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Prevalence and Quantification of *Vibrio parahaemolyticus* in Raw Salad Vegetables at Retail Level

Tunung, R.[1]*, S. P. Margaret[1], P. Jeyaletchumi[1], L. C. Chai[1], T. C. Tuan Zainazor[2], F. M. Ghazali[1], Y. Nakaguchi[3], M. Nishibuchi[3], and R. Son[1]

[1]*Center of Excellence for Food Safety Research, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*
[2]*Makmal Kesihatan Awam Kebangsaan, Kementerian Kesihatan Malaysia, Lot 1835 Kampung Melayu, 47000 Sungai Buloh, Selangor Darul Ehsan, Malaysia*
[3]*Center for Southeast Asian Studies, Kyoto University, Kyoto 606-8501, Japan*

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The purpose of this study was to investigate the biosafety of *Vibrio parahaemolyticus* in raw salad vegetables at wet markets and supermarkets in Malaysia. A combination of the most probable number – polymerase chain reaction (MPN–PCR) method was applied to detect the presence of *V. parahaemolyticus* and to enumerate their density in the food samples. The study analyzed 276 samples of common vegetables eaten raw in Malaysia (Wild cosmos=8; Japanese parsley=21; Cabbage=30; Lettuce=16; Indian pennywort=17; Carrot=31; Sweet potato=29; Tomato=38; Cucumber=28; Four-winged bean=26; Long bean=32). The samples were purchased from two supermarkets (A and B) and two wet markets (C and D). The occurrence of *V. parahaemolyticus* detected was 20.65%, with a higher frequency of *V. parahaemolyticus* in vegetables obtained from wet markets (Wet market C=27.27%; Wet Market D=32.05%) compared with supermarkets (Supermarket A=1.64%; Supermarket B=16.67%). *V. parahaemolyticus* was most prevalent in Indian pennywort (41.18%). The density of *V. parahaemolyticus* in all the samples ranged from <3 up to >2,400 MPN/g, mostly <3 MPN/g concentration. Raw vegetables from wet markets contained higher levels of *V. parahaemolyticus* compared with supermarkets. Although *V. parahaemolyticus* was present in raw vegetables, its numbers were low. The results suggest that raw vegetables act as a transmission route for *V. parahaemolyticus*. This study will be the first biosafety assessment of *V. parahaemolyticus* in raw vegetables in Malaysia.

Keywords: *Vibrio parahaemolyticus*, most probable number (MPN), polymerase chain reaction (PCR), raw vegetables, prevalence, quantification

*Corresponding author
Phone: +03 8946 8383; Fax: +03 8946 8452;
E-mail: tunungrobin@gmail.com

Fresh fruits and vegetables are perceived by customers to be healthy and nutritious foods owing to the plethora of scientifically proven and documented health benefits derived from consuming fresh products. Nevertheless, recent foodborne outbreaks throughout the world have been intensively linked to consumption of fresh fruits, vegetables, and unpasteurized juices [7]. Meldrum *et al.* [14] reported that the two recent large outbreaks in the United Kingdom demonstrated the significant health problems that could arise from consumption of contaminated salads. Okafo *et al.* [15] reported the presence of *Escherichia coli*, *Vibrio* spp., and *Salmonella* spp. in raw vegetables harvested from soils irrigated with contaminated streams in Nigeria. Another study of the prevalence of pathogens in vegetables was reported by Little *et al.* [12], in which they found *Listeria monocytogenes* in ready-to-eat mixed salads in the U.K. Several other studies on other types of foodborne pathogens in vegetables were also reported [3, 14. 15]. *V. parahaemolyticus* is known as a common foodborne pathogen in Asia [11], and has been reported to be the cause for 20–30% of food poisoning cases in Japan [6]. It is a marine bacterium that occurs naturally in coastal waters worldwide and is a cause of gastroenteritis [2] with severe abdominal pain and diarrhea [11]. The *V. parahaemolyticus* cases reported have most frequently been attributed to the consumption of raw or undercooked seafood, or the ingestion of contaminated water, but to the best of our knowledge there has been no reported study on *V. parahaemolyticus* in raw vegetables. Hence, to understand the risk of acquiring *V. parahaemolyticus* through consumption of raw vegetables, it is important to have data on the occurrence of *V. parahaemolyticus* in association with raw vegetable samples. Nowadays, the polymerase chain reaction (PCR) has been proven to be useful in detecting pathogens in food samples rapidly

Table 1. Types of raw vegetable samples selected from supermarkets and wet markets.

