VIRULENCE-ASSOCIATED GENES OF *VIBRIO ALGINOLYTICUS* ISOLATED FROM SEABASS (*LATES CALCARIFER*) IN EAST COAST, MALAYSIA

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(D17A0029)

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MAY 2022

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CERTIFICATION

This is to testify that we have read this research paper entitled '**Virulence-Associated Genes of *Vibrio alginolyticus* Isolated from Seabass (*Lates calcarifer*) in East Coast, Malaysia**' by Nurdiana Binti Ab Halim, and in our opinions it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course DVT 5436 – Research Project.

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DEDICATIONS

I dedicate my dissertation work to

Dr Ruhil Hayati Binti Hamdan

Dr Tan Li Peng

Miss Ain Aizureen Binti Mat Zin

Dr Nora Faten Afifah Binti Mohamad, Ms Nani Izreen Binti Mohd Sani, Ms C.W Salma Binti C.W. Zalati

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DVM5 class of 2017/2022

Friends

Thank you

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ABBREVIATIONS

HGT	Horizontal gene transfer
MGE	Mobile gene element
PCR	Polymerase Chain Reaction
rpm	Revolutions per minute
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
TCBS	Thiosulfate-citrate-bile salts-sucrose agar

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ABSTRACT

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

Vibriosis is one of important diseases that can infect both humans and aquatic animals. Virulence genes contribute to the virulence of the pathogens. This study aims to determine the presence of virulence-associated genes of *Vibrio alginolyticus* isolated from sea bass (*Lates calcarifer*) in East Coast, Malaysia. A total of 26 samples of *V. alginolyticus* were retrieved and revived from glycerol stocks. Boiling method was done to extract DNA from the bacteria and then polymerase chain reaction (PCR) was performed using 12 virulence genes associated with *Vibrio* spp. All 26 samples showed positive results for *chiA* gene whilst 92.3% (24/26) of the samples contains *colA* genes. None of the isolates contains $toxR_{vh}$, *hlyA*, $toxR_{vc}$, and *trh-tdh*-related hemolysin gene (*trh*). Since atypical virulence genes were detected in *V.alginolyticus*, the presence of virulence genes may pose a threat to both aquatic animals and humans.

***Keywords**: Sea bass, Polymerase Chain Reaction V. alginolyticus, virulence-associated genes,*

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ABSTRAK

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

Vibriosis adalah salah satu penyakit penting yang boleh menjangkiti kedua -dua manusia dan haiwan akuatik. Gen virulensi menyumbang kepada virulensi patogen. Kajian ini bertujuan untuk menentukan kehadiran gen yang berkaitan dengan virulensi vibrio alginolyticus yang diasingkan dari bass laut (Lates calcarifer) di Pantai Timur, Malaysia. Sebanyak 26 sampel V. alginolyticus telah diambil dan dihidupkan semula dari stok gliserol. Kaedah pendidihan telah dilakukan untuk mengekstrak DNA dari bakteria dan kemudian tindak balas rantai polymerase dilakukan menggunakan 12 gen virulensi yang berkaitan dengan *Vibrio* spp. Semua 26 sampel menunjukkan keputusan positif untuk gen *chiA* manakala 92.3% (24/26) daripada sampel mempunyai gen *colA*. Semua sampel didapati tidak mempunyai $toxR_{vh}$, *hlyA*, $toxR_{vc}$, dan *trh-tdh*-gen berkenaan hemolisin (*trh*). Memandangkan gen virulensi yang tidak tipikal telah dikesan dalam *V.alginolyticus*, kehadiran gen ini mungkin akan menimbulkan ancaman terhadap hidupan akuatik dan manusia.

Kata kunci*: gen yang berkaitan dengan virulensi siakap, tindak balas rantai polimeraes, V.alginolyticus*

1.0 Introduction

The aquaculture for sea bass began to take place in 1980 after a new spawning method for sea bass was discovered in 1971 (Liong *et al*., 1993; Mat Ali *et al*., 1996). Awang Kechik, (1995) reported that sea bass was the main marine species cultured constituting 81.5% of the total aquaculture industry at that time. The sea bass or better known as "*siakap putih*" in native language can be found in coastal marine and estuarine waters and is a carnivore by nature that feeds on fishes and some crustaceans. To rear the finfish, certain criteria must be abided to ensure quality growth of the fish. The physiochemical properties of water required for sea bass culture in Malaysia dissolved oxygen in the range of 4.0-8.0 mg/l, salinity 10-31 ppt, pH of 7.5 to 8.3, temperature of 26-32 °C, water turbidity of not more than 10ppm and ammonia nitrogen, not more than 0.02 ppm (Bijo *et al.,* 2007)

Any abnormalities with the individual or environmental conditions might predispose the fish to diseases such as vibriosis. Vibriosis is a fish disease caused by several species of bacteria from the genus *Vibrio*. Common isolated *Vibrio* spp. from Malaysia waters especially in the eastern coastal reported are *V. harveyi, V. anguillarum, V. parahaemolyticus* and *V. alginolyticus.* These bacteria species are classified as pathogenic *Vibrio* spp. as they possess virulence gene that can manifest the disease in the fish once infected (Abdullah *et al*., 2017). The aim of this study is to determine the virulence-associated genes of *Vibrio* spp. isolated from Asian sea bass (*Lates calcarifer*) in East Coast, Malaysia.

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2.0 Research Problem

Vibriosis is a common foodborne and waterborne diseases caused by pathogenic species of *Vibrio* such as *Vibrio alginolyticus* which can affect both aquatic animals and humans. Common clinical manifestations in the fishes are cloudiness of the eyes, distended mucoid and necrotic intestine as well as liver and kidney haemorrhage. In humans, the disease can cause cellulitis and otitis. Limited study done in detection of virulence-associated genes in *Vibrio alginolyticus* isolated from seabass (*Lates calcarifer*) especially in East Coast, Malaysia. Therefore, this study aims to determine the presence of virulence-associated genes isolated from sea bass (*Lates calcarifer*) in East Coast, Malaysia.

