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Prevalence of multi-antibiotics resistant (MAR) *Vibrio parahaemolyticus* in shrimp farms in Sarawak, Malaysia

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Abstract

The shrimp farming industry is constantly under threat due to outbreaks of infectious diseases and environmental problems. *Vibrio parahaemolyticus* is an important foodborne pathogen causing significant economic losses within the shrimp aquaculture industry worldwide. This research aimed to determine the prevalence of *V. parahaemolyticus* from different shrimp farms from the stocking to harvesting period and assess the antibiotic susceptibility of *V. parahaemolyticus* using the antimicrobial susceptibility test (AST). In this study, a total of 288 samples comprising twenty-four from each sample consisting of shrimp, water, effluent, and sediment samples were collected aseptically from three (n = 3) shrimp farms located at Telaga Air Farm 1 (Pond 6), Telaga Air Farm 2 (Pond 9) and Santubong Farm (Pond 7), Kuching, Sarawak. A molecular approach by polymerase chain reaction was used to confirm the presence of regulator gene, *toxR*, *V. parahaemolyticus*. A total of 14 antibiotics, including spectinomycin (SH100), imipenem (IPM10), amoxycillin/ clavulanic acid (AMC30), enrofloxacin (ENR5), bacitracin (B10), meropenem (MEM10), cephalothin (KF30), penicillin G (P10), tetracycline (TE30), kanamycin (K30), streptomycin(S25), rifampicin (RD2), erythromycin (E15), and nalidixic acid (NA30) were used. The results obtained showed that 51/288 (17.71%) of the collected samples with regulator gene, *toxR V. parahaemolyticus*. As a whole, this includes 31.25% (30/288) from sediment samples, 4.17% (4/288) from shrimp samples, 15.63% (15/288) from water samples, and 2.08% (2/288) from effluent water samples. A total of 54.9% (28/51) of *V. parahaemolyticus* acquired multiple antibiotic resistance (MAR). The resistance of antibiotics was profiled, and the multiple antibiotic resistance (MAR) indexes and classified into ten patterns. The MAR index of *V. parahaemolyticus* isolates ranged from 0.11 to 0.36. *Vibrio parahaemolyticus* isolates showed 31.38% with a MAR index > 0.2, indicating that these isolates might be originated from high-risk sources. The data obtained from this study is helpful to monitor the presence of *V. parahaemolyticus* in the aquaculture farm management system to mitigate the hazard potentially arising from the environmental factor that causes shrimp diseases and shrimp infection.

1. Introduction

Aquaculture is defined by the Food and Agriculture Organization (FAO) (2021) as the farming of aquatic organisms such as fish, molluscs, crustaceans, and aquatic plants. Farming of aquatic organisms implies some procedures to enhance the production of shrimps which involved regular stocking, feeding, and protection from predators or harmful diseases. Aquatic organisms harvested for export purposes must go through several stages before being sold to the market as aquatic organisms are considered the most traded food supply for the world. Aquaculture can meet the increasing global demand for nutritious seafood and contribute to national economies besides supporting the sustainable livelihoods of human communities. Department of Fisheries, Malaysia (2020) showed the recent analysis that the Malaysian Gross Domestic Product (GDP) for Agriculture including aquacultures is currently around

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12.5% with the trade value estimated at USD 1.75 billion (RM 7.5 billion.

Izzuddin (2020) reported that the increasing demand from the international market and decreasing percentages of shrimp, catch led to the blooming of commercial shrimp aquaculture in many countries, including Malaysia. *Penaeid* species, also known as shrimp, such as *P. vannamei* (white leg shrimp) and *P. monodon* (tiger shrimp), which are widely used products from shrimp aquaculture, were cultivated in Asia (FAO, 2021). Shrimp cultivation, usually located in the estuary and coastal areas, also provides job opportunities and better livelihood for local people.

The shrimp farming industry is constantly under threat due to outbreaks of infectious diseases and environmental problems. Bacteria are among the microorganisms that negatively impact global shrimp industries. *Vibrio* genus, including *V. cholerae, V. parahaemolyticus, V. mimicus, V. harveyi, V. alginolyticus,* and *V. vulnificus,* has been described as pathogenic species in shrimp production environment (Chatterjee and Haldar, 2012). These pathogens cause severe infections and decrease the amount of production. In addition, shrimp farmers have reported several shrimp diseases, especially from bacterial infections such as *V. parahaemolyticus* such as vibriosis which killed the shrimps by causing physical deformities, nutritional deficiencies, and unknown diseases. According to Elexson *et al*. (2014), chemical treatments such as excessive antimicrobial treatments were used to combat those diseases, which affected the long-term health of the shrimps and the consumers and even caused the rise of several antibiotic-resistant bacteria.

