Molecular Detection and Characterization of *Vibrio cholerae* from Diseased Asian Seabass Using Multi-Gene Analysis

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Molecular Detection and Characterization of *Vibrio cholerae* from Diseased Asian Seabass Using Multi-Gene Analysis

By

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ABSTRACT

Vibrio cholerae is a gram-negative bacillus with a flagellum that can cause diarrheal diseases, cholera. *Vibrio cholerae* happens to be native aquatic environment habitat that may not cause infectious diseases in fish and aquatic animals but towards human. Thus, it is important to detect and characterize the *Vibrio cholerae* using multigene analysis. Two isolates of *Vibrio cholerae* were revived from the glycerol stock on TSA, CHROMagar™ and TCBS agar. Polymerase Chain Reaction was done using 11 primers which were *pyr*H, *glp*, *gyr*B, *mdh*, *met*G, *pur*M, *dtd*S, *lys*A, *pnt*A, *pyr*C and *tna*A. The sequence information was compared with Genbank using Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). As a result, *pyr*H, *gyr*B, *met*G, *pur*M, *dtd*S, *pnt*A, and *tna*A were detected from both *V. cholerae* isolates. By analyzing these genes, the molecular characterization of *V. cholerae* isolates became more robust, revealing genetic diversity and potential evolutionary adaptations among strains.

Keywords: *Vibrio cholerae; Polymerase Chain Reaction; multi-gene analysis, housekeeping genes*

Pengesanan Molekular dan Pencirian *Vibrio cholerae* yang Diasingkan dari Ikan Siakap Berpenyakit Menggunakan Analisis Pelbagai Gen

ABSTRAK

Vibrio cholerae adalah bakteria gram-negatif berbentuk bacillus dengan flagelum yang boleh menyebabkan penyakit cirit-birit, iaitu kolera. *Vibrio cholerae* merupakan penghuni semula jadi dalam persekitaran akuatik yang mungkin tidak akan menyebabkan penyakit berjangkit pada ikan dan haiwan akuatik tetapi berpotensi menjangkiti manusia. Oleh itu, adalah penting untuk mengesan dan mencirikan *Vibrio cholerae* menggunakan analisis pelbagai gen. Dua isolat *Vibrio cholerae* dihidupkan semula daripada stok gliserol pada media TSA, CHROMagar™ dan agar TCBS. Tindak balas rantai polimerase (PCR) dilakukan menggunakan 11 primer, iaitu *pyr*H, *glp*, *gyr*B, *mdh*, *met*G, *pur*M, *dtd*S, *lys*A, *pnt*A, *pyr*C dan *tna*A. Maklumat jujukan dibandingkan dengan pangkalan data Genbank menggunakan Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Hasilnya, *pyr*H, *gyr*B, *met*G, *pur*M, *dtd*S, *pnt*A, dan *tna*A dapat dikesan pada kedua-dua isolat *V. cholerae*. Dengan menganalisis gen-gen ini, pencirian molekul bagi isolat *V. cholerae* menjadi lebih kukuh dengan mendedahkan kepelbagaian genetik dan kemungkinan adaptasi evolusi dalam kalangan strain.

Kata Kunci: *Vibrio cholerae; tindak balas rantai polimerase; analisis pelbagai gen, gen penyelenggaraan*

CERTIFICATION

This is to certify that I have read this research paper entitled 'Molecular Detection and Characterization of *Vibrio cholerae* from Diseased Asian Seabass Using Multi-Gene Analysis' by Nurulnajiha binti Ahmad Jame. In my opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course DVT55204 - Research Project.

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Lab assistants of FPV UMK

DVM5 Class of 2020/2025

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Finally, I would like to dedicate this thesis to the memory of my father, Ahmad Jame bin Yusof, who now rests in eternal peace. May this work be a testament to the values you instilled in me and the faith you had in my dreams.

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LIST OF ABBREVIATIONS

TSA : Tryptic soy agar

TCBS : Thiosulphate citrate bile salts sucrose

UMK : Universiti Malaysia Kelantan

NaCl : Sodium chloride

MLST : Multilocus sequence typing

Rpm : Revolutions per minute

Bp : Base pair

PCR : Polymerase chain reaction

BLAST : Basic Local Alignment Search Tool

CHAPTER 1

INTRODUCTION

Over the past years, world consumption of aquatic animal foods has increased significantly, at a higher annual growth rate than the world population. Between 1961 and 2021, the consumption of aquatic animal foods increased at an average annual rate of 3.0 percent compared with a world population annual growth rate of 1.6 percent for the same period ("Blue Transformation - Roadmap 2022–2030," 2022). However, infections among aquatic animals are one of the concerns that cause a potential threat to public health such as vibriosis. In public health, vibriosis is classified as an essential zoonotic disease (Helmi et al., 2020).

In a majority part of the world, *Vibrio cholerae*, responsible for the widespread diarrheal illness cholera, happens to be a native aquatic environment habitant (Halpern & Izhaki, 2017). Because *Vibrio* species are very common in the environment, they are required to be able to detect and react to a variety of adverse situations by either upregulating or downregulating the expression of particular genes (Lutz et al., 2013). *Vibrio cholerae* was known to be a successful marine organism and human pathogen because of its exceptional capacity to adapt to and to flourish in different environment settings. However, *V. cholerae* may also create disease epidemics that aren't necessarily connected to predictable water sources. Thus, to reduce human exposure towards these common aquatic bacteria, it is crucial that we learn a better understanding of the diverse environments in which *V. cholerae* can thrive globally (Daboul et al., 2020).

Vibrio cholerae strains are divided into more than 200 different serogroups based on O antigen structure. Previously, the O1 serogroup was known to be the main cause of epidemic cholerae, and in 1992 another epidemic serogroup, O139, emerged (Faruque et al., 2003). The other *V. cholerae* strains are generally referred to as "non-O1" and "non-O139" strains as they do not cause cholera epidemics. These "non-O1" and "non-O139" strains are also known as "non-toxigenic". However, the non-toxigenic strains do encode other virulence genes that can cause different kinds of disease in humans which symptoms can vary from gastroenteritis to extraintestinal infections (Dalsgaard et al., 1995; Schermeister et al., 2013; Trubiano et al., 2014).

Vibrio cholerae is commonly found in estuarine or coastal environments, usually the most toxigenic strains are found residing in subtropical climates such as Southeast Asia. *Vibrio cholerae* is one of the only *Vibrio* species with the ability to tolerate the absence of NaCl. *Vibrio cholerae* is rarely isolated in Malaysia, however, vibriosis remains a major threat to human health in many developing countries (Hassan et al., 2023). In the United States, *V. cholerae* can be isolated from coastal water environments, such as the Chesapeake Bay (Jiang et al., 2000). Frequently, these North American strains are non-toxigenic, but studies have proven that there is a high degree of genetic diversity in the isolates, and they contain a variation assortment of virulence genes that required attention (Dalsgaard et al., 1995). Therefore, this study was conducted to detect and characterize the *V. cholerae* isolated from diseased seabass around east coast Malaysia using multi-gene analysis.

1.1 Research Problem Statement

Bacteria related to public health is one of the world's main concerns nowadays. As in aquatic animals, they can also become infected with diseases such as bacterial infection. One of the most common foodborne diseases caused by aquatic life is vibriosis in which the etiological agents can be variation from the Vibrio sp., such as *Vibrio cholerae*. Thus, it is important to determine and characterize the Vibrio species that are present in diseased Asian seabass. In this study, we will conduct Molecular Detection and Characterization of *Vibrio cholerae* from diseased Asian Seabass using multi-gene analysis to determine and characterize *V. cholerae*.

1.2 Research Question

- Can multi-gene analysis help in detection and characterization of *Vibrio cholerae* isolated from diseased seabass?

1.3 Research Hypothesis

- Multi-gene analysis is useful in detection and characterization of *Vibrio cholerae*, isolated from diseased seabass.

1.4 Research Objectives

- To detect and characterize *Vibrio cholerae*, isolated from diseased seabass using multi-gene analysis.

1.5 Significance Of The Study

The rise in Aquaculture food demands has brought an interest to study possible etiology agents that can cause disease transmission in humans from the consumptions of the aquatic animals. It is commonly known that *Vibrio* sp. is present in almost all aquatic environments and organisms. However, it is important to distinguish the vibrio sp. especially *Vibrio cholerae* which have the ability to cause cholera outbreaks. By successfully detecting and characterizing the *V. cholerae* isolated from diseased Asian Seabass, further studies can be done to prevent and educate consumers in relation to *Vibrio cholerae* agents.