3.0 Research Questions

What are the virulence-associated genes present in *V. alginolyticus* isolated from seabass (*Lates calcarifer*) in East Coast, Malaysia?

4.0 Research Hypothesis

Some virulence-associated genes are present in *V. alginolyticus* isolates.

5.0 Research Objectives

To determine the virulence-associated genes in *V. alginolyticus* isolated from sea bass (*Lates calcarifer*) in East Coast, Malaysia.

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6.0 Literature Review

6.1 Background of *Vibrio* spp.

Vibrio spp. from Vibrionaceae is a family of Gram negative, facultative anaerobe, motile, either straight or curved rods bacteria with a single polar flagellum (Baker-Austin *et al*., 2018). Both pathogenic and environmental bacteria can be found ubiquitous in freshwater, estuarine and marine environments. *Vibrio* spp. can be classified in 14 different groups or clades which includes *Harveyi* clade, *Cholerae* clade, *Vulnificus* clade, *Diazotrophicus* clade, *Photobacterium* clade, *Salinivibrio* clade, *Anguillarum* clade, *Fischeri* clade and 7 other clades (Sawabe *et al.,* 2007). The most important clades would be *Harveyi* clades consisting of pathogenic species (*V. alginolyticus, V. parahaemolyticus, V. harveyi* and *V. campbelii*) and other non-pathogenic species. All *Vibrio* spp. under *harveyi* clade share similarities in genotypic and phenotypic material (Cano-Gomez *et al.,* 2009).

Upon laboratory diagnosis, the best samples to be collected in order to detect *Vibrio* spp. are liver and kidney as well as water samples. For bacterial isolation and identification, Thiosulfate-citrate-bile salts-sucrose agar or also known as TCBS agar, a selective media used for isolation of *Vibrio* spp. For *V. alginolyticus*, the colonies produced would be large yellow colonies. The bacteria can grow rapidly at 25-30°C in enriched media such as brain-heart infusion (BHI) agar for proteolytic activity. To observe the haemolytic activity, the bacteria will be inoculated on blood agar with 5% sheep red blood cells (Hernández-Robles *et al.*, 2016). Other media that can be used to detect *V. alginolyticus* includes trypticase soy agar (TSA) containing 1.5%-8% NaCl incubated for 24 hours at 28 °C in which it will produce circular, cream-coloured colonies on solid medium (Santhyia *et al.,* 2015). Molecular detection *of V.*

alginolyticus can be done using polymerase chain reaction (PCR) fingerprinting using 16S rDNA and gene sequencing (Xie *et al.,* 2020).

Vibriosis caused by *Vibrio* spp. is characterized by haemorrhagic septicaemia which will manifest general clinical sign of haemorrhage of liver and kidney that can be observed in the diseased sea bass or marine finfishes (Mohamad *et al*., 2019). Other than that, distended mucoid and necrotic intestine and petechiation as well as erosion and darkened discoloration to the skin and fins may be presented during examination. Other changes that can be seen in diseased fish are distension and cloudiness of the eye as well periorbital swelling (Anderson *et al*., 1970). In addition, several studies reported that *V. alginolyticus* causes mass mortality as well as haemorrhages and ulceration on skin with evidence of septicaemia in Gilt-had bream (*Sparus aurata*) (Kahla-Nakbi *et al.,* 2006; Ruwandeepika *et al.,* 2010).

6.2 Emergence of vibriosis in marine fish

The emergence of the bacteria that cause diseases results in severe losses in aquaculture industry. According to Rucker *et al*. (1954), disease caused by *Vibrio* species occurring in an aquaculture system without any proper discussion related to the symptoms of the disease among*, Oncorhynchus keta*, *Oncorhynchus taschawytscha* and *Oncorhynchus gorbuscha* species of salmon along the Washington coast, United States was the first to be reported. Generally, *Vibrio* spp. such as *V. alginolyticus*, *V. harveyi* are reported to cause petechial haemorrhage on the ventral and lateral area with swollen and dark skin lesions, necrosis and ulcers on the skin with mortality of 50% in sea bass (Saad & Atallah, 2014; Novriadi, 2016).

In July 2017, there was an outbreak of vibriosis reported in China that affecting farmed seahorse *Hippocampus kuda*. The major causative agents isolated during the outbreak

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were *V. harveyi* and *V. alginolyticus* in which the clinical signs observed were the presence of ascites in the abdominal cavity with addition of haemorrhagic skin and liver. Since seahorse aquaculture was blooming during the period before the outbreak due to the traditional medicine demands, the seahorse farmer later experienced a severe economic loss post-outbreak (Xie *et al.,* 2020).

Similar situation also occurs in Malaysia as an outbreak of vibriosis in hybrid groupers of Tiger Grouper (*E. fuscoguttatus)* and Camouflage Grouper (*Epinephelus polyphekadion*) was reported in September 2016 in Selangor. Based on the case study, it was reported that the disease had an impact on the daily mortality rate leading to 29% of mortality rate in 10 days. The clinical manifestations observed within the diseased fishes were necrosis of the fins, splenomegaly as well as kidney and liver congestion. Upon diagnosis, *V. harveyi* and *V. alginolyticus* were identified as the main etiological agents which caused the concurrent infections in the groupers (Mohamad *et al*., 2019). Economic impact on the aquaculture industry can be seen due to vibriosis as groupers are one of the main contributors with 92% production to the aquaculture farming worldwide (FAO, 2017).

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6.3 Virulence factors of *Vibrio* spp.

