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Prevalence and quantification of *Vibrio* species and *Vibrio parahaemolyticus* in freshwater fish at hypermarket level

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Abstract: Little is known on the biosafety level of *Vibrio* spp. in freshwater fish in Malaysia. The purpose of this study was to investigate the prevalence and concentration of *Vibrio* spp. and *V. parahaemolyticus* in freshwater fish using the Most Probable Number-Polymerase Chain Reaction (MPN-PCR) method. The study was conducted on 150 samples from two types of freshwater fish commonly sold at hypermarkets, i.e. *Pangasius hypophthalmus* (catfish) and *Oreochromis* sp. (red tilapia). Sampling was done on the flesh, intestinal tract and gills of each fish. The prevalence of *Vibrio* spp. and *V. parahaemolyticus* was found to be 98.67% and 24% respectively with higher percentages detected in samples from the gills followed by the intestinal tract and flesh. *Vibrio* spp. was detected in almost all red tilapia and catfish samples. *V. parahaemolyticus* was detected in 25% of the catfish samples compared to 22.6% of red tilapia fish. The density of *Vibrio* spp. and *V. parahaemolyticus* in the samples ranged from 0 to $1.1x10^7$ MPN/g. Although the maximum value was $1.1x10^7$ MPN/g, most samples had microbial loads ranging from 0 to $>10^4$ MPN/g. The outcome on the biosafety assessment of *Vibrio* spp. and *V. parahaemolyticus* in freshwater fish indicates another potential source of food safety issues to consumers.

Keywords: *Vibrio* spp., *Vibrio parahaemolyticus*, MPN-PCR, freshwater fish, prevalence, quantification

Introduction

Members of the genus *Vibrio* are defined as Gram negative, asporogenous rods that are straight or have a single rigid curve and are motile with a single polar flagellum when grown in liquid medium (Kaysner *et al*., 2004). The importance of *Vibrio* spp. as a contaminant of raw or undercooked seafood has been well established (Gopal *et al*., 2005; Di Pinto *et al*., 2008; Luan *et al*., 2008) and may lead to acute gastroenteritis including diarrhea, headache, vomiting, nausea and fever (Apun *et al*., 1999; Vongxay *et al*., 2008; Yang *et al*., 2008). As food safety is a major global concern that affects the consumer and those in the food service sector (Badrie *et al*., 2006; Jacxsens *et al*., 2009), serious attention has to be given to the aquaculture industry as fish can act as a vector for human pathogenic bacteria (Apun *et al*., 1999). Espineira *et al*. (2010) reported that apart from seafood, *Vibrio* spp. can be found naturally in brackish water and estuarine ecosystems with optimal salinity and temperature conditions. Almasi *et al*. (2005) reported that *Vibrio* numbers increased with temperature up to a certain limit. For food samples, temperature abuse may be due to improper storage or a long holding time on the display rack at the retail level without proper temperature control.

The World Health Organization (WHO) defines foodborne illness as a disease which is caused through the consumption of contaminated food (Velusamy *et al*., 2010). Other than *Vibrio*, pathogens such as *Campylobacter*, *Salmonella*, *Listeria monocytogenes* and *Escherichia coli* 0157:H7 have been found to be responsible for major foodborne outbreaks worldwide (Apun *et al*., 1999; Velusamy *et al*., 2010). In the Asian region, *Vibrio* spp. have been recognized as the leading cause of foodborne outbreaks in many countries including Japan (Hara-Kudo *et al*., 2001; Alam *et al*., 2003; Yang *et al*., 2008), India (Chakraborty *et al*., 2008; Gopal *et al*., 2005), China (Luan *et al*., 2008; Yang *et al*., 2008; Chen *et al*., 2010), Taiwan (Hara-Kudo *et al*., 2003), Korea (Lee *et al*., 2008) and Malaysia (Tunung *et al*., 2010). Investigations show that many outbreaks were

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Table 1. Details of fish samples analysed with the proposed methodology

Common name	Local name	Scientific name	Origin	Part of fish	No. of analyse samples
Catfish	Patin	*Pangasius hypophthalmus*	Thailand	Flesh	25
				Intestinal tract	25
				Gill	25
Red Tilapia	Tilapia merah	*Oreochromis sp.*	Taiwan	Flesh	25
				Intestinal tract	25
				Gill	25
TOTAL					**150**

caused by consumption of contaminated seafood (Jacxsens *et al*., 2008). Therefore, it is important to have data on the prevalence of *Vibrio* spp. in freshwater fish. Freshwater fish are easily available in many hypermarkets in Malaysia and are in high demand by local consumers. According to Ponniah *et al*. (2010), hypermarkets in the country offer foods under hygienic conditions as food stuffs are packed before display and their users are generally the urban population due to their location in urban areas and competitive prices.

