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MOLECULAR DETECTION OF *VIBRIO CHOLERAE* 01 ISOLATED FROM DISEASED SEABASS, *LATES CALCARIFER*

By

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(D20B0084)

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2024

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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement of the course DVT 55204 – Research Project.

Cholera remains a global health threat, causing 95,000 deaths and 2.9 million infections annually. In Malaysia, the incidence rate was 0.35 cases per 100,000 people in 2021, with no deaths. However, there is limited research on Vibrio cholerae, particularly in the aquaculture sector, hindering public awareness and understanding of its zoonotic risks. The limited genetic studies, such as PCR-based methods, restricts efforts to trace transmission patterns and control *Vibrio cholerae*-related diseases. This research objective is to determine *Vibrio cholerae* 01 from diseased seabass. *Vibrio cholera* isolates were stored in the Trypticase Soy Broth (TSB) with 50% glycerol in the Zoonotic Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. The isolates were revived in Tryptic Soy Agar, TSA. Isolates were then cultured on Thiosulphate citrate bile salts sucrose (TCSB) and CHROMagar for 24 h at 35°C. Molecular detection of *V. cholerae* 01 using PCR method, followed by nucleotides sequencing for confirmation. As a result, six out of twelve samples confirmed to be *V. cholerae* 01 were from Kuala Pahang. The virulence and antibiotic resistance of *V. cholerae* can be influenced by genetic exchange. However, there is no research available on the virulent profile of *V. cholerae* 01 in aquatic animals from Kelantan and Pahang. It's important to characterize the isolates from these areas to better understand the current status of *V. cholerae* 01 emergence.

Keywords: *Vibrio cholerae*, cholerae disease, Polymerase chain reaction, molecular detection

ABSTRAK

Abstrak kertas penyelidikan yang dibentangkan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan, sebagai sebahagian daripada keperluan kursus DVT 55204 – Projek Penyelidikan.

Kolera tetap menjadi ancaman kesihatan global, menyebabkan 95,000 kematian dan 2.9 juta jangkitan setiap tahun. Di Malaysia, kadar kejadian adalah 0.35 kes bagi setiap 100,000 orang pada tahun 2021, tanpa sebarang kematian. Walau bagaimanapun, terdapat sedikit penyelidikan mengenai *Vibrio cholerae*, terutamanya dalam sektor akuakultur, yang menyekat kesedaran awam dan pemahaman mengenai risiko zoonotiknya. Kajian genetik yang terhad, seperti kaedah berasaskan PCR, mengehadkan usaha untuk mengesan corak transmisi dan mengawal penyakit yang berkaitan dengan *Vibrio cholerae*. Objektif penyelidikan ini adalah untuk mengenal pasti Vibrio cholerae 01 daripada ikan seabass yang sakit. Isolat *Vibrio cholera* disimpan dalam Trypticase Soy Broth (TSB) dengan 50% gliserol di Makmal Zoonotik, Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan. Isolat dipulihkan dalam Tryptic Soy Agar (TSA). Isolat kemudiannya dibiakkan pada Thiosulphate citrate bile salts sucrose (TCSB) dan CHROMagar selama 24 jam pada suhu 35°C. Pengesanan molekul *V. cholerae* 01 menggunakan kaedah PCR, diikuti dengan penjujukan nukleotida untuk pengesahan. Hasilnya, enam daripada dua belas sampel yang disahkan sebagai *V. cholerae* 01 berasal dari Kuala Pahang. Virulens dan ketahanan antibiotik *V. cholerae* boleh dipengaruhi oleh pertukaran genetik. Walau bagaimanapun, tiada penyelidikan tersedia mengenai profil virulen *V. cholerae* 01 dalam haiwan akuatik dari Kelantan dan Pahang. Penting untuk mencirikan isolat dari kawasan ini untuk lebih memahami status semasa kemunculan *V. cholerae* 01.

Kata kunci: *Vibrio cholerae*, penyakit kolera, tindak balas rantai polimerase, pengesanan molekul.

CERTIFICATION

This is to certify that we have read this research paper entitled **'Molecular Detection of *Vibrio cholerae* 01 isolated from diseased seabass, *Lates Calcarifer*'** by **Nurul Lisda binti Baharudin**, and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirements for the course DVT 55204 – Research Project.

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DEDICATION

First and foremost, I would like to praise Alhamdulillah and be grateful for the health and strength granted throughout the research project, which allowed me to finish the research project successfully without any trouble.

I want to extend my deepest gratitude towards my loving parents, Baharudin bin Abdullah and Wan Maimun binti Wan Haron, who always gave me words of encouragement and utmost support throughout this process. My siblings have always been with me and provided any means necessary to help, leading me to where I am today.

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LIST OF ABBREVIATIONS

V. cholerae	-	*Vibrio cholerae*
PCR	-	Polymerase Chain Reaction
TCBS	-	Thiosulfate citrate bile salts sucrose (TCBS)
TSA	-	Tryptic Soy Agar
CTX	-	Cholera toxin
*ctx*A	-	Cholera toxin A subunit
CTXΦ	-	Cholera toxin phage
*hly*A	-	Hemolysin A
*omp*W	-	Outer membrane protein W
*tcp*A	-	Toxin-coregulated pilus A
*lol*B lipoprotein B	-	Lipoprotein outer membrane localization
NaCl	-	Sodium Chloride