Virulence factors reported to be found in *Vibrio* spp. are phospholipases, siderophages, cytotoxins, biofilm formation, hemolysins, quorum sensing, proteases and presence of phage (Aguirre-Guzman *et al*., 2004; Mohamad *et al*., 2019). The virulence-associated genes are responsible to help in expressing the virulence factors on the *Vibrio* spp. *Vibrio* spp. is reported to possess virulence gene of *toxR* that will express biofilm formation and bile resistance (Chang *et al*., 2012). The bile resistance mediated by *toxR* gene will enhance the survival and adaptation in the fish intestine. Other virulence gene expressed by *V. alginolyticus* is chitinase which is an important factor in adhesion of the bacterium to other susceptible hosts during infection (Finlay & Falkow, 1997; Aguirre-Guzman *et al*., 2004). However, it was shown that collagenase activity presented in *Vibrio* spp. has the potential in medical field for wound management as it possesses the ability to perform the enzymatic cleavage of the substrate that allows removal of necrotic tissues while sparing the healthy tissues (Di Pasquale *et al*., 2019).

Other virulence factor involved in the pathogenesis of the vibriosis would be hemolysin. Hemolysin is one of the lytic enzymes and an exotoxin present in *Harveyi* clade bacteria which plays role in lysing red blood cells of the affected hosts. Haemolytic activity of *V. alginolyticus* can be achieved by breaking down cell membranes or by forming pores on the cells of the hosts. The effect of the hemolysin can be observed by the signs of haemorrhage mainly on the liver and kidney of the aquatic animals especially fishes (Sun *et al.,* 2007).

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6.4 Public health concern towards vibriosis

Several *Vibrio* spp. are commensals in marine fishes and seawater. The most common pathogenic species of *Vibrio* in humans are *V. cholerae, V. vulnificus and V. parahaemolyticus*. Vibriosis can be contracted from the exposure of contaminated water or consumption of undercooked or raw seafood and cause many different symptoms and clinical signs in humans. (Baker-Austin *et al*., 2018).

Although pathogenic *Vibrio* spp. such as *V. alginolyticus* is commonly associated with diseases in marine fishes, shellfish and crustaceans, the bacterium can also cause disease in humans. Common infection caused by this bacterium includes superficial wound infections on the skin and ears as well as fatal infections in immunocompromised hosts (Lee *et al.,* 2008). Furthermore, a vibriosis case was reported to occur in South Korea within the year of 1998. It was reported that a young female patient was presented with diarrhoea and abdominal pain after ingestion of raw crab preserved in soy sauce. Rectal swab was collected and bacterial isolation was done in which *V. alginolyticus* was isolated after a series of diagnostic workups. This proves that although gastroenteritis can rarely happen, there is always a probability for the infection to occur due to *V. alginolyticus* in humans (Uh *et al.,* 2001).

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7.0 Materials and Methods

7.1 Bacterial collection

The collection of *Vibrio* spp. was obtained from the Aquatic Animal Health Lab, Faculty of Veterinary Medicine. The bacteria were stored in 50% glycerol stock. The *Vibrio* spp. samples were revived on Trypticase Soy Agar (TSA) with 1.5% NaCl and were incubated at 35°C for 24 hours and on Trypticase Soy Broth (TSB) concurrently. Twenty-six (26) isolates of *Vibrio alginolyticus* were proceeded for further analysis. Table 1 shows the list of *Vibrio* spp. used. Liver and kidney samples from diseased fishes were collected from 3 different farms which were from Laguna Semerak, Kelantan, Kuala Ibai and Sungai Besut, Terengganu. Based on the previous study on the blood agar, most of the isolates of *V. alginolyticus* show hemolytic activity after inoculation on blood agar.

Table 1: List of *Vibrio* spp. used in this study.

No	Bacterial isolates	Location/ organ samples	Blood Haemolysis
1.	VAK1	Laguna Semerak, Kelantan/liver	α-hemolysis
2.	VAK2	Laguna Semerak, Kelantan/liver	α-hemolysis
3.	VAK3	Laguna Semerak, Kelantan/liver	α-hemolysis
4.	VAK4	Laguna Semerak, Kelantan/liver	β-hemolysis
5.	VAK5	Laguna Semerak, Kelantan/liver	α-hemolysis
6.	VAK6	Laguna Semerak, Kelantan/liver	α-hemolysis
7.	VAK7	Laguna Semerak, Kelantan/liver	α-hemolysis
8.	VAK8	Laguna Semerak, Kelantan/liver	α-hemolysis
9.	VAK9	Laguna Semerak, Kelantan/liver	α-hemolysis
10.	VAK10	Laguna Semerak, Kelantan/liver	α-hemolysis

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11.	VAK11	Laguna Semerak, Kelantan/liver	α-hemolysis
12.	VAT1	Kuala Ibai, Terengganu/liver	β-hemolysis
13.	VAT2	Kuala Ibai, Terengganu/liver	α-hemolysis
14.	VAT3	Kuala Ibai, Terengganu/liver	α-hemolysis
15.	VAT4	Sungai Besut, Terengganu / liver	-
16.	VAT5	Sungai Besut, Terengganu / liver	-
17.	VAT6	Sungai Besut, Terengganu / liver	-
18.	VAT7	Sungai Besut, Terengganu / liver	-
19.	VAT8	Sungai Besut, Terengganu / liver	α-hemolysis
20.	VAT9	Sungai Besut, Terengganu / liver	α-hemolysis
21.	VAT10	Sungai Besut, Terengganu / liver	α-hemolysis
22.	VAT11	Sungai Besut, Terengganu / liver	β-hemolysis
23.	VAT12	Sungai Besut, Terengganu / liver	α-hemolysis
24.	VAT13	Sungai Besut, Terengganu / liver	-
25.	VAT14	Sungai Besut, Terengganu / liver	-
26.	VAT15	Sungai Besut, Terengganu / liver	-

Footnote; VAK: *Vibrio alginolyticus* isolated from Kelantan, VAT: *Vibrio alginolyticus* isolated from Terengganu.

