

## Rapid detection and enumeration of pathogenic *Vibrio parahaemolyticus* in raw vegetables from retail outlets

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**Abstract:** This study aims to determine the frequency and density of potentially pathogenic *Vibrio parahaemolyticus*, defined as those possessing thermostable-direct hemolysin (*tdh*) and/or *tdh*-related hemolysin (*trh*) genes, in raw salad vegetables at retail level in Selangor, Malaysia. A combination of Most Probable Number - Polymerase Chain Reaction (MPN-PCR) method was applied to detect the presence of *tdh* and/or *trh* gene-possessing *V. parahaemolyticus* and to enumerate their density in the samples. A total of 276 samples of vegetables commonly eaten raw in Malaysia (Cabbage = 30; Carrot = 31; Cucumber = 28; Four winged bean = 26; Indian pennywort = 17; Japanese parsley = 21; Lettuce = 16; Long bean = 32; Sweet potato = 29; Tomato = 38; Wild cosmos = 8) were analyzed. The samples were purchased from two supermarkets (A and B) and two wet markets (C and D). With the MPN-PCR technique, about 12.0% of the samples were positive for the presence of *V. parahaemolyticus* *tdh*-positive, with maximum densities of up to 39 MPN/g. The total frequency of *V. parahaemolyticus* *trh*-positive in the samples was 10.1%, with maximum concentration 15 MPN/g. *V. parahaemolyticus* *tdh*-positive was most prevalent in samples from Wet Market C (20.78%) and also in vegetable type *Oenanthe stolonifera* (Japanese parsley) with 19.0%, while *V. parahaemolyticus* *trh*-positive was predominant in samples from Wet Market D (16.7%) and was most frequent in both *Oenanthe stolonifera* (Japanese parsley) and *Cucumis sativus* (Cucumber) with 14.3% prevalence for each type. The results highlighted the fact that raw vegetables could be contaminated with virulent *V. parahaemolyticus* and could act as a transmission route, thus poses risk to consumers from the consumption of raw vegetables. To the author's knowledge, this is the first assessment of *V. parahaemolyticus* carrying *tdh* and *trh* genes in raw vegetables from retail outlets in Malaysia.

**Keywords:** *Vibrio parahaemolyticus*, most probable number (MPN), polymerase chain reaction (PCR), vegetables, thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH)

### Introduction

*Vibrio parahaemolyticus* is recognized as a common cause of foodborne illnesses in many Asian countries, including Japan, Taiwan and China (Su and Liu, 2007). For a long time, *V. parahaemolyticus* has been known to be ubiquitously present in brackish and marine waters, and infection to human is frequently associated with the consumption of contaminated seafood or raw or undercooked shellfish (Guoxiang et al., 2009). However, recent foodborne outbreaks throughout the world have been intensively linked

to consumption of fresh fruits, vegetables and unpasteurized juices (Gorny, 2006).

Fruits and vegetables, particularly those eaten raw and without peeling, have been demonstrated to be the vehicle for transmission of a range of microorganisms (Erdogrul and Sener, 2005). Okafo et al. (2003) reported the presence of *Escherichia coli*, *Vibrio* spp. and *Salmonella* spp. in raw vegetables harvested from soils irrigated with contaminated streams in Nigeria. Studies of other microorganisms found in vegetables were also reported recently (Chai et al., 2008; Learn-Han et al., 2009; Ponniah et al.,

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2009). To the author's knowledge, there is yet any report on the investigation of the contamination of pathogenic *V. parahaemolyticus* in raw vegetables, apart from our previous study on the presence of total *V. parahaemolyticus* in raw vegetables in Malaysia. The study highlighted the prevalence of 20.65% of total *V. parahaemolyticus* in raw vegetables, which indicated that there are risks posed to consumers when consuming raw vegetables (Tunung et al., 2010).

Robert-Pillot et al. (2004) stated that only members of *V. parahaemolyticus* that produce virulence factors are considered to be pathogenic and can cause acute gastroenteritis. It has been known that pathogenic *V. parahaemolyticus* produce either thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH) or both, and TDH and TRH encoded by *tdh* and *trh* genes are recognized as major virulence factors of *V. parahaemolyticus* (Ana et al., 2007). As non-pathogenic vibrios can be present in food and environmental samples, total *Vibrio* counts are not indicative enough for the presence of pathogenic vibrios. Hence, the presence of virulence genes are always considered as current markers of pathogenicity in *V. parahaemolyticus* (Robert-Pillot et al., 2004).

