

## Application of the Multiplex Polymerase Chain Reaction (M-PCR) for the Screening of *Vibrio* spp. from Rivers in Kuching, Sarawak

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### ABSTRACT

The present study was conducted to investigate the occurrence of *Vibrio* spp. from selected rivers in Kuching, Sarawak (Malaysia) using Multiplex Polymerase Chain Reaction (m-PCR). During the six month study period, 19 samples were collected monthly from seven rivers, followed by simultaneous detection of three *Vibrio* spp., *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*, in a single tube PCR reaction. Three sets of primers targeting the thermolabile (*tl*), outer membrane protein (*ompW*) and hemolysin/cytolysin genes (*vulCulsI*) of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*, respectively, were used. The results indicated that *V. parahaemolyticus* was the predominant species, occurring approximately 60.9% throughout the sampling period, followed by *V. cholerae* (23.1%) and *V. vulnificus* (16.0%). The months of July and December were found to be the months where all three *Vibrio* spp. were found to be at higher frequencies in the river samples. Results analyzed also indicated that the rivers with the highest prevalence of the three *Vibrio* spp. were Tambak Sejingkat, followed by Sungai Jernang and Sungai Tabuan. We conclude that m-PCR is a powerful and useful tool for the rapid and simultaneous detection of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* from the riverine environments without the need for isolation and culturing. Furthermore, this method is highly specific, and could be applied in diagnostic laboratories for larger scale epidemiological investigations of *Vibrio* spp.

Keywords: Multiplex PCR (m-PCR), *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio* spp., *Vibrio vulnificus*

### INTRODUCTION

*Vibrio* spp. are Gram-negative, rod or curve shaped facultative halophilic anaerobes (Vincent *et al.*, 2014; Malcolm *et al.*, 2015). These bacteria are autochthonous microbial inhabitants of temperate estuarine ecosystems, brackish water, saltwater environments and coastal areas (Micky *et al.*, 2014; Shaw *et al.*, 2015). According to Lopez-Hernandez *et al.* (2015), vibrios can remain in a viable state in the marine environment for extended periods of time. *Vibrio* spp. are resistant to alkaline environments but do not tolerate acidic conditions and will die rapidly in solutions with pH lower than six. Different ecological parameters such as nutrient, temperature, salinity, zooplankton and phytoplankton concentration also have direct influence on the presence and abundance of *Vibrio* spp. in the environment (Alam, 2003; Lopez-Hernandez *et al.*, 2015). Warmer temperature quickly increases the concentration of *Vibrio* spp. in

both molluscan shellfish and seawater. It was also reported that fewer *Vibrio* spp. were cultured when temperature drop and low salinity are recorded (Alam, 2003). However, the degree of salt tolerance varies among the species of this genus.

Outbreaks of *Vibrio* associated diseases present public health problems in many countries, such as United States, India, Bangladesh, Canada, Africa, Latin America and Malaysia (Son *et al.*, 2002; Micky *et al.*, 2014; Lopez-Hernandez *et al.*, 2015). These diseases are usually associated with contaminated or improperly cooked seafood, especially raw seafood, such as raw oysters and also domestic use of highly polluted waters (Lesley *et al.*, 2011; Shaw *et al.*, 2015; Malcolm *et al.*, 2015). *Vibrio* spp. that are implicated in these cases are numerous. However, the three species that are of major concerns are *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*.

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*Vibrio parahaemolyticus* is an opportunistic foodborne pathogen, and is often present in marine reservoirs, mainly shellfish (Vincent *et al.*, 2015). It was first discovered in the 1950s during a shirasu food poisoning outbreak (Malcolm *et al.*, 2015). Pathogenic *V. parahaemolyticus* cause severe diarrhea, abdominal cramps, nausea, vomiting, headaches, fever and chills in human via the production of several toxins such as thermo labile (*tl*) toxin, thermo stable direct hemolysin (*tdh*) and *tdh*-related hemolysin (*trh*) (Micky *et al.*, 2014). This species is reported to be the leading cause of seafood-associated bacterial gastroenteritis in the world (DePaola *et al.*, 2003).

*V. cholerae* is another member of the *Vibrio* family that is distributed worldwide in the estuarine environment (Vincent *et al.*, 2014). Pathogenic *V. cholerae*, such as *V. cholerae* O1 and *V. cholerae* O139, are important agents that cause potentially fatal diarrhea in human. The virulence factor of *V. cholerae*, especially group O1, is distinct from other genus (Bilung *et al.*, 2014). This particular species produce several toxins such as cholera toxin A (*ctxA*), cholera toxin B (*ctxB*), accessory cholera enterotoxin (*ace*) and zonula occludens toxin (*zot*) that cause bloodless, watery diarrhea, voluminous watery stools, and muscle cramps (Radu *et al.*, 1999; Son *et al.*, 2002; Vincent *et al.*, 2014). This bacterium continues to be prevalent in many underdeveloped and developing countries where it has caused major cholera epidemics. For example, 245,393 cases of cholera (3,034 deaths) were reported in 2012, and approximately 50% of these cases occurred in Africa and South America (Lopez-Hernandez *et al.*, 2015).

