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Simultaneous detection of *Vibrio* spp. in post-larvae stocking to harvest process of shrimps production in commercialised farms in Sarawak

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ABSTRACT

Aims: The shrimp farming industry faces persistent challenges stemming from infectious diseases and environmental issues. The aim of this study was to monitor and assess the risk associated with the distribution of *Vibrio* spp. throughout the entire shrimp production cycle, spanning from the first day of culture (day 1) to the 98th day of culture (day 98).
Methodology and results: The sampling was done by collecting water samples (n=48) and sediment samples (n=48) from three different locations: Telaga Air, Santubong and Tanjung Manis. Utilizing multiplex PCR with three specific primer sets, three *Vibrio* species were identified. Prevalence rates for *V. parahaemolyticus* were found to be 81.25% in water and 52.08% in sediment samples, while *V. alginolyticus* was detected in 16.67% of water samples and 27.08% of sediment samples. *Vibrio cholerae* was present in 10.42% of water samples and 25.0% of sediment samples. Furthermore, an antimicrobial susceptibility test (AST) was conducted on the *Vibrio* spp. isolates obtained from the three shrimp farms using 20 common antibiotics. Penicillin displayed the highest resistance rate at 40%, followed by streptomycin at 30%. The study also calculated the MAR index, which exhibited a range from 0.35 to 0.65.
Conclusion, significance and impact of study: These research findings provide valuable insights into the distribution of *Vibrio* spp. in Sarawak aquaculture shrimp farms, which can help in preventing and controlling the possible occurrence of vibriosis.

Keywords: Multiplex PCR, shrimp aquaculture, *V. alginolyticus, V. cholerae*, *V. parahaemolyticus*

INTRODUCTION

Modern shrimp farming, also known as marine shrimp culture in impoundments, ponds, raceways and tanks, began in the early 1970s. Today, shrimp farming enterprises are thriving in over fifty countries across the globe. The Eastern Hemisphere is dominated by key players such as Thailand, Vietnam, Indonesia, India and China, with substantial sectors also present in Malaysia, Taiwan, Bangladesh, Sri Lanka, the Philippines, Australia and Myanmar (Burma) (Debnath *et al.*, 2013). Commercial shrimp aquaculture has witnessed explosive growth in several nations, notably Malaysia, driven by the escalating global demand and dwindling percentages of shrimp caught in the wild (Ismail and Abdullah, 2013). Malaysia's extensive coastline, stretching approximately 4,809 km, provides an ideal environment for shrimp farming. Furthermore, the country's warm tropical climate and favourable environmental conditions further catalyse the advancement of shrimp farming (Chowdhury *et al.*, 2012).

Nonetheless, the shrimp farming industry remains under persistent threat due to outbreaks of infectious diseases and environmental challenges. Bacteria constitute a prevalent group of microorganisms that exert detrimental effects on the global shrimp industry. Pathogenic species within the *Vibrio* genus, including *V. cholerae*, *V. parahaemolyticus*, *V. mimicus*, *V. harveyi*, *V. alginolyticus* and *V. vulnificus*, have been identified as significant contributors to health issues in shrimp production environments (Chatterjee and Haldar, 2012). These pathogens induce severe infections, leading to a reduction in shrimp production.

Disease transmission in shrimps occurs through various means, including water, sediments, ingestion of infective material and through wounds. The collective term for infections caused by *Vibrio* spp. is vibriosis. Infected shrimps display various symptoms, including black shell disease, tail rot, septic hepatopancreatic necrosis, brown gill disease, swollen hindgut syndrome and luminous bacterial disease. These infections manifest with clinical signs such as lethargy, loss of appetite,

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Figure 1: Sampling locations in the state of Sarawak, Malaysia. Location 1: Santubong, Location 2: Tanjung Manis and Location 3: Telaga Air (Map Source: Google Map, 2020).

luminescence, yellowing of the gill tissue and red discolouration of the body (Peddie and Wardle, 2005).

Consequently, routine identification of *Vibrio* spp. in shrimp farms stands as a vital prerequisite for sound management decisions. Typically, preventive measures entail the application of disinfectants and antimicrobial solutions (Verschuere *et al.*, 2000). However, the employment of antibiotics within shrimp farms carries potential adverse repercussions, affecting both the shrimp and the surrounding environment. In the past, research on antimicrobial resistance (AMR) primarily concentrated on human health. In recent years, the focus has shifted to encompass agricultural and environmental factors due to their intricate interplay with human health. As per the Center for Disease Control and Prevention (CDC, 2013), the excessive use of antibiotics has raised substantial concerns, emerging as the primary contributor to the surge in resistant infections. The overuse of antibiotics could foster resistance among shrimp pathogens and have implications for nearby ecosystems. This heightened antibiotic use might exacerbate the challenge of treating bacterial infections within shrimp ponds. Therefore, careful consideration of antibiotics, including fluoroquinolones, tetracyclines and sulphonamides, is imperative to mitigate potential environmental and human health risks (Holmström *et al.*, 2003).

Hence, the primary objective of this research is to enhance monitoring and risk mitigation efforts. It aims to achieve this by identifying three common disease-causing *Vibrio* spp. in water and sediment samples using multiplex PCR, employing three primer sets designed to target *V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae*. Additionally, the study seeks to assess the antibiotic susceptibility of *Vibrio* species within three commercial shrimp farms in different locations.

MATERIALS AND METHODS

Samples collection

Water and sediment samples were collected from three commercial shrimp farms in Sarawak, namely, Location 1: Santubong, Location 2: Tanjung Manis and Location 3: Telaga Air (Figure 1). Sampling was carried out every two weeks from shrimp stocking until the harvesting of adult shrimp, spanning an approximate cycle of 100 days. Sampling activities were performed on the 1st, 14th, 28th, 32nd, 56th, 70th, 84th and 98th day of the culture period. The 14-day intervals were selected since they aligned with the operational schedule and production cycle of the shrimp farms, which makes it easier to integrate them into their existing routines without disrupting their activities. Besides, the standardized two-week intervals were practical and allowed an adequate timeframe for the preparation of the material, sample processing and MPN analysis. This ensured that the samples could be processed promptly without overwhelming laboratory resources.

In each sampling location, two ponds (Pond A and Pond B) were chosen for each sampling session, with the prerequisite that these two selected ponds underwent the same stocking process duration. The sampling point selection for each pond was contingent upon the location of the pond's inlet and outlet.

Water samples were obtained utilizing 250 mL sterile Schott bottles, while sediment samples were procured using an ethanol-sterilized polyvinyl chloride (PVC) pipe. Water samples were retrieved from each sampling point, ensuring a depth of at least 50 cm below the water surface, whereas sediment samples were collected with a minimum distance of 10 cm from the pond's bottom layer.

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Table 1: Multiplex-PCR Primers (Kim *et al.*, 2015).

Primer name	Target species	PCR product size (bp)	Primer sequences (5' - 3')	Protein of target gene
VP 1155272 F VP 1155272 R	*Vibrio parahaemolyticus*	297	AGCTT ATTGG CGGTT TCTGT CGG CKCAA GACCA AGAAA AGCCG TC	Hypothetical protein VPA1095
VC C634002 F VC C634002 R	*Vibrio cholerae*	154	CAAGC TCCGC ATGTC CAGAA GC GGGGC GTGAC GCGAA TGATT	Hypothetical protein VCA0694
VA 1198230 F VA 1198230 R	*Vibrio alginolyticus*	199	ACGGC ATTGG AAATT GCGAC TG TACCC GTCTC ACGAG CCCAA G	Whole-genome shotgun sequence

Physical parameters of water, including pH, temperature (°C) and salinity (PSU), were promptly recorded for every selected pond sampling point. Following collection, the samples were transported to the laboratory in a cool box containing ice and were processed within 24-h (Alfiansah *et al.*, 2018).

