

**DETECTION OF VANCOMYCIN RESISTANCE GENES IN
VIBRIO ALGINOLYTICUS ISOLATED FROM SEABASS, *LATES
CALCARIFER* ON EAST COAST, MALAYSIA.**

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A RESEARCH PAPER SUBMITTED TO THE FACULTY OF VETERINARY
MEDICINE, UNIVERSITI MALAYSIA KELANTAN
IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR
THE DEGREE OF DOCTOR OF VETERINARY MEDICINE
FEBRUARY 2023

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CERTIFICATION

This is to certify that we have read this research paper entitled '**Detection of vancomycin resistance genes in *Vibrio alginolyticus* isolated from seabass, *Lates calcarifer* on East Coast, Malaysia**' by Nur Faqihah Binti Mohd 'Asri and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course DVT55204- Research Project.



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ACKNOWLEDGEMENT

**Greatly appreciated to those who have given their fully support, guidance,
advice for the completion of this project paper:**

Dr. Ruhil Hayati Binti Hamdan

Dr. Tan Li Peng

Ain Auzureen Binti Mat Zin

Dr. Nora Faten Afifah Binti Mohamad

Lab Assistants of FPV UMK

Family Members

DVM Class of 2018/2023

Thank you.

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DEDICATIONS

I dedicate this thesis to God the Almighty, the omnipotent, and to Prophet Muhammad SAW. This work is also dedicated to My dearest parents, my sister, Nur Dini, and my brothers for always supporting, and encouraging me in my ups and downs.

I dedicate this thesis to my supervisor Dr. Ruhil Hayati Hamdan and co-supervisor Dr. Tan Li Peng and Kak Ain Auzureen for aiding and guiding me in my final year project.

Special thanks to Dr. Nora Faten and the lab assistants for helping me throughout my journey.

I dedicate this thesis to my housemate Rahmah and Najla for always supporting, me and having joyous moments together with me.

Thank you for my classmates 2018/2023.

God Bless.

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List of abbreviations

AMP - Ampicillin

AMR - Antimicrobial resistance (AMR)

ARB - Antibiotic resistant bacteria

ARG - Antibiotic resistance genes

AST - Antibiotic susceptibility test

C - Chloramphenicol

CIP - Ciprofloxacin

CN - Gentamicin

CTT - Cefotetan

CTX - Cefotaxime

DNTP - Deoxyribonucleotide triphosphate

DVS - Department of Veterinary Services

E - Erythromycin

FAO - Food and Agriculture Organization

FEP - Cefepime

K - Kanamycin

KF - Cephalothin

MAR - Multiple antibiotic resistance

MHA- Mueller Hinton Agar

NA - Nalidixic acid

NaCl - Sodium chloride

NGS - Next-generation sequencing

NPRA - National Pharmaceutical Regulatory Agency

OT - Oxytetracycline

PCR - Polymerase Chain Reaction

RD - Rifampicin

SXT - Trimethoprim/sulfamethoxazole

TE - Tetracycline

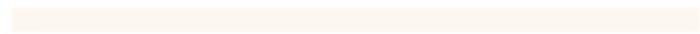
TSA - Tryptic Soy Agar

TSB - Trypticase Soy Broth

VA - Vancomycin



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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 55204-Research Project.

Vibrio alginolyticus is a pathogenic gram-negative bacterium that can cause infection in both aquatic animals and humans. The main objectives of this study are (i) to investigate the antibiotic profiles of *V. alginolyticus* isolated from seabass on the East Coast, Malaysia, and (ii) to determine vancomycin resistance genes towards *V. alginolyticus*. A total of 19 samples of *V. alginolyticus* were isolated from seabass fish or known as *Lates carcarifer* from East Coast, Malaysia. Antibiotic susceptibility test (AST) was conducted using Kirby-Bauer method by using antibiotics like ampicillin (AMP10), cefotaxime (CTX30), cefotetan (CTT30), chloramphenicol (C30), ciprofloxacin (CIP5), erythromycin (E15), cefepime (FEP30), gentamicin (CN10), kanamycin (K30), cephalothin (KF30), nalidixic acid (NA30), rifampicin (RD5), streptomycin (S10), tetracycline (TE30), oxytetracycline (OT30), trimethoprim/sulfamethoxazole (SXT1.25/23.75), vancomycin (VA30). Polymerase Chain Reaction (PCR) was carried out for the detection of vancomycin resistance genes (*vanA*, *vanB*, *vanC1*, *vanC2/C3*, *vanR*, *vanS*, *vanH*, *vanD* and *vanE*). As a result, 18 resistance patterns were observed in this study. Multiple antibiotic resistance (MAR) index values for Laguna Semerak, Kuala Ibai, and Sungai Besut were 0.29, 0.19, and 0.23, respectively. Laguna Semerak and Sungai Besut show a significant risk of contamination with the high usage of antibiotics. None of the vancomycin resistance genes were found. This study demonstrates a report on multidrug resistance of *V. alginolyticus* in seabass that could be of concern to the fish farmers. In addition, data from this study can be further used in fish disease management plans.

