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Detection of Cholera Toxin-Producing *Vibrio cholerae* in Phytoplankton from Santubong and Samariang Estuaries

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ABSTRACT

Many cholera outbreaks worldwide were associated with cholera toxin-producing *Vibrio cholerae*. The bacteria are ubiquitous in aquatic environment, whilst phytoplankton is associated with adaptation of the *Vibrio* species. This study was conducted to detect cholera toxin-producing *Vibrio cholerae*, and to determine association of the selected water physicochemical parameters with the number of the bacteria. In this study, a total of ten phytoplankton samples were collected at Santubong and Samariang Estuaries in Kuching, Sarawak. Water physicochemical parameters (temperature, pH and salinity) were recorded. *Vibrio* bacteria were cultivated on thiosulfate citrate bile-salts sucrose selective agar and analysed for cholera toxin-producing *Vibrio cholerae* using polymerase chain reaction by targeting *ctx*A gene that encodes for virulence *cholera* enterotoxin subunit A. The result revealed that a range of $1.0 \times 10^7 – 8.0 \times 10^7$ CFU/ml of yellow colonies growing on the thiosulfate citrate bile-salts sucrose agars. Inversely, no samples were positive with cholera toxin-producing *Vibrio cholerae*. The physicochemical parameters at Samariang Estuary were more associated with the number of bacteria in the samples compared to Santubong Estuary.

Keywords: cholera toxin-producing, *ctx*A gene, *Vibrio cholerae*

INTRODUCTION

Vibrio species is gram-negative bacteria, rod-curved shaped, halophile, non-spore forming, autochthonous living in marine and estuaries. The bacteria thrive in saline aquatic environments, that either be free in water or attached to animate surface like phytoplankton or zooplankton (Cavallo & Stabili, 2002). The presence of *Vibrio* bacteria in the environment is influenced by different ecological parameters such as temperature, salinity, nutrient, and concentration of zooplankton and phytoplankton (Alam *et al*., 2003). *Vibrio cholerae* is one of the common species causing infection. In past record, cholera outbreaks occurred in Miri and Northern Division in Sarawak, Malaysia from November 1997 to April 1998 were due to the contaminated water supplies (Vincent *et al*., 2015).

Phytoplankton is responsible as natural reservoirs for *Vibrio* bacteria especially *V. cholera*. Furthermore, phytoplankton blooms can promote the increase in *Vibrio* spp. density (Huq *et al*., 2012). Direct influence of phytoplankton with growth of *Vibrio* is explained as the number of different phytoplankton species that can contribute to the growth of *Vibrio* (Peterson *et al*., 2010). Human risk from this event exists where the presence of *Vibrio* spp. in water and phytoplankton will lead to accumulation of *Vibrio* in the shellfish after filter feeding process (Huq *et al*., 2012).

Cholera toxin (CT) is one of critical virulence factors involved in enteropathogenicity by certain strains of the species (Radu *et al*., 2002). This toxin is strong heat-labile and encoded by *ctxA* gene. It was first discovered by Koch in 1884 (Broeck *et al*., 2007). On the other hand, O1 and O139 serogroups are the only two *V. cholerae* serogroups critical for worldwide outbreaks. Other strains have been reported causing infections but being deemed as rare and trivial (Reidl & Klose, 2002). Pathogenicity of *V. cholerae* is influenced by several virulence factors including potent enterotoxin (CT) (Sharma & Chaturvedi, 2009), which is also carried by O1 and O139 groups (Dutta *et al*., 2013). Nonetheless, other non-O1 and non-139 strains may also produce cholera toxin during infection. Clinical manifestations of

cholera infection includes massive excretion of rice-water stool that can amount to over ten litres a day (Broeck *et al*., 2007). Despite frequent of such reports in Sarawak, to the best of our knowledge this is the first study on the occurrence of *V. cholerae* in phytoplankton samples in the state. This preliminary study was conducted as to detect the presence of cholera toxin-producing *V. cholerae* in phytoplankton and to determine the association between physical parameters and the presence of *V. cholerae*.