CHAPTER 1

INTRODUCTION

Vibrio cholerae is a gram-negative aerobic or facultative anaerobic bacillus bacterium. The bacteria appeared in a comma-shaped size range from 1-3 µm in length by 0.5-0.8 µm in diameter (Montero *et al*., 2023). Worldwide aquaculture production keeps rising to fulfil the increasing demand from the world's growing population for fish meals. In Malaysia, the aquaculture industry generated about 411,781 tonnes of total aquaculture production in 2019, with an estimate to be worth USD 700 million (Tan *et al.,* 2023). The fishery industry's products include deep-sea and inshore as well as inland fishing activities (Safa, 2004). Animal diseases have the potential to negatively impact aquaculture production by lowering the essential resources, physical output, or unit value of a production process, its efficiency, and directly affecting human health (Marc C.J. Verdegem *et al*., 2023). Ultimately, this could result in financial losses for the aquaculture industry. This study will be focused on *Vibrio cholerae* bacteria that have been isolated from diseased seabass, *Lates calcarifer* with the aim to determine the molecular group of *Vibrio cholerae*. In Malaysia,in the early 1990s, cultured marine fish were said to have been impacted by vibriosis, resulting in a USD 7.4 million loss (Bondad-Reantaso *et al.,* 2005). Meanwhile, in the case of endemic vibriosis for an Asian seabass floating net-cage on Peninsular Malaysia's west coast, the expenses of treating and diagnosing the diseased seabass were calculated to be USD 0.24 per tail (Mohd Yazid *et al.,* 2021).

Vibriosis is generally referred to as a systemic bacterial infection caused by the bacteria from the family of *Vibrionaceae* which are frequently associated with both wild and

farmed marine fishes (Mohamad *et al.,* 2019). The causative agent of pandemic cholera, *Vibrio cholerae*, is widespread in freshwater and marine habitats. This species' natural reservoirs are copepods and chironomids. It is currently uncertain how *V. cholerae* is spread throughout the world (Halpern & Izhaki, 2017). However, Halpern et al. (2008) presented the theory that there might be involvement of waterbirds that also may feed on these invertebrates and consume fish as well, hence acting as the reservoirs and vectors of *V. cholerae*. Thirty fish species have been shown to harbour *V. cholerae* that include 22 from freshwater and 9 from marine (Halpern & Izhaki, 2017).

Vibrio cholerae possesses a flagellar H antigen and a somatic O antigen (Handa, 2019). The serogroup classification of *V. cholerae* is based on the composition of the O antigen of Lipopolysaccharides (LPS). Strains in the O1 serogroup are further divided into three serotypes which are known as Ogawa, Hikojima, and Inaba. Also, serogroup O1 is classified into the Classical and El Tor biotypes based on phenotypic and genetic markers. The Classical and El Tor biotypes' genetic traits have been associated with the increasing number of *V. cholerae* strain reports during the previous 20 years, which has resulted in the creation of hybrid or variant strains (Montero *et al.,* 2023). To summarize, *V. cholerae* O1 and *V. cholerae* O139 are the principal ones associated with epidemic cholera worldwide. Of these, serogroups O1 and O139 have been linked to cholera epidemics, whereas serogroups non-O1/non-O139 are known to cause sporadic outbreaks (Sack *et al.,* 2003).

1.1 Research problem statement

Globally, cholera causes 95,000 deaths annually. In addition, it infected 2.9 million individuals annually, causing mild to severe infections (CDC, 2021). In Malaysia, the cholera case incidence rate in the year 2021 is at 0.35 per 100.000 populations with no mortality rate (Malaysia Health Fact, 2021). Therefore, this made cholera a public health threat worldwide including Malaysia. In Malaysia, there is a lack of study on *Vibrio cholerae* molecular group classification done for data reference that depict current *Vibrio cholerae* status in the aquaculture industry. Due to the lack of information on this study subject, the public awareness towards this zoonotic disease cannot be emphasized since insufficient data available to be used for justification. Moreover, there is also lack of study on *Vibrio cholerae* genotypes using the PCR method in the country in identifying clusters, understanding phylogeny, tracking spread and transmission, and tracing the origin of the source of infection. Adequate knowledge is essential for control over diseases linked to *Vibrio cholerae*.

1.2 Research question

Can *Vibrio cholerae* 01 be determined by the PCR method?

1.3 Research hypothesis

Vibrio cholerae 01 can be differentiated from non 01 using PCR.

1.4 Research objective

To determine *Vibrio cholerae* 01 from diseased seabass.

1.5 Significance of expected research findings

The emergence of new strains within bacterial populations has always posed significant challenges, and *Vibrio cholerae* is no exception. This bacterium persists globally and evolves over time, exhibiting a dual characteristic of surviving in both environmental (less infectious) and infectious states. Its ability to adapt by acquiring or modifying virulence genes and mobile genetic elements complicates efforts to understand its pathogenesis. Factors such as increased poverty, declining water quality, overuse of antibiotics, and climate change further promote genetic exchange, enhancing both virulence and antibiotic resistance. (Sharma et al., 2021). No published literature has been found describing the virulent profile of *V. cholerae* specifically in the 01 *V. cholerae* group of aquatic animals in Kelantan and Pahang. Characterization of the isolates is important to better understand the current status for pathogenesis of *V. cholerae* 01 from these areas.

1.6 Scope of study

The study was conducted in Kota Bharu, Kelantan. *Vibrio cholera* isolates were obtained from archive samples stored in the Trypticase Soy Broth (TSB) (Oxoid, England) which had been added with 50% glycerol stocks in a -80˚C freezer in the Zoonotic Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan.