Keywords: *Vibrio alginolyticus*, *Lates calcarifer*, vancomycin, antibiotics, resistance gene.

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 55204- Projek Penyelidikan.

Vibrio alginolyticus ialah bakteria patogenik gram-negatif yang boleh menyebabkan jangkitan pada haiwan akuatik dan manusia. Objektif utama kajian ini adalah (i) untuk menyiasat profil antibiotik *V. alginolyticus* yang diisolasi daripada ikan siakap di pantai Timur, Malaysia dan (ii) untuk menentukan gen rintangan vancomycin terhadap *V. alginolyticus*. Sebanyak 19 sampel *V. alginolyticus* telah diisolasi daripada ikan siakap atau dikenali sebagai *Lates carcarifer* di Pantai Timur, Malaysia. Ujian kerintangan antibiotik (AST) telah diuji untuk menggunakan antibiotik kaedah Kirby-Bauer seperti ampicillin (AMP10), cefotaxime (CTX30), cefotetan (CTT30), chloramphenicol (C30), ciprofloxacin (CIP5), erythromycin (E15), cefepime (FEP30), gentamicin (CN10), kanamycin (K30), cephalothin (KF30), nalidixic acid (NA30), rifampicin (RD5), streptomycin (S10), tetracycline (TE30), oxytetracycline (OT30), trimethoprim/sulfamethoxazole (SXT1.25/ 23.75), vankomisin (VA30). Tindak balas berantai polimerase (PCR) telah dijalankan untuk pengesanan gen rintangan vancomycin (*vanA*, *vanB*, *vanC1*, *vanC2/C3*, *vanR*, *vanH*, *vanD* dan *vanE*). Sebagai hasilnya, 18 corak gen rintangan telah diperhatikan dalam kajian ini. Nilai indeks perintang perlbagai dadah (Multidrug antibiotic resistance) bagi Laguna Semerak, Kuala Ibai dan Sungai Besut masing-masing adalah 0.29, 0.19 dan 0.23. Laguna Semerak dan Sungai Besut menunjukkan risiko pencemaran yang besar dengan penggunaan antibiotik yang tinggi. Tiada gen rintangan vancomycin ditemui. Kajian ini menunjukkan kajian terhadap ikan siakap dan laporan tentang perintang perlbagai dadah (MAR) terhadap *V. alginolyticus* yang boleh membimbangkan penternak ikan. Selain itu, data daripada kajian ini boleh digunakan selanjutnya dalam rancangan pengurusan penyakit ikan.

Kata kunci: *Vibrio alginolyticus*, *Lates calcarifer*, vancomycin, antibiotik, gen rintangan.

1.0 INTRODUCTION

Aquaculture industry demand has increased year by year in order to satisfy the demand for seafood in society. Noted by the United Nations Food and Agriculture Organization (FAO), there are 1,381,423 tonnes of marine capture fisheries production recorded in 2007. From the data shown, the consumption of fisheries and aquaculture is one of the major food industries in Malaysia. But marine life will always be exposed to pathogenic bacteria such as *Vibrio* spp., with pathogenic strains such as *Vibrio alginolyticus*. To prevent such infection from occurring, a drug known as antibiotic is used.

V. alginolyticus is a gram-negative bacteria that can be found widely in marine environments. It is a member of gammaproteobacteria in *Vibrionaceae* family (Jones, 2014), oxidase-positive rod-shaped halophilic bacteria with motile single polar flagellum when grown in a liquid medium (Palit & Nair, 2014; Jones 2014). It is a pathogenic bacterium that causes infections in both humans and animals leading to serious seafood poisoning or fatal extra-intestinal infections such as necrotizing soft-tissue infections, bacteraemia, and septic shock that are accompanied by multiple organ failure (Fu et al., 2016). Another clinical syndrome connected with *V.alginolyticus* are otitis externa and conjunctivitis obtained from seawater during swimming (Mukherji et al. 2000).

Antibiotics are drugs that are used to prevent bacterial infections that act as medication to prevent disease in marine environments. In addition, it comes with other roles as a feed additive and growth promoter to increase the growth rate. *Vibrio* spp. is present worldwide with broad temperature values and salinity (Ramalingam & Ramarani, 2006), and due to the pathogenic strains of *Vibrio* spp. It has led to the usage of various antibiotics in marine environments including Vancomycin. Vancomycin acts as a bactericidal for cell wall synthesis inhibitor that can interfere with the production of bacterial cell walls.

