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Occurrences of *Vibrio parahaemolyticus* in Retailed Freshwater Fish

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ABSTRACT

Vibrio parahaemolyticus is a causative agent of foodborne outbreaks associated with the consumption of raw or under-cooked seafood. This study aimed to quantify and detect the occurrence of *V. parahaemolyticus* in freshwater fish by performing Most Probable Number (MPN) method in combination with Polymerase Chain Reaction (PCR). In this study, a total of 20 red tilapia (*Oreochromis* sp.) were collected from nearby local wet markets. PCR assay targeting the *tox*R gene in *V. parahaemolyticus* was performed, with the expected DNA amplification size of 368 bp. MPN analysis showed that the estimated microbial load of *V. parahaemolyticus* were more than 1100 MPN/g. The result of the PCR assay confirmed the presence of *V. parahaemolyticus* in 90% of the isolates. This positive detection elucidated the presence of food-borne bacteria in freshwater fish from local wet-market which may affect not only the health of fish stocks but also raise public health concerns.

Keywords: Freshwater Fish, MPN, PCR, *tox*R, *Vibrio parahaemolyticus*

INTRODUCTION

Fisheries and aquaculture industries have become a significant pillar to uplift the national economic status. Southeast Asian Fisheries Development Center (SEADEC, 2015), reported that the fisheries sector in Malaysia has provided employment to 175,980 people and contributed to national GDP at 1.1% in 2015. Freshwater fish production in Malaysia is at 782,855.5 MY/year and the estimated average consumption of fish is 56.8kg/person/year (SEADEC, 2015). However, the increase in freshwater fish products has brought and caused unintended problematic scenarios such as the transmission of food-borne bacteria and the emergence of food-borne outbreaks. Apun, Asiah, and Jugang (1999) stated that serious attention must be given to the aquaculture industry as fish can act as a vector for human pathogenic bacteria.

Vibrio parahaemolyticus has accounted for 25% of foodborne diseases as compared to other *Vibrio* spp. (Feldhusen, 2000). This gram-negative halophilic bacterium is a natural inhabitant of marine and estuarine environments around the world (Meador *et al*., 2007). It is capable of causing fish and human diseases (IFAS, 2009). In Taiwan and Japan, reported outbreaks of food poisoning caused by *V. parahaemolyticus* were due to the consumption of two popular Japanese food, sashimi and sushi (Novotny, Dvorska, Lorencova, Beran & Pavlik, 2004). In Malaysia, a study by Letchumanan, Yin, Lee and Chan (2015) had demonstrated a high level of *V. parahaemolyticus* contamination in shrimps purchased from a local wet market. The presence of human pathogenic bacteria in fish can be linked to direct contact with a contaminated water environment and ingestion of bacteria from sediments or contaminated feed. Thus, bacteria detection in fish reflects the condition and safety of aquatic environments.

In Sarawak, limited studies on pathogens in fish products have been conducted and the potential of freshwater fish as a vector for *V. parahaemolyticus* transmission is not well documented. It is necessary to study the prevalence of pathogens in freshwater fish products to ensure a better understanding of the ecology and distribution of pathogens in the food chain. Combination of Most Probable Number with species-specific Polymerase Chain Reaction (PCR) method provides a better alternative for the enumeration and detection of pathogens in food products as compared to the conventional method (e.g. gram staining and glucose fermentation). *toxR* gene is used to identify various *Vibrio* species due to its properties to activate cfx expression, which resulting in the direct binding to a specific element at the promoter. Hence, this study aims to enumerate and detect the presence of *V. parahaemolyticus* in freshwater fish from the local wet market.

MATERIALS & METHODS

Sample Collection and Processing
Ten samples of red tilapia (*Oreochromis* sp.) were purchased from every two locations which were Kota Sentosa and Stutong wet market in Kuching. The fish samples were placed in an icebox after collection and brought to the Molecular Microbiology Laboratory, Faculty Resource Science and Technology, Universiti Malaysia Sarawak (UNIMAS) for processing. Briefly, 10g-portion of sample intestine was homogenized with 90 ml of Tryptic Soy Broth (TSB) with 3% sodium chloride (NaCl), in a sterile stomacher bag (Kaysner, DePaola & Jones, 2004). The homogenized sample was pre-enriched at 37ºC for 18 h to 24 h.

