

## SHORT COMMUNICATION

### Application of Real Time PCR for the Detection and Enumeration of *Vibrio parahaemolyticus* from Seafood in Sarawak (Malaysia)

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#### ABSTRACT

*Vibrio parahaemolyticus* has been associated as the major cause of food poisoning and gastroenteritis in many countries including Southeast Asia. Their human infection is regularly associated with the consumption of raw or undercooked seafood and contaminated water supplies. A rapid method of detection is important to monitor the occurrence of *V. parahaemolyticus* in both food and environment samples especially in Malaysia. Thus, the aim of this study was to detect and enumerate *V. parahaemolyticus* from seafood by using the real-time PCR based on the SYBR green assay, targeting the thermolabile (tl) gene. The assay was applied on 24 seafood samples comprised of six cockles, six prawns, six squids and six fishes. In this study, all of the six cockles and prawns were positive for the presence of *V. parahaemolyticus* while fish samples have only two positive isolates. However, this study recorded no presence of *V. parahaemolyticus* in squids. Overall, the real-time PCR assay was proven to be highly specific, and is sensitive in detecting and enumerating *V. parahaemolyticus* in the seafood samples. In addition, this study has proven that seafood (especially cockles and prawns) are potential sources for *V. parahaemolyticus*.

Keywords: Detection, enumeration, real-time PCR, seafood, *Vibrio parahaemolyticus*

Seafood makes up one of the largest phyla of organisms in the animal kingdom. The term seafood covers fish, shellfish and mollusks from all waters – fresh and marine, warm and cold (Adams & Moss, 2002). Seafood is known for its nutritious content which is needed to make a person healthy. Nevertheless, there is health risks associated with the consumption of seafood. One of the major risks involves the consumption of raw or undercooked seafood that may be naturally contaminated by foodborne pathogens present in the marine environment. In Japan, consuming a variety of raw seafood or lightly cooked is highly selected as a favourite style of consumption. However, this practice of eating seems to provide a justification for many cases of foodborne illness by *V. parahaemolyticus* in the country (Elexson *et al.*, 2013). Such risk is further increased if the food is mishandled during processing where

pathogens could multiply exponentially under favourable conditions. In contrast to most of the other foodborne pathogens, *Vibrio* species has the aquatic habitat as their natural niche. As a result, vibrios are most commonly associated with seafood as natural contaminants. Among the potentially pathogenic vibrios occurring naturally on fish and shellfish, *V. parahaemolyticus* is the most widespread.

Early studies demonstrated that the Kanagawa phenomenon, a beta type haemolysis on Watsugama agar medium, was associated with most clinical strains but with very few environmental strains (Dileep *et al.*, 2003). The haemolysis was due to the production of the thermostable direct haemolysin (TDH) (Lee & Pan, 1993). Production of a TDH-related haemolysin (TRH) is also known as one of the virulence

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traits of *V. parahaemolyticus*. The *tdh* and *trh* genes shares approximately 70% nucleotide sequence identity (Nishibuchi *et al.*, 1985). These genes have been widely applied to identify the presence of pathogenic *V. parahaemolyticus*. There are other commonly used marker genes such as *toxR* (Zulkifli *et al.*, 2009) and *tl* (Zhang *et al.*, 2005; Micky *et al.*, 2014) that provides better quantification of the total non-pathogenic and environmental *V. parahaemolyticus* population (Rosec *et al.*, 2009; Theethakaew *et al.*, 2013).

Several studies conducted in the recent years highlighted the rise of the prevalence of the organism and underlines the need for adequate consumer protection against pathogenic *V. parahaemolyticus* in Malaysia since seafood is one of the important ingredient and diet in this country. Export of seafood is an important economic activity for many developing countries, including Malaysia. Failure to meet the bacteriological standards of importing may cause severe economic losses.