1.6 Scope of Study

The study was conducted on the East Coast, Malaysia. The samples were collected from Kuala Pahang, Pahang.

CHAPTER 2

LITERATURE REVIEW

2.1 Vibrio cholerae in aquatic animals and humans

Vibrio cholerae is a gram-negative bacillus with a flagellum. This organism is a non-pathogenic agent for fish. Unfortunately for humans, it can cause severe cholera (Farzadnia & Naeemipour, 2019). *Vibrio cholerae* distribution in the environment depends on temperature, salinity and nutrient concentration. This bacterium is a halotolerant microorganism mostly isolated from environmental sites where NaCl concentrations are between 0.2% and 3.0% and the optimal temperature for growth ranges from 30°C to 40°C while the optimal pH is 8 (Kaper et al., 1995) Based on Daboul et al. (2020), *V. cholerae* have more than 200 different serogroups based on O antigen. In aquatic habitat, the bacteria frequently harbour non-O1/non-O139 strains. Consuming water or raw fish contaminated with *V. cholerae* and marine life (such as oysters) can transmit the disease to humans, and sometimes, the infections can be fatal (Blake et al. 1980).

2.2 Molecular characterization of Vibrio cholerae

Accurate identification and characterization of *V. cholerae* are crucial for epidemiological surveillance, outbreak investigation, and the implementation of effective control strategies. Traditional identification methods based on phenotypic and biochemical characteristics have limitations in terms of sensitivity and specificity, especially for the detection of non-O1/non-O139 serogroups (Yaashikaa et al., 2016). In recent years, molecular techniques, such as multi-gene analysis, have emerged as powerful tools for the reliable identification and characterization of *V. cholerae* isolates.

Several studies have reported the use of multi-gene analysis for the molecular characterization and identification of *V. cholerae*. These studies have focused on the analysis of various genes, including virulence-related genes, housekeeping genes, and genes involved in toxin production and serogroup determination.

Multilocus sequence typing (MLST) is a powerful molecular tool used to accurately characterize bacterial species, including *Vibrio* species (Urwin et al., 2003). MLST is important for studying Vibrio because multiple housekeeping genes are analyzed to offer high-resolution data on genetic variants. It is essential for epidemiological research and comprehending the genetic diversity within the species that closely related strains of Vibrio can be distinguished from one another (Huan Li et al., 2017). On the other hand, researchers can follow the evolution of Vibrio strains over time or in different geographic regions by comparing their MLST characteristics. Monitoring disease outbreaks, such as cholera, which is caused by *Vibrio cholerae*, and locating the origins of infection for other harmful *Vibrio* species, such as *V. parahaemolyticus* and *V. vulnificus*, are particularly dependent on this (Teh et al., 2011).

Vibrio species population dynamics and evolutionary links can be investigated using MLST data. It aids in comprehending the relationships between various strains and their historical evolution. Having this knowledge is helpful in creating infection control and prevention plans. For comparative studies, Vibrio strains from a variety of habitats, such as marine, estuarine, and human hosts, can be compared thanks to MLST (Li et al., 2017). Through the analysis of genetic distinctions and affinities, scientists can acquire a deeper understanding of the adaptation strategies employed by *Vibrio* species across diverse ecological contexts (Lin et al., 2018).

Due to the great degree of standardization of MLST, data from various laboratories and studies may be readily compared. This standardization makes international surveillance and cooperative research projects easier, which results in more thorough and reliable understandings of *Vibrio* epidemiology (Liang et al., 2020). MLST data is frequently kept in databases that are open to the public, like PubMLST. Researchers can compare their isolations with a huge number of previously defined strains using these databases, which are a valuable resource. This aids in the identification of novel strains and the comprehension of their public health implications (Liang et al., 2020). Researchers also can monitor the emergence and spread of resistant *Vibrio* strains by combining MLST data with information on antibiotic resistance. This is essential for developing antibiotic resistance treatment plans and public health regulations (Lü et al., 2021). Certain genetic profiles can be correlated with features of pathogenicity and virulence using MLST. Understanding the genetic underpinnings of virulence in *Vibrio* species and identifying virulent strains are crucial for the development of vaccines and other preventive strategies (Mohamad, et al., 2021).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Bacterial collection

Two isolates of *Vibrio cholerae* from Kuala Pahang, Pahang were obtained from archived samples stored in the Trypticase Soy Broth (TSB) (Oxoid, England) added with 50% glycerol stocks in -80℃ freezer in Zoonotic Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. The isolates were revived in Tryptic Soy Agar, TSA (Oxoid, England) and incubated at 30℃ for 24 hours (Amel et al., 2006).

3.2 DNA extraction

Boiling method was used in this study with modification (Dashti et al., 2009). A single bacterial colony was suspended in a 1 ml of 0.85% NaCI in a 1.5 ml centrifuge tube. The tube was centrifuged at 12000 rpm for 5 min and the supernatant was discarded. The pellet was resuspended in 500 ul of sterile distilled water and vortexed vigorously. The bacterial suspension was incubated for 15 min at 95°C in a water bath. The bacterial suspension was immediately cooled on ice for 15 min and it was centrifuged at 12000 rpm for 5 min. The clear supernatant was transferred into a new 1.5ml centrifuge tube and was kept at -20℃ for further use. The tube was centrifuged to sediment the debris and the supernatant was collected.

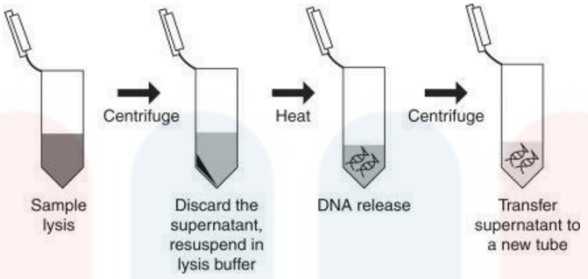

Figure 1: Bacterial DNA extraction using boiling method (Barbosa et al., 2016)

3.3 Polymerase Chain Reaction (PCR)

The primers that were used in this study to detect and characterize *Vibrio cholerae* were selected based on the previously conducted research. 11 primers were used in this study. 1 primer had a different PCR protocol than the rest of the 10 primers. The other 10 primers had the same PCR protocol.

Table 1: The list of primers and PCR protocol for identification of *Vibrio cholerae*

Locus	Name and sequence of primer		Bp	Protocol
	Forward primer	Reverse primer		
pyrH	5'- GAT CGT ATG GCT CAA GAA G-3'	5'-TAG GCA TTT TGT GGT CAC G-3'	440	Denaturation at 95°C for 1 min, primer annealing at 59°C for 2 min, and extension at 72°C for 1 min 15 secs, for 33 cycles (Amalina et al., 2019)
glp	glp-S1, AGT CGA TCC TGA GAC AAC ACT C	glp-S2, GCC TTG GTG GAT	480	Denaturation at 94°C for 1 min, primer

		CAG TTG GTA		annealing at 50°C for 45 sec, and extension at 72°C for 1 min, for 30 cycles (*PubMLST*, n.d.)
*gyr*B	gyrB-S1, ACA TCC TAG CGA AGC GTC TAC G	gyrB-S2, AGA ACA AAC GGT TTT CGC TTC G	459	
mdh	mdh-S1, ACT AGA AGG TGC TGA TGT GGT T	mdh-S2, TGT GCA AAG AAG CTA GCA TGC T	489	
*met*G	metG-S1, CGC TCT GGC TCT CTG CAA ACT G	metG-S2, TTG CCT TCA AAG CGT TTC GCA A	429	
*pur*M	purM-S1, ACT GAT GGC GTT GGC ACC AAG	purM-S2, CAG AAA CCG CCG CCA GTG	444	
*dtd*S	dtdS-S1, ATT GGT GTG GGC GTA AAC CG	dtdS-S2, CAT TTC ACG GCC ATA GAT ACC	417	
*lys*A	lysA-S1, GGA GTT GGT CGC TTC TGA AG	lysA-S2, GAT AAA GCG ACC CGG TTC AG	465	
*pnt*A	pntA-S1, GTT ATC GAG CGG TTG TTG AAG	pntA-S2, CCG ATC ACT TTT ACG CCG TT	396	
*pyr*C	pyrC-S1, GAT CGC ATT	pyrC-S2, GTC	423	

	CAA GCA CAT AAC C	CGT ACC CAA AAA GAA CTT C		
*tna*A	tnaA-S1, ATC ATG GAC TCA(T) GCC CGT TT	tnaA-S2, CTA AAG CAT GGG CAG GGA ACT	324	

3.4 Agarose Gel Electrophoresis

Amplified PCR products were visualized on 1.5% agarose gel and stained with Midori green. The electrophoresis set was run at 100V and 400 mA for 45 min. The electrophoresis gel was photographed using a gel documented system, UV Transilluminator (Bio-Rad, USA).

