley	-	-	-	-	7.3	19.0
Cucumber	-	-	-	-	-	-
Carrot	-	-	3.6	-	-	3.0
Tomato	-	3.0	6.1	-	3.0	9.1
Romaine Lettuce	-	-	-	-	-	9.1

Dash (-) = less than 3.0 MPN/g

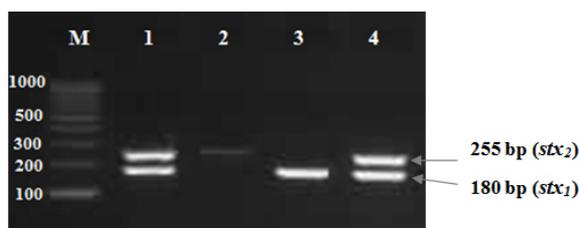


Figure 1. The determination of the presence of *stx1* and *stx2* gene of STEC in raw vegetables by multiplex-PCR. Lane M: 100bp DNA Marker. Lane 1: positive control for *stx1* and *stx2* genes. Lane 2 to 4: STEC detected in raw vegetables using multiplex PCR method.

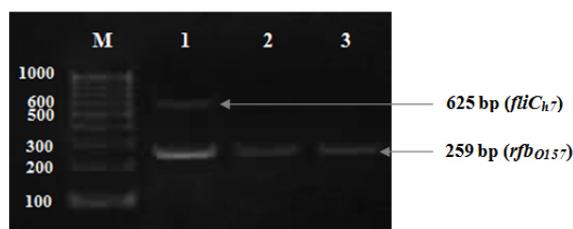


Figure 2: Multiplex-PCR assay for the detection of *rfbO₁₅₇* and *fliC_{h7}* genes of STEC isolated from raw vegetables. Lane M: 100bp DNA Marker. Lane 1: positive control for *rfbO₁₅₇* and *fliC_{h7}* genes. Lane 2 and 3: STEC isolated from raw vegetables.

wet market (3.0%).

The density of STEC in raw vegetable is summarized in Table 2. The microbial loads of STEC in raw vegetables were ranged from <3.0 to 5.3 MPN/g. Data showed that most of the raw vegetable had the lower distribution which is <3.0 MPN/g of STEC. The number of STEC in raw vegetable from Wet Market was ranged from <3.0 to 6.1 MPN/g while for Hypermarket, the range was determined from <3.0 to 19.0 MPN/g.

Discussion

Shiga-toxin producing *Escherichia coli* (STEC) are well recognized as the foodborne pathogens which is able to cause many outbreaks. STEC can cause bloody diarrhea, hemorrhagic colitis (CS) and hemolytic uremic syndrome (HUS) (Amézquita-López *et al.*, 2012). STEC can cause the life-threatening HUS due to the production of shiga toxin by *stx 1* gene, *stx 2* gene or both (Sarimehmetoglu *et al.*, 2009). Raw vegetables are consumed daily by most of the people because of the high nutritional value. However, there is limited data on the prevalence of STEC in raw vegetables in Malaysia.

The results show the prevalence of STEC in raw vegetable from hypermarket was higher than the raw vegetable from wet market. It is likely that the contamination happened during the holding period and the packaging process in the hypermarket. Cross contamination from the handler, the utensil used for cutting, and the water used for washing could be one of the reasons for the high prevalence of STEC in raw vegetable from hypermarket. Poor hygiene practice and the working environment is often contributing to the cross contamination (Tan *et al.*, 2008; Tang *et al.*, 2011). The different handling and processing method in both wet market and hypermarket may be one of the contributing factor in cross-contamination (Ponniah *et al.*, 2010).

Besides that, STEC are able to survive under harsh and stressed environment. Carey *et al.* (2009) carried out the studies of *E. coli* O157:H7 stress and the virulence gene expression. In the study, it showed that the virulence genes of STEC (*stx1* and *stx2*) were upregulated when stored at 4°C. The high expression of the virulence genes may cause by the concentration of iron content and the presence of micro flora in the vegetables. Thus, this could be the reason for the highest prevalence value of STEC in raw vegetable from hypermarket as most of the vegetables are kept under 4°C.

Furthermore, microbial contamination will be affected during pre-harvest and post-harvest process.

Soil is one of the sources of contamination during pre-harvest process. However, the duration of survival for the pathogens in the soil is depending on different conditions such as temperature, moisture level and type of soils (Lang and Smith, 2007; Zhang *et al.*, 2009; Erickson *et al.*, 2010; Olaimat and Holley, 2012). Other pre-harvest contamination sources are animal manures, reconstructed pesticides, irrigation water, dust and feces (Larry, 2002).

Microorganisms are naturally occurring contaminants in fresh products and processed fresh-cut products (Beuchat, 1996; Rico *et al.*, 2007; Lehto *et al.*, 2011). Fresh product are more susceptible to microorganisms after harvesting (Ippolito and Nigro, 2003). This is because the harvesting and processing of the fresh product affect their microbiological safety. Further process such as cutting, slicing and peeling may cause tissue damage. The release of nutrients will promote the growth and multiplication of the microorganisms in the fresh product (Harris *et al.*, 2003; Olaimat and Holley, 2012).

However, it was interesting to found out that there are isolates which showed positive result for the O157-antigen detection but negative result for the flagella H-antigen detection in this study. These isolates could be considered as non-motile STEC O157 (STEC O157:NM). STEC O157:NM are pathogenic as *E. coli* O157:H7 due to the ability of shiga-like toxin production. The characteristic of STEC O157:NM are similar to *E. coli* O157:H7 but STEC O157:NM are lack of flagella H-antigen which made them non-motile (Falkenstein, 2011). Multistate outbreak of STEC O157:NM which involved the consumption of raw beef (Ammon *et al.*, 1999) and alfafa sprouts (Ferguson *et al.*, 2005) had been reported.

A combination of a traditional enumeration method, the most probable number (MPN) with multiplex-PCR assay was used in enumeration and identification of the bacteria in this study. MPN-PCR is one of the rapid techniques in enumeration and identification. The conventional methods such as plate counting and biochemical test are consuming a lot of time compare to this technique. The conventional methods may used up more than one day compare to MPN-PCR method (Mäntynen *et al.*, 1997). PCR is able to give a higher value of estimates than the conventional plating. MPN-PCR technique had been successfully applied in many studies.

Conclusion

In conclusion, the virulence genes of STEC were detected in the raw vegetables. Severe diarrhea may happen once consuming the contaminated foods.

Therefore, a good hygiene practice such as washing the vegetables before consuming should be applied by every individual to prevent the outbreak of foodborne diseases. Further research is required to give a better understanding in the occurrence of STEC before harvesting and the source of contamination while processing the raw vegetables.

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