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FULL PAPER

Detection and quantification of *Vibrio parahaemolyticus* in vegetables and environmental samples at farm level

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Abstract

The purpose of this study was to detect and quantify total and pathogenic *Vibrio parahaemolyticus* from vegetables and environmental samples at the farm level in Cameron Highlands, Pahang, Malaysia. Most Probable Number (MPN) – Polymerase Chain Reaction (PCR) method was used to detect *toxR, tdh* and *trh* genes and to quantify their concentration in samples. Samples obtained were cabbage (20), carrot (10), cucumber (10), lettuce (31), tomato (18), manure (10), soil (12), surface swab (21) and water (14), with a total of 146 samples. Sampling locations involved were three vegetable farms, two packing houses and one loading bay. Based on the results, overall, 13.7% of samples were present with *V. parahaemolyticus toxR* (maximum concentration 1100 MPN/g), with the highest detection in cabbage (6%). *Vibrio parahaemolyticus tdh* was detected in 1.4% samples (maximum concentration 7.3 MPN/g), and *V. parahaemolyticus trh* could not be detected in any samples. No *tdh* and *trh* genes could be detected from the recovered isolates. This finding highlighted that vegetables and environmental samples could potentially be contaminated with *V. parahaemolyticus* which poses risk to consumers. This study could be useful in future food safety risk communication and management programmes.

1. Introduction

Fruits and vegetables play a significant role in human nutrition by supplying nutrients such as vitamins, minerals and dietary fibre. The production and processing of fruits and vegetables involve a complex supply chain from the farm to the point of consumption (Pilizota, 2013). The number of produce-related outbreaks has increased in the past decade (Kalantar *et al.*, 2018). These products could be contaminated with biological hazards at any point throughout the supply chain. Consumers eating fresh fruits and vegetables are at risk because this product may be grown on contaminated soil. These bacterial-tainted fruits and vegetables may have come from fields that used to contain animals. Their faeces, faeces-laced irrigation water, or raw manure may have been used as soil additives. Other contributing factors may include changes in agronomic and processing practices, increased international trade and distribution, and an increase in the number of immuno-compromised consumers. Contamination of produce is a concern in developing countries that lack sanitary basic conditions

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(Western Farm Press, 2007; Pilizota, 2014).

Vibrio parahaemolyticus is a halophilic bacterium that has been known to cause foodborne disease through the consumption of contaminated raw or undercooked seafood (Prabhakaran *et al.*, 2020; Martinez-Urtaza and Baker-Austin, 2020). However, there have been reports of vibrio gastroenteritis associated with the consumption of raw vegetables. *V. parahaemolyticus* could be found in freshwater or streams and is occasionally isolated from the infected skin of fish handlers. This shows that *V. parahaemolyticus* could cross-contaminate and survive on non-marine sources. Vibrio gastroenteritis has sometimes been associated with the consumption of raw vegetables which have been contaminated through kitchen utensils (Sakazaki *et al.*, 2006). An outbreak in Kedah, Malaysia in 2003 was reported to be caused by *V. parahaemolyticus* linked to 'kerabu tauge', a local dish mixed with raw vegetables (Mohamad *et al.*, 2006). In Wenzhou, China, Li (2007) reported a 6.12% (20/112) presence of *V. parahaemolyticus* in vegetable fruit salad and other food types. Besides that, Okafo *et al.* (2003) reported the presence of *Vibrio* spp. in raw vegetables harvested from soils irrigated with contaminated streams in Nigeria (Sakazaki *et al.*, 2006; Mohamad *et al.*, 2006; Li, 2007). This indicated that there is a risk for consumers when consuming raw vegetables and fruits, and can cause acute gastroenteritis in immunocompromised individuals. Therefore, it is important to investigate the presence of *V. parahaemolyticus* in raw vegetables and fruits, especially in Malaysia.