Type	Local name	English name	Scientific name	Total
Leafy	Ulam raja	Wild cosmos	*Cosmos caudatus*	8
	Selom	Japanese parsley	*Oenanthe stolonifera*	21
Soil	Kubis	Cabbage	*Brassica oleracea*	30
	Lettuce	Lettuce	*Lactuca sativa*	16
	Pegaga	Indian pennywort	*Centella asiatica*	17
Roots	Lobak merah	Carrot	*Daucus carota*	31
	Ubi kayu	Sweet potato	*Ipomoea batatas*	29
Fruits	Tomato	Tomato	*Solanum lycopersicum*	38
	Timun	Cucumber	*Cucumis sativus*	28
Beans	Kacang botol	Four-winged bean	*Psophocarpus tetragonolobus*	26
	Kacang panjang	Long bean	*Vigna unguiculata*	32
TOTAL				276

and accurately. *V. parahaemolyticus* possesses a regulatory gene, *toxR*, which is present in all strains, and PCR based on *toxR* reported to be specific for *V. parahaemolyticus* have been found useful for confirmation of this species [6, 10]. PCR methods have been developed for specific detection of *V. parahaemolyticus*, but they are limited to qualitative determination of the organism unless they are used in conjunction with the most probable number (MPN) procedure [17]. The MPN method is commonly used to measure the concentration of a target microbe in samples [9]. However, apart from being labor-intensive and time-consuming, a major disadvantage of the MPN method is that the thiosulfate–citrate–bile salts–sucrose (TCBS) agar used in the method cannot differentiate *V. parahaemolyticus* from some strains of *V. vulnificus* or *V. mimicus* [17]. This is where PCR will complement the MPN procedure, through accurate confirmation of the presence of *V. parahaemolyticus* in the sample tested. There are many epidemiological reports and qualitative studies of prevalence related to food poisoning caused by *V. parahaemolyticus* in seafood, but there is a lack of quantitative or enumeration studies on this that have been published [11], especially on *V. parahaemolyticus* in raw vegetables. Therefore, the aims of this study were to determine the prevalence and to enumerate the numbers of *V. parahaemolyticus* in vegetables that are usually eaten raw in Malaysia. This study will be the first biosafety assessment of *V. parahaemolyticus* in raw vegetables in Malaysia, and it will provide an insight on the Malaysian scenario. The findings will serve as useful data in future risk assessment for *V. parahaemolyticus*.

Materials and Methods

Sample Collection

The study included the analysis of 276 raw vegetable samples collected from two supermarkets (A and B) and two wet markets (C and D) in Selangor, Malaysia, which were randomly selected, over a one-year period (February 2008 to January 2009). During collection, all the samples were transferred to sterile plastic bags for transportation and were analyzed immediately on arrival to the laboratory. The types of raw vegetable samples obtained were as shown in Table 1.

Most Probable Number Procedure

The sampling method performed in this study was based on the *Bacteriological Analytical Manual* standard method [9], with modification according to the procedures by Hara-Kudo *et al.* [8] and Chai *et al.* [4]. A 10-g portion of each sample was placed in a stomacher bag added with 90 ml of Tryptic Soy Broth (TSB; Bacto, France) with 3% sodium chloride (NaCl; Merck, Germany) and pummeled in a stomacher (Interscience, France) for 60 s, followed by pre-enrichment by incubation at 37°C for 6 h. For three-tube Most MPN analysis, 100-fold and 1,000-fold dilutions of the stomacher fluid were prepared with Salt Polymyxin Broth (SPB; Nissui, Japan). A portion of each dilution was 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 subjected to PCR for the detection of *toxR* gene specific for *V. parahaemolyticus*.