Even though the gastroenteritis caused by *V. parahaemolyticus* infection is often self-limited, it may cause septicaemia which is life-threatening to immunocompromised individuals (Su and Liu, 2007), and thus, investigation of *V. parahaemolyticus* virulent cells in food is of primary importance and is required for evaluation of the safety of food. In order to establish effective control measures to reduce the risk of *V. parahaemolyticus* infection and to ensure the safety of foods, efficient analytical methods for detecting *V. parahaemolyticus* in foods and environment are needed.

The most common conventional method for routine analysis usually includes a two-step protocol, in which selective enrichment with Alkaline Peptone Water (APW) is followed by plating of the enrichment culture on thiosulfate citrate bile salts (TCBS) agar (Hara-Kudo et al., 2001). Nonetheless, in recent years there are reports on several limitations associated with the use of TCBS agar or culture method for the correct identification of *V. parahaemolyticus*, in which *V. parahaemolyticus* shows similar morphology and characteristics as other *Vibrio* spp. Thus, this lack of specificity and limited selectivity thereby mask and complicate the identification of *V. parahaemolyticus* (Blanco-Abad et al., 2009). Recent work done by Hara-Kudo et al. (2001) suggested a two-step enrichment using a nonselective medium followed by a selective medium and plating on chromogenic

agar for a more sensitive and accurate identification of *V. parahaemolyticus*.

The more traditional enumeration method, the most probable number (MPN), is usually combined with this identification method to detect and enumerate *V. parahaemolyticus* (Nishibuchi, 2006). The major drawbacks of the MPN method coupled with traditional confirmation techniques are the amount of workload, the material, and the time needed to complete identification which usually takes 7 to 10 days. However, the combination of MPN with a species-specific polymerase chain reaction (PCR) method enables the completion of enumeration within 2 days (Martin et al., 2004).

It has been demonstrated that the PCR has proved to be very useful because of its ability to amplify a specific DNA segment by a factor of  $10^6$  or more within hours, therefore potentially permitting detection of very limited amounts of cells (Alam et al., 2003). Other researchers have reported the success of MPN combined with PCR for detection and quantification of pathogens (Savill et al., 2001; Fredslund et al., 2001; Martin et al., 2004; Chai et al., 2007; Lee et al., 2009). In this study, the MPN-PCR technique will be used to detect particularly *tdh* and *trh* genes to enumerate the virulent *V. parahaemolyticus* cells in the vegetable samples.

Since vegetables are often consumed raw in local dishes ('ulam') and usually as an accompaniment to popular dishes such as 'nasi lemak', a traditional rice dish that is often consumed for breakfast in Malaysia, Singapore and Indonesia (Ponniah et al., 2009), the presence of potentially pathogenic *V. parahaemolyticus* in raw vegetables would be of high public concern. Therefore the objective of this study was to provide quantitative data on the contamination of *tdh* and *trh* gene-possessing *V. parahaemolyticus* in raw vegetables in Selangor, Malaysia which will provide an insight on Malaysian scenario and will be useful for future risk assessment.

## Materials and Methods

### Sample collection

Supermarkets and wet markets in Selangor, Malaysia were listed, and two supermarkets (A and B) and two wet markets (C and D) were chosen with a random number table for this study. This study included the analysis of 276 raw vegetable samples which were collected over a one-year period (February 2008 to January 2009). A total of 11 types of vegetables which were commonly eaten raw in Malaysia were selected, and these were classified into 5 groups (Table 1). Efforts were made to collect

**Table 1.** Total number and types of raw vegetables collected from sampling locations.

Vegetable types	Local name	Scientific name	n <sup>a</sup>
<b>Leafy-type</b>			
Japanese parsley	Selom	<i>Oenanthe stolonifera</i>	21
Wild cosmos	Ulam raja	<i>Cosmos caudatus</i>	8
<b>Touching soil</b>			
Cabbage	Kubis	<i>Brassica oleracea</i>	30
Indian pennywort	Pegaga	<i>Centella asiatica</i>	17
Lettuce	Lettuce	<i>Lactuca sativa</i>	16
<b>Tuber-type</b>			
Carrot	Lobak merah	<i>Daucus carota</i>	31
Sweet potato	Ubi kayu	<i>Ipomoea batatas</i>	29
<b>Fruit-type</b>			
Cucumber	Timun	<i>Cucumis sativus</i>	28
Tomato	Tomato	<i>Solanum lycopersicum</i>	38
<b>Bean-type</b>			
Four winged bean	Kacang botol	<i>Psophocarpus tetragonolobus</i>	26
Long bean	Kacang panjang	<i>Vigna unguiculata</i>	32
<b>TOTAL</b>			<b>276</b>