V. parahaemolyticus regulatory gene *toxR* is present in all strains, and PCR based on *toxR* reported to be specific for *V. parahaemolyticus* has been found useful for confirmation of this species. 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* (Zhang *et al.*, 2018; Prabhakaran *et al.*, 2020; Martinez-Urtaza and Baker-Austin, 2020). Most Probable Number (MPN) combined with PCR has been successfully applied in enumerating various pathogens in food samples including raw vegetables (Chai *et al.*, 2009; Sandra *et al.*, 2012; Wong *et al.*, 2012).

In order to understand the risk of acquiring *V. parahaemolyticus* from the consumption of raw vegetables, it is necessary to assess the prevalence and quantity of total and pathogenic *V. parahaemolyticus* in raw vegetables. This study will assess *V. parahaemolyticus* in vegetables and environmental samples collected at the farm level in Cameron Highlands, Pahang, Malaysia. This will provide an insight into the scenario in Malaysia and will be useful for further risk assessment studies.

2. Materials and methods

2.1 Sample collection

Samples were collected from three vegetable farms, two packing houses and one loading bay, in Cameron Highlands, Pahang. The location is one of the main sources and distributors of fresh produce throughout Peninsular Malaysia. The sampling locations were located at three of the eight sub-districts and were randomly chosen. The samples collected were freshly cut vegetables (n = 89), soil (n = 12), animal manure (n = 10), irrigation water from the reservoir and pipes (n = 14), and swabs (n = 21). Vegetable types collected from the three farms were conducted at random and were dependent on the vegetables that were available at the time of the visit. Soil and animal manure were only collected from Farms 2 and 3 which practised organic farming. The samples collected from the loading bay were freshly harvested vegetables and about to be transported. The samples collected from the packing houses were freshly harvested vegetables, surface swabs from vats, food sorting equipment and packaging tools. Vegetable samples were freshly harvested and placed in sterile plastic bags. Soil and animal manure samples (approximately 200 g each) were collected from different points of the planting site and placed in sterile plastic sampling tubes. Water samples were collected from different points at the main reservoir, distribution tanks, taps and irrigation pipes, and were placed into sterile tubes. Surface swabs were taken from baskets, vehicles, knives, food sorting equipment and vats using sterile cotton swabs, and were placed in sterile plastic tubes. All samples were transported to the laboratory immediately and analyzed within 24 hrs of sample collection.

2.2 Sample preparation

The sampling method performed in this study was based on methods by Tunung *et al.* (2010) and Tunung *et al.* (2011). A 10 g portion of each vegetable 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 with pre-enrichment by incubation at 37°C for 6 hrs.

2.3 MPN-PCR

A 100-fold and 1000-fold dilutions of the stomacher fluid were prepared with Salt Polymyxin Broth (SPB; Nissui, Japan). Portions of each dilution were transferred

into three tubes and incubated at 37°C for 18 to 24 hrs. After incubation, a loopful of culture from each tube was streaked onto CHROMagar™ Vibrio. The MPN tubes were first preceded by DNA extraction, which was carried out using the boil cell method (Tunung *et al.*, 2011) with slight modifications. A 1 mL portion of each MPN broth was centrifuged at 13400×*g* for 1 min and the pellet was resuspended in 500 µL of sterile distilled water. The mixture was boiled for 10 mins and then immediately cooled at -20°C for 10 mins before it was centrifuged at 13400×*g* for 3 mins. The supernatant was kept for use in PCR for detection of *toxR*. The reference *V. parahaemolyticus* strains (*V. parahaemolyticus* strain coded 1808, 1896, 2053) used for positive control in the PCR reaction was obtained from Kyoto University, Japan.