Samples processing

The method for sample processing was adapted from Briquaire *et al.* (2017) with modifications. Both water and sediment samples were collected at the same sampling point. Sampling points were chosen based on the position of the water inlet and outlet. In Pond A and Pond B, at least two sampling points were designated. For water samples, no less than 1 L of water samples were gathered from each point. The water samples obtained from each point were mixed at a 1:9 ratio in a sterile bijou bottle and labelled accordingly. Subsequently, 1 mL of the homogenized water sample was pipetted into 9 mL of alkaline peptone water (APW) and designated as dilution 10^{-1}.

As for sediment samples, a minimum amount of 100 g of sediment samples was collected from each point. The sediment samples collected from each point were combined at a ratio of 1:9 in a sterile plastic bag, and they were labelled based on Pond A and Pond B. One gram of the homogenized sediment sample was mixed with 9 mL of APW in a sterile bijou bottle and labelled as dilution 10^{-1}. Triplicates of samples were created for each dilution. All incubation was carried out at a temperature range of 28 °C to 30 °C for 6 to 8 h (Briquaire *et al.*, 2017).

Bacterial DNA extraction

DNA extraction was done by using the boiling-centrifugation method, as detailed by Wang *et al.* (2013), with a few modifications. Initially, 500 mL of overnight bacterial culture was transferred into a sterile 1.5 mL microcentrifuge tube and subsequently centrifuged at 10,000 rpm for 5 min. The supernatant from the microcentrifuge tube was discarded, leaving only the pellet inside the tube. Subsequently, 200 µL of sterile distilled water (dH_2O) was added into the microcentrifuge tube to dissolve the pellet. The suspension was then boiled for 10 min. Following this, the boiled suspension was promptly cooled at -20 °C for 5 min. After cooling, the suspension underwent centrifugation at 10,000 rpm for 10 min. The tube was then stored at -20 °C for use in subsequent procedures.

Multiplex PCR

In order to identify the specific *Vibrio* species (*V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae*) isolated from the three commercial aquaculture shrimp farms, a multiplex PCR assay was conducted. This method enabled the detection and targeting of specific genes, employing the VP 1155272 F, VP 1155272 R, VC C634002F, VC C634002R, VA 1198230 F and VA 1198230 R primers. Details regarding the primers are summarized in Table 1.

The primer sets used in this study were based on prior research conducted by Kim *et al.* (2015). The multiplex PCR protocol closely followed the methodology outlined by Kim *et al.* (2015) with minor adjustments. A premixed master mix (exTEN 2× PCR Master Mix) containing dNTPs, magnesium chloride, Taq DNA polymerase, a proofreading enzyme and reaction buffer was acquired from 1st BASE (Singapore). Each 15 µL of the PCR mixture consisted of the following components: 7.5 µL exTEN 2× PCR Master Mix, 0.6 µL of each primer with a concentration of 10 µM, 2.0 µL DNA template and 1.9 µL sterile distilled water.

The PCR amplification method followed the following parameters: initial denaturation step at 94 °C for 5 min, followed by 25 cycles consisting of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 30 sec, concluding with a final extension step at 72 °C for 10 min and storage at 4 °C (Kim *et al.*, 2015).

Agarose gel electrophoresis (AGE)

For gel electrophoresis, 5 mL of PCR products were loaded onto a 2% agarose gel in 1× Tris-Borate-EDTA (TBE) buffer and subjected to electrophoresis at 100 V for 55 min. Subsequently, the agarose gel was soaked in an ethidium bromide (EtBr) solution for 40 min before visualizing the gel using a UV transilluminator (MaestroGen, Australia). A 100 bp DNA ladder (Promega, USA) was used as a reference to determine the sizes of the PCR products.

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Table 2: Antibiotics and their concentrations used in this study.

Antimicrobial class	Antimicrobial agent	Concentration
Aminoglycosides	Amikacin (AK)	30 µg
	Kanamycin (K)	2 µg
	Streptomycin (S)	25 µg
	Gentamicin (CN)	10 µg
Penicillins & β-Lactam/β-Lactamase inhibitor combinations	Amoxycillin-clavulanic acid (AML)	10 µg
	Ampicillin (AMP)	10 µg
Penicillin	Penicillin (P)	10 IU
Phenicols	Chloramphenicol (C)	30 µg
Carbapenems	Meropenem (MEM)	10 IU
	Imipenem (IPM)	10 µg
Cephems	Ceftazidime (CAZ)	30 µg
Rifamycins	Rifampicin (RD)	2 µg
Aminocyclitol	Spectinomycin (SH)	100 µg
Tetracyclines	Tetracycline (TE)	30 µg
Quinolones	Nalidixic acid (NA)	30 µg
Macrolides	Erythromycin (E)	15 µg
Fluoroquinolones	Norfloxacin (NOR)	10 µg
	Enrofloxacin (ENR)	5 µg
Cephalosporin	Cephalotin (KF)	30 µg
Bacitracin	Bacitracin (B)	30 µg

Table 3: Summary of sampling locations and their parameters throughout sampling durations.

Sampling location	Duration of sampling	Average pH of pond water	Average temperature of pond water (°C)	Average salinity of pond water (g/kg)
Santubong	March-June	7.0	31.0	24.5
Tanjung Manis	April-July	8.0	28.0	27.0
Telaga Air	August-November	6.5	34.5	25.0

Antibiotic susceptibility test (AST)

A comprehensive assessment of antimicrobial susceptibility was conducted for *V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae* isolates obtained during the sampling at the three locations. To determine the percentages of resistant isolates to various antibiotics, a total of ten (n=10) representative isolates (if any) for each of *V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae* from each location were used. In this experiment, *Escherichia coli* ATCC® 25922 was used as the positive control for quality control. Meanwhile, negative controls consisted of sterile paper discs containing fresh Mueller-Hinton Broth (MHB).

Antimicrobial susceptibility testing (AST) was conducted on the putative isolates using the disc-diffusion method following the guidelines outlined by the Clinical and Laboratory Standards Institute (CLSI, 2015). Overnight cultures of the isolates were aerobically cultivated in 10 mL of Mueller-Hinton Broth (MHB) supplemented with 2% sodium chloride (NaCl) at 37 °C with agitation at 170 rpm until reaching a turbidity of 0.5 McFarland standard.

A bacterial culture lawn was then prepared by swabbing the culture onto Mueller-Hinton Agar (MHA) supplemented with 2% NaCl using a sterile cotton bud. The plate was allowed to air-dry for 5 min before three antibiotic discs were placed on the swabbed agar, equidistant from each other, using sterile forceps. The antibiotics used, and their respective concentrations are detailed in Table 2. These antibiotics were selected from a list of antibiotics representing various antimicrobial classes outlined in the CLSI-M45 guidelines, which are commonly employed for the treatment of *Vibrio* spp. (CLSI, 2015).

Following this, the plates were inverted and incubated at 37 °C for 24 h, and the size of the inhibition zone was measured the next day. The results were presented as the mean value of triplicates and the susceptibility level (sensitive, intermediate or resistant) of each isolate was determined. Additionally, the multiple antibiotic resistance (MAR) index was calculated from the AST result (Krumperman, 1983).

