DETECTION OF VIRULENCE GENES IN *VIBRIO CHOLERAE* ISOLATED FROM DISEASED SEABASS, *LATES CALCARIFER* IN EAST COAST, MALAYSIA

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CERTIFICATION

This is to certify that we have read this research paper entitled **'Detection of Virulence Genes in *Vibrio cholerae* Isolated from Diseased Seabass, *Lates Calcarifer* in East Coast, Malaysia'** by Ilya Anaty Faiqah binti Ismail, and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course DVT 5436-Research Project.

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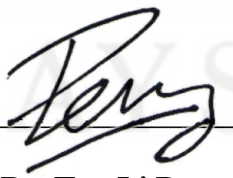

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List of Abbreviation

HGT	Horizontal Gene Transfer
PCR	Polymerase Chain Reaction
TSA	Trypticase Soy Agar
TCBS	Thiosulfate- citrate-bile salts-sucrose Agar
CTX	Cholera Toxin
TCP	Toxin Co-regulated Pilus

ABSTRACT

An abstract of the research paper was presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement for the course DVT 5436 – Research Project.

Vibrio cholerae is the aetiological agent for cholera disease, a major public health concern due to the zoonotic ability of the infection. *Vibrio cholerae* encodes two major virulence factors which is the cholera toxin (CT) and toxin co-regulated pilus (TCP). It also encodes other virulence factors, such as the hemolysin encoded by the *hly*A gene and *Tox*R$_{VC}$. This study was conducted to detect the presence of virulence genes in *Vibrio cholerae* in East Coast Malaysia. A total of thirty-eight isolates of Vibrio cholera were revived from glycerol stock stored in -80˚C freezer. Blood hemolysis test was done by inoculating the bacterial samples on 5% sheep blood agar and incubated at 35˚C for 24h. The boiling method was done to extract the bacterial DNA. Then, a Polymerase chain reaction (PCR) was done to detect the presence of twelve types of virulence genes, namely *tdh*, *trh-tdh*, *tlh*, *vvh*a, *chi*A, *lux*R, *tox*R(Vh), *tox*R(Vc), *hly*A, cola, VcgEP2 and VcgP1 Interestingly, 95% (19/20) of the *Vibrio cholerae* isolated from Kuala Pahang showed beta hemolysis. The bacteria isolates were grouped into 10 groups of virulence genes frequency. Group I consist of *Tox*R$_{VC,}$ *lux*R, *hly*A, and *col*A were the most abundant and detected in 70% (14/20) of *Vibrio cholerae* isolates from Pahang. The environmental condition and horizontal gene transfer might be responsible for the presence of virulence genes in *Vibrio cholerae.*

Keywords: *Vibrio cholerae, Cholera, Virulence factors, hlyA gene, ToxR$_{VC}$ gene*

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 5436 – Projek Penyelidikan.

Vibrio cholerae adalah agen etiologi bagi penyakit kolera yang menjadi sebuah kebimbangan bagi kesihatan awam disebabkan oleh kebolehannya untuk menjangkiti manusia. *Vibrio cholerae* mempunyai dua faktor virulensi utama iaitu *cholera toxin* (CT) dan *toxin co-regulated pilus* (TCP). Ia juga mempunyai faktor virulensi tambahan lain seperti *hemolysin* yang dikod oleh gen *hly*A dan juga gen virulensi lain seperti *Tox*R$_{VC}$. Kajian ini dijalankan bagi mengesan kewujudan gen virulensi pada *Vibrio cholerae* di Kawasan Pantai Timur, Malaysia. Oleh itu, sejumlah 38 sampel *Vibrio cholerae* diambil daripada stok gliserol yang disimpan pada suhu sejuk beku -80˚C. Ujian hemolisis juga telah dilakukan dengan menginokulasi sampel-sampel bakteria pada 5% agar darah bebiri yang kemudiannya diinkubasi pada suhu 35˚C selama 24 jam. DNA bakteria diekstrak melalui metode pendidihan. Seterusnya, *Polymerase chain reaction* (PCR) dilakukan bagi mengesan kewujudan dua belas gen virulensi daripada sampel yang diperolehi iaitu, gen *tdh, trh-tdh, tlh, vvh*a, *chi*A, *lux*R, *tox*R(Vh), *tox*R(Vc), *hly*A, cola, VcgEP2 dan VcgP1. Menariknya, hasil daripada kajian ini mendapati 95% (19/20) *Vibrio cholerae* yang diperolehi daripada Kuala Pahang adalah beta hemolisis bagi ujian hemolisis darah. Isolat bakteria ini dibahagikan kepada 10 kumpulan mengikut kekerapan gen virulensi. Kumpulan I terdiri daripada gen *Tox*R$_{VC,}$ *lux*R, *hly*A dan *cola* yang merupakan gen paling banyak dikesan pada 70% (14/20) *Vibrio cholerae* yang diisolasi daripada Pahang. Keadaan persekitaran dan juga *horizontal gene transfer* (HGT) merupakan antara faktor yang mempengaruhi kewujudan gen virulensi pada *Vibrio cholerae.*

Kata kunci: *Vibrio cholerae, Penyakit Kolera, faktor virulensi, gen hlyA, gen ToxR$_{VC}$*

1.0 Introduction

Asian seabass culture was initiated in Thailand in the 1970s, which then expanded to neighboring countries such as Malaysia, Philippines, Taiwan, Vietnam, Indonesia, and, Singapore (FAO, 2020). In Malaysia, seabass is commonly cultured in floating net cages, ponds, tanks, and enclosures (FAO, 2020). Based on the Economic Transformation Program (ETP) 2010, sea bass production in aquaculture was listed under this program with the aim to increase the sea bass production and become more productive, competitive, and knowledge intensive (PEMANDU, 2010). However, aquaculture production has decreased by 10.3% to almost 290,000 tons from 324,000 tonnes in 2017 (DOSM, 2019). Therefore, to ensure the success in the development of the aquaculture, Department of Fisheries Malaysia (DOFM) has introduced several incentives such as a training for the farmer to develop their skills, subsidies in the form of farm equipment and infrastructure as well as founding a fish seed centers (Aripin *et al.*, 2019).