Extensive usage of antibiotics has led to the emergency level causing antibiotic resistance toward the bacterial strains notably in *Vibrio* spp. Reportedly, the resistance of pathogenic level in *Vibrio* spp. has increased year by year (Letchumanan et al., 2015) thus, this is a worrying phenomenon as the level of pathogenicity increases, the disease will be harder to be treated as there is a small option left to be used for treatment. There is a report regarding *V. alginolyticus* resistance toward vancomycin (Kang et al., 2016). Antibiotic resistance genes can be obtained by the bacteria due to overexposure. The acquisition can be via horizontal gene transfer or

vertical gene transfer. In bacteria, there is one structure known as a plasmid that carries genes and it acts as the one that carries the antibiotic resistance genes because it contains the genetic determinants of antibiotic resistance.

In this study, the antibiotic resistance patterns and vancomycin resistance genes were determined in *V. alginolyticus* isolated from *Lates calcarifer*, seabass on East coast of Malaysia.

1.1 Research problem

It is crucial to determine the resistance and susceptibility towards the vancomycin genes of *V. alginolyticus*. There are various reports regarding the detection of the vancomycin resistance gene for an instant, Çardak et al. (2016), were able to detect *vanA* from the bacterial isolation of Enterobacteriaceae isolated from the Istanbul Strait, the Canakkale Strait, and the Sea of Marmara. In this study, we try to detect vancomycin resistance genes of *vanA*, *vanB*, *vanC1*, *vanC2/C3*, *vanR*, *vanS*, *vanH*, *vanD* and *vanE*. Furthermore, antimicrobial resistance (AMR) is also important to be determined in order to prepare the optimal treatment for the infected.

1.2 Research questions

- (i) What are the antibiotic profiles of *V. alginolyticus* isolated from seabass on the East Coast, Malaysia?
- (ii) What vancomycin resistance genes can be detected in *V. alginolyticus* isolates?

1.3 Research hypothesis

- (i) *Vibrio alginolyticus* isolated from seabass on the East coast, Malaysia has different antibiotic profiles.
- (ii) Vancomycin-resistance genes can be detected in *Vibrio alginolyticus* isolates.

1.4 Objectives

- (i) To investigate the antibiotic profiles of *Vibrio alginolyticus* isolated from seabass on the East coast, Malaysia
- (ii) To determine the vancomycin resistance genes towards *Vibrio alginolyticus* isolated from seabass on East Coast Malaysia.



2.0 LITERATURE REVIEW

2.1 *Vibrio alginolyticus* infection in humans and fish

Vibrio alginolyticus is a gram-negative, rod-shaped, halophilic bacteria with wide temperature and salinity ranging from 17 to 35 °C and 5 to 25%, respectively (Hörmansdorfer et al., 2000). This bacteria is also related to high mortality in the aquaculture industry if late treatment is provided. *Vibrio alginolyticus* is pathogenic for marine *Vibrio* spp. (Fu et al., 2016). In human-related infection, *V. alginolyticus* can be spread through undercooked or raw consumption of sea products and cause clinical syndromes like severe gastroenteritis and extraintestinal disease related to wounds, intracranial infection in immunocompromised and cirrhotic patients) (Kang et al., 2016). In fish, it can cause septicaemia, darken skin and loose scales on the skin, and congestion of the liver, swim bladder, and intestines walls (Colorni et al., 1981).

2.2 *Lates calcarifer*

Lates calcarifer, also known as barramundi, Asian sea bass, or giant sea perch is catadromous where they will spend their life in freshwater and migrate to salt water for breeding purposes. Asian sea bass can survive in a broad range of salinity but they need to be introduced slowly into the salt water to prevent shock. Habitat regions can be varied from tropical, saltwater or marine, or freshwater. Asian sea bass is serially hermaphroditic where they can transform from male to female. The transformation can usually occur from 3 to 8 years (Guiguen et al., 1994; FAO, 1999). Based on the report, in 2014 there were 30,440 tons of both pond and cages sea bass production contributing to RM 539 million but by 2016, there was declining in sea bass production by 50% with a decrease in profit with only 15,024 tons production (Aripin et al., 2019).

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2.3 Antibiotic resistance and susceptibility in *Vibrio alginolyticus*.