Therefore, this study aimed to isolate and detect *V. parahaemolyticus* from different commercial shrimp farms and determine the multiple antibiotics resistant (MAR) index of the *V. parahaemolyticus* isolates.

2. Materials and methods

2.1 Measurement of physiochemical parameters

During each sampling trip, the environmental parameters such as temperature, pH, and salinity, were measured using a thermometer (G H Zeal ASTM), a pH meter (pH510, Eutech Instrument), and a salinity refractometer (Hisamatsu Atago S/Mill), respectively.

2.2 Sampling

Sampling was performed in collaboration with Persatuan Nelayan Satang Biru and Seahorse Sdn Bhd shrimp aquaculture farms. The Shrimps farm involved were Telaga Air Farm 1 (Pond 6), Telaga Air Farm 2 (Pond 9) and Santubong Farm (Pond 7). (0"N 110° 11'51., 1°40'59, 1, 93250 Kuching, Sarawak).

A total of 288 (n = 288) samples were collected from three different shrimp farm locations. This included twenty-four (n = 24) samples from each pond in the shrimp farm water, sediment, shrimps, and effluents. Samples were taken every two weeks for 98 days, encompassing one cycle of production, which starts from post-larvae stocking and ends with shrimp harvesting. One sampling point was selected for each pond. The samples were transported to the laboratory in an icebox and were processed within 24 hrs (Alfiansah *et al.,* 2018).

2.3 Enrichment of Vibrio parahaemolyticus isolates

Each water and effluent sample taken from the different sampling points in the same pond was mixed in a ratio of 1:9 in a sterile bijou bottle. Then, 1 mL of the homogenized water and effluent samples was added into 9 mL alkaline peptone water (APW). For sediment and shrimp samples, sediments and shrimps were mixed in a ratio of 1:9 in a sterile plastic bag. After thorough mixing, 1 g of the homogenized sediment sample was added to 9 mL of APW. Firstly, five sterile centrifuge tubes were set up and numbered with sequential 10-fold dilutions and 9 ml of APW media was added to the series of the tube. Approximately, 1 ml of the processed sample was added to the first tube and mixed thoroughly. Then, 1 mL of the sample mixture from the first tube was transferred to the second tube, and the process was repeated with the subsequent tubes. A new and sterile pipette tip was used for each transfer up to the last tube in which the sample is diluted to 10^{-5}. The mixtures were then incubated at 37°C for 24 hrs.

2.4 Isolation of Vibrio parahaemolyticus

A loopful of the broth culture was then plated onto Thiosulfate citrate bile-salts sucrose (TCBS) agar (HiMedia, India) in each plate and incubated at 37°C for 24 hrs. After incubation, colonies formed on the agar were observed. *Vibrio* spp. will appear as yellow and green colonies on TCBS agar. Then green colonies marked for *V. parahaemolyticus* were picked for plating on Chrom Agar Vibrio (CHROMagar™, France) to confirm the species. The violet colonies on Chrom Agar Vibrio will be used for PCR test.

2.5 Extraction of bacterial DNA by boiling method

Bacterial nucleic acid was extracted from isolates by boiling method as described by Dashti *et al*. (2018) with modification. Briefly, 100 µL of an overnight culture of a *Vibrio* isolate in LB broth medium was centrifuged at 13,000×*g* for 1 min. The supernatant was discarded and

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the pellet was washed with 100 µL of sterile distilled water. The suspension was vortexed vigorously for 10 s, centrifuged again at 13,000×*g* for 1 min, and the supernatant was replaced with 100 µL of fresh sterile distilled water. They were then vortexed again at 10 s and boiled for 10 mins. After boiling, the tubes were placed on ice for 5 mins. The tubes were then centrifuged at 13,000×*g* for 5 mins, and the DNA extracts were stored at -20°C for the next step.