MATERIALS AND METHODS

Background of the Study

Two estuaries in Sarawak were studied namely Santubong (1°117'50" N 110°19'34" E) and Samariang (1°36'32" N 110°19'37" E) (Figure 1).

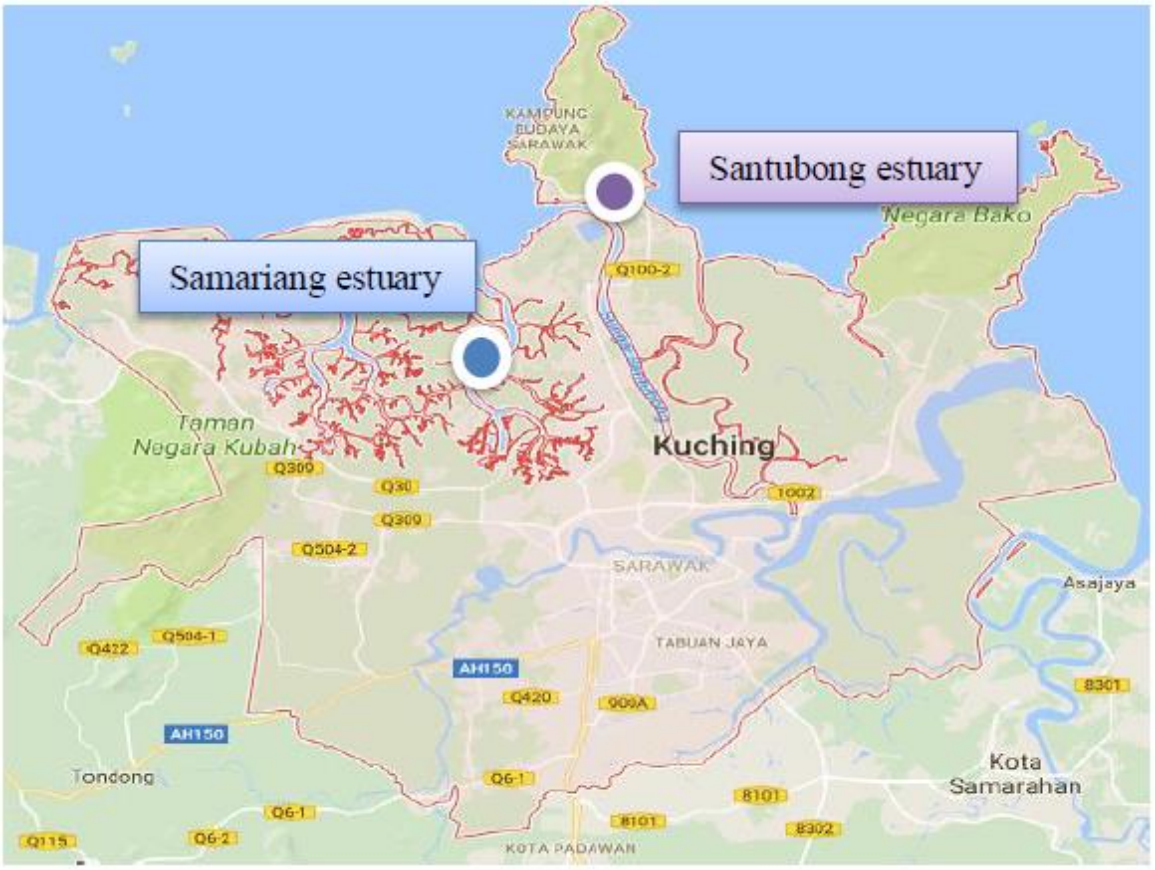

Figure 1. A map of Kuching, Sarawak showing the location of sampling sites in Santubong and Samariang Estuaries where phytoplankton samples were collected.

