Genomic Characterization of *Vibrio* spp. in Asian Seabass (*Lates calcarifer*, Bloch, 1790) Following Field Vaccination Using a Feed-Based Inactivated Vaccine against Vibriosis

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Abstract

Vibriosis outbreaks pose a significant threat to the productivity of Asian seabass culture, causing substantial losses. Nevertheless, the excessive utilization of antimicrobials exacerbates the issue by fostering the development of antimicrobial resistance (AMR). Consequently, exploring alternative disease management strategies, such as the introduction of oral vaccines into Asian seabass culture, has become a subject of ongoing investigation. This study aims to compare the genomic characteristics of different *Vibrio* species isolated from both orally vaccinated and unvaccinated Asian seabass populations. Archived samples of vaccinated and unvaccinated Asian seabass from one site in Selangor, Malaysia, were utilized in this sample. Briefly, the vaccinated group was administered the feed-based vaccine on week 0 (prime vaccination), 2 (booster), and 6 (second booster) at 4% body weight. At the same time, the non-vaccinated fish were fed with a commercially formulated pellet without the vaccine. *Vibrio* isolates identified from the gut samples were used in this study. The samples were stored at -80°C before being subjected to genomic DNA extraction, PCR, gel electrophoresis, and sequencing using Illumina and Nanopore platforms. Universal 16s primer and *pyrH* primer were used to identify *Vibrio* species. Bioinformatic analysis was done using NCBI BLAST, QUAST, BUSCO 5, CGE, and J Species. The isolates of *Vibrio* species exhibited smooth, convex, round, and entire colonies on TCBS agar plates, which were yellow and green. Twenty-two isolates were sent for 16s rRNA sequencing and revealed *Vibrio alginolyticus* (54.54%), followed by *V. diabolicus* (13.63%) and *V. parahaemolyticus* and *V. harveyi* (9.09% respectively). Of the 22 samples, 7 were selected for further Illumina sequencing. The whole genome sequences of the six *Vibrio* species isolated exhibited good coverage percentage, N50 value, Average Nucleotide Identity (ANIb), single-copy percentage, and GC content, while one sample showed low single-copy percentage and high duplicated percentage, which suggested contamination during DNA extraction. Eight novel alleles were discovered, three from the vaccinated group and five from the unvaccinated group, including the *Rec*, *atpA*, *gyrB*, and *pyrH*. A virulence factor database analysis search revealed 58 virulent genes from the unvaccinated samples and 39 virulent genes from the vaccinated samples. Overall, this research provides valuable insights into the genomic characteristics between orally vaccinated and unvaccinated cultured Asian seabass in the locality.

Keywords: Asian seabass, bioinformatics, next-generation sequencing, vaccination, vibriosis

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INTRODUCTION

Asian seabass *(Lates calcarifer*) is a highly sought-after species in aquaculture due to its excellent flavor, rapid growth, and high market demand (Jerry, 2013). Asian seabass aquaculture has experienced significant growth over the years, driven by domestic and international markets. One of the key challenges in Asian seabass aquaculture is the management of bacterial infections caused by *Vibrio* spp. Vibrio infections can lead to severe mortalities and economic losses in aquaculture operations (Sanches-Fernandes *et al*., 2022). *Vibrio*sis in Asian seabass can be caused by *Vibrio alginolyticus*, *Vibrio harveyi*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus*

(Eleonor, 2002; Sulumane *et al*., 2012; Kenconojati *et al*., 2023), which are ubiquitous in the marine environment (Ina-Salwany *et al*., 2019; Low *et al*., 2014). Vibriosis is a common occurrence at numerous aquaculture farms in Malaysia and nearby countries, where the climate remains tropical throughout the year, with temperatures averaging 28°C (Mohamad *et al*., 2022). In many outbreaks, *V. harveyi* was most frequently isolated, followed by *V. parahaemolyticus, V. alginolyticus*, and *V. anguillarum*, affecting Asian seabass *Lates calcarifer*, brown-marbled grouper *Epinephelus fuscoguttatus*, orange-spotted grouper *Epinephelus coioides*, snappers *Lutjanus* sp., and hybrid grouper (Ina-Salwany *et al*., 2019). The mortality of Asian seabass due to vibriosis was recorded to be 16.23%, with a higher risk at the hatchery level. It may increase mortality risk in the grow-out phase (Mohd Yazid *et al*., 2021).

Vaccination is a valuable tool for disease management in aquaculture, and there have been efforts to develop vaccines for the Asian seabass (*Lates calcarifer*) in Southeast Asia, India, and Australia (Mohd Yazid *et al*., 2021). Vaccines developed specifically for *Vibrio* spp. have shown promise in reducing the incidence and severity of bacterial infections (Kumar *et al*., 2008; Mohamad *et al*., 2021). Oral vaccination against *V. harveyi* has been reported using inactivated vaccine (Mohamad *et al*., 2022; Amir-Danial *et al*., 2023), showing the potential to protect the host fish in controlled and field conditions against vibriosis. Rapid and precise diagnosis of aquatic pathogens enables stakeholders to strategize disease-free fish broodstock with potentially healthy seeds, screening pathogens and preventing outbreaks (Delamare-Debouttеville *et al*., 2021). Therefore, next-generation sequencing, such as Illumina and Nanopore platforms, especially aquaculture, can be an avenue for rapid identification with reduced cost (Delamare-Debouttеville *et al*., 2021).

