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Research Article

Characterization of the Virulency of *Vibrio* spp. in Giant Tiger Shrimp (*Penaeus monodon*) from Peninsular Malaysia

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Nowadays, bacterial diseases caused by *Vibrio* spp. pose a significant obstacle to aquaculture growth, affecting socioeconomic development. The presence of pathogenic *Vibrio* spp. are gaining attention due to widespread mortality in fish and shellfish worldwide. This study investigates the distribution of virulence genes and their pathogenicity by experimentally infecting a healthy group of cultured shrimp, specifically *Penaeus monodon*. A total of 225 *Vibrio* spp. isolated from 210 cultured shrimp from various geographical regions in Peninsular Malaysia were examined, resulting in 13 *Vibrio* spp. being identified and characterized. According to virulence gene assessment, only eight of the 11 virulence genes were detected in the isolates, including *chiA* (89%), *flaC* (74%), *tlh* (72%), *toxR* (70%), *luxR* (40%), *pirA* (11%), *pirB* (11%), and *hlyA* (0.4%). Other virulence genes, such as *tdh*, *trh*, and *ctxA*, were not found in any of the isolates (0%). Then, three *Vibrio* isolates, including *V. parahaemolyticus* S12-3, *V. campbellii* S10-4, and *V. rotiferianus* S24-4, were selected for pathogenicity test based on highest number of virulence genes in each species. These species were subjected to immersion challenge test against postlarvae (PL) *P. monodon* approximately PL-20 with three different concentrations of 1×10^6, 1×10^7, and 1×10^8 CFU/mL. The *V. parahaemolyticus* S12-3 was determined to have lethal concentration 50 (LC_{50}) 1×10^5 CFU/mL after 168 hr of exposure. The immersion challenge test resulted in a pale hepatopancreas and empty guts in *P. monodon*. Histopathological examination showed sloughing of epithelial cells in the hepatopancreatic tubule, hemocytic infiltration, massive vacuolation, and loss of hepatopancreatic tubule structure. Overall, the study demonstrated the virulence and pathogenicity of *V. parahaemolyticus* S12-3 toward *P. monodon*, which could be associated with its virulence properties. Furthermore, the current findings will be useful in developing microbiological risk assessments for shrimp farming and aquaculture management.

1. Introduction

Shrimp aquaculture contributed to 15% of all worldwide traded seafood products [1] According to FAO [2], the shrimp industry contributes USD 250 billion to regional, national, and local economies. The main producers of giant tiger shrimp (*Penaeus monodon*) and whiteleg shrimp (*Penaeus vannamei*) are China, Vietnam, India, Thailand, and Indonesia, which contributing production values of USD 5.59 billion and USD 26.7 billion [3, 4]. However, the Asia-Pacific region's shrimp industry, as the world's leading producer of aquaculture products, has been affected by emerging diseases, resulting in high mortality rates and economic losses. The common diseases such as luminous vibriosis, white spot disease (WSD), yellow head disease

(YHD), Taura syndrome (TS), and acute hepatopancreatic necrosis disease (AHPND) have devastated the region's shrimp aquaculture, resulting in the shrimp industry's collapse [5]. Therefore, maintaining the animals' health is crucial to prevent the outbreak.

Vibriosis is a common bacterial disease that affects a wide range of marine fish and shellfish. It has been reported that vibriosis caused severe production losses in shrimp farms, with mortality rates of up to 100%, particularly in postlarvae and juvenile populations across all producing countries [6]. *Vibrio* spp. such as *V. alginolyticus*, *V. anguillarum*, *V. harveyi*, *V. vulnificus*, and *V. parahaemolyticus* are the most common species that cause vibriosis in shrimp [7]. Furthermore, *V. parahaemolyticus* is one of the several causative agents of shrimp disease that has become more prevalent as global shrimp demand and production have increased [8]. The presence of pathogenic bacteria in the global marine environment lead to high mortality rates and can result in significant economic losses. The bacteria enter into aquaculture system through contaminated water, feed, and equipment [9]. Therefore, a good management practices should be implemented to promote a healthy and productive industry, particularly shrimp industry.

There are a variety of virulence genes that encode virulence factors that contribute to *Vibrio* strains' pathogenicity [10]. The virulence factors enable pathogens to infect and harm hosts by allowing adhesion and entry, establishment and multiplication, evading host defenses, causing host damage, and eventually leaving the host [11]. Besides, five major virulence factors, which play a role in microbial pathogenesis, found in *Vibrio* such as capsular polysaccharides, adhesive factors, cytotoxins, lipopolysaccharides, and flagella [9, 12]. Furthermore, these virulence factors may be required for virulence against various hosts because vibrios infect a diverse range of aquatic species, including fish, shrimp, and mollusks [13]. According to Menezes et al. [14], the aquaculture environment provides a suitable condition for virulent lineages of *Vibrio*. This is primarily due to the high level of nutrients and the presence of antibiotics, providing the optimum environment for growth and development of the microorganisms.

There has been little research on virulence and pathogenicity of several *Vibrio* spp. that cause vibriosis in shrimp, particularly in Malaysia. This makes it difficult to identify, treat, and prevent, causing significant losses in the shrimp farming industry. Therefore, a study on virulence genes profiling and their pathogenicity was conducted to provide a better knowledge of their potential virulence. In addition, further study is required to understand the differences in pathogenicity across *Vibrio* spp. and to develop effective management techniques for vibriosis in shrimp.

2. Materials and Methods

2.1. Collection of Vibrio spp. A total of 225 *Vibrio* isolates that were previously obtained from shrimp were used. The isolates were identified using *pyrH* gene in previous experiment [15] as *V. parahaemolyticus* ($n = 124$), *V. communis* ($n = 20$), *V. campbellii* ($n = 18$), *V. owensii* ($n = 15$), *V. rotiferianus* ($n = 12$), *Vibrio* spp. ($n = 8$), *V. alginolyticus* ($n = 7$), *V. natriegens* ($n = 5$), *V. brasiliensis* ($n = 4$), *V. xuii* ($n = 2$), *V. harveyi* ($n = 2$), *V. hepatarius* ($n = 1$), and *Photobacterium damselae* ($n = 7$). The isolates were streaked onto tryptic soy agar (TSA) (Oxoid, Hampshire, UK) supplemented with 1.5% NaCl and incubated at 30°C for 24 hr. Then, the single colony on TSA was further subcultured on TCBS agar.