PCR Detection

The MPN tubes were subjected to DNA extraction, which was carried out using the boil cell method [19] with slight modifications. A 1-ml portion of each MPN broth was subjected to centrifugation at 13,400 ×*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

Table 2. Oligonucleotide primer sequences for the detection of *V. parahaemolyticus*.

Primer	Primer sequence	Length	Amplicon size	Reference
toxR4 (forward)	5'-GTCTTCTGACGCAATCGTTG-3'	20 bp	368 bp	[10]
toxR7 (reverse)	5'-ATACGAGTGGTTGCTGTCATG-3'	21 bp		

13,400 ×*g* for 3 min. The supernatant was kept for use in PCR for detection of *toxR* that is specific for *V. parahaemolyticus* [10]. The reference *V. parahaemolyticus* strains (*V. parahaemolyticus* strains 1808, 1896, 2053) used for the PCR reaction were obtained from Kyoto University, Japan.

PCR amplification was performed in a 20-μl reaction mixture containing 4.0 μl of 5× PCR buffer, 2 mM $MgCl_2$, 0.4 mM of deoxynucleoside triphosphate mix, 0.4 μM of each primer (as listed in Table 2), 0.5 U/μl *Taq* polymerase, and 2.0 μl of DNA template. All PCR reagents were from Promega, U.S.A., and the primers were synthesized by Invitrogen. The following thermocycler conditions were used: predenaturation at 96°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and extension at 72°C for 30 s, and followed by a final extension at 72°C for 7 min. Three μl of the PCR products was loaded and electrophoresed in 1.0% agarose gel with 100 V and were stained and viewed using the Gel Documentation System (SynGene).

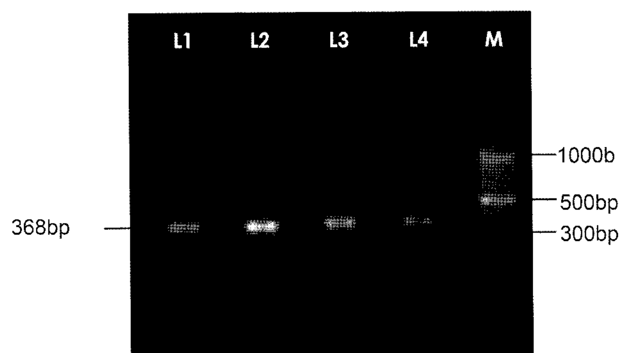

Fig. 1. Agarose gel electrophoresis of the *toxR* gene of *V. parahaemolyticus* (368 bp).
L1 to L3=representative *V. parahaemolyticus* positive samples; L4=positive control; M=100-bp DNA marker.

RESULTS

The presence of *V. parahaemolyticus* in the samples was identified using the PCR technique targeting the *toxR* gene, which is specific for *V. parahaemolyticus*, producing a PCR product of size 368 bp (as shown in Fig. 1). From the PCR detection, the frequency of *V. parahaemolyticus* occurrence in the 276 raw salad vegetable samples could be determined (as summarized in Table 3). The total prevalence rate of *V. parahaemolyticus* in raw vegetables obtained from the retail level in Selangor, Malaysia was 20.65%. The frequency of occurrences in vegetables from wet market C (27.27%) and wet market D (32.05%) were particularly higher compared with supermarket A (1.64%) and supermarket B (16.67%).

The prevalence of *V. parahaemolyticus* in each type of vegetables varied, where Indian pennywort contained the highest percentage (41.18%), followed by Wild cosmos (37.5%), Japanese parsley (28.57%), Cabbage (26.67%), Four-winged bean (19.23%), Tomato (18.42%), Cucumber (17.86%), Carrot (16.13%), Long bean (15.63%), Sweet potato (13.79%), and Lettuce (12.5%).

From the MPN–PCR method, the density of *V. parahaemolyticus* in the vegetable samples was summarized (Table 4). The total number of *V. parahaemolyticus* in the

Table 3. Frequency of occurrence of *V. parahaemolyticus* in raw vegetable samples using the MPN–PCR method.