V. parahaemolyticus is a halophilic pathogen which can be subtyped based on its somatic (O) and capsular (K) antigen patterns. The O3:K6 serovar is a predominant strain that is distributed globally (Bhunia, 2008). Identification and detection of *Vibrio* spp. and *V. parahaemolyticus* through conventional culture and biochemical test methods is a laborious and time consuming process. Currently, deoxyribonucleic acid (DNA) based typing techniques are frequently used to generate strain-specific fingerprinting and have proven to be useful tools in detecting a single copy of a target DNA sequence of cells that are present in very limited amounts (Chakraborty *et al*., 2008; Velusamy *et al*., 2010). They are able to differentiate up to the serogroup and biotype level (Espeneira *et al*., 2010) against a large background of the prokaryotic and eukaryotic cells that may be present in the samples (Alam *et al*., 2003) thus providing high specificity, sensitivity and accuracy within hours in the laboratory (Velusamy *et al*., 2010). The recent study of Ponniah *et al*. (2010) and Tunung *et al*. (2010) reported that the polymerase chain reaction (PCR) can be successfully used with MPN for the quantitative determination of foodborne pathogens. In addition to this, isolation can be carried out using a chromogenic agar medium such as CHROMagar™ *Vibrio* (CV) combined with a selective enrichment of salt polymyxin broth (SPB) (Hara-Kudo *et al*., 2001) for qualitative analysis. According to Su and Liu., (2007) a major disadvantage of the MPN method is that thio-citrate-bile-salt (TCBS) agar cannot differentiate *V. parahaemolyticus* from some strains of *Vibrio vulnificus* and *Vibrio mimicus*. Therefore, several presumptive positive colonies formed on the TCBS agar plates needs to be analysed with lengthy biochemical tests for confirmation and results may not be available for four to five days compared to the MPN-PCR method which only takes two days to complete.

For this study, we utilised the MPN-PCR enumeration method for *Vibrio* spp. and *V. parahaemolyticus* using species-specific PCR techniques. While much research has been done and published on the prevalence of *Vibrio* spp. in epidemiological samples related to food poisoning and outbreak cases worldwide (Yang *et al*., 2008; Tunung *et al*., 2010), there is a lack of quantitative or enumeration studies on the prevalence of *Vibrio* spp. especially *V. parahaemolyticus* in freshwater fish. Therefore, this study was to investigate the prevalence of *Vibrio* spp. and *V. parahaemolyticus* in freshwater fish sold at the hypermarket level using a combination of the most-probable-number (MPN) procedure and PCR technique. The findings will provide insight on the biosafety assessment of *Vibrio* spp. and *V. parahaemolyticus* in freshwater fish.

Materials and Methods

Samples collection

A total of 150 samples of freshwater fish were collected over a six month period (June to December 2009). Samples of catfish (*Pangasius hypophthalmus*) and red tilapia (*Oreochromis* sp.) were purchased from several hypermarkets in the state of Selangor, Malaysia. During collection, all of the samples were placed in sterile, labelled, sealed plastic bags prior to transportation and were analysed immediately on the day of sampling. The details of fish samples analysed with the proposed methodology are shown in Table1.

MPN-PCR

The analytical method performed in this study was based on the *Bacteriological Analytical Manual* standard method (Kaysner *et al*., 2004) with modification according to the procedures by Tunung *et al*. (2010). For detection and enumeration of *Vibrio* spp. and *V. parahaemolyticus*, a 10g-portion of each sample was homogenized with 90 ml of Tryptic Soy Broth (TSB; Bacto, France) with 1% and 3% of sodium chloride (NaCl; Merck, Germany) respectively, in a sterile stomacher bag for 60 s. The

homogenized samples were pre-enriched at 37^{0}C for 18h. For the three tube MPN method, serial dilution was carried out up to 10^{-7} with Salt Polymyxin Broth (SPB; Nissui, Japan). One ml of the 10^{-5}, 10^{-6} and 10^{-7} fold dilutions was transferred into three sterile tubes and incubated at 37^{0}C for 18 to 24 h. The contents of the turbid MPN tubes were subjected to PCR for the detection of 16S rRNA gene specific for *Vibrio* spp. and *tox*R gene specific for *V. parahaemolyticus*.