Enumeration by Most Probable Number (MPN)
The pre-enriched samples were subjected to three-tube MPN analysis where dilution series of up to 10^{-7} was performed using Salt Polymyxin Broth (SPB). One millilitre of the 10^{-5}, 10^{-6}, and 10^{-7}fold dilutions were transferred into new three sterile tubes and incubated at 37ºC for 18 h to 24 h. After incubation, the turbid tubes were chosen for DNA extraction

DNA Extraction
Approximately 1 ml of the enriched bacterial cultures in the broth was centrifuged (Hettich EBA 21 Zentrifugen, Germany) at 12,000 rpm for 2 min. The supernatant was discarded, and the cell pellet resuspended in 500 µl of sterile distilled water and was vortexed (Labnet International, USA). The cell suspension was boiled for 10 min and immediately cooled at -20 °C for 10 min before being centrifuged again (Hettich EBA 21 Zentrifugen, Germany) for 3 min at 13,000 rpm.

Detection of *V. parahaemolyticus* by PCR Assay
PCR assay was performed as described by Tunung *et al.* (2010). The primer set for the detection of *tox*R gene is summarized in Table 1.

Table 1. Primers used for the detection of *V. parahaemolyticus*

Targeted gene	Primer	Nucleotide sequences (5' – 3')	Amplicon Size (bp)	Reference
*tox*R	*tox*R-F	GTCTTCTGACGCAATCGTTG	368	(Tunung *et al.,* 2010)
	*tox*R-R	ATACGAGTGGTTGCTGTCATG		

The amplification was performed in an Eppendorf Mastercycler® Personal (Hamburg, Germany). An initial denaturation was started at 96 °C for 5 min, followed by 35 cycles each of denaturation at 94 °C for 1 min, primer annealing at 63 °C for 1 min and 30 sec and extension at 72 °C for 1 min and 30 sec. A final extension was performed at 72ºC for 7 minutes. The presence of amplified *tox*R gene fragment was detected by using gel electrophoresis with 1% of agarose gel, 1X TBE buffer at 85 V for 1 h before visualization under UV transilluminator.

RESULTS & DISCUSSION

The findings from pre-enrichments indicated that all fish samples (n=20) showed turbidity in TSB. MPN analysis revealed that all samples were contaminated with more than 1100 MPN/g of presumptive *V. parahaemolyticus*. Table 2 summarizes the results of *V. parahaemolyticus* enumeration and detection from the samples.

Table 2. Enumeration and detection of *V. parahaemolyticus* from freshwater fish.

Sampling site (Total isolates)	MPN/g (No. of positive isolates)	PCR Detection of *V. parahaemolyticus* (Percentage of Positive Isolates)
7th Milem (n=10) Stutong (n=10)	>1100 (n=20)	18/20 (90%)
	Total: 20/20 (100%)	Total: 18/20 (90%)

The agarose gel image for the detection of *toxR* (*V. parahaemolyticus*) gene in freshwater fish samples is shown in Figure 1. The result of gel electrophoresis shows the product of DNA amplification at 368 bp.