<sup>a</sup> = Number of samples collected.

equal number of samples for each type of vegetables at each location, however this was not possible for *Cosmos caudatus* (Wild cosmos), which is one of the more indigenous local vegetable, as they were totally unavailable at supermarkets and also Wet Market D. During collection, all the samples were transferred to sterile plastic bags for transportation and were analyzed immediately on arrival to the laboratory.

#### Most Probable Number (MPN) procedure

The sampling method performed in this study was described previously by Tunung et al. (2010) and was based on the Bacteriological Analytical Manual standard method (Kaysner and DePaola, 2004), with modification according to the procedure by Hara-Kudo et al. (2001) and Chai et al. (2007). 10 g portion of each sample was placed in a stomacher bag added with 90 ml of Tryptic Soy Broth (TSB; BactoTM, France) with 3% sodium chloride (NaCl; Merck, Germany) and pummeled in a stomacher (Interscience, France) for 60 s, followed with pre-enrichment by incubation at 37°C for 6 h prior to Most Probable Number (MPN) analysis. For the three-tube MPN analysis, a 100 fold and a 1000 fold dilutions of the pre-enriched samples were prepared with Salt Polymyxin Broth (SPB; Nissui, Japan). Portions of each dilution were transferred into three tubes, with each tube containing 1 ml, and then the tubes were incubated at 37°C for 18 to 24 h. The MPN tubes were then subjected to PCR for the detection of *tdh* and *trh* genes.

#### PCR detection

DNA extraction of the MPN tubes were carried out using boil cell method (Tunung et al., 2007; Chai et al., 2007) with slight modifications. A 1 ml portion of each MPN broth was subjected to centrifugation at 13, 400 x g for 1 min and the pellet was resuspended in 500 µl of sterile distilled water. The mixture was boiled for 10 min and then immediately cooled at -20°C for 10 min before it was centrifuged at 13, 400 x g for 3 min. The supernatant was kept for use in PCR for the detection of *tdh* and *trh* genes. The *V. parahaemolyticus* reference strains used as positive control for the PCR reactions (coded 1808, 1896, and 2053) were obtained from Kyoto University, Japan. The primer sequences used for the detection of *tdh* gene were *tdh*-D3 (5'-ccactaccactctcatatgc-3') and *tdh*-D5 (5'-ggtactaaatggctgacatc-3'), while for the *trh* gene were *trh*-R2 (5'-ggetcaaatggtaagcg-3') and *trh*-R6 (5'-cattccgctctcatatgc-3'), and the amplicon sizes for the PCR amplifications of *tdh*<sup>+</sup> and *trh*<sup>+</sup> are 251 bp and 250 bp, respectively (Tada et al., 1992; Lesley et al., 2005). The PCR for *tdh* and *trh* detections were carried out separately.

PCR amplification was performed in a 20 µl reaction mixture containing 4.0 µl of 5X PCR buffer, 2.25 mM MgCl<sub>2</sub>, 0.2 mM of deoxynucleoside triphosphate mix, 0.2 µM of each primers, 0.5 U *Taq* polymerase and 2.0 µl of DNA template. All PCR reagents were from Promega, USA, and the primers were synthesized by Invitrogen (Japan). The following

**Table 2.** Frequency of pathogenic *V. parahaemolyticus* (*tdh*-positive) in raw vegetables at retail level using MPN-PCR.