2.6 PCR master mixture

The components of the standard master mix protocol using *Taq* DNA polymerase were as follows; 10× *Taq* buffer (1st Base, Singapore), dNTP mix (10 mM) (1st Base, Singapore), 25 mM $MgCl_2$ (1st Base, Singapore), 1.0 µL of each primer (1st Base, Singapore), *Taq* Polymerase (1st Base, Singapore), DNA template and ultra-pure water. Approximately, 5 µL of the DNA extract was used as a DNA template for Polymerase Chain Reaction (PCR).

2.7 Detection of Vibrio parahaemolyticus using PCR method

The primers used to detect the presence of *V. parahaemolyticus* were (5'-GTCTTCTGACGCAATCGTTG-3') forward primers and (5'-ATACGAGTGGTTGCTGTCATG-3') reverse primers which were adopted from Elexson *et al*. (2014). The PCR amplification for *V. parahaemolyticus* was carried out based on the method described by Zulkifli *et al*. (2009) with slight modifications in 25 mL PCR mixture containing 4.0 µL of 10× PCR buffer, 3.0 µL of 25 mM $MgCl_2$, 1.0 µL of 10 mM dNTPs, 1.0 µL of each 10 pmol primer, 1.0 µL of 2.5 U *Taq* DNA polymerase, 2.0 µL of DNA template and 200 mL sterile distilled water.

2.8 PCR amplification

All thermal cycling reactions were performed with a Thermocycler (Esco Swift MiniPro, country) using the following parameters: Initial denaturation at 94°C for 3 mins; followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1.5 mins and extension at 72°C for 1.5 mins before the final extension at 72°C for 7 min. For visualization, 5 µL of each PCR product was run on 1.2% agarose gel at 85V for 1 hr in 1X Tris Borate EDTA (TBE) buffer. The gel was stained with ethidium bromide for 15 mins and viewed under a UV transilluminator.

2.9 Antibiotic susceptibility test

A total of fourteen antibiotics that are commonly used for aquaculture have been used in this test are as follows; spectinomycin (SH100, 100 mcg), imipenem (IPM10, 10 mcg), amoxycillin/clavulanic acid (AMC30, 30 mcg), enrofloxacin (ENR5, 5 mcg), bacitracin(B10, 10 mcg), meropenem (MEM10, 10 mcg), cephalothin (KF30, 30 mcg), penicillin G (P10, 10 mcg), tetracycline (TE30, 30 mcg), kanamycin (K30, 30 mcg), streptomycin (S25, 25 mcg), rifampicin (RD2, 2 mcg), erythromycin (E15, 15 mcg) and nalidixic acid (NA30, 30 mcg).

The *V. parahaemolyticus* isolates were cultured for the preparation of an antibiotic susceptibility test and grown on Mueller Hinton agar (MHA). Antibiotic discs were placed on the surface of the agar in each plate. Then the plates were incubated at 37°C for 24 hrs. After the incubation period have ended, the zone of inhibition for each antimicrobial disk was measured and the results of AST were interpreted to determine whether the isolate is resistant or susceptible towards the tested antibiotics.

3. Results

3.1 Samples collection

As shown in Table 1, the physiochemical parameters including water temperature (°C), water pH, and water salinity readings during the stocking period (August to November), while Table 2 shows the water temperature, water pH, and water salinity readings during the harvesting period. The total average values of the three physiochemical parameters (pH, temperature, and salinity) of the water samples collected from the stocking period till the harvesting stage was shown in Table 3. As shown in Table 3, the mean pH of pond water for two farms ranged between pH 9 to pH 11. The average temperature of pond water recorded during sampling for all three locations ranged between 29°C to 32°C while the salinity of the pond water for all farms ranged between 28 to 34 ppt.

Table 1. Water temperature, water pH and water salinity readings during the stocking period.

Parameters	Pond 6 (Telaga Air Farm 1)	Pond 9 (Telaga Air Farm 2)	Pond 7 (Santubong Farm)
Water temperature (°C)	31	30	29
Water pH	10	9	11
Water Salinity (ppt)	32	28	34

Table 2. Water temperature, water pH and water salinity readings during the harvesting period.

Parameters	Pond 6 (Telaga Air Farm 1)	Pond 9 (Telaga Air Farm 2)	Pond 7 (Santubong Farm)
Water temperature (°C)	32	30	31
Water pH	11	11	9
Water Salinity (ppt)	30	29	33

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Table 3. Total average of physical parameters (pH, temperature, and salinity) in sampling locations.