The current study focuses on the characterisation of the *Vibrio* species isolated from oral vaccinated and unvaccinated Asian seabass populations with the data analysis aimed at genomic statistics from Illumina sequencing, novel alleles, resistance genes, antimicrobial resistance profile between vaccinated and unvaccinated Asian seabass of *Vibrio* species in the locality. Vibriosis is a significant threat to the aquaculture of Asian seabass. By identifying specific virulence genes and genomic markers in vaccinated versus unvaccinated fish, particularly from oral vaccines, the research could guide more effective vaccination programs and inform biosecurity measures to mitigate the impact of vibriosis in aquaculture.

MATERIALS AND METHODS

Ethical Approval

The Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia, granted the feed-based vaccine preparation under approval number UPM/IACUC/AUP-R078/2019.

Study Period and Location

This research was partially performed at the AquaHealth Laboratory, Institute of Bioscience, Universiti Putra Malaysia and the molecular sequencing was carried out at Patriot Biotech Sdn. Bhd. during September 2023 until September 2024.

Feed-based Vaccine Preparation

The preparation of the feed-based inactivated *V. harveyi* vaccine in Asian seabass *Lates calcarifer* in a private fish farm in Malaysia as described by Amir-Danial *et al*. (2023). Briefly, *V. harveyi* strain VH1, a bacterial strain, was obtained from tiger grouper *E. fuscoguttus*, in deep-sea cages in Langkawi, Malaysia (Mursidi, 2018). This strain was noted for inducing potent antigenic responses to both a similar strain and exhibiting cross-reactivity with different species like *V. parahaemolyticus* strain VPK1, *V. alginolyticus* strain VA2, and *Photobacterium damselae* strain PDS1 (Mursidi, 2018). The initial cultivation and maintenance of *V. harveyi* strain VH1 involved a 24-hour incubation at 30°C on selective thiosulfate-citrate-bile-salts-sucrose (TCBS) agar (Oxoid, Hampshire, England), followed by inoculation and another 24-hour

incubation at 30 °C with 150 rpm in tryptone soy broth (TSB) (Oxoid) + 1.5% NaCl.

To create fine mesh feed powder, a commercial feed pellet for marine fish (Star Feed, Star Feed Mills SDN. BHD., Klang, Malaysia; with 43% protein content) was ground using a blending machine. Formalin-killed *Vibrio harveyi* strain VH1 cells were produced following the method outlined in the reference (Mohamad *et al*., 2022). In short, bacterial suspensions were treated with 0.5% formalin and stored at 4°C for 12 hours or overnight. The inactivated bacterial suspensions underwent a 15-minute centrifugation at 6000× g, followed by three sterile phosphate buffer saline (PBS) washes. The resulting bacterial pellets were then adjusted to a 10^8 CFU/mL concentration. The vaccine mixture, with the addition of palm oil (Vesawit, Malaysia) as an adjuvant at a concentration of 10%, was thoroughly blended and incorporated into the fish feed powder using an industrial mixer (Golden Bull-B10-A Universal Mixers, Johor Bahru, Malaysia). This mixture was loaded into a mini feed pellet machine (Golden Avill, Guangdong Province, China) to create feed pellets sized at 1 cm × 0.5 cm, which were then dried for 48 hours at 30˚C. A patent application has been submitted for the composition and procedure of the oral vaccine (MyIPO Malaysia, patent No. PI2021000105). In contrast, the group that was not vaccinated received commercial feed containing PBS and 10% palm oil.

A field-scale experiment was carried out at a private commercial fish farm in the western region of Malaysia, where cases of vibriosis had been reported. A total of 4800 Asian seabass (*Lates calcarifer*) with an average body weight of 182 ± 31 g were selected for the study (Amir-Danial *et al*., 2023). Before commencing the study, 15 Asian seabass were randomly dissected to ensure their health and the absence of bacteria. These fish were found to be healthy, with no bacteria detected. The experimental design and vaccine schedule are illustrated in Figure 1. The experiment was duplicated by feeding two cages with the prepared feed-based vaccine and the other two with untreated control (PBS). At the beginning of the trial, the fish underwent a 12-hour fasting period before vaccination to enhance the uptake of the feed-based vaccine. Subsequently, the fish were fed the respective feed for three consecutive days in weeks 0 (primary vaccination), 2 (first booster), and 6 (second booster) at a rate of 4% of their body weight. On all other days, the fish were fed regular untreated commercial feed pellets (Star Feed, Klang, Malaysia) until the conclusion of the 16-week experimental period.

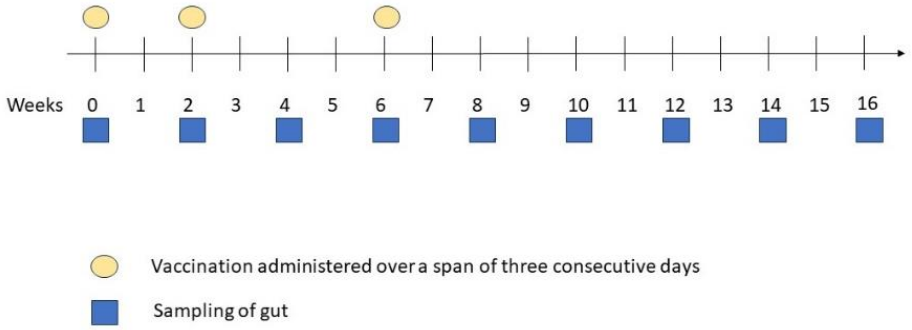

Figure 1. The administration of oral vaccine and sampling points of the Asian seabass during the field-scale experiment at Selangor, Malaysia.