RESULTS

Average parameters in sampling locations

The sampling details are summarized in Table 3 below. The average pH of pond water for most farms ranged from 6.5 to 8.0, which falls within the optimal range for shrimp growth. The temperature of pond water was observed to range between 28 °C and 35 °C during sampling at all three locations. The salinity of the pond water for the farms ranged from 25 to 27 g/kg. Statistical analysis using the one-way ANOVA showed that there

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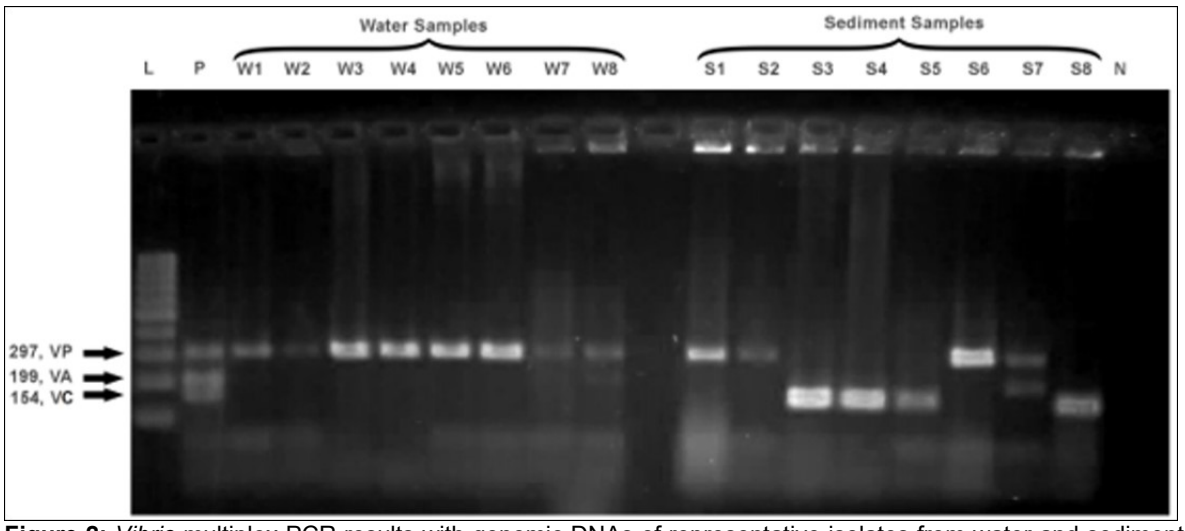

Figure 2: *Vibrio* multiplex PCR results with genomic DNAs of representative isolates from water and sediment samples collected at Pond A (Telaga Air) from the first day of culture until the 98th day of culture. Lane L: 100 bp DNA ladder (Promega, USA), P: Positive control (mixture of *Vibrio cholerae* KCDC 13589, *V. alginolyticus* ATCC 17749, *V. parahaemolyticus* ATCC 27969), W1-W8: Water samples day 1-98, S1-S8: Sediment samples day 1-98, N: Negative control.

Table 4: Summary of detection of *V. parahaemolyticus* (VP), *V. alginolyticus* (VA) and *V. cholerae* (VC) isolated from water and sediment samples in Location 1 (Santubong).

Pond	*Vibrios*	Samples	1	2	3	4	5	6	7	8	Total
A	VP	Water	+	-	+	-	+	+	+	-	5
		Sediment	-	-	-	+	-	+	+	-	3
	VA	Water	-	-	-	+	-	-	-	+	2
		Sediment	-	-	-	-	-	-	-	+	1
	VC	Water	-	+	-	+	-	-	-	+	3
		Sediment	+	+	+	-	+	-	-	-	4
	VP	Water	-	+	+	-	+	+	+	+	6
B		Sediment	+	-	-	+	+	-	+	-	4
	VA	Water	-	-	-	-	-	-	-	-	0
		Sediment	-	-	-	-	-	-	-	-	0
	VC	Water	+	-	-	+	-	-	-	-	2
		Sediment	-	+	+	-	-	+	-	+	4

(+) Present; (-) Not present.

Table 5: Summary for detection of *V. parahaemolyticus* (VP), *V. alginolyticus* (VA) and *V. cholerae* (VC) isolated from water and sediment samples in Location 2 (Tanjung Manis).

Pond	*Vibrios*	Samples	1	2	3	4	5	6	7	8	Total
A	VP	Water	+	+	+	+	+	+	+	+	8
		Sediment	-	+	+	-	+	-	+	-	4
	VA	Water	-	-	-	-	-	-	-	-	0
		Sediment	+	-	-	+	+	+	-	+	5
	VC	Water	-	-	-	-	-	-	-	-	0
		Sediment	-	-	-	-	-	-	-	-	0
	VP	Water	+	+	+	-	+	+	-	-	5
B		Sediment	+	+	+	+	+	+	+	+	8
	VA	Water	-	-	-	+	-	-	+	+	3
		Sediment	-	-	-	-	-	-	-	-	0
	VC	Water	-	-	-	-	-	-	-	-	0
		Sediment	-	-	-	-	-	-	-	-	0

(+) Present; (-) Not present.

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Table 6: Summary of detection of *V. parahaemolyticus* (VP), *V. alginolyticus* (VA) and *V. cholerae* (VC) isolated from water and sediment samples in Location 3 (Telaga Air).

Pond	*Vibrio* spp.	Samples	Day of culture								Total
			1	14	28	32	56	70	84	98	
A	VP	Water	+	+	+	+	+	+	+	+	8
		Sediment	+	+	-	-	-	+	+	-	4
	VA	Water	-	-	-	-	-	-	-	+	1
		Sediment	-	-	-	-	-	-	+	-	1
	VC	Water	-	-	-	-	-	-	-	-	0
		Sediment	-	-	+	+	+	-	-	+	4
B	VP	Water	+	+	+	+	+	-	+	+	7
		Sediment	-	+	-	-	+	-	-	-	2
	VA	Water	+	-	-	-	-	+	-	-	2
		Sediment	+	-	+	+	-	+	+	+	6
	VC	Water	-	-	-	-	-	-	-	-	0
		Sediment	-	-	-	-	-	-	-	-	0

(+) Present; (-) Not present.

Table 7: Prevalence of *V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae* in water and sediment samples collected from the three locations.

Sample	n[a]	Prevalence (%)		
		V. parahaemolyticus	*V. alginolyticus*	*V. cholerae*
Water	48	81.25[b] (68.06-89.81)[c]	16.67 (8.70-29.58)	10.42 (4.53-22.17)
Sediment	48	52.08 (38.33-65.53)	27.08 (16.57-41.0)	25.0 (14.92-38.78)
TOTAL	96	66.67 (56.76-75.29)	21.88 (14.78-31.14)	17.71 (11.36-26.54)

[a]Number of samples; [b]Percentage of positive samples; [c]95% confidence interval (%).

were no significant differences across the three parameters throughout the entire sampling period in the three farms (P>0.05).

Identification of *Vibrio* species using multiplex PCR

The expected sizes of the PCR products of *V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae* using the primer sets are 297 bp, 199 bp and 154 bp, respectively. Bands that corresponded with the expected sizes were observed when the PCR products were run on agarose gel electrophoresis (AGE) (Figure 2). Based on these results, summaries of the detection of specific Vibrio spp. on the samples from different locations were presented in Table 4, Table 5 and Table 6.

