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Antibiotic Resistance and Virulence Genes of *Vibrio alginolyticus* from Asian Seabass in the East Coast of Peninsular Malaysia

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Abstract | *Vibrio alginolyticus* caused vibriosis and contributed to a major problem in seabass farming. This study aims to determine the antibiotic resistance and virulence genes of *V. alginolyticus* isolates from Asian seabass in the East Coast of Peninsular Malaysia. A total of 180 Asian seabass was dissected and a loopful of kidney and liver samples were inoculated on thiosulphate-citrate bile-salt sucrose (TCBS) agar and CHROMagar™ Vibrio. Polymerase chain reaction (PCR) was used to identify the isolates and detect the twelve virulence genes associated with *V. alginolyticus*. Antibiotic resistance tests were conducted using 17 antibiotics. The difference in index scores between locations was tested using one-way ANOVA, while the index scores between (multidrug resistance index) MARi and multi-virulence gene indexes (MVGi) were compared using the Mann-Whitney test. Total of 26 (7.2%) isolates out of 360 organ samples were determined as *V. alginolyticus*. The observation of external lesions was haemorrhagic eyes, pale skin, detachable scales, pale liver and enlarged spleen. Twenty-six isolates of *V. alginolyticus* were containing *chi*A (100%) and 96% of *col*A genes. None of the isolates possess *hly*A, *tox*R_{VH}, *tox*$R_{VC,}$ and *trh-tdh* genes. MVGi were varying from 0.16 to 0.33. In addition, 18 antibiotic resistance patterns were identified, and the MARi ranged from 0.05 to 1. It is alarming that seven out of 12 isolates from Sungai Besut, Terengganu were resistant to all antibiotics tested. The statistical analysis revealed that the mean MARi in Besut, Terengganu was significantly higher than in other locations (P=0.032). Additionally, the index score between MARi and MVGi was found to be significantly different (P=0.013), indicating a possible relationship between the two variables. The outcome of this study may be helpful in elucidating *V. alginolyticus* pathogenicity and evaluating antibiotics misuse for the development of sustainable disease control methods.

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Introduction

The Asian seabass (*Lates calcarifer*, Bloch) also known as barramundi has been widely growing in Southeast Asia, and Australia, and has been in Malaysian since the 1970s and essentially cultured for its hardy, fast-growing, and high salinity tolerant (Idris *et al*., 2022). In the early 1920s, aquaculture in Malaysia has become an important sector in increasing food in local production including in brackish, freshwater, and marine water aquaculture (FAO, 2020). Mohd-Yazid *et al*. (2021) estimated the economic loss caused by Vibriosis in Asian seabass from the east coast of peninsular Malaysia was at $0.19 per tail per kilogram equal to 7.06% of the total production cost of Asian seabass per kilogram. In recent years, the challenges in maintaining great systems seabass farm were discussed by Ong *et al*. (2016) such as farmers letting the dead fish in the cages instead of using proper disposal, bacterial disease and protozoan infection, sudden climate changes, and bottom of laguna covered with silt.

Vibriosis has been commonly reported as a marine pathogen that can infect a variety of aquatic animals, specifically *Vibrio alginolyticus capable* of carrying pathogenic genes that can cause serious septicaemia (Mustapha *et al*., 2013). *Vibrio alginolyticus*, a Gram-negative rod bacterium, was considered a harmful infection and pathogenic to humans and aquatic animals (Dong *et al*., 2020). Many mortalities cases of aquatic animals associated were revealed, including Pacific oyster, sea cage cultured cobia, cultured hybrid grouper, Asian seabass, and cultured European seabass (Krupesha *et al*., 2012; Korun *et al*., 2013; Mao *et al*., 2021; Mohamad *et al*., 2019; Rameshkumar *et al*., 2017). In addition, the evolution of *V. alginolyticus* was associated with human infections, leading to fatal food poisoning, necrotizing soft-tissue, bacteraemia, septic shock, and organ failures (Fu *et al*., 2016).

The aquaculture sector has become the main economy contributing to farmers and rapidly increasing to fulfill the protein demands around the world. WHO described that antimicrobial resistance (AMR) was caused by the misuse and overuse of antimicrobials in the development of drug-resistance pathogens, lack of clean water and sanitization, and inadequate infection prevention (WHO, 2021). The misuse and abuse of antibiotic drugs among fish farmers in Malaysia are one of the factors contributing to the increasing cases of antimicrobial resistance particularly in aquaculture sectors. The challenging factor arising in aquaculture was fish healthcare like disease control, outbreak infection and antibiotics still being the main tools to prevent the presence of bacteria using multiple types of antibiotics (Kathleen *et al*., 2016). The expansion of antimicrobial resistance may spread to humans from one ecological niche and result in an increasing number of failures in treatment and life-threatening diseases (Aich *et al*., 2018). The same authors added that the residual antimicrobial may contribute to human health leading to allergy, toxicity, intestinal flora variation, and health hazards.