Bacterial Culture

The sampling occurred every two weeks, commencing from week 0 and continuing until the study's conclusion at week 16. Information regarding incidence rates (Raju *et al*., 2023) and general clinical indicators was recorded at each sampling point. Subsequently, 15 fish from each replicate were chosen randomly and promptly euthanized through pithing. Necropsy was carried out immediately to investigate noticeable abnormalities. The gastrointestinal tract was

sampled to collect lavage fluid (Firdaus-Nawi *et al*., 2013).

Twenty-two (n = 22) isolates from weeks 4 and 10 were chosen for further identification and genomic characterization in this study. Isolate samples of *Vibrio* strains were incubated 24-hour at 30°C on thiosulfate-citrate-bile-salts-sucrose (TCBS) agar (Oxoid, Hampshire, England), followed by inoculation and another 24-hour incubation at 30°C in tryptone soy broth (TSB) (Oxoid Hampshire, England) + 1.5% NaCl.

DNA Extraction

Approximately 0.25 mg or 250 uL of the sample were homogenized in a DNA/RNA shield (Zymoresearch, California, USA), followed by DNA purification using the Zymobiomics DNA Extraction Kit (Zymoresearch, California, USA) according to the manufacturer's instructions. DNA was purified by binding it to a Zymo-spinTM column, washing it, and then eluting it using DNA buffer. After DNA was extracted, the sample was run through a JenwayTM Genova Nano Micro-Volume Spectrophotometer (Thermo Scientific, Massachusetts, USA) to assess its concentration, purity, and amount in preparation for sequencing. DNA with a concentration of at least 50 ng/L and an optical density of 180 and 220 nanometers was selected for PCR.

PCR was carried out using a mini8 thermal cycler (miniPCR bio, Boston, USA), and the reaction mixture of the respective primers universal 16s and *pyrH* was mixed with Bioline MyTaqTM Red Mix PCR master mix (Meridian Bioscience, Ohio, USA). The PCR reaction was initially done with the 16s universal primer with the forward primer 5'-AGA GTT TGA TCC TGG CTC AG-3' dan reverse primer 5'-GGT TAC CTT GTT ACG ACT T -3' with denaturation at 95°C in 3 minutes and 15 seconds, annealing at 50°C in 15 seconds followed by extension at 72°C in 2 minute for 31 cycles. Then the reaction is followed by the use of *pyrH* with the forward primer 5'- GAT CGT ATG GCT CAA GAA G-3' and reverse primer 5'-TAG GCA TTT TGT GGT CAC G- 3' with denaturation 95°C in 7 minutes and 1 minute, annealing 59°C in 2 minutes 15 seconds and extension 72°C in 1 minutes 15 seconds and 72°C for 1 minute with 33 cycles. Gel electrophoresis for all reactions was conducted with 2% agarose gel in TBE buffer and SYBR green stain (Invitrogen, Massachusetts, USA). Gel visualization was done using the FluorChem E System (ProteinSimple, San Jose, USA).

16s rRNA Sequencing

DNA samples were then sent for confirmation of identification using 16s rRNA assay and analysed using NCBI-BLAST.

Library Preparation for Illumina Sequencing

A total of 100 ng gDNA as measured using Qubit (Thermo Fisher Scientific, Massachusetts, USA) high sensitivity dsDNA assay was fragmented to 300 bp using Bioruptor (Diagenode, New Jersey, USA). The fragmented DNA was subsequently processed using the NEBUltra II Illumina Library Preparation Kit (Illumina, California, USA) according to the manufacturer's instructions. Briefly, the fragmented DNA was converted to blunt-ended DNA with 5' phosphates and 3'-hydroxyls, followed by A-tailing to incorporate a single A overhang base at the 3'end. Then, Illumina adapters were ligated to the A-tailed DNA fragments, followed by index PCR to incorporate sample-specific barcode index and enrich DNA fragments with ligated adapters at both ends. The constructed library was analyzed using a fragment analyzer (Agilent, California, USA) to estimate its concentration and fragment size distribution. Sequencing of the constructed library used NovaSEQ6000 (Illumina, California, USA) at 2 × 150 bp configuration.

Data Analysis

Paired-end reads were adapter-trimmed using fastp v0.21 (Chen *et al*. 2018). The filtered reads were assembled de novo using Unicycler v0.5.0 (Wick *et al*. 2017). Assessment of the assembly statistics and genome completeness used QUAST v5.2.0 and BUSCO v5.4.3, respectively (Gurevich *et al*. 2013; Simão *et al*. 2015). Protein prediction was carried out using

Prodigal v2.6.0 with the default setting to produce protein sequences that can be submitted to the eggnogmapper or kofamkoala webserver for detailed annotation (Cantalapiedra *et al*. 2021; Aramaki *et al*. 2020). The genome assembly was searched against various Antimicrobial Resistance databases using Abricate (https://github.com/tseemann/abricate). For taxonomic classification, the pair-wise ANI of genome assembly was calculated against the reference genomes in GTDB r207 database using fastANI v1.33 (Jain *et al*. 2018; Parks *et al*. 2020).

RESULTS AND DISCUSSION

Phenotypic Identification

All the colonies observed (n = 22) showed consistent morphology of 1–2 mm diameter with smooth, convex, round colony, which appears cloudy in TSA NaCl agar and yellow on TCBS agar. Gram staining revealed negatively curved rods.