The geographical location of Santubong Estuary is located near to Damai Beach, whilst Samariang Estuary is located upstream of Samariang Estuary (Batang Salak). Furthermore, in these selected estuaries, the phytoplankton samples from Santubong sampling site were associated with sea water while the phytoplankton samples from Samariang sampling site were associated with brackish water. Areas of Santubong are local tourism places that are surrounded with resorts, whereas areas of Samariang Estuary are surrounded with mangrove forests that represent a habitat for aquatic living and several shrimp and fish cage cultures for protein source demands.

Phytoplankton Collection and Water Physicochemical Parameter Assessment

Phytoplankton sample collection was conducted as described by Tuney and Maroulakis (2014). Surface water samples were collected using a plankton net (10-µm mesh size). The sampling was conducted from November 2017 until January 2018 with a total of ten plankton samples were collected (500 ml each). The samples were stored in an ice box and were transported to Molecular Microbiology Laboratory at Universiti Malaysia Sarawak for further analysis. Besides, the selected water physicochemical parameters (i.e. pH, temperature

and salinity) were measured *in-situ* using a pH meter (Hanna Instrument, USA) to measure pH and temperature, and a hand refractometer (Agato, Japan) to measure salinity.

Sample Processing

The sample processing procedure was conducted as described by Huq *et al*. (2012). All samples were concentrated through filtration by using polycarbonate membrane filter (0.45 µm pore size; 47 mm). The trapped phytoplankton on the membrane filter were washed with 25 ml of sterile 1× phosphate buffer solution in 50 ml of centrifuge tube before being vortexed. Next, 1 ml of the sample was transferred into 25 ml of alkaline peptone water (1% peptone; 1% NaCl; pH 8.6) and was incubated 24 h at 35 °C.

Enumeration of Bacteria Using Spread Plate Method

A five-tube serial dilution was conducted in Falcon tube containing 9 ml of PBS. Afterwards, 100 µl of each dilution solution was spread plated on thiosulfate citrate bile-salts sucrose (TCBS) agar (Oxoid, Canada) using a sterile hockey stick and the agars were incubated at 35 °C for 24 h. Yellow colonies on TCBS agar were counted and expressed in colony forming unit (CFU) per millilitre of sample. This was calculated using Eq. 1. The bacteria isolates were used for subsequent analysis of bacteria identification.

$$\text{CFU/ml} = \frac{no\ of\ colonies\ x\ dilution\ factor}{volume\ of\ culture\ plate} \quad \text{Eq. 1}$$

Identification of Bacteria via Gram Staining

The bacteria isolates were identified by morphological test via Gram staining. Gram staining procedure was conducted according to the Gram staining protocol (Smith & Hussey, 2005).

The heated-fixed bacteria smear was subjected to crystal violet, iodine solution, alcohol or decolorizing agent and safranin respectively. The slide then was observed under light microscope at both 400× and 1000× with oil immersion.

DNA Extraction and Polymerase Chain Reaction

The boil cell method was conducted based on Bilung *et al*. (2005) with some modifications. Afterwards, the extracted DNA was used in the subsequent PCR assay for detecting *V. cholerae* by targeting the *ctx*A gene. This procedure was in accordance to the method by Nandi *et al*. (2000) with some modifications. The PCR assay was conducted in 25 µl final volume, containing 5.0 µl of 5× Green Go*Taq* reaction buffer (Promega, USA), 0.3 µl of 5 U *Taq* DNA polymerase, 2.0 µl of 25 mM magnesium chloride, 1.0 µl of 10 mM deoxynucleotide triphosphates, 1.0 µl of each 10 pmol/µl *ctxA* primer, 0.3 µl of 5 U/µl Go*Taq* DNA polymerase (Promega, USA), 5.0 µl DNA template and 9.7 µl distilled water. Details of the primers used is shown in Table 1.

The amplification reaction was run in a DNA thermal cycler (Perkin Elmer, USA) with a cycle of initial denaturation at 94 °C for 3 min, 35 cycles each of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 2 min, and a cycle of final extension at 72 °C for 5 min. DNA of *V. cholerae* was used as positive control, whilst distilled water was used as negative control.