Common antibiotic that was resistant was beta-lactam antibiotics like ampicillin, penicillin G, and penicillin (Hernández-Robles *et al*., 2016; Nurliyana *et al*., 2019; Zavala-Norzagaray *et al*., 2015). Thus, bacteria were allowed to exchange their genetic materials to adapt to antibiotic attacks by horizontal gene transfer and gene mutations (Sun *et al*., 2019). The resistance mechanism of beta-lactams in Gram-negative bacteria was achieved by modifications of their target site, the penicillin-binding protein (PBPs) (Munita and Arias, 2015). Haemolysis, hemagglutination, protease production, polar and lateral flagella, and outer membrane protein A are known to contribute to the virulence of Vibrio (Mao *et al*., 2021; Zanetti *et al*., 2000). *Vibrio alginolyticus* carries a few virulence genes such as collagenase, *omp*K, *tox*R in Malaysia (Nor Najwa *et al*., 2015), *tox*R, *tlh*, *asp*A, clg, *fla*B, fur, *omp*W (Yang *et al*., 2021).

Table 1 was showing the previous studies that have found a high prevalence of *V. alginolyticus* in aquatic life in Malaysia. Elhadi *et al*. (2004) mentioned that the seafood markets in Malaysia potentially infected with pathogenic *Vibrio* species nevertheless the changing season and need for capable consumer measures. For instance, Lee and Wee (2012) reported the correlation between antibiogram of *V. alginolyticus* bacteria with heavy metal resistance genes were developed after being exposed to heavy metal residues for ambiguous period. Vibriosis has been reported in various fundamental knowledge including history, taxonomy, and epidemiological aspects and control measures of vibriosis (Amalina *et al*., 2019). The extensive and misuses usage of antibiotic in aquaculture as prevention options in controlling the spread of disease has become the big issues to emergence of antibiotic drug resistance in *V. parahaemolyticus* (Tan *et al*., 2020).

Table 1: *List of the previous studies that stated the high prevalence of Vibrio infection in aquatic animal in Malaysia.*

Disease	Type of aquatic animal	Location	References
V. alginolyticus	Shrimp, squid, crab, cockles, and mussels	Selangor, Negeri Sembilan, Pulau Pinang, Sarawak (Malaysia)	Elhadi *et al.*, 2004
V. alginolyticus	White leg shrimp (*Litopenaeus vannamei*)	Farms at Selangor, Malaysia	Lee and Wee, 2012
Vibrio spp.	Cultured groupers (*Epinephelus* spp.)	Peninsular Malaysia	Amalina *et al.*, 2019
Vibrio parahaemolyticus	Blood clams, shrimps, surf clams, and squids	Wet markets in Selangor, Malaysia	Tan *et al.*, 2020
Vibrio alginolyticus	Cockles (*Anadora granosa)*	Seri Kembangan, Seremban, Kuala Terengganu (Malaysia)	Shahimi *et al.*, 2021

In other way to identify the presence of *V. alginolyticus* strain in cockles, the authors using four antibiotic resistance genes such as penicillin (*pbp2a*), ampicillin (*bla*OXA), erythromycin (*erm*B) and vancomycin (*van*B) and the results showed the low percentage due to the *V. alginolyticus* isolates were not exposed to high-risk sources of antibiotic contamination where antibiotic were often used (Shahimi *et al.*, 2021).

The presence of pathogenic Vibrio infection in varying organisms in Malaysia is a sign of the need for continuous monitoring for food safety and public health. The occurrence of *V. alginolyticus* infection carrying several virulence genes was crucial globally. *V. alginolyticus* may cause different clinical symptoms such as septicemia, ophthalmia, corneal opaqueness, melanosis, white spot syndrome on *Penaeus vannamei*, necrosis and mortality. Hence, this study aimed to identify the drug resistance profile and determine the distribution of virulence genes in *Vibrio alginolyticus* that was potentially pathogenic and isolated from the Asian seabass in the East Coast of Peninsular Malaysia. Our results may guide local antibiotic use and regulations of disease infection for sustaining a healthy aquaculture environment.

Materials and Methods

Study sites of sampling

Hundred and eighty (180) samples of Asian seabass were randomly collected from six locations in the East Coast region of Peninsular Malaysia. Two commercial farms located in Kelantan are Laguna Semerak (5.86405, 102.497) and Laguna Tumpat (6.21358, 102.13032). Three selected farms in Terengganu are Kuala Setiu (5.64338, 102.75405), Kuala Ibai (5.27562, 103.16258) and Sungai Besut (5.81430, 102.55669). Only one farm in Kuala Pahang, Pahang (3.52887, 103.45147). Samples weighing approximately 300-500g were transferred into an ice box in sterile condition and transported immediately to the Aquatic Animal Health Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan.

Isolation of Vibrio alginolyticus

The Vibrio species were isolated by streaking the loopful of liver and kidney (n = 360) of Asian seabass onto TCBS (Oxoid, UK) and CHROMagar™ Vibrio (CHROMagar, France) agar. The plates were incubated at 35°C for 24 to 48 hours. The isolates were preserved in TSB with 50% of glycerol and kept at -80°C for further use. Single presumptive *V. alginolyticus* colonies were picked for Gram staining, catalase, and oxidase tests.