2.2. Virulence Genes Assessment. A total of 11 virulence-associated genes, including *pirA*, *pirB*, *chiA*, *luxR*, *flaC*, *hlyA*, *toxR*, *ctxA*, *tlh*, *tdh*, and *trh*, were amplified by polymerase chain reaction (PCR), and the sequence of primers used were shown in Table 1. The PCR mixtures were performed in a final volume of 25 μL containing 2.5 μL of the DNA template (10–100 ng) that was mixed with 12.5 μL of PCR MasterMix (First Base, Kuala Lumpur, Malaysia) comprised of 60 U/mL of Taq DNA polymerase, 3 mM $MgCl_2$, 400 μM dNTP mix, and 10 μM each forward and reverse primer.

The amplification was performed under the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min; 48.9°C for 1 min (*chiA*), 50°C for 1 min (*luxR*), 60°C for 1 min (*hlyA*), 55°C for 1 min (*flaC*) and 72°C for 1 min, and a final extension of 72°C for 10 min. The amplification of *tlh*, *trh*, and *tdh* was performed under the following conditions: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 10 min. The amplification of *pirA* and *pirB* was performed under the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 58.5°C for 30 s, and 72°C for 30 s, and a final extension of 72°C for 10 min. For *ctxA* and *toxR*, the conditions were initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 60°C for 40 s (*ctxA*), 52°C for 45 s (*toxR*), and 72°C for 1 min, and a final extension of 72°C for 10 min. Then, the PCR amplifications were carried out using a PCR thermocycler (Bio-Rad, California, USA). The PCR products were visualized with a gel documented system (Acinterlab, Miami, USA) after gel electrophoresis (70 V, 60 min) using 1.5% agarose gel electrophoresis that had been preadded with FloroSafe DNA Stain (First Base Laboratories).

2.3. Acclimatization of Giant Tiger Shrimp (P. monodon). A 20-day postlarvae (PL-20) cultures with 0.09 ± 0.03 g in weight were obtained from a local commercial shrimp hatchery. The shrimp were acclimatized in a 500-L tank with constant aeration for 7 days. Approximately 30% of the seawater was replaced, and water quality measures were evaluated on a daily basis. The mean $\pm$ SD of water parameters, including pH, temperature, salinity, and dissolved oxygen, were determined a using a handheld YSI meter (YSI Incorporation, Ohio, USA). During the entire experiment, the shrimp were fed with commercial feed containing 35% crude protein three times daily [23].

2.4. Preparation of Different Concentration of Vibrio spp. The *Vibrio* isolates were selected based on the presence of the

Table 1: Virulence factors, sequence of primers, references, and expected amplicon size of target gene used in this study.

Gene	Virulence factor	Primer sequence (5–3)	Amplicon size (bp)	References
pirA	AHPND strains of *V. parahaemolyticus*	F: TGACTATTCTCACGATTGGACTG R: CACGACTAGCGCCATTGTTA	284	Han et al. [16]
pirB	AHPND strains of *V. parahaemolyticus*	F: TGATGAAGTGATGGGTGCTC R: TGTAAGCGCCGTTTAACTCA	392	Han et al. [16]
chiA	Chitinase	F: GGAAGATGGCGTGATTGACT R: GGCATCAATTTCCCAAGAGA	232	Ruwandeepika et al. [17]
luxR	Quorum sensing factors	F: ATGGACTCAATTGCAAAGAG R: TTAGTGATGTTCACGGTTGT	618	Ruwandeepika et al. [17]
flaC	Flagella	F: AAATCATTCCAAATCGGTGC R: TCTTTGATTCGGCTCTTA	580	Bai et al. [18]
hlyA	Hemolysin	F: GGCAAACAGCGAAACAAATACC R: CTCAGCGGGCTAATACGGTTTA	738	Saravanan et al. [19]
toxR	Transmembrane transcription regulator	F: ATACGAGTGGTTGCTGTCATG R: GTCTTCTGACGCAATCGTTG	368	Letchumanan et al. [20]
ctxA	Cholera toxin	F: CGGGCAGATTCTAGACCTCCTG R: CGATGATCTTGGAGCATTCCCAC	563	Sechi et al. [21]
tlh	Thermolabile hemolysin	F: AAAGCGGATTATGCAGAAGCACTG R: GCTACTTTCTAGCATTTTCTCTGC	450	Bej et al. [22]
tdh	Thermostable direct hemolysin (TDH)	F: GTAAAGGTCTCTGACTTTTGGAC R: TGGAATAGAACCTTCATCTTCACC	269	Bej et al. [22]
trh	TDH-related hemolysin (TRH)	F: TTGGCTTCGATATTTTCAGTATCT R: CATAACAAACATATGCCCATTTCCG	500	Bej et al. [22]

highest number of virulence genes in each of the isolates. Briefly, five colonies formed on TCBS agar were randomly picked and inoculated into 1 L of TSB supplemented with 1.5% NaCl and incubated at 30°C for 24 hr, with a constant shaking at 150 rpm. Then, serial dilutions were performed up to 10-fold and 0.1 mL of TSB from each dilution was plated on TSA in triplicate, and further incubated at 30°C for 24 hr. The colony-forming units per milliliter (CFU/mL) was calculated using standard plate count.