Agarose Gel Electrophoresis

The PCR products were resolved by electrophoresis in 1.5% agarose gel. A 1 kb molecular weight DNA ladder was used in each reaction. The electrophoresis was run in 1× TBE buffer with a constant voltage of 90 V for 1 h. The gel was stained with ethidium bromide for 20 min and visualised using transmitted ultraviolet illumination (Vilber Lourmat).

Table 1. Details of *ctxA* gene for detection of *V. cholerae*.

Target gene	Primer	Sequences (5'- 3')	Amplicon size (bp)	Sources
*ctx*A	Forward: *ctx*A-*F*	CTCAGACGGGATTTGTTAGGCACG	302	Nandi *et al*. (2000)
	Reverse: *ctx*A-*R*	TCTATCTCTGTAGCCCCTATTACG		

RESULTS

Bacterial Enumeration and PCR assay

Enumeration of yellow colonies growing on the TCBS agar revealed that the number of bacteria in the phytoplankton samples from Samariang Estuary (1.0×10^7 - 8.0×10^7 CFU/ml) was higher than from Santubong Estuary (1.5×10^7 – 4.0×10^7 CFU/ml) as shown in Table 2. Whilst, PCR analysis targeting the virulence cholera enterotoxin subunit A (*ctx*A) gene found that no samples of phytoplankton from both localities were positive with pathogenic strains of *V. cholerae* in phytoplankton samples as displayed in Table 2, Figures 2 and 3.

Table 2. Results of the spread plating and PCR assay for the phytoplankton samples

Santubong Estuary

Sampling date	Sample	Colony forming unit (CFU/ml) of yellow colonies	PCR assay
16/11/2017	A1	1.2×10^7	Negative
30/11/2017	A2	2.8×10^7	Negative
14/12/2017	A3	3.4×10^7	Negative
28/12/2017	A4	1.5×10^7	Negative
24/01/2018	A5	4.0×10^7	Negative

Samariang Estuary

Sampling date	Sample	Colony forming unit (CFU/ml) of yellow colonies	PCR assay
16/11/2017	B1	1.4×10^7	Negative
30/11/2017	B2	1.0×10^7	Negative
14/12/2017	B3	8.0×10^7	Negative
28/12/2017	B4	2.6×10^7	Negative
24/01/2018	B5	6.0×10^7	Negative

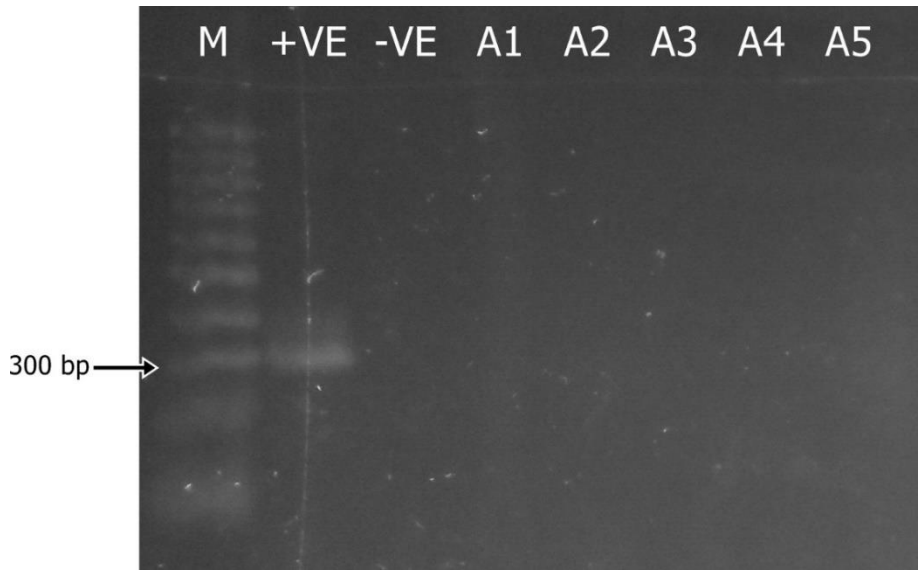

Figure 2. Agarose gel electrophoresis of the *ctx*A gene of *V. cholerae* (302bp). Lane M: Molecular weight DNA ladder (100 bp); Lane +VE: *V. cholerae* strain O1 positive control; Lane -VE: Negative control; Lane A1 – A5: PCR amplification for samples from Santubong Estuary.