DNA extraction

The bacterial DNA was extracted using a boiling method by Ahmed and Dablool (2017). Briefly, 1 ml of bacterial culture was centrifuged at 12, 000 rpm for 5 min and discarded the supernatant. The pellet was resuspended with 500 µl of sterile distilled water and vortexed vigorously. Then, the suspension was boiled in a 95°C water bath for 15 min. After that, it was immediately cooled in ice for 15 min. The suspension was centrifuged at 12,000 rpm for 5 min and transferred the supernatant into a new 1.5 ml microcentrifuge tube. The supernatant was stored at -20°C until further use. The DNA was quantified using a nanophotometer P360 class (IMPLEN, Germany).

Bacterial identification using pyrH and designated species-specific primers

The genus identity confirmation of presumptive *Vibrio* isolates was based on the *pyr*H gene (Byers *et al.*, 1998; Nurliyana *et al.*, 2019). Then, species-specific primers were used to classify the isolates into *V. alginolyticus* as shown in Table 2.

Table 2: *Primers for the confirmation of Vibrio genus and species.*

Primers	Sequences (5' - 3')	Amplicon size (bp)	Tm	References
pyrH	F: GATCGTATGGCTCAAGAAG R: TAGGCATTTTGTGGTCACG	440	59	Nurliyana *et al.* (2019)
Vibrio alginolyticus specific	F: TCCGTGGTGCAGGCCTTGCT R: TCAACTTTCGTCGCTTTTAGT	324	60	In this study

Tm: Melting temperature, F: Forward, R: Reverse, bp: base pair

Antimicrobial resistance test and multiple antibiotic resistance index (MARi)

Antimicrobial resistance assay was performed with the disk diffusion method according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2015). A total of 17 antibiotic agents (Oxoid, UK) were used such as ampicillin (AMP; 10 µg), cefotaxime (CTX; 30 µg), cefotetan (CTT; 30 µg), chloramphenicol (C; 30 µg), ciprofloxacin (CIP; 5 µg), erythromycin (E; 15 µg), cefepime (FEP; 30 µg), gentamicin (CN; 10 µg), kanamycin (K; 30 µg), cephalothin (KF; 30 µg), nalidixic acid (NA; 30 µg), rifampicin (RD; 5 µg), streptomycin (S; 10 µg), tetracycline (TE; 30 µg), oxytetracycline (OT; 30 µg) trimethoprim/sulfamethoxazole (SXT; 1.25 µg and 23.75 µg), vancomycin (VA; 30 µg). Briefly, the bacterial culture was suspended in a sterile 0.85 % sodium chloride solution and then coated on Mueller Hinton agar plates. Different antibiotic discs were placed onto the plates and incubated at 35°C for at least 24 hours. The diameter of the inhibition zone was measured, and the results were interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2015).

The multiple antibiotic resistance index (MARi) was according to Krumperman (1983), when applied to single isolate was defined A/B, where A represent the number of antibiotics to resistant isolates and B represent the total number of antibiotics tested which the exposed to isolate. MAR indexing of 0.200 was to differentiate between low and high risk of contamination inconsistent. The indices were in a range of ambiguity and careful inspection. MAR value equals or less than 0.2 were determine that the antibiotic seldom or never used on treatment purpose while, MAR index higher than 0.2 stated the animals in high risk of exposure of these antibiotics.

Detection of virulence genes and multiple virulence gene index (MVGi)

Twelve virulence genes were used to evaluate the distribution of virulence genes in *V. alginolyticus*. The virulence genes used in this study were chitinase (*chi*A), collagenase (*col*A), transcriptional regulator (*lux*R), thermostable direct hemolysin (*tdh*), thermolabile hemolysin (*tlh*), *trh–tdh* related hemolysin, *E. coli* α-hemolysin (*hyl*A), virulence-correlated gene ($vcgEP_2$ and $vcgCP_1$), recombinant *V. vulnificus* cytolysin (*vvh*A), and toxin transcriptional activator; $toxR_{Vc}$ and $toxR_{Vh}$ (Table 3). The multiple virulence gene indexes by Deng *et al.* (2020), MVGIs = the number of virulence genes detected in one strain/total number of detected virulence genes).

The PCR was performed in 25 µl per reaction containing 12.5 µl of Master Mix (Promega, USA), 1.0 µl of each primer (10 µM), 2.0 µl of DNA template, and 8.5 µl of nucleus-free water. The amplification was performed using a thermal cycler (BioRad, USA), and the PCR products were assessed with 1.5% agarose gel electrophoresis.

Data analysis

To test for differences in the index scores between multiple locations, one-way ANOVA was performed, while the Mann-Whitney test was used to compare the index scores between two variables, namely MARi and MVGi. Normality and equal variance assumptions were checked using the graphical histogram and Levene's test, respectively. The mean and standard deviation were calculated for the normally distributed data while the mean rank was calculated for the skewed distribution data. Error bars plot was used to visualise the variability of the data according to the locations. The data was analysed using IBM SPSS version 27 with a statistical significance level set as 0.05.