3.5 Sequencing and Analysis of PCR Product

The products of PCR were submitted to Apical Sdn. Bhd. for further sequencing. The sequence information was compared with Genbank using Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

CHAPTER 4

RESULTS

Vibrio cholerae isolates on Tryptic soy agar (TSA) was a clear pinpoint colonies of the isolates of sample 1(KP16LY(1)) while for sample 2 (KP16LY(2)), there is presence of creamy pinpoint colonies (Figure 2). On the other hand, in both samples CHROMagar™ and TCBS, both appear greenish pinpoint and yellow pinpoint colonies, respectively (Figure 3 & 4).

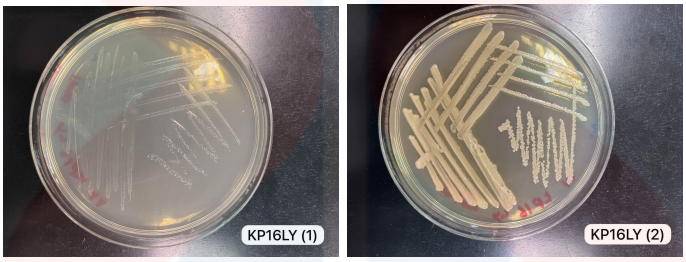

Figure 2: *Vibrio cholerae* on TSA agar.

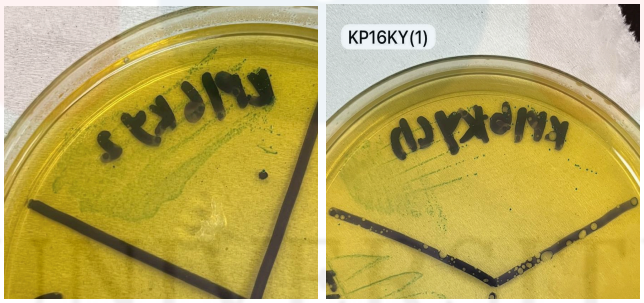

Figure 3: *Vibrio cholerae* on CHROMagar™.

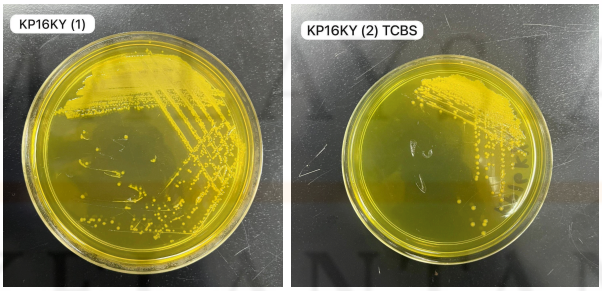

Figure 4: *Vibrio cholerae* on TCBS agar.

From this study, *pyr*H, *gyr*B, *met*G, *pur*M, *dtd*S, *pnt*A, and *tna*A genes were detected from both *V. cholerae* isolates using PCR (Figure 5 and 6). None of the isolates have *glp*, *mdh*, *lys*A and *pyr*C genes.

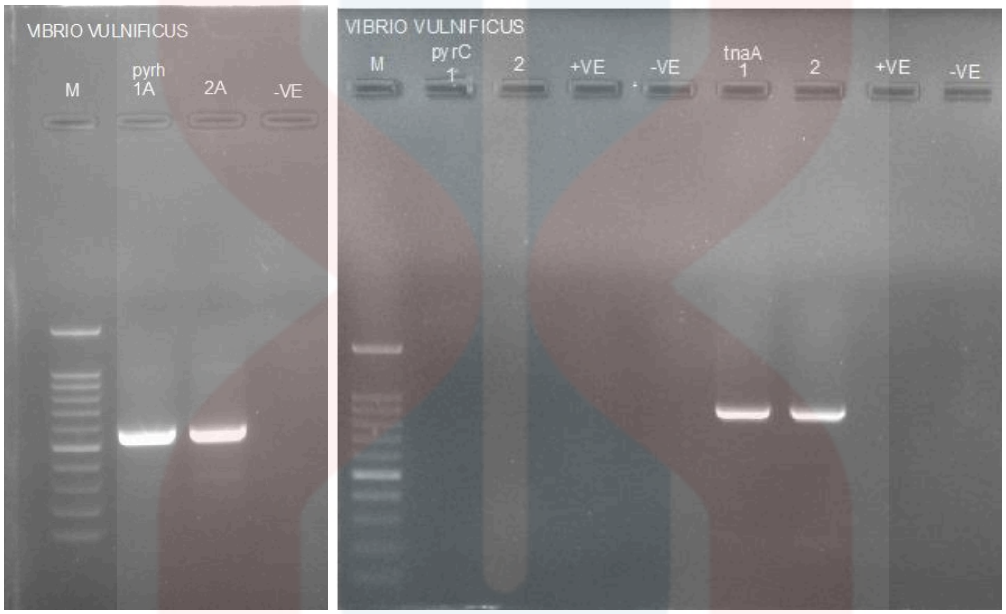

Figure 5: *Vibrio cholera* using *pyr*H (559bp), *pyr*C and *tna*A (919bp) gene on agarose gel

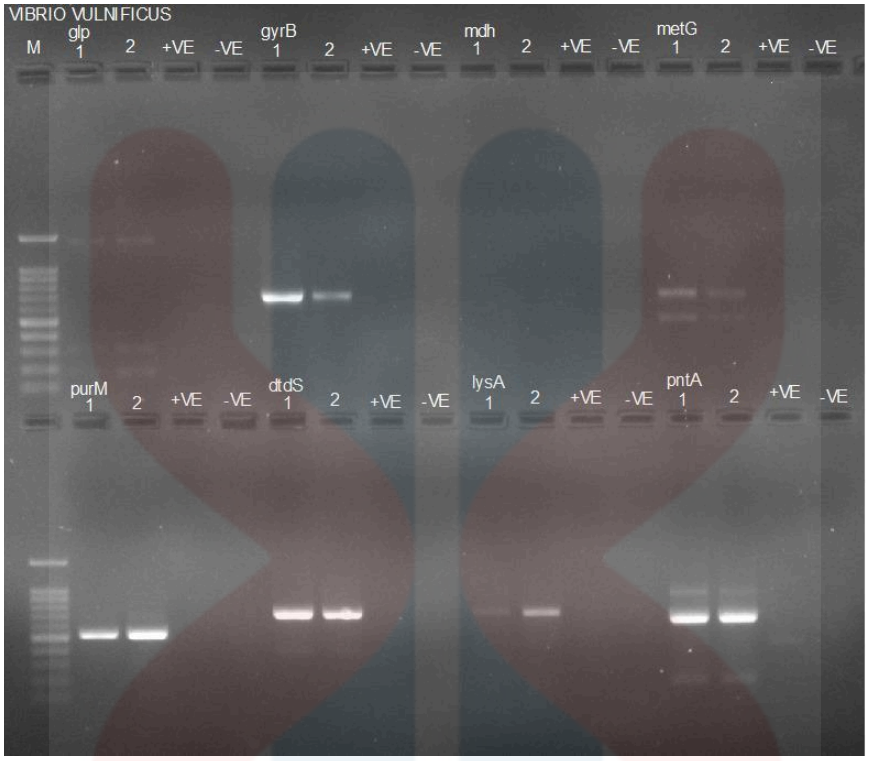

Figure 6: *Vibrio cholera* using *glp, gyr*B (700bp), *mdh, met*G (695bp), *pur*M (694bp), *dtd*S (724bp), *lys*A (862bp) and *pnt*A (396bp) gene on agarose gel

CHAPTER 5

DISCUSSION

The molecular detection and characterization of *Vibrio cholerae* in diseased Asian seabass (*Lates calcarifer*) were conducted using a multi-gene analysis approach. In this study, we used two bacterial samples (n=2) of *Vibrio cholerae* isolated from diseased Asian seabass in Kuala Pahang, Pahang. Both isolates were detected and characterized through multi-gene analysis in which eleven genes were targeted. The targeted genes, including *pyr*H, *glp*, *gyr*B, *mdh*, *met*G, *pur*M, *dtd*S, *lys*A, *pnt*A, *pyr*C and *tna*A were chosen for their essential roles in bacterial physiology, taxonomy, and pathogenicity. This multi-gene approach provided a powerful and comprehensive framework for precise identification and characterization of *V. cholerae* strains isolated from the diseased fish.