Vegetable	Supermarket A			Supermarket B			Wet Market C			Wet Market D			Total		
	[a]n	PCR positive	[c]%	[a]n	PCR positive	[c]%	[a]n	PCR positive	[c]%	[a]n	PCR positive	[c]%	[a]n	PCR positive	[c]%
Wild cosmos	[b]-	[b]-	[b]-	[b]-	[b]-	[b]-	8	3	37.50	[b]-	[b]-	[b]-	8	3	37.50
Japanese parsley	2	0	0.00	2	1	50.00	9	3	33.33	8	2	25.00	21	6	28.57
Cabbage	7	1	14.29	9	1	11.11	6	2	33.33	8	4	50.00	30	8	26.67
Lettuce	4	0	0.00	1	0	0.00	3	0	0.00	8	2	25.00	16	2	12.50
Indian pennywort	1	0	0.00	1	1	100.00	7	2	28.57	8	4	50.00	17	7	41.18
Carrot	7	0	0.00	8	1	12.50	8	2	25.00	8	2	25.00	31	5	16.13
Sweet potato	6	0	0.00	8	1	12.50	7	2	28.57	8	1	12.50	29	4	13.79
Tomato	10	0	0.00	9	1	11.11	9	2	22.22	10	4	40.00	38	7	18.42
Cucumber	9	0	0.00	6	2	33.33	7	1	14.29	6	2	33.33	28	5	17.86
Four-winged bean	6	0	0.00	8	1	12.50	6	2	33.33	6	2	33.33	26	5	19.23
Long bean	9	0	0.00	8	1	12.50	7	2	28.57	8	2	25.00	32	5	15.63
TOTAL	61	1	**1.64**	60	10	**16.67**	77	21	**27.27**	78	25	**32.05**	276	57	**20.65**

[a]n=Number of sample.
[b](-)=Sample not available.
[c](%)=Percentage.

Table 4. *V. parahaemolyticus* densities (MPN/g) in raw vegetable samples using the MPN–PCR method.

Vegetable	Supermarket A			Supermarket B			Wet Market C			Wet Market D		
	[a]Min	[b]Med	[c]Max	[a]Min	[b]Med	[c]Max	[a]Min	[b]Med	[c]Max	[a]Min	[b]Med	[c]Max
Wild cosmos	[d]-	-	-	-	-	-	<3	<3	19	-	-	-
Japanese parsley	<3	<3	<3	<3	9.5	19	<3	<3	1,100	<3	<3	>2,400
Cabbage	<3	<3	3	<3	<3	>2,400	<3	<3	>2,400	<3	7.5	>2,400
Lettuce	<3	<3	<3	<3	<3	<3	<3	<3	<3	<3	<3	>2,400
Indian pennywort	<3	<3	<3	15	15	15	<3	<3	>2,400	<3	3.65	>2,400
Carrot	<3	<3	<3	<3	<3	9.2	<3	<3	120	<3	<3	>2,400
Sweet potato	<3	<3	<3	<3	<3	>2,400	<3	<3	>2,400	<3	<3	53
Tomato	<3	<3	<3	<3	<3	>2,400	<3	<3	>2,400	<3	<3	>2,400
Cucumber	<3	<3	<3	<3	<3	>2,400	<3	<3	>2,400	<3	<3	>2,400
Four-winged bean	<3	<3	<3	<3	<3	>2,400	<3	<3	>2,400	<3	<3	>2,400
Long bean	<3	<3	<3	<3	<3	<3	<3	<3	>2,400	<3	<3	460
AVERAGE	<3	<3	3	<3	<3	>2,400	<3	<3	>2,400	<3	<3	>2,400

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

276 samples analyzed ranged widely, from <3 to >2,400 MPN/g. The MPN/g estimate of *V. parahaemolyticus* in the samples from Supermarket A was the lowest among all the locations selected, with a minimum value of <3 MPN/g and maximum 3 MPN/g. Samples from the other locations contained varied numbers of *V. parahaemolyticus* detection, but mostly showed a minimum of <3 MPN/g, median of <3 MPN/g, and maximum of >2,400 MPN/g.