Real-time PCR systems offer a wide range of capabilities. The system provides an ability to monitor amplicon accumulation as a reaction proceeds and allows the operator to evaluate product specificity without opening the reaction chamber, time-saving, and reduces carry-over contamination risk (Cai *et al.*, 2006). The amount of DNA is measured after each cycle by the use of fluorescent markers included into the PCR mixture. Fluorescent reporters used can be a nonspecific dsDNA binding dye, or dye molecules attached to PCR primers or probes that generate a signal only in the presence of the target DNA sequence. SYBR Green is a nonspecific dsDNA binding dye that is commonly used in real-time PCR assay due to the advantages of being easy to design, relatively low setup and running costs (Arikawa *et al.*, 2008; Barkallah *et al.*, 2013). SYBR Green real-time PCR assays have been successfully used for the detection of food-borne pathogens such as Enterobacteriaceae (Martinon *et al.*, 2011), *Vibrio* species (Zhou *et al.*, 2007; Sujewa *et al.*, 2009; Martinon *et al.*, 2011; Wang *et al.*, 2013), *Salmonella* species (Rakesh *et al.*, 2010) and *Listeria* species (Barbau-Piednoir *et al.*, 2013). This

technique has also been applied for the rapid detection of virus such as sheep pox virus (Tian *et al.*, 2012) and Avian influenza A (H7N9) virus (Zhu *et al.*, 2013).

In this study, detection and enumeration of *V. parahaemolyticus* was performed by using the real-time PCR based on the SYBR green assay, particularly targeting the thermo labile hemolysin (*tl*) gene. SYBR Green is chosen due to the fact that it is more economical, rapid and sensitive (Dorak, 2007). Our study report the first observation of detection and enumeration of *V. parahaemolyticus* in seafood using real-time PCR in Sarawak, Malaysia.

Sample collection and preparation – Four types of seafood products were analyzed in this study. A total of 24 fresh samples consisted of bloody cockle (*Anadara granosa*), prawn (*Penaeus* species), Indian mackerel (*Rastrelliger kanagurta*) and squid (*Loligo opalescen*) were obtained from a local wet market in Sarawak, from September 2013 till February 2014. During collection, the samples were placed in sterile bags and were transported to the laboratory under refrigerated conditions. The samples were analyzed within 24 hours of collection.

Enumeration and detection of *Vibrio parahaemolyticus* from seafood samples by streak plate – Briefly, a 25 g-portion of each seafood sample were homogenized with 225 ml of alkaline peptone water (APW), in a sterile stomacher bag for 60 seconds (Noorlis *et al.*, 2011). The homogenized samples were pre-filtered before being subjected to a series of ten-fold dilution (with the highest dilution at  $10^{-4}$ ) in sterile phosphate-buffered saline (PBS). Aliquots of each dilution (except at  $10^{-4}$ ) were spread plated in duplicates onto Thiosulphate-Citrate-Bile Salt Sucrose (TCBS; Merck, Germany) agar and CHROMAGAR™ *Vibrio* (CHROMagar 4, France) respectively. The plates were then incubated overnight at 37°C. The filtered samples broths were used for bacterial genomic extraction.

Standard curve construction and bacterial cell count by hemocytometer – A series of ten-fold dilution (with the highest dilution at  $10^{-7}$ )

was prepared with 9 ml of sterile saline solution in each tube. An overnight culture of *V. parahaemolyticus* (laboratory collection) in APW with 3% NaCl was also prepared. Dilutions were performed in triplicates. The overnight and diluted cultures were subjected for DNA extraction. A hemocytometer was used to count bacterial cell presence in each dilution series. Seven hundred microliter of the overnight and diluted culture was transferred into microcentrifuge tubes. Then, 300  $\mu$ l of Crystal Violet dye solution was added into each tube. The tubes were then vortexed thoroughly. Ten microliter of the cell suspension was loaded onto hemocytometer chamber which was covered with a glass cover and then counted under a Polarizing microscope (Olympus BX51, USA).

DNA extraction of *Vibrio parahaemolyticus* from seafood samples and serial dilution samples – Bacterial genomic DNA was extracted from the seafood samples and serial dilution samples by using DNeasy Blood & Tissue Kit (Qiagen, Hilden; Germany) according to the manufacturer's instructions.

Real-time PCR reaction condition and cycling parameters – All real-time PCR reactions were performed using a Rotor Gene 6000 RT-PCR thermocycler (Corbett Research Australia). Real-time PCR amplification was performed in a 25  $\mu$ l volume containing 20 ng of genomic DNA, 12.5  $\mu$ l SYBR supermix and 5 pmol primer. The amplifications were carried out with cycling parameters 5 min at 95°C for pre-denaturation, 40 cycles each of 5 sec at 95°C for denaturation, 10 sec at 6°C for annealing, 25 sec at 72°C for extension. Melt curve analysis was performed at the end of each PCR run to evaluate the amplification specificity.