Single-gene approaches, such as the use of 16S rRNA gene sequencing, often lack the resolution to distinguish between closely related bacterial species or strains (Janda & Abbott, 2007). By incorporating multiple housekeeping genes (*pyr*H, *gyr*B, *met*G, *pur*M, *dtd*S, *pnt*A, and *tna*A), this study achieved higher discriminatory power and resolution in identifying and characterizing *V. cholerae* isolates. Lee et al. (2007) stated that *pyr*H encodes UMP kinase catalyzing UMP phosphorylation. It will convert Uridine Monophosphate (UMP) to Uridine -5'-triphosphate (UDP) and the produced UDP will serve as the key precursor in the pathway to form pyrimidines. *Glp* was characterized as glycolipoprotein, it is important for the reduction and stabilization of silver nanoparticles (glycolipoprotein) (Gahlawat et al., 2016). Next, the *gyrB* gene consists of subunit B protein of DNA gyrase, it is a type II DNA topoisomerase. This plays a crucial part in DNA replication which can be found in almost all bacteria species (Watt &

Hickson, 1994; Huang, 1996; Yin et al., 2008). Different studies have shown *gyrB* as a suitable phylogenetic marker for the study of phylogenetic and taxonomic relationships of a bacteria at species level (Wang et al., 2007). Meanwhile for *mdh*, it is used as housekeeping gene malate dehydrogenase (Jermyn & Boyd, 2005).

Aside from that, Slattery et al., (2013) and Fridman et al., (2014) mentioned that *met*G is a gene that produces methionyl-tRNA synthetase (MetRS). The MetRS will affect the levels of microbial antibiotic resistance. By that, it will have different responses in translation elongation and aminoacylation (Ibba and Soll, 2000; Laursen et al., 2005). The gene *pur*M is cyclo-ligase, which involves the biosynthesis of purines or to be specific, it is important for the fifth steps of the purines biosynthesis (Miyamoto & Meighen, 2006). Next, *dtdS* gene is used to encodes threonine dehydrogenase (Fu et al., 2020) and *lysA* gene to encodes diaminopimelate decarboxylase (Stragier et al., 1983; Tuntufye et al., 2012). Teh et al., (2009) mentioned that *pnt*A gene creates transhydrogenase alpha subunit which is one of the housekeeping genes in most of the *Vibrio* species such as *V. parahaemolyticus, V. cholerae, V. vulnificus* and other bacteria. This is substantial to couple the proton transport across the membrane to the reversible transfer of hydride ion equivalents between NAD and NADP. Finally, *pyrC* gene encodes a putative dihydroorotase that formed carbamoyl N-Carbamoyl-L-aspartate and changed it into 4,5-dihydroorotate (Nakamura et al., 2022; Ramos et al., 2015). Lastly, Tryptophanase or *tna*A is a pyridoxal 5′phosphate-dependent enzyme that catalyses the hydrolytic β-elimination of tryptophan to indole, pyruvate, and ammonia. All of them play different roles within organisms as well as the environment. Most importantly, it is an enzyme crucial for amino acid and nitrogen metabolism (Boya et al., 2021).

The detection of multiple housekeeping genes further provided insights into the physiological capabilities of *V. cholerae*. Genes such as *tnaA* and *gyrB* are associated with survival in aquatic environments and stress adaptation, suggesting a potential role in the persistence of *V. cholerae* within fish hosts and aquaculture systems (Chen et al., 2010; Yamamoto & Harayama, 1995). The presence of these genes in isolates from diseased seabass underlines their pathogenic potential and highlights the need for routine monitoring of aquaculture systems.

Multi-gene analysis using MLST is a standardized method that basically uses a collection of six or seven well-defined housekeeping genes. In *Vibrio cholerae* study, common housekeeping genes used are *gyrB*, *mdh*, *adk*, *metE*, *purM*, *pntA* and *pyrC* (Octavia et al., 2013; Liang et al., 2020). The gene fragment sequences of the seven housekeeping genes ranged in length from 416 bp –591 bp (Octavia et al., 2013). In our study, the gene range varies from 396 bp -919 bp and based on the results, we managed to identify 7 out of 11 vibrio cholerae genes which are *pyr*H, *gyr*B, *met*G, *pur*M, *dtd*S, *pnt*A, and *tna*A. Unfortunately, the common housekeeping genes for *V. cholerae* studies such as *mdh* and *pyr*C do not show any significant results. Octavia et al. (2013) mentioned that two genes (*mdh* and *pyrC*) may show a difference by recombination causing an error on the strain relationship with the pandemic lineage. As for *glp* and *lys*A, results may be incorrect as both *glp* and *lys*A can be used in identification of *V. cholerae*, although uncommonly used in studies of *V. cholerae*, but both genes can be found in the microbial.

The observed genetic variability highlights the adaptability of *V. cholerae* to different environmental conditions, particularly in aquaculture systems, where stress factors such as high stocking densities and poor water quality can promote pathogen emergence (Haldar et al., 2010). The multi-gene analysis thus serves as an essential tool for distinguishing pathogenic strains from non-pathogenic counterparts.

The results of this study underscore the importance of molecular tools, such as multi-gene analysis, in the early detection and characterization of *Vibrio cholerae* in aquaculture. Targeting multiple genes enhances the reliability of pathogen identification and provides critical insights into genetic diversity and pathogenicity. These findings can inform aquaculture management practices, including improved water quality monitoring, stress reduction, and targeted antimicrobial interventions (Austin & Austin, 2012).

CHAPTER 6

CONCLUSION AND RECOMMENDATION

In conclusion, further studies should be conducted using multi-gene analysis for identification and characterization of *Vibrio cholerae*. In this study, 4 out of 11 gene variations cannot be detected in *V. cholerae* using MLST. While most genes show differences in the base pair from the expected findings. The molecular detection and characterization of *Vibrio cholerae* using multi-gene analysis (targeting *pyrH*, *gyrB*, *metG*, *purM*, *dtdS*, *pntA*, and *tnaA*) provided a comprehensive understanding of the genetic diversity and pathogenic potential of isolates from diseased Asian seabass. This approach offers a reliable and high-resolution method for pathogen identification, contributing to the development of effective disease management strategies in aquaculture. As for recommendation, PCR should be repeated for confirmation of the results. Next, glycerol stocks used in research studies should be less than 3 years and for precaution, the isolates should be restocked every year to ensure the survivability of the isolates. Lastly, some housekeeping genes and universal genes related to *Vibrio cholerae* should be added to get accurate results.

APPENDICES

pyrH

Sample	Base	Blast	%
Sample 1	AGTGGGCGTTGTGATTGGTGGCGGCA ACCTATTCCGTGGTGCTGGCCTTGCCA AAGCGGGTATGAACCGTGTTGTTGGGG ATCATATGGGCATGCTTGCCACGGTAAT GAATGGTTTGGCAATGCGTGATGCGCT GCATCGTGCTTACGTCAATGCACGTCT GATGTCTGCTATCCCACTAAATGGTGTG TGTGACGATTACAGCTGGTCTGATGCG ATTCGTGAATTGCGCCAAGGCCGAGTG GTAATTTTTGCTGCGGGTACAGGCAAT CCTTTCTTTACTACGGATTCGGCTGCGT GTCTGCGTGGTATTGAAATTGAAGCTG ATGTCGTTCTCAAAGCGACCAAAGTAG ATGGTGTTTACAGCGCTGACCCGGTAG CCAACCCGGATGCACAACTGTATGATA AGCTGGCTTACAACGATGTG	*Vibrio cholerae*	Isolated from a water sample in USA:California,San Pedro Creek Strain - SP7G
Sample 2	AGTGGGCGTTGTGATTGGTGGCGGCA ACCTATTCCGTGGTGCTGGCCTTGCCA AAGCGGGTATGAACCGTGTTGTTGGGG ATCATATGGGCATGCTTGCCACGGTAAT GAATGGTTTGGCAATGCGTGATGCGCT GCATCGTGCTTACGTCAATGCACGTCT GATGTCTGCTATCCCACTAAATGGTGTG TGTGACGATTACAGCTGGTCTGATGCG ATTCGTGAATTGCGCCAAGGCCGAGTG GTAATTTTTGCTGCGGGTACAGGCAAT	*Vibrio cholerae*	Isolated from a water sample in USA:California,San Pedro Creek Strain - SP7G