2.5. Pathogenicity Study. For the pathogenicity test, a total of 540 PL of *P. monodon* were randomly distributed into 10 L of tank. The shrimp were divided into three groups (three groups × three bacterial dilutions × 15 shrimp/tank) of bacterial isolates and three group of control (15 shrimp × three replicates). Each of the treatment was conducted in triplicate ($n = 3$). The experiment was conducted using immersion challenge test as described by Tran et al. [24] with some modifications. The bacterial cells were harvested from the overnight incubation by centrifugation at 7,000 rpm for 10 min. Then, the shrimp were immersed into fresh bacterial suspension for 15 min. Next, the shrimp were transferred into the experimental tank with bacterial suspension to obtain final concentration of 1×10^6, 1×10^7, and 1×10^8 CFU/mL. Control shrimp were not exposed to any of the isolates. The shrimp were maintained with commercial shrimp feed at 5% body weight throughout the period. Moribund and dead shrimp were removed daily from the tanks. During the experiment, all tanks were aerated with zero water exchange for the duration of 168 hr. In all challenges, the moribund shrimp were anesthetized using MS-222 (Western Chemical Inc., Ferndale, WA, USA) at a concentration of 100 ng/mL [25]. The abnormalities and mortalities of shrimp were observed and expressed as the cumulative mortality. The lethal concentration 50 (LC_{50}) was calculated using probit analysis with SPSS version 25. The freshly dead shrimp were subjected to bacterial isolation and identification [26].

2.6. Histopathology Analysis. Histological analysis was performed to study the pathologic changes. The hepatopancreas of shrimp was taken for the analysis at the end of the experiment. Shrimp were immediately fixed in 10% (v/v) phosphate-buffered formalin composed of 100 mL of 40% formalin, 900 mL of distilled water, 4 g/L of NaH_2PO_4, and 6.5 g/L of Na_2HPO_4 for 24 hr. Then, the shrimp were preserved in 70% ethanol until further processing [27, 28]. The preserved samples were submitted to the Veterinary Histopathology Laboratory at Universiti Putra Malaysia for further processing. Briefly, the samples were trimmed and were routinely processed and embedded using the paraffin-embedded technique. These tissues were sectioned at 4 μm thickness, dried overnight, and were routinely stained using hematoxylin and eosin (H&E) staining. All tissue sections

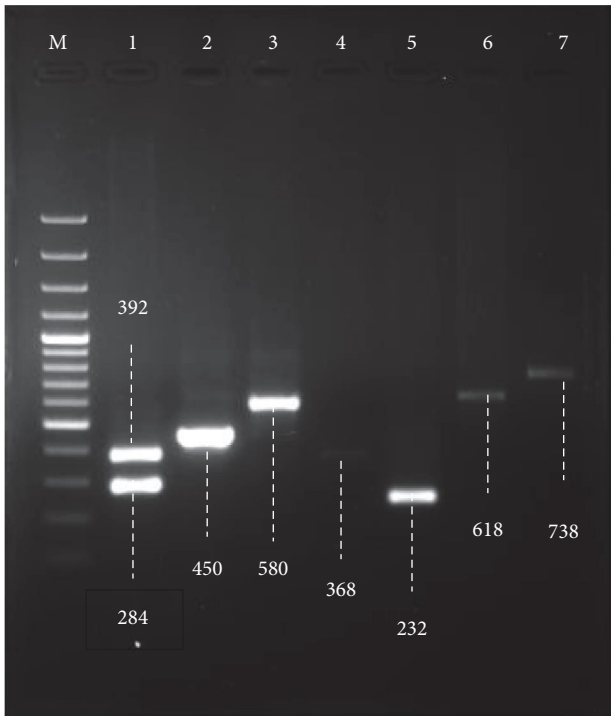

FIGURE 1: The presence of virulence genes of *Vibrio* spp. Lane M: 100-bp ladder, lane 1: *pirA* (284 bp) and *pirB* (392 bp), lane 2: *tlh* (450 bp), lane 3: *flaC* (580 bp), lane 4: *toxR* (368 bp), lane 5: *chiA* (232 bp), lane 6: *luxR* (618 bp), and lane 7: *hlyA* (738 bp).

were examined under a light microscope to describe and compare the histopathological features.

2.7. Statistical Analysis. The statistical significance of data was measured using the analysis of variance (ANOVA) test. A p-value <0.05 was set as a statistically significance. Mean, standard deviation, and graph for cumulative mortalities were constructed using Microsoft Excel.

3. Results

3.1. Virulence Genes Assessment. The assessment of the virulence genes was based on the presence of specific sizes of bands in gel electrophoresis. Amplification of *pirA*, *pirB*, *tlh*, *flaC*, *toxR*, *chiA*, *luxR*, and *hlyA* produced 284, 392, 450, 580, 368, 232, 618, and 738 bp, respectively (Figure 1). There were a total of 11 virulence-associated genes, including *flaC*, *pirA*, *pirB*, *tlh*, *tdh*, *trh*, *toxR*, *luxR*, *chiA*, *hlyA*, and *ctxA*, that were evaluated against the *Vibrio* isolates. In total, eight out of the 11 targeted virulence genes were found in the isolates.

According to the findings, 99% of the isolates included possessed at least one virulence gene, whereas 1% do not possessed any virulence genes. The highest number of *Vibrio* isolates harbored seven out of 11 virulence genes, including *pirA*, *pirB*, *tlh*, *flaC*, *toxR*, *chiA*, and *luxR*. In addition, the majority of the *Vibrio* isolates had four virulence genes

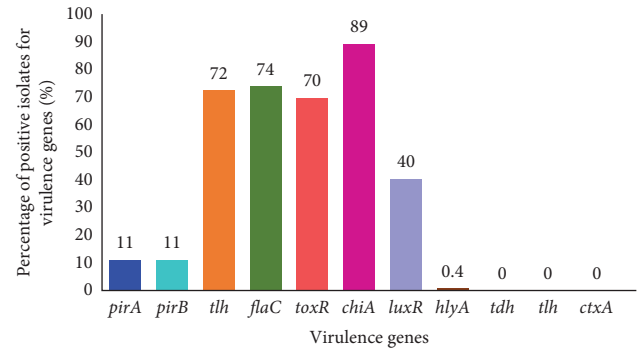

FIGURE 2: The distribution pattern of virulence genes detected in the isolates of *Vibrio* spp.