CHAPTER 2

LITERATURE REVIEW

2.1 *Vibrio cholera* in aquaculture and public health

Vibrio cholerae is a gram-negative, comma-shaped bacterium responsible for cholera. It is highly motile, possessing a single polar flagellum, and is primarily transmitted via the fecal-oral route, typically through contaminated water, food, fomites, or direct contact with infected individuals. This species exhibits a notable tolerance to salt, requiring sodium chloride for optimal growth. *V. cholerae* is commonly found in natural aquatic environments, particularly coastal and brackish waters contaminated with human or animal waste (Ojeda Rodriguez & Kahwaji, 2020). Such conditions contribute to the occasional transmission of the pathogen through the consumption of aquaculture products, including shellfish (Ojeda Rodriguez & Kahwaji, 2020). There are three main *Vibrio* species that pose a potential pathogenic risk to humans which include *V. cholera, V. parahaemolyticus, and V. vulnificus*. These *Vibrio* spp. are responsible for the elevation of seafood-borne infections that occur throughout the world as they can contaminate the seafood consumed by people (Mougin *et al.,* 2020). Human infection with *V. cholerae* begins with ingestion of contaminated food or water with the bacteria. *V. cholerae* O1 and *V. cholerae* O139 produces enterotoxin that promotes the efflux of fluid and electrolytes into the lumen of the small intestine (Percival & Williams, 2014). Humans infected by these strains commonly will manifest the clinical signs of watery diarrhea with electrolyte values that are isotonic to plasma levels (Handa, 2019).

2.2 Diagnostic methods for *Vibrio cholerae* detection

The first validated diagnostic test for detecting *Vibrio cholerae* was Bandi's test, which identifies the pathogen within 3 to 4 hours (Keddy *et al.,* 2013). This method involves using cultures grown in alkaline peptone water (APW) mixed with large volumes of *V. cholerae* O1 antiserum to induce agglutination. However, the test yielded 38% fewer positive results compared to the culture method and was inconsistent when other organisms were present in the sample. The radial passive immune hemolysis assay, while effective for screening large numbers of cholera toxin (CT)-producing *V. cholerae* strains is limited by its complexity. It requires a specialized medium, prolonged incubation, anti-CT antibodies, and serum complement, making it cumbersome to use. The rapid visual test (RVT) relies on an enzymatic reaction involving lysates of *V.cholerae* O1, which alters a redox indicator (Ramamurthy *et al.,* 2019). RVT can distinguish between *V. cholerae* O1 and non-O1 strains, but its effectiveness is influenced by the medium composition. *Vibrio cholerae* O1 was identified from stool samples using a multi-step process (Huq *et al*., 2012). The procedure involved initially fixing the sample on a solid agar medium, followed by cultivation in a liquid medium for 2 to 4 hours, and finally performing agglutination with O1 antiserum within an hour. This method was time-consuming and required several steps. To improve detection, monoclonal antibodies (MAbs) targeting the lipopolysaccharide (LPS) of V. cholerae O1 have been developed and used in whole-cell enzyme-linked immunosorbent assays (ELISA) or agglutination tests.

2.3 Molecular detection of virulent genes in *Vibrio cholerae*

The primary virulence factors of the toxigenic *Vibrio cholerae* serotypes O1 and O139 are the cholera toxin (CTX), encoded by the *ctxA* and *ctxB* genes, and the toxin-coregulated pilus (TCP), which facilitates bacterial motility and adherence (Lindmark, 2009). Additionally, the *hlyA* genes, which encode *V. cholerae* hemolysin, represent a highly conserved genetic element in *Vibrio cholerae* (B. Lindmark, 2009). Moreover, the *hly*A virulence gene has also been associated with causing tissue damage to the infected host (Sukrama *et al.,* 2017). In addition to the *hlyA* gene, *toxRVC*, a regulatory gene, is also a characteristic virulence gene of *Vibrio cholerae*, playing a crucial role in the expression of its virulence factors. (Deng *et al.,* 2020). Meanwhile, the *Vibrio cholera* non-O1 and non-O139 is the non-toxigenic type whose lack of the major virulence factor CTX by 95% which is responsible for the manifestation of the cholera clinical signs such as acute explosive and watery diarrhea (Chakraborty *et al.,* 2000). In PCR detection of *Vibrio cholerae*, *ctx*A is the common target gene, which encodes the A subunit of cholera toxin. This gene is a hallmark of toxigenic strains of *Vibrio cholerae* (O1 and O139 serogroups), which are responsible for cholera disease. PCR assays designed for detecting *ctx*A genes are highly specific and sensitive for identifying toxigenic *V. cholerae* strains in clinical and environmental samples. (Martín-Rodríguez *et al.,* 2022). The specific primers that recognize all serogroups of *V. cholerae* were designed based on the *lol*B gene, while the primers for detecting the virulence gene *ctx*A were based on the sequence of the *ctx* operon. (Chua *et al*., 2011)

CHAPTER 3

METHODOLOGY

3.1 Bacterial sample

Vibrio cholera isolates were obtained from archive samples stored in the Trypticase Soy Broth (TSB) (Oxoid, England) with 50% glycerol stocks in a -80˚C freezer in the Zoonotic Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. The isolates were revived in Tryptic Soy Agar, TSA (Oxoid, England), and were incubated at 35°C for 24 hours. Table 1 shows the list of *V. cholerae* isolates that were used in this study.

Table 1: *Vibrio cholerae* sample from Kelantan and Pahang.

No.	**Bacterial isolates**	**Sampling site**
1.	VCK 1	Laguna Tumpat, Kelantan
2.	VCK 2	
3.	VCK 3	
4.	VCK 4	
5.	VCK 5	
6.	VCK 6	
7.	VCK 7	
8.	VCP 8	Kuala Pahang,

9.	VCP 9	Pahang
10.	VCP 10	
11.	VCP 11	
12.	VCP 12	
13.	VCP 13	
14.	VCP 14	

3.2 DNA isolation

The 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 then immediately cooled on ice for 15 min and centrifuged at 12000 rpm for 5 min. The clear supernatant was transferred into a new 1.5ml centrifuge tube and kept at -20C for further use. The tube had been centrifuged to sediment the debris and the supernatant was collected.