PCR amplification (Tunung *et al.*, 2011) for the detection of *toxR* gene 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 (*toxR4* 5'-GTCTTCTGACGCAATCGTTG-3', *toxR7* 5'-ATACGAGTGGTTGCTGTCATG-3'), 0.5 U/µL *Taq* polymerase and 2.0 µL of DNA template. Meanwhile, PCR amplification for the detection for *tdh* and *trh* genes were performed in a 20 µL reaction mixture containing 4.0 µL of 5× PCR buffer, 2.25 mM $MgCl_2$, 0.2 mM of deoxynucleoside triphosphate mix, 0.2 µM of each primer (*tdh-D3* 5'-CCACTACCACTCTCATATGC-3', *tdh-D5* 5'-GGTACTAAATGGCTGACATC-3', *trh-R2* 5'-GGCTCAAAATGGTTAAGCG-3', *trh-R6* 5'-CATTTCCGCTCTCATATGC-3'), 0.5 U/µL *Taq* polymerase and 2.0 µL of DNA template. All PCR reagents were from Promega, USA, and the primers were synthesized by Invitrogen. The following thermocycler conditions were used: pre-denaturation at 96°C for 5 mins, 35 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s (for *toxR*) or 55°C for 30 s (for *tdh* and *trh*), and extension at 72°C for 30 s, and followed by final extension at 72°C for 7 mins. 3 µL of the PCR products were loaded and electrophoresed in 1.0% agarose gel with 0.5× TBE using 100 V, and were stained with ethidium bromide, and viewed using Gel Documentation System (SynGene).

2.4 Direct PCR

The direct-PCR detection method was used only for environmental samples such as soil, animal manure, swab and water samples (Chai *et al.,* 2009). For soil and animal manure samples, 10 g of the sample was weighed into a 50 mL Falcon tube, mixed with sterile distilled water up to a final volume of 40 mL and incubated at 37° C for 1 hr. The mixture was centrifuged at 600×*g* for 15 mins and the supernatant was filtered through a sterile cheesecloth. The supernatant was discarded and 400 µL of sterile distilled water was added. The mixture was then transferred to a sterile 1.5 mL microcentrifuge tube and was boiled for 10 mins. Then the sample was cooled at -20°C for 5 mins before it was centrifuged at 12000×*g* for 10 mins. The supernatant was transferred to a new 1.5 mL centrifuge tube and 800 µL of 95% ethanol was added. The tube was inverted several times and left on ice for 5 mins prior to centrifugation at 12000×*g* for 10 mins. The supernatant was discarded and the pellet was washed with 1 mL of 97% ethanol. Then it was centrifuged at 12000×*g* for 10 mins and the pellet was dried under laminar airflow. The DNA was resuspended with 200 µL of sterile distilled water and proceeded for PCR analysis.

For water samples, 50 mL of the sample was poured into a 50 mL Falcon tube and centrifuged at 600×*g* for 15 mins. The supernatant was transferred to a new tube and subjected to centrifugation at 12000×*g* for 30 mins. The supernatant was discarded and the pellet was resuspended with 400 µL of sterile distilled water. The mixture was transferred to a sterile 1.5 mL centrifuge tube and boiled for 10 mins. The sample was cooled at -20°C for 5 mins before centrifugation at 15000×*g* for 10 mins. The supernatant was transferred to a new 1.5 mL centrifuge tube and 800 µL of 95% ethanol was added. The tube was inverted several times and was left on ice for 5 mins prior to centrifugation at 12000×*g* for 10 mins. The supernatant was discarded and the pellet was washed with 1 mL of 97% ethanol. Centrifugation was carried out again at 12000×*g* for 10 mins and the pellet was dried under laminar airflow. The DNA was resuspended with 200 µL of sterile distilled water and proceeded for PCR analysis.

2.5 Isolates recovery

A loopful of culture from each MPN tubes was streaked onto CHROMagar™ Vibrio (CV). The plates were then incubated at 37°C for 18 to 24 hrs. Presumptive colonies of *V. parahaemolyticus* (mauve purple colour) was picked and subcultured onto Tryptic Soy Agar (TSA; Bacto™, France) with 3% sodium chloride (NaCl; Merck, Germany). Isolates were confirmed by gram-strain, colonial and microscopic morphology, catalase test, oxidase test (data not shown), and specific PCR targeting *toxR, tdh* and *trh* genes of *V. parahaemolyticus*. Pure confirmed isolates were maintained on TSA 3% NaCl agar slants and stock cultred in 20% glycerol.