(44%), followed by 20% with three virulence genes, 16% with five virulence genes, 7% with six virulence genes, 7% with two virulence genes, and 4% with one virulence gene. Notably, no *Vibrio* isolates possessed virulence genes such as *tdh*, *trh*, or *ctxA* (Table S1).

From a total of eight virulence genes, *chiA* was the most virulence genes found from the isolates (89%), followed by *flaC* (74%), *tlh* (72%), *toxR* (70%), *luxR* (40%), *pirA* (11%), and *pirB* (11%). However, none of the isolates tested positive for *tdh*, *trh*, or *ctxA*. The findings also revealed that *hylA* was the least frequent of the virulence gene detected in this study (0.4%) (Figure 2).

Figure 3 illustrates the distribution of virulence genes for each of the *Vibrio* spp. The *chiA* gene was detected in all of the *Vibrio* spp., including *V. parahaemolyticus* (53%), *V. campbellii* (8%), *V. owensii* (6%), *V. rotiferianus* (5%), *V. communis* (5%), *V. alginolyticus* (3%), *Vibrio* spp. (3%), *V. natriegens* (2%), *V. brasiliensis* (2%), *V. xuii* (1%), *V. harveyi* (1%), and *V. hepatarius* (0.4%). However, none of the *chiA* gene was detected in *P. damselae* (0%). In addition, *flaC* was detected in 11 types of *Vibrio* spp., including *V. parahaemolyticus* (46%), *V. owensii* (5%), *V. rotiferianus* (5%), *V. campbellii* (4%), *Vibrio* spp. (4%), *V. communis* (2%), *V. alginolyticus* (2%), *V. natriegens* (2%), *V. brasiliensis* (1%), *V. xuii* (1%), and *V. harveyi* (1%), while *luxR* was detected in 10 types of *Vibrio* spp., including *V. parahaemolyticus* (12%), *V. owensii* (7%), *V. campbellii* (5%), *V. communis* (5%), *V. rotiferianus* (4%), *Vibrio* spp. (4%), *V. alginolyticus* (3%), *V. brasiliensis* (1%), *V. xuii* (1%), *V. harveyi* (1%), and *P. damselae* (1%).

However, none of the isolates tested positive for *tdh*, *trh*, or *ctxA*. The findings also revealed that *hlyA* was the least frequent of the virulence gene detected in *V. campbellii* (0.4%). The virulence genes of *pirA* were found in five *Vibrio* spp., including *V. campbellii* (4%), *V. parahaemolyticus* (2%), *V. alginolyticus* (1%), *V. rotiferianus* (1%), and *Vibrio* spp. (3%). Similar findings were made with *pirB*, which was revealed in *V. campbellii* (4%), *V. parahaemolyticus* (2%), *V. alginolyticus* (1%), *V. rotiferianus* (1%), and *Vibrio* spp. (3%). Other virulence genes, such as *toxR*, were found *in V. parahaemolyticus* (52%), *V. owensii* (4%), *V. communis* (4%), *V. rotiferianus* (2%), *V. campbellii* (2%), *V. alginolyticus* (1%), *V. brasiliensis* (1%), *Vibrio* spp. (2%),