Antibiotic resistance is a crisis that occurs worldwide where it leads to the limitation of alternative treatment for the infected. It is also related to high morbidity and mortality (Akova, 2016). There is a report regarding the occurrence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) that did occur naturally in ancient times back in millions or billions of years (Hall and Barlow, 2004; Wright, 2007). According to D'Costa et al. (2011), antibiotic resistance genes of vancomycin and other antibiotics like β -lactams and tetracycline were found in the cores of the frozen sediment of 30,000 years old. The bacteria found in aquatic animals like sea bass which is major for human consumption has risen public health. Asian sea bass is not excluded from the infection for example vibriosis. Vibriosis is a common infection in aquatic animals that causes loss to the farmer (Yazid et al., 2021). Vibriosis is caused by the *Vibrio* spp. like *Vibrio alginolyticus*. Hence to treat vibriosis, antibiotics are used. According to Shahimi et al. (2021), the example of antibiotics that is susceptible in *Vibrio alginolyticus* are kanamycin, tetracycline, and also streptomycin. The over usage of antibiotics can lead to antibiotic resistance like in vancomycin and penicillin (Shahimi et al., 2021). Antibiotic resistance can be detected with the multiple antibiotic resistance (MAR) index, where the value that is greater than 0.2 shows high usage of the antibiotic in the respective place.

2.4 Vancomycin resistance genes in *Vibrio* spp.

Vancomycin is a drug of glycopeptide that is used to treat bacterial infections. It is derived from *Streptomyces orientalis* which is a glycopeptide related to ristocetin (Drugbank, 2018). Vancomycin acts as a bactericidal by interfering with the synthesis of cell walls (Dasgupta, 2012). In Malaysia, The National Pharmaceutical Regulatory Agency (NPRA) and the Department of Veterinary Services (DVS) have banned the usage of vancomycin in order to avoid the high level of antibiotic resistance strains in bacteria (Wada et al., 2022). Antibiotic resistance genes are genes that are located on plasmid or transposons of the gram-negative bacteria and they can be transferred from cell to cell through conjugation, transformation, or transduction process. The genetic element can be transmitted through horizontal gene transfer where the exchange of genes occurs in a population and spread to a different species of bacteria or vertical gene transfer where the genes can be transmitted from the mother to the offspring (Coleman & Smith, 2014; Lucey, 2022). Reported studies showed that there was a vancomycin resistant gene found in *Vibrio alginolyticus* isolated in Korea (Kang et al., 2016)

3.0 MATERIALS AND METHODS

3.1 Bacterial collection

Vibrio alginolyticus isolates were obtained from archive samples stored in the Trypticase Soy Broth (TSB) (Oxoid, England) added with 50% glycerol stocks in -80°C freezer in Zoonotic Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. The isolates were revived in Tryptic Soy Agar, TSA (Oxoid, England) and incubated at 30°C for 24 hours. Table 1 shows the list of *V. alginolyticus* used in this study.

Table 1: The list of *Vibrio alginolyticus* isolates from East Coast, Malaysia.

No	Bacterial isolates	Bacterial strain code	Sampling Site
1.	VAK 1	LS3LY	Laguna Semerak, Kelantan
2.	VAK 2	LS4LY	
3.	VAK 3	LS5LY	
4.	VAK 4	LS6LG	
5.	VAK 5	LS10LY	
6.	VAK 6,	LS17LY,	
7.	VAK 11	LS28LY	
8.	VAK 7	LS18LY	
9.	VAK 8	LS21LY	
10.	VAK 9	LS24LY	
11.	VAK 10	LS25LY	

12.	VAT 1	KI4LY	Kuala Ibai, Terengganu
13.	VAT 2	KI15LY	
14.	VAT 3	KI26LY	
15.	VAT 9	SB17LY	Sungai Besut, Terengganu
16.	VAT 8	SB13LY	
17.	VAT 10	SB20LY	
18.	VAT 11	SB26LY	
19.	VAT 12	SB28LY	

3.2 Antibiotic sensitivity testing (AST)

Antibiotic sensitivity testing was carried out to identify and determine which of the antimicrobial regimen is working on the patients. The inoculum of bacterial suspension was equivalent to 0.5 of McFarland standard. The inoculum was prepared in 0.85% of NaCl (normal saline). Through, Kirby-Bauer disk diffusion susceptibility test protocol, the medium used was Mueller Hinton Agar (MHA). The bacterial sample was then inoculated on the MHA using a sterile swab. The antimicrobial disk was dispensed by using forceps on the MHA. Once all of the disks have been dispensed, the plates were inverted and incubated at 35°C for 16 h (CLSI, 2015). The antibiotic used were ampicillin (AMP10), cefotaxime (CTX30), cefotetan (CTT30), Chloramphenicol (C30), ciprofloxacin (CIP5), erythromycin (E15), cefepime (FEP30), gentamicin (CN10), kanamycin (K30), cephalothin (KF30), nalidixic acid (NA30), rifampicin (RD5), streptomycin (S10), tetracycline (TE30), oxytetracycline (OT30), trimethoprim/sulfamethoxazole (SXT1.25/23.75), vancomycin (VA30).