2.6 Statistical analysis

To determine if there was any significant difference

between the prevalence of total and pathogenic *V. parahaemolyticus* among the samples, sample types, sampling locations, temperatures during sampling, and hygiene levels of the sampling locations, SPSS software (version 16.0) was utilised to analyze the data. The level of significance was set at $P<0.05$. Whenever there was a significant difference, post-hoc comparison was done using Tukey's Honestly Significant Difference (HSD) to identify the groups that are significantly different (data not shown).

3. Results

The total frequency of *V. parahaemolyticus* detected in 146 samples collected at pre-harvest level was 13.7% (Table 1). *Vibrio parahaemolyticus* was found highest in cabbage (30%), followed by soil (25%), cucumber and animal manure (20% respectively), tomato (16.7%), carrot (10%), lettuce (6.5%), and a surface swab of the basket (4.8%). *Vibrio parahaemolyticus* in water samples was not detected (0%). For sampling locations, the prevalence of *V. parahaemolyticus* was highest in samples collected from Farm 3 (21.2%), followed by Farm 2 (20%), 18.75% from Packing House 1, 11.5% from Loading Bay, and 3.9% from Farm 1. No *V. parahaemolyticus* was detected in samples from Packing House 2. The concentrations of *V. parahaemolyticus* in the samples ranged from <3 MPN/g up to 1100 MPN/g. The highest maximum numbers of *V. parahaemolyticus* were in samples from Farm 2 and Farm 3 (1100 MPN/g respectively). Statistical analysis was carried out to determine any significant differences in MPN/g values of *V. parahaemolyticus toxR* between the samples, sample types, sampling locations and temperatures during sampling (data not shown). The results showed no significant differences between all the categories tested.

The prevalence of *V. parahaemolyticus tdh* was 1.4% (2/146) overall, as shown in Table 2. *Vibrio parahaemolyticus tdh* was found in one sample from Loading Bay (3.85%) and one sample from Farm 2 (3.33%), while none were detected in samples from the rest of the sampling locations. *V. parahaemolyticus tdh* was most predominant in sample type cabbage with 10% (2/20) prevalence; while no *V. parahaemolyticus tdh* could be detected in other types of samples. The MPN/g values of *V. parahaemolyticus tdh* detected in the samples ranged from <3 MPN/g up to 7.3 MPN/g. When the data were analyzed statistically, there was no significant difference in MPN/g values between the samples, sample types, sampling locations and temperatures during sampling. As for *V. parahaemolyticus trh*, it was not detected in all of the samples.

For the recovery of isolates from plating, only 1 isolate (0.7%) was confirmed positive *V. parahaemolyticus toxR* (Table 3). The sample was cabbage from Packing House 1. The confirmed *V. parahaemolyticus* isolate was further tested for the presence of virulent genes *tdh* and *trh*, however, none was detected (data not shown).

4. Discussion

This study revealed the presence of *V. parahaemolyticus* in vegetables and environmental samples at the pre-harvest stage in vegetable farms. In this study, *V. parahaemolyticus* was detected in non-marine samples. The issue of how *V. parahaemolyticus* could survive and grow on these unnatural sources raises questions and requires explanation and further research. However, previous outbreaks and research have reported the presence of *Vibrio* spp. in vegetables and other non-marine samples (Okafo *et al.*, 2003; Mohamad *et al.*, 2006; Sakazaki *et al.*, 2006; Li, 2007; Noorlis *et al.*, 2011). Changes in dietary habits, methods of fruit and vegetable production and processing, sources of produce, and the emergence of pathogens previously not recognized for their association with raw produce, could have enhanced the potential for outbreaks.