	CCTTTCTTTACTACGGATTCGGCTGCGT GTCTGCGTGGTATTGAAATTGAAGCTG ATGTCGTTCTCAAAGCGACCAAAGTAG ATGGTGTTTACAGCGCTGACCCGGTAG CCAACCCGGATGCACAACTGTATGATA AGCTGGCTTACAACGATGTGCT		

MLST

Primers	Sample	Base	Blast	
dtdS	Sample 1	GTGGTCGGCATTGGCCAAGAAGT GCGTGGTTTCCAAATTGGTGATCG CGTTTCTGGCGAAGGCCACATCA CTTGTGGTCACTGCCGTAACTGTC GTGGTGGCCGTACGCACTTGTGC CGCAACACCATTGGTGTGGGCGT AAACCGCACCGGTTGCTTCTCTG AATACTTGGTGATCCCAGCGTTTA ACGCATTCAAAATTCCAGAAGGG ATTTCCGACGATCTGGCGTCTATC TTCGATCCGTTTGGCAACGCTGTA CACACTGCGCTTTCATTCGACTTA GTGGGTGAAGATGTGCTGATCAC CGGTGCTGGCCCAATCGGTATTAT GGCTGCTGCGGTTGCAAAACACG TAGGTGCGCGCCATGTGGTGATCA CCGATGTGAACGAATATCGCCTCG ATTTAGCCCGTAAAATGGGCGTGA CTCGCGCTGTGAACGTTGCGGAG	*Vibrio cholerae*	Samples isolated from intestinal content of Mastacembelus in Ho Chi Minh, Vietnam. Strains - VN152

		CAAAACCTAGAAGAGGTGATGAA AGAGCTCGGCATGACCGAAGGTT TTGATGTGGGCTTAGAGATGTCTG GCGTACCAAGCGCATTCAGCGCC ATGCTCAAAACCATGAACCACGG TGGCCGCATCGCTTTGCTAGGTAT TCCACCGTCATCGATGGCGATTGA TTGGAACCAAGTG		
	Sample 2	GTGGTCGGCATTGGCCAAGAAGT GCGTGGTTTCCAAATTGGTGATCG CGTTTCTGGCGAAGGCCACATCA CTTGTGGTCACTGCCGTAACTGTC GTGGTGGCCGTACGCACTTGTGC CGCAACACCATTGGTGTGGGCGT AAACCGCACCGGTTGCTTCTCTG AATACTTGGTGATCCCAGCGTTTA ACGCATTCAAAATTCCAGAAGGG ATTTCCGACGATCTGGCGTCTATC TTCGATCCGTTTGGCAACGCTGTA CACACTGCGCTTTCATTCGACTTA GTGGGTGAAGATGTGCTGATCAC CGGTGCTGGCCCAATCGGTATTAT GGCTGCTGCGGTTGCAAAACACG TAGGTGCGCGCCATGTGGTGATCA CCGATGTGAACGAATATCGCCTCG ATTTAGCCCGTAAAATGGGCGTGA CTCGCGCTGTGAACGTTGCGGAG CAAAACCTAGAAGAGGTGATGAA AGAGCTCGGCATGACCGAAGGTT TTGATGTGGGCTTAGAGATGTCTG	*Vibrio cholerae*	Samples isolated from intestinal content of Mastacembelus in Ho Chi Minh, Vietnam. Strains - VN152

		GCGTACCAAGCGCATTCAGCGCC ATGCTCAAAACCATGAACCACGG TGGCCGCATCGCTTTGCTAGGTAT TCCACCGTCATCGATGGCGATTGA TTGGAACCAAGTG		
gyrB	Sample 1	TTCCATTACGACATTTTGGCTAAA CGCCTGCGTGAGCTGTCATTCCTG AACTCTGGCGTGTCGATCAAGCT GACCGATGAACGTGAAGAAGATA AAAAAGACCACTTCATGTATGAA GGGGGTATTCAAGCGTTTGTGACC CACTTGAATCGTAACAAAACGCC GATCCATGAAAAAGTCTTCCACTT CAACCAAGAGCGTGAAGATGGCA TCAGCGTGGAAGTGGCAATGCAG TGGAATGATGGTTTCCAAGAAAA CATCTACTGCTTTACCAACAACAT CCCACAGCGTGATGGTGGTACCC ACTTAGCCGGTTTCCGTGGTGCGT TGACTCGTACTTTGAACAACTACA TGGACAAAGAAGGCTTCTCGAAG AAAGCCCAAGCGGCAACCTCGGG TGATGATGCGCGTGAAGGCTTAAC GGCTGTGGTATCGGTGAAAGTGC CAGATCCTAAATTCTCAAGCCAAA CCAAAGATAAGCTGGTTTCTTCGG AAGTGAAATCCGCGGTTGAGTCA GCCATGAATGAGAAGCTGGCAGA TTTCCTAGCTGAAAACCCAAGCG AAGCGAAAAACGTTTGTTCGAAG	*Vibrio cholerae*	Samples isolated from coastal marine water in California, USA. Strains - SA1106-91

		ATTATTGATGCGGCGCGCGCT		
	Sample 2	TTCCATTACGACATTTTGGCTAAA CGCCTGCGTGAGCTGTCATTCCTG AACTCTGGCGTGTCGATCAAGCT GACCGATGAACGTGAAGAAGATA AAAAAGACCACTTCATGTATGAA GGGGGTATTCAAGCGTTTGTGACC CACTTGAATCGTAACAAAACGCC GATCCATGAAAAAGTCTTCCACTT CAACCAAGAGCGTGAAGATGGCA TCAGCGTGGAAGTGGCAATGCAG TGGAATGATGGTTTCCAAGAAAA CATCTACTGCTTTACCAACAACAT CCCACAGCGTGATGGTGGTACCC ACTTAGCCGGTTTCCGTGGTGCGT TGACTCGTACTTTGAACAACTACA TGGACAAAGAAGGCTTCTCGAAG AAAGCCCAAGCGGCAACCTCGGG TGATGATGCGCGTGAAGGCTTAAC GGCTGTGGTATCGGTGAAAGTGC CAGATCCTAAATTCTCAAGCCAAA CCAAAGATAAGCTGGTTTCTTCGG AAGTGAAATCCGCGGTTGAGTCA GCCATGAATGAGAAGCTGGCAGA TTTCCTAGCTGAAAACCCAAGCG AAGCGAAAAACGTTTGTTCGAAG ATTATTGATGCGGCGCGCGC	*Vibrio cholerae*	Samples isolated from coastal marine water in California, USA. Strains - SA1106-91
metG	Sample 1	GCCATGCGCTTGCGCAGCATGAA ACACGCGTTCAGTTTGCGCCACG	*Vibrio cholerae*	Samples isolated from homo

		CTAAAGCCAATCGACTCACAAAA CACATCCACGCTGGTGGCCAGTT GCTCTTCGGCAACCTGAGGAATG ATCTCTTGGCAAATATGCGTGATAT AGTCATCACTACGGCCTTGAAATT CAGGAGGCAGTGCGTGCGCGGCA AGTAATGTGGTGGTGATTTTGACG CGGCGATGCTGCTCAAGCGCTTTG GCGGCGCGCAGCATTTTCAGCTC ATCCTTGAGCGTTAAACCGTAGCC AGATTTCACTTCAACGGAAGTGA CACCGCTACGCAGCAGGCCATCC AAACGCGGTAGCGCGAGTTCGAT CAACGCTTCTTCACTGGCCTTACG AGTGGCATTCACGGTAGAAAGAA TGCCGCCACCTTGGGCGGCGATA GTT		sapiens in Japan. Strain - RIMD 2214340
	Sample 2	GCCATGCGCTTGCGCAGCATGAA ACACGCGTTCAGTTTGCGCCACG CTAAAGCCAATCGACTCACAAAA CACATCCACGCTGGTGGCCAGTT GCTCTTCGGCAACCTGAGGAATG ATCTCTTGGCAAATATGCGTGATAT AGTCATCACTACGGCCTTGAAATT CAGGAGGCAGTGCGTGCGCGGCA AGTAATGTGGTGGTGATTTTGACG CGGCGATGCTGCTCAAGCGCTTTG GCGGCGCGCAGCATTTTCAGCTC ATCCTTGAGCGTTAAACCGTAGCC AGATTTCACTTCAACGGAAGTGA	*Vibrio cholerae*	Samples isolated from homo sapiens in Japan. Strain - RIMD 2214340 Serotype - O102