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7.2 Detection of virulence-associated genes in *Vibrio alginolyticus*

7.2.1 DNA extraction

Boiling method was used for DNA extraction. One millilitre (ml) of bacterial culture from TSB were put in a test tube containing 1 ml of normal saline. The mixture was then centrifuged at 12,000 rpm for 5 minutes and the supernatant was discarded. The pellet left then were resuspended in 500 µL distilled water and vortexed vigorously and boiled at 100°C for 10 mins. Then, it was cooled on ice for 10 mins and later centrifuged again at 12,000 rpm for 5 mins. The supernatant was obtained and stored in new tubes and kept in a freezer at -20°C.

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7.2.2 Polymerase chain reaction (PCR) of virulence-associated genes

Polymerase chain reaction was done using a T100™ Thermal Cycler (Bio-Rad, USA) following the DNA extraction to amplify the DNA from the bacterial isolates. Polymerase Chain Reaction (PCR) mixtures were incubated 3 min at 94 °C, followed by 30 cycles of amplification. Each cycle consisted of denaturation at 94 °C for 1 min, annealing at 55 °C for primer *colA, hlyA, vcgCP1,* $toxR_{vh}$, *luxR, chiA*, 47°C for *vcgEP2* and 53°C for $toxR_{Vc}$ for 1 min, and extension at 72 °C for 1 min plus a final extension at 72 °C for 10 min.

Another PCR protocol was performed for the remaining primer. Initial denaturation 94 °C for 5 min, followed by 35 cycles of amplification. Each cycle consisted of denaturation at 94 °C for 30 s, annealing at 58 °C for primer *tlh* and *tdh*, 46°C for *trh* and 60°C for *vvhA* for 45s and extension at 72 °C for 30s with a final extension at 72 °C for 10 min.

The primer sequences used in this study for PCR to confirm the virulence-associated genes in *Vibrio* spp., selected based on previous studies conducted as listed in the Table 2.

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Table 2: Primers used, target bp and PCR protocol.

GENE	VIRULENCE FACTOR	PRIMER	PROTOCOL	bp	REFERENCES
colA	Collagenase	F: 5’-CGAGTACAGTCACTTGAAAGCC-3’ R :5’-CACAACAGAACTCGCGTTACC -3’	94°C for 3 min, 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 72°C for 10 min	737	Di Pinto *et al*., (2005)
*hly*A	Hemolysin of *Vibrio cholera*	F:5’-GGCAAACAGCGAAACAAATACC-3’ R:5’-CTCAGCGGGCTAATACGGTTTA -3’	94°C for 3 min, 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 72°C for 10 min	738	Mohamad *et al*., 2019/Schroeder *et al*., 2017/ Ruwandeepika *et al*., 2010
*Vcg*EP2/*vcg*P3	Virulence-correlated gene of *Vibrio vulnificus*	F:5’- CTCAATTGACAATGATCT-3’ R:5’-CGCTTAGGATGATCGGTG -3’	94°C for 3 min, 30 cycles of 94°C for 1 min, 47°C for 1 min, 72°C for 1 min, 72°C for 10 min	278	Fri *et al*., 2017
*Vcg*CP1/ *vcg*P3	Virulence-correlated gene of *Vibrio vulnificus*	F:5’-AGCTGCCGATAGCGATCT-3’ R:5’- CGCTTAGGATGATGGTG-3’	94°C for 3 min, 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 72°C for 10 min	278	
tdh	Thermostable direct hemolysin	F:5’-GTAAAGGTCTCTGACTTTTGGAC-3’	94°C for 5 mins, 35 cycles of 94°C for 30s, 58°C for	269	Bej *et al*., 1999/ Mohamad *et al*., 2020

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		R:5'-TGGAATAGAACCTTCATCTTCACC-3'	45s, 72°C for 30s, 72°C for 10 min		
tlh	Thermolabile hemolysin	F:5'-AAAGCGGATTATGCAGAAGCACTG-3' R:5'-GCTACTTTCTAGCATTTTCTCTGC-3'	94°C for 5 mins, 35 cycles of 94°C for 30s, 58°C for 45s, 72°C for 30s, 72°C for 10 min	450	
trh	Thermostable-related hemolysin	F:5'-TTGGCTTCGATATTTTCAGTATCT-3' R:5'-CATAACAAACATATGCCCATTTTCCG-3'	94°C for 5 mins, 35 cycles of 94°C for 30s, 46°C for 45s, 72°C for 30s, 72°C for 10 min	500	
$toxR_{vh}$	Toxin of *Vibrio harveyi*	F:5'-GAAGCAGCACTCACCGAT-3' R:5'- GGTGAAGACTCATCAGCA-3'	94°C for 3 mins, 30 cycles of 94°C for 1 min, 55°C for 45s, 72°C for 1 min, 72°C for 10 min	82	Deng *et al*., 2019 Mohamad N. *et al*., 2019 Liao & Leano, 2008
luxR	Quorum sensing factor	F:5'-GTGGTTCGTCAATTCTCGAAC 3' R:5'-CGAATAGTGGCCACACTTC-3'		178	
chiA	Chitinase	F:5'-CTCAAGGTGTTTGGGAAGATG-3' R:5'-GTTGATGCCAGTGTTGTTCG-3'		82	
vvhA	*Vibrio vulnificus* exotoxin-hemolysin	F:5'-TTCCAACTTCAAACCGAACTATGAC-3' R:5'-ATTCCAGTCGATGCGAATACGTTG-3'	94°C for 5 mins, 35 cycles of 94°C for 30s, 60°C for 45s, 72°C for 30s, 72°C for 10 min	205	Bonny *et al*., 2018
$toxR_{vc}$	Toxin of *V. cholera*	F: 5'-ATGTTCGGATTAGGACAC-3' R :5'-TACTCACACACTTTGATGGC -3'	94°C for 3 min, 30 cycles of 94°C for 1 min, 53°C for 1 min, 72°C for 1 min, 72°C for 10 min	883	Mohamad M. *et al*., 2019/ Deng *et al*., 2019

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7.2.3 Agarose gel electrophoresis

Gel electrophoresis was done to visualize the PCR products on a 1.5% agarose gel in 1x Tris-borate-EDTA (TBE) buffer. The electrophoresis was run on 100 V, 300 A for 40 to 45 mins and visualized by SYBR Green and an UV transilluminator (Bio-Rad, USA). A molecular weight was measured using a VC 100bp Plus DNA Ladder. (Gennari *et al.,* 2012).