The risk of disease occurrence in Malaysia is directly proportional to the demand for fisheries products due to intensifying the culture system to meet the demand (Mohamed et al., 2000). In the aqueous environment, aquatic animals are constantly challenged by a wide variety of pathogen and antimicrobial load carried by slimy layers on the skin surface, gastrointestinal tract, and gills. When the fish is physiologically disturbed due to an unfavorable environment, invasion of bacteria occurs and leads to disease formation in fish (Austin, 2011).

Vibriosis is one of the most common infections in marine fish. Certain species of *Vibrio*, such as *Vibrio aginolyticus* and *Vibrio harveyi*, may cause infection where the infected fish will exhibit clinical signs such as skin ulceration, scale drops on the

abdomen, and necrosis of the caudal fin (Mohd Yazid *et al.,* 2021). This disease was one of the factors contributing to the fall of seabass production in 2018. Vibriosis was also reported to cause global losses in aquaculture industries (Wei *et al.,* 2014).

On the other side, there are several *Vibrio* spp. that pose a threat to public health too due to the ability of the disease to be transmitted from animal to human. There are three main species of *Vibrio* spp. that pose a potential pathogenic risk to humans: *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 (Jusserand *et al.,* 2017). Ingestion of food contaminated with vibrio is linked with gastrointestinal infections and septicemia. For example, *Vibrio cholera* infection can occur through ingesting food or water contaminated with the bacterium, primarily via faeces or vomitus of infected persons, directly or indirectly. *Vibrio cholera* has been shown to cause acute watery diarrhea in humans in the form of an occasional outbreak or sporadic outbreak, thus, it was declared a public health problem in Malaysia (Vadivelu *et al.,* 2000).

Each year cholera is responsible for the cause of death of 95,000 people throughout the world. It also infected 2.9 million people yearly, causing them to develop mild or severe illnesses (CDC, 2021). In Malaysia, a statistic by Malaysia Health Fact has shown that the cholera case incidence rate in the year 2021 is at 0.35 per 100,000 populations with a mortality rate of 0.00 (Malaysia Health Fact, 2021). Therefore, this made cholera a public health threat worldwide, not only in Malaysia.

2.0 Research problem

In Malaysia, the main cultured brackish water aquaculture fish species were grouper (*Epinephelus fuscoguttatus*), red snapper (*Lutjanus argentimaculatus*), and seabass (*Lates calcarifer*) (Wan Norhana *et al.,* 2020). Following the expansion of these industries was the increment of vibriosis among the fish (Najiah *et al.,*2002).

In addition to the elevation of the seafood production, it has also dramatically increased the zoonotic infection, thus, leading to public health concerns. The increase in the disease transmission might be due to the importation of seafood contaminated with pathogenic *Vibrio* spp. such as *Vibrio cholera* and *Vibrio parahaemolyticus*. *Vibrio cholera* infection can lead to various clinical signs such as vomiting, tachycardia, and watery diarrhea followed by dehydration. However, despite the risk of zoonotic disease by pathogenic *Vibrio* spp., there was only limited data regarding virulence genes in *Vibrio* spp. isolated from seabass in East Coast, Malaysia. Therefore, this study was conducted to fill the knowledge gap in these issues.

3.0 Research question

Do virulence genes present in *Vibrio spp*. isolated from the seabass in East Coast, Malaysia?

4.0 Research hypothesis

The virulence genes are detected in *Vibrio* spp. isolated from the seabass in East Coast, Malaysia.

5.0 Objectives

To determine the presence of virulence genes of *Vibrio spp.* in East Coast, Malaysia.

6.0 Literature review

6.1Aquaculture in Malaysia

Aquaculture in Malaysia began in the 1920s with the extensive polyculture of Chinese carps in ex-mining pools (Tim *et al.*, 2013). However, as of 2009, Malaysian fisheries' performance is still dependent on capture as aquaculture sources are still low at 25% (Tim *et al.*, 2013). In Malaysia, caged aquaculture is one of the most popular methods used in aquaculture, especially within an area with a sheltered coast or estuary (LKIM, 2020). The type of fish species suitable for fish farming is seabass (*Lates calcarifer),* snapper (*Lutjanus johni),* and grouper (*Epinephelus taurina)* (LKIM, 2020). Figure 1 shows a pie chart of common marine species in cage aquaculture in Malaysia.

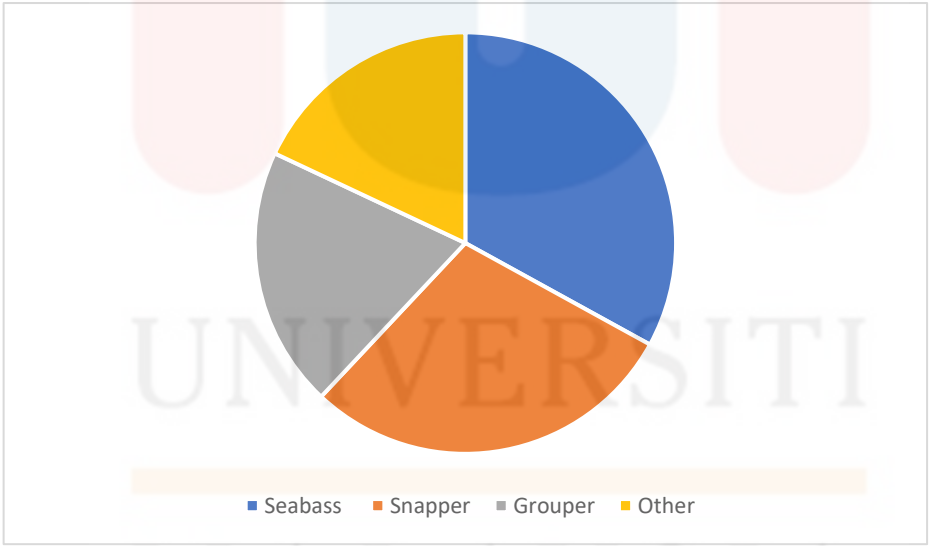

Figure 1: Cultured species in marine cage aquaculture in Malaysia (Tim *et al.*, 2013).