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3.3 Multiple antibiotic resistance (MAR) index determination

The determination of multiple antibiotic resistance index was determined through the calculation of the formula by using the formula $MAR = a / (y \times x)$, where a: the total of cases of antibiotic resistance, y: the number of antibiotics used, x: the total of isolates. MAR value that is greater than 0.2 implies that the isolates are from high risk contaminated sources, where antibiotics used, are frequent (Ayandele et al., 2020). The MAR value is equal to or less than 0.2 meaning that the antibiotics are rarely or never used.

3.4 DNA extraction

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

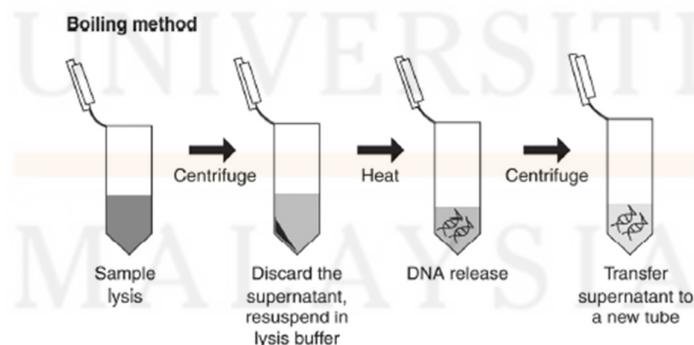


Figure 1. Boiling method (Barbosa et al., 2016).

3.5 Polymerase Chain Reaction (PCR) of antibiotic resistance genes.

The primers that were used in this study to detect the presence of vancomycin resistance genes were selected based on the previously conducted research. There were 9 primers and specific PCR protocols for each primer were used in this study based on table 2. The reagents used and the volume 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 12.5 μ l, forward primer 1 μ l, reverse primer 1 μ l, nuclease free water 8.5 μ l, and DNA template 2 μ l. The primers used for the vancomycin resistance gene were listed in table 2. Each of the primers has its own protocol based on the reference listed.

Table 2: The list of primers and PCR protocol for detection of vancomycin resistance genes in *Vibrio alginolyticus*.

Gene	Primer (5'-3')	Protocol	Tm	Bp	Reference
<i>vanA</i>	F - GGGAAAACGACAA TTGC R 5'- GTACAATGCGGCC GTTA-3'	94°C for 3 min, 30 cycles at 94°C 60 sec, 54°C 60 sec, 72°C 60 sec, and 72°C 10 min.		732	Jackson et al., 2004
<i>vanB</i>	F - CATCGCCGTCCCCG AATTTCAA R - GATGCGAAGATAC GCGTGGCT	94°C 3 min, 30 cycles at 94°C 60 sec, 54°C 60 sec, 72°C 60 sec, and 72°C 10 min.		667	Shahimi et al., 2021

		extension at 72°C for 2 min. After the last cycle, the reaction was terminated by incubation at 72°C for 6 min.			
<i>vanS</i>	<p>F- AACGACTATTCCA AACTAGAAC</p> <p>R- GCTGGAAGCTCTA CCCTAAA</p>	95 °C for 3 min (initial denaturation), followed by 35 cycles of 95 °C for 1 min , 60 °C for 40 sec, 72 °C for 50 sec and a final extension of 72 °C for 7 min.	1094	1155	Whitener et al., 2004
<i>vanH</i>	<p>F- GTGAGCAGGATGA GGCAGA-3'</p> <p>R- GCTGCGACTATAA GCCAACAC-3'</p>	95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 1 min, followed by a final elongation step at	644	503	Rezvani et al., 2016

		72 °C for 7 min.			
<i>vanD</i>	F- TAAGGCGCTTGCAT ATACCG-3) R- TGCAGCCAAGTAT CCGGTAA-3)	30 sec at 94°C, 15 sec at 54°C, and 15 sec at 72°C (30 cycles)	545	461	Perichon et al., 1997
<i>vanE</i>	E1 (5- TGTGGTATCGGAG CTGCAG-3) E2 (5- GTCGATTCTCGCTA ATCC-3)	30 sec at 94°C, 30 sec at 52°C, and 30 sec at 72°C (30 cycles).		513	Fines et al., 1999

3.6 Agarose Gel Electrophoresis

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

4.0 RESULTS

4.1 Antimicrobial sensitivity testing (AST)

A total of 19 isolated samples of *Vibrio alginolyticus* were tested with antimicrobial sensitivity testing (AST). Eighteen different antibiotic resistance patterns were observed in this study (Table 3). Most of the bacterial isolates that were isolated were resistant towards vancomycin (89.47%) and ampicillin (84.21%) followed by cephalothin (52.63%), cefotaxime (36.84%), erythromycin and gentamicin with 26.31%, rifampicin and cefepime with 21.05%, kanamycin and nalidixic acid with 10.52%, ciprofloxacin, trimethoprim/sulfamethoxazole and oxytetracycline with 5.26%. All of the isolates were susceptible to tetracycline, cefotetan, and chloramphenicol. MAR index values for Laguna Semerak, Kelantan, Kuala Ibai, and Sungai Besut Terengganu are 0.29, 0.19, and 0.23, respectively (Table 4).