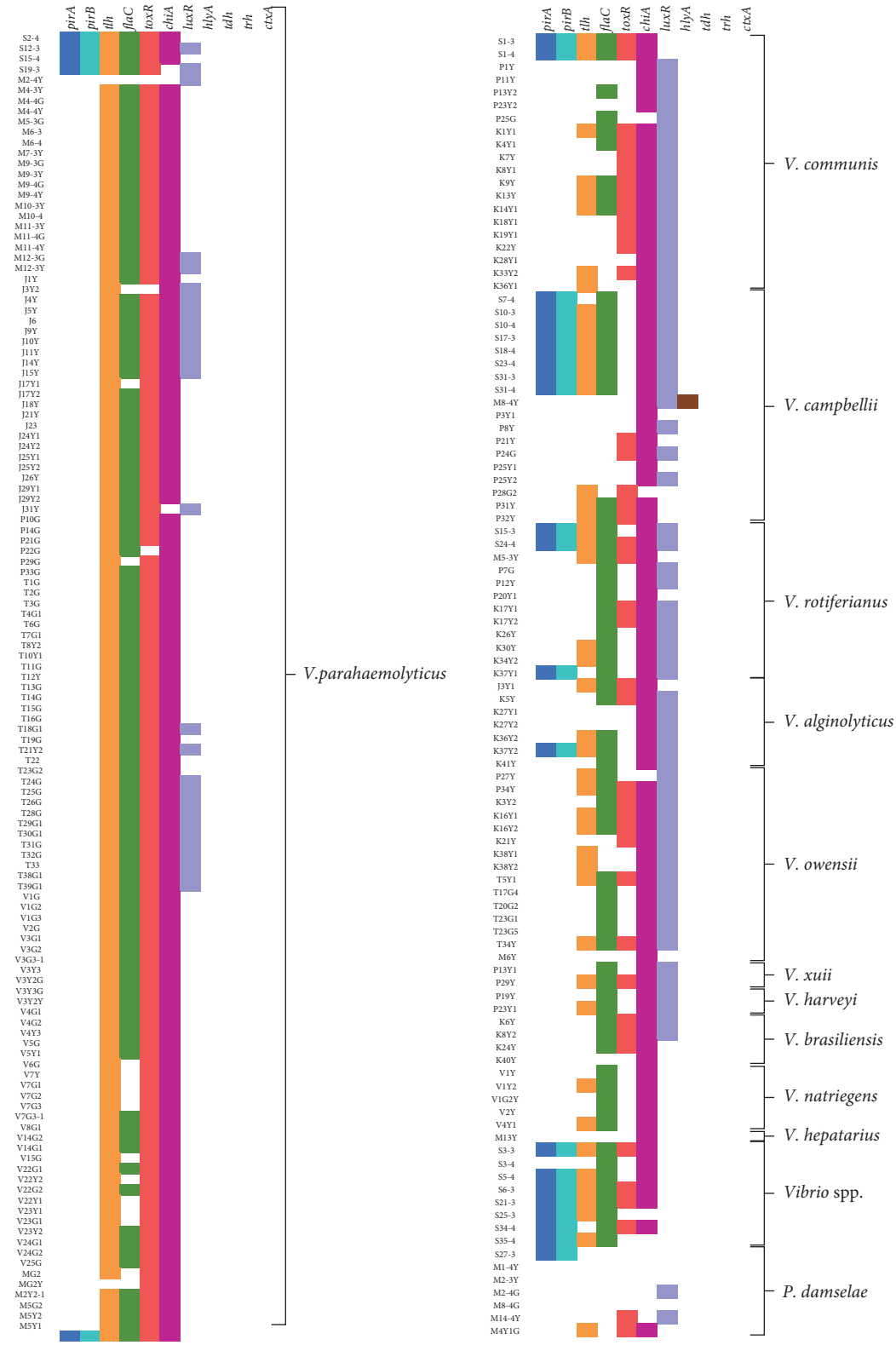

FIGURE 3: The distribution of virulence genes of *Vibrio* spp. Different colors represent different virulence genes study.

and *P. damselae* (1%). Furthermore, the *tlh* gene was found in *V. parahaemolyticus* (53%), *V. campbellii* (4%), *V. owensii* (4%), *V. rotiferianus* (2%), *V. communis* (2%), *V. alginolyticus* (1%), *V. natriegens* (1%), and *Vibrio* spp. (3%).

3.2. Pathogenicity Study. In pathogenicity study, three isolates were selected based on the number of highest virulent genes present in each isolate, as shown in Table S1. The isolates were *V. parahaemolyticus* S12-3, *V. campbellii* S10-

4, and *V. rotiferianus* S24-4. Since these isolates carried the highest number of virulence genes, therefore, these isolates were selected for the pathogenicity test. Table 2 compares the percentage of mortality from pathogenicity tests of *V. parahaemolyticus* S12-3, *V. campbellii* S10-4, and *V. rotiferianus* S24-4. The findings demonstrate that *V. parahaemolyticus* S12-3 isolate was the most virulent with LC_{50} 1×10^5 CFU/mL. On the other hand, the LC_{50} for *V. campbellii* S10-4 was 1×10^{13} CFU/mL and *V. rotiferianus* S24-4 was 1×10^{11} CFU/mL.

As shown in Table 2, at higher concentration of *V. parahaemolyticus* S12-3 (1×10^8 CFU/mL), the first mortality began as early as 12 hr (11%) and reached 50% of the mortality at 48 hr. The shrimp reached 100% mortality within 72 hr. In comparison to 1×10^8 CFU/mL, it takes a few hours for shrimp challenged with *V. parahaemolyticus* S12-3 to die at a concentration of 1×10^7 and 1×10^6 CFU/mL. The shrimp obtained a complete mortality (100%) at 108 hr when challenged with 1×10^7 CFU/mL and 132 hr when challenged with 1×10^6 CFU/mL. There was a significant difference ($p < 0.05$) from 0 to 108 hr when the shrimp were challenged with 1×10^7 CFU/mL of *V. parahaemolyticus* S12-3.

The shrimp also were challenged with *V. campbellii* S10-4. The findings revealed that mortality began within 72 hr but with a low percentage of mortality (2%) in the concentration of 1×10^8 CFU/mL, which was the highest concentration used in this experiment. Moreover, when shrimp were challenged with 1×10^8 CFU/mL of *V. campbellii* S10-4, 50% of the shrimp died within 168 hr (56%) ($p < 0.05$). However, at the concentration of 1×10^7 and 1×10^6 CFU/mL, the mortality begins at 96 hr with a mortality rate of 4% and 7%, respectively. At 168 hr, the mortality rate for 1×10^7 and 1×10^6 CFU/mL did not reach 50% of mortality but for a concentration of 1×10^8 CFU/mL, the mortality rate obtained was 56%. The results showed that there was a significant difference ($p < 0.05$) from 96 hr onward at the concentration of 1×10^7 CFU/mL. When the shrimp challenged with *V. rotiferianus* S24-4, the mortality begins within 72 hr for 1×10^8 (11%) and 1×10^7 CFU/mL (7%), respectively. However, the mortality for 1×10^6 CFU/mL begins at 84 hr (7%). When the PL of *P. monodon* were challenged with different concentrations of *V. rotiferianus* S24-4, 50% of the shrimp died within 120 hr at concentrations of 1×10^8 (60%) and 1×10^7 CFU/mL (56%) ($p < 0.05$). However, at the concentration of 1×10^6 CFU/mL, 50% of death occurred within 132 hr (53%). The experiment ended after 168 hr showing that the mortality rates for the three concentrations of *V. rotiferianus* S24-4 were higher than *V. campbellii* S10-4. As for a control group, no mortality was detected from the start through the end of the experiment.

Following daily monitoring, the shrimp between control and challenged groups were observed for the clinical signs. The clinical signs observed in moribund shrimp were similar to those observed in infected shrimp from farms. The common clinical signs include lethargy and abnormal swimming behavior. In addition, infected shrimp may show a decrease in feeding activity and a lack of interest in food, which contribute to decreased growth and weight gain (Figure 4(a)). This would give a negative impact on their overall health and performance. Aside from that, hemorrhaging was characterized by red or brown discoloration or appendages caused by bleeding beneath the exoskeleton, which can indicate a serious bacterial infection (Figure 4(b)). The affected area was red and swollen due to the inflammation. In addition, the exoskeleton also became soften and brittle, making the shrimp more vulnerable to damage and disease. The gross lesion displayed by the challenged shrimp includes an empty stomach, as well as white and atrophied hepatopancreas (Figure 4(c)) compared with the normal shrimp (unchallenged) with large pigmented hepatopancreas (Figure 4(d)). At the end of the experiment, reisolation of the bacteria indicated that the growth of the colony was the same as the colony isolated from the farms (Figure 4(e)–4(g)), and the *Vibrio* spp. were confirmed by PCR.