DNA extraction was carried out on turbid tubes using a boiled cell method. In brief, a 1 ml portion of each tube was subjected to centrifugation at 12 000 rpm for 2 min to pellet the microorganisms. The pellet was resuspended in 500 µl of sterile distilled water and boiled for 10 min. The boiled cell lysate was immediately cooled at -20^{0}C for 10 min. before it was centrifuged at 13 000 rpm for 3 min. The boiled cell lysate was used as the DNA template for PCR detection of 16S rRNA for *Vibrio* spp. (Gonzalez-escalona *et al*., 2006) and *tox*R for specific detection of *V. parahaemolyticus* (Vongxay *et al*., 2008). The space synthetic oligonucleotide primer pairs used for detection was 16S rRNA (5'-GTCAAAGCGATGCAGGTG-3' and 5'-CTTCGCCACCGGTATTCCTT-3') (Gonzalez-escalona *et al*., 2006) and *tox*R (5'-GTCTTCTGACGCAATCGTTG-3' and 5'-ATACGAGTGGTTGCTGTCATG-3') (Tunung *et al*., 2010).

PCR amplification for detection of *Vibrio* spp. was performed in a 20 µl reaction mixture containing 4.0 µl of 5X PCR buffer, 2.4 µl 3 mM $MgCl_2$, 0.4 µl of 0.2 mM of deoxynucleoside triphosphate mix, 1.0 µl of 0.5 µM of each primer, 0.1 µl of 0.5U/µl of Taq polymerase and 2.0 µl of DNA template. Reaction mixtures were heated at 94^{0}C for 4 min in the initial denaturation step, followed by 35 cycles of denaturation at 94^{0}C for 1 min, primer annealing at 60^{0}C for 1 min, and primer extension at 72^{0}C for 1 min. A final extension was performed at 72^{0}C for 5 min. Gel electrophoresis was carried out for the PCR products using 1% agarose with 0.5X Tris-borate-EDTA buffer at 100 V for 22 min. The gel was visualized using the Gel Documentation System (SynGene, USA). A 100bp ladder (Promega, USA) was used as a molecular size marker.

PCR amplification for detection of *V. parahaemolyticus* was carried out in 20 µl comprising 4.0 µl of 5X PCR buffer, 1.6 µl 2 mM $MgCl_2$, 0.4 µl of 0.4 mM of deoxynucleoside triphosphate mix, 0.4 µl of 0.4 µM of each primer, 0.1 µl of 0.5U/µl of Taq polymerase and 2.0 µl of DNA template. Reaction mixture was heated at 96^{0}C for 5 min in the initial denaturation step, followed by 35 cycles of denaturation at 94^{0}C for 30 s, primer annealing at 63^{0}C for 30 s, and primer extension at 72^{0}C for 30 s. A final extension was performed at 72^{0}C for 7 min. Gel electrophoresis was carried out on the PCR products using 1% agarose with 0.5X Tris-borate-EDTA buffer at 100 V for 22 min. The gels were visualized using the Gel Documentation System (SynGene, USA). A 100bp ladder (Promega, USA) was used as a molecular size marker. The *V. cholerae* 01 Inaba and *V. parahaemolyticus* 1896 used as the positive control in every PCR reaction were obtained from Kyoto University, Japan.

Results

A total of 150 samples (as shown in Table 1) of freshwater fish were analysed for the presence of *Vibrio* spp. and *V. parahaemolyticus* using MPN-PCR. The 16S rRNA gene (162bp) was targeted for detection of *Vibrio* spp. and the *tox*R gene (368 bp) was specifically targeted for detection of *V. parahaemolyticus* (as shown in Table 2). Figure 1 shows a representative gel electrophoresis image of the PCR amplification of the 16S rRNA gene and *tox*R gene. From the PCR detection, the prevalence of *Vibrio* spp. and *V. parahaemolyticus* from freshwater fish sample could be determined.