Vegetable types	Supermarket A		Supermarket B		Wet Market C		Wet Market D		TOTAL						
	<i>tdh</i> <sup>+</sup>	n <sup>a</sup>	% <sup>b</sup>	<i>tdh</i> <sup>+</sup>	n	%	<i>tdh</i> <sup>+</sup>	n	%	<i>tdh</i> <sup>+</sup>	n	%			
Cabbage	0	7	0.0	1	9	11.1	1	6	16.7	2	8	25.0	4	30	13.3
Carrot	0	7	0.0	0	8	0.0	2	8	25.0	1	8	12.5	3	31	9.7
Cucumber	0	9	0.0	1	6	16.7	0	7	0.0	1	6	16.7	2	28	7.1
Four winged bean	0	6	0.0	0	8	0.0	2	6	33.3	2	6	33.3	4	26	15.4
Indian pennywort	0	1	0.0	0	1	0.0	1	7	14.3	2	8	25.0	3	17	17.6
Japanese parsley	0	2	0.0	0	2	0.0	3	9	33.3	1	8	12.5	4	21	19.0
Lettuce	0	4	0.0	0	1	0.0	0	3	0.0	1	8	12.5	1	16	6.3
Long bean	0	9	0.0	0	8	0.0	2	7	28.6	2	8	25.0	4	32	12.5
Sweet potato	0	6	0.0	1	8	12.5	2	7	28.6	1	8	12.5	4	29	13.8
Tomato	0	10	0.0	0	9	0.0	2	9	22.2	1	10	10.0	3	38	7.9
Wild cosmos	NA	NA	NA	NA	NA	NA	1	8	12.5	NA	NA	NA	1	8	12.5
<b>TOTAL</b>	<b>0</b>	<b>61</b>	<b>0.0</b>	<b>3</b>	<b>60</b>	<b>5.0</b>	<b>16</b>	<b>77</b>	<b>20.8</b>	<b>14</b>	<b>78</b>	<b>17.9</b>	<b>33</b>	<b>276</b>	<b>12.0</b>

*tdh*<sup>+</sup> = Number of sample positive for *V. parahaemolyticus* harbouring *tdh* gene

<sup>a</sup> = Number of samples tested

<sup>b</sup> = Percentage (number of positive samples/number of samples tested)

NA = Sample not available

**Table 3.** Frequency of pathogenic *V. parahaemolyticus* (*trh*-positive) in raw vegetables at retail level using MPN-PCR.

Vegetable types	Supermarket A		Supermarket B		Wet Market C		Wet Market D		TOTAL	
	<i>trh</i> <sup>+</sup>	n <sup>a</sup>	<i>trh</i> <sup>+</sup>	n						
Cabbage	0	7	1	9	0	6	2	8	3	30
Carrot	0	7	0	8	1	8	1	8	2	31
Cucumber	0	9	2	6	0	7	2	6	4	28
Four winged bean	0	6	0	8	1	6	1	6	2	26
Indian pennywort	0	1	0	1	1	7	1	8	2	17
Japanese parsley	0	2	0	2	2	9	1	8	3	21
Lettuce	0	4	0	1	0	3	1	8	1	16
Long bean	0	9	0	8	2	7	2	8	4	32
Sweet potato	0	6	1	8	2	7	1	8	4	29
Tomato	0	10	0	9	1	9	1	10	2	38
Wild cosmos	NA	NA	NA	NA	1	8	NA	NA	NA	8
<b>TOTAL</b>	<b>0</b>	<b>61</b>	<b>4</b>	<b>60</b>	<b>11</b>	<b>77</b>	<b>13</b>	<b>78</b>	<b>28</b>	<b>276</b>

*trh*<sup>+</sup> = Number of sample positive for *V. parahaemolyticus* harbouring *trh* gene

<sup>a</sup> = Number of samples tested

<sup>b</sup> = Percentage (number of positive samples/number of samples tested)

NA = Sample not available

**Table 4.** Concentration (MPN/g value)<sup>a</sup> of *V. parahaemolyticus tdt<sup>+</sup>* and *trh<sup>+</sup>* genes in raw vegetables at retail level using MPN-PCR.