Like other members of the genus *Vibrio*, *V. vulnificus* is also found in the estuarine environments. This bacterium is pathogenically more severe as infections occur not only through the oral route, but also via open wounds that are exposed to warm seawater infested with this bacterium (Hlavsa *et al.*, 2011). Upon entry, localised septicemia symptoms such as swelling, erythema and intense pain would manifest between four hours to four days. According to Shaw *et al.* (2015), approximately 93 serious cases of *V. vulnificus* infections are reported in the United

States annually. The same study also stated that *V. vulnificus* alone was responsible for 35% of all non-foodborne *Vibrio* infections (NFVIs) and 78% of NFVI deaths in the United States from 1997 to 2006. Moreover, an estimated 50% of mortality rate is reported in immuno compromised patients infected with *V. vulnificus*, topping the fatality list among the three species (Oliver, 2005).

The state of Sarawak (Malaysia), experienced a major cholera outbreak in Miri and the Northern Division from November 1997 to April 1998 (Son *et al.*, 2002). According to Son *et al.* (2002), contaminated water supplies from the surrounding rivers were the main mode of transmission for the outbreak which involved *V. cholerae* O1. During this outbreak, more than 1,000 cases were reported (Son, personal communication). This outbreak and possible unreported ones are strong justifications for a need to constantly monitor the distribution of *Vibrio* spp. in rivers, especially its estuarine environment, where these bacteria are generally found in abundance. One recommended monitoring procedure for the detection of *Vibrio* spp. is to use polymerase chain reaction (PCR)-based method (Lesley *et al.*, 2011, Micky *et al.*, 2014). This method has proven to speed the detection of even non-culturable *Vibrio* spp. (Vincent *et al.*, 2014). A more recent improvement of the conventional PCR called Multiplex PCR (m-PCR) is a technique that uses multiple pairs of primers to amplify three or more target organisms simultaneously in a single reaction (Bej *et al.*, 1999; Lee *et al.*, 2003). m-PCR has proven to be a rapid and highly sensitive method for specific detection of *Vibrio* spp. either from aquatic environments or seafood (Lee *et al.*, 2003). In this study, m-PCR was performed on water samples collected from seven rivers during a six month study period from July, to December, 2003 in attempts to detect and monitor the distribution of several *Vibrio* spp. simultaneously. m-PCR reactions were performed in one-tube PCR mixtures utilizing the use of three primer pairs (*tl*, *ompW* and *vulCulst*), specifically targeting individual *Vibrio* spp. such as *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* (Lee *et al.*, 1998; Bej *et al.*, 1999; Lopez-Hernandez *et al.*, 2015; Vincent *et al.*, 2015).

## MATERIALS & METHODS

### Collection of Samples

Water samples were collected from several areas in Kuching, Sarawak (Malaysia) from the following sites; Sungai Bako, Sungai Jernang, Sungai Sejingkat, Tambak Sejingkat, Sungai Tabuan, Sungai Kuap and Sungai Melaban. Two to three surface water samples were collected from each sampling site, totaling to a number of 19 samples collected monthly during the six month study period (July-December, 2003). The water samples were collected and stored at 4°C in pre-sterilized 250 ml Schott bottles, then transported to the laboratory and processed within 2 hours of collection.

### Enrichment of Water Samples

The water samples were diluted to a 1:5 ratio in alkaline peptone water (APW, pH 8.5-8.6), as modified from a procedure by Atlas (1993). For each water samples, 50 ml was added to 200 ml of APW, mixed well and incubated at 37°C for 16-18 hours. Upon enrichment, 1.5 ml of the upper layer was used for bacterial DNA extraction. In addition, 200 µl of the enrichment homogenate were pipetted out from the upper layer and mixed with a mixture of LB broth with 15% glycerol (Fluka, Switzerland) when immediate DNA extraction was not performed. These cultures were then stored at -20°C until further use.

### Genomic DNA Extraction

Genomic DNA isolation was performed by modifying the procedure described by Ausubel *et al.* (1987). Overnight cultures from the processed water samples were transferred into sterile Eppendorf tubes and were centrifuged at 10,000 rpm for 1 min. After removing the supernatant, 700 µl of TE buffer was added, followed by a short vortexing before adding 5 µl of Proteinase K (25 mg/ml) and 10 µl of 10% SDS. Next, the mixtures were incubated at 60°C for about 1 hr. Phenol:Chloroform:Isoamyl propanol was then added at 500 µl to the mixture, followed by centrifuging at 12,000 rpm for 1 min. After centrifugation, 200 µl of the upper layer of the supernatant was

transferred into a new tube before adding 200 µl of 3 M KAc and 400 µl of isopropanol, followed by a thorough mixing. The solution was then centrifuged at 12,000 rpm for another 7 min and the supernatant was discarded. The DNA pellet collected was then washed with 500 µl of 70% ethanol and centrifuged for 5 min at 12,000 rpm. After the centrifugation, the supernatant was discarded and the pellet was dried for approximately 30 min before dissolving in 30 µl of sdH<sub>2</sub>O and kept frozen at -20°C until further use.

