



Faculty of Resource Science and Technology

The Association of *Vibrio* species and Cyanobacteria in Selected Local Shrimp Farm

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The Association of *Vibrio* species and Cyanobacteria in Selected Local
Shrimp Farm

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.


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ABSTRACT

The discovery of cyanobacteria supporting the persistence of *Vibrio* species in the environment have bridged the gap for the discovery of *Vibrio* related association, however, the existing evidence are mixed. Some studies showed that cyanobacteria may be the side-kick to *Vibrio* species in marine environments, while others have found little or no association between the two groups. In this research, the association between *Vibrio* species and cyanobacteria in a selected local shrimp farm in Sarawak was examined. Over the course of 5 months starting from August 2021 to December 2021 with 2 weeks intervals between each sampling starting from the stocking of shrimp larvae up to the harvesting time, thirty-two ($n=32$) samples were collected from two shrimp ponds labelled as Pond A and Pond B, the effluent, and influent water of the shrimp farm from Persatuan Nelayan Kawasan Satang Biru, Telaga Air. *Vibrio* species and cyanobacteria population density were observed, and canonical Correspondence Analysis (CCA) was used to evaluate their correlation. Molecular method called duplex-Polymerase Chain Reaction (duplex-PCR) was used to identify *Vibrio parahaemolyticus* and *Vibrio cholerae* at molecular levels. An inverted microscope with a DIC filter that can produce pseudo-3D images was used for the morphological identification of cyanobacteria. As a result, *V. parahaemolyticus* showed 78%, 100%, 90%, and 90% prevalence while *V. cholerae* showed 72%, 61%, 90% and 70% prevalence from Pond A, Pond B, effluent, and influent, respectively. The morphological identification of cyanobacterial species revealed the presence of *Pseudanabaena*, *Chroococcus*, *Phormidium*, *Oscillatoria* and *Lyngbya* genus in our local shrimp farm. Results from CCA showed an insignificant correlation between *Vibrio* species and cyanobacteria with p -values of 0.847, 0.255, 0.288, and 0.304 for Pond A, Pond B, effluent, and influent water, respectively. This lack of association suggests that other factors such as species-specific association, regional

factors, and other multidimensional landscapes may play a role in the persistence of *Vibrio* species in shrimp farms. Additionally, this research also examined the virulence genes and antibiotic susceptibility of *V. cholerae* and *V. parahaemolyticus*. Ten ($n=10$) *V. cholerae* and ten ($n=10$) *V. parahaemolyticus* isolates were selected from the same study. The primers used were *tdh*, *trh* for *V. parahaemolyticus* isolates and *ctxB*, *rtxA* and *rtxC* for *V. cholerae* isolates. 70% of *rtxA* and 90% of *rtxC* virulence genes were present in *V. cholerae* isolates while *tdh* and *trh* were absent in *V. parahaemolyticus* isolates. Antibiotic susceptibility testing showed that all *V. cholerae* and *V. parahaemolyticus* isolates were resistant to at least one antibiotic with the mean MAR indices of 0.34 for *V. cholerae* and 0.24 for *V. parahaemolyticus*. The MAR index of 0.20 and greater indicates that antibiotics are heavily contaminating the shrimp farm water. *V. cholerae* isolates showed resistance towards 25 µg sulfamethoxazole (100%), 30 µg cephalothin (80%), 30 µg neomycin (70%), 10 µg streptomycin (70%) and 30 µg ceftriaxone (60%). On the other hand, *V. parahaemolyticus* showed resistance towards is 30 µg cephalothin (100%), 10 µg streptomycin (100%), 25 µg sulfamethoxazole (100%) and 300 µg compound sulphonamides (80%). With no evidence of resistance from *V. cholerae* isolates, 10 µg imipenem, 30 µg tetracycline, 10 µg norfloxacin appeared to be the most potent antibiotics. All of *V. parahaemolyticus* isolates were susceptible with 30 µg ceftazidime, 30 µg ceftriaxone, 30 µg amikacin, 30 µg neomycin, 30 µg nalidixic acid, 5 µg ciprofloxacin, 25 µg sulfamethoxazole-trimethoprim and 30 µg chloramphenicol. For the treatment against *V. cholerae* and *V. parahaemolyticus*, 5 µg ciprofloxacin was found to be the best with the exception of banned antibiotics in food animals. This study contributes to the better understanding of *Vibrio* species' distribution level in shrimp farm, and the possibility of them in causing diseases. The need for proper antibiotic administration and water treatment in shrimp farming environments is

recommended to reduce the risk of antibiotic-resistant infections caused by *V. cholerae* and *V. parahaemolyticus* and minimize negative impact on the environment.