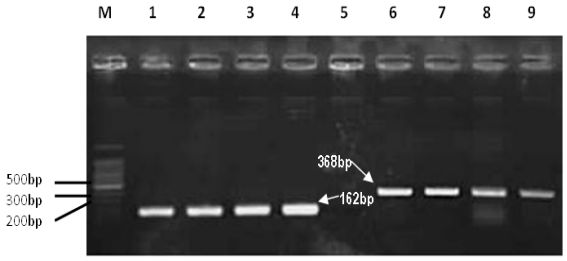

Figure 1. Agarose gel electrophoresis of the 16s rRNA gene of *Vibrio* spp. (162 bp) and *tox*R gene of *V. parahaemolyticus* (368 bp). Lane 1 to lane 3 = representatives *Vibrio* positive samples; lane 4 = *Vibrio* spp. positive control; lane 5 = blank; lane 6 to lane 8 = *V. parahaemolyticus* positive samples; lane 9 = *V. parahaemolyticus* positive control ; M= 100bp DNA marker

From the study, it was found that *Vibrio* spp. could be detected at a prevalence of 98.67% (148/150) whereas *V. parahaemolyticus* was detected at a prevalence of 24% (36/150) from both types of freshwater fish obtained from hypermarkets in Malaysia. *Vibrio* spp. could be detected in the flesh, intestinal tract and gills of all the red tilapia samples (100%). For catfish, all samples indicated the presence of *Vibrio* spp. in the gills whereas it was detected at a prevalence of 96% (24/25) in the flesh and intestinal tract. The prevalence of *V. parahaemolyticus* in parts of catfish samples varied, with intestinal tract and gills giving the highest percentage of 24% (6/25) followed by flesh with only 20% (5/25). The

Table 2. Prevalence of *Vibrio* spp. and *Vibrio parahaemolyticus* from freshwater fish at hypermarket level

Type of fish	Part of fish	[a]n	*Vibrio* spp.		*Vibrio parahaemolyticus*	
			PCR positive	%	PCR positive	%
Catfish	Flesh	25	25	100.00	5	20.00
	Intestinal tract	25	25	100.00	6	24.00
	Gill	25	25	100.00	6	24.00
Red tilapia	Flesh	25	24	96.00	4	16.00
	Intestinal tract	25	24	96.00	5	20.00
	Gill	25	25	100.00	10	40.00
TOTAL		150	148	98.67	36	24.00

[a]n = Number of sample.
[b](%) = Percentage.

Table 3. Microbial load (MPN/g) of *Vibrio* spp. and *Vibrio parahaemolyticus* from freshwater fish at hypermarket level

Type of fish	Part of fish	*Vibrio* spp.			*Vibrio parahaemolyticus*		
		[a]Min	[b]Med	[c]Max	[a]Min	[b]Med	[c]Max
Catfish	Flesh	$6.1X10^4$	$1.1X10^7$	$1.1X10^7$	0	0	$1.1X10^7$
	Intestinal tract	$7.2X10^4$	$1.1X10^7$	$1.1X10^7$	0	0	$1.1X10^7$
	Gill	$1.2X10^5$	$1.1X10^7$	$1.1X10^7$	0	0	$1.1X10^7$
Red tilapia	Flesh	0	$1.1X10^7$	$1.1X10^7$	0	0	$1.1X10^7$
	Intestinal tract	0	$1.1X10^7$	$1.1X10^7$	0	0	$1.1X10^7$
	Gill	$6X10^4$	$1.1X10^7$	$1.1X10^7$	0	0	$1.1X10^7$

[a]Min = Minimum MPN/g value.
[b]Med = Median MPN/g value.
[c]Max = Maximum MPN/g value.

prevalence data from red tilapia samples indicate that *V. parahaemolyticus* were often detected in the gills at 40% (10/25), followed by intestinal tract 20% (5/25) and flesh at 10% (4/25).

The estimated quantity of *Vibrio* spp. and *V. parahaemolyticus* in freshwater fish varied from 0 to $1.1x10^7$ MPN/g (Table 3). The highest quantity of *Vibrio* spp. and *Vibrio parahaemolyticus* in freshwater fish from hypermarket was $1.1x10^7$ MPN/g. Most of the samples from the hypermarket show a minimum of 0 MPN/g and maximum value of $1.1x10^7$ MPN/g respectively.

Discussion

Igbinosa and Okoh (2008) reported that *Vibrio* spp. are widespread in marine and estuarine environments and several pathogenic species are known to be commonly associated with outbreaks of *Vibrio* infections due to consumptions of food and water contaminated with human faeces or sewage, raw fish and seafood or with exposure of skin lesion such as cuts, open wounds and abrasions to aquatic environments and marine animals. *V. parahaemolyticus* is an important food-borne pathogen and therefore it is essential to obtain data on the presence of this organism in freshwater fish for future biosafety assessment.