16s rRNA Sequencing

Twenty-two samples were analysed using NCBI BLAST, and the highest percentage identity for all samples confirmed the identity of the *Vibrio* spp. (Table 1).

Overall for both vaccinated and unvaccinated groups, the main isolated bacteria identified *V. alginolyticus* as the primary species discovered (54.54%), followed by *V. diabolicus* (13.63%), *V. harveyi* (9.09%), *V. parahaemolyticus* (9.09%) followed by *V. harveyi, V. taketomensis and V. owensii* which were the least species discovered in the vaccinated samples (5.45%) (Figure 2).

Table 1. Percent identity of the *Vibrio* samples obtained (n = 22) when compared to the BLAST database

Vibrio species	Sample ID	Percent identity %	Accession No	Strain
Vibrio alginolyticus	C1	100	AP022865.1	YM4
Vibrio alginolyticus	C2	100	CP046814.1	2010V-1102
Vibrio harveyi	C3	99.41	KC871684.1	PEL36D
Vibrio alginolyticus	C4	100	OP198319.1	K41Y
Vibrio owensii	C5	87.88	MN938185.1	3-5
Vibrio taketomensis	C6	99.01	AP019649.1	-
Vibrio alginolyticus	C7	98.32	KC751249.1	A-290
Vibrio alginolyticus	C8	88.15	CP046814.1	2010V-1102
Vibrio harveyi	C9	97.45	OQ210054.1	SB2022091703
Vibrio harveyi	C10	98.50	OQ210054.1	SB2022091703
Vibrio alginolyticus	V1	99.80	OP198313.1	K36Y2
Vibrio ponticus	V2	98.06	MK482004.1	CSTC-Y1
Vibrio alginolyticus	V3	99.80	CP054700.1	GS_MYPK1
Vibrio alginolyticus	V4	97.88	CP054700.1	GS_MYPK1
Vibrio diabolicus	V5	100	LR860833.1	B1STR6
Vibrio alginolyticus	V6	99.21	OP198319.1	K41Y
Vibrio parahaemolyticus	V7	97.97	EU660319.1	CM2
Vibrio diabolicus	V8	97.42	CP042451.1	FA3
Vibrio alginolyticus	V9	99.60	OP198319.1	K41Y
Vibrio parahaemolyticus	V10	98.96	MT860504.1	3-33
Vibrio diabolicus	V11	98.83	CP042451.1	FA3
Vibrio harveyi	V12	98.58	MN578144.1	SETBT4

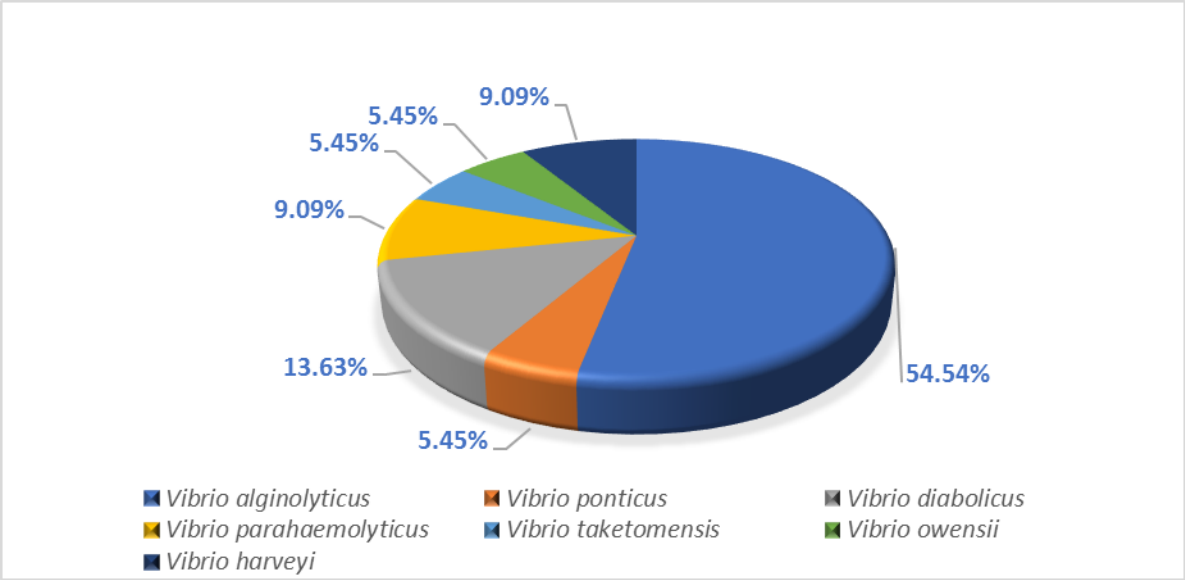

Figure 2. Percentage (%) of *Vibrio* spp. Isolated from Samples of Vaccinated and Unvaccinated Asian seabass (*Lates calcarifer*).