### Multiplex PCR (m-PCR) Reactions

Table 1 below shows the properties of the specific primers, target genes, amplicon sizes, melting temperature and sources of gene sequences used for m-PCR amplification in detecting the presence of all three *Vibrio* spp. All primers were obtained from MWG, Germany. m-PCR amplifications were performed in a 25 µl final volume consisting of 2.5 µl of 10X PCR reaction buffer (Promega Corporation, WI, USA), 1.0 µl of each of the 5 pmol/µl forward and reverse primers for *tl*, *ompW* and *VulcIs*, 0.5 µl of 10 µM dNTPs, 1.5 µl of 25 µM MgCl<sub>2</sub>, 0.3 µl of 5 U/µl Taq DNA polymerase and 9.2 µl volume of sterile distilled water. After 20 µl of the m-PCR mixtures were transferred into 0.2 µl PCR tubes, 5 µl of DNA template from each water sample was pipetted into the respective reaction tubes. The m-PCR reactions were run in a DNA thermal cycler (Perkin Elmer, USA), by setting the following temperature-cycling parameters: initial denaturation at 94°C for 3 min, then 30 cycles of amplification of each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min and primer extension at 72°C for 2 min. Following the amplification cycles, samples were kept at 72°C for 7 min to allow final extension of incompletely DNA synthesized.

### Visualization of m-PCR Products

All m-PCR products were separated in a 1.7% (w/v) agarose gel electrophoresis. A DNA ladder (Promega Corporation, WI, USA) of 1 kbp was used to determine the DNA sizes. Electrophoresis was performed using 1 X TBE buffer and a constant voltage of 60 V at 2

**Table 1.** Characteristics of the specific primers for the detection of *Vibrio* spp.

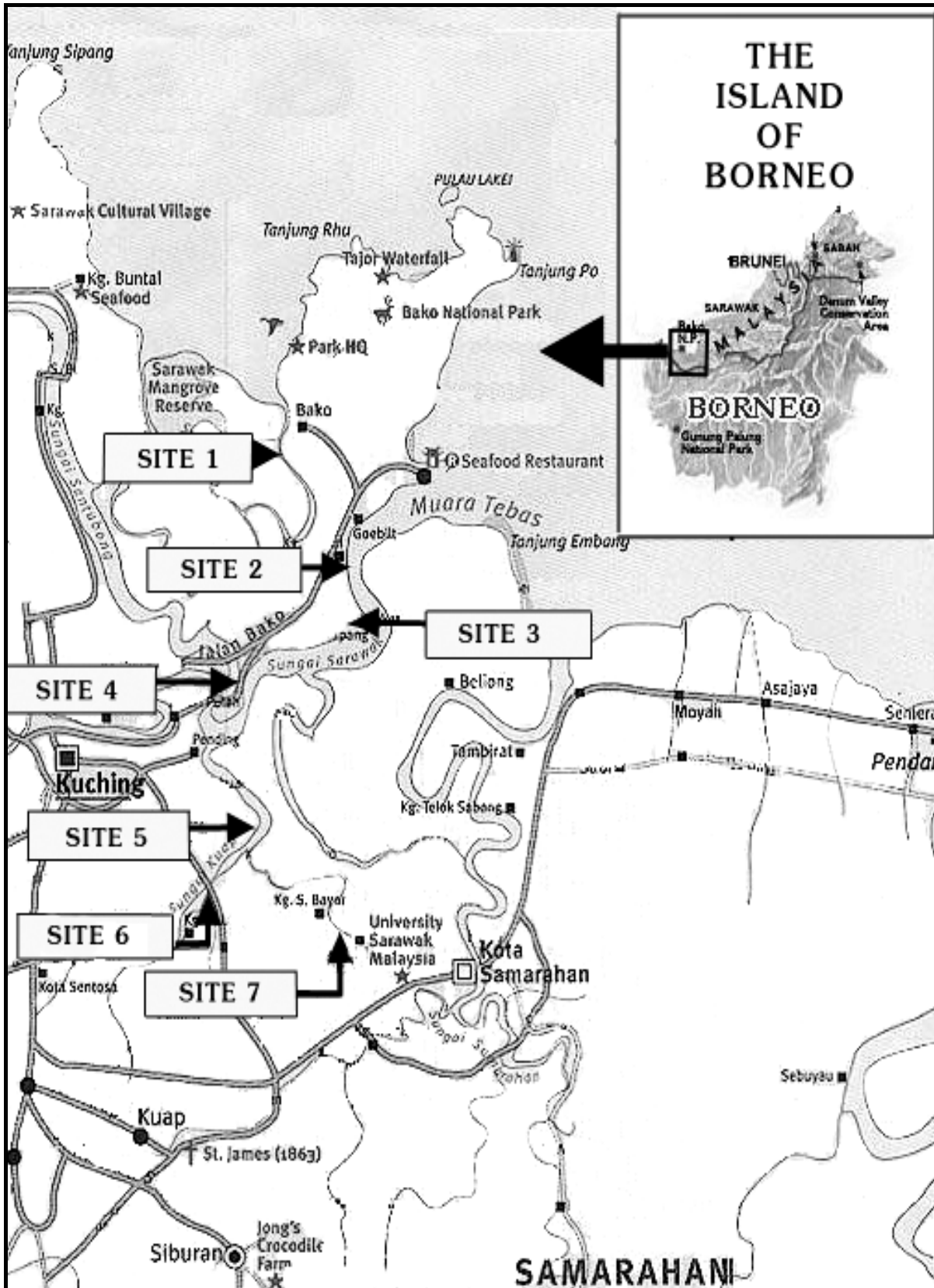
<i>Vibrio</i> spp.	Target gene	Primer sets	T <sub>m</sub> (°C)	Amplicon Size (bp)	Source
<i>V. parahaemolyticus</i>	<i>tl-F</i>	AAAGCGGATTATGCAGAAGC ACTG	65	450	Bej <i>et al.</i> , (1999)
	<i>tl-R</i>	GCTACTTTCTAGCATCATTTTC TCTGC	63		
<i>V. cholerae</i>	<i>ompW-F</i>	CACCAAGAAGGTGACTTTATT GTG	63	588	Lopez- Hernandez <i>et al.</i> , (2015)
	<i>ompW-R</i>	GAA CTT ATA ACC ACC CGC G	53		
<i>V. vulnificus</i>	<i>vulClsl-F</i>	GCT ATT TCA CCG CCG CTC AC	59	222	Lee <i>et al.</i> , (1998)
	<i>vulClsl-R</i>	CCG CAG AGC CGT AAA CCG AA	59		