Keywords: Antibiotics, cyanobacteria, shrimp farm, *Vibrio cholerae*, *Vibrio parahaemolyticus*

Hubungan antara Spesies Vibrio dan Cyanobacteria di Ladang Udang Tempatan

ABSTRAK

Penemuan cyanobacteria dalam menyokong pembiakan spesies Vibrio terhadap alam sekitar telah ditemui, namun begitu, bukti yang sedia ada didapati pelbagai. Sesetengah kajian menunjukkan spesies Vibrio boleh ditemui bersama dengan cyanobacteria dalam habitat marin, manakala dapatan lain menemui sedikit atau tiada kaitan antara kedua-dua bakteria. Dalam penyelidikan ini, perkaitan antara spesies Vibrio dan cyanobacteria di dua kolam udang tempatan di Sarawak telah dikaji. Sepanjang tempoh 5 bulan bermula daripada bulan Ogos 2021 sehingga Disember 2021 dengan selang 2 minggu antara setiap persampelan bermula dari penyimpanan larva udang sehingga masa penuaian, tiga puluh dua (n=32) sampel telahpun dikumpul. Sampel telah dilabel sebagai Kolam A dan Kolam B, efluen, dan influen daripada Persatuan Nelayan Kawasan Satang Biru, Telaga Air. Analisis Koresponden Kanonikal (AKK) telah digunakan untuk menilai perkaitan antara spesies Vibrio dan cyanobacteria. Kaedah molekular yang dipanggil tindak balas rantai polimerase dupleks (dupleks-PCR) telah digunakan untuk mengenal pasti spesies Vibrio parahaemolyticus dan Vibrio cholerae dalam kolam-kolam tersebut. Pengenalpastian morfologi cyanobacteria pula dikaji menggunakan mikroskop dengan filter DIC untuk menghasilkan imej pseudo-3D. Hasilnya, V. parahaemolyticus menunjukkan kelaziman 78%, 100%, 90%, dan 90% manakala V. cholerae menunjukkan kelaziman 72%, 61%, 90% dan 70% daripada Kolam A, Kolam B, efluen, dan influen masing-masing. Pengenalpastian morfologi spesies cyanobacteria telah membuktikan kewujudan genus Pseudanabaena, Chroococcus, Geitlerinema, Phormidium, Oscillatoria dan Lyngbya dalam kolam-kolam tersebut. AKK menunjukkan korelasi yang tidak signifikan antara spesies Vibrio dan cyanobacteria dengan nilai p ialah 0.847, 0.255, 0.288, dan 0.304 untuk Kolam A, Kolam

*B, efluen, dan air influen masing-masing. Kekurangan perkaitan ini menunjukkan bahawa faktor lain seperti perkaitan khusus spesies, faktor serantau dan landskap multidimensi lain mungkin memainkan peranan dalam pertumbuhan spesies Vibrio dalam kolam udang. Selain itu, penyelidikan ini juga mengkaji gen virulensi dan ketahanan terhadap antibiotik daripada V. cholerae dan V. parahaemolyticus. Sepuluh koloni (n=10) V. cholerae dan sepuluh (n=10) koloni V. parahaemolyticus telah dipilih daripada kajian yang sama. Primer yang digunakan ialah *tdh*, *trh* untuk koloni V. parahaemolyticus dan *ctxB*, *rtxA* dan *rtxC* untuk koloni V. cholerae. 70% daripada *rtxA* dan 90% daripada gen virulens *rtxC* telah ditemui dalam koloni V. cholerae manakala *tdh* dan *trh* tidak ditemui dalam koloni V. parahaemolyticus. Ujian ketahanan terhadap antibiotik menunjukkan bahawa semua koloni V. cholerae dan V. parahaemolyticus tahan terhadap sekurang-kurangnya satu antibiotik dengan purata indeks MAR 0.34 untuk V. cholerae dan 0.24 untuk V. parahaemolyticus. Indeks MAR 0.20 yang lebih tinggi menunjukkan bahawa antibiotik banyak mencemari air kolam udang. Koloni V. cholerae tidak menunjukkan sebarang ketahanan terhadap 10 µg imipenem, 30 µg tetracycline dan 10 µg norfloxacin. Bagi koloni V. parahaemolyticus pula, ianya tidak menunjukkan sebarang ketahanan terhadap 30 µg ceftazidime, 30 µg ceftriaxone, 30 µg amikacin, 30 µg neomycin, 30 µg nalidixic acid, 5 µg ciprofloxacin, 25 µg sulfamethoxazole-trimethoprim dan 30 µg chloramphenicol. Bagi rawatan Vibriosis di kolam udang, 5 µg ciprofloxacin didapati terbaik (dengan pengecualian terhadap antibiotik yang terlarang dalam haiwan makanan). Kajian ini menyumbang kepada pemahaman yang lebih baik tentang tahap taburan spesies Vibrio pada kolam udang, dan kemungkinan ianya penyebab penyakit. Penggunaan antibiotik yang betul dan rawatan air dalam kolam udang disyorkan bagi mengurangkan risiko jangkitan ketahanan terhadap antibiotik yang*