		CACCGCTACGCAGCAGGCCATCC AAACGCGGTAGCGCGAGTTCGAT CAACGCTTCTTCACTGGCCTTACG AGTGGCATTCACGGTAGAAAGAA TGCCGCCACCTTGGGCGGCGATA GTT		
pntA	Sample 1	CTGCACTGACGATCAACGAAAGC ATCCAGCTTAAAGCTGCCACCCAT GCTGCGATGCAGCAACAGGAAGT GGCTCAATTCTCCGCCCACCAATC CCCAAATCGCTTGCTCGGGGATCA CTCGTCCTAGCGTTAATCCCGCCG ACACCGCCATGCTTTGCAACTCAA GGATAGTACGCTTGCTGATCACAT AGGCTTGTACTTTACCACTGCCGG GAAGAGGGTGAGCATCCGCCACT ACATCCGTCACTTTCTCACTCAAC ATATCTTTGAGCAGGAAAGGCAA GGCTGCCGACCACTCTTCACGCG GAATGCTCGGCTGTTCAATTTGAT AGGTTTGATAAAGCTGTGAATGCA GTACAAGGTGGATTTGTACATCAT GGACGGCGTGCTTTTGCAGCGTTT GCACCAATACCGCTTGCCAACTTT GTTGCTGTAACGGCTGGGGAGGA ATAGGCGACAAATCATCGGATGTG AAGTACAGGTGCTCTGGCTGCAC CACAACATATAACTGTTGCGAGGC AACCTTAGGGGCAATCAGTTTCTC TATCCAACTCGGTT	*Vibrio cholerae*	Samples isolated from water samples in USA:California,Old Salinas River Strain - SA7G

	Sample 2	CTGCACTGACGATCAACGAAAGC ATCCAGCTTAAAGCTGCCACCCAT GCTGCGATGCAGCAACAGGAAGT GGCTCAATTCTCCGCCCACCAATC CCCAAATCGCTTGCTCGGGGATCA CTCGTCCTAGCGTTAATCCCGCCG ACACCGCCATGCTTTGCAACTCAA GGATAGTACGCTTGCTGATCACAT AGGCTTGTACTTTACCACTGCCGG GAAGAGGGTGAGCATCCGCCACT ACATCCGTCACTTTCTCACTCAAC ATATCTTTGAGCAGGAAAGGCAA GGCTGCCGACCACTCTTCACGCG GAATGCTCGGCTGTTCAATTTGAT AGGTTTGATAAAGCTGTGAATGCA GTACAAGGTGGATTTGTACATCAT GGACGGCGTGCTTTTGCAGCGTTT GCACCAATACCGCTTGCCAACTTT GTTGCTGTAACGGCTGGGGAGGA ATAGGCGACAAATCATCGGATGTG AAGTACAGGTGCTCTGGCTGCAC CACAACATATAACTGTTGCGAGGC AACCTTAGGGGCAATCAGTTTCTC TATCCAACTCGGTT	*Vibrio cholerae*	Samples isolated from water samples in USA:California,Old Salinas River Strain - SA7G
purM	Sample 1	GCGCTCTTGGAAGCACATCACAC CAAACTAAACACTTATCAACACA AAACAGCATAAATGATTACAGTGT TGTTGCAAATTGCCGTTTTGTAAG TATCGCCAAGATAATAAATGCGAG TCGAATCACACAATCAAGCGCGA	*Vibrio cholerae*	Samples isolated from water samples in USA:California,Old Salinas River Strain - SA3G

		AAACGACATTCTTCTGCGCAAAAT CGTTCCGGCTCGAACTAGTTGTAA GCATTTTAAGCCCTAAGTGATAGG ATTAGGCGATTTTAAAACAGATGG ATAGGAACATCATAATGACCGAGA CGCCGATTCGCATTGCAACCCGCC AGAGTCCACTGGCTTTGTGGCAA GCCAACTATGTTAAAGACGCCTTA ATGGCAGCCCACCCCGGACTGCA GGTGGAGTTGGTGACTATGGTCA CGCGCGGCGATGTGATTCTGGATA CTCCACTGGCTAAAGTG		
	Sample 2	GCGCTCTTGGAAGCACATCACAC CAAACTAAACACTTATCAACACA AAACAGCATAAATGATTACAGTGT TGTTGCAAATTGCCGTTTTGTAAG TATCGCCAAGATAATAAATGCGAG TCGAATCACACAATCAAGCGCGA AAACGACATTCTTCTGCGCAAAAT CGTTCCGGCTCGAACTAGTTGTAA GCATTTTAAGCCCTAAGTGATAGG ATTAGGCGATTTTAAAACAGATGG ATAGGAACATCATAATGACCGAGA CGCCGATTCGCATTGCAACCCGCC AGAGTCCACTGGCTTTGTGGCAA GCCAACTATGTTAAAGACGCCTTA ATGGCAGCCCACCCCGGACTGCA GGTGGAGTTGGTGACTATGGTCA CGCGCGGCGATGTGATTCTGGATA CTCCACTGGCTAAA	*Vibrio cholerae*	Samples isolated from water samples in USA:California,Old Salinas River Strain - SA3G

tnaA	Sample 1	GGTAACTTCGACTTAGAAAAGCT CGAACAAGCCATCCTTGAAGCGG GCCCAGCAAACGTCCCATATATTG TCAGCACCATCACTTGTAACTCTG CGGGTGGCCAGCCAGTTTCGATC GCCAACTTAAAAGCCGTGTATGA GATTGCCCAGCGTTACGACATTCC CGTGATCATGGATTCTGCTCGTTT TGCTGAAAATGCGTATTTTATTCA GCAACGTGAGCGCGATTATCGCA ACTGGAGTATCGAAGAGATCACG CGTGAAGCTTACAAATACGCCGAT GGACTCGCGATGTCGGCCAAAAA GGATGCCATGGTGCAAATGGGCG GTTTACTCTGCTTCAAAGACGAA AGCTTCTTTGACGTGTACACCGAA TGCCGAACCCTGTGTGTGGTGCA AGAAGGCTTCCCTACATACGGAG GCTTAGAAGGTGGTGCGATGGAG CGGTTGGCGGTTGGCTTGTATGAC GGTATGCGCCAAGATTGGCTCGCT TATCGCATTAACCAAGTGGAGTAT CTGGTCAATGGTTTAGAAGCGATT GGGGTTATTTGCCAACAAGCTGG CGGCCATGCCGCGTTTGTCGATGC GGGTAAACTGCTGCCTCACATCCC AGCAGATCAATTCCCTGCTCACGC TTTAGCTTGTGAACTCTATAAAGT CGCAGGCATTCGCGCAGTAGAAA TTGGCTCATTACTACTAGGACGCG	*Vibrio cholerae*	Samples were collected from frogs located in China. Strain - ICDC-VC702

		ATCCTGCAACCGGAAAACAGCAT CCTTGCCCAGCCGAATTGCTCCGT TTAACCATTCCACGCGCGACTTAT ACGCAAACGCACATGGATT		
	Sample 2	GGTAACTTCGACTTAGAAAAGCT CGAACAAGCCATCCTTGAAGCGG GCCCAGCAAACGTCCCATATATTG TCAGCACCATCACTTGTAACTCTG CGGGTGGCCAGCCAGTTTCGATC GCCAACTTAAAAGCCGTGTATGA GATTGCCCAGCGTTACGACATTCC CGTGATCATGGATTCTGCTCGTTT TGCTGAAAATGCGTATTTTATTCA GCAACGTGAGCGCGATTATCGCA ACTGGAGTATCGAAGAGATCACG CGTGAAGCTTACAAATACGCCGAT GGACTCGCGATGTCGGCCAAAAA GGATGCCATGGTGCAAATGGGCG GTTTACTCTGCTTCAAAGACGAA AGCTTCTTTGACGTGTACACCGAA TGCCGAACCCTGTGTGTGGTGCA AGAAGGCTTCCCTACATACGGAG GCTTAGAAGGTGGTGCGATGGAG CGGTTGGCGGTTGGCTTGTATGAC GGTATGCGCCAAGATTGGCTCGCT TATCGCATTAACCAAGTGGAGTAT CTGGTCAATGGTTTAGAAGCGATT GGGGTTATTTGCCAACAAGCTGG CGGCCATGCCGCGTTTGTCGATGC GGGTAAACTGCTGCCTCACATCCC	*Vibrio cholerae*	Samples were collected from frogs located in China. Strain - ICDC-VC702