hour. Then, the gel was stained with ethidium bromide for 15 min and visualized on a UV transilluminator (Fotodyne, WI, USA) and photographed with Polaroid™ Type 667 film.

## RESULTS AND DISCUSSION

In this study, the division of Kuching, Sarawak (Figure 1) in the South Western Island of Borneo was chosen because the rivers in the northern part of Kuching, such as the Sungai Bako, Sungai Jernang, Sungai Sejingkat, Sejingkat Causeway, Sungai Tabuan, Sungai Kuap and sungai Melaban are in close proximity to the sea, where the intermixing of freshwater and saltwater is common. Also, developments along the selected rivers such as constructions of factories and the blooming of human population make these areas prime sites for bacterial population studies, as river pollutions from sewage, rubbish and industrial wastes are often dumped directly into the rivers, which may be conducive to the existence and propagation of pathogenic microbes such as *Vibrio* spp. During the six month study period, three water samples were taken from each Sungai Bako, Sejingkat Causeway, Sungai Tabuan, Sungai Kuap and Sungai Melaban. While only two samples were taken from the smaller tributaries of Sungai Jernang, Sungai Sejingkat. In total, 114 water samples were collected and processed from the seven rivers during the period of study.

The results of the m-PCR monthly screenings of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* from the selected rivers are shown in Figure 2, Figure 3 and Table 2. From Table 2, 15 of the surface water samples (SB1, SB3, SJ1, SJ2, SS1, SS2, TS1, TS2, TS3, ST1, ST3, SK1, SK2, SM1 and SM2) collected were positive for *V. vulnificus*, 13 samples (SB1, SB2, SB3, SJ2, SS1, SS2, TS1, TS2, ST2, ST3, SK1, SK2 and SM2) were positive for the presence of *V. parahaemolyticus*, while 10 samples (SB2, SJ1, SS1, TS1, TS2, ST2, ST3, SM1 and SM2) were positive for the presence for *V. cholerae* in July. In August, 17 samples were positive for the presence of *V. parahaemolyticus* (SB1, SB2, SB3, SJ1, SJ2, SS2, TS1, TS2, TS3, ST1, ST2, ST3, SK1, SK2, SK3, SM1 and SM2), 7 samples showed positive result for *V. vulnificus* (SB3, SJ1, SJ2, SS2, TS1, TS2 and ST3) with only one sample showing the presence of *V. cholerae* (TS3). While in September, 15 samples collected (SB3, SJ1, SJ2, SS2, TS1, TS2, TS3, ST1, ST2, SK1, SK2, SK3, SM1, SM2 and SM3) were positive for the presence of *V. parahaemolyticus*, and three samples (TS1, TS2 and TS3) were positive for *V. cholerae*. However, all 19 samples collected in September were negative for the presence of *V. vulnificus*. In October, all 19 samples were positive for the amplification of the *tl* gene indicating the presence of *V. parahaemolyticus* but none were positive for *V. cholerae* and *V. vulnificus*. The 19 samples (SB1, SB2, SB3,