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8.0 Results

Based on the result from Table 3, most of the isolates from Kelantan and Terengganu were shown to have *colA* and *chiA* gene. Isolates that have the most genes are 5 isolates from Kelantan; VAK1, VAK3, VAK4, VAK8 and VAK11. The least gene possessed by *V. alginolyticus* was VAT11 isolated in Terengganu.

Figure 1: Schematic diagram of gel electrophoresis for virulence-associated genes for *V.alginolyticus*. M represents molecular ladder and other wells represent other genes detected by PCR.

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Table 3: The virulent-associated genes detected by PCR

No	Bacterial isolates	Location	Blood Haemolysis	Virulence-associated genes
1.	VAK1	Laguna Semerak, Kelantan	α	*colA, vcgEP2, chiA, vvhA*
2.	VAK2	Laguna Semerak, Kelantan	α	*tdh, chiA*
3.	VAK3	Laguna Semerak, Kelantan	α	*colA, vcgP1, tlh, chiA*
4.	VAK4	Laguna Semerak, Kelantan	β	*colA, tdh, tlh, chiA*
5.	VAK5	Laguna Semerak, Kelantan	α	*colA, tlh, chiA*
6.	VAK6	Laguna Semerak, Kelantan	α	*colA, chiA*
7.	VAK7	Laguna Semerak, Kelantan	α	*colA, chiA*
8.	VAK8	Laguna Semerak, Kelantan	α	*colA, vcgEP2, tlh, chiA*
9.	VAK9	Laguna Semerak, Kelantan	α	*colA, chiA*
10	VAK10	Laguna Semerak, Kelantan	α	*colA, chiA*
11	VAK11	Laguna Semerak, Kelantan	α	*colA, vcgEP2, tlh, chiA*
12	VAT1	Kuala Ibai, Terengganu	β	*colA, chiA*
13	VAT2	Kuala Ibai, Terengganu	α	*colA, tlh, chiA*
14	VAT3	Kuala Ibai, Terengganu	α	*colA, chiA*
15	VAT4	Sungai Besut, Terengganu	-	*colA, chiA*
16	VAT5	Sungai Besut, Terengganu	-	*colA, chiA*
17	VAT6	Sungai Besut, Terengganu	-	*colA, chiA, luxR*
18	VAT7	Sungai Besut, Terengganu	-	*colA, chiA*
19	VAT8	Sungai Besut, Terengganu	α	*colA, chiA*
20	VAT9	Sungai Besut, Terengganu	α	*colA, chiA*
21	VAT10	Sungai Besut, Terengganu	α	*colA, chiA*
22	VAT11	Sungai Besut, Terengganu	β	*chiA*
23	VAT12	Sungai Besut, Terengganu	α	*colA, chiA, tlh*

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24	VAT13	Sungai Besut, Terengganu	-	*colA, chiA*
25	VAT14	Sungai Besut, Terengganu	-	*colA, chiA*
26	VAT15	Sungai Besut, Terengganu	-	*colA, chiA*

Footnote; VAK: *Vibrio alginolyticus* isolated from Kelantan; VAT: *Vibrio alginolyticus* isolated from Terengganu; α: Alpha hemolysis; β:beta hemolysis

Table 3 shows the result of the molecular detection of virulence-associated genes in *Vibrio alginolyticus* using Polymerase Chain Reaction (PCR).

All 26 samples show positive result for the presence of *chi*A gene. While 92.3% (24/26) of the samples possess *colA* genes. None of the isolates contain $toxR_{vh}$, *hlyA,* $toxR_{vc}$, *trh-tdh*-related hemolysin gene (Table 3).

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Table 4: Frequency of virulence-associated genes from V. alginolyticus isolated in East Coast, Malaysia

Virulence-associated genes	*Vibrio alginolyticus*					
	Kelantan		Terengganu		Total	
	n	(%)	n	(%)	n	(%)
ColA	10	38.5	14	53.8	24	92.3
*hly*A	0	0	0	0	0	0
*Vcg*CP1/*vcg*P3	1	3.8	0	0	1	3.8
*Vcg*EP2/ *vcg*P3	3	11.5	1	3.8	4	15.4
tdh	2	7.7	0	0	2	7.7
tlh	5	19.2	2	7.7	7	26.9
trh	0	0	0	0	0	0
$toxR_{vh}$	0	0	0	0	0	0
luxR	0	0	1	3.8	1	3.8
chiA	11	42.3	15	57.7	26	100
vvhA	1	3.8	0	0	1	3.8
$toxR_{vc}$	0	0	0	0	0	0
Total	**33**	**126.8**	**33**	**126.8**	**66**	**253.7**

Based on the result tabulated in Table 4, all *V. alginolyticus* samples were revealed to possess *chi*A gene, 24/26 (92.3%) samples revealed to have *colA*, 7 samples show positive result for *tlh* gene, 4 samples show positive result for *Vcg*EP2/*vcg*P3 gene, 2 samples show positive for the presence of *tdh* gene and 1 sample was found to out express *vcg*CPI/*vcg*P3, *luxR* and *vvhA*. All samples of *V. alginolyticus* revealed negative results for *toxrRvh*, *hlyA*, $toxR_{vc}$, *trh* genes. All samples were confirmed with PCR amplification using targeted virulence-associated genes commonly found in *Vibrio* spp.