disebabkan oleh V. cholerae dan V. parahaemolyticus di samping dapat mengurangi kesan negatif terhadap alam sekitar.

Kata kunci: *Antibiotik, cyanobacteria, ladang udang, Vibrio cholerae, Vibrio parahaemolyticus*

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LIST OF ABBREVIATIONS

°C	Degree Celcius
µL	Microlitres
AGE	Agarose Gel Electrophoresis
AHPND	Acute Hepatopancreatic Necrosis
APW	Alkaline Peptone Water
CCA	Canonical Correspondence Analysis
CTX	Cholera Toxin
DIC	Differential Interference Contrast
EMS	Early Mortality Syndrome
mL	Millilitre
mm	Millimetre
PAST	PAleontological STatistics
PCR	Canonical Correspondence Analysis
PPT	Parts per Thousand
TCBS	Thiosulfate-Citrate-Bile-Sucrose Agar
TDH	Thermostable Direct Hemolysin
TLH	Thermolabile Hemolysin
TRH	Thermostable Direct Hemolysin-Related Hemolysin
rpm	Rotation per Minute
RTX	Repeat in Toxin
UNIMAS	Universiti Malaysia Sarawak
UV	Ultra Violet

CHAPTER 1

INTRODUCTION

1.1 Study Background

Shrimp is one of the most popular seafood in the world's animal protein production alongside salmon and tilapia in aquaculture (Nunes et al., 2022). Due to the high demand for shrimp, governments in developing countries such as China, Thailand, Indonesia, India, Vietnam, Ecuador and Bangladesh have widely encouraged shrimp aquaculture to minimize poverty (World Wildlife Fund, 2022). In Malaysia, the data from the Fisheries Development of Authority Malaysia (LKIM), (2020) reported that shrimp export volumes in 2017 and 2018 totalled 27,674.10 and 33,667.54 metric tonnes, respectively, contributing RM 833,283,764 in profit in 2017 and RM 1,001,325,595 in 2018. Despite the high demand for shrimp and the significant profit generated from shrimp farming, the susceptibility of shrimp towards diseases, specifically vibriosis, remains a major concern in the industry. Often reared in a packed environment with water parameters fluctuations, relying of innate immune system and lack in species diversity in reared shrimps can make shrimps highly susceptible towards diseases.

Vibriosis is one of the common diseases that were caused by *Vibrio* species and belongs to the *Vibrionaceae* family. They are frequently found in warm seawater bodies. *Vibrio* species can infect shrimp at any stage of development and cause almost 100% death when an outbreak happens (Aguirre-Guzman et al., 2004; Harris & Owens, 1999; Prayitno & Latchford, 1995). Shrimp diseases that can be caused by *Vibrio* species in shrimp farms are White Spot Syndrome Virus (WSSV), Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis (AHPND), White Faeces Disease (WFD), loose shell syndrome,

white gut disease, red disease and luminous disease (de la Peña et al., 2015; Jayasree et al., 2006; Lavilla-Pitogo et al., 2000). Common *Vibrio* species that can be found in diseased shrimps are *Vibrio harveyi*, *V. parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio vulnificus* and *Vibrio splendidus* (Chatterjee & Haldar, 2012).

Vibrio species often coexist with cyanobacteria due to their similarity in their living habitat and nutritional needs. According to research conducted by Islam et al., (2004), cyanobacteria can act as a long-term reservoir for *V. cholerae* O139 for up to 15 months due to the cyanobacteria's ability to encase *V. cholerae* cells within mucilaginous sheaths, causing *V. cholerae* to enter a viable but non-culturable state (VBNC). Another research by Ward (2014) that studied the association between cyanobacteria and *V. parahaemolyticus* found out that the strain G445 was able to last long with the presence of cyanobacteria because the cells seemed to cluster randomly within the cyanobacterial mats. Eiler et al. (2007) also stated that cyanobacterial organic matters play an essential role in the growth and abundance of *V. cholerae* and *V. vulnificus*, strengthening the possibility of the interaction between these two bacterial species.