Table 3: Antibiotic resistance patterns of *Vibrio alginolyticus*.

No	Bacterial isolates	Bacterial strain code	Sampling Site	Antibiotic resistance patterns
1.	VAK 1	LS3LY	Laguna Semerak, Kelantan	AMP, KF, S, VA
2.	VAK 2	LS4LY	Laguna Semerak, Kelantan	AMP, CTX, KF, VA
3.	VAK 3	LS5LY	Laguna Semerak, Kelantan	AMP, CN, CTX, KF, S, VA
4.	VAK 4	LS6LG	Laguna Semerak, Kelantan	AMP, CN, CTX, E, FEP, K, RD, S, VA

5.	VAK 5 VAT 9	LS10LY SB17LY	Laguna Semerak, Kelantan Sungai Besut, Terengganu	AMP, KF, VA
6.	VAK 6, VAK 11	LS17LY, LS28LY	Laguna Semerak, Kelantan	AMP, S, VA
7.	VAK 7	LS18LY	Laguna Semerak, Kelantan	AMP, CN, CTX, E, KF, VA
8.	VAK 8	LS21LY	Laguna Semerak, Kelantan	AMP, CN, CIP, KF, S, VA
9.	VAK 9	LS24LY	Laguna Semerak, Kelantan	AMP, K, KF, VA
10.	VAK 10	LS25LY	Laguna Semerak, Kelantan	AMP, CTX, E, FEP, KF, S, VA
11.	VAT 1	KI4LY	Kuala Ibai, Terengganu	CTX, S, SXT
12.	VAT 2	KI15LY	Kuala Ibai, Terengganu	AMP, E, NA, RD, VA
13.	VAT 3	KI26LY	Kuala Ibai, Terengganu	CTX, FEP
15.	VAT 8	SB13LY	Sungai Besut, Terengganu	NA, VA, AMP, VA
16.	VAT 10	SB20LY	Sungai Besut, Terengganu	OT

17.	VAT 11	SB26LY	Sungai Besut, Terengganu	AMP, KF, RD, S, VA
18.	VAT 12	SB28LY	Sungai Besut, Terengganu	AMP, CN, E, FEP, RD, S, VA

Table 4. MAR index value of *Vibrio alginolyticus*

Places	Bacterial isolates	Antibiotic resistant	MAR index
Laguna Semerak, Kelantan	VAK 1	AMP, KF, S, VA	0.29
	VAK 2	AMP, CTX, KF, VA	
	VAK 3	AMP, CN, CTX, KF, S, VA	
	VAK 4	AMP, CN, CTX, E, FEP, K, RD, S, VA	
	VAK 5	AMP, KF, VA	
	VAK 6	AMP, S, VA	
	VAK 7	AMP, CN, CTX, E, KF, VA	
	VAK 8	AMP, CN, CIP, KF, S, VA	
	VAK 9	AMP, K, KF, VA	

	VAK 10	AMP, CTX, E, FEP, KF, S, VA	
	VAK 11	AMP, S, VA	
Kuala Ibai, Terengganu	VAT 1	CTX, S, SXT	0.19
	VAT 2	AMP, E, NA, RD, VA	
	VAT 3	CTX, FEP	
Sungai Besut, Terengganu	VAT 8	NA, VA, AMP, VA	0.23
	VAT 9	AMP, KF, VA	
	VAT 10	OT	
	VAT 11	AMP, KF, RD, S, VA	
	VAT 12	AMP, CN, E, FEP, RD, S, VA	

4.2 Polymerase Chain Reaction (PCR)

None of the vancomycin resistance genes of *vanA*, *vanB*, *vanC1*, *vanC2/C3*, *vanR*, *vanS*, *vanH*, *vanD*, *vanE* were detected in this study.