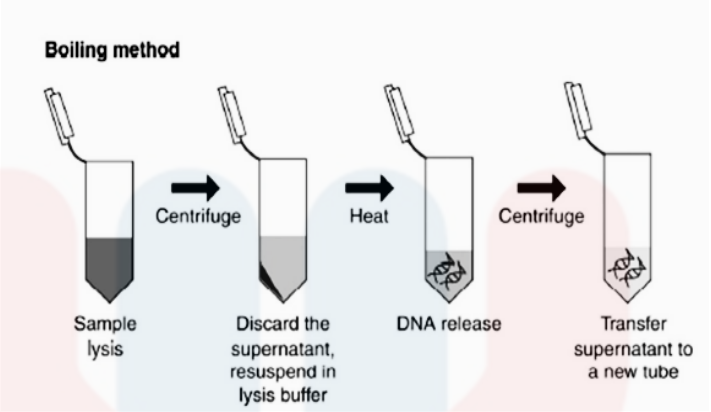


Figure 1. Boiling method (Barbosa et al., 2016)

3.3 PCR of *Vibrio cholerae*

PCR protocol for designated *V.cholerae* specific primer conducted for identification of *V. cholerae* using forward primer; TTGAGCTGGGTGTTCAAGTG, reverse primer; AGCTTATCATACAGTTGTGCA starting with initial denaturation at 95°C for 3 min, 30 cycles of denaturation, 95°C for 30 s, annealing temperature for 30 s at either 54°C, 55°C, or 60°C (Table 3.4), extension at 72°C for 30 s, and a final extension at 72°C for 5 min.

3.4 PCR of *Vibrio cholerae* 01 and non 01

The primers that were used in this study were selected based on the previously conducted research. There were 2 sets of primers and specific PCR protocols for each primer used in this study based on table 2. Each PCR master reagent mix comprises 12 pmol of each primer for the *lol*B gene, 16 pmol of each primer for the *ctx*A gene, 4 pmol of each primer for the IC. The reagents were used for a single PCR reaction using T100 Thermocycler (Bio-Rad, USA) were master mix (PCR buffer, Mg+, Cl-, DNTP, Taq polymerase) with the volume of 12.5μl, forward primer 1μl, reverse primer 1μl, nuclease free water 8.5μl, and DNA template 2μl. The primers that are used for the detection of *Vibrio cholerae* genes are listed in table 2. Reagents concentration per reaction tube: 1.5 mM MgCl2, 0.125 mM dNTPs (Qiagen), 0.5

mM each primer, 1 U Taq polymerase (Taq Platinum High Fidelity, Invitrogen). DNA used was 1ng per reaction tube. The initial denaturation step at 95 ˚C for 2 min, 35 cycles of denaturation at 95 ˚C for 30 s, annealing at 60 ˚C for 30 s and extension at 72 ˚C for 30 s, followed by an extra annealing step at 60˚C for 30 s and a final extension step at 72 ˚C for 5 min.

Table 2: The list of primers and PCR protocol for detection of 01 *Vibrio cholerae* gene

Gene	Direction	Primer sequence	Protocol	Reference
lolB	Forward	GTGCATCTTGGTCGC GGTAG	ID: 95˚C for 2mins D: 95˚C for 30 sec A: 60˚C for 30 sec E: 72˚C for 30 sec D + A+ E: 35 cycles EA: 60˚C for 30 sec FE:72˚C for 5 min	(Chua *et al*., 2011)
	Reverse	GGCAATCACACCAAG TCACTC		
ctxA	Forward	AACTCAGACGGGATT TGT TAGGC		
	Reverse	TCTCTGTAGCCCCTA TTACGATGT		
Internal control	Forward	GGTACCTTTACCACA AGTTACAC		
	Reverse	TTCTCGATCGTCTTCTGG AT		

ID: initial denaturation; D: denaturation; A: Annealing; E: extension; EA: extra extension FE: final extension.

3.5 Agarose gel electrophoresis

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

3.6 Sequencing and analysis

PCR products were sent to Apical Sdn. Bhd. for further sequencing. The sequence information then was compared with Genbank using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.7 Ethical considerations

This study does not require any ethical approval.

CHAPTER 4

RESULTS

4.1 Bacterial Culture

Primary culture was performed on Tryptic soy agar (TSA), the results for bacterial colony morphology are shown and described in the supplementary document in appendix. Twelve out of 14 isolates were suspected as *V. cholerae* from bacterial culture on TCBS and Chromagar. *Vibrio cholerae* colony characteristics on TSA, TCBS & CHROMagar. *Vibrio bacteria* can be identified based on the color, shape, and size of colonies that grow on TCBS media. Typical *V. cholerae* will show a positive catalase-oxidase test reaction. In this study, the isolates were positive for catalase and oxidase, which meet the characteristics described. Meanwhile, on TCBS agar, initially, TCBS agar appears green; after 18 to 24 hours of incubation, typical *Vibrio cholerae* appear as large, slightly flattened yellow colonies (2 to 4 mm in diameter) with opaque centers and translucent edges. The yellow coloration results from sucrose fermentation in the medium. *Vibrio cholerae* can sometimes appear turquoise or pale blue on TCBS (Thiosulfate-Citrate-Bile Salts-Sucrose) agar (Figure 2). These characteristics are present in 12 bacteria culture samples from KP6LY(1), KP13LY(2), KP1KG(3), LT11KG(4), LT5LG(5), KP2KY(6), KP25LY(7), KP4KY(8), LT23KG(9), LT18KG(10), KP3LY(11), and LT20LY(12). All the 12 bacterial isolates were selected for further *Vibrio cholerae* confirmation by PCR, meanwhile the rest of the samples were excluded from the molecular detection (PCR). Suggestively, sucrose non-fermenting organisms like *Vibrio parahaemolyticus* may also produce green to blue-green colonies. Colonies suspected of being pathogenic *V.cholerae* are further tested by subculturing onto selective CHROMagar for *Vibrio* species isolation and identification. 24 hours post

incubation on CHROMagar, 6 isolates show green-turquoise colonies that match the description for typical *V. cholerae.*