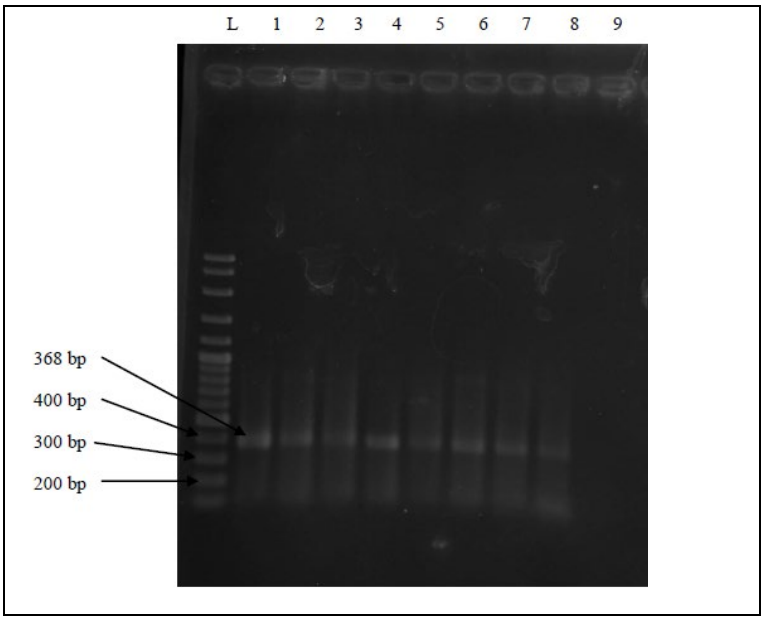

Figure 1. Representative gel image of PCR amplification of *toxR* gene detection from fish samples collected from two wet markets. M1: 100-bp DNA ladder; Lane 1: *V. parahaemolyticus* positive control, lane 2 to 8: isolates from fish samples, lane 9: *V. parahaemolyticus* negative control.

Fish from natural environments are known to harbour various bacterial species (Pillay, 1990). Bacterial colonization can be observed on fish skin and gills due to constant exposure to contaminated water, while the digestive tract may be affected through contaminated feed or water. Hence, our first goal in this study was to enumerate the concentration of presumptive *V. parahaemolyticus* from fish samples by the MPN method. The result indicated that 100% (20/20) of the samples were showing a microbial load of more than 1100 MPN/g. The isolates were subjected to PCR assay to confirm the presence of *V. parahaemolyticus* by targeting *tox*R gene. The findings from PCR analysis show that 90% (18/20) of the isolates harboured *tox*R gene and confirmed the presence of *V. parahaemolyticus* on the fish samples.

Vibrios are abundant in aquatic environments, and these bacteria were also observed on the skin, gills, and the intestinal tracts of fish or shellfish. High numbers of *V. vulnificus* and *V. parahaemolyticus* were described in fish intestines in comparison to water and sediment samples (Givens, Bowers, DePaola, Hollibaugh & Jones, 2014). Novotny *et al.* (2004), discussed the influence of seasonal variation in natural reservoirs towards the distribution of *V. parahaemolyticus*. It is frequently isolated from fish, molluscs, and crustaceans throughout the year in tropical climates and can be found in warm water with a lower salinity (Huehn *et al*., 2014). The tropical climate of Sarawak encouraged the proliferation of *V. parahaemolyticus* in the freshwater habitat. Our study proved the presence of this bacteria from local freshwater fish through the positive detection of *tox*R gene. *Vibrios* most frequently are found in marine fish; however, these pathogens are also observed in freshwater fish. Noorlis *et al.* (2011) reported that 24 % of catfish and 40% of red tilapia samples were contaminated with *V. parahaemolyticus*. When fish and fish products are consumed raw or undercooked, they can cause food-borne illnesses. However, the pathogenicity of *V. parahaemolyticus* strains detected in our study was not determined.

Cross-contamination is considered as a contributing factor for the presence of *V. parahaemolyticus* in this study. The use of contaminated ice to cover fresh fish in the wet market may act as a vector of transmission from handler to the fish samples. This is supported by Yang *et al*. (2008), which reported that 14.9% of frozen and iced seafood samples were contaminated with *V. parahaemolyticus.* The pivotal role of the natural environment as the source of bacterial infection in fish should also not be ruled out. The presence of *V. parahaemolyticus* in our study can be linked to direct contact of freshwater fish with contaminated water environment and ingestion of bacteria from

sediments or contaminated feed. Positive detection of *V. parahaemolyticus* in our study reflects the condition and safety of aquatic environments. Novoslavskij *et al.* (2016) highlight the presence of human pathogenic microorganisms in fish and fish products may be affected by various factors, including cultural practices, environmental conditions, processing, and distribution of products. For the case of unhygienic conditions, the increasing possibilities of contamination the fish and the fish products are may due to poor sanitary handling, rotation of unassigned tasks of the workers, and surrounding airborne microorganism during packing of the product (Novotny *et al*., 2004).

CONCLUSION

In summary, our study detected the presence of *V. parahaemolyticus* in 90% (18/20) of the total samples, highlighting the possibility of this bacteria to entering the food chain and lead to cross-contamination of premises, equipment, and end-product, ultimately facilitating the distribution of this pathogenic bacteria. However, more studies are needed to understand the pathogenicity of this *V. parahaemolyticus* and comparative studies of different parts of the fish need to be conducted in order to better understand the mode of contamination of the fish samples.