		AGCAGATCAATTCCCTGCTCACGC TTTAGCTTGTGAACTCTATAAAGT CGCAGGCATTCGCGCAGTAGAAA TTGGCTCATTACTACTAGGACGCG ATCCTGCAACCGGAAAACAGCAT CCTTGCCCAGCCGAATTGCTCCGT TTAACCATTCCACGCGCGACTTAT ACGCAAACGCACATGGATTTCATC ATCGAAGCATT		

REFERENCES

Amalina, N. Z., Santha, S., Zulperi, D., Amal, M. N. A., Yusof, M. T., Zamri-Saad, M., & Ina-Salwany, M. Y. (2019). Prevalence, antimicrobial susceptibility and plasmid profiling of *Vibrio* spp. isolated from cultured groupers in Peninsular Malaysia. *BMC Microbiology*, *19*(1). https://doi.org/10.1186/s12866-019-1624-2

Amel, B. K., Amine, B., & Amina, B. (2006). Survival of *Vibrio fluvialis* in seawater under starvation conditions. *Microbiological Research*, *163*(3), 323–328. https://doi.org/10.1016/j.micres.2006.06.006

Austin, B., & Austin, D. A. (2012). Bacterial fish pathogens: Disease of farmed and wild fish. Springer Science & Business Media.

Barbosa, C., Nogueira, S., Gadanho, M., & Chaves, S. (2016). DNA extraction: finding the most suitable method. Molecular Microbial Diagnostic Methods, 135–154. https://doi.org/10.1016/b978-0-12-416999-9.00007-1

Blake, P.A., D.T. Allegra, J.D. Snyder, T.J. Barrett, L. McFarland, C.T. Caraway, J.C. Feeley, J.P. Craig, J.V. Lee, N.D. Puhr, and R.A. Feldman (1980) Cholera ± a possible endemic focus in the United States. New Eng. J. Med. 302(6): 305±309.

Blue Transformation - Roadmap 2022–2030. (2022). In *FAO eBooks*. https://doi.org/10.4060/cc0459en

Boya, B. R., Kumar, P., Lee, J., & Lee, J. (2021). Diversity of the Tryptophanase Gene and Its Evolutionary Implications in Living Organisms. Microorganisms, 9(10), 2156. https://doi.org/10.3390/microorganisms9102156

Chen, C. Y., Nace, G. W., & Irwin, P. L. (2010). A 16S rRNA gene sequence analysis of Vibrio species. Journal of Microbiological Methods, 82(1), 76-83.

Daboul, J., Weghorst, L., DeAngelis, C., Plecha, S. C., Saul-McBeth, J., & Matson, J. S. (2020b). Characterization of *Vibrio cholerae* isolates from freshwater sources in northwest Ohio. *PLoS ONE*, *15*(9), e0238438. https://doi.org/10.1371/journal.pone.0238438

Dalsgaard, A., Albert, M. J., Taylor, D. N., Shimada, T., Meza, R., Serichantalergs, O., & Echeverria, P. (1995). Characterization of *Vibrio cholerae* non-O1 serogroups obtained from an outbreak of diarrhea in Lima, Peru. *Journal of Clinical Microbiology*, *33*(10), 2715–2722. https://doi.org/10.1128/jcm.33.10.2715-2722.1995

Dashti, A. A., Jadaon, M. M., Abdulsamad, A. M., & Dashti, H. (2009, June). Heat Treatment of Bacteria: A Simple Method of DNA Extraction for Molecular Techniques. ResearchGate; unknown.

https://www.researchgate.net/publication/266888615_Heat_Treatment_of_Bacteria_A_Simple_Method_of_DNA_Extraction_for_Molecular_Techniques

Faruque, S. M., Sack, D. A., Sack, R. B., Colwell, R. R., Takeda, Y., & Nair, G. B. (2003). Emergence and evolution of *Vibrio cholerae* O139. *Proceedings of the National Academy of Sciences*, *100*(3), 1304–1309. https://doi.org/10.1073/pnas.0337468100

Farzadnia, A., & Naeemipour, M. (2019). Molecular techniques for the detection of bacterial zoonotic pathogens in fish and humans. *Aquaculture International*, *28*(1), 309–320. https://doi.org/10.1007/s10499-019-00462-7

Fridman, O., Goldberg, A., Ronin, I., Shoresh, N., & Balaban, N. Q. (2014). Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature*, *513*(7518), 418–421. https://doi.org/10.1038/nature13469

Fu, S., Wei, D., Yang, Q., Xie, G., Pang, B., Wang, Y., Lan, R., Wang, Q., Dong, X., Zhang, X., Huang, J., Feng, J., & Liu, Y. (2020). Horizontal Plasmid Transfer Promotes the Dissemination of Asian Acute Hepatopancreatic Necrosis Disease and Provides a Novel Mechanism for Genetic Exchange and Environmental Adaptation. *mSystems*, *5*(2). https://doi.org/10.1128/msystems.00799-19

Gahlawat, G., Shikha, S., Chaddha, B. S., Chaudhuri, S. R., Mayilraj, S., & Choudhury, A. R. (2016). Microbial glycolipoprotein-capped silver nanoparticles as emerging antibacterial agents against cholera. *Microbial Cell Factories*, *15*(1). https://doi.org/10.1186/s12934-016-0422-x

Haldar, S., Maharajan, A., Chatterjee, S., Hunter, S. A., & Chowdhury, N. (2010). *Vibrio cholerae* infections in aquaculture: A critical review. Aquaculture, 309(1-4), 1-10.

Halpern, M., & Izhaki, I. (2017). Fish as Hosts of Vibrio cholerae. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.00282

Hassan, M., Ali, M. R. M., Zamri, H. F., Amdan, N. a. N., Azmai, M. N. A., Maniam, S., Alipiah, N. M., & Hashim, R. (2023). Distribution, Prevalence, and Antibiotic Susceptibility Profiles of Infectious Noncholera Vibrio Species in Malaysia. *Journal of Tropical Medicine*, *2023*, 1–7. https://doi.org/10.1155/2023/2716789

Huang, W. M. (1996). BACTERIAL DIVERSITY BASED ON TYPE II DNA TOPOISOMERASE GENES. *Annual Review of Genetics*, *30*(1), 79–107. https://doi.org/10.1146/annurev.genet.30.1.79

Helmi, A. M., Mukti, A. T., Soegianto, A., & Effendi, M. H. (2020). A Review of Vibriosis in Fisheries: Public Health Importance. *Systematic Reviews in Pharmacy*, *11*(8), 51–58. https://doi.org/10.31838/srp.2020.8.8

Ibba, M., & Söll, D. (2000). Aminoacyl-tRNA Synthesis. *Annual Review of Biochemistry*, *69*(1), 617–650. https://doi.org/10.1146/annurev.biochem.69.1.617

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. *Journal of Clinical Microbiology*, *45*(9), 2761–2764. https://doi.org/10.1128/jcm.01228-07

Jermyn, W. S., & Boyd, E. F. (2005). Molecular evolution of Vibrio pathogenicity island-2 (VPI-2): mosaic structure among *Vibrio cholerae* and *Vibrio mimicus* natural isolates. *Microbiology*, *151*(1), 311–322. https://doi.org/10.1099/mic.0.27621-0

Jiang, S. C., Louis, V., Choopun, N., Sharma, A., Huq, A., & Colwell, R. R. (2000). Genetic Diversity of *Vibrio cholerae* in Chesapeake Bay Determined by Amplified Fragment Length Polymorphism Fingerprinting. *Applied and Environmental Microbiology*, *66*(1), 140–147. https://doi.org/10.1128/aem.66.1.140-147.2000

Kaper, J. B., Morris, J. G., & Levine, M. M. (1995). Cholera. *Clinical Microbiology Reviews*, *8*(1), 48–86. https://doi.org/10.1128/cmr.8.1.48