Results and Discussion

Isolation and identification of V. alginolyticus from Asian seabass

In this study, *Vibrio* species were isolated from farmed Asian seabass in three regions such as Kelantan, Terengganu, and Pahang in the East Coast of

Table 3: *Primers were used for the identification of virulence factors of Vibrio species in this study.*

Gene	Primer sequences (5' - 3')	Amplicon size (bp)	Tm	References
tdh	F: GTAAAGGTCTCTGACTTTTGGAC R: TGGAATAGAACCTTCATCTTCACC	269	58	Nurliyana *et al.* 2019
tlh	F: AAAGCGGATTATGCAGAAGCACTG R: GCTACTTTCTAGCATTTTCTCTGC	448	58	
trh-tdh	F: TTGGCTTCGATATTTTCAGTATCT R: CATAACAAACATATGCCCATTTCCG	500	46	
hlyA	F: GGCAAACAGCGAAACAAATACC R: CTCAGCGGGCTAATACGGTTTA	738	55	
vcgEP$_2$	F: CTCAATTGACAATGATCT R: CGCTTAGGATGATCGGTG	278	47	Fri *et al.* 2017
vcgCP$_1$	F: AGCTGCCGATAGCGATCT R: CGCTTAGGATGATCGGTG	278	56	
chiA	F: CTCAAGGTGTTTGGGAAGATG R: GTTGATGCCAGTGTTGTTCG	83	55	Deng *et al.* 2020
luxR	F: GTGGTTCGTCAATTCTCGAAC R: CGAATAGTGGCCACACTTC	178	55	
toxR$_{VC}$	F: ATGTTCGGATTAGGACAC R: TACTCACACACTTTGATGGC	883	53	
toxR$_{VH}$	F: GAAGCAGCACTCACCGAT R: GGTGAAGACTCATCAGCA	382	55	
colA	F: CGAGTACAGTCACTTGAAAGCC R: CACAACAGAACTCGCGTTACC	737	55	Di Pinto *et al.* 2006
vvhA	F: TTCCAACTTCAAACCGAACTATGAC R: ATTCCAGTCGATGC-GAATACGTTG	205	60	Bonny *et al.* 2018

Tm: Melting temperature, F: Forward, R: Reverse, bp: base pair.

Peninsular Malaysia, performed antibiotic resistance tests, and the presence of the virulence genes. Besides that, *V. alginolyticus* is a natural disease in environment that could causes clinical symptoms such as septicemia, haemorrhaging, dark skin, and ulcers on the skin surface Balebona *et al.* (1998). A total of 26 (7.2%) isolates from 360 organ samples such as the liver and kidney of Asian seabass were determined as *V. alginolyticus*. Most of the pathogen of *V. alginolyticus* was isolated from skin ulcers, and internal organs of diseased or freshly died fish (Emam *et al.*, 2019). *Vibrio alginolyticus* caused mortality rate were high in the egg-rearing and larval stages, symptoms of anorexia, low vitality, epidermal bleeding, ulcerated tail shaft into the muscles, swollen spleens, are able to trigger the immune system (Yanuhar *et al.*, 2022).

Generally, *V. alginolyticus* is a halophilic organism and categorised as biotype 2 of *V. parahaemolyticus* (Citil *et al.*, 2015). It is widely found in seafood, seawater, and undercooked foods that may cause serious diseases including acute gastrointestinal malfunctions. Malaysia has a tropical climate of 28°C, has the most Vibriosis outbreaks recorded and *V. alginolyticus* was one of the most frequently isolated (Deng *et al.*, 2020). The fish sampling was conducted randomly for each farm thus, clinical signs were observed only on diseased or unhealthy fish. The external lesions observed included haemorrhagic eyes, pale skin, detachable scales, pale liver, and enlarged spleen (Figure 1).

These bacteria are transmitted through water contamination or direct contact and cause exophthalmia, wounds, septicemia, opaque eyes, and fatal disease (Yanuhar *et al.*, 2022). In this study, these bacterial species were evaluated from TCBS and CHROMagar™ Vibrio agar which showed yellow and whitish colonies (Figure 2). PCR with specific primers targeting the housekeeping gene *pyr*H was used to detect the *Vibrio* spp., with a focus on the selected species *V. alginolyticus*. Ahmed *et al.* (2015) mentioned the differences of markers that were suitable in the detection of *Vibrio* spp. are *gro*EL, and heat shock protein gene. The sequences of the *pyr*H gene act as a degree of relatedness and detect the possible transmission routes in *Vibrio* spp. isolates (Nurliyana *et al.*, 2019). *pyr*H genes were common markers used

Figure 1: *Picture (A) showed a scale image of Asian seabass and a zoomed picture of detachable scales and haemorrhage on skin (B).*

in PCR and multi-locus sequence analysis (MLSA) in determining the taxonomy diversity of *Vibrio* spp. (Amalina *et al.*, 2019). Among the strains of *V. alginolyticus*, 11 samples were isolated from Laguna Semerak while 3 were isolated from Kuala Ibai and 12 from Sungai Besut (Table 5). Previous study showed that many cases of *V. alginolyticus* infection in marine and aquaculture developed from various organisms including farmed seahorse in China (Xie *et al.*, 2020), Pacific oyster in China (Yang *et al.*, 2021), *Oreochromis niloticus* in Egypt (El-Sayed *et al.*, 2019) seabass in Malaysia (Auzureen *et al.*, 2022) and *Penaeus monodon* in Bangladesh (Hannan *et al.*, 2019). Letchumanan *et al.* (2019) added that the food safety in seafood and environmental samples has been declining since the increasing cases involving the detection of antibiotic resistance strains in Malaysia.