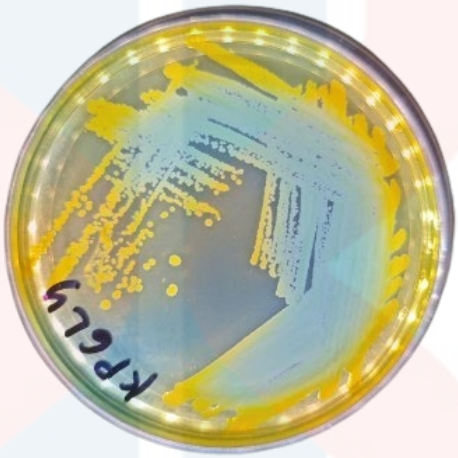


Figure 2: *Vibrio cholera* on TCBS agar showed agar turn yellow and circular colonies.

Figure 3: *Vibrio cholerae* on CHROMagar™ showed green turquoise colonies.

4.2 Confirmation of *V. cholerae* 01 using PCR

Using designated *V. cholerae* specific genes primer, six out of twelve were confirmed as *V. cholerae* (Figure 4). Consequently, all the positive *V. cholerae* samples were detected

positive for the *lol*B gene, negative *ctx*A for the gene and absent of an internal control band (Figure 5). So, for further confirmation, all the isolates were sent for sequencing. The results were confirmed as *V. cholerae* O1. The sequence information then was compared with Genbank using BLAST, and results were summarized in Table 4.

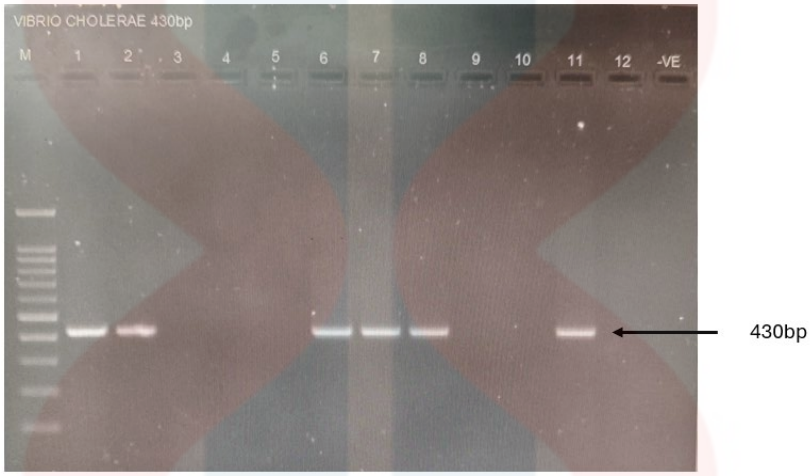


Figure 4: Six (Sample ID; 1,2,6,7,8,11) isolates of *Vibrio cholerae* detected the expected band on polymerase chain reaction with length size, 430bp with absence of Internal Control band.

Figure 5: All six isolates Non-toxigenic *V. cholerae* are detected when only amplicons of 237 bp (*lol*B gene) are present, without the presence of a 300 bp amplicon of the *ctx*A gene. Somehow, the 150 bp (IC) is also absent.

Table 4: Isolates sequencing result with 100% complete sequence query identity, location and types of study sample.

Sample ID	ID label	Query identical (%)	Location	Type of sample
KP6LY(1)	VCP 1	100	Brazil	River
KP13LY(2)	VCP2	100	Brazil	River
KP2KY(6)	VCP6	100	Japan	Feces
KP25LY(7)	VCK7	100	Japan	Feces
KP4KY(8)	CVP8	100	Democratic Republic of Congo	Feces
KP3LY(11)	VCP11	100	Brazil	River

CHAPTER 5

DISCUSSION

CHROMagar is a selective and differential medium designed for the isolation and differentiation of Vibrio species, including *Vibrio cholerae, Vibrio parahaemolyticus*, and *Vibrio vulnificus* (Lee *et al*., 2020). Typical Characteristics of *Vibrio cholerae* on CHROMagar usually appear turquoise blue depending on the strain and its ability to ferment sucrose with medium-sized, smooth, and circular colonies (Huq *et al.,* 2012). The specific turquoise coloration helps distinguish *V. cholerae* from other *Vibrio* species such as *V. parahaemolyticus* and *V. vulnificus* which appear in purple and green colonies respectively (Yeung & Thorsen, 2016). PCR demonstrated high specificity and effectively distinguished *Vibrio cholerae* from other *Vibrio* species (Lalitha *et al.,* 2008). PCR is the method of choice for detecting *Vibrio cholerae* due to its ability to amplify specific DNA sequences such as *ctx*A, *lol*B, *omp*W, and *tcp*A, which are conserved and associated with virulence. It offers exceptional sensitivity, detecting as few as 1-10 DNA copies, and high specificity through the use of primers that anneal to unique sequences in the *Vibrio cholerae* genome. PCR is rapid, delivering results within 2-4 hours compared to the 24-48 hours required for traditional culture methods. Additionally, it can detect *Vibrio cholerae* in a viable but non-culturable (VBNC) state, overcoming limitations of culture-based diagnostics. Multiplex PCR further enhances its utility by allowing simultaneous detection of multiple genes, providing comprehensive profiling of the pathogen's virulence and serogroup. Real-time PCR (qPCR) enables quantification of bacterial load, aiding in assessing infection severity and environmental contamination. Its adaptability to various sample types, including