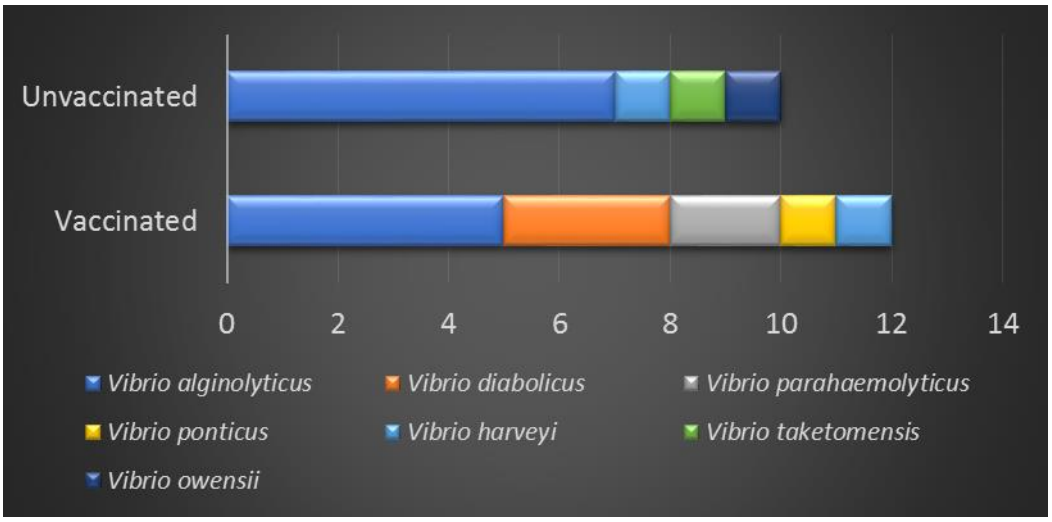

Figure 3. Frequency of *Vibrio* spp, discovered of vaccinated Asian seabass compared to unvaccinated fish through NCBI BLAST.

In comparison, the unvaccinated group's unique species isolated were *V. taketomensis* and *V. owensii*, while in the vaccinated group, the unique species were *V. diabolicus*, *V. parahaemolyticus* and *V. ponticus*. In both groups, *V. alginolyticus* was the main species discovered with *V. harveyi* (Figure 3).

Figure 4 shows the phylogenetic tree of *Vibrio* spp. generated using Phylogeny Analyses website One-click Mode enabled alignment by MUSCLE, Phylogeny by PhyML, and Tree Rendering by TreeDyn performed in one website (Dereeper *et al*., 2010; Edgar, 2004; Guindon and Gascuel, 2003; Anisimova and Gascuel, 2006; Chevenet *et al*., 2006) and further analysed using FigTree v1.4.4 for better visualization of phylogenetic tree. The species nearest to the sample determine the highest possibility for the samples to be identified as the species.

Whole Sequencing Analysis: **Illumina Novaseq 6000**

The whole genome sequencing information was further analysed the quality using the Quality Assessment Tool for Genome Assemblies (QUAST) and Assessing Genomic Data Quality and Beyond (BUSCO) v5.5.0 to discover the completeness and quality of the whole genome;

the information was further analysed using one-way ANOVA analysis methods, stated vaccination status as the independent variable and other parameters such as %ANI, %cov, and GC(%) as the dependent variable to investigate the differences of quality of whole genome information in between vaccination and unvaccinated group (Table 2).