Isolation of *Vibrio parahaemolyticus* from seafood samples by streak plate – Yellow and green colonies were isolated on the TCBS agar plates. On the other hand, blue, mauve and white colonies were isolated on the CHROMAGAR™ Vibrio. Cockle and prawn samples showed a constant growth of bacteria on both agar medium over the six month periods with varied colony counts. In contrast, fish and squid samples showed a periodic growth of bacteria on both selective medium.

However, the isolation of suspected *V. parahaemolyticus* in all the seafood samples were very few to almost none as indicated with the presence of green colonies on TCBS and purple colonies on CHROMAGAR™ Vibrio.

A total of 24 seafood samples comprised of six cockles, six prawns, six squids and six fishes were tested in this study. All the cockle and prawn samples showed a consistent presence of *V. parahaemolyticus* for the duration of six months while only two fish samples showed positive presence of *V. parahaemolyticus* (Table 1). However, there was no detection of *V. parahaemolyticus* recorded in all the squid samples.

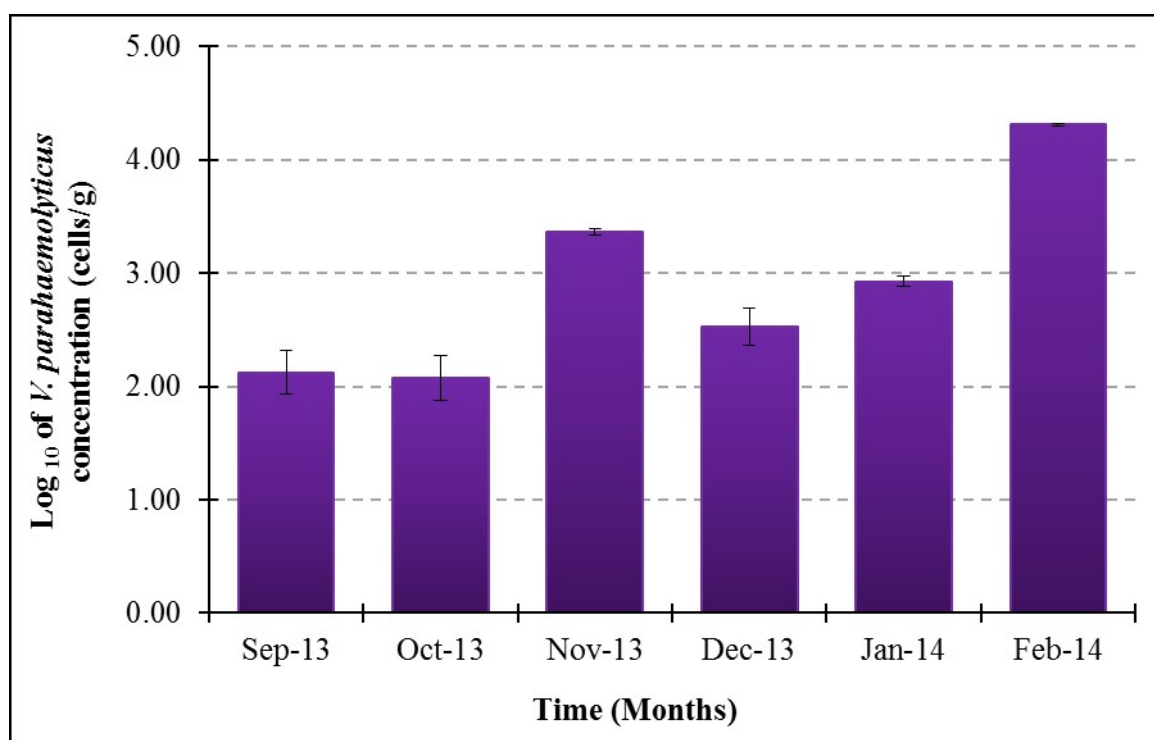
The average concentration of *V. parahaemolyticus* cells gained from the standard curve for the seafood samples were as followed: Cockle samples ( $4.03 \times 10^3$  cells/g), prawns ( $1.44 \times 10^4$  cells/g) and fish ( $8.65 \times 10^1$  cells/g). Cockle samples recorded its highest amount of *V. parahaemolyticus* concentration in February 2014 with  $2.04 \times 10^4$  cells/g (Figure 1). For prawn samples, it recorded its highest amount of *V. parahaemolyticus* in September 2013 with  $8.42 \times 10^2$  cells/g and the least amount of  $3.31 \times 10^1$  cells/g was recorded in January 2014 (Figure 2). On the other hand, *V. parahaemolyticus* was detected in fish samples on September 2013 and February 2014 with  $1.28 \times 10^2$  cells/g and  $4.52 \times 10^1$  cells/g respectively (Figure 3).