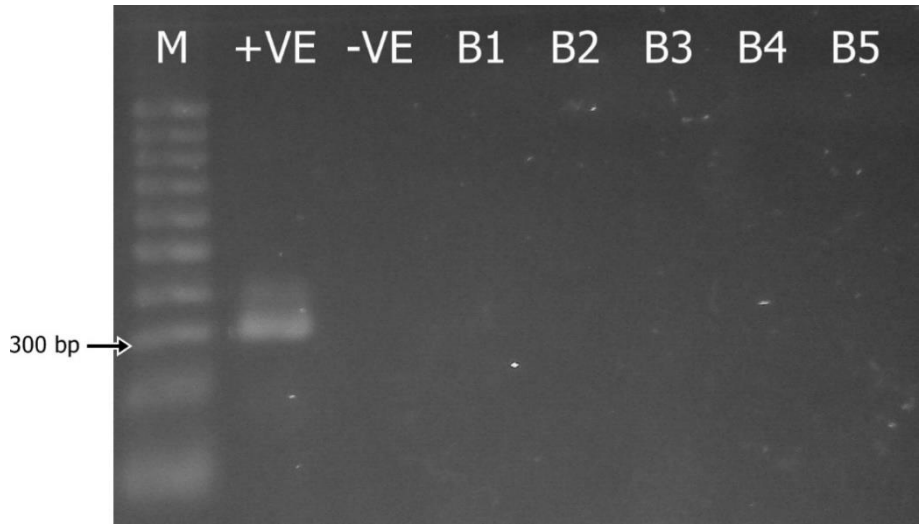

Figure 3. Agarose gel electrophoresis of the *ctx*A gene of *V. cholerae* (302bp). Lane M: Molecular weight DNA ladder (100 bp); Lane +VE: *V. cholerae* strain O1 positive control; Lane -VE: Negative control; Lane B1 – B5: PCR amplification for samples from Samariang Estuary.

Water Physicochemical Parameters

The physical parameters such as temperature, salinity and pH were used to study the association between physical parameters and the presence of *V. cholerae*. The results of water physicochemical parameters of the sampling sites recorded during the five samplings at Santubong and Samariang Estuaries are shown in Table 3. At Santubong Estuary, temperature of the surface water ranged from 29.2 - 36.0 ºC, salinity ranged from 1.3 - 3.0 % and pH ranged from 6.3 - 8.1, whereas, at Samariang Estuary the physicochemical parameters of surface water samples were temperature that ranged from 27.8 - 34.5 ºC, salinity ranged from 1.3 - 2.3 % and pH ranged from 5.9 - 7.6.

Table 3. Water physicochemical parameters of the phytoplankton samples

Santubong Estuary				
Date of sampling	Sample	Temperature (ºC)	Salinity (%)	pH
16/11/2017	A1	34.3	2.5	8.1
30/11/2017	A2	31.8	3.0	7.6
14/12/2017	A3	33.2	1.3	6.3
28/12/2017	A4	29.2	2.7	7.3
24/01/2018	A5	36.0	2.2	7.1
Samariang Estuary				
Date of sampling	Sample	Temperature (ºC)	Salinity (%)	pH
16/11/2017	B1	30.6	1.9	7.3
30/11/2017	B2	29.8	2.3	6.9
14/12/2017	B3	29.5	2.2	5.9
28/12/2017	B4	27.8	2.2	6.8
24/01/2018	B5	34.5	1.3	7.6