The results of *Vibrio* spp. detection in water and sediment samples from Ponds A and B from Santubong (Location 1) in Table 4 below revealed that *V. parahaemolyticus* was the most consistently detected species, with 18 positive detections across both ponds and sample types. *Vibrio parahaemolyticus* was prevalent in both water and sediment samples from Pond B, particularly in the latter. *Vibrio cholerae* exhibited sporadic presence, with three positive detections in Pond A water samples, four detections in Pond A sediment samples, and two and four detections in water and sediment samples of Pond B, respectively. *Vibrio alginolyticus* was less frequently identified, with only two positive detections in Pond A water samples. Notably, Pond B water and sediment samples showed no detections of *V. alginolyticus* throughout the study period. Overall, these findings underscore the dominance of *V. parahaemolyticus* in the shrimp farm in Santubong, with *V. cholerae* and *V. alginolyticus* being less prevalent.

In the analysis of *Vibrio* spp. across water and sediment samples from Ponds A and B in Tanjung Manis (Location 2) shown in Table 5, *V. parahaemolyticus* again emerged as the predominant species with high detections in both water and sediment samples. Pond A water samples consistently exhibited positive detections across all eight sampling periods, while Pond B water samples showed *V. parahaemolyticus* presence in five periods. Sediment samples from Pond B consistently contained *V. parahaemolyticus* throughout all eight periods. *Vibrio alginolyticus* was intermittently detected in Pond A sediment samples over four periods and appeared in Pond B water samples during two periods. Notably, *V. cholerae* remained undetected in all samples from both ponds during the entire study period. In summary, *V. parahaemolyticus* was the most prevalent species in Tanjung Manis, with sporadic presence of *V. alginolyticus* and an absence of *V. cholerae* in all samples.

Table 6, presented below, displays the results of *Vibrio* spp. detection in water and sediment samples collected from the Telaga Air (Location 3) shrimp farm. It is noteworthy that *V. parahaemolyticus* was consistently

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Table 8: Prevalence of *V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae* across the three sampling locations.

Location	n[a]	Prevalence (%)		
		V. parahaemolyticus	*V. alginolyticus*	*V. cholerae*
Santubong	32	56.25[b] (39.33-71.84)[c]	9.38 (3.24-24.22)	40.63 (25.52-57.74)
Tanjung Manis	32	78.13 (61.25-88.98)	25.0 (13.25-42.11)	0 (0-10.72)
Telaga Air	32	65.63 (48.31-79.59)	31.25 (17.95-48.57)	12.50 (4.97-28.07)
TOTAL	96	66.67 (56.76-75.29)	21.88 (14.78-31.14)	17.71 (11.36-26.54)

[a]Number of samples; [b]Percentage of positive samples; [c]95% confidence interval (%).

detected in most water samples from both Pond A and Pond B totalling 15 detections, spanning from the initial sampling period (day 1) to the conclusion of the sampling period (day 98). Conversely, sediment samples from Pond B exhibited a notably high detection rate of *V. alginolyticus* with six detections. *Vibrio cholerae*, however, remained undetected in water samples from both ponds and was only identified four times in sediment samples from Pond A. In summary, the findings indicate that *V. parahaemolyticus* was the most prevalent species in both ponds, followed by *V. alginolyticus* and *V. cholerae*.

Prevalence of *Vibrio* species in water and sediment samples

The overall prevalence data, presented as the percentage of positive samples with 95% confidence intervals, reveals the occurrence of Vibrio spp. in both water and sediment samples in all three locations (Table 7). In water samples, *V. parahaemolyticus* had the highest prevalence, accounting for 81.25% (95% CI: 68.06-89.81%) of positive samples, followed by *V. alginolyticus* at 16.67% (95% CI: 8.7-29.58%) and *V. cholerae* at 10.42% (95% CI: 4.53-22.17%). In sediment samples, *V. parahaemolyticus* still dominated with a prevalence of 52.08% (95% CI: 38.33-65.53%), while *V. alginolyticus* and *V. cholerae* had a prevalence of 27.08% (95% CI: 16.57-51.64%) and 25.0% (95% CI: 14.92-38.78%), respectively. When considering the overall prevalence across both sample types (water and sediment), *V. parahaemolyticus* remained the most prevalent species at 66.67% (95% CI: 56.76-75.29%), followed by *V. alginolyticus* at 21.88% (95% CI: 14.78-31.14%) and *V. cholerae* at 17.71% (95% CI: 11.36-26.54%).

Prevalence of *Vibrio* species between sampling locations

The prevalence data for *Vibrio* species across different locations, along with their respective 95% confidence intervals, reveals variations in the occurrence of these species (Table 8). In Santubong, *V. parahaemolyticus* was the dominant species, with a prevalence of 56.25% (95% CI: 39.33-71.84%), while *V. alginolyticus* and *V. cholerae* were detected in 9.38% (95% CI: 3.24-24.22%) and 40.63% (95% CI: 25.52-57.74%) of samples, respectively. In Tanjung Manis, *V. parahaemolyticus* showed the highest prevalence at 78.13% (95% CI: 61.25-88.98%), followed by *V. alginolyticus* at 25.0% (95% CI: 13.25-42.11%), while *V. cholerae* was not detected. In Telaga Air, *V. parahaemolyticus* was also the most prevalent species, detected in 65.63% (95% CI: 48.31-79.59%) of samples, followed by *V. alginolyticus* at 31.25% (95% CI: 17.95-48.57%) and *V. cholerae* at 12.50% (95% CI: 4.97-28.07%).

Antibiotic susceptibility test (AST) in Location 1 (Santubong)

In this study, antibiotic susceptibility testing (AST) was conducted to determine antibiotic resistance in aquaculture shrimp farms and to compare antibiotic resistance patterns between Location 1 (Santubong), Location 2 (Tanjung Manis) and Location 3 (Telaga Air). A total of 20 antimicrobial classes were tested against *V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae* isolated during sampling in each location. Ten (n=10) isolates of each *Vibrio* spp. were used to determine the percentages of resistant isolates based on antibiotics.

Table 9 shows the percentages of resistant *V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae* isolates from Location 1 (Santubong) based on different types of antibiotics.

The antimicrobial resistance patterns of *Vibrio* spp. isolated from the Santubong shrimp farm indicate varying levels of resistance to different antimicrobial agents (Table 9). Among *V. parahaemolyticus* isolates, the highest resistance was observed for Penicillin (P10) and Ampicillin (AMP10) at 40% and 30%, respectively. *Vibrio alginolyticus* displayed notable resistance to Amoxycillin-clavulanic acid (AML10), Penicillin (P10), Ceftazidime (CAZ30), Cephalotin (KF30) and Bacitracin (B30), each with 20% resistance. *Vibrio cholerae* exhibited the highest resistance to Penicillin (P10) and Tetracycline (TE30), both at 40% resistance.

The overall result showed that all three *Vibrio* spp. showed resistance towards Penicillin. Meanwhile, some antimicrobial agents showed no resistance across all three *Vibrio* spp., including Meropenem (MEM10, Erythromycin (E15), Norfloxacin (NOR10) and Enrofloxacin (ENR5), which means that they were effective against all isolates.

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Table 9: Antimicrobial-resistant profile of *V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae* isolates from Location 1 (Santubong).