Fig. 2 show's the distribution of MPN–PCR counts of *V. parahaemolyticus* in the samples. The results showed that more than 50 samples from each sampling location contained <3 MPN/g of *V. parahaemolyticus*. Samples that harbored 3 to 53 MPN/g of *V. parahaemolyticus* were less than 10 samples from each location. Less than 4 samples from Wet markets C and D contained 54 to 2,400 MPN/g of *V. parahaemolyticus*, whereas none of the samples from Supermarkets A and B contained this range of *V. parahaemolyticus*. Samples that contained >2,400 MPN/g *V. parahaemolyticus* were lower than 16 samples in Supermarket B, and Wet market C and D, whereas no samples from Supermarket A contained >2,400 MPN/g *V. parahaemolyticus*.

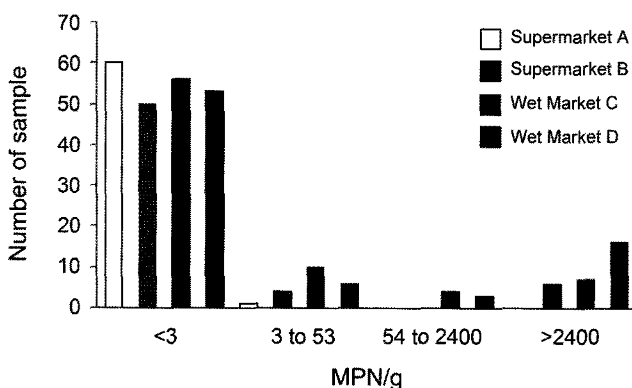

Fig. 2. Distribution of MPN–PCR counts of *V. parahaemolyticus* in raw vegetable samples from four retail outlets.

DISCUSSION

This study employed a highly sensitive PCR-based method to detect and quantify *V. parahaemolyticus* in vegetables, which combined the high sensitivity of the MPN method with a *V. parahaemolyticus*-specific PCR assay. This method has been successfully applied in enumerating *V. parahaemolyticus* in seafood [8], *Campylobacter* spp. in drinking waters [16], *Listeria monocytogenes* in fermented sausages [13], *Campylobacter* spp. in ready-to-eat sushi [18], and *Campylobacter* spp. in raw vegetables [3, 4]. The major disadvantages of the MPN method coupled with traditional confirmation techniques were the amount of material, the workload, and the time needed for complete identification (usually 7 to 10 days); however, the combination with the species-specific PCR method enables the completion of enumeration in approximately 2 days [13]. Alam *et al.* [1] also described the advantages of using the MPN–PCR technique over the traditional techniques such as the MPN–culture method.

The presence of *V. parahaemolyticus* in the raw vegetable samples is probably a reflection of the nature of the four retail outlets in the study. This result highlights the fact that raw vegetables could be contaminated with *V. parahaemolyticus* and thus could possibly act as a transmission vehicle of *V. parahaemolyticus*. The detection of *V. parahaemolyticus* in particularly 20.65% of the samples (Table 3) is of concern; however, this concern is largely alleviated because the concentration of *V. parahaemolyticus* was low, with a majority of the samples containing <3 MPN/g (Fig. 2). Although in low concentration, this showed that *V. parahaemolyticus*, which naturally occurs in seafood, could also be found in vegetables samples.

The fact that samples from wet markets contained higher percentage of *V. parahaemolyticus* compared with supermarkets (Table 3) were understandable, as it could be seen that the way of handling vegetables at wet markets was less hygienic compared with supermarkets. The surroundings and places for vegetable displays at wet markets were not clean and tidy, and the handlers were not wearing gloves while handling the vegetables when compared with supermarkets in which most of their handlers wear gloves. The vegetables stands in wet markets were also located quite near to the fishes and meat stands. This could contribute to cross-contamination and could be the possible cause of prevalence of *V. parahaemolyticus* in vegetables. Tan *et al.* [18] also described that the most possible source of contamination of *Campylobacter* spp. in sushi was cross-contamination from other products in the sampling location. However, cross-contamination could occur at any stage during the long processing and distribution chain. At the harvesting stage and post-harvest, contamination might occur through a contaminated container for transporting and improper handling [3].