Vegetable types	Supermarket A			Supermarket B			Wet Market C			Wet Market D					
	Med <sup>b</sup>	Max <sup>c</sup>	<i>trh<sup>+</sup></i>	Med	Max	<i>tdt<sup>+</sup></i>	Med	Max	<i>trh<sup>+</sup></i>	Med	Max	<i>tdt<sup>+</sup></i>	Med	Max	<i>trh<sup>+</sup></i>
Cabbage	— <sup>d</sup>	—	—	15	—	3	—	23	—	—	15	—	—	6.2	—
Carrot	—	—	—	—	—	—	—	9.1	—	3.6	—	—	6.1	—	3
Cucumber	—	—	—	14	—	11	—	—	—	—	14	—	—	—	3.6
Four winged bean	—	—	—	—	—	—	—	39	—	15	—	—	12	—	3.6
Indian pennywort	—	—	—	—	—	—	—	9	—	3.6	—	—	9.1	—	3.6
Japanese parsley	—	—	—	—	—	—	—	20	—	9.1	—	—	9.1	—	3
Lettuce	—	—	—	—	—	—	—	—	—	—	—	—	9.3	—	3.6
Long bean	—	—	—	—	—	—	—	14	—	9.1	—	—	15	—	3.6
Sweet potato	—	—	—	11	—	3	—	6.2	—	3.6	—	—	15	—	3
Tomato	—	—	—	—	—	—	—	6.1	—	6.2	—	—	6.2	—	3.6
Wild cosmos	NA	NA	NA	NA	NA	NA	—	15	—	3	—	NA	NA	NA	NA

<sup>a</sup> = Most Probable Number of cells in 1 g of sample (MPN/g).

<sup>b</sup> = Median MPN/g value

<sup>c</sup> = Maximum MPN/g value

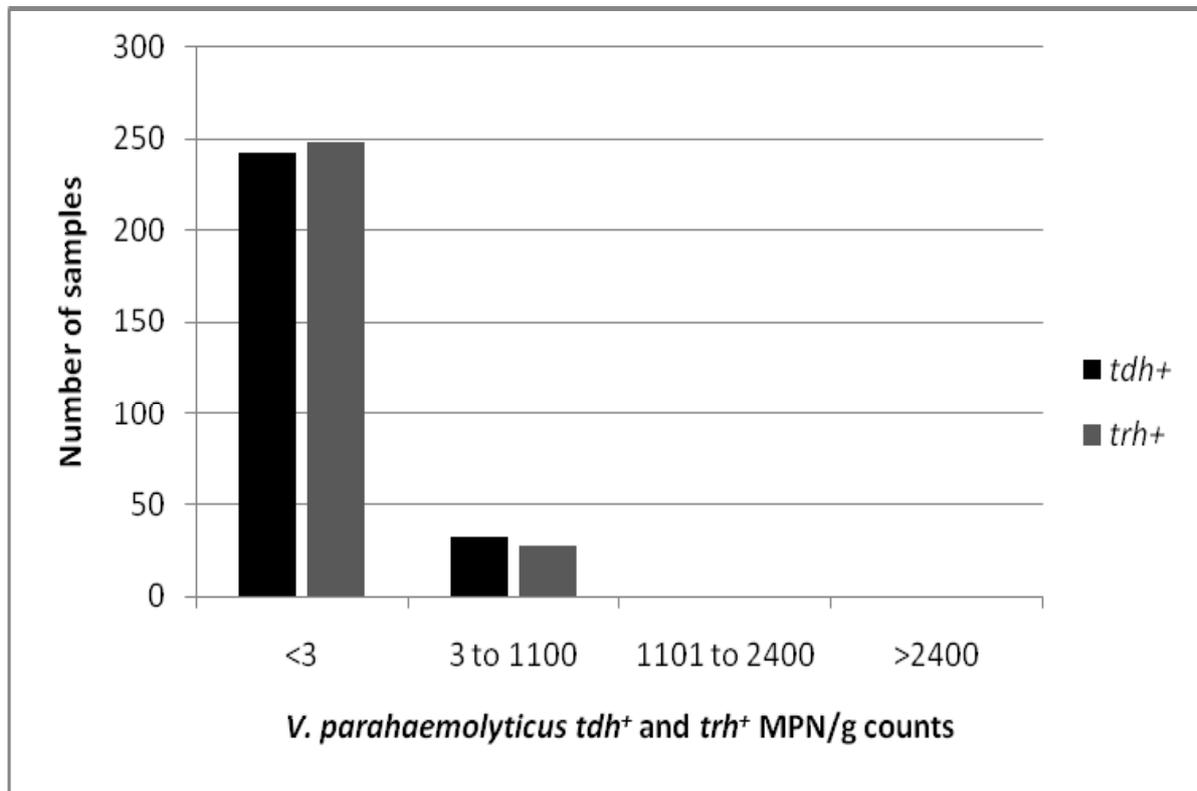
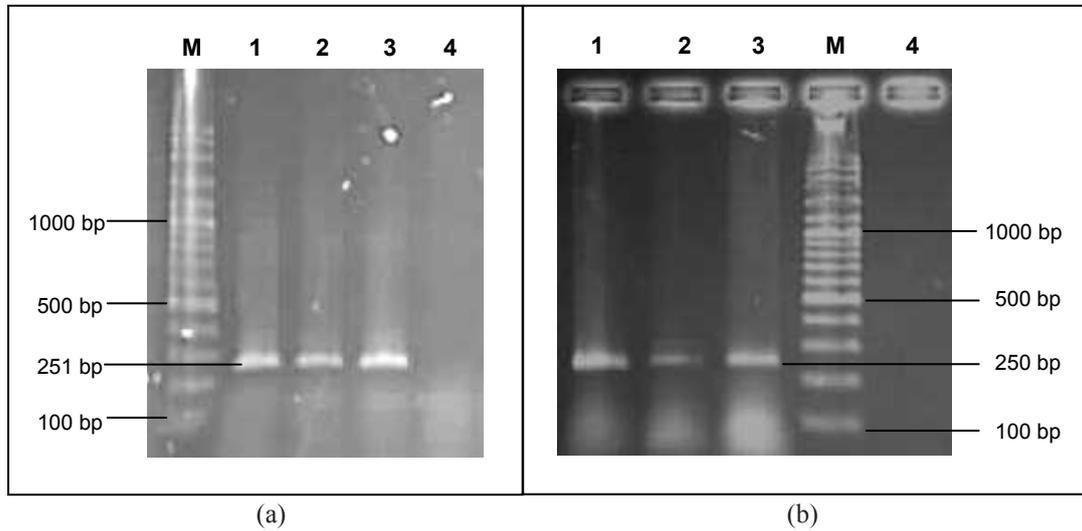
<sup>d</sup> = Less than 3 MPN/g.