Vibrio spp.	Antimicrobial agent	Number of resistant isolates	Percentage resistance (%)
V. parahaemolyticus (n=10)	Amikacin (AK30)	0	0
	Kanamycin (K2)	0	0
	Streptomycin (S25)	2	20
	Gentamicin (CN10)	2	20
	Amoxycillin-clavulanic acid (AML10)	0	0
	Ampicillin (AMP10)	3	30
	Penicillin (P10)	4	40
	Chloramphenicol (C30)	0	0
	Meropenem (MEM10)	0	0
	Imipenem (IPM10)	0	0
	Ceftazidime (CAZ30)	1	10
	Rifampicin (RD2)	0	0
	Spectinomycin (SH100)	2	20
	Tetracycline (TE30)	2	20
	Nalidixic acid (NA30)	1	10
	Erythromycin (E15)	0	0
	Norfloxacin (NOR10)	0	0
	Enrofloxacin (ENR5)	0	0
	Cephalotin (KF30)	1	10
	Bacitracin (B30)	0	0
V. alginolyticus (n=10)	Amikacin (AK30)	0	0
	Kanamycin (K2)	0	0
	Streptomycin (S25)	1	10
	Gentamicin (CN10)	1	10
	Amoxycillin-clavulanic acid (AML10)	2	20
	Ampicillin (AMP10)	1	10
	Penicillin (P10)	2	20
	Chloramphenicol (C30)	0	0
	Meropenem (MEM10)	0	0
	Imipenem (IPM10)	0	0
	Ceftazidime (CAZ30)	2	20
	Rifampicin (RD2)	0	0
	Spectinomycin (SH100)	1	10
	Tetracycline (TE30)	1	10
	Nalidixic acid (NA30)	1	10
	Erythromycin (E15)	0	0
	Norfloxacin (NOR10)	0	0
	Enrofloxacin (ENR5)	0	0
	Cephalotin (KF30)	2	20
	Bacitracin (B30)	2	20
V. cholerae (n=10)	Amikacin (AK30)	1	10
	Kanamycin (K2)	1	0
	Streptomycin (S25)	1	10
	Gentamicin (CN10)	1	10
	Amoxycillin-clavulanic acid (AML10)	0	0
	Ampicillin (AMP10)	2	20
	Penicillin (P10)	4	40
	Chloramphenicol (C30)	1	10
	Meropenem (MEM10)	0	0
	Imipenem (IPM10)	1	10
	Ceftazidime (CAZ30)	0	0
	Rifampicin (RD2)	1	10
	Spectinomycin (SH100)	0	0
	Tetracycline (TE30)	3	30
	Nalidixic acid (NA30)	1	10

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(Continued)

Erythromycin (E15)	0	0
Norfloxacin (NOR10)	0	0
Enrofloxacin (ENR5)	0	0
Cephalotin (KF30)	1	10
Bacitracin (B30)	3	30

Antibiotic susceptibility test (AST) in location 2 (Tanjung Manis)

Table 10 shows the percentages of resistant *V. parahaemolyticus* and *V. alginolyticus* isolates from Location 2 (Tanjung Manis) based on different types of antibiotics. It's worth noting that *V. cholerae* isolates were not included in the analysis because none were isolated from the Tanjung Manis shrimp farm samples. Therefore, the results and analysis pertain only to *V. parahaemolyticus* and *V. alginolyticus* isolates.

The antimicrobial susceptibility testing results for *V. parahaemolyticus* and *V. alginolyticus* isolates from Tanjung Manis reveal distinct resistance patterns to various antimicrobial agents (Table 10). In the case of *V. parahaemolyticus*, notable resistance was observed with 40% resistance to both Penicillin (P10) and Ampicillin (AMP10), followed by 30% resistance to Amikacin (AK30), Ampicillin (AMP10) and Bacitracin (B30). Additionally, 20% resistance was observed for Tetracycline (TE30), while Ceftazidime (CAZ30), Rifampicin (RD2), Spectinomycin (SH100), Enrofloxacin (ENR5) and Cephalotin (KF30) each exhibited 10% resistance. In contrast, *V. alginolyticus* displayed resistance levels of 30% to Penicillin (P10) and Ampicillin (AMP10), as well as 20% resistance to Ampicillin (AMP10), Rifampicin (RD2), Tetracycline (TE30), Ceftazidime (CAZ30) and Cephalotin (KF30).

Similar to the Santubong shrimp farm, *Vibrio* isolates from Tanjung Manis also showed high resistance towards Penicillin. Other antimicrobial agents, including Gentamicin (CN10), Amoxycillin-clavulanic acid (AML10), Chloramphenicol (C30), Meropenem (MEM10), Imipenem (IPM10), Erythromycin (E15) and Norfloxacin (NOR10) were effective against isolates of both *Vibrio* species.

Antibiotic susceptibility test (AST) in location 3 (Telaga Air)

The results of the antibiotics susceptibility test (AST) of isolates from Location 3 (Telaga Air) are shown in Table 11 below.

The results of antimicrobial susceptibility testing for *V. parahaemolyticus* (n=10) isolated from Telaga Air revealed varying resistance patterns (Table 11). Notably, 20% of isolates showed resistance to Amikacin (AK30), Streptomycin (S25) and Amoxycillin-clavulanic acid (AML10), while Penicillin (P10) exhibited the highest resistance with 30% of isolates being resistant. Ceftazidime (CAZ30), Nalidixic acid (NA30) and Cephalotin (KF30) each displayed 10% resistance. Among the resistant isolates of *V. alginolyticus* (n=10), the most notable resistance was observed towards Penicillin (P10), with 30% of isolates being resistant, followed by Amoxycillin-clavulanic acid (AML10), Ceftazidime (CAZ30), Cephalotin (KF30) and Bacitracin (B30), each exhibiting 20% resistance. Additionally, Ampicillin (AMP10), Nalidixic acid (NA30) and Amikacin (AK30) showed 10% resistance, while all other tested antimicrobial agents displayed no resistance among the tested isolates. Meanwhile, Penicillin (P10), Streptomycin (S25) and Bacitracin (B30) each displayed 20% resistance when tested with the *V. cholerae* isolates, while Amikacin (AK30), Gentamicin (CN10), Ampicillin (AMP10), Imipenem (IPM10), Nalidixic acid (NA30), Cephalotin (KF30) showed 10% resistance.

The overall result showed that the antimicrobial agent with the most resistant isolate is Penicillin (P10), with 30% of each *V. parahaemolyticus* and *V. alginolyticus*, and 20% of *V. cholerae* showing resistance towards the antibiotics. Among all antimicrobial agents, Kanamycin (K2), Chloramphenicol (C30), Meropenem (MEM10), Rifampicin (RD2), Spectinomycin (SH100), Tetracycline (TE30), Erythromycin (E15), Norfloxacin (NOR10) and Enrofloxacin (ENR5) are 100% effective against all three *Vibrio* spp. with none of the isolates showing resistance towards them.

Multiple antibiotics resistance (MAR)

Multiple antibiotic resistant (MAR) Index analyses of each isolate were determined to monitor the spread of antibiotic resistance in three different sampling locations. It was introduced by Krumperman (1983) to group isolates according to the sources from the area where the bacteria were recovered using those antibiotics tested (Krumperman, 1983; Osundeya *et al.*, 2013). Table 6 shows the MAR index for the sampling locations; Santubong, Tanjung Manis and Telaga Air.