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9.0 Discussion

Vibrio spp. is one of the most common bacteria found in aquaculture systems which causes vibriosis in aquatic organisms. One of the clades of *Vibrio* spp. is *Harveyi* clades which comprised of *V. alginolyticus, V. harveyi, V. rotiferianus, V. natriegens, V. parahaemolyticus* and *V. mytili*, known to be pathogenic to aquatic animals (Sawabe *et al*., 2007). All of the species listed under *Harveyi* clade share similar genotypic and phenotypic homology at a certain level (Cano-Gomez *et al*., 2009). Certain *Vibrio* spp. from *Harveyi* clade such as *V. parahaemolyticus* is an enteric human pathogen which is known to cause pandemic cholera while most *Harveyi* clades may manifest vibriosis in aquatic animals such as in fishes, mollusks or crustaceans which highly likely to cause over high mortality rate, economic losses and severe outbreaks (Ina-Salwany *et al*., 2019). Hence, this study aims to investigate the presence of typical virulence genes and atypical virulence genes as well as factors contributing to the presence of those genes in *V. alginolyticus* in East Coast, Malaysia.

Generally, Typical virulence genes in *V. alginolyticus* is *chiA* and *colA* which encodes for chitinase and collagenase while atypical virulence genes of *V. alginolyticus* are *hlyA*, *toxRvc, tdh* and *trh* (Mohamad *et al*., 2019). Thus,

Based on the result from the molecular detection described in Table 3 and 4, most of the samples were revealed to possess *colA* gene. As proven from previous studies done, *colA* gene is identified as species-specific gene for *V. alginolyticus* and most abundantly found in this specific species (Di Pinto *et al*., 2005; Gennari *et al*., 2012). Thus, *colA* gene can be used as the genetic marker for biochemical identification of *V. alginolyticus* in future studies (Di Pinto *et al*., 2005).

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There are several factors as to why most of the genes which are not specific to *V. alginolyticus* present within the said bacteria recovered from the East Coast, Malaysia. One of the possibilities of the atypical virulence genes to be transferred to *V. alginolyticus* is horizontal gene transfer (HGT) or mobile gene elements (MGE) which can lead to virulent infections caused by *V. alginolyticus* (Chibani *et al.,* 2020). Horizontal gene transfer can be defined as the exchange of the genetic material within species without any sexual mechanism. Horizontal gene transfer (HGT) is achieved for survival benefits in a variety of host organisms and environments which can be achieved by mobile gene elements. It is more often to find HGT between strains of the same species whilst MGEs are frequent to occur interspecies due to its broad host range which eventually lead to difficulty to distinguish between different species (Hazen *et al.,* 2010; Chibani *et al.,* 2020). According to Deng *et al.,* (2019), HGT can be influenced by several factors such as climate change and environmental pollution which is believed to cause effects such as alteration in bacterial pathogenicity and cause genetic communication.

Regarding the environmental factor, poor water quality parameters play an important role in increasing the growth of pathogens in farmed fish and their habitat (Abdullah *et al.,* 2017). Other study stated there is correlation with fluctuating physiochemical properties of marine water such as dissolved oxygen (DO), SO_4, pH, temperature, salinity and turbidity which predispose the fishes to stress thus promoting the proliferation of *Vibrio* spp. (Mohamad *et al.,* 2019). Another factor would be the presence of marine leech (*Zeylanicobdella arugamensis*) which is said to be the vector for *V. alginolyticus*. Under experimental conditions in sea bass, there is an estimation of 70% fry in a cage infected simultaneously by both leeches and *V. alginolyticus* (Kua

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et al., 2010). These factors supported that the bacteria may proliferate and undergo adaptation to survive in various environmental conditions.

According to Sujeewa *et al.* (2009), *tlh* (thermolabile hemolysin) gene has widely been used as specific marker for the detection of *V. parahaemolyticus* at species level however, the result from this study may contradict with the statement as 7 isolates of *V. alginolyticus* are positive for this gene. Hence, *tlh* gene cannot be used as species specific marker due to probability of false positive result. According to Di Pinto *et al*. (2005), *tdh* and *trh* gene which encodes for virulence factor thermostable direct hemolysin and thermostable-related hemolysin are recognized as the alternative genetic marker apart from *toxR* detection (Fri *et al*., 2017) for the detection for *Vibrio parahaemolyticus*. This study is also supported by several different studies conducted by Sujeewa *et al*. (2009) and recently by Mohamad *et al*. (2019), which explained about the presence of hemolysin genes which commonly cause enterotoxic and cytotoxic effects found in elevated level of strains of environmental and pathogenic *V. parahemolyticus* even though other *Vibrio* species such as *V. cholerae, V. mimicus* and *V. hollisae* may also possess similar genes (Nishibuchi & Kaper, 1995). Both *trh* and *tdh* majorly contributes to the pathogenicity of *V. parahaemolyticus* instead of *V. alginolyticus* based on the lack of presence of those genes in this study.

In addition, negative result for *vvhA* in most *V. alginolyticus* isolates can be linked to the role of the said gene as a species-specific target gene in *Vibrio vulnificus*. Hence, the result is consistent with the findings from previous study along with this study. *vvhA* gene express exotoxin-hemolysin virulence factor which promotes iron release from haemoglobin as well as producing cytotoxic effects due to the binding to cell membrane which form pores that leads to activation of inflammatory responses in the infected hosts (Sugiyama *et al*., 2011, Yuan *et al*., 2020). Other virulence-associated

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genes confirmed by molecular detection are *vcg*EP2 and *vcg*CP1 which express virulence factor of virulent correlated gene (*vcg*). Based on the earlier studies done by Bier et al. (2015) in Germany, *vcg*EP2 is associated with isolates recovered from environment whilst *vcg*CP1 is linked with isolates recovered from clinical strains which considered avirulent and virulent strains of *V. vulnificus* respectively.