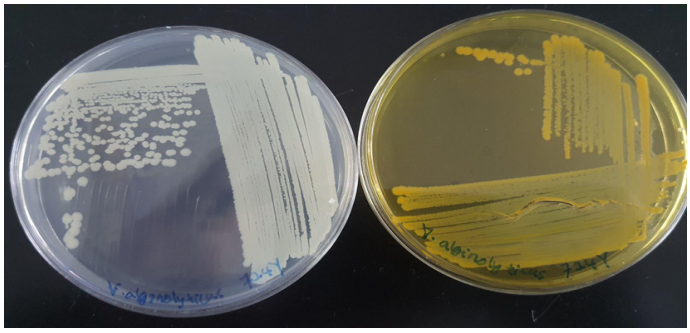

Figure 2: *V. alginolyticus shows whitish colonies on CHROMagar™ Vibrio (white agar plate), yellow colonies on TCBS agar (yellow agar plate).*

Antimicrobial susceptibility profile of *V. alginolyticus*
Seventeen types of antibiotics were used to regulate the antibiotic resistance profiles. The results showed high resistance of more than 50% to vancomycin (88.4%), ampicillin (84.6%), cephalothin (65.3%), streptomycin (61.5%) and cefotaxime (53.8%); moderate resistance (10-50%) were all the remaining,

Table 4: *Antimicrobial profiles of V. alginolyticus towards difsferent classes of antibiotics.*

Antibiotic class	Antimicrobials	Antimicrobial profiles (N)			Resistance (%)
		Resistant	Intermediate	Sensitive	
Penicillin	Ampicillin (AMP)	22	0	4	84.6
Phenicols	Chloramphenicol (C)	7	1	18	26
Fluoroquinolones	Ciprofloxacin (CIP)	8	3	15	30.7
Aminoglycoside	Gentamicin (CN)	11	3	12	42.3
	Kanamycin (K)	9	4	13	34.6
	Streptomycin (S)	16	5	5	61.5
Cephalosporins	Cefotetan (CTT)	7	2	17	26
	Cefotaxime (CTX)	14	6	6	53.8
	Cefepime (FEP)	9	10	7	34.6
	Cephalothin (KF)	17	2	7	65.3
Macrolide	Erythromycin (E)	11	12	3	42.3
Quinolone	Nalidixic acid (NA)	8	2	16	30.7
Antimycobacterials	Rifampicin (RD)	10	10	6	38.4
Tetracyclines	Tetracycline (TE)	7	1	18	26
	Oxytetracycline (OT)	8	1	17	30.7
Sulfonamide	Trimethoprim/sulfamethoxazole (SXT)	8	1	17	30.7
Glycopeptide	Vancomycin (VA)	23	1	2	88.4

N: Total isolates of antimicrobial profile, %: Percentage.

Table 5: *Resistance phenotype with multiple antibiotic resistance (MAR) index, presence of virulence genes and multiple virulence gene (MVG) index in V. alginolyticus isolated from the East Coast of Peninsular Malaysia.*