5.0 DISCUSSION

In Malaysia, the Asian seabass has been one of the farmed aquaculture marine fish since 1970. Asian seabass is hardy and easily domesticated where they can tolerate variations of salinity. The main Asian seabass farming in Malaysia focuses more on the East Coast (Kelantan, Terengganu, Pahang) and the northern part of peninsula Malaysia (Perak, Penang, and Kedah) (IDRIS, 2022). However, Asian seabass are not excluded from being infected with diseases like Vibriosis. Due to this, antibiotics are one of the ways of treating them. In addition to that, the vibriosis incidence is also increasing where climate change and also rising of the sea water temperature contribute to the spreading of the *Vibrio* spp. (Baker-Austin et al., 2018). There was also a report in Malaysia regarding the outbreak of vibriosis caused by *Vibrio harveyi* and *Vibrio alginolyticus* isolated from the diseased fish where the mortality rate is 29% within 10 days (Mohamad et al., 2019).

There were 19 bacterial samples (n=19) of *V. alginolyticus* isolated from Laguna Semerak in Kelantan and also in Kuala Ibai and Sungai Besut in Terengganu, Malaysia. According to Baker-Austin et al. (2018), the significance of non-cholera *Vibrio* spp. like *V. alginolyticus* in humans, it can cause gastroenteritis, septicemia, and wound infection and these pathogens can be transmitted through ingestion of the infected aquatic animals. Non-cholera *Vibrio* spp. are crucial environmental human pathogens that emerge from aquatic and marine habitats and antibiotics are widely used to treat the infection. In addition, the vibriosis incidence is also increasing where climate change and also rising sea water temperatures contribute to the spreading of the *Vibrio* spp. (Baker-Austin et al., 2018). There was also a report in Malaysia regarding the outbreak of vibriosis caused by *V. harveyi* and *V. alginolyticus* isolated from the diseased fish where the mortality rate is 29% within 10 days (Mohamad et al., 2019).

Antibiotic susceptibility testing (AST) used 17 antibiotics on *Vibrio alginolyticus* (n=19). *V. alginolyticus* demonstrated the highest level of vancomycin resistance in this study. Ampicillin had the second-highest rate of resistance in bacteria followed by cephalothin, cefotaxime, erythromycin and gentamicin, rifampicin and cefepime, kanamycin and nalidixic acid and ciprofloxacin, trimethoprim/sulfamethoxazole and oxytetracycline. The isolated bacteria were susceptible towards tetracycline, cefotetan, and chloramphenicol. Nevertheless, resistance to ampicillin is not uncommon in *Vibrio* spp. and has been used since 1960 (Venggadasamy et al., 2021). As a result of excessive and uncontrolled use of antibiotics in treating human disease and many agricultural practices, high incidences of ampicillin-

resistant *Vibrio alginolyticus* have been reported in the previous report from Shahimi et al., (2021). Although ampicillin is not used in the management of Vibriosis, these findings are of great concern as it impedes the role of ampicillin in the empirical management of bacterial infections (Venggadasamy et al., 2021).

The bacteria isolated from Laguna Semerak, Kelantan, has the highest MAR index (0.29), followed by Sungai Besut (0.23), and Kuala Ibai (0.19), with a range of 0.19 to 0.29. Although the MAR index provides a good measure of the severity of antibiotic resistance in the samples, comparisons of MAR indices between studies are impossible to make due to the variation in the types of antibiotics tested and the total number of antibiotics used in individual studies (Venggadasamy et al., 2021). For instance, a comparison between the studies done by Narayanan et al. (2020) and Siddique et al. (2021) demonstrates the highest MAR indices of *V. parahaemolyticus* isolates are 0.71 and 0.27, respectively. Narayanan et al. (2020) showed that the isolates were resistant to 17 out of 24 antibiotics, while Siddique et al. (2021), were only resistant to four out of 15 antibiotics. In addition, antibiotic resistance levels are influenced by the difference in geographical locations and selective pressures (Lesley et al., 2011; Tunung et al., 2012; Shahimi et al., 2021).

In this study, there were 9 primers were used to detect the vancomycin resistance genes. The genes for the detection of vancomycin resistance were *vanA*, *vanB*, *vanC1*, *vanC2/C3*, *vanR*, *vanS*, *vanH*, *vanD*, *vanE*. The result showed all of the *Vibrio alginolyticus* that were isolated from the liver of *Lates calcarifer* on the East Coast, Malaysia was negative towards the vancomycin resistance genes where there was no presence of a positive band on the photographed gel electrophoresis on UV Transilluminator. The previous study detected vancomycin resistant genes, *vanA*, and *vanB* in *Vibrio alginolyticus* (Ateba et al., 2013). The complex glycopeptide of vancomycin disrupts the development of growing gram-positive bacteria from synthesising cell wall peptidoglycans. This antibiotic interacts with the D-alanine-Dalanine termini of the pentapeptide side chains, which interferes sterically with the formation of the bridges between peptidoglycan chains (Murray et al. 2016). This antibiotic is inactive against Gram-negative bacteria because the molecule is too large to pass through the outer membrane pores and reach the peptidoglycan target site. The genes for vancomycin resistance are primarily *vanA* and *vanB* which mediate changes in the pentapeptide terminus (Coombs et al. 1999; Murray et al. 2016).