Based on the previous studies done on *Vibrio* spp., it is common to find typical virulence genes of *chiA* and *luxR* to be present in most isolates of *Harveyi* clade bacteria (Aguirre-Guzman *et al.,* 2004; Mohamad *et al.,* 2019). Nevertheless, only *chiA* gene was presented in all isolates and *luxR* was found to be lacking in those isolates. *chiA* gene which encodes for chitinase is found responsible for the adhesion function of the bacteria to the host surfaces. Apart from the role in adhesion, the chitinase activity may aid the bacteria to penetrate the tissues that contain chitin such as tissues of crustaceans (Finlay & Falkow, 1997). Thus, it is common to isolate *Harveyi* clades, especially *V. alginolyticus, presented* with lesions through epicuticle crabs. *luxR* encodes for quorum sensing factor which regulates virulence factor expression for serine protease, extracellular polysaccharide, flagella and hemolysin in *V. alginolyticus* (Rui *et al.,* 2009; Ruwandeepika, 2010). Earlier studies reported that *V. harveyi* quorum-sensing system shares similarity with *luxR* of *V. alginolyticus*. however, *luxR* of *V. harveyi* may differ from *luxR* of *V. alginolyticus* has proven from the recent study done in Terengganu which described *luxR V. alginolyticus* EF596781 strain is similar to *luxR* of *V. alginolyticus* SNA 212-S1 strain. In contrast, *luxR* of *V. harveyi* SEA 131-K1 shared high similarity with *luxR* of *V. parahaemolyticus* GRO180-K1 strain (Mohamad *et al.,* 2019). These findings explained the lack of positive result in molecular detection of *luxR* gene in *V. alginolyticus* due to the distinguished *luxR* genetic material within interspecies of *Harveyi* clade.

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In this study, *toxR* was found to be negative in all isolates. The toxR regulon which regulates the expression of gene *ctx* responsible for cholera toxin has also been found to be present in *V. alginolyticus* (Okuda *et al.,* 2001), *V. parahaemolyticus* and *V. harveyi* (Ruwandeepika *et al.,* 2010). A few reports stated that *V. cholerae* $toxR_{vc}$ has been found to be similar to *V. parahaemolyticus toxR* in terms of function and structure. However, there is less study to support whether the $toxR_{vc}$ and $toxR_{vh}$ *(V. harveyi toxR*) can co-exist within *V. alginolyticus* isolates (Lin *et al*., 1993).

As described from previous studies, *V. alginolyticus* has been found to cause Vibriosis in animals and humans. Common habitat for the bacteria apart from warm sea water and brackish water with moderate to high salinity were fish, free living organisms in the sea, crustaceans and algae. Therefore, ingestion of undercooked or raw contaminated sea or brackish water occupants will lead to septicemia and mild gastroenteritis (Baker-Austin et al., 2021). Other than that, *V.alginolyticus* which contains various virulence factors can be transmitted to human via wound exposure towards contaminated water was proved to cause chronic non-healing. This finding was supported by occurrence of the condition in a woman in her 70s from Guernsey, British Isles who was presented with lesion of non-healing wound for several days after a swimming activity in the ocean upon having an open wound on her leg (Reilly *et al*., 2011). Based on a study conducted by Jones & Oliver in 2009, the causative agent present in *V.alginolyticus* causing wound infections in humans was due to collagenase and phospholipase produced by the bacteria which results in the massive tissue destruction upon inoculation of host's tissues.

Several preventive methods can be imposed to curb the disease. One of them is to monitor the quality of fish to be marketed to the consumers by performing microbiological analysis (Iwamoto *et al*., 2010). Other approach that can be done is

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increasing awareness amongst public especially to those working in food industry in terms of ideal cooking temperature to avoid ingesting undercooked food (Potasman *et al*., 2002). Since the bacteria can be transmitted through wound exposure to contaminated water, Centers for Disease Control (CDC) has provided a guideline to restrict shellfish and water exposure to immunocompromised and susceptible individuals to prevent the occurrence of the disease.

10.0 Conclusion

In conclusion, diseased kidney and liver samples collected from Asian sea bass in Kelantan and Terengganu were detected using Polymerase Chain Reaction (PCR). All *V.alginolyticus* were presented with two major and several atypical virulence-associated genes of *Vibrio* spp. These results indicate that most of the diseased samples were contaminated with *V. alginolyticus* that contained virulence genes that contributed to the pathogenicity of the bacteria. Since most of the isolates contains collagenase as virulence factors which was expressed by *colA*, this may explain massive tissue destruction leading to tissue necrosis manifested by skin infections. Therefore, in the present study, *V. alginolyticus* can pose threat for both aquatic animals and humans by the activity of its virulence factors hence awareness should be raised among Kelantan and Terengganu citizens.

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11.0 Recommendations

Several recommendations can be made to improve this study in the future. One of the recommendations is to use a larger sample size obtained from East Coast states such as Kelantan, Terengganu and Pahang. Bigger sample size would provide more diverse result in terms of detection of virulence genes which will provide better prevalence of the genes. Since this study design is case control with non-probability with bias sampling hence, samples collected were only from diseased fish. The sampling method can be changed to random sampling in which samples can be obtained from asymptomatic fishes with addition to the other fish species farmed within the same location. Other than that, many other pathogens can cause similar clinical signs as Vibriosis, therefore bacteria detected during primary bacterial identification must be reported.

For this study, conventional monoplex PCR were performed. The virulence-associated genes were detected once at a time due to the difference in PCR protocol hence the result must be obtained several times. Therefore, for future work, it is suggested that the molecular detection of virulence-associated genes in *Vibrio* spp. to be done in multiplex PCR so that more primers can be used for rapid detection of many genes within similar isolates and it is preferably to use the method it less time-consuming.

Other than that, next generation sequencing (NGS) can be utilized for molecular detection as it is used for the sequencing of whole exomes, genomes, transcriptomes and targeted gene regions. NGS also provides high sensitivity, accuracy and

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specificity compared to conventional and real-time PCR despite being less cost-effective and more time consuming for sequencing low numbers of targets.

