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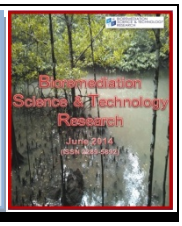

Incidence of *Vibrio parahaemolyticus* at Estuarine Region of Sg. Sarawak (Sarawak) Influenced by Physicochemical Parameters

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Abstract

This study was aimed to determine the correlation between physicochemical parameters namely salinity, pH and temperature with the occurrence of *V. parahaemolyticus* in estuarine river of Sg. Sarawak. A total of 72 water samples (surface and deep waters) were collected from the study site. A combination of MPN and PCR to detect and quantify the presence of *V. parahaemolyticus*, targeting the regulatory gene, *toxR* was performed. Over the 12-weeks sampling period, site 2 and site 3 recorded 100% presence of *V. parahaemolyticus* while site I was only 17%. The conditions found in site 2 (9.75‰, pH 7.2, 29.65 °C) and 3 (10.3‰, pH 7.36, 30 °C) favored the presence of *V. parahaemolyticus* (669 cells 10 ml^{-1} and 922 cells 10 ml^{-1} respectively) than site 1 (0.68‰, pH 6.75, 29.2 °C). Statistical analysis showed that the abundance of total *V. parahaemolyticus* counts was correlated well with the water salinity (Pearson correlation coefficient, $r = 0.699$; $P < 0.01$) and water temperature ($r = 0.531$; $P < 0.01$) but less correlated with water pH ($r = 0.343$; $P < 0.01$).

INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative, halophilic bacterium occurring naturally in estuarine and coastal marine water. It causes illness in humans and commonly associated to various gastroenteritis outbreaks worldwide [1]. It is also known to cause septicemia particularly in patients with underlying medical conditions [2]. Infection may be transmitted by consuming contaminated seafood, vegetables, water and through open wounds [3-6]. Their presences are usually influenced by the season and environment conditions, where it is higher in warmer months [7] and sensitive to salinity of the freshwater [8]. The presence of *V. parahaemolyticus* has been successfully confirmed by PCR based method targeting on *toxR* gene, as the regulatory gene of the organism [9-11].

In Malaysia, *V. parahaemolyticus* has been isolated and identified from the coastal seawater in Peninsular Malaysia recently [12]. This has raised the probability of *V. parahaemolyticus* outbreak by consumed contaminated seafood. The distribution of *V. parahaemolyticus* depends on several parameters such as temperature, dissolved oxygen and salinity [13, 14]. Thus, further study is very crucial to understand the distribution of *V. parahaemolyticus* in relation to several environmental parameters since information on the physiochemical status of the water in association with this bacterium is unclear, especially in the estuarine region of Sg. Sarawak. Hence, the present study was carried out to gain more knowledge on the relationship of parameters such as salinity, pH and temperature with the distribution of *V. parahaemolyticus* in the estuarine region of Sg. Sarawak.

MATERIALS AND METHODOLOGY

Sample collection, processing and enrichment

Sample collection was performed according to the methodology by Garay *et al*. [15] and Randa *et al*. [16]. The water samples were collected from three different sites (S1, S2 and S3) at the estuarine region of Sg. Sarawak (GPS Coordination: 1.584233, 110.447088) (**Figure 1**). Site 1 is nearer to the freshwater region while both site 2 and site 3 are nearer to the seawater region. Sg. Sarawak is a main river in the Kuching Division of Sarawak's State whereby the river water serves as the main water source for the villagers for drinking and daily purposes. At each site, samples were taken from the surface and deep region (1.3 meter below). The water samples from the surface and deep regions were collected using bottle and ballast, respectively. The sampling was carried out weekly for 12 consecutive weeks. Water temperature and salinity were recorded *in situ* while the pH readings were performed in the laboratory.