Melting curve analysis was conducted at the end of each PCR run for the SYBR green based PCR detection to acquire an accurate  $T_m$  value. This was conducted to confirm the amplification of the targeted gene segment. SYBR green dye will intercalate to the double stranded PCR product at low temperature and cause the increase in fluorescence intensity. However, due to the denaturation and degradation of PCR products during high temperature, there was a rapid decrease in fluorescence intensity. A single dissociation peak in the melting curve analysis indicated that there was positive PCR amplification exhibited with an expected  $T_m$  value. The specific  $T_m$  value for thermolabile hemolysin (tl) gene of *V. parahaemolyticus* was 86.5°C.

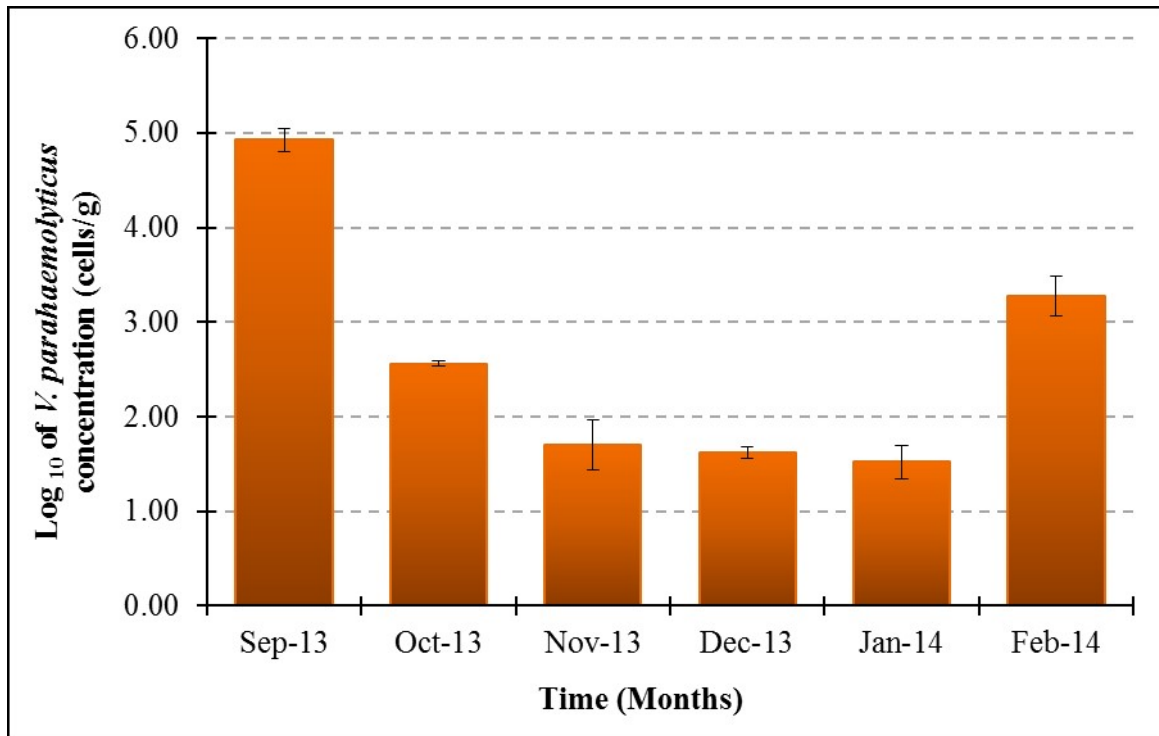
**Table 1.** Detection of *V. parahaemolyticus* in four types of seafood samples from September 2013 to February 2014 on Real-Time Quantitative PCR (RTq-PCR).