6.2Vibriosis in fish

In Malaysia, the main problem which inhibits the development of aquaculture activities is the fish diseases though it is also a worldwide problem (Wei *et al.*, 2014). Most of the diseases are caused by bacterial pathogens followed by viruses, parasites, and fungi which are likely to infect cultured fish. Vibriosis, which is caused by *Vibrio spp.,* is one of the most common fish diseases which threaten the marine aquaculture farm in Malaysia and worldwide (Wei *et al.*, 2014).

Vibrio spp. is a ubiquitous bacterium that usually lives in coastal and estuarine water bodies. It can be isolated from seawater, fish, and shellfish. According to research, most of the *Vibrio spp.* that have been associated with diseases in animals and humans often possess virulence factors, which are not available or present in the environmental *Vibrio* (Mohamed *et al.*, 2019). However, they are a probability of virulence genes transfer between pathogenic *Vibrio* to environmental *Vibrio* as the *Vibrio* possess a highly plastic genome, thus, leading to elevation of the pathogenic strain of *Vibrio spp*. (Xu Y *et al.*, 2017).

Vibrio parahaemolyticus is one of the most common *Vibrio spp.* isolated globally from the coastal and estuarine water bodies (Faja *et al.*, 2019). *Vibrio parahaemolyticus* is a gram-negative halophilic bacterium that can be found abundantly in environments (Alagappan *et al.*, 2010). Gastrointestinal infection due to ingestion of contaminated food *with Vibrio parahaemolyticus* has been reported. The number of cases reported is in the coastal areas where the amount of seafood consumption is high (Ralston *et al.*, 2011). The virulence factor of *Vibrio parahaemolyticus*, which contributes to its pathogenicity, includes its ability to encode the thermostable direct hemolysin (*tdh*) with *tdh*-related hemolysin (*trh*) (Faja *et al.*, 2019).

Besides *Vibrio parahemolyticus*, *Vibrio cholera* is also one of the most concerning species due to its ability to cause cholera disease. *Vibrio cholera* can be further divided into more than 200 serogroups based on its O antigen. However, only serotype O1 and O139 were said to be responsible for the sporadic cases of the seafood-borne outbreak in humans.

6.3 Detection of virulence genes in *Vibrio* spp.

Vibrio cholera serotype O1 and O139 are the toxigenic types, where the important virulence genes are the cholera toxin (CTX) with the *ctx* genes of *ctx*A and *ctx*B and the toxin-coregulated pilus (TCP), which is responsible for the bacteria mobility and adherences (Lindmark, 2009). In addition, the virulence *hly*A genes which encode the *Vibrio cholera* hemolysin is a highly conserved genetic element for *Vibrio cholera* (B. Lindmark, 2009). Moreover, the *hly*A virulence gene also has been associated to cause tissue damage to the infected host (Sukrama *et al.*, 2017). Besides the *hlyA* gene, $toxR_{VC}$ which is the regulon, is also a typical virulence gene for *Vibrio cholerae* are responsible for expressing the virulence factors in *Vibrio cholera* (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 are responsible for the manifestation of the cholera clinical signs such as acute explosive and watery diarrhea(Chakraborty *et al.*, 2000).

7.0 Materials and methods

7.1 Bacterial collection and blood hemolysis test

The collection of *Vibrio spp.* will be obtained from the Aquatic Animal Health Lab, Faculty of Veterinary Medicine. The bacteria were stored in 50% glycerol stock. The *Vibrio cholera* samples were revived on TSB with 1.5% NaCl and incubated at 35℃ for 24 h. Thirty-eight (38) isolates of *Vibrio cholera* were chosen for further study. Table 1 shows the list of *Vibrio* spp. used in this study.

For blood hemolysis test, all isolates were inoculated on 5% sheep blood agar (BML, Malaysia) and incubated at 35℃ for 24 h.

Table 1: List of Vibrio cholera used in this study.

No.	Bacteria isolates	Location
1	VCK 1	
2	VCK 2	
3	VCK 3	Laguna Semarak,
4	VCK 4	Kelantan
5	VCK 5	
6	VCK 6	
7	VCK 7	
8	VCK 8	
9	VCK 9	
10	VCK 10	
11	VCK 11	
12	VCK 12	Laguna Tumpat,
13	VCK 13	Kelantan
14	VCK 14	
15	VCK 15	
16	VCK 16	
17	VCK 17	
18	VCK 18	
19	VCP 1	
20	VCP 2	
21	VCP 3	
22	VCP 4	Kuala Pahang, Pahang
23	VCP 5	
24	VCP 6	
25	VCP 7	
26	VCP 8	

27	VCP 9	
28	VCP 10	
29	VCP 11	
30	VCP 12	
31	VCP 13	
32	VCP 14	Kuala Pahang, Pahang
33	VCP 15	
34	VCP 16	
35	VCP 17	
36	VCP 18	
37	VCP 19	
38	VCP 20	

7.2 DNA extraction

The presumptive *Vibrio cholera* colonies were selected for DNA extraction using boiling. The suspended samples were incubated at 95°C for 15 minutes and immediately cooled on ice for another 15 minutes. The extracted DNA was stored at -20°C.

7.3 Polymerase Chain Reaction (PCR) of virulence genes

The primers used in this study to confirm the *Vibrio* spp. and the presence of virulence genes were selected based on the previously conducted research. Table 2 shows the list of primers and PCR protocol used in this study. Table 3 shows the list of reagents used and the volume for a single PCR reaction using a T100 Thermocycler (Bio-Rad, USA).

Table 2: The list of primers and PCR protocol for detection of virulence genes in Vibrio spp.