6.0 CONCLUSION AND RECOMMENDATION,

In conclusion, antimicrobial sensitivity testing (AST) also shows a diverse degree of antimicrobial resistance where the majority of the isolated *Vibrio alginolyticus* were resistant towards highly on vancomycin and secondly towards ampicillin. The bacterial isolated also showed resistance towards cephalothin, cefotaxime, erythromycin and gentamicin, rifampicin and cefepime, kanamycin and nalidixic acid, ciprofloxacin, trimethoprim/sulfamethoxazole and oxytetracycline. In this study, eighteen resistance patterns were found. There were respective MAR index values of 0.29, 0.19, and 0.23 for Laguna Semerak, Kuala Ibai, and Sungai Besut. Due to the widespread use of antibiotics, Laguna Semerak and Sungai Besut exhibit a major danger of contamination. None of the vancomycin resistance genes (*vanA*, *vanB*, *vanC1*, *vanC2/C3*, *vanR*, *vanS*, *vanH*, *vanD* and, *vanE*) were found. This study demonstrates a report on multidrug resistance of *Vibrio alginolyticus* in seabass that could be of concern to the fish farmers. In addition, data from this study can be further used in fish disease management plans.

For recommendation, further research and study like next-generation sequencing (NGS) can be conducted to confirm the presence of the resistance gene towards vancomycin in *Lates calcarifer* on the East Coast for the detection of nucleotide order in the entire genome. Time constraint is also one of the factors that need to be considered causing the lack of lab work done. Due to the limited timeframe for lab work, further confirmation for resistance genes cannot be done in this study, like further optimization for annealing temperature for PCR. Optimization of annealing temperature is essential due to specificity and sensitivity based on the PCR product (Anwar & Sutanto, 2021). Also, in this study, there was no positive control that is used for each of the genes. The positive control is used to alleviate the false negative result and as a marking that the work done is meticulous.

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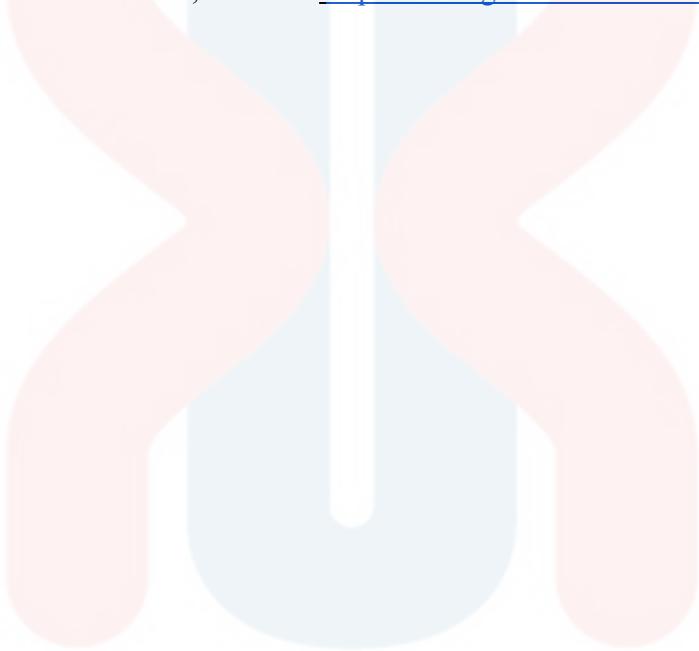
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Appendix A



Figure 2. Gel electrophoresis of *vanD* and *vanH*

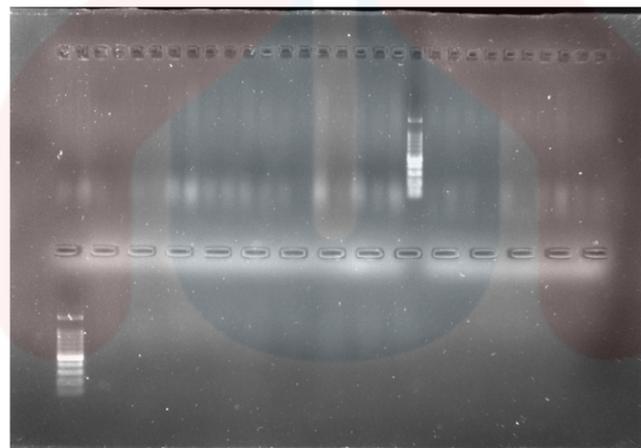


Figure 3. Gel electrophoresis of *vanR* and *vanS*



Figure 4. Gel electrophoresis of *vanE*, *vanR*, *vanC2/C3*