REFERENCE

Apun, K., Asiah, M. Y., & Jugang, K. (1999). Distribution of bacteria in tropical freshwater fish and ponds. *International Journal of Environmental Health Research*, *9*, 285-292.

Feldhusen, F. (2000). The role of seafood in bacterial food borne diseases. *Microbes and Infection*, *2*, 1651-1660.

Givens, C. E., Bowers, J. C., DePaola, A., Hollibaugh, J. T., & Jones, J. L. (2014). Occurrence and distribution of *Vibrio vulnificus* and *Vibrio parahaemolyticus* – potential role for fish, oyster, sediment and water. *Letters in Applied Microbiology*, *58*, 503-510.

Huehn, S., Eichhorn, C., Urmersbach, S., Breidenbach, J., Bechlars, S., Bier, N., Alter, T., Bartelt, E., Frank, C., Oberheitmann, B., Gunzer, F., Brennholt, N., Böer, S., Appel, B., Dieckmann, R., & Strauch, E. (2014). Pathogenic *Vibrios* in environmental, seafood and clinical sources in Germany. *International Journal of Medical Microbiology*, *304*, 843-850.

Institute of Food and Agriculture Sciences, IFAS (2009). *Preventing food borne and nonfood borne illness*. Retrieved October 3, 2012, from http://edis.ifas.ufl.edu.

Kaysner, C., DePaola, A. J., & Jones, J. (2004). Vibrio. In *Bacteriological Analytical Manual*. Retrieved March 25, 2019, from http://www.fda.gov/Food/scienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm070830.htm.

Letchumanan, V., Yin, W. F., Lee, L. H., & Chan, K. G. (2015). Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from retail shrimps in Malaysia. *Frontiers in Microbiology*, *6*, 33.

Meador, C. E., Parsons, M. M., Bopp, C. A., Gerner-Smidt, P., Painter, J. A., & Vora, G. J. (2007). Virulence gene-and pandemic group-specific marker profiling of clinical *Vibrio parahaemolyticus* isolates. *Journal of Clinical Microbiology*, *45*(4), 1133-1139.

Noorlis, A., Ghazali, F. M., Cheah, Y. K., Tuan Zainazor, T. C., Ponniah, J., Tunung, R., Tang, J. Y. H., Nishibuchi, M., Nakaguchi, Y., & Son, R. (2011). Prevalence and quantification of *Vibrio* species and *Vibrio parahaemolyticus* in freshwater fish at hypermarket level. *International Food Research Journal*, *18*, 689-695.

Novoslavskij, A., Margarita, T., Inga, E., Olga, V., Vadims, B., & Aivars, B. (2016). Major foodborne pathogens in fish and fish products: a review. *Annals of Microbiology*, *66*, 1-15.

Novotny, L., Dvorska, L., Lorencova, A., Beran, V., & Pavlik, I. (2004). Fish: a potential source of bacterial pathogens for human beings. *Veterinary Medicine*, *49*(9), 343-358.

Pillay, T. V. R. (1990). Fish and public health and disease. In *Aquaculture, principles and practices* (pp. 174–215). Oxford, UK: Fishing News Books.

Southeast Asian Fisheries Development Center, SEADEC. (2015). *Fisheries country profile: Malaysia*. Retrieved June 10, 2018, from http://www.seafdec.org/fisheries-country-profileMalaysia/.

Tunung, R., Margaret, S. P., Jeyaletchumi, P., Chai, L. C., Zainanor, T. C., Ghazali, F. M., Nakaguchi, Y., Nishibuchi, M., & Son, R. (2010). Prevalence and quantification of *Vibrio* in raw salad vegetables at retail level. *Journal of Microbiology and Biotechnology*, *20*(2), 391-396.

Yang, Z., Jiao, X., Zhou, X., Cao, G., Fang, W., & Gu, R. (2008). Isolation and molecular characterization of *Vibrio parahaemolyticus* from fresh, low-temperature preserved, dried and salted seafood products in two coastal areas of eastern China. *International of Food Microbiology*, *125*, 279-285.