Locations	Duration of sampling	Mean for pH of pond water	Mean temperature of pond water (°C)	Mean salinity of pond water (ppt)
Telaga Air Farm 1	August - November 2020	10.5	31.5	31.0
Telaga Air Farm 2	August - November 2020	10 .0	30.0	28.5
Santubong Farm	August - November 2020	10.0	30.0	33.5

3.2 Detection of Vibrio parahaemolyticus in different farm locations

Molecular detection using PCR *V. parahaemolyticus* was carried out to detect gene with amplicon at 200 bp specific for detecting *V. parahaemolyticus*. Figure 1 showed the gel images obtained from the specific PCR for samples. For all the samples collected, out of the total number of eighty-eight (n = 88) samples collected, there were 51 over 288 samples (17.71%) are *V. parahaemolyticus* positive. This is including 31.25% (30/288) from sediment samples, 4.17% (4/288) from shrimp samples, 15.63% (15/288) from water samples and 2.08% (2/288) from effluent samples as shown in Table 4.

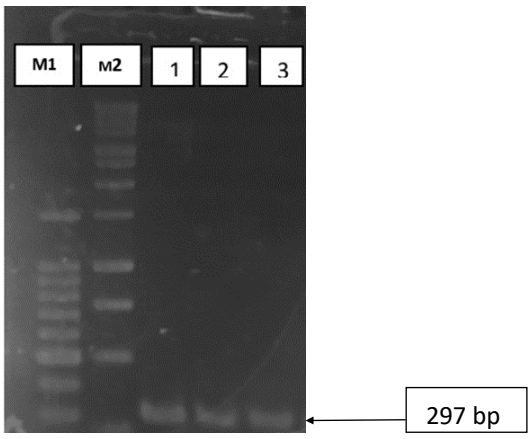

Figure 1. PCR identification of *V. parahaemolyticus* at 297 bp. M1: 100 bp DNA ladder, M2: 1000 bp, Lane 1: *V. parahaemolyticus* ATCC 1708 Lane 2: Shrimps samples, Lane 3: Sediment samples.

For the Telaga Air Farm 1, *V. parahaemolyticus* was detected in 20.8% (5/24) in water samples, 4.17% in shrimp samples (1/24), 41.7% (10/24) in sediment samples, and 4.17% (1/24) in effluent samples. Meanwhile, Telaga Air Farm 2, *V. parahaemolyticus* was detected 25% (6/24) in water samples, 4.17% in shrimps' samples (1/24), 37.5% (9/24) in sediment samples and 0% 01/24) in effluent samples.

In Santubong Farm, *V. parahaemolyticus* was detected 16.7% (4/24) in water samples, 8.33% in shrimp samples (2/24), 45.8% (11/24) in sediment samples, and 4.17% (1/24) in effluent samples. The percentage of positive detection based on farm location were shown in Figure 2. It showed that the Santubong Farm had higher detection of *V. parahaemolyticus* (18.75%), followed by Telaga Air Farm 1 (17.7) and Telaga Air Farm 2 (16.7%).

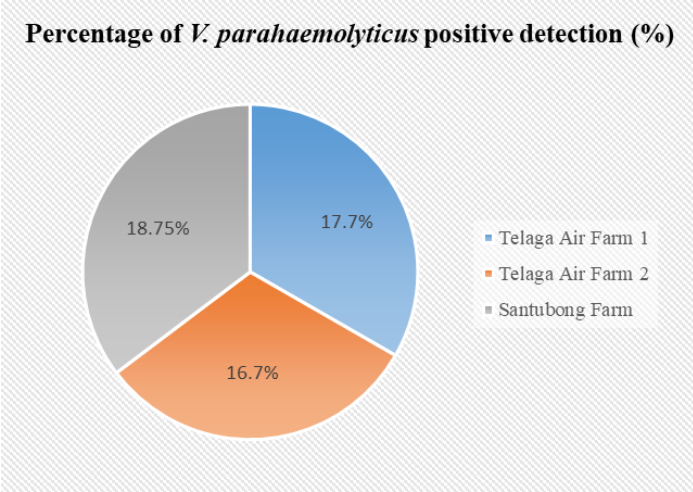

Figure 2. Number of *V. parahaemolyticus* positive detection (%) based on farm location.