Overall, the percentage of samples with the presence of *toxR* and *tdh* were 13.7% and 1.4% respectively, while *trh* could not be detected in any of the samples. Higher prevalence and concentration of *toxR* were found in samples from Farm 2 and 3 compared to samples from Farm 1. This could be due to the fact that Farms 2 and 3 practised organic farming. It is tempting to speculate that the environmental samples were the most possible sources of contamination of *V. parahaemolyticus* to the vegetables since there was the presence of *toxR* in manure, soil and swab samples from Farm 2 and 3. Chai *et al.* (2009) also reported the possibility of *Campylobacter* spp. contamination in vegetables in their study was associated with the presence of *Campylobacter* spp. in soil and manure samples. However, vegetable samples from Loading Bay and Packing House 1 harboured *V. parahaemolyticus toxR* although none could be detected in environmental samples from the same location. The possible explanation is most probably due to cross-contamination introduced by other sources which were not sampled in this study, which include the handler's hands. Pickers, handlers, packers, and other individuals involved in the handling of produce may have the potential to contaminate the procedure along the harvesting chain.

In this study, the number of *V. parahaemolyticus toxR* and *tdh* in the samples were mostly <3 MPN/g, with the maximum number reaching only 1100 MPN/g and 7.3 MPN/g (respectively). Although the counts were

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Table 1. Detection and concentration of *V. parahaemolyticus tox*R in samples.

Location	Cabbage	Carrot	Cucumber	Lettuce	Tomato	Manure	Soil	Swab	Water	Total
Farm 1										
% (positive/total)	NA	NA	NA	5.9 (1/17)	NA	NA	NA	0 (0/4)	0 (0/5)	3.9 (1/26)
Med (MPN/g)	NA	NA	NA	<3	NA	NA	NA	<3	<3	<3
Max (MPN/g)	NA	NA	NA	3	NA	NA	NA	<3	<3	3
Farm 2										
% (positive/total)	50 (3/6)	NA	66.7 (2/3)	0 (0/6)	NA	0 (0/4)	20 (1/5)	0 (0/3)	0 (0/3)	20 (6/30)
Med (MPN/g)	1.8	NA	3	<3	NA	<3	<3	<3	<3	<3
Max (MPN/g)	1100	NA	7.2	<3	NA	<3	3	<3	<3	1100
Farm 3										
% (positive/total)	NA	NA	NA	NA	25 (2/8)	33.3 (2/6)	28.6 (2/7)	16.7 (1/6)	0 (0/6)	21.2 (7/33)
Med (MPN/g)	NA	NA	NA	NA	<3	<3	<3	<3	<3	<3
Max (MPN/g)	NA	NA	NA	NA	1100	3	3	3	<3	1100
Packing House 1										
% (positive/total)	25 (1/4)	33.3 (1/3)	0 (0/3)	NA	50 (1/2)	NA	NA	0 (0/4)	NA	18.8 (3/16)
Med (MPN/g)	<3	<3	<3	NA	3.6	NA	NA	<3	NA	<3
Max (MPN/g)	3.6	3.6	<3	NA	7.2	NA	NA	<3	NA	7.2
Packing House 2										
% (positive/total)	0 (0/4)	NA	0 (0/4)	NA	0 (0/3)	NA	NA	0 (0/4)	NA	0 (0/15)
Med (MPN/g)	<3	NA	<3	NA	<3	NA	NA	<3	NA	<3
Max (MPN/g)	<3	NA	<3	NA	<3	NA	NA	<3	NA	<3
Loading Bay										
% (positive/total)	33.3 (2/6)	0 (0/7)	NA	12.5 (1/8)	0 (0/5)	NA	NA	NA	NA	11.5 (3/26)
Med (MPN/g)	<3	<3	NA	<3	<3	NA	NA	NA	NA	<3
Max (MPN/g)	11	<3	NA	3.6	<3	NA	NA	NA	NA	11
Total										
% (positive/total)	30 (6/20)	10 (1/10)	20 (2/10)	6.5 (2/31)	16.7 (3/18)	20 (2/10)	25 (3/12)	4.8 (1/21)	0 (0/14)	13.7 (20/146)

(positive/total) = no. of positive sample/total sample; Med = Median MPN/g value, Max = Maximum MPN/g value, NA = Sample not available

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Table 2. Detection and concentration of *V. parahaemolyticus tdh* in samples.