In Location 1 (Santubong), the MAR index ranged from 0.45 to 0.65, indicating potential high-risk sources. Notably, penicillin exhibited the highest resistance (40%) against *V. parahaemolyticus* and *V. cholerae*. A small percentage (5%) of pairings displayed 30% resistance, primarily *V. parahaemolyticus* against ampicillin and V. *cholerae* against tetracycline and bacitracin. Additionally, 18.33% of pairings exhibited 20% resistance, including *V. alginolyticus* against multiple antibiotics, *V. parahaemolyticus* against several antibiotics, and *V. cholerae* against streptomycin and ampicillin. A substantial portion (43.33%) of pairings remained susceptible to the antibiotics used (Table 12).

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Table 10: Antimicrobial-resistant profile of *V. parahaemolyticus*, *V. alginolyticu* and *V. cholerae* isolates from Location 2 (Tanjung Manis).

Vibrio spp.	Antimicrobial agent	Number of resistant isolates	Percentage resistance (%)
V. parahaemolyticus (n=10)	Amikacin (AK30)	3	30
	Kanamycin (K2)	1	10
	Streptomycin (S25)	4	40
	Gentamicin (CN10)	0	0
	Amoxycillin-clavulanic acid (AML10)	0	0
	Ampicillin (AMP10)	3	30
	Penicillin (P10)	4	40
	Chloramphenicol (C30)	0	0
	Meropenem (MEM10)	0	0
	Imipenem (IPM10)	0	0
	Ceftazidime (CAZ30)	1	10
	Rifampicin (RD2)	1	10
	Spectinomycin (SH100)	1	10
	Tetracycline (TE30)	2	20
	Nalidixic acid (NA30)	0	0
	Erythromycin (E15)	0	0
	Norfloxacin (NOR10)	0	0
	Enrofloxacin (ENR5)	1	10
	Cephalotin (KF30)	1	10
	Bacitracin (B30)	3	30
V. alginolyticus (n=10)	Amikacin (AK30)	1	10
	Kanamycin (K2)	0	0
	Streptomycin (S25)	0	0
	Gentamicin (CN10)	0	0
	Amoxycillin-clavulanic acid (AML10)	0	0
	Ampicillin (AMP10)	2	20
	Penicillin (P10)	3	30
	Chloramphenicol (C30)	0	0
	Meropenem (MEM10)	0	0
	Imipenem (IPM10)	0	0
	Ceftazidime (CAZ30)	1	10
	Rifampicin (RD2)	2	20
	Spectinomycin (SH100)	0	0
	Tetracycline (TE30)	3	30
	Nalidixic acid (NA30)	1	10
	Erythromycin (E15)	0	0
	Norfloxacin (NOR10)	0	0
	Enrofloxacin (ENR5)	0	0
	Cephalotin (KF30)	2	20
	Bacitracin (B30)	2	20

In Location 2 (Tanjung Manis), the MAR index ranged from 0.45 to 0.60. *Vibrio parahaemolyticus* showed the highest resistance (40%) against streptomycin and penicillin. A minor percentage (10%) of pairings displayed 30% resistance, including *V. alginolyticus* against penicillin and tetracycline, and *V. parahaemolyticus* against amikacin, ampicillin and bacitracin. Furthermore, 12.5% of pairings exhibited 20% resistance, with *V. alginolyticus* against multiple antibiotics and *V. parahaemolyticus* against tetracycline. A significant portion (47.5%) of pairings remained susceptible to the antibiotics used. Notably, no *V. cholerae* isolate was included due to the absence of *V. cholerae* in samples collected from Tanjung Manis shrimp farm (Table 12).

In Location 3 (Telaga Air), the MAR index ranged from 0.35 to 0.45, suggesting potential high-risk sources. Approximately 3.33% of antibiotic-isolate pairings exhibited 30% resistance, notably V. alginolyticus against penicillin and *V. parahaemolyticus* against penicillin. Furthermore, 15% of pairings showed 20% resistance, including *V. alginolyticus* against multiple antibiotics and *V. parahaemolyticus* against several antibiotics. The majority (55%) of pairings were susceptible to the antibiotics used (Table 12).

Based on the resistance patterns observed, it was determined that resistance to a minimum of seven antibiotics was exhibited by all *Vibrio* species. Among the *Vibrio* spp. collected from the three different locations, a

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Table 11: Antimicrobial-resistant profile of *V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae* isolates from Location 3 (Telaga Air).

Vibrio spp.	Antimicrobial agent	Number of resistant isolates	Percentage resistance (%)
V. parahaemolyticus (n=10)	Amikacin (AK30)	2	20
	Kanamycin (K2)	0	0
	Streptomycin (S25)	2	20
	Gentamicin (CN10)	0	0
	Amoxycillin-clavulanic acid (AML10)	2	20
	Ampicillin (AMP10)	0	0
	Penicillin (P10)	3	30
	Chloramphenicol (C30)	0	0
	Meropenem (MEM10)	0	0
	Imipenem (IPM10)	0	0
	Ceftazidime (CAZ30)	1	10
	Rifampicin (RD2)	0	0
	Spectinomycin (SH100)	0	0
	Tetracycline (TE30)	0	0
	Nalidixic acid (NA30)	1	10
	Erythromycin (E15)	0	0
	Norfloxacin (NOR10)	0	0
	Enrofloxacin (ENR5)	0	0
	Cephalotin (KF30)	1	10
	Bacitracin (B30)	0	0
V. alginolyticus (n=10)	Amikacin (AK30)	0	0
	Kanamycin (K2)	0	0
	Streptomycin (S25)	0	0
	Gentamicin (CN10)	0	0
	Amoxycillin-clavulanic acid (AML10)	2	20
	Ampicillin (AMP10)	1	10
	Penicillin (P10)	3	30
	Chloramphenicol (C30)	0	0
	Meropenem (MEM10)	0	0
	Imipenem (IPM10)	0	0
	Ceftazidime (CAZ30)	2	20
	Rifampicin (RD2)	0	0
	Spectinomycin (SH100)	0	0
	Tetracycline (TE30)	0	0
	Nalidixic acid (NA30)	1	10
	Erythromycin (E15)	0	0
	Norfloxacin (NOR10)	0	0
	Enrofloxacin (ENR5)	0	0
	Cephalotin (KF30)	2	20
	Bacitracin (B30)	2	20
V. cholerae (n=10)	Amikacin (AK30)	1	10
	Kanamycin (K2)	0	0
	Streptomycin (S25)	2	20
	Gentamicin (CN10)	1	10
	Amoxycillin-clavulanic acid (AML10)	0	0
	Ampicillin (AMP10)	1	10
	Penicillin (P10)	2	20
	Chloramphenicol (C30)	0	0
	Meropenem (MEM10)	0	0
	Imipenem (IPM10)	1	10
	Ceftazidime (CAZ30)	0	0
	Rifampicin (RD2)	0	0
	Spectinomycin (SH100)	0	0
	Tetracycline (TE30)	0	0
	Nalidixic acid (NA30)	1	10

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	Erythromycin (E15)	0	0
	Norfloxacin (NOR10)	0	0
	Enrofloxacin (ENR5)	0	0
	Cephalotin (KF30)	1	10
	Bacitracin (B30)	2	20

Table 12: MAR index of *Vibrio* spp. isolated from the three locations.