In addition, for future study, plasmid profiling can be performed to identify the potential spread of virulence genes. In bacteria, genes can be found within plasmid DNA and it is found that plasmids are important for horizontal gene transfer (HGT). The mechanism of HGT can be achieved conjugation, transformation and transduction. When a typing method known as plasmid profiling, or plasmid fingerprinting, is applied, plasmids can potentially be used as markers of different bacterial strains. By using agarose gel electrophoresis, these techniques separate the various species of deoxyribonucleic acid from partially purified plasma based on their molecular sizes. Plasmid profiling is helpful in tracing intra- and inter-species spread of virulence genes and aiding in investigation of bacterial disease outbreaks.

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Appendix A

Appendix A.1: Presence of virulent-associated genes detected by PCR

Bacterial isolates	Virulence genes											
	ColA	*hlyA*	*Vcg*CP1	*Vcg*EP2	*tdh*	*tlh*	*trh*	toxR(Vh)	*luxR*	*chiA*	*vvhA*	toxR(Vc)
Kelantan												
VAK 1	+	-	-	+	-	-	-	-	-	+	+	-
VAK 2	-	-	-	-	+	-	-	-	-	+	-	-
VAK 3	+	-	+	-	-	+	-	-	-	+	-	-
VAK 4	+	-	-	-	+	+	-	-	-	+	-	-
VAK 5	+	-	-	-	-	+	-	-	-	+	-	-
VAK 6	+	-	-	-	-	-	-	-	-	+	-	-
VAK 7	+	-	-	-	-	-	-	-	-	+	-	-
VAK 8	+	-	-	+	-	+	-	-	-	+	-	-
VAK 9	+	-	-	-	-	-	-	-	-	+	-	-
VAK 10	+	-	-	-	-	-	-	-	-	+	-	-
VAK 11	+	-	-	+	-	+	-	-	-	+	-	-
Terengganu												
VAT 1	+	-	-	-	-	-	-	-	-	+	-	-
VAT 2	+	-	-	-	-	+	-	-	-	+	-	-
VAT 3	+	-	-	-	-	-	-	-	-	+	-	-
VAT 4	+	-	-	-	-	-	-	-	-	+	-	-
VAT 5	+	-	-	-	-	-	-	-	-	+	-	-
VAT 6	+	-	-	+	-	-	-	-	+	+	-	-

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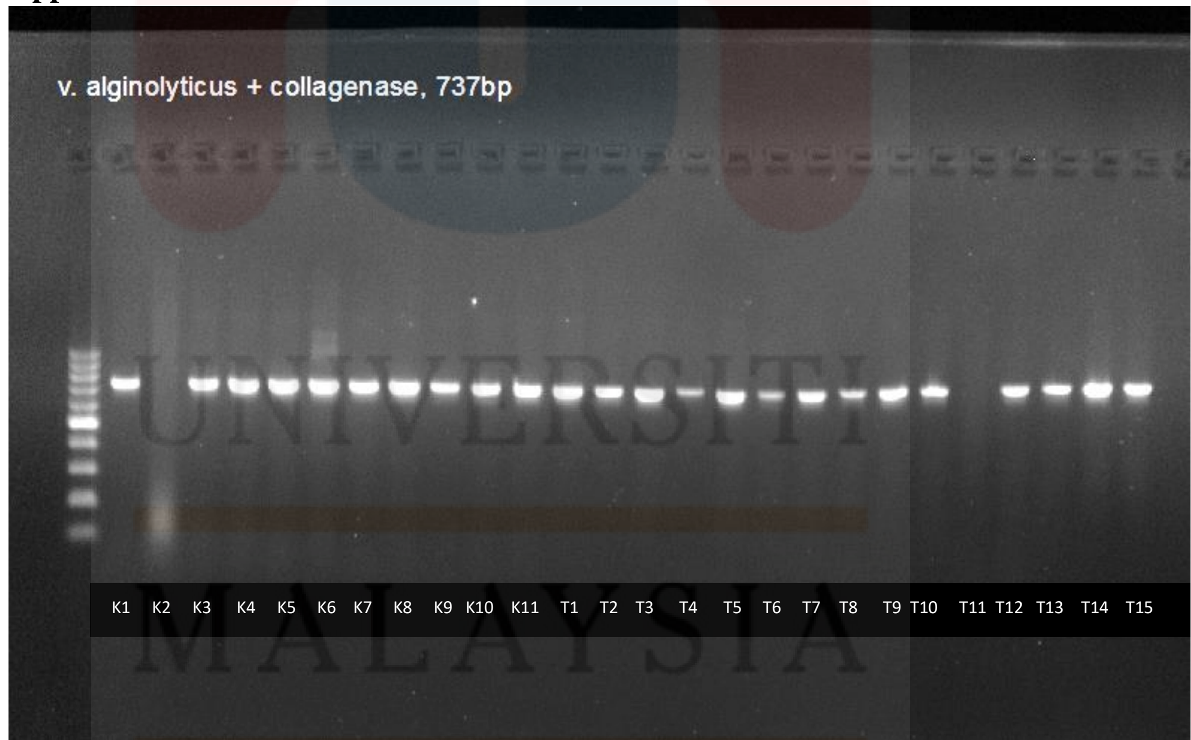
VAT 7	+	-	-	-	-	-	-	-	-	+	-	-
VAT 8	+	-	-	-	-	-	-	-	-	+	-	-
VAT 9	+	-	-	-	-	-	-	-	-	+	-	-
VAT 10	+	-	-	-	-	-	-	-	-	+	-	-
VAT 11	-	-	-	-	-	-	-	-	-	+	-	-
VAT 12	+	-	-	-	-	+	-	-	-	+	-	-
VAT 13	+	-	-	-	-	-	-	-	-	+	-	-
VAT 14	+	-	-	-	-	-	-	-	-	+	-	-
VAT 15	+	-	-	-	-	-	-	-	-	+	-	-

(+) denotes positive; (-) denotes negative

Appendix B



Footnote; K denotes as VAK1 (*V.alginolyticus* Kelantan 1), T denotes as VAT1 (*V.alginolyticus* Terengganu 1)

Appendix B.1: Gel electrophoresis of collagenase gene detected in *V.alginolyticus*

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