**Figure 1.** Location of sampling sites. Site 1 - Sg. Bako, Site 2 - Sg. Jernang, Site 3 - Sg. Sejingkat, Site 4 - Tambak Sejingkat, Site 5 - Sg. Tabuan, Site 6 - Sg. Kuap, Site 7 - Sg. Melaban (Source: Google Map).

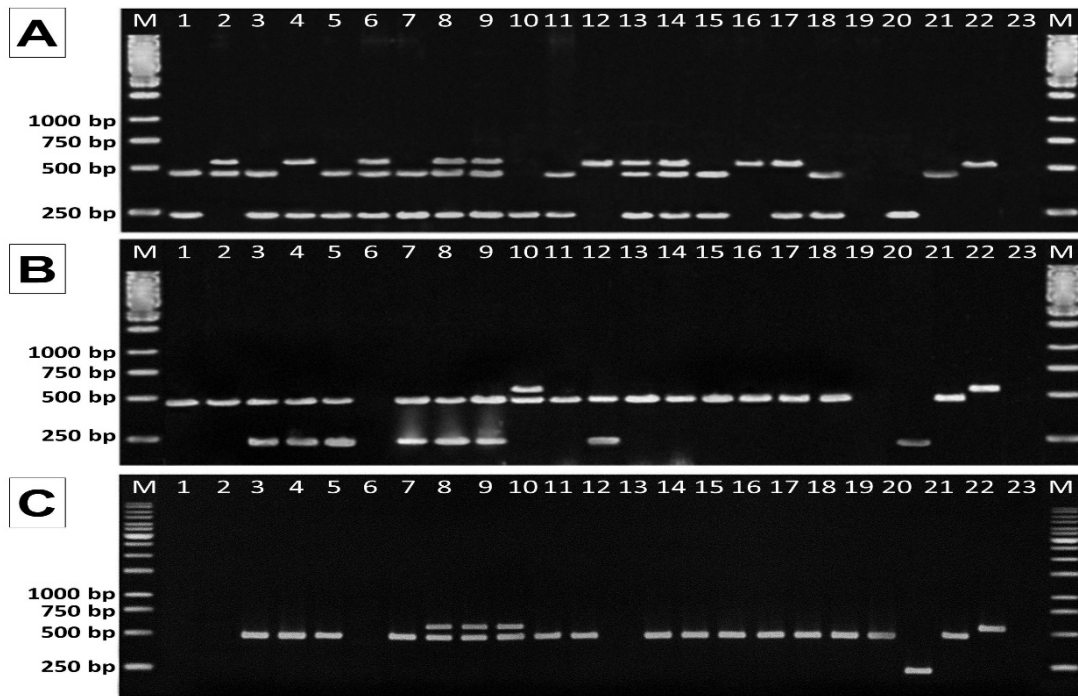
SB3, SJ1, SJ2, SS1, SS2, TS1, ST1, ST2, ST3, SK1, SK2, SK3, SM1, SM2 and SM3) processed in November also showed that almost all samples (17 samples) were positive for the presence of *V. parahaemolyticus* with nine samples being positive for the presence of *V. cholerae* from TS1, TS2, TS3, ST1, ST2, ST3, SK1, SK3 and SM1 but none were positive for the presence of *V. vulnificus*. In December, all three *Vibrio* spp. were present from the collective river water samples. The majority of the samples (SB1, SB2, SB3, SB3, SJ2, TS1, TS2, ST1, ST3, SK1, SK2, SK3, SM1, SM2 and SM3) showed positive result for the presence of *V. parahaemolyticus*, followed by 13 positive results for *V. cholerae* (SB1, SB2, SB3, SB3, TS1, TS2, ST1, ST2, ST3, SK1, SK2, SK3 and SM3) and three samples (SB2, SB3 and SM1) were positive for the presence of *V. vulnificus*.

Table 3 indicates that the majority of the water samples collected from each river during the study period were positive for the presence for *V. parahaemolyticus*. Results also showed that the river with the highest prevalence of the three *Vibrio* spp. was Tambak Sejingkat (*V. parahaemolyticus*-77.8%, *V. cholerae*-66.7%, *V. vulnificus*-27.8%), Sungai Jernang (*V. parahaemolyticus*-83.3%, *V. cholerae*-8.3%, *V. vulnificus*-33.3%), Sungai Tabuan (*V. parahaemolyticus*-83.3%, *V. cholerae*-38.9%, *V. vulnificus*-16.7%) and Sungai Kuap (*V. parahaemolyticus*-94.4%, *V. cholerae*-33.3%, *V. vulnificus*-11.1%) during the six month period.