Location	Cabbage	Carrot	Cucumber	Lettuce	Tomato	Manure	Soil	Swab	Water	Total
Farm 1										
% (positive/total)	NA	NA	NA	0 (0/17)	NA	NA	NA	0 (0/4)	0 (0/5)	0 (0/26)
Med (MPN/g)	NA	NA	NA	<3	NA	NA	NA	<3	<3	<3
Max (MPN/g)	NA	NA	NA	<3	NA	NA	NA	<3	<3	<3
Farm 2										
% (positive/total)	16.7 (1/6)	NA	0 (0/3)	0 (0/6)	NA	0 (0/4)	0 (0/5)	0 (0/3)	0 (0/3)	3.3 (1/30)
Med (MPN/g)	<3	NA	<3	<3	NA	<3	<3	<3	<3	<3
Max (MPN/g)	7.3	NA	<3	<3	NA	<3	<3	<3	<3	7.3
Farm 3										
% (positive/total)	NA	NA	NA	NA	0 (0/8)	0 (0/6)	0 (0/7)	0 (0/6)	0 (0/6)	0 (0/33)
Med (MPN/g)	NA	NA	NA	NA	<3	<3	<3	<3	<3	<3
Max (MPN/g)	NA	NA	NA	NA	<3	<3	<3	<3	<3	<3
Packing House 1										
% (positive/total)	0 (0/4)	0 (0/3)	0 (0/3)	NA	0 (0/2)	NA	NA	0 (0/4)	NA	0 (0/16)
Med (MPN/g)	<3	<3	<3	NA	<3	NA	NA	<3	NA	<3
Max (MPN/g)	<3	<3	<3	NA	<3	NA	NA	<3	NA	<3
Packing House 2										
% (positive/total)	0 (0/4)	NA	0 (0/4)	NA	0 (0/3)	NA	NA	0 (0/4)	NA	0 (0/15)
Med (MPN/g)	<3	NA	<3	NA	<3	NA	NA	<3	NA	<3
Max (MPN/g)	<3	NA	<3	NA	<3	NA	NA	<3	NA	<3
Loading Bay										
% (positive/total)	16.7 (1/6)	0 (0/7)	NA	0 (0/8)	0 (0/5)	NA	NA	NA	NA	3.9 (1/26)
Med (MPN/g)	<3	<3	NA	<3	<3	NA	NA	NA	NA	<3
Max (MPN/g)	3.6	<3	NA	<3	<3	NA	NA	NA	NA	3.6
Total										
% (positive/total)	10 (2/20)	0 (0/10)	0 (0/10)	0 (0/31)	0 (0/18)	0 (0/10)	0 (0/12)	0 (0/21)	0 (0/14)	1.4 (2/146)

(positive/total) = no. of positive sample/total sample; Med = Median MPN/g value, Max = Maximum MPN/g value, NA = Sample not available

Table 3. Positive *V. parahaemolyticus* (*toxR*+) isolates recovered from plating onto CV.

Location	Cabbage	Carrot	Cucumber	Lettuce	Tomato	Manure	Soil	Swab	Water	Total
Farm 1										
% (positive/total)	NA	NA	NA	0 (0/17)	NA	NA	NA	0 (0/4)	0 (0/5)	0 (0/26)
Farm 2										
% (positive/total)	0 (0/6)	NA	0 (0/3)	0 (0/6)	NA	0 (0/4)	0 (0/5)	0 (0/3)	0 (0/3)	0 (0/30)
Farm 3										
% (positive/total)	NA	NA	NA	NA	0 (0/8)	0 (0/6)	0 (0/7)	0 (0/6)	0 (0/6)	0 (0/33)
Packing House 1										
% (positive/total)	25 (1/4)	0 (0/3)	0 (0/3)	NA	0 (0/2)	NA	NA	0 (0/4)	NA	16.67 (1/16)
Packing House 2										
% (positive/total)	0 (0/4)	NA	0 (0/4)	NA	0 (0/3)	NA	NA	0 (0/4)	NA	0 (0/15)
Loading Bay										
% (positive/total)	0 (0/6)	0 (0/7)	NA	0 (0/8)	0 (0/5)	NA	NA	NA	NA	0 (0/26)
Total										
% (positive/total)	5 (1/20)	0 (0/10)	0 (0/10)	0 (0/31)	0 (0/18)	0 (0/10)	0 (0/12)	0 (0/21)	0 (0/14)	0.68 (1/146)