Nevertheless, *V. parahemolyticus* could be found in vegetables from supermarkets, although in lower prevalence compared with wet markets (Table 3). From our observation and collection of information during sampling at supermarkets, the vegetables were first washed and packaged before being sold, unlike vegetables handled at wet markets, which were sent directly from the farms every morning and were sold without further washing or packaging. During the various handling for vegetables at supermarkets, there is a possibility that cross-contamination could occur from the handlers or the instruments and utensils used. Chai *et al.* [4] reported the possibility of handling utensils as potential risk factors in *C. jejuni* transmission to consumers. Apart from that, the vegetables at supermarkets sometimes have a long holding time, which could contribute to the accumulation of *V. parahaemolyticus*.

In this study, the vegetable samples analyzed showed a lower density of *V. parahaemolyticus* in Supermarket A compared with the other locations, with a range of only <3 to 3 MPN/g (Table 4). The more hygienic conditions of the surrounding and handling of vegetables in Supermarket A might account for the low concentration, as seen from our observation during sample collection. This is supported by Tan *et al.* [18], which reported that cross-contaminations are often due to poor hygiene and sanitation practice of the workers. Although Supermarket B contained almost the same concentration level of *V. parahaemolyticus* with vegetables from the wet markets (Table 4), the total prevalence percentage for Supermarket B was still lower compared with the wet markets (Table 3). However, Supermarket B had a high density (maximum >2,400 MPN/g) and prevalence (16.67%) of *V. parahaemolyticus* in vegetables compared with Supermarket A. From our observation, the vegetables sold at Supermarket B were far many compared with Supermarket A, and the vegetables at Supermarket B tended to finish slower than at Supermarket A. This could probably contribute to a longer holding time for the vegetables at Supermarket B (average holding time observed for Supermarket A was approximately one to three days, and for Supermarket B one to five days, depending on the type of vegetables), and therefore giving more time for *V. parahaemolyticus* to grow in the vegetables [3].

Apart from hygiene and holding time, temperature is also known to relate to the distribution of *V. parahaemolyticus* [17]. We observed that the surrounding temperatures in Wet Markets C and D were mostly higher compared with Supermarkets A and B (data not shown). Compared with the refrigerated vegetables at the supermarkets, contaminations of vegetables from Wet Markets C and D were more prevalent and had relatively higher *V. parahaemolyticus* cells (Tables 3 and 4, and Fig. 2), probably mainly because *V. parahaemolyticus* cells increase rapidly under unrefrigerated conditions [17]. Duan and Su [5] reported a positive correlation between *V. parahaemolyticus* in seawater and water temperatures, with the highest populations of *V. parahaemolyticus* in seawater in summer months.

From the samples analyzed, *V. parahaemolyticus* was most predominant in vegetable type Indian pennywort with a 41.18% prevalence rate (Table 3). The leafy structure and form of the Indian pennywort, which allowed more surfaces for attachments, could contribute to the higher rate of survival of *V. parahaemolyticus* on the vegetable. The other types of vegetables with a high prevalence rate of *V. parahaemolyticus* (Wild cosmos 37.5%, Japanese parsley 28.57%, Cabbage 26.67%, and Four-winged bean 19.23%) were also leafy in structure and form, compared with the rest of the vegetable types.

This study showed that MPN–PCR is a very useful tool for detection of *V. parahaemolyticus* in vegetables. Monitoring *V. parahaemolyticus* in vegetables is important in preparation of a risk assessment plan relating to vegetables. The detectable prevalence and concentration of *V. parahaemolyticus* in raw vegetables from all the locations sampled indicated that *V. parahaemolyticus*

could contaminate vegetables usually consumed raw in Malaysia and thus poses a risk to consumers. Hence, it is recommended to pay attention to handling raw vegetable products at the retail level to safeguard public health.

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