environmental and clinical specimens, makes PCR a versatile, accurate, and efficient tool for both diagnosis and monitoring of *Vibrio cholerae* (Yan *et al.,* 2022). Previous studies have shown that bacterial species often share highly conserved housekeeping genes with substantial nucleotide similarity, which limits their effectiveness as diagnostic tools by increasing the likelihood of false-positive results. To overcome this limitation, whole genome sequencing has emerged as a more effective approach for selecting diagnostic targets. This method enables the identification of genes that can reliably differentiate between populations, strains, or closely related species (Arif *et al.,* 2021).

Cholera toxin (CT) produced by *Vibrio cholerae*, is the primary virulence factor responsible for the disease. It is encoded by the *ctx*AB operon, which is a component of the CTX genetic element. The CTX genetic element corresponds to the genome of cholera toxin phage (CTXΦ), which is integrated into the chromosome of *V. cholerae* (Ramamurthy *et al*., 2019). The disease phenotype is represented by CT A subunit, whereas the B subunit catalyses ADP (adenosine diphosphate)-ribosylation, which activates adenylate cyclase and raises intracellular cyclic adenosine monophosphate (cAMP) and serves as a vehicle to deliver subunit A to target cells causing increased intracellular cAMP, impairs salt intake and increases chloride outflow that result in clinical presentation of diarrhoea in human (Ramamurthy *et al*., 2019). The *lol*B gene of *V. cholerae* is part of the lipoprotein outer membrane localization system, which ensures proper localization of lipoproteins to the outer membrane of Gram-negative bacteria (Garrido-Maestu *et al*., 2015).

In this study, all the *V. cholerae* isolates were detected positive for the *lol*B gene, negative ctxA gene and absence of internal control band (Figure 5). For further confirmation, all the isolates were sent for sequencing and the results were confirmed to be *V. cholerae* O1. PCR test targeting the primer targeting outer membrane lipoprotein (*lol*B) and cholerae toxin

(*ctx*A) genes were used in this study to detect toxigenic *V. cholerae* and non-toxigenic *V. cholerae* of O1/non-O139 groups. For PCR result interpretation, positive non-toxigenic *V. cholerae* when amplicons of 237 bp (*lol*B gene) and 150 bp (IC) are present, without the presence of a 300 bp (*ctx*A) amplicon of the *ctx*A gene. Whereas positive toxigenic *V. cholera* when all 3 amplicons of 237 bp (lolB gene) 300 bp (ctxA), and 150 bp (IC) are present (Chua *et al.,* 2011). Contrary, based on a study conducted for *V. cholerae* detection using *lol*B gene, result showed all the *Vibrio cholerae* species belonging to O1, O139 and non O1/non O139 that positive for *lol*B PCR gave a species specific of *V. cholerae* (Lalitha *et al.,* 2008). This proved that the *lol*B gene is highly specific for *V. cholera* confirmation, but not able to differentiate between O1, O139 and non O1/non O139 groups since all the species groups mentioned possessed the *lol*B gene. This may explain the negative toxigenic *V. cholerae* from PCR, but sequencing proved otherwise with positive toxigenic *V. cholera* 01. To some extent, the matter raised concerns when from all the 6 positives, only amplicons of 237 bp (*lol*B gene) are present, without the presence of a 300 bp (*ctx*A) amplicon and 150 bp (IC) which requires further discussion.

For the absence of the Internal Control (IC) band in both PCR tests, the PCR result is considered a valid negative when only the PCR IC amplicon is observed. If all three amplicons are absent, the PCR is defined as inhibited, and the test should be repeated. (Chua *et al.,* 2011). Internal controls (ICs) are used to ensure the successful amplification and detection of clinical specimens. The nucleic acids of the IC contain primer binding regions that are identical to those of the target sequence, along with a unique probe binding region that distinguishes the IC from the amplified target nucleic acid (Rosenstraus *et al.,* 1998).

There are few possible causes for negative IC amplification. One of the factors is due to the presence of inhibitory specimens. By directly interacting with DNA or interfering with the

action of the polymerase or other components of the PCR mixture such as MgCl2, inhibitors which can be found in a variety of specimen matrices can interfere with PCR operations and eventually prevent target amplification. Heme in blood, urea in urine, and bile salts in faeces are a few examples of the inhibitors (Kermekchiev *et al*., 2009). Furthermore, some parts of widely used laboratory collection tools, like heparin, formalin, viral transport media, or swabs that contain gel or charcoal, are also known to be PCR inhibitors (Buckwalter *et al.,* 2014). Negative amplification test results do not necessarily confirm the absence of infection unless inhibitory specimens are detected. Inhibitory specimens can be identified by tracking the amplification of a second target nucleic acid, which acts as an internal control (IC). A positive signal from this second target confirms successful amplification, thus validating a negative result for the primary target. (Rosenstraus et al., 1998). However, in this study, the PCR tests were rerun but similar results obtained by the absence of the internal control band appearance as shown in Figure 4 and Figure 5.

A negative internal control (IC) result in specimens that test positive for one target suggests that competition or inhibition has reduced amplification efficiency to a level below the threshold needed to detect a low-level target. In such cases, negative results for other targets are considered invalid. The data presented in the study by Rosenstraus et al., (1998), indicate that competition will not lead to a false-negative result unless the concentration of one target exceeds 104-fold greater than the concentration of the second target.