The presence of *Vibrio* spp. and *V. parahaemolyticus* in samples of freshwater fish in this study suggests that foodborne illness could arise if these fish are consumed in the uncooked or undercooked state. They could also cross-contaminate ready-to-eat foods that are in the same environment. The high prevalence of *Vibrio* spp. in 98.67% of freshwater fish samples (Table 2) at the hypermarket level is of concern because it can cause illnesses in humans. This high incidence probably reflects the nature of *Vibrio* spp. which is known as a halophilic waterborne bacterium that commonly inhabits environmental water sources worldwide. It has been found that freshwater rivers as well as brackish water and marine environments may support the growth of these organisms which are also pathogenic to humans (Janda, 1987). The temperature of the water is considered as the most important factor of *Vibrio* distribution (Apun *et al*., 1999; Luan *et al*., 2008; Yang *et al*., 2008; Chen *et al*., 2010). The high prevalence of *Vibrio* spp. in the samples could be due to temperature abuse, use of contaminated ice to cover the fish on the display bench, mishandling and the presence of conditions that favour the growth of the *Vibrio* spp.

V. parahaemolyticus was first recognised as the cause of foodborne illness in Osaka, Japan in 1951 and was identified as a common cause of food-borne illness due to consumption of seafood in many Asian countries (Apun *et al*., 1999). Using the MPN-PCR technique, about 24% of freshwater fish samples were found to harbour *V. parahaemolyticus* and has been identified as a potential reservoir for this pathogen. The findings of this study with regards to the high contamination of *V. parahaemolyticus* in fish gills is in concurrence with a previous study which also detected *V. parahaemolyticus* in fish gills (Luan *et al*., 2008). As reported by Chen *et al*. (2010), the Food Hygiene Regulations of Japan require the *V. parahaemolyticus* level to be below $<10^2$ MPN/g in seafood for raw consumption whereas the level of concern established by Food and Drug Administration (FDA) for *V. parahaemolyticus* in molluscan shellfish is 10^4 MPN/g. The maximum microbial loads for *V. parahaemolyticus* in most samples were $1.1x10^7$ MPN/g (Table 3). Therefore, although *V. parahaemolyticus* is generally regarded as a marine organism, it can be found in samples of

freshwater fish.

The presence of *V. parahaemolyticus* in freshwater fish sold at hypermarkets is a cause of concern since this type of freshwater fish is always available in most hypermarkets in Malaysia and is in high demand. Cross contamination could be the cause of its presence since fish on the display bench is always covered with ice to maintain its freshness. The proximity of the freshwater fish display area to that of other seafood could also contribute to its cross-contamination. Yang *et al*. (2008) had previously reported that 14.9% of frozen and iced seafood samples were contaminated with *V. parahaemolyticus*. Food handling practices, the location where samples are displayed, the absence of gloves for handling fish and the use of contaminated ice and containers during transportation could contribute to the high prevalence of *V. parahaemolyticus* in this study. Several researchers (Yang *et al*., 2008; Ponniah *et al*., 2010; Tunung *et al*., 2010; Usha *et al*., 2010a) have considered improper handling and poor hygienic practices to be a major source of contamination of food in hypermarkets in the country.

It is imperative that monitoring and routine screening of freshwater fish samples for the presence of *Vibrio* spp. and *V. parahaemolyticus* be conducted at the retail level to reduce the incidence of *Vibrio* spp. infections. Contaminated food that is stored at ambient temperature can reach the infectious dose in only a few hours (Luan *et al*., 2008). So, it is important to detect the presence of *Vibrio* spp. and *V. parahaemolyticus* with a sensitive, simple, fast, less laborious, cheap and reliable method. MPN-PCR has been successfully used for enumerating many organisms (Tunung *et al*., 2010). In this study, it was found that the MPN-PCR was also a suitable and useful tool for detecting *Vibrio* spp. and *V. parahaemolyticus* in freshwater fish. Most recently, several prevalence studies have detected the presence of various foodborne pathogens in raw foods (Cahi *et al*., 2008; Tang *et al*., 2009; Usha *et al*., 2010b; Suzita *et al*., 2010; Jeyaletchumi *et al*., 2010). This study has detected *Vibrio* spp. and *V. parahaemolyticus* in two types of freshwater fish (*P. hypopthalmus* and *Oreochromis* spp.).

In conclusion, it was found that *Vibrio* spp. and *V. parahaemolyticus* was present in freshwater fish sold at hypermarkets in Malaysia. This could pose a threat to those who consume or handle freshwater fish. The study also showed that the MPN-PCR is quite useful in quantitative detection of *Vibrio* spp. and *V. parahaemolyticus*. The data presented here will be useful for the microbiological risk assessment of *Vibrio* spp. and *V. parahaemolyticus* associated with freshwater fish consumption in Malaysia.

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