3.3 Antibiotic resistance profiles and multiple antibiotic resistance index of Vibrio parahaemolyticus

A total of 54.9% (28/51) *V. parahaemolyticus* isolates are multiple antibiotic-resistant (MAR). The resistance of antibiotics was profiled and the multiple

Table 4. Occurrence of *V. parahaemolyticus* from there different location.

Sample	Pond 6 (Telaga Air Farm 1)	Pond 9 (Telaga Air Farm 2)	Pond 7 (Santubong Farm)	Total positive *V. parahaemolyticus* (%) in samples
	Occurrence of positive *V. parahaemolyticus* (%)	Occurrence of positive *V. parahaemolyticus* (%)	Occurrence of positive *V. parahaemolyticus* (%)	
Water	(20.8)	(25)	(16.7)	(15.63)
Shrimp	(4.17)	(4.17)	(8.33)	(4.17)
Sediment	(41.7)	(37.5)	(45.8)	(31.25)
Effluent	(4.17)	0	(4.17)	(2.08)
Total positive *V. parahaemolyticus* (%)	17.7	16.7	18.75	

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antibiotic resistance (MAR) indexes are shown in Table 5. From the results, the isolates were classified into 10 patterns. Pattern 1 consisted of seven isolates (VP001, VP002, VP003, VP004, VP010, VP022, VP035), which showed the MAR index of 0.21. These strains were resistant towards bacitracin, penicillin and cephalothin, While VP009, VP016, VP017, VP018, VP019, VP036 (Pattern II) strains were resistant towards bacitracin and penicillin with 0.38 MAR index.0.14.

Notably, Pattern group III (VP011) has 0.29 MAR index, which was resistant to four antibiotics (bacitracin, cephalothin, penicillin and streptomycin), while Pattern IV (VP012) was resistant towards rifampicin and bacitracin (0.14 MAR Index) and pattern VI (VP014, VP015) were resistant towards penicillin, tetracycline and bacitracin with 0.21 MAR index. Group patterns VII (VP023, VP033, VP037) with 0.14 MAR index were resistant toward bacitracin and cephalothin. Pattern group VIII (VP034) with a MAR index of 0.29 was resistant toward streptomycin, tetracycline, penicillin and cephalothin.

Pattern group XI consisted of 0.21 MAR index resistant to two antibiotics: bacitracin and cephalothin, while Group X was resistant towards rifampicin and penicillin. Pattern group XI had a higher MAR Index of 0.36 which were resistant toward antibiotics; cephalothin, tetracycline, streptomycin, rifampicin and bacitracin.

Figure 3 shows the number of *V. parahaemolyticus* antibiotics multi-resistant (%) detected in the samples. The highest detection was in shrimp samples which are 100%, followed by the water sample (66.7%), sediment sample (50%), and no detection (0%) of antibiotics multi -resistant in the effluent samples.

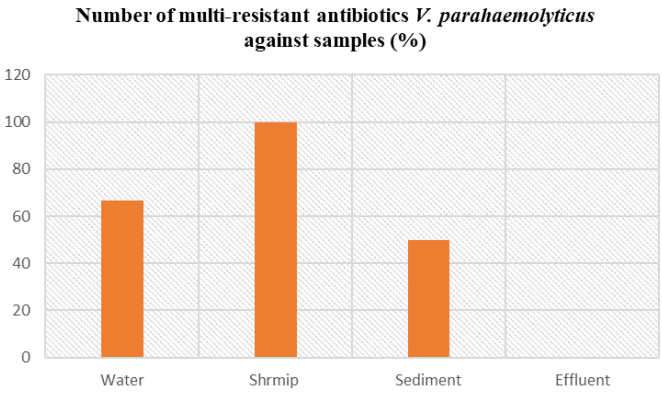

Figure 3. Number of *V. parahaemolyticus* antibiotics multi-resistant (%) against water, shrimp, sediment and effluent samples.

4. Discussion

Microbiological hazard assessment of aquaculture systems will be able to determine the growth of an aquaculture shrimp industry. Microbiological assessment in aquaculture systems is essential to ensure consumers' hygienic and safe seafood products (Rakhshandehroo *et al*., 2017).