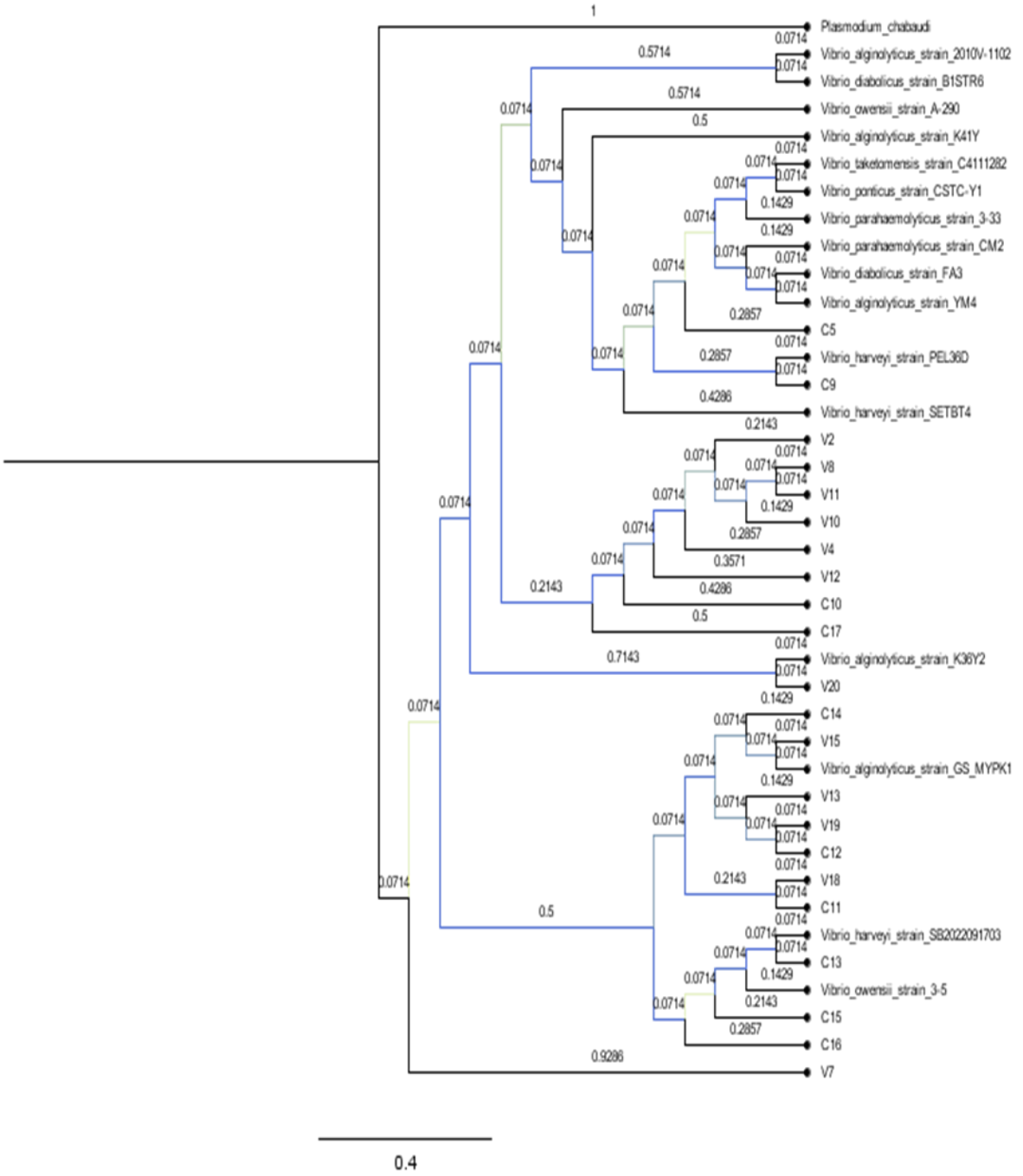

Figure 4. Phylogenetic tree generated by maximum likelihood computation analyses of the 16S rRNA sequences of *Vibrio* spp. Numbers at nodes indicate bootstrap confidence values (100 replicates). The scale bar indicates a branch length of 0.4 inferred substitutions per site.

Table 2. Assembly statistics and genome completeness of 7 selected samples using the Illumina platform

ID	Reads (millions)	Bases (Mb)	Read length (bp)	GC%	Filtered Reads (millions)	Filtered Bases (Mb)	Filtered read length (bp)	Filtered GC%
C3	10.72	1608.46	150	44.91	10.27	1540.71	149.95	45.01
C4	9.98	1496.92	150	43.56	9.57	1435.50	149.95	43.65
C5	14.47	2170.95	150	45.33	14.09	2113.07	150.00	45.38
V1	11.75	1762.86	150	44.63	11.26	1688.34	149.95	44.72
V4	13.83	2075.02	150	44.77	13.46	2018.23	150.00	44.82
V5	9.87	1480.50	150	44.88	9.46	1418.32	149.95	44.97
V10	11.19	1677.86	150	45.26	10.70	1603.78	149.95	45.35

Seven good-quality selected DNA samples were sent for whole genome sequencing using the Illumina Novaseq 6000 sequencer. The Bioruptor (Diagenode, Denville, NJ) was used to fragment 100 ng of gDNA to 300 bp, as assessed by the Qubit high sensitivity dsDNA test (Thermo Fisher Scientific, Waltham, MA). After the DNA was fragmented, it was prepared for sequencing using the NEB Ultra II Illumina Library Preparation Kit. In a nutshell, 5' phosphates, 3' hydroxyls, and A-tailing were used to transform the DNA fragments into blunt-ended DNA with a single A overhang base added to the 3' end. Illumina adapters were ligated to the A-tailed DNA fragments following index PCR to incorporate sample-specific barcode index and enrich DNA fragments with ligated adapters at both ends. The assembled library's concentration and fragment size distribution were calculated using a Fragment Analyzer (Agilent, Santa Clara, CA). NovaSEQ6000 (2 × 150 bp configuration) was used for sequencing the finished library. Adapter trimming was performed on paired-end reads with fastp v0.21 (Chen *et al*., 2018). Unicycler v0.5.0 (Wick *et al*. 2017) was used to perform a de novo assembly of the filtered readings. Gurevich *et al*. (2013) and Simo *et al*. (2015) employed QUAST v5.2.0 and BUSCO v5.4.3 to evaluate the assembly statistics and genome completeness. Protein sequences predicted by Prodigal v2.6.0 were sent to the eggnogmapper or kofamkoala website for in-depth annotation (Cantalapiedra *et al*. 2021; Aramaki *et al*. 2020). Abricate (https://github.com/tseemann/abricate) was used to search multiple databases for information on antimicrobial resistance and compare it to the genome assembly. Using fastANI v1.33 (Jain *et al*. 2018; Parks *et al*. 2020), the pair-wise ANI of the genome assembly was estimated compared to the reference genomes in the GTDB r207 database for taxonomic classification. The information was obtained and subsequently assessed (Table 3). The C4 sample was excluded from further analysis as it has low reads and a high duplication percentage.

The whole genome sequencing information was further analysed, such as virulence factors, number of novel alleles discovered, and percentage of average nucleotide identity blast (%ANIb) similarity using web-based tools such as Centre for Genomic Epidemiology-Resfinder (CGE-ResFinder), Virulence Factor Database (VFDB), Multi Locus Sequence Typing (MLST) and J species software.