Gene	Primer	Protocol	bp	Reference
tdh- (thermostable direct hemolysin)	F: GTAAAGGTCTCTGACTTTTGGAC R:TGGAATAGAACCTTCATCTTCACC	94°C for 3 min, 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, 72°C for 10 min	269	Bej *et al.*, 1999/ Nurliyana *et al.*, 2020
Trh- tdh-related hemolysin)	F: TTGGCTTCGATATTTTCAGTATCT R: CATAACAAACATATGCCCATTTCG	94°C for 2 min, 35 cycles of 94°C for 30 sec, 58°C for 1 min, 72°C for 10 min	500	Bej *et al.*, 1999/ Nurliyana *et al.*, 2020
Tlh (thermolabile hemolysin)	F: AAAGCGGATTATGCAGAAGCACTG R: GCTACTTTCTAGCATTTTCTCTGC	94°C for 5 min, 35 cycles of 94°C for 30 sec, 54°C for 45 sec, 72°C for 1 min, 72°C for 5 min	448	Woodring *et al.*, 2012/ Bej *et al.*, 1999/ Nurliyana *et al.*, 2020
vvha *(hemolysin)*	F 5-TTCCAACTTCAAACCGAACTATGAX-3 R 5-ATTCCAGTCGATGC-GAATACGTTG-3	94°C for 4 min, 35 cycles of 94°C for1 min, 60°C for 1 min, 72°C for 1 min, 72°C for 7 min	205	Bonny *et al.*, 2018
Forward VcgEP2 Reverse VCGP3 (*virulence-correlated gene)*	F 5'-CTCAATTGACAATGATCT-3' R 5'-ATTCCAGCGATGC-GAATACGTTG-3	94°C for 5 min, 35 cycles of 94°C for 40 sec, 49°C for 40 sec, 72°C for 1 min, 72°C for 7 min	278	Justin Fri *et al.*, 2017/ Rosche TM *et al.*, 2008
Forward VcgP1 Reverse VCGP3	F 5'-CTCAATTGACAATGATCT-3' R 5'-CGCTTAGGATGATCGGTG-3'	94°C for 5 min, 35 cycles of 94°C for 40 sec, 56°C	278	Justin Fri *et al.*, 2017/ Rosche TM *et al.*, 2008

		for 40 sec, 72°C for 1 min, 72°C for 7 min		
*chi*A (*encode chitinase*)	F 5'-CTCAAGGTGTTTGGGAAGATG-3' R 5'-GTTGATGCCAGTGTTGTTCG-3'	95°C for 5 min, 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, 72°C for 10 min	83	Deng Yiqin *et al.*, 2020/ Mohamad N. 2019
*lux*R (*transcriptional activator protein*)	F 5'-GTGGTTCGTCAATTCTGAAC-3' R 5'-CGAATAGTGGCCACACTTC-3'		178	Deng Yiqin *et al.*, 2020/ Mohamad N. 2019
*tox*R(Vh)	F 5'-GAAGCAGCACTCACCGAT-3' R 5'-GGTGAAGACTCATCAGCA-3'		382	Deng Yiqin/ Liao and Leano, 2008
*tox*R(Vc) *(toxin transcriptional activator)*	F 5'-ATGTTCGGATTAGGACAC-3' R 5'-TACTCACACACTTTGATGGC-3'	94°C for 3 min, 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, 72°C for 10 min	883	Nurliyana M. *et al.*, 2019/ Schroeder *et al.*, *2020*/ Deng Yiqin *et al.*, 2020
*hly*A (*E.coli α hemolysin)*	F 5'-GGCAAACAGCGAAACAAATACC-3' R 5'-CTCAGCGGGCTAATACGGTTTA-3'	94°C for 3 min, 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, 72°C for 10 min	738	Nurliyana M. *et al.*, 2019/ Deng Yiqin *et al.*, 2020/ Ruwandeepika *et al.*, 2010
*col*A (*Collagenase activity*)	F 5'-CGAGTACAGTCACTTGAAAGCC-3' R 5'-CACAACAGAACTCGCCTTACC-3'	94°C for 2 min, 35 cycles of 94°C for 30 sec, annealing for 58 sec, 72°C for 10 min	737	Di pinto *et al.*, 2005

Table 3: Reagent concentration and volumes for a single PCR reaction.

Reagents	**Volume (μl)**
Master mix (PCR buffer, Mg+, Cl-, DNTP, Taq polymerase)	12.5
Forward primer	1
Reverse primer	1
Nuclease free water	8.5
DNA template	2
Total	25

7.4 Agarose Gel Electrophoresis

Amplified PCR products were visualized on 1.5% Agarose gel and was stained with Midori green, run at 100V and 400 mA for 45 minutes. It was then photographed using gel documented system, UV Transilluminator (Bio-Rad, USA)

8.0 Result

Table 4 shows the result of the virulence genes, and the blood hemolysis results. Interestingly, 95% (19/20) of the VCP isolates showed beta hemolysis on sheep blood agar. To detect typical *tox*R_{VC} virulence gene, 5.56% (1/18) of the VCK samples was positive for *tox*R_{VC}, while 95.0%. (19 /20) of the VCP samples were positive for *tox*R_{VC}. For detection of *hly*A gene, all VCP isolates were detected with *hly*A gene and no VCK isolates were detected with *hly*A gene.

For detection of atypical genes, only *chi*A, *col*A and *lux*R genes were detected among VCP and VCK isolates with frequencies of, 13.16%, 60.53% and 42.11%, respectively.

The detection of the *lux*R gene showed 5.56% (1/18) of the VCK isolates was positive while 70.0% (14/20) of the VCP isolates were detected with *lux*R gene. While for the result of *chi*A detection, it shows that 27.78% (5/18) of the VCK isolates were positive while none of the VCP isolates were positive for *chi*A. Then, *col*A virulence gene detection shows 22.22% (4/18) of the VCK isolates were positive while 90.0% (18/20) of the VCP isolates were positive for PCR.

