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. *Pangasiidae* 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 1.1x10⁷ MPN/g. Although the maximum value was 1.1x10⁷ MPN/g, most samples had microbial loads ranging from 0 to >10⁸ 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; Jaecxens 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
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., 2007). 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™ (Jacxsens et al., 2008). In addition to this, isolation can be carried out using a chromogenic agar medium such as CHROMagar™ (Jacxsens et al., 2008). In addition to this, isolation can be carried out using a chromogenic agar medium such as CHROMagar™ (Jacxsens et al., 2008). In addition to this, isolation can be carried out using a chromogenic agar medium such as CHROMagar™ (Jacxsens et al., 2008).

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 Table 1.

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

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

<table>
<thead>
<tr>
<th>Common name</th>
<th>Local name</th>
<th>Scientific name</th>
<th>Origin</th>
<th>Part of fish</th>
<th>No. of analyse samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catfish</td>
<td>Patin</td>
<td>Pangasius hypophthalmus</td>
<td>Thailand</td>
<td>Flesh</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Intestinal tract</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Gill</td>
<td>25</td>
</tr>
<tr>
<td>Red Tilapia</td>
<td>Tilapia merah</td>
<td>Oreochromis sp.</td>
<td>Taiwan</td>
<td>Flesh</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Intestinal tract</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Gill</td>
<td>25</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>150</td>
</tr>
</tbody>
</table>
homogenized samples were pre-enriched at 37°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 $10^{-2}$, $10^{-6}$ and $10^{-7}$ fold dilutions was transferred into three sterile tubes and incubated at 37°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 12000 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°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’-CTTCGCCAGCTCGATACTT-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°C for 4 min in the initial denaturation step, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 1 min. A final extension was performed at 72°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 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.

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
parahaemolyticus contaminate ready-to-eat foods that are in the same or undercooked state. They could also cross-illnesses in humans. This high incidence probably hypermarket level is of concern because it can cause 98.67% of freshwater fish samples (Table 2) at the skin lesion such as cuts, open wounds and abrasions sewage, raw fish and seafood or with exposure of and water contaminated with human faeces or 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 Vibrio spp. and Vibrio parahaemolyticus in freshwater fish for pathogen and therefore it is essential to obtain data 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 seawage, 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 <10^5 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 10^5 MPN/g. The maximum microbial loads for V. parahaemolyticus in most samples were 1.1x10^5 MPN/g (Table 3). Therefore, although V. parahaemolyticus is generally regarded as a marine organism, it can be found in samples of

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

<table>
<thead>
<tr>
<th>Type of fish</th>
<th>Part of fish</th>
<th>n</th>
<th>PCR positive</th>
<th>%</th>
<th>PCR positive</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catfish</td>
<td>Flesh</td>
<td>25</td>
<td>25</td>
<td>100.00</td>
<td>5</td>
<td>20.00</td>
</tr>
<tr>
<td></td>
<td>Intestinal tract</td>
<td>25</td>
<td>25</td>
<td>100.00</td>
<td>6</td>
<td>24.00</td>
</tr>
<tr>
<td>Red tilapia</td>
<td>Flesh</td>
<td>25</td>
<td>25</td>
<td>100.00</td>
<td>4</td>
<td>16.00</td>
</tr>
<tr>
<td></td>
<td>Intestinal tract</td>
<td>25</td>
<td>24</td>
<td>96.00</td>
<td>5</td>
<td>20.00</td>
</tr>
<tr>
<td></td>
<td>Gill</td>
<td>25</td>
<td>25</td>
<td>100.00</td>
<td>10</td>
<td>40.00</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>125</td>
<td>118</td>
<td>94.67</td>
<td>36</td>
<td>28.00</td>
</tr>
</tbody>
</table>

an = Number of sample. 
(% ) = Percentage.

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

<table>
<thead>
<tr>
<th>Type of fish</th>
<th>Part of fish</th>
<th>Min</th>
<th>Med</th>
<th>Max</th>
<th>Min</th>
<th>Med</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catfish</td>
<td>Flesh</td>
<td>6.1X10^4</td>
<td>1.1X10^4</td>
<td>1.1X10^4</td>
<td>0</td>
<td>0</td>
<td>1.1X10^4</td>
</tr>
<tr>
<td></td>
<td>Intestinal tract</td>
<td>1.2X10^4</td>
<td>1.1X10^4</td>
<td>1.1X10^4</td>
<td>0</td>
<td>0</td>
<td>1.1X10^4</td>
</tr>
<tr>
<td>Red tilapia</td>
<td>Flesh</td>
<td>0</td>
<td>1.1X10^4</td>
<td>1.1X10^4</td>
<td>0</td>
<td>0</td>
<td>1.1X10^4</td>
</tr>
<tr>
<td></td>
<td>Intestinal tract</td>
<td>6X10^4</td>
<td>1.1X10^4</td>
<td>1.1X10^4</td>
<td>0</td>
<td>0</td>
<td>1.1X10^4</td>
</tr>
</tbody>
</table>

Min = Minimum MPN/g value.
Med = Median MPN/g value.
Max = Maximum MPN/g value.
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. hypophthalmus* 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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