Therefore, prevention of the risk of infection should be highlighted by avoiding the consumption of raw seafood and ensuring proper preparation and cooking are done to kill the bacteria that might inhabit them (Novoslavskij *et al*., 2015). Moreover, it is also essential to manage the storage and handling process of the seafood according to the standards that align with the food safety and quality assurance programme set by local authorities. Contamination could cause significant economic loss, and besides, it may lead to the consumption of unsafe shrimp by consumers. Pathogenic bacteria can adhere to seafood from contaminated aquaculture environments such as water sources or the pond bottom (Hamilton *et al*., 2018). According to FAO (2019), the high levels of *Vibrio* spp. are associated with poor environmental management systems in terms of

Table 5. The patterns of antibiotic resistance profiles and multiple antibiotic resistance (MAR) indexes of *V. parahaemolyticus*.

Pattern	Strain No.	Antibiotic Resistant Profiles[a]	MAR Index	No. of isolate/Total Isolates (% of Occurrence)
I	VP001, VP002, VP003, VP004, VP010, VP022, VP035	B10P10KF30	0.21	7/51 (13.73%)
II	VP009, VP016, VP017, VP018, VP019, VP036	B10P10	0.14	6/51 (11.7%)
III	VP011	B10KF30P10S25	0.29	1/51 (1.97%)
IV	VP012	RDB10	0.14	1/51(1.97%)
VI	VP014, VP015	P10TE30B10	0.21	2/51(3.92%)
VII	VP023, VP033, VP037	B10KF30	0.14	3/51(5.88%)
VII	VP034	S25TE30P10KF30	0.29	1/51(1.97%)
IX	VP025, VP026, VP040, VP041	P10KF30	0.21	4/51(7.84%)
X	VP027	RDP10	0.14	1/51(1.97%)
XI	VP031	KF30TE30S25RD2B10	0.36	1/51(1.97%)

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hygiene and safety, which cause the shrimp to become weak and susceptible to bacterial infection.

Environmental and physiochemical factors influence the growth of the shrimps and the growth of the co-existing bacterial populations (Alfiansah *et al.*, 2020). When referring to Table 1 and Table 2, the result showed no significant difference in the physical parameters of the stocking process to the harvesting process. This is due to the farm management constantly monitoring the parameter SOP implemented by the management. In this study, the pH values recorded from all sites were slightly acidic (pH 6.08 - 7.98) for almost every week. However, *V. parahaemolyticus* was detected even at pH 6.47. The difference in water pH level was due to influx and decay in the estuarine region and the hydrogen ions that run out during the rainy season. Besides, the pH of the water was less correlated to the abundance of *V. parahaemolyticus*. This may be due to only low variable values of pH (6.08 - 7.98) over the twelve-week sampling.

In Table 3, the mean pH observed in all three locations ranged from pH 9 to pH 11. While maintaining the water pH between this range is crucial to ensure the healthy growth of shrimp, this range is also optimum for the growth of most bacteria. Even though most of the *Vibrio* spp. will grow on a pH between 6.5 and 9.0, it also tends to propagate best under alkaline surroundings (Percival *et al*., 2014)

Vibrio spp. can tolerate a wide range of temperatures (20˚C to more than 40˚C), and the ideal temperature for the growth of *Vibrio* spp. is between 28°C to 32°C (Sampaio *et al*., 2022). The temperature and pH levels of the water in the pond provide an optimal conditions for the *Vibrio* spp. to proliferate abundantly inside these farms. Temperature between 28°C to 35°C, which was recorded in all three sampling locations, is also optimum for the growth of *Vibrio* species. Temperature is associated with the growth of *V. parahaemolyticus*. The findings of this study showed that as the temperature increases, the number of bacteria also increases. Previous researchers have reported that temperatures between 10° C and 43°C with an optimum of 37°C are suitable for the growth of *V. parahaemolyticus, w*ith a short generation time of 8 – 9 minutes (Elexson *et al*., 2014). The findings showed positive results only in the first two weeks when the temperature was lower than the rest.