Bioinformatics Analysis: Novel Genes and Novel Alleles

In the vaccinated group samples, three novel alleles were discovered, including *atp_36*, *pyrh_15*, and *gyrB_164*, meanwhile in the

Table 3. Genomic profiling metrics of 7 selected samples using the Illumina platform

ID	Reads (millions)	Bases (Mb)	Read length (bp)	Top hit (Accession Number)	% ANI	% cov	Contigs	Assembled length (Mbp)	N50 (kbp)	GC (%)	Sing Copy (%)	Duplicated (%)	Fragmented (%)
C3	10.72	1608.46	150	*Vibrio harveyi* (GCF_001591145.1)	98.6932	95.3328	43	5.91	488.34	44.83	97.6	2.4	0.0
C4	9.98	1496.92	150	*Vibrio alginolyticus* (GCF_000354175.2)	98.2074	92.8642	150	9.56	182.99	42.72	7.3	92.7	0.0
C5	14.47	2170.95	150	*Vibrio owensii* (GCF_008017815.1)	96.5046	80.1914	74	5.92	179.99	45.54	96.8	3.2	0.0
V1	11.75	1762.86	150	*Vibrio alginolyticus* (GCF_000354175.2)	98.4455	91.2389	66	5.21	186.93	44.62	99.2	0.8	0.0
V4	13.83	2075.02	150	*Vibrio alginolyticus* (GCF_000354175.2)	98.4312	93.2012	58	5.26	326.67	44.61	99.2	0.8	0.0
V5	9.87	1480.50	150	*Vibrio diabolicus* (GCA_001048675.1)	98.0258	91.7065	44	5.05	359.88	44.83	99.2	0.8	0.0
V10	11.19	1677.86	150	*Vibrio parahaemolyticus* (GCF_900460535.1)	98.4195	90.4856	28	5.14	818.20	45.24	98.4	1.6	0.0

unvaccinated group, five novel alleles, namely *rec_141*, *atpA_45*, *gyrB_120*, *pyrH_57*, and *rec_204* were discovered. The related functions of the novel alleles discovered are presented in Table 4.

Table 4. Novel alleles of five samples of *Vibrio* spp. from vaccinated and unvaccinated samples

ID	*Vibrio* spp.	Novel allele
C3	*Vibrio harveyi*	*rec_141*
C5	*Vibrio owensii*	*atpA_45*
		gyrB_120
		pyrH_57
		rec_204
V1	*Vibrio parahaemolyticus*	*atp_36*
V4	*Vibrio diabolicus*	*gyrB_164*
V5	*Vibrio alginolyticus*	*pyrH_155*

A virulence factor database analysis search revealed 58 virulent genes from the unvaccinated samples and 39 virulent genes from the vaccinated samples using the database from Virulence Factors of Pathogenic Bacteria (VFDB), as presented in Table 5.

In this study, the vaccine was derived from a local isolate *V. harveyi*, which was previously isolated from brown-marbled grouper *E. fuscoguttatus* upon infection with vibriosis in deep-sea cages in Langkawi, Malaysia (Mohamad *et al*., 2021). The fish survival rate of hybrid grouper in the vaccinated group in a field trial was higher than that of the unvaccinated group, giving potential for oral *Vibrio* vaccine that could provide good protection and stimulate immune responses. Oral vaccination is a good candidate for aquaculture fishes as it can reduce stress due to handling fish that are too small for injection vaccination or immersion (Embregts and Forlenze, 2016). Oral vaccination with a feed-based *V. harveyi* vaccine is advantageous for caged Asian seabass, as it reduces the occurrence of vibriosis and the severity of external lesions (Amir-Danial *et al*., 2023). The vaccine's mechanisms involve enhancing gut immunity through the production of specific IgM against *V. harveyi*, increased lysozyme activity, and stimulation of the size and density of GALT

regions along with their lymphocyte population (Amir-Danial *et al*., 2023).

The *Vibrio* spp. isolated from the fish's gastrointestinal tract, which was revived in the present study, revealed *V. alginolyticus* as the primary species discovered (54.54%), followed by *V. diabolicus* (13.63%), *V. harveyi* (9.09%), *V. parahaemolyticus* (9.09%) followed by *V. taketomensis and V. owensii* which were the least species discovered (5.45% respectively). Both groups revealed the detection of *V. alginolyticus* followed by *V. harveyi.*

Table 5. Virulent genes of five samples of *Vibrio* spp. of vaccinated and unvaccinated samples

ID	***Vibrio* spp.**	**No of virulent genes**	**Virulent genes**
C3	*Vibrio harveyi*	5	*vscF, L, D, H* *VPA0450*
V3	*Vibrio alginolyticus*	12	*vopD, B, R* *vcrH* *tyeA* *vscN, R, S, V, L, F* *tlh*
V4	*Vibrio alginolyticus*	11	*tlh* *vscF,L, S, R,N* *vopR* *tyeA* *vcrH* *vopB,D*
V5	*Vibrio diabolicus*	16	*vopD,B, R* *vcrH, D* *tyeA* *vscN, O, R, S, U, L, I, F, C* *tlh*
V10	*Vibrio parahaemolyticus*	41	*VPA0450* *tlh* *vscB, C, D,F,G,H,L,I K, L U, T, S, R,Q, P, O, N, X, Y* *virC* *exsA,D* *vopS, R, Q, N, B, D* *vecA* *tyeA* *svcN* *vcrD, R, G V, H* *VP 1611*

Both isolates have been found to cause pathogenic vibriosis in fish. They are responsible for disease outbreaks in Malaysia (Mohamad *et al*., 2019), with *V. alginolyticus* being a known opportunistic pathogen (Chen *et al*., 2019). This condition coincides with the farm's history, which has reported vibriosis in the field trial experimentation (Solfaine *et al*., 2024). In addition, an earlier challenge study on the farm, conducted using the same oral vaccine produced to investigate the immunoprotective effects, revealed the relative percentage survival (RPS) values obtained from the vaccinated group against *V. harveyi*, *V. alginolyticus*, and *V. parahaemolyticus* in Asian seabass, *L. calcarifer* was higher (70–80%) compared with

the control (0%) (Mohamad *et al*., 2022). The initial aim for selecting the seven samples for Illumina sequencing was to compare the same species with different vaccination statuses. However, due to variability in the success of growing and culturing the original 22 samples, the selection criteria shifted towards species variability. This allowed for broader coverage of different *Vibrio* species and their pathogenicity in marine culture, providing greater value to the genomic data by revealing novel alleles and virulence genes (Lin *et al*., 2018).