*tdt<sup>+</sup>* = MPN/g value of sample positive for *V. parahaemolyticus* harbouring *tdt* gene

*trh<sup>+</sup>* = MPN/g value of sample positive for *V. parahaemolyticus* harbouring *trh* gene

NA = Sample not available

**Figure 1.** Agarose gel electrophoresis of PCR products corresponding to amplifications of *tdh* and *trh* genes. (a) Lane M: 100 bp DNA ladder; Lanes 1 to 3: positive control for *V. parahaemolyticus* *tdh*-positive (251 bp); Lane 4: negative control. (b) Lanes 1 to 3: positive control for *V. parahaemolyticus* *trh*-positive (250 bp); Lane M: 100 bp DNA ladder; Lane 4: negative control.



**Figure 2.** Distribution of *V. parahaemolyticus* *tdh*<sup>+</sup> and *trh*<sup>+</sup> MPN/g counts.

*tdh*<sup>+</sup> = number of sample positive for *V. parahaemolyticus* harbouring *tdh* gene  
*trh*<sup>+</sup> = number of sample positive for *V. parahaemolyticus* harbouring *trh* gene

thermocycler conditions were used: pre-denaturation at 96°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, and followed by final extension at 72°C for 7 min. An aliquot of 3 µl of the PCR products were loaded and electrophoresed in 1.0% agarose gel with 0.5X Tris-Base EDTA (TBE) at 100 V, and were stained and viewed using Gel Documentation System (SynGene).

#### Statistical analysis

To determine if there was any significant difference between the prevalence of *V. parahaemolyticus tdh*<sup>+</sup> and *trh*<sup>+</sup> among the sampling locations and the vegetable types, the data was analyzed using SPSS software (version 16.0). The level of significance was set at  $P < 0.05$ .

### Results and Discussions

Results for the prevalence of *V. parahaemolyticus* harbouring *tdh* and *trh* genes in raw vegetable samples from wet markets and supermarkets in Selangor, Malaysia were summarized in Table 2 and 3. Amplifications of *tdh* and *trh* genes were shown in Figure 1. Out of the 276 samples examined, the presence of *V. parahaemolyticus tdh*-positive was found to be 12.0%, with the highest detection in samples from Wet Market C (20.8%) followed by Wet Market D (17.9%). The prevalence was lower in supermarkets with Supermarket B showing 5.0% detection in samples, whereas none were detected in samples from Supermarket A (0.0%). Among the 11 types of vegetables, *V. parahaemolyticus tdh*<sup>+</sup> was most prevalent in *Oenanthe stolonifera* (Japanese parsley) with 19% prevalence, while the least was detected in *Lactuca sativa* (Lettuce) with only 6.3% prevalence.