Location	*Vibrio* spp.	Total antibiotics tested	Number of resistance antibiotics	Antibiotic resistance profiles	MAR index[a]
Santubong	*V. parahaemolyticus*	20	9	SCNAMPPCAZSHTENAKF	0.45
	V. alginolyticus	20	11	SCNAMLAMPPCAZSHTENAKFB	0.55
	V. cholerae	20	13	AKKSCNAMPPCIPMRDTENAKFB	0.65
Tanjung Manis	*V. parahaemolyticus*	20	12	AKKSAMPPCAZRDSHTEENRKFB	0.60
	V. alginolyticus	20	9	AKAMPPCAZRDTENAKFB	0.45
Telaga Air	*V. parahaemolyticus*	20	7	AKSAMLPCAZNAKF	0.35
	V. alginolyticus	20	7	AMLAMPPCAZNAKFB	0.35
	V. cholerae	20	9	AKSCNAMPPMEMNAKFB	0.45

[a] MAR index = Number of resistance antibiotics/total number of antibiotics tested.

total of eight distinct antibiogram patterns were identified. When the different locations were compared based on the species, it was observed that the highest MAR index of 0.60 was recorded for *V. parahaemolyticus* isolates from Tanjung Manis, as opposed to *V. parahaemolyticus* isolates from Telaga Air and Santubong. Meanwhile, the highest MAR index of 0.55 was exhibited by *V. alginolyticus* isolates from Santubong when compared to their counterparts from the other two locations. Among all *Vibrio* spp. in all sampling locations, it was found that the highest MAR index value of 0.65 was displayed by *V. cholerae* isolates from the Santubong shrimp farm, and they were found to be resistant to 13 antibiotics (AK-K-S-CN-AMP-P-C-IPM-RD-TE-NA-KF-B).

Based on the established criteria, where a MAR index of <0.2 signifies isolation from a low-contaminated area and a MAR index of >0.2 indicates isolation from a highly contaminated area, the isolates can be categorized accordingly (Krumperman, 1983). None of the MAR indices in this study were below 0.2, as the lowest recorded value was 0.35 and the highest was 0.65. Therefore, all isolates in this study can be classified as originating from highly contaminated areas, as their MAR indices exceeded the threshold of 0.2. This suggests that the sampled locations were characterised by a significant level of antibiotic resistance, indicative of high contamination levels in these environments.

DISCUSSION

The Malaysian aquaculture shrimp industry encounters a range of issues and challenges, primarily centred around disease outbreaks, environmental pollution and fluctuating international market demand. One of the alarming diseases is Early Mortality Syndrome (EMS), a shrimp ailment commonly caused by *V. parahaemolyticus*, leading to significant mortality rates within the initial 30 days of shrimp cultivation. Additionally, there are many reported occurrences of *Vibrio*-related food poisoning cases due to consumption of raw and undercooked seafood products (Wright and Harwood, 2013).

Identification of *Vibrio* spp., specifically *V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae* by molecular detection using multiplex PCR was carried out to detect specific target genes of the species. These three species were identified from water and sediment samples collected from the 1st day of culture (day 1) until the 98th day of culture (day 98). *Vibrio* spp. can be found in the water utilized in shrimp culture facilities, as well as in the biofilm that forms on various structures in contact with water, such as hatcheries and farms (Lavilla-Pitogo *et al.*, 1990). Given the ubiquitous presence of *Vibrio* spp. in the natural environment, it is imperative to vigilantly monitor their population within a shrimp farming system to proactively mitigate the potential for outbreaks capable of causing extensive harm to the entire shrimp population.

Based on the prevalence results, it was observed that although the occurrence of specific *Vibrio* spp. shows significant variation across different geographical areas, *V. parahaemolyticus* is still the most prevalent in all three shrimp farm locations. Since the shrimp farms are all located in coastal areas, the prevalence of *V. parahaemolyticus* over *V. alginolyticus* and *V. cholerae* may be attributed to their halophilic nature, which enables them to thrive in high-salinity environments like seawater. Conversely, *V. cholerae* exhibited significantly lower detection rates in two locations and none in one location due to its non-halophilic nature. While *V. cholerae* was still detectable in the water samples from the shrimp farms, it is reported to be more prevalent in freshwater environments (Tey *et al.*, 2015).

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In addition to geographical factors, several other considerations are essential for ensuring the quality of shrimp production with respect to bacterial distribution. The management practices in the ponds at the three selected sampling sites vary significantly. Specifically, Santubong shrimp farm employs a no water exchange system, Telaga Air shrimp farm employs a partial water exchange system, and Tanjung Manis shrimp farm employs both systems, depending on the physio-chemical parameters obtained from the farm, including water pH, temperature and salinity. These physio-chemical factors not only impact the growth of the shrimp but also influence the growth of co-existing bacterial populations. In this study, the physio-chemical factors obtained did not exhibit significant differences across the parameters, from stocking to harvesting processes ($P>0.05$). This uniformity is attributed to the farms' monitoring and management practices, which adhere to the recommended physio-chemical standards as suggested by the Ministry of Agriculture (MOA).

In this study, Table 3 presents the average pH values from all three sampling locations, revealing that they fell within the range of slightly acidic to slightly alkaline. Despite this pH range, *Vibrio* spp. remained detectable. The variations in pH levels in the estuarine region, influenced by factors like influx, decay and fluctuations in hydrogen ion concentrations during the rainy season, likely contributed to these pH differences (Raj and Viswanathan, 2023). However, the pH of the water exhibited a weak correlation with the abundance of *Vibrio* species. This observation suggests that *Vibrio* spp. exhibit a certain level of tolerance to slightly acidic conditions, allowing them to persist in these environments. The average (±) pH readings for pond water in all three locations were found to consistently fall within the critical range of 6.5 to 8.0. Maintaining this pH range is crucial for promoting the healthy growth of shrimp. However, it's worth noting that this pH range is also conducive to the growth of most bacteria. Many *Vibrio* spp. can thrive within a broader pH range, even up to pH 11 and tend to flourish in alkaline environments, particularly at ideal temperatures. Typically, *Vibrio* spp. thrive best within the pH range of 6.5 to 9.0, with their highest propagation rates occurring in alkaline conditions (Alfiansah *et al.*, 2018).

Another crucial physio-chemical factor to consider is the pond water temperature. The average temperatures recorded at all three sampling locations ranged from 28 °C to 34.5 °C, which is notably conducive to the growth of *Vibrio* species. *Vibrio* spp. exhibit a wide tolerance range for optimal temperature, typically spanning from 20 °C to 40 °C (Sheikh *et al.*, 2022). The temperatures and pH levels observed in the pond water create an ideal environment for *Vibrio* spp. to proliferate within the farming systems. This relationship between temperature and bacterial growth is in line with the findings of Percival and Williams (2014), who established that temperature significantly influences bacterial population growth. In this study, it was observed that as the temperature rises, the bacterial population, which is the *Vibrio* spp., also increases.

In addition to pH and temperature, the salinity of pond water is another crucial environmental factor that can significantly impact both shrimp and bacterial distribution. In shrimp farming, pond salinity variation is recognized as a determining factor for successful shrimp production. The samples analysed in this study were collected during the rainy season, a period known to affect pond salinity. Rainfall during this season dilutes the water, reducing the salinity of estuarine water compared to warmer seasons. The analysis revealed a significant relationship between salinity levels and the abundance of *Vibrio* species. Table 3 displayed high salinity readings ranging from 24.5 to 27 g/kg. As reported by Givens *et al.* (2014), this range has been shown to support higher densities of *Vibrio* species. Most *Vibrio* spp., including *V. parahaemolyticus* and *V. alginolyticus* are classified as halophiles, meaning they require a certain level of salt for growth. This statement elucidates the prevalence of *V. parahaemolyticus* and *V. alginolyticus* in all three locations, as it is attributed to the elevated salinity levels observed in the water samples. The presence of sodium ions in the water can stimulate their proliferation (Wright and Harwood, 2013). Based on the data collected from all three sampling locations, the average pH, temperature and salinity levels observed throughout the sampling periods created conditions conducive to the proliferation of *Vibrio* spp. in the shrimp ponds.