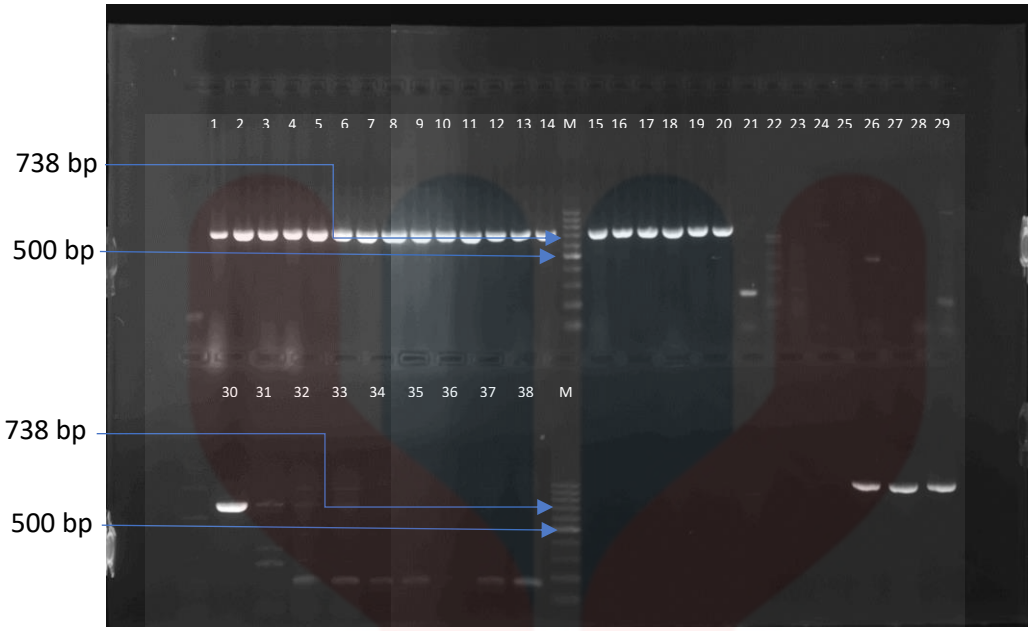

Figure 2: Detection of hlyA gene in Vibrio cholerae using agarose gel electrophoresis where M represents ladder, and well 1 to 38 represent samples.

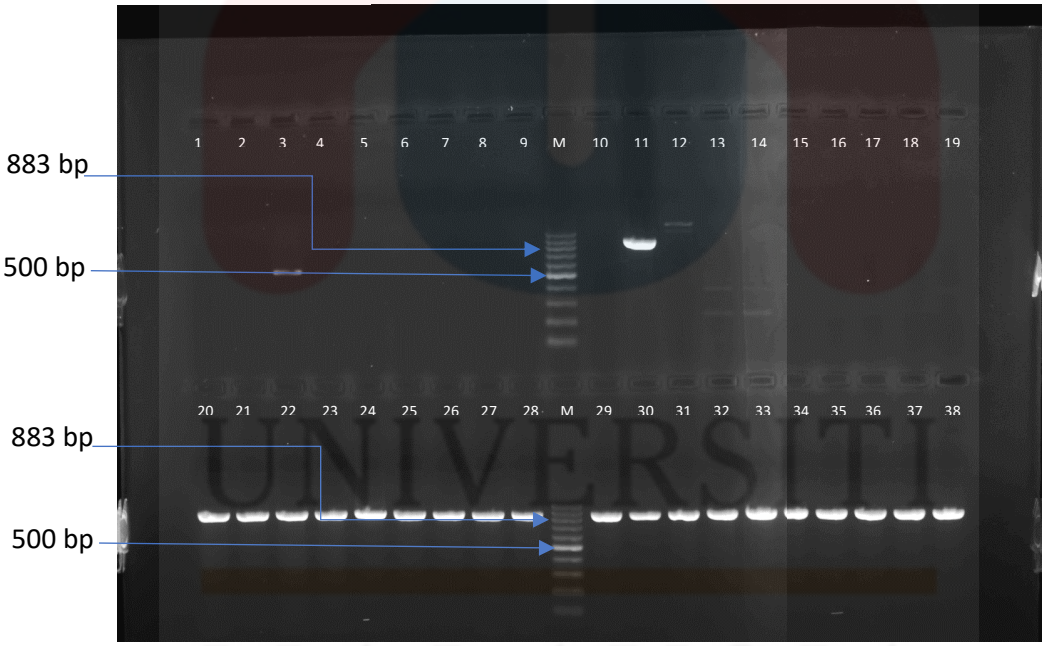

Figure 3: Detection of toxRVC gene in Vibrio cholerae using agarose gel electrophoresis where M represents ladder, and well 1 to 38 represent samples.

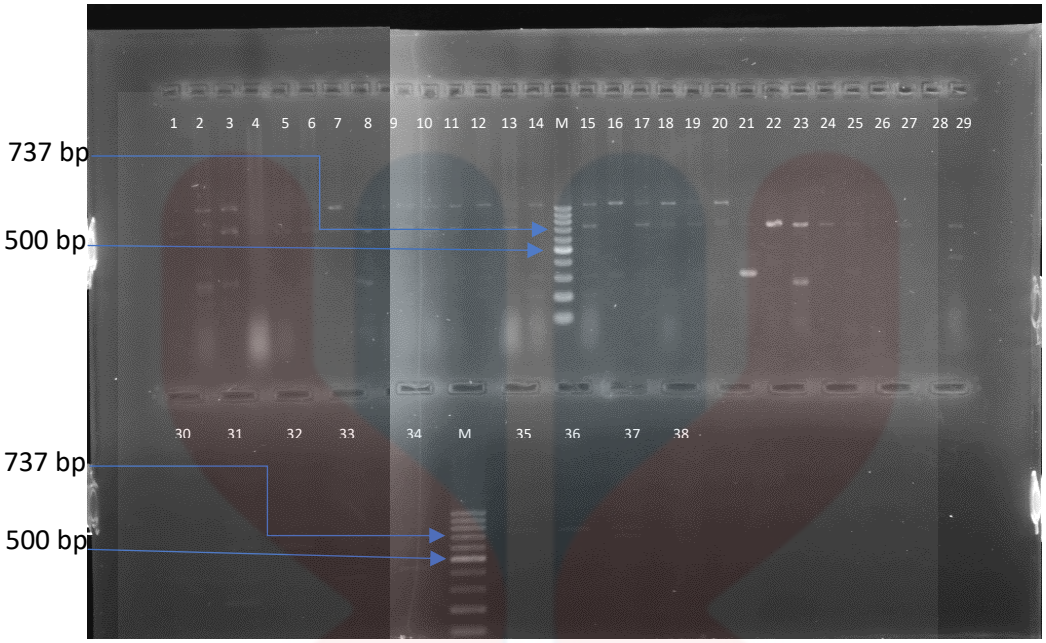

Figure 4: Detection of colA gene in Vibrio cholerae using agarose gel electrophoresis where M represents ladder, and well 1 to 38 represent samples.