Nonetheless, the prevalence of *V. parahaemolyticus trh*-positive in vegetables from Supermarket B was 6.7% and none was detected from Supermarket A (0.0%), while retailed vegetables from Wet Market C and Wet Market D revealed higher prevalence, with 14.3% and 16.7%, respectively. *V. parahaemolyticus trh*<sup>+</sup> was most predominant in both vegetable type *Oenanthe stolonifera* (Japanese parsley) and *Cucumis sativus* (Cucumber) with 14.3% prevalence for each type, while the least prevalence was found in vegetable type *Solanum lycopersicum* (Tomato) with only 5.3% detection.

The number of *V. parahaemolyticus* harbouring *tdh* gene in the various retail vegetables as determined by MPN-PCR technique ranged from <3 to 39 MPN/g for Wet Market C, and <3 to 15 MPN/g for Wet Market D, <3 to 15 MPN/g for Supermarket B, whereas for

Supermarket A, the maximum number was <3 MPN/g (Table 4). Wet Market C showed the broadest range of MPN number of pathogenic *V. parahaemolyticus* in its retailed vegetables, while vegetables from Supermarket A was the least contaminated.

For *V. parahaemolyticus trh*-positive cells, the range of density in vegetables from Wet Market C was <3 to 15 MPN/g, while in Wet Market D from <3 to 6.2 MPN/g. Meanwhile for supermarkets, the density ranged from <3 to 11 for Supermarket B, while for Supermarket A the maximum number was up to <3 MPN/g. The distribution of MPN/g counts of *V. parahaemolyticus tdh*<sup>+</sup> and *trh*<sup>+</sup> were shown in Figure 2, highlighting the fact that the concentration of *V. parahaemolyticus tdh*<sup>+</sup> and *trh*<sup>+</sup> in all samples were mostly <3 MPN/g.

When analyzed statistically, there was no significant difference among the vegetable types and sampling locations for the contamination of *V. parahaemolyticus tdh*<sup>+</sup> and *trh*<sup>+</sup>. However, for sampling locations, the mean difference between Supermarket A and Wet Market C was significant at the 0.05 level.

The increasing and growing importance of *V. parahaemolyticus* infections on a global scale has promoted the implementation of improved and rapid analytical procedures for the detection and enumeration of this pathogen (Blanco-Abad et al., 2009). A highly sensitive PCR-based method to detect and quantify *V. parahaemolyticus tdh*<sup>+</sup> and *trh*<sup>+</sup> in raw vegetable samples was applied, in which this method combines the high sensitivity of the MPN method with *V. parahaemolyticus tdh*<sup>+</sup> and *trh*<sup>+</sup> specific PCR assay. Detection of *tdh* and *trh* genes by PCR has been proven successful by previous researchers (Tada et al., 1992; Lesley et al., 2005; Gwendelyne et al., 2005; Marlina et al., 2007; Zulkifli et al., 2009).

The presence of *V. parahaemolyticus tdh*<sup>+</sup> and *trh*<sup>+</sup> in 12% and 10.1%, respectively, of the raw vegetable samples are probably a reflection of the scenario of retail outlets in the study. From the 276 samples, the results revealed higher prevalence in samples from wet markets compared to supermarkets. Since *V. parahaemolyticus* occur naturally in marine and estuarine aquatic ecosystems (Gopal et al., 2005), thus its presence detected in raw vegetables are most possibly due to cross-contamination introduced from several factors or sources. From observations during sample collection, the level of hygiene practiced by handlers at wet markets was lower compared to supermarkets, and the cleanliness of the display location at the wet market itself was unsatisfactory. Fresh seafood items were seen to be sold nearby the vegetables area, which could be the obvious

probable source of cross-contamination since *V. parahaemolyticus* occurs naturally in the marine environments and frequently isolated from a variety of seafood (Su and Liu, 2007).