DISCUSSION

Bacterial Enumeration and Detection of *V. cholerae* Targeting the Virulence Cholera Enterotoxin Subunit A (*ctxA*) Gene

The detection of virulence cholera enterotoxin subunit *A* (*ctxA*) gene, that is only conserved in toxigenic *V. cholerae* (i.e. cholera-toxin producing), by utilising PCR assay is useful to differentiate toxigenic and non-toxigenic *V. cholerae* strains. In this study, no samples from Santubong and Samariang Estuaries were contaminated with cholera toxin-producing *V. cholerae*. The discrepancy of colonies growing on TCBS agar and the finding from PCR assay might indicate the presence of non-toxigenic *V. cholerae* (Lipp *et al*., 2003) or other non-cholera *Vibrio* species (e.g. *V. alginolyticus, V. fluvialis, V. furnissii*) (USFDA, 2004) which may still cause infections in humans.

Based on previous studies in Sarawak such as by Norazah *et al*. (2001) found *V. cholerae* O1 in eight out of 80 samples (10%) upon an outbreak in Daro and Bintulu. Afterwards, Radu *et al*. (2002) reported 33 isolates of *V. cholerae* O1 were linked to an outbreak in Miri. In an earlier study by Benjamin *et al*. (2005), 1,672 people in Sarawak were infected with *V. cholerae* from 1994 – 2003. In comparison, this study did not obtain any CT-producing isolates.

In retrospect of India subcontinent being cholera endemic, the negative finding of CT-producing *V. cholerae* in this study showed that the water at both estuaries did not receive (or with low level) of human waste contamination (Jiang *et al.,* 2003). The absence of this event (i.e. human waste contamination) may explain the low circulation of the virulence gene among environmental strains. Besides, it could also be low predation of CTX φ in the environmental strains that may cause emergence of new toxigenic *V. cholerae* by horizontal gene transfer (Faruque & Mekalanos, 2012).

Association Between Selected Physicochemical Parameters (pH, Temperature and Salinity) and the Occurrence of *V. Cholerae* in Sampled Phytoplankton

In this study, the physicochemical parameters recorded in all surface water samples of both Santubong and Samariang Estuaries indicated favourable condition for the distribution of *V. cholerae* in the environment. In natural habitat, *V. cholerae* prefers salinity from the range of 2 to 20% and temperature between 20 and 35 ºC (Janda *et al*., 1988). There was no association of pH on the presence of *V. cholerae* in the environment. Nevertheless, the presence of plankton in the surface water may provide vital support for population of *V. cholerae* especially during unfavourable conditions such as low salinity which may lead to their existence in the "free living" state (Neogi *et al*., 2012). These association is also supported by Lutz *et al*. (2013) mentioning that the presence of *Vibrio* spp. in the environment is associated with the salinity, temperature, as well as zooplankton and phytoplankton.

Based on Table 3, the average physicochemical parameters of surface water samples from Santubong Estuary were more ideal for the presence of *V. cholerae* compared to Samariang Estuary. However, the number of variable yellow colonies appeared on the TCBS agar was lower in Santubong Estuary compared to Samariang Estuary. This could be due to the habitat of the surrounding Samariang Estuary that is surrounded with mangrove forests. Mangroves are environments naturally rich in chitin and the degradation of chitinous and mucilaginous parts of plankton by certain enzymes provides nutrients for survival of *V. cholerae* (Neogi *et al.,* 2012).

CONCLUSION

This is the first study investigating the presence of cholera-producing *Vibrio cholerae* in phytoplankton in Sarawak. The absence of the toxigenic *V. cholerae* in all samples somehow still highlights the presence of other non-CT-producing *Vibrio cholerae* strains and non-*cholerae Vibrio* species that is still indisputable, that this study did not perform on such detections. As Santubong and Samariang Estuaries are local tourism places, the risks should not be underestimated. Further researches on the presence *Vibrio* spp. in phytoplankton need to be conducted and evaluated frequently according to the monsoon season in Malaysia.

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