No	ID	Location	Resistance phenotype	MARi	Virulence gene	MVGi
1.	VAK1	Laguna Semerak, Kelantan	AMP-KF-S-VA	0.23	*chi*A-*colA*-*vcg*EP$_2$-*vvh*A	0.33
2.	VAK2		AMP-CTX-KF-VA	0.23	*chi*A-*tdh*	0.16
3.	VAK3		AMP-CN-CTX-KF-S-VA	0.35	*chi*A-*colA*-*vcg*CP$_1$- *tlh*	0.33
4.	VAK4		AMP-CN-CTX-E-FEP-K-RD-S-VA	0.52	*chi*A- *colA- tdh- tlh*	0.33
5.	VAK5		AMP- KF-VA	0.17	*chi*A- *colA- tlh*	0.25
6.	VAK6		AMP-S-VA	0.17	*chi*A- *colA*	0.16
7.	VAK7		AMP-CN-CTX-E-KF-VA	0.35	*chi*A- *colA*	0.16
8.	VAK8		AMP-CN-CIP-KF-S-VA	0.35	*chi*A-*colA*-*vcg*EP$_2$- *tlh*	0.33
9.	VAK9		AMP-K-KF-VA	0.23	*chi*A- *colA*	0.16
10.	VAK10		AMP-CTX-E-FEP-KF-S-VA	0.41	*chi*A- *colA*	0.16
11.	VAK11		AMP-S-VA	0.17	*chi*A-*colA*-*vcg*EP$_2$- *tlh*	0.33
12.	VAT12	Kuala Ibai, Terengganu	CTX-S-SXT	0.17	*chi*A- *colA*	0.16
13.	VAT13		AMP-E-NA-RD-VA	0.29	*chi*A- *colA- tlh*	0.25
14.	VAT14		CTX-FEP	0.12	*chi*A- *colA*	0.16
15.	VAT15	Besut, Terengganu	AMP-C-CN-CIP-CTT-CTX-E-FEP-K-KF-NA-OT-RD-S- SXT-TE-VA	1	*chi*A- *colA*	0.16
16.	VAT16		AMP-C-CN-CIP-CTT-CTX-E-FEP-K-KF-NA-OT-RD-S-SXT- TE-VA	1	*chi*A- *colA*	0.16
17.	VAT17		AMP-C-CN-CIP-CTT-CTX-E-FEP-K-KF-NA-OT-RD-S-SXT-TE-VA	1	*chi*A-*colA*-*luxR*-*vcg*EP$_2$	0.33
18.	VAT18		AMP-C-CN-CIP-CTT-CTX-E-FEP-K-KF-NA-OT-RD-S-SXT- TE-VA	1	*chi*A- *colA*-*luxR*	0.25
19.	VAT19		NA-VA	0.12	*chi*A- *colA- luxR*	0.25
20.	VAT20		AMP-KF-VA	0.17	*chi*A- *colA*	0.16
21.	VAT21		AMP-VA	0.12	*chi*A- *colA*	0.16
22.	VAT22		OT	0.05	*chi*A- *vcg*CP$_1$	0.16
23.	VAT23		AMP-KF-RD-S-VA	0.29	*chi*A- *colA- tlh*	0.25
24.	VAT24		AMP-C-CN-CIP-CTT-CTX-E-FEP-K-KF-NA-OT-RD-S-SXT-TE-VA	1	*chi*A- *colA*	0.16
25.	VAT25		AMP-C-CN-CIP-CTT-CTX-E-FEP-K-KF-NA-OT-RD-S-SXT-TE-VA	1	*chi*A- *colA*	0.16
26.	VAT26		AMP-C-CN-CIP-CTT-CTX-E-FEP-K-KF-NA-OT-RD-S-SXT-TE-VA	1	*chi*A- *colA*	0.16

42.3% (erythromycin and gentamicin), 38.4% (rifampicin), 34.6% (cefepime and kanamycin), 30.7% (ciprofloxacin, oxytetracycline, trimethoprim/sulfamethoxazole, and nalidixic acid), and 26.9% (tetracycline, cefotetan, and chloramphenicol). Even though *V. alginolyticus* infection is still sensitive to most antibiotics, it is highly resistant to penicillin, erythromycin, vancomycin, ampicillin, sulfisoxazole, and sulphonamides antibiotics (Cai *et al.*, 2022). Our result agreed with Kang *et al.* (2016) and Cai *et al.* (2022) that stated *V. alginolyticus* is highly resistant to ampicillin and vancomycin. In other study by Sun *et al.* (2024) revealed that *V. alginolyticus* has similar outcome for high level of ampicillin resistance with 93.75% from 128 samples included seafood and freshwater products that was collected across China. In addition, more than 80% of *Vibrio* spp. isolates including *V. alginolyticus* were susceptible to tetracycline and streptomycin (Amalina *et al.*, 2019).

Seventeen different antibiotic resistance profile patterns were observed (Table 5). In this present study, the MAR indexes ranged between 0.05 to 1, and the MAR index of more than 0.2 was considered as exposure to high-risk infection sources like humans, commercial poultry farms, swine, and dairy cattle

(Noorlis *et al*., 2011). Srinivasan and Ramasamy (2009) suggested that the high resistance percentage in the penicillin group, 97% in aquaculture of *Vibrio* spp. may be due to the continuous use of medicated feeds which may lead to the dissemination of virulent and resistant pathogens into the environment and can affect the antibiotic resistance patterns of other microorganism. The excessive use of antibiotics on microorganisms in aquatic environments may cause the drugs to remain in natural environments through bioaccumulation and have a gradual deposition in sediments or aquatic surfaces (Caroline *et al*., 2021). Different with Shahimi *et al*. (2021) had a low percentage of antibiotic resistance in *V. alginolyticus* due to the lack of information from animal sources or aquaculture in their study area.

Regarding the antibiotic sensitivity test of *V. alginolyticus,* it was observed for chloramphenicol and tetracycline with 69.2% and this result was in accordance with the finding of Korun *et al*. (2013). This study indicated that chloramphenicol and tetracycline antibiotics were still effective in counteracting this infectious disease (Yuidiati *et al*., 2021) but chloramphenicol was banned in Malaysia. Table 4 of the MAR index value showed seven isolates have a full resistance to antibiotics. Antibiotic resistance characterization analysis indicated that 65.3% of the total isolates had a MAR index of more than 0.2. Yu *et al*. (2023) showed that a similar 63.5% of *Vibrio* isolates showed a MAR index of more than 0.2 under the shrimp (*P. vannamei*) breeding system in China.

Antibiotic resistance was emerging mechanism happened worldwide, it occurred when there are changes of bacteria that antibiotic have no ability to destroy nor stop the infection growth. Thus, it is becoming extremely harder to treat. The study by Colavecchio *et al*. (2017) stated that the viability of phage-mediated transduction by bacteriophages was found to be a contributor to the propagation of antibiotic-resistance genes as well as environmental factors in foodborne pathogens.