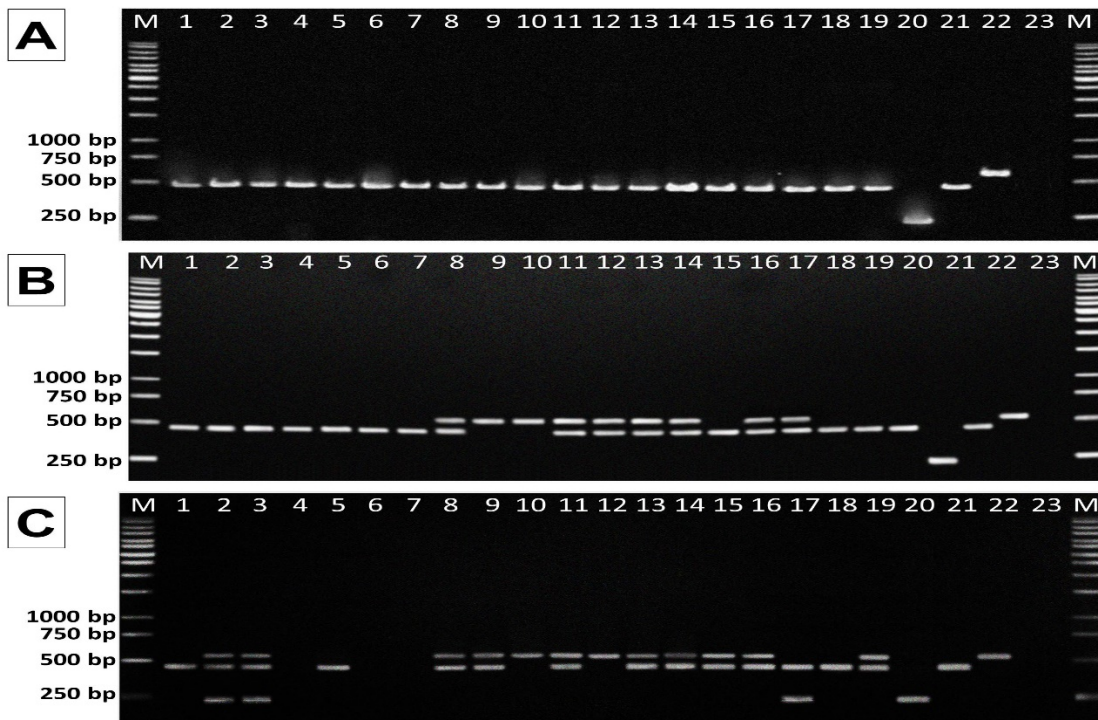
The results also showed a correlation between the weather conditions with the presence of several *Vibrio* spp. in the river samples. These observations are also supported and have been discussed at length in a study by Lopez-Hernandez *et al.* (2015) and Malcom *et al.* (2015). During the dryer months of July and August (data unpublished), the samples collected showed the presence of almost all three *Vibrio* spp. Dryer climate conditions increase the level of salinity which is conducive for the growth of the three species. Interestingly, the presence of several *Vibrio* spp. in the rainy month differed from the dryer month. Most of the water samples collected during the rainy season (September-December)

indicated the presence of *V. parahaemolyticus* and *V. cholerae*. But only few samples (3 of 76 processed samples) showed the presence of *V. vulnificus*. From Table 3 and Table 4, samples from the month of September, October, November, and December showed the presence of *V. parahaemolyticus* to be at 70%-90%, with 15% and 68% of water samples collected in September and December being positive for the presence of *V. cholerae*. These observations suggest that *V. parahaemolyticus* and *V. cholerae* are more prevalent and dominant in the brackish waters during rainy season when, theoretically, water salinity is lower. However, water salinity level was not recorded during the course of this study. We suggest that future studies on the detection of *Vibrio* spp. to be done concurrently with the nutrient influx, real-time weather and river abiotic data to generate clearer correlations between the various parameters and *Vibrio* spp. population dynamics, as according to Lopez-Hernandez *et al.* (2015), concerted studies and efforts are needed to understand how biotic and abiotic factors influence *Vibrio* spp. population dynamics in the environments that contribute to disease epidemiology.