Novel Alleles

Unknown or undocumented genetic variations, such as a genetic marker, can potentially be applied to disease diagnosis (Pham *et al*., 2021). The present study discovered eight novel alleles, three from the vaccinated group and five from the unvaccinated group. *Rec* allele has been associated with resistance to the alkylating agent methyl methanesulfonate, resistance to UV irradiation, promotion of homologous recombination in Hfr mating experiments, and catalysed hydrolysis of ATP (Dumann *et al*., 2023). Meanwhile, the *atpA* allele has been associated with the synthesis of ATP for transportation (Dun and Grider, 2023). The *gyrB* allele has been associated with DNA replication (Nishiki *et al*., 2018) and the rejoining of double-stranded DNA (Dahanayake *et al*., 2020). The *pyrH* allele catalyses the reversible phosphorylation of uridine-5′-diphosphate (UMP) to uridine-5′-monophosphate (UDP) (Liu *et al*., 2015).

Virulent Genes

The identification of virulence factors is important in evaluating bacterial pathogenicity as these factors allow bacteria to infect and damage hosts (Deng *et al*., 2020) and serve as a basis for developing vaccines and diagnosing vibriosis (Cai *et al*., 2007). Virulence-associated genes of *Vibrio* spp. isolated from cultures of marine fishes in Malaysia, which was previously reported by Mohamad *et al*. (2019), where 63 *Vibrio* spp. isolated from Peninsular Malaysia revealed 66.7% were positive for *chiA*, *luxR*, and *vhpA*, which are the virulent genes produced by *V. harveyi*. Other virulent genes, *tdh, trh, hlyA* and *toxRvc*, were absent in the reported study. The *pyrH* gene in *Vibrio parahaemolyticus* employs various toxins and two type 3 secretion systems (T3SS-1 and T3SS-2) to manipulate host cells during infection (Getz and Thomas, 2018). The study found that previous findings indicated that the activity of T3SS-1 in *V. parahaemolyticus* is enhanced by the elevated expression of the master regulator ExsA (Getz and Thomas, 2018). Type III secretion system 1 (T3SS1) is a major virulence factor that delivers effectors into the host eukaryotic cytoplasm. The T3SS is necessary for the virulence of many Gram-negative bacterial species (Atkinson and Williams, 2016), and in *V. parahaemolyticus*, the T3SS has been shown to affect the biofilm formation, motility, and cytotoxicity and contributes to the survival of bacterial in the environment (Calder *et al*., 2014). The previously mentioned study also revealed that structural and regulatory mutations in *V. parahaemolyticus* type III secretion systems display variable effects on virulence. Meanwhile, VPA0450 is a type III effector protein causing host cell death caused by autophagy, cell rounding, and cell lysis (Waddell *et al*., 2014). The expression of T3SS1 is precisely controlled by the ExsA-ExsC-ExsD regulatory system (Zhang *et al*., 2016). While T3SS1-specific chaperone, *VecA* binds to *VopQ, f*or its secretion and stability in the host cells (Li *et al*., 2019). The *vscF* gene encodes the needle protein of T3SS1 (Lian *et al*., 2021). Virulence factor genes encoding thermostable direct hemolysin (*tdh*) and thermostable direct hemolysin-related hemolysin (*trh*) are closely associated with the virulence of the emergent human pathogen *Vibrio parahaemolyticus*. The gene encoding thermolabile hemolysin (*tlh*) is recognized as a key molecular marker for identifying *V. parahaemolyticus* (Gutierrez *et al*., 2013). Another virulent gene, *virC,* is a novel gene essential for Va's virulence in *V. anguillarum* (Zheng *et al*., 2017). In *Yersinia* species, TyeA is located on the bacterial surface and plays a role in regulating Yop release with the extracellular *Yersinia* disarming the immune system by

injecting effector *Yersinia* outer proteins (*Yops*) into target cells (Grabowski *et al*., 2017). The *Vcr* and the *vop* genes have been described in a study of population genomics study of *Vibrio alginolyticus* (Zheng *et al*., 2021), and *VscC* and *vscD* genes have been described in a study of functional characterization of *VscCD,* which are important components of the type III secretion system of *V. harveyi* (Zhang, 2021).

While the virulent properties of a pathogen are crucial in the onset of fish disease, environmental and nutritional factors are considered more significant in determining the severity of disease outbreaks (Jun and Woo 2003). Key factors influencing the development of vibriosis include chemical stress (e.g., diet composition, water quality, and pollution), physical stress (e.g., salinity and temperature), and biological stress (e.g., population density and the presence of other macro- or micro-organisms (Huicab-Pech *et al*. 2016).

CONCLUSION

Overall, this research provides valuable insights into the genomic characteristics and differences between vaccinated and unvaccinated Asian seabass regarding the *Vibrio* species in the locality, highlighting several novel alleles and virulent genes.

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AUTHORS' CONTRIBUTIONS

NDMT: designed, conceptualized, supervised, analyzed data and wrote the manuscript. SYY, NHN, ISMY, MNAA, NIUZ and HMG: performed the experiment, analyzed data and wrote the manuscript. All authors have read and approve the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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