	Cockles	Prawns	Fishes	Squids
September 2013	+	+	+	x
October 2013	+	+	x	x
November 2013	+	+	x	x
December 2013	+	+	x	x
January 2014	+	+	x	x
February 2014	+	+	+	x
Totals	6	6	2	N/A

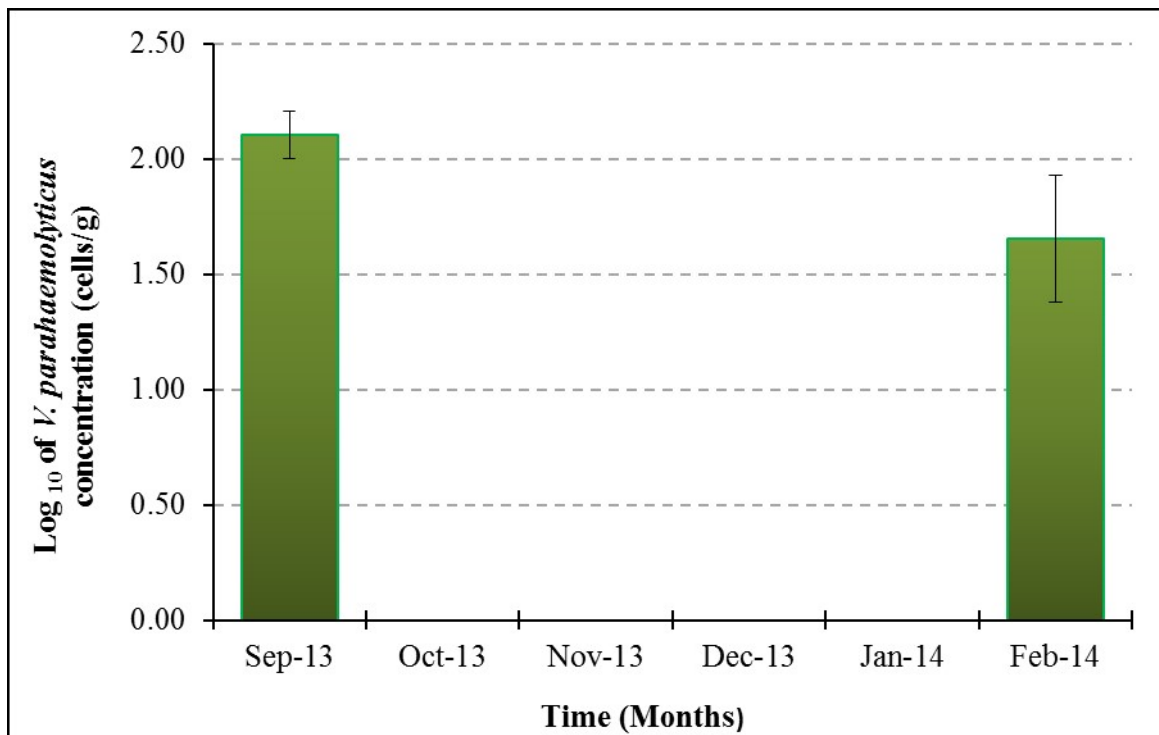
+ = Indicate positive detection, x = Indicate no detection, N/A = Not Available.



**Figure 1.** *Vibrio parahaemolyticus* density in cockle samples. Cell densities detected in each sample are the means of three replications. Error bars represent standard error of the mean.



**Figure 2.** *Vibrio parahaemolyticus* density in prawn samples. Cell densities detected in each sample are the means of three replications. Error bars represent standard of the mean.



**Figure 3.** *Vibrio parahaemolyticus* density in fish samples. Cell densities detected in each sample are the means of three replications. Error bars represent standard of the mean.

In this study, *V. parahaemolyticus* were detected and enumerated in seafood samples with species-specific thermolabile haemolysin (tl) primers with the sequence 5'- GTT GCA CTC GGT GAC AGC TTG-3' (MV2B-TLF) and 5'- AGT TTT GCG TAG GTT AAG TAC-3' (MV2B-TLR) (Micky *et al.*, 2014) to amplify a 248 bp PCR product. Besides that, isolation and detection of *V. parahaemolyticus* were also performed on two selective agars (TCBS and CHROMagar). The results of this study indicated that seafood is a potential reservoir for *V. parahaemolyticus* in the study area, and in Malaysia generally. Overall, about 58% (14/24) samples were positive for the presence of *V. parahaemolyticus*.