3.3. Histopathology Analysis. The histopathological features of hepatopancreas were analyzed at the end of the experiment. Histopathological examination of the normal shrimp hepatopancreas revealed no histopathological changes. In general, the hepatopancreatic tubules were intact, with relatively uniform size and a large central lumina. The tubules were lined by viable and healthy B and R cells, whereby the B cells showed a single large vacuole, while the R cells showed numerous small intracytoplasmic vacuoles (Figure 5(a)). In contrast, few abnormalities were noted in the hepatopancreas of PL infected with pathogenic *Vibrio* spp. The hepatopancreatic tubules were of various sizes and usually distant from one another. The lumens were usually small, largely incriminated to hepatopancreatic epithelial changes. Most of the B and R cells lacked intracytoplasmic vacuoles. Instead, their cytoplasm showed intracytoplasmic eosinophilia, while the nuclei were round, basophilic, and centrally located. Stacking of these epithelial cells was observed, accompanied by elongation, leading to prominence that partially occluded the hepatopancreatic tubular lumen (Figure 5(b)).

Other changes included the sloughing of epithelial cells (Figure 5(c)) and enlarged of nuclei (Figure 5(d)), leading to observation of individual cells or cellular debris in the lumen of the hepatopancreas. Severe and a more advanced histopathological changes included structural loss, largely due to severe necrosis of the epithelial cells, as well as rupture of the hepatopancreatic tubules. Regardless of the lesion severity involving the hepatopancreatic tubules, hemolytic infiltrates were seen in moderate to high number (Figure 5(e)). Moreover, some hepatopancreatic tubules showed presence of epithelial cells that exhibited vacuolation resulting from inflammatory reaction (Figure 5(f)).

4. Discussion

Vibriosis is a major disease problem in shrimp farming resulting in high mortality and significant economic losses in all producing countries [29]. *Vibrio* spp. are one of the most important bacterial pathogens of cultured shrimp, causing a variety number of diseases and up to 100%

Table 2: Percentage of mortality observed from the pathogenicity test after 168 hr.

Vibrio spp.	Concentration (CFU/mL)	Percentage of mortality at specific time (hr)															LC_{50} (CFU/mL)
		0	12	24	36	48	60	72	84	96	108	120	132	144	156	168	
V. parahaemolyticus S12-3	1×10^8	0^{d}	11^{cd}	20^{c}	47^{b}	89^{a}	98^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	1×10^5
	1×10^7	0^{g}	0^{g}	13^{fg}	20^{f}	38^{e}	60^{d}	71^{cd}	84^{bc}	91^{ab}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	
	1×10^6	0^{g}	0^{g}	9^{fg}	18^{ef}	27^{de}	38^{d}	62^{c}	67^{c}	82^{b}	89^{b}	91^{b}	100^{a}	100^{a}	100^{a}	100^{a}	
V. campbellii S10-4	1×10^8	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	2^{e}	7^{e}	16^{e}	18^{d}	22^{Ac}	31^{b}	44^{a}	44^{a}	56^{a}	1×10^{13}
	1×10^7	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	4^{e}	13^{de}	22^{cd}	24^{bcd}	31^{bc}	38^{ab}	47^{a}	
	1×10^6	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	7^{cd}	7^{cd}	9^{cd}	16^{bc}	16^{bc}	27^{b}	42^{a}	
V. rotiferianus S24-4	1×10^8	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	0^{e}	11^{de}	13^{de}	22^{cde}	47^{cd}	60^{bc}	80^{ab}	87^{ab}	87^{a}	93^{a}	1×10^{11}
	1×10^7	0^{f}	0^{f}	0^{f}	0^{f}	0^{f}	0^{f}	7^{f}	9^{f}	24^{e}	40^{d}	56^{c}	73^{b}	80^{ab}	82^{ab}	89^{a}	
	1×10^6	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	7^{d}	7^{cd}	18^{cd}	31^{bcd}	53^{bc}	71^{ab}	76^{ab}	76^{a}	

Note: Mean that does not share the same letter shows a significant difference ($p<0.05$).