There have been several reports of studies being developed using PCR-based methods to directly detect only single strain of *Vibrio* spp. (Lee *et al.*, 1993; Blackstone *et al.*, 2003). However, very limited studies have used m-PCR for simultaneous detection of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* from water or environmental samples. The major challenge when using Multiplex PCR is the presence of many PCR primers in a single tube that can cause several problems such as the formation of multiple mismatching of primers, primer-dimers and other nonspecific products that may interfere with the amplification of specific products. To avoid the mismatching of the primers, all primers were pair analyzed against each other using the BlastN program from the GenBank database to rule out cross species priming. Computer analyses confirmed that all the oligonucleotide primer pairs showed significant affinities only for their target species and non-mismatch were obtained from the primers used. Furthermore, to assist in the PCR product interpretation, the sizes of the amplicons (Table 2) were initially fixed to be



**Figure 2.** Agarose gel electrophoresis showing the results from PCR amplification product from samples collected in (A) July, (B) August, (C) September, 2003. Lane M – 1 kbp ladder; lane 1, 2, 3 - Sungai Bako; lane 4, 5 - Sungai Jenang; lane 6, 7 - Sungai Sejingkat; lane 8, 9, 10 - Tambak Sejingkat; lane 11, 12, 13 - Sungai Tabuan; lane 14, 15, 16 - Sungai Kuap; lane 17, 18, 19 - Sungai Melaban; lane 20 - *V. vulnificus* positive control; lane 21 - *V. parahaemolyticus* positive control; lane 22 - *V. cholerae* positive control; lane 23 - Negative control.



**Figure 3.** Agarose gel electrophoresis showing the results from PCR amplification product from samples collected in (A) October, (B) November, (C) December, 2003. Lane M – 1 kbp ladder; lane 1, 2, 3 - Sungai Bako; lane 4, 5 - Sungai Jenang; lane 6, 7 - Sungai Sejingkat; lane 8, 9, 10 - Tambak Sejingkat; lane 11, 12, 13 - Sungai Tabuan; lane 14, 15, 16 - Sungai Kuap; lane 17, 18, 19 - Sungai Melaban; lane 20 - *V. vulnificus* positive control; lane 21 - *V. parahaemolyticus* positive control; lane 22 - *V. cholerae* positive control; lane 23 - Negative control.

**Table 2.** Detailed data of *Vibrio* spp. occurrence collected from July-December, 2003.

Site Location	Samples	<i>Vibrio</i> spp.	Jul	Aug	Sept	Oct	Nov	Dec
Sg. Bako	SB1	<i>V. parahaemolyticus</i>	+	+	-	+	+	+
		<i>V. cholerae</i>	-	-	-	-	-	+
		<i>V. vulnificus</i>	+	-	-	-	-	-
	SB2	<i>V. parahaemolyticus</i>	+	+	-	+	+	+
		<i>V. cholerae</i>	+	-	-	-	-	+
		<i>V. vulnificus</i>	-	-	-	-	-	+
	SB3	<i>V. parahaemolyticus</i>	+	+	+	+	+	+
		<i>V. cholerae</i>	-	-	-	-	-	+
		<i>V. vulnificus</i>	+	+	-	-	-	+
Sg. Jernang	SJ1	<i>V. parahaemolyticus</i>	-	+	+	+	+	-
		<i>V. cholerae</i>	+	-	-	-	-	-
		<i>V. vulnificus</i>	+	+	-	-	-	-
	SJ2	<i>V. parahaemolyticus</i>	+	+	+	+	+	+
		<i>V. cholerae</i>	-	-	-	-	-	-
		<i>V. vulnificus</i>	+	+	-	-	-	-
Sg. Sejingkat	SS1	<i>V. parahaemolyticus</i>	+	-	-	+	+	-
		<i>V. cholerae</i>	+	-	-	-	-	-
		<i>V. vulnificus</i>	+	-	-	-	-	-
	SS2	<i>V. parahaemolyticus</i>	+	+	+	+	+	-
		<i>V. cholerae</i>	-	-	-	-	-	-
		<i>V. vulnificus</i>	+	+	-	-	-	-
T. Sejingkat	TS1	<i>V. parahaemolyticus</i>	+	+	+	+	+	+
		<i>V. cholerae</i>	+	-	+	-	+	+
		<i>V. vulnificus</i>	+	+	-	-	-	-
	TS2	<i>V. parahaemolyticus</i>	+	+	+	+	-	+
		<i>V. cholerae</i>	+	-	+	-	+	+
		<i>V. vulnificus</i>	+	+	-	-	-	-
	TS3	<i>V. parahaemolyticus</i>	-	+	+	+	-	-
		<i>V. cholerae</i>	-	+	+	-	+	+
		<i>V. vulnificus</i>	+	-	-	-	-	-
Sg. Tabuan	ST1	<i>V. parahaemolyticus</i>	+	+	+	+	+	+
		<i>V. cholerae</i>	-	-	-	-	+	+
		<i>V. vulnificus</i>	+	-	-	-	-	-
	ST2	<i>V. parahaemolyticus</i>	-	+	+	+	+	-
		<i>V. cholerae</i>	+	-	-	-	+	+
		<i>V. vulnificus</i>	-	-	-	-	-	-
	ST3	<i>V. parahaemolyticus</i>	+	+	-	+	+	+
		<i>V. cholerae</i>	+	-	-	-	+	+
		<i>V. vulnificus</i>	+	+	-	-	-	-
Sg. Kuap	SK1	<i>V. parahaemolyticus</i>	+	+	+	+	+	+
		<i>V. cholerae</i>	+	-	-	-	+	+
		<i>V. vulnificus</i>	+	-	-	-	-	-
	SK2	<i>V. parahaemolyticus</i>	+	+	+	+	+	+
		<i>V. cholerae</i>	-	-	-	-	-	+
		<i>V. vulnificus</i>	+	-	-	-	-	-
	SK3	<i>V. parahaemolyticus</i>	-	+	+	+	+	+
		<i>V. cholerae</i>	-	-	-	-	+	+
		<i>V. vulnificus</i>	-	-	-	-	-	-
Sg. Melaban	SM1	<i>V. parahaemolyticus</i>	-	+	+	+	+	+
		<i>V. cholerae</i>	+	-	-	-	+	-
		<i>V. vulnificus</i>	+	-	-	-	-	+
	SM2	<i>V. parahaemolyticus</i>	+	+	+	+	+	+
		<i>V. cholerae</i>	+	-	-	-	-	-
		<i>V. vulnificus</i>	+	-	-	-	-	-
	SM3	<i>V. parahaemolyticus</i>	-	-	+	+	+	+
		<i>V. cholerae</i>	-	-	-	-	-	+
		<i>V. vulnificus</i>	-	-	-	-	-	-