While many studies have found an association between *Vibrio* and cyanobacteria, there are also research that contradicts this, pointing to limited or no correlation between the two. For instance, the association is thought to differ according to regional factors, seasonal temperature and species involved (Greenfield et al., 2017; Paranjpye et al., 2015; Turner et al., 2009). Greenfield et al., (2017) reported that the presence of *Vibrio* species (*V. vulnificus* and *V. parahaemolyticus*) is dependent on seasonal temperature when they observed very low to no correlation between *Vibrio* species numbers with phytoplankton during winter. Chlorophyll a, essential for photosynthesis in cyanobacteria, also lacks correlation with *Vibrio* populations (Greenfield et al., 2017). Aside from seasonality, Turner et al., (2009)

also found out that species-specific taxa play a role in the determination of *Vibrio* species' population density and their associations with cyanobacteria. The study observed the antagonistic relationship between cyanobacteria, copepods and diatoms (Turner et al., 2009). Paranjpye et al., (2015) gathered no evidence of the association between the two bacteria groups with low density of cyanobacteria in Pacific Northwest in the USA in 2015 as opposed to the majorly stagnant *Vibrio* population density. Overall, the evidence on the association between cyanobacteria and *Vibrio* species is limited and inconclusive and involves complex multidimensional landscape to understand the relationship between these two groups.

The ability of a *Vibrio* species to cause diseases is determined by the presence of virulent genes in their genome. Several virulent genes that were known to be associated with *Vibrio* species are *tdh*, *trh*, *tlh*, *ctxB*, *rtxA* and *rtxC*. Thermostable direct haemolysin (*tdh*) and thermostable related haemolysin (*trh*) are majorly related to the toxicity of *V. parahaemolyticus* (Raghunath, 2015). The expression of *tdh* causes the formation of pore in red blood cell (Matsuda et al., 2010). *trh* genes are 70% similar to that of *tdh* and the expression results in abnormal secretion of chloride ion in human colon (Takahashi et al., 2000). *rtxA* and *rtxC* genes expression induces cell death in human intestine (Lee et al., 2008) while cholera toxin subunit B (*ctxB*) is the gene responsible for the watery diarrhoea symptom causing dehydration and severe electrolyte imbalance in human (Satitsri et al., 2016). *ctxB* was also detected in shrimp and is hazardous towards public health when consumed (Madhusudana & Surendran, 2013). Due to the high mortality rate of these illnesses, it is crucial to monitor them by looking for any possible virulence genes that the *Vibrio* species may have.

In shrimp farms, illnesses brought on by *Vibrio* species are typically treated with antibiotics. They were added in shrimp feed, or in the rearing pond water. However, due to the improper administration of antibiotics, the cases of antibiotic resistant bacteria have emerged (Fletcher, 2015; Holmström et al., 2003). Over the past few decades, the resistance towards antibiotics has developed and spread throughout many bacterial species as a result of the overuse of antibiotics in agricultural and aquaculture systems (Cabello, 2006). Antibiotic resistance can cause longer treatment duration, increase healthcare costs that can become a burden for families, society and the economy (Ventola, 2015). It is estimated that 10 million people will die from antibiotic resistance globally with the loss of £60 trillion by 2050 at the current rate of antibiotic resistance spread, making it more lethal than cancer (O’neill, 2016). When infection can no longer be treated by first-line antibiotic, more expensive and toxic medication will come in play (Ventola, 2015).

This research is vital to understand the biotic factor that supports the growth population of *Vibrio* and to facilitate better understanding on the species of *Vibrio* that exist in local shrimp farm. It is hypothesized that the association between *Vibrio* species and cyanobacteria in shrimp farms, is highly dependent with the environmental factors such as water temperature, salinity and pH. Additionally, specific virulent genes associated with *Vibrio* species, such as *tdh*, *trh*, *ctxB*, *rtxA*, and *rtxC*, are expected to be present in the predominant *Vibrio* isolates in shrimp farms. Furthermore, the study hypothesizes that the widespread administration of antibiotics in shrimp farms contributes to the emergence of antibiotic-resistant *Vibrio* species. Methods such as Most Probable Number (MPN), Sedgwick-Rafter cell counting, Canonical Correspondence Analysis (CCA), Duplex-Polymerase Chain Reaction (D-PCR) were implemented in this study to achieve the objectives of quantifying and enumerating *Vibrio* species and cyanobacteria, determining