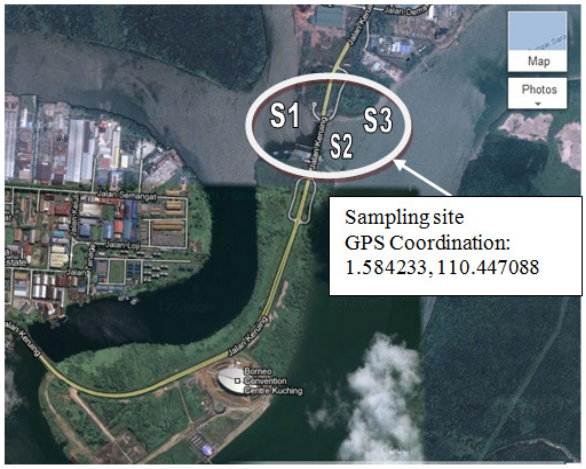


Figure 1: Estuarine region of Sg. Sarawak, Sarawak

Enumeration with Most Probable Number (MPN) method

This method was carried out according to Lee *et al.* [17]. A serial dilution of alkaline peptone water (APW) was prepared and used in Most Probable Number (MPN) method, in which the river water samples were serially diluted to 1:10, 1:100 and 1:1000. These tubes were incubated at 37 °C for 18 to 24 h for enrichment stage. After overnight incubation, the tubes with positive turbidity were recorded and the results were compared with the three tubes MPN **Table**.

DNA extraction and PCR amplification

MPN tubes showing turbid growth were then subjected to specific PCR to determine the presence of *V. parahaemolyticus*. DNA extraction was carried out using a boiled-cell method [11] with some modification. Briefly, 1 ml of positive samples were transferred into 1.5 ml microcentrifuge tubes and centrifuged at 12, 000 rpm for 3 min. The supernatants were discarded. Five hundred microlitres of sterile distilled water were added and vortexed to mix. The samples were boiled for 15 min. After boiling, the samples were cooled on ice for 15 min and re-centrifuged at 13,000 rpm for 1 min. The supernatants containing the genomic DNA were used as template for PCR reaction.

The primers used to detect the presence of *V. parahaemolyticus* were stated are as shown in **Table** 1. The PCR amplification for *V. parahaemolyticus* was carried out based on Zulkifli *et al.* [11] with slight modifications in 25 ml PCR mixture containing 4.0 µl of 10x PCR buffer, 3.0 µl of 25 mM $MgCl_2$, 1.0 µl of 10 mM dNTPs, 1.0 µl of each 10 pmol primer, 1.0 µl of 2.5 U *Taq* DNA polymerase, 2.0 µl of DNA template and sterile distilled water. All thermal cycling reactions were performed with a Thermocycler (Esco Swift MiniPro) using the following parameters: Initial denaturation at 94 °C for 3 min; followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 63 °C for 1.5 min and extension at 72 °C for 1.5 min before final extension at 72 °C for 7 min. For visualization, 5 µl of each PCR product was run on 1.2% agarose gel at 85V for 1 h in 1X Tris Borate EDTA (TBE) buffer. The gel was stained with ethidium bromide for 15 min and viewed under UV transilluminator.

Table 1: Primers sequences for the detection of *V. parahaemolyticus.*

Primers	Primer sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
ToxR-4 *ToxR-7*	5'-GTCTTCTGACGCAATCGTTG-3' 5'-ATACGAGTGGTTGCTGTCATG-3'	*ToxR*	368	[10]

Statistical analysis

One-way ANOVA test was performed to analyze the correlation of the sampling sites on the *V. parahaemolyticus* counts and physiochemical parameters. The correlations between *V. parahaemolyticus* counts and the environmental parameters measured were analyzed by Pearson correlation coefficient. All the statistical analysis was performed using the Minitab version 14.0.

RESULTS AND DISCUSSION

Average value of the physicochemical parameters and *V. parahaemolyticus* count

The average value of physicochemical parameters found in site 2 (9.75‰, pH 7.2, 29.65 °C) and 3 (10.3‰, pH 7.36, 30 °C) favored the presence of *V. parahaemolyticus* with 669 cells 10 ml^{-1} and 922 cells 10 ml^{-1} respectively (**Table 2**). The conditions in site 1 (0.68‰, pH 6.75, 29.2 °C) were not favored the presence of *V. parahaemolyticus* with only 190 cells 10 mL^{-1}.

Statistics of physicochemical parameters

The statistical analyses for each physicochemical parameter (pH, salinity, and temperature) at the three different sites were calculated, as displayed in **Table 2**. Salinity, pH and temperature at Site 1 were much lower (0.708 ± 0.690 ‰, pH 6.7442 ± 0.4806, 28.938 ± 1.288 °C) than site 2 and site 3. However, physiochemical parameters at site 2 (9.792 ± 1.503 ‰, pH 7.2029 ± 0.4530, 29.500 ± 0.780 °C) were comparable with site 3 (10.292 ± 1.732 ‰, pH 7.3646 ± 0.3993, 29.667 ± 0.637 °C).

Table 2: descriptive statistics of physiochemical parameters and *V. parahaemolyticus* bacterial counts with respected to sampling sites at sg.