**Table 3.** Percentages of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* occurrences based on sampling sites.

Site Location	<i>Vibrio</i> spp.	% occurrence
Sg. Bako	<i>V. parahaemolyticus</i>	33.3 (6/18)
	<i>V. cholerae</i>	22.2 (4/18)
	<i>V. vulnificus</i>	27.8 (5/18)
Sg. Jernang	<i>V. parahaemolyticus</i>	83.3 (10/12)
	<i>V. cholerae</i>	8.3 (1/12)
	<i>V. vulnificus</i>	33.3 (4/12)
Sg. Sejingkat	<i>V. parahaemolyticus</i>	66.7 (8/12)
	<i>V. cholerae</i>	5.6 (1/12)
	<i>V. vulnificus</i>	16.7 (3/12)
T. Sejingkat	<i>V. parahaemolyticus</i>	77.8 (14/18)
	<i>V. cholerae</i>	66.7 (12/18)
	<i>V. vulnificus</i>	27.8 (5/18)
Sg. Tabuan	<i>V. parahaemolyticus</i>	83.3 (15/18)
	<i>V. cholerae</i>	38.9 (7/18)
	<i>V. vulnificus</i>	16.7 (3/18)
Sg. Kuap	<i>V. parahaemolyticus</i>	94.4 (17/18)
	<i>V. cholerae</i>	33.3 (6/18)
	<i>V. vulnificus</i>	11.1 (2/18)
Sg. Melaban	<i>V. parahaemolyticus</i>	83.3 (15/18)
	<i>V. cholerae</i>	22.2 (4/18)
	<i>V. vulnificus</i>	16.7 (3/18)

different from each other in sizes making size discrimination easier by using gel electrophoresis.

When a large number of samples for screening purposes are processed to examine, individual species, the analyses may be lengthier, with higher cost for identification and confirmation. Thus identification of *Vibrio* spp. using m-PCR with three or more pairs of primers in the same reaction volume reduces labor, saves time, cost effective not only for specific detection of several species simultaneously but also for rapid analysis of large number of samples. In this experiment,

every batch of 19 samples can be processed within 24 hour to obtain results after sampling, processing and enrichment, suggesting that m-PCR method of identification is faster compared with traditional biochemical tests which are useful for diagnostic laboratories to detect *Vibrio* spp. and also for epidemiological investigations. This study has also successfully generated data on the monthly distribution of *Vibrio* spp. in selected rivers within the Kuching district for clinical and diagnostic laboratories use in the monitoring of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*.

**Table 4.** Overall number and prevalence of *Vibrio* spp. samples from July–December, 2003.

	<i>V. parahaemolyticus</i>	<i>V. cholerae</i>	<i>V. vulnificus</i>	Total
July	13	10	15	38
August	17	1	7	25
September	15	3	0	18
October	19	0	0	19
November	17	9	0	26
December	14	13	3	30
Total	95	36	25	
Percentages of prevalence in river samples (%)	83.3 (95/114)	31.6 (36/114)	21.9 (25/114)	
Percentages of prevalence among <i>Vibrio</i> spp. (%)	60.9 (95/156)	23.1 (36/156)	16.0 (25/156)	

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