In addition to pH value and temperature, salinity is another environmental factor affecting the growth of both shrimp and bacteria. In shrimp farming, the salinity variation of water is considered a determinant factor in shrimp production. The samples examined in this study were collected during the rainy season, which might indirectly affect the salinity of the ponds. During the rainy season, the rainfall dilutes the water, causing the estuarine water to be less saline than in the warmer season. From the analysis, it was found that there is a significant relationship between the level of salinity and the abundance of the *V. parahaemolyticus*. Table 3 showed the mean of the salinity factor was in the range of 28.5 to 33.5 ppt. As reported by Givens *et al*. (2014), this range has been reported to have yielded higher densities of *Vibrio* spp. Like other *Vibrios*, *V. parahaemolyticus* is a halophile, and sodium ions stimulate their growth (Pumipuntu and Indrawattana, 2017). Noteworthy, *V. parahaemolyticus* is often found in tropical and sub-tropical countries (Nakaguchi, 2013). Thus, it can be shown in the detection of *V. parahaemolyticus* in all the samples collected. Based on the data obtained from all three sampling locations, the average pH, temperature, and salinity for the entire sampling period are all conducive to the proliferation of *V. parahaemolyticus* in the shrimp ponds.

There is a high presence of *V. parahaemolyticus* with 31.25% detection in sediment samples, followed by water samples with 15.63% detected starting from shrimp post-larvae stocking until the harvesting of mature shrimp. Thus, it is speculated that the observed prevalence may also be produced by seasonal variation of the *V. parahaemolyticus* population due to salt and oxygen concentrations, interactions with the plankton, the presence of sediment, and organic matter (Elexson *et al*., 2014). Lower *V. parahaemolyticus* detection (2.08%) in effluent samples due to the strict wastewater treatment practices by the farm management, which were, reduces the abundance and diversity as they are meeting the requirement of the local authority.

According to the latest finding by Andrews (2020) referring to Table 1 to Table 3, ecological parameters such as temperature, salinity, and pH affect the abundance of *Vibrio* spp. Shrimp farmers shall conduct essential monitoring of water physiochemical and microbiological characteristics and sustain the daily basis of record-keeping for immediate and easy reference. In addition to the subsequent cropping and essential troubleshooting problems, shrimp farmers should monitor the water depth, temperature, salinity, and pH twice daily, such as morning and in the evening, to maintain the optimum conditions for growth and avoid any drastic changes in the parameters.

Diseases caused by *V. parahaemolyticus* are referred to as vibriosis. Infection in shrimps can be detected with several symptoms: black shell disease, tail rot, septic hepatopancreatic necrosis, brown gill swollen hindgut syndrome, and luminous bacterial disease. This infection also causes several clinical signs such as lethargy, loss of

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

The level of *V. parahaemolyticus* in each sampling location experienced fluctuations and increments throughout the year, depending on the weather. These changes may be due to drastic changes in the water salinity and temperature caused by rain. In Malaysian weather that varies from day to day, drastic changes in these environmental factors can occur readily. Table 4 shows the presence of *V. parahaemolyticus* with 17.71% of positive detection in all collected samples. To reduce or eliminate the presence of this bacteria, a sanitization and disinfection process on the facilities and equipment used should be routinely performed. The personal hygiene of the workers and the water quality should also be monitored (Nakaguchi, 2013).

The presence of *V. parahaemolyticus* indicates a poor environmental management system in terms of control and monitoring of hygiene and safety towards biological hazards. Improvements to the management system can be made to curb this problem by ensuring sanitization and disinfection of facilities and equipment before culturing. Microbiological assessment in aquaculture systems is essential to mitigate the disease infection problems in shrimps, leading to food safety issues. Contamination can cause significant economic loss and may lead to the consumption of unsafe shrimp by consumers (Novoslavskij *et al*., 2015).

In order to prevent diseases from spreading from one pond to another, breeders shall cultivate and provide specific pathogen-free (SPF) shrimp stocks to the farmers. SPF shrimp stocks have improved in shrimp production in many countries. However, strict management procedures such as physical barriers, water treatment control, carrier exclusion, and feed management system are also essential (Flegel, 2019). In addition, routine determination of *Vibrio* spp. in farms is essential before management decisions. According to Verschuere *et al*. (2000), preventive measures are usually applied to disinfect and provide antimicrobial solutions.

The harvesting process of the shrimp cultures is operated partially until the level of water inside the aquaculture farm is reduced and all the mature shrimps are harvested. Hence, the amount of the *Vibrio* spp. is reduced due to the reduction of shrimp cultures. This technique involves water exchange using clean water, which can lessen *Vibrio* spp. loads and decrease the impacts of stressors by washing out organic material and other metabolites such as ammonia (Barraza-Guardado *et al*., 2013).