As noted by Vincent *et al.* (2016), the distribution of *Vibrio* spp. is closely associated with ecological parameters, including temperature, salinity and environmental pH. Consequently, it is crucial to conduct daily monitoring of physio-chemical and microbiological aspects within shrimp ponds. This practice is essential for promptly identifying and addressing issues and serves as a valuable reference. Monitoring should ideally occur at least twice daily, both in the morning and evening, to uphold optimal growth conditions and prevent abrupt parameter fluctuations (Vincent *et al.*, 2016).

In addition to tracking prevalence, it is imperative to monitor the antibiotic-resistant profiles of the presumptive *Vibrio* spp. isolated from each location. Antibiotic resistance within aquaculture bacteria can emerge due to the excessive use of antibiotics and alterations in the physiochemical characteristics of ecosystems (Pepi and Focardi, 2021). Elevated antibiotic usage poses increasing challenges for the treatment of bacterial infections in aquaculture ponds. In assessing antibiotic resistance, the contamination levels of antibiotics reflect the frequency of their usage in the studied area. A low resistance level indicates minimal antibiotic use within the specific niche (McPhearson *et al.*, 1991; Wang *et al.*, 2006; Samuel *et al.*, 2011; Hua and Apun, 2013), whereas a high resistance level suggests heightened antibiotic utilization. Particular attention must be given to various antibiotics, including fluoroquinolones, tetracyclines and sulphonamides, to mitigate environmental and human health risks (Holmström *et al.*,

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2003). This study has unveiled that *Vibrio* isolates from shrimp pond samples exhibit resistance to at least seven different antibiotics, emphasizing the significance of monitoring and managing antibiotic usage in aquaculture. Farmers at the aquaculture farms where the samples were collected asserted that they did not use antibiotics in their operations. However, antibiotic resistance was detected in the bacterial isolates, particularly against antibiotics such as streptomycin, ampicillin and penicillin, despite the absence of antibiotics as treatments or in feed. This finding may be attributed to historical antibiotic use, as ampicillin and streptomycin were among the earliest antibiotics introduced following the discovery of penicillin, as noted in previous studies (Davies and Davies, 2010).

Even though antibiotic use has been discontinued on these farms for many years, the possibility of antibiotic contamination persists due to the presence of residual antibiotics in the environment from past usage. Bacteria retrieved from the sediment of the aquaculture ponds might have acquired antibiotic resistance traits from unconsumed feed and the faeces of cultured organisms, which could contain residual antibiotics (Tu *et al.*, 2009; Noorlis *et al.*, 2011; Tamminen *et al.*, 2011). These unconsumed materials and faeces accumulate in the sediment, altering the composition of the sediment microbiota under selective pressure (Tu *et al.*, 2009). It is also plausible that multiple antibiotic-resistant bacteria may have entered the aquaculture farms via water originating from agricultural farms. In support of this possibility, Buschmann *et al.* (2012) noted in their study that antibiotic-resistant bacteria detected in a multi-culture farm could potentially be transmitted through water currents originating from farms with extensive antibiotic use to the multi-culture site. It is worth emphasizing that antibiotic-resistant microorganisms are a widespread concern both locally and globally, as highlighted by Lesley *et al.* (2011).

Out of all the *Vibrio* isolates obtained from the three farms, only one isolate of *V. cholerae* from the Santubong farm exhibited resistance to chloramphenicol. The prohibition of chloramphenicol usage within the Malaysian aquaculture sector could potentially influence the relatively low occurrence of chloramphenicol-resistant bacteria observed in this study. Apart from Malaysia, chloramphenicol has been prohibited in Europe since 1994 and in Korea and Japan since 1983 (Vassort-Bruneau *et al.*, 1996). Even at relatively low doses, chloramphenicol is lethal to humans. It can cause irreversible aplastic anaemia in humans, severe or deadly bone marrow depression and 'grey syndrome', a cyanosis and cardiovascular collapse syndrome that primarily affects neonates. Due to this, workers handling antibiotic-containing items are in danger (Shariff *et al.*, 2000; Li *et al.*, 2007). Antibiotic prohibition has aided in the reduction of antibiotic-resistant microorganisms in the environment.

Pathogenic *Vibrio* strains should be closely monitored to conduct thorough investigations into their potential antibiotic resistance. The antibiotic resistance patterns tend to vary depending on geographical regions and the selective pressures exerted (Satyanarayana, 2009; Lesley *et al.*, 2011) and these patterns evolve rapidly over time. Therefore, it is crucial to continually monitor these pattern shifts due to their significant implications for public health. To prevent the continued transmission of antibiotic resistance genes, improved management practices should be established and implemented across a majority of aquaculture operations (Samuel *et al.*, 2011).

One of the challenges faced in Malaysia is the absence of a robust surveillance system to monitor the incidence of food poisoning cases. In 1981, the reported cases of food poisoning in Malaysia were 9.62 per 100,000 population, with *V. parahaemolyticus* identified as one of the most common causative agents (Todd, 1997). The incidence of food poisoning in Malaysia experienced a significant increase from 2005 to 2013, with Selangor reported as the state with the highest number of cases in 2016. However, between 2012 and 2019, there was a reduction in the incidence (Mshelia *et al.*, 2022). As of 2020, no recent data is available regarding the occurrence of food poisoning. It is important to note that most food poisoning cases are underreported, with only a few individuals seeking medical treatment (Mshelia *et al.*, 2022). Therefore, the available data may not accurately reflect the true incidence.

In this study, the Telaga Air aquaculture farm demonstrated the most efficient management systems and maintained high standards of cleanliness compared to the other two aquaculture farms under investigation. This farm employs a partial system as its standard operating procedure, which includes the installation of netting over each aquaculture pond. According to the farm manager, the installation of these nettings serves the dual purpose of preventing birds from preying on the shrimp and, consequently, averting the potential dissemination of antibiotic resistance by birds. Birds, due to their mobility, can act as potential carriers of antibiotic-resistant genes, which they may acquire from various sources during their travels. The mobility of these animals poses a significant risk to human health, as it can contribute to the accelerated spread of antibiotic resistance within the environment. It is worth noting that most of the antibiotic data previously published in Sarawak pertained to sources other than aquaculture, such as swiftlet faeces (Sien *et al.*, 2013), clinical patients (Radu *et al.*, 2002; Kho *et al.*, 2013) and various wildlife or food animals (Son *et al.*, 1997; Apun *et al.*, 2008).

CONCLUSION

In conclusion, multiplex PCR effectively amplified *V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae*. *Vibrio parahaemolyticus* showed robust amplification across all sampling locations, indicating its prevalence in both water and sediment samples. Meanwhile, *V. alginolyticus* and *V. cholerae* were primarily prevalent in sediment samples. The MAR index ranged from 0.35 to 0.65, suggesting that these isolates may originate from high-risk sources. Implementing corrective measures is crucial to mitigate potential foodborne illness outbreaks associated with

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shrimp consumption. Early detection of *Vibrio* species in shrimp farms is essential for prompt intervention, enabling targeted treatment and management strategies to minimize the spread of infection and economic losses for shrimp farmers. This supports the health and sustainability of shrimp farming operations, ensuring a consistent supply of seafood to consumers worldwide.

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CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

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