Distribution of virulence genes in V. alginolyticus isolates *Vibrio* spp. disease infection was arising and caused the losses and economic impact thus it is important to study the virulence factors and pathogenic mechanism of the bacteria since there are no effective methods of controlling the infection and these factors allows bacteria to infect and damage hosts (Deng *et al*., 2020). The pathogenicity characters of bacteria was essential and were fixed on the identification of virulence factors. The releases microbiological product that can enter the host cell and use their mechanisms to contribute to the acquisition of infection (Yu *et al*., 2023). The previous study done by Yu *et al*. (2023) detected *chi*A and *lux*R genes in *V. alginolyticus* which agree with our study.

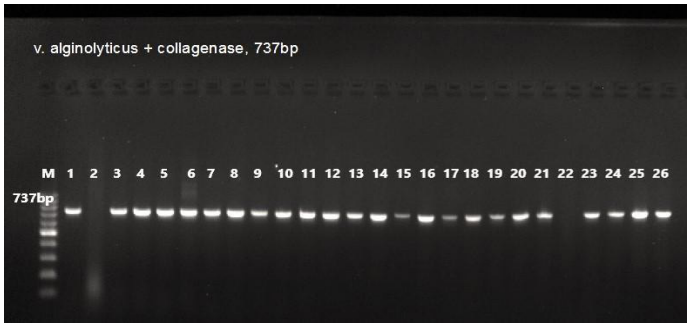

Figure 3: *The colA virulence gene of Vibrio alginolyticus was detected by using 1.5% of gel electrophoresis with 737bp size. Notes: M: 100 bp DNA ladder.*

From this study, eight virulence genes were detected among *V. alginolyticus* isolates out of 12 (Table 5) that showed a risk potential of infection in Asian seabass. Yu *et al*. (2023) had a high detection rate between 11.4 and 100% of *Vibrio* spp. in *P. vannamei* breeding system in China that had a potential of carrying and transmitting virulence genes. *chi*A, *lux*R, *tox*R and *vhh* of virulence genes were widely found among pathogenic *V. harveyi* (Ruwandeepika *et al*., 2010). Meanwhile, *chi*A2 were found in *V. cholerae* mainly on the chitinous surface of copepods and utilise chitin as the sole carbon and nitrogen source to survive in intestines and in pathogenesis (Mondal *et al*., 2014). All isolates of *V. alginolyticus* possess of *chi*A, while 92% isolates have detected *col*A among virulence genes tested (Figure 3). Chitinase are chitin-degrading enzymes, it was produced by numerous bacterial species synthesize chitinases to decompose ambient chitin for nutritional purposes (Devlin and Behnsen, 2023). The coexistence of chitinase production (virulence genes) and antibiotic resistance in bacteria especially like in *Vibrio* played a significant role in ecological and pathogenicity. Simultaneously, these bacteria may possess antibiotic resistance genes, which grant a survival advantage in environments where antibiotics are present (Thompson *et al*., 2001). Similarly, Gobarah *et al*. (2022) mentioned that 100% of *V. alginolyticus* isolates from different types of fish carried the collagenase gene. Collagenase

were utilized as biomarker in identification of *V. alginolyticus* in molecular specifically, and enable to degrading the conjunctive tissue, the basal epithelial membrane could lead to pathological of intestinal and blood stream of dissemination. The high detection of *colA* virulence genes could be due to pathogenic Vibrio species that was able to accelerate the bacterial dissemination and facilitate the diffusion of other toxins through hydrolysis of collagenous components (Galvis *et al.*, 2021).

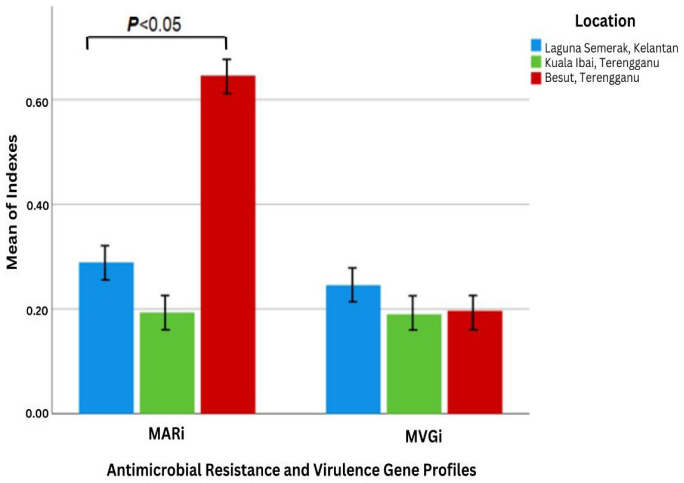

Figure 4: *The resistance phenotype with multiple antibiotic resistance (MAR) index and presence of virulence genes and multiple virulence genes (MVG) index based on the location from East Coast of Peninsular Malaysia.*