(positive/total) = no. of positive sample/total sample

below the accepted level based on the recommended level by USFDA, which stated that the infectious dose in a healthy person is 10^6 cells, it is still dependent on the host and is risky for those who are immunocompromised.

Several researchers (Park and Sanders, 1992; Chai *et al.*, 2009) have reported the prevalence of pathogens in leafy and roots vegetables, suggesting that vegetables in close contact with soil have a higher possibility of contamination by pathogens. Previous research by Tunung *et. al.* (2010) also reported a higher prevalence of *V. parahaemolyticus* in leafy vegetables, suggesting that the leafy structure, which allowed more surfaces for attachments, could contribute to the higher rate of survival of *V. parahaemolyticus* on the vegetables. However, in this study, there was no significant difference in the prevalence of *V. parahaemolyticus toxR* between the different types of vegetables. Both leafy-type vegetable samples consisted of the highest (cabbage 30%) and lowest (lettuce 6.5%) prevalence of *V. parahaemolyticus* compared to root-type and smooth-surface vegetables samples. In addition to that, cucumber and tomato, which are smooth-surface vegetables, contained a higher prevalence of *V. parahaemolyticus* (20% and 16.7% respectively) compared to carrot (10%), which is a root-type vegetable. However, it may be worthy to note that in this study; only sample type cabbage contained virulent *V. parahaemolyticus tdh* (1.4%). Bruised and cut surface tissue discharge fluids containing nutrients and numerous phytoalexins and other antimicrobials that may improve or retard the growth of naturally occurring microflora and pathogens (Thompson, 2009). Soil or faecal material present on the surface of products that may permeate bruised tissues may alter the ecological environment and the behaviour of pathogens and microflora (Beuchat, 2002; Alegbeleye *et al*., 2018). Mould growths in these environments may result in increased pH and enhance the probability of growth of pathogenic bacteria. Colonization and biofilm development may occur, resulting in conditions that would protect against the death of pathogens or promote the growth of pathogenic microorganisms. Their viability as affected by extrinsic and intrinsic factors unique to fruits and vegetables is unknown (Beuchat, 2002). Natural and food processing environments often impose various stresses on foodborne pathogens and cause morphological changes and adaptation to stress. In an investigation done by (Chen *et al.,* 2009), *V. parahaemolyticus* was incubated under starvation conditions for 3 days and revealed a characteristic morphological change called viable but nonculturable (VBNC) state. Yoon *et al.* (2020) also published a report on the VBNC characteristic of *V. parahaemolyticus*.

The MPN-PCR detection method used in this study was effective compared to the conventional plating method. The plating method was only able to detect viable cells which resulted in a very low prevalence (0.7%), while the MPN-PCR technique was able to detect non-viable cells in which the total prevalence detected was 13.7%.

5. Conclusion

This study provided data on the presence of *V. parahaemolyticus* at the farm level in Cameron Highlands, Pahang, Malaysia, and indicated a 13.7% overall prevalence in vegetables and environmental samples. The findings in this study highlighted the potential of vegetables and environmental samples to be contaminated with *V. parahaemolyticus* and may pose risk to consumers, especially through the consumption of

raw vegetables. This study could be useful in future risk communication and management programmes. Comparison to other research was difficult to be made as information on *V. parahaemolyticus* in vegetable and environmental samples is still lacking. More studies on this subject need to be carried out in order to assess the risk of acquiring pathogenic *V. parahaemolyticus* when consuming raw vegetables.

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