The wet markets and supermarkets in Malaysia generally differ in their holding time and in the way food items are processed prior to sale. Vegetables in wet markets do not undergo any processing, were sent directly from farms every morning and were sold without further washing or packaging, while vegetables in supermarkets were usually washed and packaged before being displayed (Chai et al., 2007), which could be the reason for the higher prevalence of *V. parahaemolyticus tdh<sup>+</sup>* and *trh<sup>+</sup>* in vegetables at wet markets. A study by Ponniah et al. (2010) on the prevalence of *Listeria monocytogenes* in raw vegetables from wet markets and hypermarkets also revealed higher prevalence in samples at wet market compared to hypermarket.

However, the various processing steps for vegetables at supermarkets could also introduce contaminants to the vegetables, through cross-contamination from handlers, from the water used for washing and utensils used for packaging. The longer holding time for vegetables at supermarkets also aid in proliferation of the pathogens introduced to the vegetables, thus resulting in detectable level of *V. parahaemolyticus tdh<sup>+</sup>* and *trh<sup>+</sup>*. This could be the explanation for the *V. parahaemolyticus tdh<sup>+</sup>* and *trh<sup>+</sup>* detected in samples at Supermarket A and B, although the hygiene level at these locations was considered high from our observations. Chao et al. (2009) also reported in their study on *V. parahaemolyticus* from various sources that markets, hotels and restaurants, which seem hygienic, became the key spots causing cross-contamination in the aquatic products that they tested. Cross contamination is often due to poor hygiene and sanitation practice of the workers involved (Tan et al., 2008).

As for the types of vegetables, *V. parahaemolyticus tdh<sup>+</sup>* was most predominant in *Oenanthe stolonifera* (Japanese parsley), while *V. parahaemolyticus trh<sup>+</sup>* was mostly found in *Oenanthe stolonifera* (Japanese parsley) and *Cucumis sativus* (Cucumber). However, there was no specific pattern or obvious preferred vegetable type for *V. parahaemolyticus tdh<sup>+</sup>* and *trh<sup>+</sup>* to attach or accumulate, as there was no significant difference among the vegetable types ( $P < 0.05$ ). Yet there could be a lot of reason as to how the pathogen could attach and survive on the vegetables, since *V. parahaemolyticus* do not occur naturally in vegetables, and the temperature during sampling (data not shown), the condition and the food matrix of vegetables were unfavourable for *V. parahaemolyticus*

to grow. Several aspects of adaptation that have been suggested to play a role in the survival of vibrios in these conditions are starvation adaptation, the viable but nonculturable (VBNC) response, and biofilm formation (McDougald and Kjelleberg, 2006).

The standard minimum allowable level of *V. parahaemolyticus* in seafood destined for raw consumption in New Zealand and Japan is 100 MPN/g, while the US FDA requires less than 10,000 MPN/g (Lee et al., 2008). The standard level of *V. parahaemolyticus* in foods in Malaysia was however unavailable to the author. In this study, the number of *V. parahaemolyticus tdh<sup>+</sup>* and *trh<sup>+</sup>* in raw vegetables were mostly  $< 3$  MPN/g, with maximum number reaching only until 39 MPN/g for *tdh<sup>+</sup>* and 15 MPN/g for *trh<sup>+</sup>*. Although the counts were low and still below the accepted level based on the recommended level by US FDA, which stated that the infectious dose in a healthy person is  $10^6$  cells, still it is dependent on the host and thus is risky especially for those who are immunocompromised.

Classic methods used to monitor the microbiological quality of food may be insufficient in that they reveal only culturable cells, thus new methods based on PCR should be applied to reveal non-culturable bacterial forms including those which are still viable (VBNC) or injured (Alam et al., 2002). Since vegetables can be eaten raw, it can be of high risk to consumers. Although the prevalence and concentration of *V. parahaemolyticus tdh<sup>+</sup>* and *trh<sup>+</sup>* in this study are considered low, the fact that high risk individuals may be exposed to virulent *V. parahaemolyticus* from consuming raw vegetables should have the public to be aware of the risk involved and of the preventive measures that should be taken. Further studies are required to determine the sources of contamination of *V. parahaemolyticus* and at which stage that the contamination occurs during food preparation. In an effort to reduce the chances of contamination, the hygiene practiced in retail outlets particularly at supermarkets and wet markets must be monitored closely.

## Acknowledgements

This study was supported by Science Fund (project no. 02-01-04-SF0390) from the Ministry of Science, Technology and Innovation, Malaysia and in part by Grant-in-Aid for Scientific Research (KAKENHI 191010) from Japan Society for the Promotion of Sciences.

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