This study showed that *tlh* were distributed among *V. alginolyticus* with seven isolates (26.9%), five from Semerak and one from both Kuala Ibai and Sungai Besut. The *tlh* gene encodes thermostable direct hemolysin, while the *tdh* gene is thermostable direct haemolysin that has the virulence factors in *V. parahaemolyticus* (Hasrimi *et al.*, 2018). *Vibrio alginolyticus* was formerly regarded as biotype 2 of *V. parahaemolyticus* and both infections are known as increasing pathogenic aquatic vibrio disease including *V. vulnificus* (Xu *et al.*, 2017). Four isolates (15.3%) were detected to carry *vcg*EP$_2$, three isolates detected on *lux*R, two isolates (7.69%) carried *tdh* and one sample isolates was detected in *vcg*CP$_1$ and *vvh*A. The remaining virulence genes (*hly*A, *trh-tdh*, *tox*R$_{Vc}$ and *tox*R$_{Vh}$) were not detected from all isolates. Up to four virulence genes were detected in six isolates, with the MVG index ranging from 0.16 to 0.33 (Table 5). Virulence factors found on mobile genetic components can transform harmless bacteria into dangerous pathogens by horizontal gene transfer (Deng *et al.*, 2020). High detection rate of virulence genes showed that *V. alginolyticus* can be seen carrying virulence genes and may be widespread specifically in aquatic animal and environment, this should be taken seriously from all perspective.

Statistical analysis

Comparison of multiple antibiotic resistance (MAR) indexes and presence of virulence genes and multiple virulence genes (MVG) index based on locations.

A total of 11, 3, and 12 resistance phenotypes were found in Laguna Semerak, Kuala Ibai, and Besut, respectively. It was shown that the mean of MARi was statistically higher in Besut, Terengganu (Mean = 0.65, SD = 0.44) compared to the mean of MARi in Laguna Semerak, Kelantan (Mean=0.29, SD=0.11), with P = 0.032. There was no statistically significant difference in MVGi based on locations (P > 0.05) (Figure 4). Statistical analysis showed that the host species which is *V. alginolyticus* and sampling location showed negative correlation between the presence of virulence gene detected and antibiotic resistance profiles.

Table 6: *Comparison of index score between MARi and MVGi (n= 52).*

Group	Mean rank	z-statistics	P-value[b]
MARi	31.65	-2.490	0.013
MVGi	21.35		

[b]*Mann-whitney test sapplied; normality assumption not fulfilled.*

Comparison of multiple antibiotic resistance (MAR) index and presence multiple virulence genes (MVG) index seen in V. alginolyticus isolates in East Coast of Peninsular Malaysia

There was a statistically significant difference in an index score of three locations and antibiotic resistance between MARi and MVGi (P<0.05) on Table 6. The result indicated that the mean of MARi was higher compared to MVGi, with 31.65 and 21.35, respectively. The significantly higher mean of MARi suggested that the *Vibrio alginolyticus* bacteria being analyzed possess a considerable level of antibiotic resistance compared to their virulence capacity as indicated by MVGi. This could have several implications such as clinical implications and environmental factors. High antibiotic resistance challenged for treatment purposes that could be difficult to manage the survival of antibiotic therapies thus, the continuing monitor and other approaches of treatment were highlighted. The extensive use of antibiotics in aquaculture may lead to a predominance of resistant strains, while the

relatively lower MVGi indicates that these strains might not have a high capacity for virulence (Bobate *et al*., 2023).

Conclusions and Recommendations

The distribution, antimicrobial resistance profile and virulence genes of *V. alginolyticus* isolated from the Asian seabass cultured in the East Coast of Peninsular Malaysia were documented. *Vibrio alginolyticus* was detected in a high number of pathogenic infections and had a potential risk of carrying and transmitting virulence genes. The highest antimicrobial resistance were vancomycin and ampicillin, this could be the future global threat of antibiotic usage and must be taken into consideration. These findings highlight the importance of sustainable and responsible practices in aquaculture for food safety and public health.

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Novelty Statement

The novelty of the research resides in the contribution of *Vibrio alginolyticus* in the Asian seabass from the East Coast of Peninsular Malaysia. This study also focused on antibiotic resistance distribution and the variance of detected virulence genes of *V. alginolyticus*.

Author's Contribution

Mat Zin Ain-Auzureen, Kamaruddin Mardhiah and Ruhil Hayati Hamdan: Writing the manuscript.
Mat Zin Ain-Auzureen, Nora Faten Afifah Mohamad, Ruhil Hayati Hamdan and Maizan Mohamed: Planned and conducted the experiment.
Mohd Faizal Ghazali, Chai Min Hian, Choong Siew Shean, Najiah Musa and Tan Li Peng: Data interpretation.
Choong Siew Shean, Najiah Musa and Tan Li Peng: Review of literature.
Kamaruddin Mardhiah: Statistical analysis.
Nora Faten Afifah Mohamad and Ruhil Hayati Hamdan: Supervision.

Ethics statement

This research was conducted under the ethical evaluation of the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. The ethical code number is UMK/FPV/ACUE/PG/4/2021.

Conflict of interest

The authors have declared no conflict of interest.

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