Overuse of antibiotics may develop resistance among shrimp pathogens and adjacent ecosystems. An increase in the use of antibiotics would increase the difficulty of treating bacterial infections in shrimp ponds. Several antibiotics, such as fluoroquinolones, tetracyclines, and sulphonamides, may cause a high risk to the environment and human health (Holmström *et al*., 2019). This study showed that *V. parahaemolyticus* isolates from shrimp farm samples consisted of strains with multiple resistance to the tested antibiotics at 54.9%, as groups III, VIII, and XI were resistant to at least four antibiotics. These results were not surprising since other reports on the multi-drug resistance pattern in *V. parahaemolyticus* isolates from raw seafood were reported in Indonesia and Nigeria (Elexson *et al.*, 2014).

The MAR index of *V. parahaemolyticus* isolates ranged from 0.11 to 0.36. Besides, the 51 *V. parahaemolyticus* isolates showed 31.38% of the *V. parahaemolyticus* with MAR index > 0.2, indicating that these isolates might originate from high-risk sources. The high-risk sources include human and farm animals (e.g., poultry, swine, and dairy cattle) frequently exposed to antibiotics. The wide use of antibiotics in human therapy resulted in the emergence of MAR pathogenic microorganisms in human faeces and, subsequently, contamination of aquatic systems and environments. The resistance level may arise from various selective pressure for the uncontrolled use of antibiotics. Besides, animal farms release their effluent and water following antibiotic therapy on the farms (Schwach *et al*., 1982).

Besides that, wastewater from industrial plants containing microbial agents has been prepared and washed. The residual water is discharged directly into the sewage system (Guardabassi *et al*., 1998), which eventually flows into the sea and is connected to the aquaculture seafood farm. Thus, the MAR of *V. parahaemolyticus* introduced into the marine environment can easily contaminate shellfish, which concentrates on the marine microflora through filter-feeding. This subsequently causes a mutation in the cellular DNA (Zulkifli *et al*., 2009). The results of this study provided valuable information in finding safe and efficient antibiotics. In addition, it also provided some insights into the problems faced by the shrimp industry.

One preventive measure that has been implemented in shrimp farms before the stocking process is removing the surface layer of the land that has been in contact with the broth cultures of the shrimp. This method removes *Vibrio* spp. or other bacterial colonies that are inhabiting the sediment. Limestone powder is then spread to the whole area to neutralize the sediment. This process aims to upsurge the availability of nutrients, sterilize the pond

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before the stocking process, increase the pH and act as a buffer against the daily fluctuations of the pH (Queiroz *et al*., 2004). The limestone application can help raise the alkalinity by about 50 mg/L to develop the production through ponds fertilization and improve the water quality of the ponds for feed-based aquaculture (Boyd, 2017). Thus, this preventive measure caused (68.63%) 35/51 of *V. parahaemolyticus* isolates to demonstrate MAR index < 0.2, which were considered to be obtained from low-risk sources (seldom or never exposed to antibiotics).

Although antimicrobial resistance can be a significant problem for therapy directed against organisms with a MAR index < 0.2, the importance of antibiotics in managing human infection caused by *V. parahaemolyticus* is not defined. Therefore, appropriate antimicrobial therapy is required for more effective management of severe infections to investigate those with antibiotic resistance potential. *V. parahaemolyticus* strains should be monitored carefully.

All water samples were taken with the sediment because the virus exists in greater quantities in bulk sediment than in pore water (Drake *et al*., 1998). In particle-rich conditions, most viruses will be adsorbed to the sediments, and only a tiny portion of them will be located in pore water. They are immobilized until viruses are adsorbed to sediments, contributing to the aggregation and concentration of viruses in sediments (De Flora *et al*., 1975). Therefore, sediments containing both the particle-adsorbed virus and virus in pore water were collected in this sample to extract bacteriophage effectively.

5. Conclusion

This study reported on the importance of environmental parameters such as salinity, pH, and temperature, in influencing the distribution and abundance of *V. parahaemolyticus* in commercial shrimp farms. *Vibrio parahaemolyticus* has been found in all commercial shrimp farms, highly detected in sediment samples followed by water, shrimp, and effluent samples. This study has suggested effective shrimp pond management in order to help in controlling the numbers of *V. parahaemolyticus* in aquaculture shrimp farms.

Conflict of interest

The authors declare no conflict of interest.

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