Laursen, B. S., Sørensen, H. P., Mortensen, K. K., & Sperling-Petersen, H. U. (2005). Initiation of Protein Synthesis in Bacteria. *Microbiology and Molecular Biology Reviews*, *69*(1), 101–123. https://doi.org/10.1128/mmbr.69.1.101-123.2005

Lee, S. E., Kim, S. Y., Kim, C. M., Kim, M., Kim, Y. R., Jeong, K., Ryu, H., Lee, Y. S., Chung, S. S., Choy, H. E., & Rhee, J. H. (2007). The *pyr*H Gene of *Vibrio vulnificus* Is an Essential In Vivo Survival Factor. *Infection and Immunity*, *75*(6), 2795–2801. https://doi.org/10.1128/iai.01499-06

Liang, K. Y. H., Orata, F. D., Islam, M. T., Nasreen, T., Alam, M., Tarr, C. L., & Boucher, Y. F. (2020). A *Vibrio cholerae* Core Genome Multilocus Sequence Typing Scheme To Facilitate the Epidemiological Study of Cholera. *Journal of Bacteriology*, *202*(24). https://doi.org/10.1128/jb.00086-20

Li, H., Tang, R., Lou, Y., Cui, Z., Chen, W., Hong, Q., Zhang, Z., Malakar, P. K., Pan, Y., & Zhao, Y. (2017). A Comprehensive Epidemiological Research for Clinical *Vibrio parahaemolyticus* in Shanghai. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.01043

Lin, H., Yu, M., Wang, X., & Zhang, X. H. (2018). Comparative genomic analysis reveals the evolution and environmental adaptation strategies of vibrios. *BMC Genomics*, *19*(1). https://doi.org/10.1186/s12864-018-4531-2

Lü, H., Zhang, H., Liu, T., Hao, W., & Yuan, Q. (2021). MLST/MVLST Analysis and Antibiotic Resistance of *Vibrio cholerae* in Shandong Province of China. *Iranian Journal of Public Health*. https://doi.org/10.18502/ijph.v50i9.7053

Lutz, C., Erken, M., Noorian, P., Sun, S., & McDougald, D. (2013). Environmental reservoirs and mechanisms of persistence of *Vibrio cholerae*. *Frontiers in Microbiology*, *4*. https://doi.org/10.3389/fmicb.2013.00375

Miyamoto, C. M., & Meighen, E. A. (2006). Involvement of LuxR, a quorum sensing regulator in *Vibrio harveyi*, in the promotion of metabolic genes: *arg*A, *pur*M, *lys*E and *rlu*A. *Biochimica Et Biophysica Acta (BBA) - Gene Structure and Expression*, *1759*(6), 296–307. https://doi.org/10.1016/j.bbaexp.2006.05.005

Mohamad, A., Zamri-Saad, M., Amal, M. N. A., Al-Saari, N., Monir, M. S., Chin, Y. K., & Yasin, I. S. M. (2021). Vaccine Efficacy of a Newly Developed Feed-Based Whole-Cell Polyvalent Vaccine against Vibriosis, Streptococcosis and Motile Aeromonad Septicemia in Asian Seabass, *Lates calcarifer*. *Vaccines*, *9*(4), 368. https://doi.org/10.3390/vaccines9040368

Nakamura, T., Shimizu, T., Ikegaya, R., Uda, A., Watanabe, K., & Watarai, M. (2022). Identification of *pyr*C gene as an immunosuppressive factor in *Francisella novicida* infection. Frontiers in Cellular and Infection Microbiology, 12. https://doi.org/10.3389/fcimb.2022.1027424

Octavia, S., Salim, A., Kurniawan, J., Lam, C., Leung, Q., Ahsan, S., Reeves, P. R., Nair, G. B., & Lan, R. (2013). Population Structure and Evolution of Non-O1/Non-O139 *Vibrio cholerae* by Multilocus Sequence Typing. *PLoS ONE*, *8*(6), e65342. https://doi.org/10.1371/journal.pone.0065342

PubMLST. (n.d.). PubMLST. https://pubmlst.org/organisms/vibrio-vulnificus/primers

Ramos, L., Sinn, J., Lehman, B., Pfeufer, E., Peter, K., & McNellis, T. (2015). *Erwinia amylovora pyr*C mutant causes fire blight despite pyrimidine auxotrophy. Letters in Applied Microbiology, 60(6), 572–579. https://doi.org/10.1111/lam.12417

Schirmeister, F., Dieckmann, R., Bechlars, S., Bier, N., Faruque, S. M., & Strauch, E. (2013). Genetic and phenotypic analysis of *Vibrio cholerae* non-O1, non-O139 isolated from German and Austrian patients. *European Journal of Clinical Microbiology & Infectious Diseases*, *33*(5), 767–778. https://doi.org/10.1007/s10096-013-2011-9

Slattery A, Victorsen AH, Brown A, Hillman K, Phillips GJ.2013.Isolation of Highly Persistent Mutants of *Salmonella enterica* Serovar Typhimurium Reveals a New Toxin-Antitoxin Module. J Bacteriol195:.https://doi.org/10.1128/jb.01397-12

Stragier, P., Danos, O., & Patte, J. (1983). Regulation of diaminopimelate decarboxylase synthesis in *Escherichia coli*. *Journal of Molecular Biology*, *168*(2), 321–331. https://doi.org/10.1016/s0022-2836(83)80021-7

Teh, C., Chua, K., & Thong, K. (2009). Simultaneous differential detection of human pathogenic and nonpathogenic *Vibrio* species using a multiplex PCR based on *gyr*B and

*pnt*A genes. *Journal of Applied Microbiology*. https://doi.org/10.1111/j.1365-2672.2009.04599.x

Teh, C. S. J., Chua, K. H., & Thong, K. L. (2011). Genetic variation analysis of *Vibrio cholerae* using multilocus sequencing typing and multi-virulence locus sequencing typing. *Infection, Genetics and Evolution*, *11*(5), 1121–1128. https://doi.org/10.1016/j.meegid.2011.04.005

Trubiano, J. A., Lee, J. Y. H., Valcanis, M., Gregory, J., Sutton, B. A., & Holmes, N. E. (2014). Non‐O1, non‐O139 *Vibrio cholerae* bacteraemia in an Australian population. *Internal Medicine Journal*, *44*(5), 508–511. https://doi.org/10.1111/imj.12409

Tuntufye, H. N., Lebeer, S., Gwakisa, P. S., & Goddeeris, B. M. (2012). Identification of Avian Pathogenic *Escherichia coli* Genes That Are Induced In Vivo during Infection in Chickens. *Applied and Environmental Microbiology*, *78*(9), 3343–3351. https://doi.org/10.1128/aem.07677-11

Urwin, R., & Maiden, M. C. (2003). Multi-locus sequence typing: a tool for global epidemiology. *Trends in Microbiology*, *11*(10), 479–487. https://doi.org/10.1016/j.tim.2003.08.006

Wang, L., Lee, F., Tai, C., & Kasai, H. (2007). Comparison of *gyr*B gene sequences, 16S rRNA gene sequences and DNA–DNA hybridization in the *Bacillus subtilis* group. *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY*, *57*(8), 1846–1850. https://doi.org/10.1099/ijs.0.64685-0

Watt, P. M., & Hickson, I. D. (1994). Structure and function of type II DNA topoisomerases. *Biochemical Journal*, *303*(3), 681–695. https://doi.org/10.1042/bj3030681

Yaashikaa, P., Saravanan, A., & Kumar, P. S. (2016). Isolation and identification of *Vibrio cholerae* and *Vibrio parahaemolyticus* from prawn (*Penaeus monodon*) seafood: Preservation strategies. *Microbial Pathogenesis*, *99*, 5–13. https://doi.org/10.1016/j.micpath.2016.07.014

Yamamoto, S., & Harayama, S. (1995). PCR amplification and direct sequencing of *gyr*B for bacterial phylogeny. Journal of Bacteriology, 177(10), 2565-2572.

Yin, H., Cao, L., Xie, M., Chen, Q., Qiu, G., Zhou, J., Wu, L., Wang, D., & Liu, X. (2008). Bacterial diversity based on 16S rRNA and gyrB genes at Yinshan mine, China. *Systematic and Applied Microbiology*, *31*(4), 302–311. https://doi.org/10.1016/j.syapm.2008.05.003