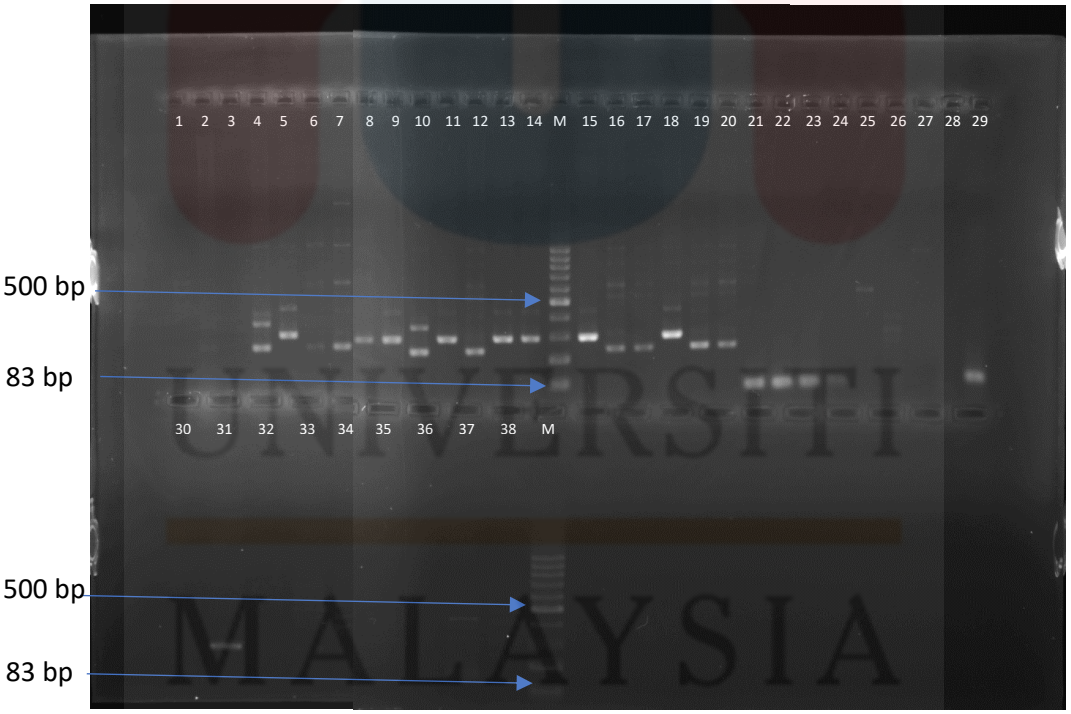

Figure 5: Detection of chiA gene in Vibrio cholerae using agarose gel electrophoresis where M represents ladder, and well 1 to 38 represent samples.

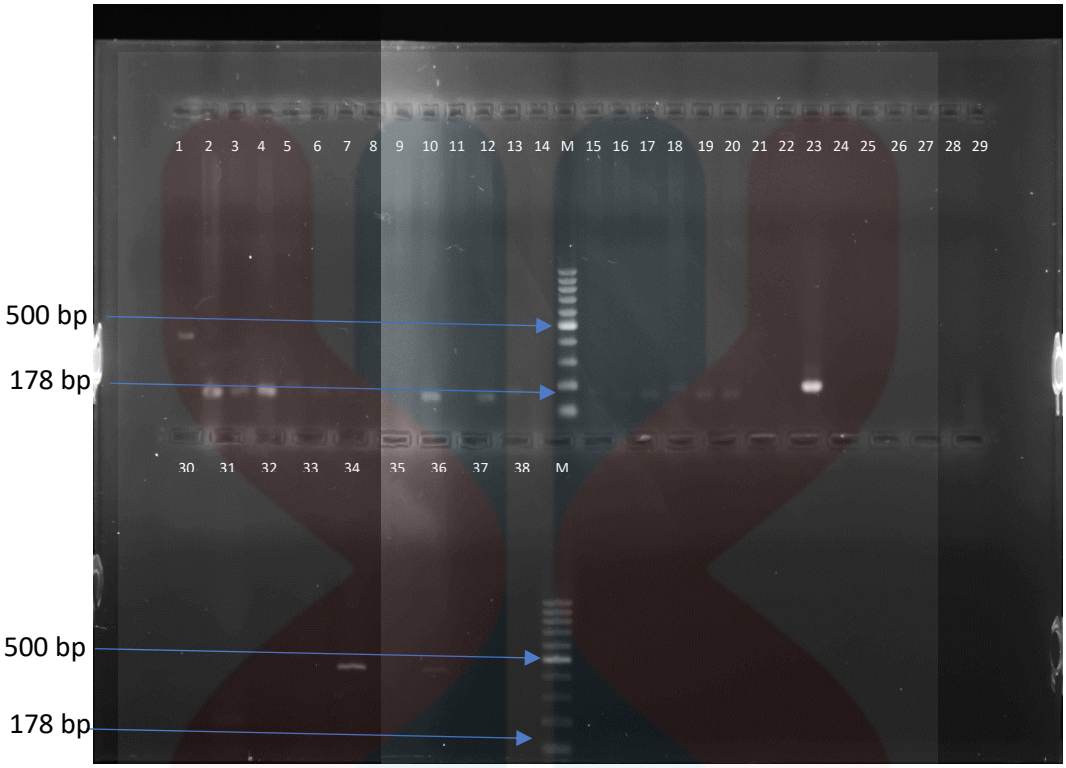

Figure 6: Detection of lux*R gene *in Vibrio cholerae using agarose gel electrophoresis where M represents ladder, and well 1 to 38 represent samples.