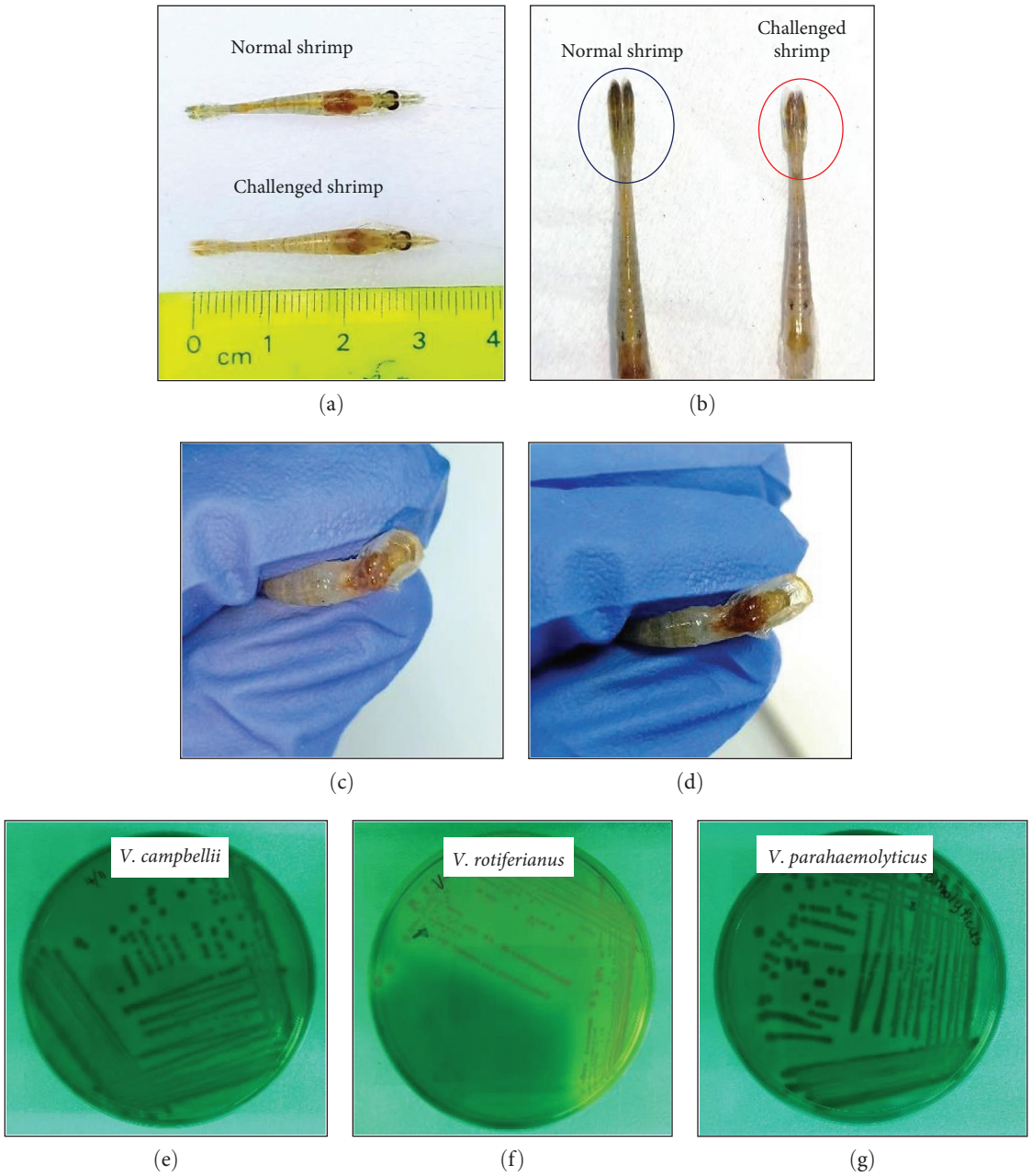

FIGURE 4: Clinical signs in *P. monodon* resulted from immersion challenge test of three *Vibrio* spp: (a) comparison between normal and challenged shrimp, (b) normal shrimp showed black uropod (blue circle), while challenged shrimp showed reddening in uropod (red circle), (c) infected hepatopancreas after challenged, (d) normal hepatopancreas after challenged, (e) the growth of *V. campbellii* on TCS agar after reisolation, (f) the growth of *V. rotiferianus* on TCS agar after reisolation, and (g) the growth of *V. parahaemolyticus* on TCS agar after reisolation. All the isolates were confirmed by PCR.

mortality rates [30]. The most pathogenic *Vibrio* spp. found in shrimp, including *V. harveyi*, *V. penaeicida*, *V. parahaemolyticus*, and *V. vulnificus*, which possessed various important virulence factors [6, 31]. Therefore, identification of virulence factors is essential for evaluating bacterial pathogenicity because these factors allow bacteria to infect and damage hosts [32]. In this study, we demonstrated the presence of virulence genes associated with pathogenic strains that may contribute to the pathogenicity of *Vibrio* spp., which were previously isolated from cultured shrimp in Peninsular Malaysia.

The main factor in *Vibrio* pathogenicity is virulence factors, which are expressed by virulence genes [10]. *Vibrio* spp. infect and damage the host through these five elements of virulence factors such as capsular polysaccharide, adhesion factor, cytotoxin, lipopolysaccharide, and flagellum [9]. Then, they allow pathogens to adhere, enter, establish, and multiply while evading the host's immune defense system [11]. Furthermore, *Vibrio* can acquire atypical virulence genes, which can further increase its pathogenicity [33]. It has been discovered that these atypical virulence genes can be acquired from the environment or from other bacteria