Another possible cause is protease activity. If protease remains active, it can degrade the polymerase during the PCR reaction, leading to a failed PCR run (Lorenz, 2012). Without IC reaction for reference, this failure could be mistaken for a standard negative result. Another possible cause is user error during the sample preparation process (Kalle *et al*., 2013). Deviations in the IC reaction may occur when using an incorrect lysis buffer, hydrating PCR

tablets with the wrong volume of lysate, or accidentally removing the PCR tablet from the tube during pipetting or mixing (Kalle *et al.,* 2013). Hence, a positive IC reaction is crucial to validate the conditions for successful amplification even for negative samples. Without this comparison, distinguishing between a failed run and a true negative sample would be challenging.

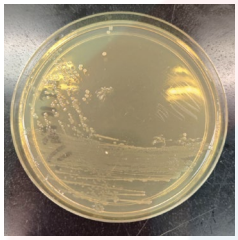
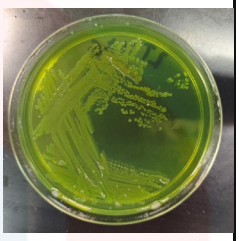
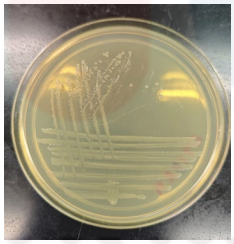
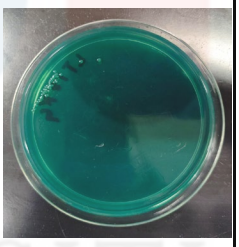
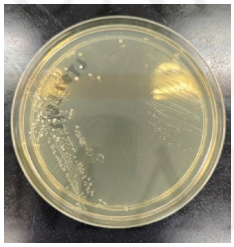
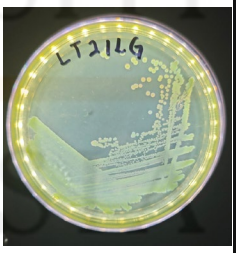
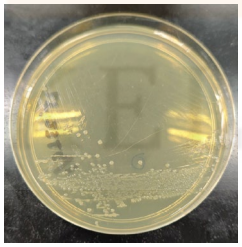
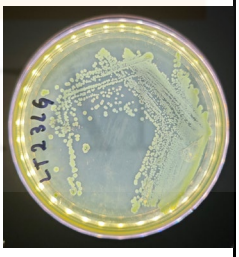
CHAPTER 6

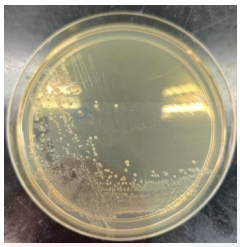
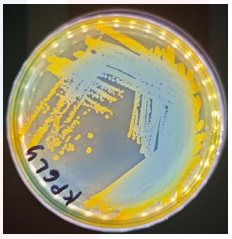
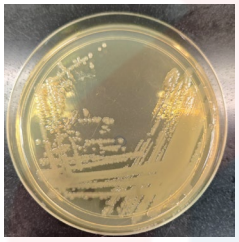
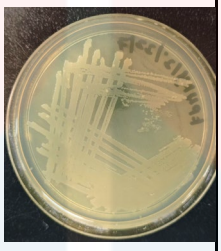
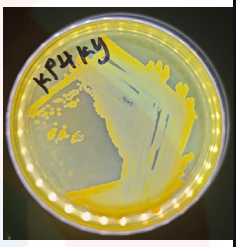
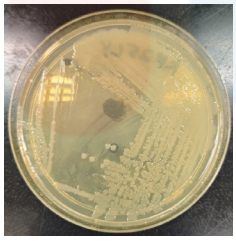
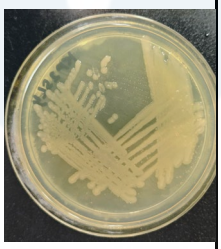
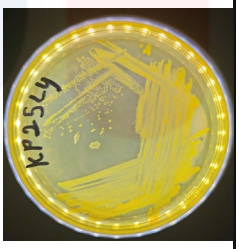
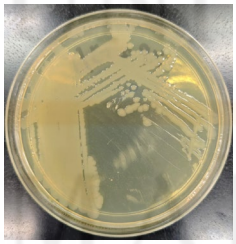
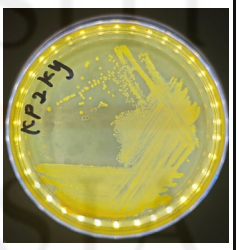
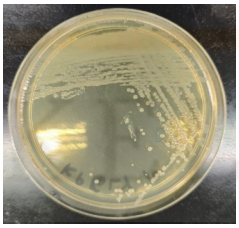
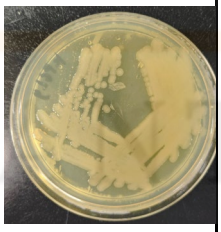
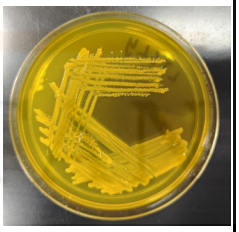
CONCLUSION AND RECOMMENDATION

In this study, PCR confirmed six out of twelve positive isolates positive for non-toxigenic *V. cholerae* which all originated from Kuala Pahang. Contrary to sequencing confirmed the isolates as *V. cholerae* O1 and the possible causes as in discussion. This research paper information will undoubtedly serve as a valuable reference and guideline for future studies on *V. cholerae* investigations, as limited data are currently available in Malaysia. Hence, further specific and comprehensive studies regarding *V. cholerae*, such as the molecular detection and characterization of circulating strains in Malaysia, are required. Lastly, it is critically necessary to improve laboratory diagnostic methods for *V. cholerae* detection, as well as the reporting systems for clinical manifestations of cholera, such as acute watery diarrhea and dehydration, to support public health efforts and inform effective interventions.