Table 4: Result of the molecular detection of virulence genes and blood hemolysis

No.	Bacterial isolates	Virulence genes	Blood hemolysis
1	VCK 1	*chi*A	α
2	VCK 2	*chi*A, *col*A	γ
3	VCK 3	*lux*R, *chi*A, *col*A	α
4	VCK 4	*chi*A, *col*A	β
5	VCK 5	-	γ
6	VCK 6	-	β
7	VCK 7	-	γ
8	VCK 8	*chi*A	β
9	VCK 9	*col*A	α
10	VCK 10	-	α
11	VCK 11	-	β
12	VCK 12	--	β
13	VCK 13	-	α
14	VCK 14	-	β
15	VCK 15	-	β
16	VCK 16	*Tox*R_{VC}	β
17	VCK 17	-	α
18	VCK 18	-	α
19	VCP 1	*hly*A, *col*A	α
20	VCP 2	*Tox*$R_{VC,}$ *hly*A, *lux*R	β

21	VCP 3	*Tox*R$_{VC,}$ *hly*A, *lux*R, *col*A	*β*
22	VCP 4	*Tox*R$_{VC,}$ *hly*A, *lux*R, *col*A	*β*
23	VCP 5	*Tox*R$_{VC,}$ *hly*A, *lux*R, *col*A	*β*
24	VCP 6	*Tox*R$_{VC,}$ *hly*A, *lux*R, *col*A	*β*
25	VCP 7	*Tox*R$_{VC,}$ *hly*A, *lux*R, *col*A	*β*
26	VCP 8	*Tox*R$_{VC,}$ *hly*A, *lux*R, *col*A	*β*
27	VCP 9	*Tox*R$_{VC,}$ *hly*A, *col*A	*β*
28	VCP 10	*Tox*R$_{VC,}$ *hly*A, *lux*R, *col*A	*β*
29	VCP 11	*Tox*R$_{VC,}$ *hly*A, *lux*R, *col*A	*β*
30	VCP 12	*Tox*R$_{VC,}$ *hly*A, *lux*R, *col*A	*β*
31	VCP 13	*Tox*R$_{VC,}$ *hly*A, *lux*R, *col*A	*β*
32	VCP 14	*Tox*R$_{VC,}$ *hly*A, *col*A	*β*
33	VCP 15	*Tox*R$_{VC,}$ *hly*A, *lux*R, *col*A	*β*
34	VCP 16	*Tox*R$_{VC,}$ *hly*A	*β*
35	VCP 17	*Tox*R$_{VC,}$ *hly*A, *lux*R, *col*A	*β*
36	VCP 18	*Tox*R$_{VC,}$ *hly*A, *lux*R, *col*A	*β*
37	VCP 19	*Tox*R$_{VC,}$ *hly*A, *lux*R, *col*A	*β*
38	VCP 20	*Tox*R$_{VC,}$ *hly*A, *lux*R, *col*A	*β*

Table 5 shows the result of the virulence genes detection using PCR. The bacteria isolates were grouped into 10 groups of virulence genes frequency. Group I consist of *Tox*R$_{VC,}$ *lux*R, *hly*A and *col*A were the most abundance and detected in 70% (14/20) of *Vibrio cholera* isolates from Pahang.

Table 5: The frequency of virulence genes detected among bacteria isolates.

Group	Virulence genes	Bacterial isolates
I	*Tox*R$_{VC,}$ *lux*R, *hly*A, *col*A	VCP 3, VCP 4, VCP 5, VCP 6, VCP 7, VCP 10, VCP 11, VCP 12, VCP 13, VCP 15, VCP 17, VCP 18, VCP 19, VCP 20
II	*Tox*R$_{VC}$,*hly*A,*col*A	VCP 8, VCP 9, VCP 11, VCP 14
III	*Tox*R$_{VC}$, luxR, *hly*A	VCP 2
IV	*lux*R, *chi*A, *col*A	VCK 3
V	*chi*A, *col*A	VCK 2, VCK 4
VI	*hly*A, *col*A	VCP 1
VII	*Tox*R$_{VC}$, *hlyIA*	VCP 16
VIII	*chi*A	VCK 1, VCK 8
IX	*col*A	VCK 9
X	*Tox*R$_{VC}$	VCK 16

Notes: VCP1-20= *Vibrio cholera* isolated from Kuala Pahang; VCK1-5= *Vibrio cholera* isolated from Laguna Semerak, Kelantan; VCK6-18= *Vibrio cholera* isolated from Laguna Tumpat, Kelantan

9.0 Discussion

In this study, 38 samples of *Vibrio cholera* were used to detect the virulence genes of *Vibrio* spp. Two typical virulence genes of *Vibrio cholera* were used which are the *Tox*R_{VC} and *lux*R genes. *Tox*R_{VC} is a virulence regulon that responsible for the expression and activation of *Vibrio cholerae* virulence factor (Bina *et al.,* 2013). Thus, this gene plays a huge role that determine the pathogenicity of *Vibrio cholerae.* For *hly*A gene, it is a cholera hemolysin which is responsible for extracellular pore-forming toxin. It also responsible for several biological activity in *Vibrio cholerae* such as possessing the hemolytic, cytotoxic and enterotoxic activity of the bacteria (Gao *et al.,* 2018). This gene also has the ability to induce apoptosis, a program cell death, and cells vacuolation of the host cells (Chakraborty *et al.,* 2011). The result showed that all *hly*A gene was detected in Pahang samples are tally with the blood hemolysis test result obtained which is beta hemolysis. This is due to the ability of *hly*A to cause lysis of the erythrocytes of most mammals species including sheep, which blood were used for the hemolysis test (Mizuno *et al.,* 2019).

The inability for certain *Vibrio cholerae* isolates, mostly the bacteria isolate from Kelantan to detect the *Tox*R_{VC} and *hly*A genes could possibly be due to the environmental condition as VCK and VCP isolates are obtained from 2 different locations. These environmental condition differences were important as the *Vibrio cholera* is able to undergo evolution with a variety of gene regulation systems and signal transduction to adapt to the environment that has different pH, temperature, concentration of CO_2, and many other stimuli which affect the transcription regulation of the virulence factors (Lindmark, 2009).

In this study, three atypical virulence genes of *Vibrio cholerae* which are *lux*R gene, *chi*A gene and *col*A genes were detected. *lux*R is a typical virulence gene for *Vibrio harveyi,* it is a Quorum sensing-related gene that is also responsible for the bacterial survivability and transmission in the environment by the formation of biofilm (Chen and Xie, 2011). *chi*A is a typical virulence gene for Harveyi clade which consist of *Vibrio harveyi* and *Vibrio campbellii* (Ruwandeepika *et al.*, 2010). This virulent factor chitinase enhances the survivability of the bacteria inside the host through mucin enhancement as the nutrient source (Mondal *et al.*, 2014). Collagenase is an important virulent factor of the *Vibrio* species. It is responsible for bacterial dissemination and is also able to aid in the diffusion of toxins through the process of hydrolysis of the collagenous components of the extracellular matrix (Wang *et al.*, 2022). It is a typical virulent gene for *Vibrio parahaemolyticus* which is responsible for invasiveness and tissue injury (Kassegne *et al.*, 2014).