Site	Parameter	Mean ± Standard deviation (SE)
S1	Count (cells 10 ml^{-1})	188.2 ± 74.3
	Salinity (‰)	0.708 ± 0.690
	pH	6.7442 ± 0.4806
	Temperature (°C)	28.938 ± 1.288
S2	Count (cells 10 ml^{-1})	669.2 ± 382.5
	Salinity (‰)	9.792 ± 1.503
	pH	7.2029 ± 0.4530
	Temperature (°C)	29.500 ± 0.780
S3	Count (cells 10 ml^{-1})	921.7 ± 319.9
	Salinity (‰)	10.292 ± 1.732
	pH	7.3646 ± 0.3993
	Temperature (°C)	29.667 ± 0.637

Enumeration of *V. parahaemolyticus*

About 188.2 ± 74.3 (cells 10 ml^{-1}) of *V. parahaemolyticus* count was recorded from site 1, 669.2 ± 382.5 (cells 10 ml^{-1}) from site 2 and slightly higher from site 3 which was 921.7 ± 319.9 (cells 10 ml^{-1}) of *V. parahaemolyticus* count (**Table** 2).

PCR result

A total of 72 water samples were subjected to PCR analysis targeting the *toxR* gene for *V. parahaemolyticus.* Forty eight water samples from site 2 and site 3 showed the presence of *V. parahaemolyticus* consistently over the twelve weeks (100% presence of *V. parahaemolyticus*). Water sample from site 1 only showed positive *V. parahaemolyticus* for the first two weeks (17% presence of *V. parahaemolyticus*) while negative for the following 10 weeks. **Figure** 2 presented the representative agarose gel electrophoresis of PCR products from six water samples for detection of *V. parahaemolyticus*.

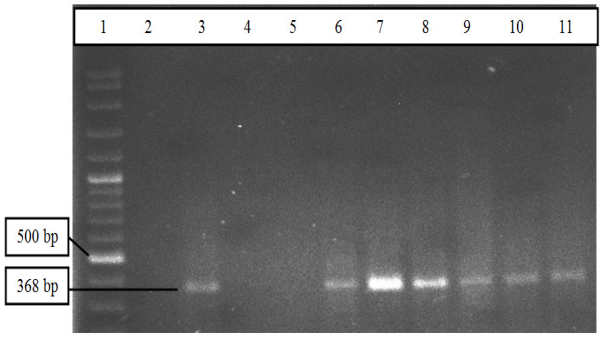


Figure 2: Representative agarose gel electrophoresis of PCR products from six water samples for detection of *V. parahaemolyticus.* Lane 1: 100bp DNA ladder, Lane 3: positive control of *Vibrio parahaemolyticus*, Lane 4: negative control, Lane 6: site 1 (surface), Lane 7: site 2 (surface.), Lane 8: site 3 (surface), Lane 9: site 1 (deep), Lane 10: site 2 (deep), Lane 11: site 3 (deep).

Statistical analysis

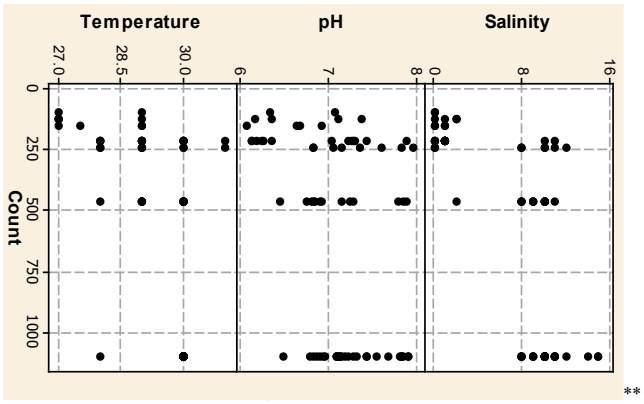
There is a significant effect of sampling locations on the total *V. parahaemolyticus* counts, water salinity and pH reading (P<0.001; separate one-way ANOVA test) (**Table** 3). Tukey pairwise comparison test based on 95% simultaneous confidence intervals showed that the mean total bacterial counts, water salinity and pH reading at sampling site 1 are significantly lower than 2 and 3, but comparable between sampling sites 2 and 3. There is also an effect of sampling location on the water temperature reading (P=0.024, one-way ANOVA test) in which the mean reading at sampling site 1 is significantly lower than site 3. However, there is no effect on the bacterial counts and the physicochemical parameters measured either at the surface or 1.3 m below the water surface.