7.0 APPENDICES

Table 5: Bacteria culture results from TSA and TCBS agar and catalase-oxidase test.

Sample	TSA 18/7/24 (primary culture)	TSA 22/7 (secondary culture)	TCBS 21/7/24	TCBS bacteria colony description	Oxidase	Catalase
LT11KG (VCK4)		No secondary culture		Agar turn yellow Translucent pinpoint colony	+ve	+ve
LT18KG (VCK9)		No secondary culture		No agar color change Few white colony Green color	+ve	+ve
LT21LG (VCK10)		No secondary culture		Agar turn yellow Colony color green turquoise with dark green centre	-ve	+ve
LT23LG (VCK9)		No secondary culture		Agar turn yellow Circular pin point Green turquoise colony with dark green centre	+ve	+ve

LT20LY (VCK12)		No secondary culture		Agar turn yellow Colony color green turquoise Circular pinpoint	+ ve	+ ve
KP6LY (VCP1)		No secondary culture		Agar turn yellow Circular cream and green turquoise colony	- ve	- ve
KP4KY (VCP8)				Agar turn yellow Circular cream colony Mixed with green turquoise	+ ve	+ ve
KP25LY (VCP7)				Agar turn more yellow Cream color pinpoint colony	+ ve	+ ve
KP2KY (VCP6)				Agar turn more yellow Pinpoint colony with cream color	+ ve	+ ve
LT6LY (VCK12)				*Discarded due different color on chromagar of purplish colonies TCBS:Agar turn from green to yellow Sz:0.1mm	+ ve	+ ve

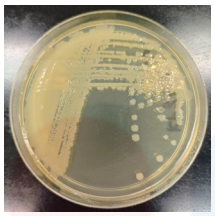
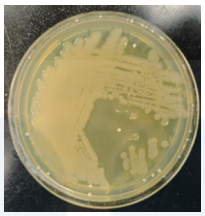
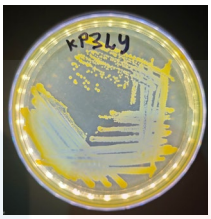
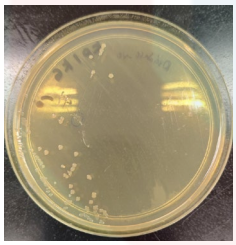
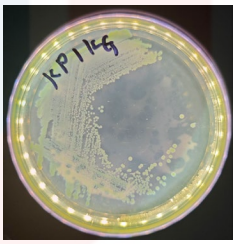
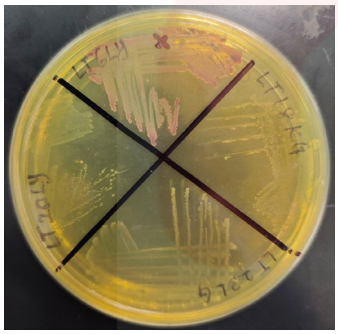
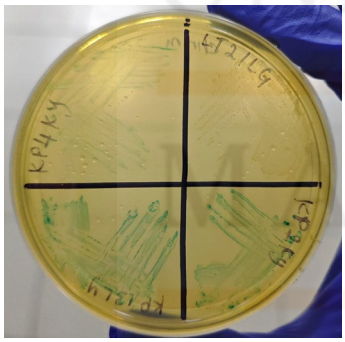
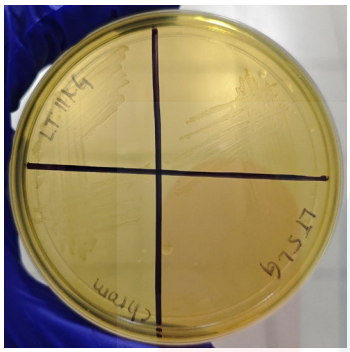
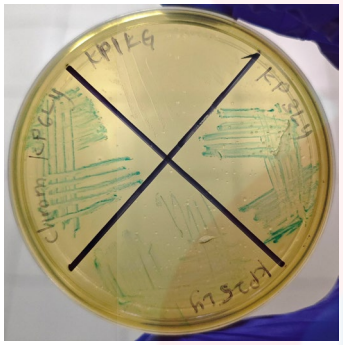
KP3LY (VCP11)				Agar turn yellow Circular pinpoint colony Cream color with mixed green turquoise	+ ve	+ ve
KP1KG (VCP3)		No secondary culture		Agar turn yellow Circular pinpoint green turquoise with dark green centre	- ve	+ ve

Table 6: Bacterial culture results from Chromagar agar.

CHROMagar 21/7/24	LT6LY	LT18KG (VCK10)	LT23LG (VCK9)	LT20LY (VCK12)
	mauve color colony *excluded for research	Cream colony	Cream colony	cream colony
CHROMagar	LT21LG	KP2KY (VCP6)	KP13LY (VCP2)	KP4KY (VCP8)
	Cream pinpoint colony *excluded for research	green turquoise colony	green turquoise colony	green turquoise colony
CHROMagar	LT11KG (VCK4)	LT5LG (VCK5)		

	white cream colony *excluded for research	white cream colony		
CHROMagar	KP6LY (VCP1)	KP1KG (VCP3)	KP3LY (VCP11)	KP25LY (VCP7)
	green turquoise colony	colorless colony	green turquoise colony	green turquoise colony

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