The detection of atypical virulence genes in *Vibrio cholera* such as *lux*R, *chi*A and *col*A in this study were probably due to the occurrence of horizontal gene transfer (HGT) of the virulence genes from the environmental and pathogenic *Vibrio* species in order to improve their survivability and virulence (Deng *et al.*, 2020; Mohamad *et al.*, 2019). This HGT process is likely to occur in *Vibrio* species as *Vibrio spp.* possess a highly plastic genome, it increases the possibility of HGT leading to the elevation of the pathogenic strain of *Vibrio spp.* (Xu Y *et al.*, 2017; Mohamad *et al.*, 2019).

Meanwhile, none of both VCP and VCK isolates were positive for *tox*R_{VH}, *tdh, tlh, trh, vvh*A *vcg*EP and *vcg*CP. However, these atypical virulence genes were included in this study as it possesses a pathogenic risk in both human and fish (Mohamad *et al.*, 2019). These genes are a hemolysin which is a typical virulence factor for *Vibrio parahaemolyticus, Vibrio vulnificus.* It is a potent toxin that can induce cytolytic and

hemolytic activity which will cause a harmful effect toward the infected host (Ruwandeepika *et al.,* 2010).

10.0 Conclusion

In conclusion, *hly*A, *Tox*R$_{VC}$, *lux*R, *chi*A and *col*A genes were detected using a molecular method from the *Vibrio cholerae* isolated from the diseased seabass. The environmental condition and the probability of horizontal gene transfer have a significant impact on the presence of the virulence genes in the *Vibrio cholerae* species.

11.0 Recommendation and future work

For future work, the samples have been sent for nucleotide sequencing to confirm the presence of the virulence genes by determining the nucleic acids sequence of the virulence genes with the sequences available in the GenBank of National Centre for Biotechnology Information (NCBI-www.ncbi.nlm.nih.gov). In addition, multiplex Polymerase Chain Reaction (PCR) was recommended to be performed for bacteria isolates with the most virulence genes detected which are isolates; VCP 3, VCP 4, VCP 5, VCP 6, VCP 7, VCP 10, VCP 11, VCP 12, VCP 13, VCP 15, VCP 17, VCP 18, VCP 19, VCP 20 for group *Tox*R$_{VC,}$ *lux*R, *hly*A, *col*A. Multiplex PCR can detect more than targeted genes just by using multiple primers in a single reaction (Elniro *et al.,* 2000).

Appendix

Appendix A.1: shows the result of the virulence genes detected from the Vibrio cholera samples.

Bacteria isolates	ID	*toxR*$_{VC}$	*luxR*	*hlyA*	*chiA*	*toxR*$_{VH}$	*colA*	*tdh*	*tlh*	*trh*	*vhA*	*vcgEP*	*vcgCP*
VCK1	LS23LY	-	-	-	+	-	-	-	-	-	-	-	-
VCK2	LS24LG	-	-	-	+	-	+	-	-	-	-	-	-
VCK3	LS26LY	-	+	-	+	-	+	-	-	-	-	-	-
VCK4	LS27LY	-	-	-	+	-	+	-	-	-	-	-	-
VCK5	LS29LY	-	-	-	-	-	-	-	-	-	-	-	-
VCK6	LT5LG	-	-	-	-	-	-	-	-	-	-	-	-
VCK7	LT6KY	-	-	-	-	-	-	-	-	-	-	-	-
VCK8	LT6LY	-	-	-	+	-	-	-	-	-	-	-	-
VCK9	LT11KG	-	-	-	-	-	+	-	-	-	-	-	-
VCK10	LT15LY	-	-	-	-	-	-	-	-	-	-	-	-
VCK11	LT17KY	-	-	-	-	-	-	-	-	-	-	-	-
VCK12	LT17LG	-	-	-	-	-	-	-	-	-	-	-	-
VCK13	LT18KG	-	-	-	-	-	-	-	-	-	-	-	-

VCK14	LT20LY	-	-	-	-	-	-	-	-	-	-	-	-
VCK15	LT21LG	-	-	-	-	-	-	-	-	-	-	-	-
VCK16	LT22KG	+	-	-	-	-	-	-	-	-	-	-	-
VCK17	LT23LG	-	-	-	-	-	-	-	-	-	-	-	-
VCK18	LT24LG	-	-	-	-	-	-	-	-	-	-	-	-
VCP1	KP1KG	-	-	+	-	-	+	-	-	-	-	-	-
VCP2	KP2LY (M)	+	+	+	-	-	-	-	-	-	-	-	-
VCP3	KP2LY (S)	+	+	+	-	-	+	-	-	-	-	-	-
VCP4	KP2KY	+	+	+	-	-	+	-	-	-	-	-	-
VCP5	KP3LY	+	+	+	-	-	+	-	-	-	-	-	-
VCP6	KP4LY	+	+	+	-	-	+	-	-	-	-	-	-
VCP7	KP4KY	+	+	+	-	-	+	-	-	-	-	-	-
VCP8	KP5KY	+	-	+	-	-	+	-	-	-	-	-	-
VCP9	KP6LY	+	-	+	-	-	+	-	-	-	-	-	-
VCP10	KP7LY	+	+	+	-	-	+	-	-	-	-	-	-
VCP11	KP9LY	+	-	+	-	-	+	-	-	-	-	-	-
VCP12	KP10LY	+	+	+	-	-	+	-	-	-	-	-	-
VCP13	KP11LY	+	+	+	-	-	+	-	-	-	-	-	-

VCP14	KP13LY	+	-	+	-	-	+	-	-	-	-	-	-
VCP15	KP13KY	+	+	+	-	-	+	-	-	-	-	-	-
VCP16	KP14LY	+	-	+	-	-	-	-	-	-	-	-	-
VCP17	KP17LY	+	+	+	-	-	+	-	-	-	-	-	-
VCP18	KP20LY	+	+	+	-	-	+	-	-	-	-	-	-
VCP19	KP25LY	+	+	+	-	-	+	-	-	-	-	-	-
VCP20	KP29LY	+	+	+	-	-	+	-	-	-	-	-	-

(+) denotes positive (-) denotes negative

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