Cockle and prawn showed consistent presence of *V. parahaemolyticus* with an average density of  $4.03 \times 10^3$  and  $1.44 \times 10^4$  respectively for the six months of sampling. Cockles and prawns are two of the most consumed seafood product. The results are in agreement with other studies that indicated the higher concentrations of *V. parahaemolyticus* cells in seafood samples as compared to water or environmental origins, mainly due to the ability of marine organisms to accumulate *V. parahaemolyticus* cells within their tissues (Nelapati *et al.*, 2012; Quiroz-Guzmán *et al.*, 2013). Cockles are filter feeders and they tend to accumulate microorganisms in the surrounding waters which may also contain vibrios (Iwamoto *et al.*, 2010). On the other hand, only two samples of fish were positive for *V. parahaemolyticus*. Healthy live fish is protected by its immune system and therefore bacteria cannot grow in its flesh. However, when the fish dies, the immune system is no longer functioning causing the bacteria present to be able to proliferate freely. Studies have shown that *V. parahaemolyticus* may be found on the skin, gills as well as the intestinal tracts of fish (Adebayo-Tayo *et al.*, 2011). Besides that, freshwater fish such as catfish and tilapia were among the fish that has shown prevalence of *V. parahaemolyticus*. A study conducted by Noorlis *et al.* (2011) has detected *V. parahaemolyticus* in 25% of the catfish samples compared to 22.6% of red tilapia fish. The density of *Vibrio* species and *V. parahaemolyticus* in the samples ranged from 0 to  $1.1 \times 10^7$  MPN/g.

A study conducted by Chitov *et al.* (2009) has documented a significant level of *V. parahaemolyticus* contamination from raw squid samples. This is in contradiction with the result obtained in this study as none of the squid samples showed occurrence of *V. parahaemolyticus*. Although the food group (gastropods) are rarely the main cause of gastroenteritis, there have been 55 and 27 confirmed cases of food poisoning due to octopus, squids and jellyfish, respectively, in Hong Kong from 1999 to 2003 (Food and Environmental Hygiene Department, 2005). In addition, there are other research that studies the symbiosis between the squid (*Euprymna scolopes*) and its luminous bacterial symbiont *Vibrio fischeri* (Nyholm & McFall-Ngai, 2004). Most seafood is caught from wild populations, later, handled and processed without any use of additives or chemical preservatives and distributed on the same day to the markets or freezing as the only means of preservation. This condition propagates the growth of *V. parahaemolyticus* in raw seafood, thus, maybe harmful to the costumers. Besides the method of handling and preserving seafood, contamination of seafood with *V. parahaemolyticus* could be due to the faecal contamination in the areas where seafood is harvested.

The incidence of illness from the consumption of contaminated seafood is a primary concern of the seafood industry, public health agencies and the public. As a result, demand for a rapid and sensitive method of isolation, detection and enumeration of pathogenic *V. parahaemolyticus* is on the rise. The present study demonstrated that the application of SYBR Green based real-time PCR assay can specifically and accurately quantify *V. parahaemolyticus* from genomic DNA preparation of various sources, including artificially and naturally infested samples over a large concentration range (Micky *et al.*, 2014). The standard curve of the serial dilution of *V. parahaemolyticus* was constructed to compare Ct value and to correlate the amount of *V. parahaemolyticus* present in the seafood samples. The standard curve graph of the serial dilution of DNA showed that the Ct value was inversely

proportional to the initial template of DNA concentration and the correlation coefficient ( $R^2$ ) value was equal to 0.99.

Because the number of isolates used in this study was limited, a comprehensive collection of isolates with detailed information on samples collected, sample locations, sampling frequencies and sampling periods from harvesting sites or farms, production movement and processing plants is warranted to identify the source and possible transmission. Knowledge of pre-harvest and post-harvest sources responsible for *V. parahaemolyticus* infection may help intergrators and producers to define critical, pre- and post-harvest control points on the harvesting sites/ farm and processing units because intervention at these points may reduce and/ or eliminate horizontal transmission and *V. parahaemolyticus* positive seafood arriving at the processing plants. It is also important to design hazard analysis and critical control point (HACCP) protocols as well conducting Risk Assessment to safeguard fresh seafood against *V. parahaemolyticus* contamination during processing and deliverance of seafood to consumers. More importantly, this knowledge would facilitate *V. parahaemolyticus* control by means other than sub therapeutic use of antibiotics.

The ubiquitous nature of *V. parahaemolyticus* in the aquatic environment and marine life creates a significant challenge for our country to produce seafood free of pathogens. This study was conducted to determine the occurrence of *V. parahaemolyticus* in raw seafoods in Sarawak, Malaysia. The results presented here provided information on the enumeration and detection of *V. parahaemolyticus*, targeting the species-specific gene (thermolabile haemolysin; *tlh* gene) by using a rapid SYBR green based real- time quantitative PCR assay. This study also demonstrated that the real- time PCR assay could be completed within 3 hours and has proven to be a promising tool to screen *V. parahaemolyticus* from food and environmental samples.

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