The associations of total *V. parahaemolyticus* counts with the physicochemical parameters were compared using Pearson correlation test (**Figure** 3).

The abundance of total *V. parahaemolyticus* counts was correlated well with the water salinity (Pearson correlation coefficient, r = 0.699; P < 0.01) and water temperature (r = 0.531; P < 0.01) but less correlated with water pH (r = 0.343; P < 0.01).

Table 3: The correlations between bacterial counts and physiochemical parameters.

		Count	Salinity	pH	Temperature	*Site*	Level
Count	Pearson Correlation	1	.699**	.343**	.531**	.718**	-.025
	Sig. (2-tailed)		.000	.003	.000	.000	.836
	N	72	72	72	72	72	72
Site	Pearson Correlation	.718**	.849**	.497**	.305**	1	.000
	Sig. (2-tailed)	.000	.000	.000	.009		1.000
	N	72	72	72	72	72	72
Level	Pearson Correlation	-.025	.015	.045	.021	.000	1
	Sig. (2-tailed)	.836	.900	.706	.859	1.000	
	N	72	72	72	72	72	72



. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Figure 3: The associations of total *V. parahaemolyticus* counts with the physicochemical parameters in Sungai Sarawak.

DISCUSSION

Salinity plays an important role in the distribution of *V. parahaemolyticus* in the estuarine environment and the bacteria are stated to be sensitive to salinity due to the fact that *V. parahaemolyticus* is halophile [8]. In this study, there was a big difference in the level of salinity among site 1, 2, and 3. This is due to the characteristic of the estuarine environment as the transition region between freshwater and the sea. Site 1 is nearer to the freshwater region while both site 2 and site 3 are nearer to the seawater region. In addition, study by McLusky [18] stated that the different levels of salinity in the estuarine environment are related to the rainy season. During the rainy season, the water had been diluted by the rainfall and caused the estuarine water to be less saline than in the warmer season.

From the statistical analysis, it was found that there was a positive significant relationship between level of salinity in Sg. Sarawak with the abundance of the *V. parahaemolyticus*. Previous studies that analyzed the relationship between salinity and *V. parahaemolyticus* abundance had been contradictory. Martinez-Urtaza *et al*. [19] found that the abundance of *V. parahaemolyticus* was inversely correlated to salinity (>30 ‰). Lip *et al*. [20] on the other hand, found that the abundance of *V. parahaemolyticus* was positively correlated to salinity when the salinity was less than 15 ‰ and inversely correlated to salinity when the salinity was greater than 15 ‰. This report was consistent and supports our findings as the value of salinity recorded over twelve weeks ranged from 0 to 15 ‰.

V. parahaemolyticus is able to grow well in alkaline condition with high pH value [21]. In this study, the pH values recorded from all sites were slightly acidic (pH 6.08-7.98) for almost every week. However, *V. parahaemolyticus* was able to be detected even at pH 6.47. The different in the level of pH in the water was due to influx and decay in the estuarine region and also the hydrogen ions which run-out off during rainy season [22]. Besides, the pH of the water was less correlated to the abundance of *V. parahaemolyticus*. This may be due to only low variable values of pH were recorded (6.08-7.98) over the twelve weeks sampling.

Temperature is associated with the growth of *V. parahaemolyticus*. The findings of this study showed that as the temperature increases, the number of bacteria also increases, and it gives positive PCR result. Previous researchers have reported that temperature between 10 °C – 43 °C with optimum at 37 °C is suitable for the growth of *V. parahaemolyticus* which has short generation time ranging from 8 – 9 minute [23]. Based on the findings, samples from site 1 showed positive result only in the first two weeks when the temperature was lower than the rest of the weeks. As the temperature increased, site 1 showed negative PCR result but increased in the number of bacteria. From the statistical analysis, temperature was found to be significant and positive correlated to the abundance of *V. parahaemolyticus*. These results were consistent with other study which recorded a high occurrence of *V. parahaemolyticus* in the estuarine with the increase in temperature [24].

CONCLUSION

In conclusion, this study reported the importance of specific environmental parameters namely salinity, pH and temperature in influencing the distribution and abundance of *V. parahaemolyticus* in Sungai Sarawak. This study also confirmed some previous findings (impacts of salinity, pH and temperature of water to the occurrence or densities of *V. parahaemolyticus*) which improve the chances of identifying environmental situation that can used to predict and possibly prevent *V. parahaemolyticus* outbreaks in a wide range of locations along Sungai Sarawak.

ACKNOWLEDGEMENT

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