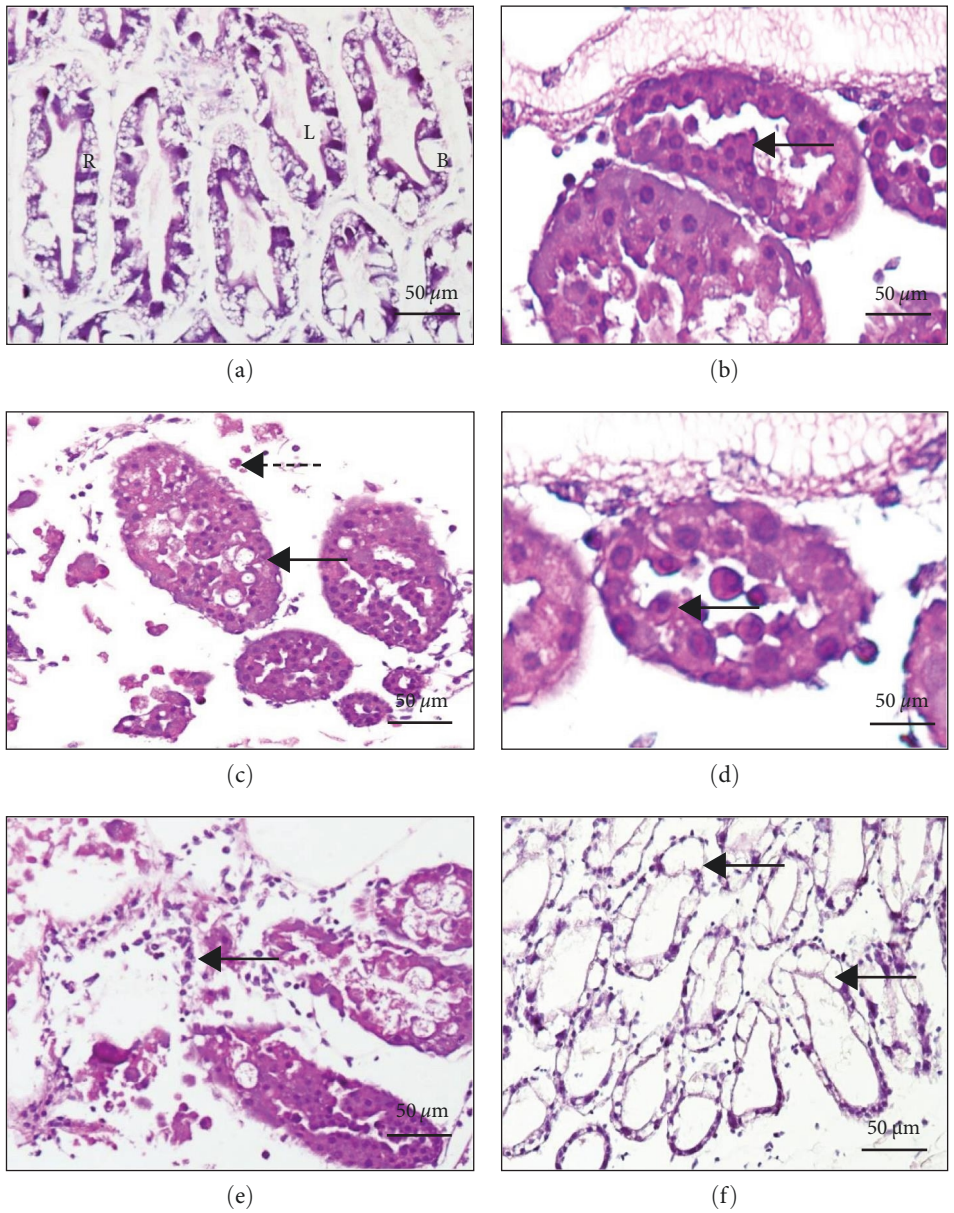

FIGURE 5: Transversal view of hepatopancreas sections of non-infected (a) and infected (b–f) shrimp (H&E). (a) Normal appearance of hepatopancreas tubules observed in unchallenged shrimp. The tubules are lined by viable R and B cells, while the lumen is large and clear (L). (H&E, bar = 50 μm). (b) Tubular epithelium lacked intracytoplasmic vacuolation, exhibiting intense eosinophilic cytoplasm. Note that the epithelial cells are elongated, producing prominence into the lumen (black arrow) (H&E, bar = 50 μm). (c) Necrosis (dash arrow) and sloughing of tubular epithelial cells (black arrow), leading to presence of cellular debris in the partially occluded tubular lumen. The number of vacuoles in the affected R and B cells are generally low (H&E, bar = 50 μm). (d) Observation of sloughed tubular epithelial cells in lumen of hepatopancreatic tubules and enlargement of nucleus (karyomegaly) (black arrow) (H&E, bar = 50 μm). (e) Infiltration of hemocytes infiltration (black arrow) in the interstitial space (H&E, bar = 50 μm). (f) Massive vacuolation (black arrow) (H&E, bar = 50 μm). HP, hepatopancreas.

through horizontal gene transfer (HGT) [34]. In addition, other factors such as global climate change, antibiotics, heavy metals, and environmental eutrophication might affect bacteria stress response mechanism, which could induce mutagenesis and HGT, and ultimately increasing their pathogenicity and antibiotic resistance [35–37]. The observation through the gross lesion also serves as a sign of the pathogen's virulence. More virulent pathogens, for example, may create larger and more severe lesions, whereas less virulent pathogens may cause smaller and less severe lesions [38]. As a result, it is essential for comprehending disease mechanisms and developing disease control.

In this study, *chiA* was found to be the most prevalent gene among the isolates (89%). In addition, *chiA* was widely distributed in *V. parahaemolyticus* compared to another *Vibrio* spp. The result was in concordance with the findings reported by Mohamad et al. [39] where all 63 isolates of *Harveyi* clade, including *V. parahaemolyticus*, *V. alginolyticus*,

V. harveyi, and *V. campbellii*, possessed virulence gene of *chiA* (100%). Another study by Ruwandeepika et al. [17] also revealed that 48 isolates of *Harveyi* clades, including 47 isolates of *V. harveyi* and one isolate of *V. campbellii* from diverse range of hosts and geographical locations, were found to carry *chiA* gene. The *chiA* gene is associated with the production of chitinases, which are the enzymes capable of degrading chitin. Chitin is a substrate that can be found abundantly in water, and the presence of *chiA* gene in *Harveyi* clades suggests that these bacteria may have the ability to degrade chitin. The ability to utilize chitin could promote pathogen's survivability in nutrient-scarce environment, while contributing to their pathogenicity by altering host's immune response [40, 41].

Flagella play an important role in infecting host because it increases the motility of bacteria for colonization or act as adhesive [42]. Other than *chiA*, this study revealed that *flaC* also possessed a high number of percentage (74%) where it was dominantly found in *V. parahaemolyticus*. On the contrary, a study conducted by Bai et al. [18] revealed that most of the *flaC* was detected in *V. harveyi*. However, the *flaC* was also detected in various *Vibrio* spp. such as *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. campbellii*, *V. fischeri*, *V. fluvialis*, *V. mimicus*, and *V. natriegens*, aligning with the *Vibrio* spp. identified in our study. In addition, another study discovered that 68% of the *Vibrio* isolates possessed for *flaC* [39]. Other virulence gene such as thermolabile hemolysin gene, *tlh*, has previously been used to identify *V. parahaemolyticus* [22]. However, it has been reported that *flaC* not only found in *V. parahaemolyticus* but also could be found in *V. alginolyticus*, *V. harveyi*, *V. fischeri*, *V. mimicus*, *V. natriegens*, *V. proteolyticus*, *V. anguillarum*, and *V. vulnificus* [43]. In this study, 72% of the isolates possessed for *tlh*, including *V. parahaemolyticus*, *V. alginolyticus*, *V. campbellii*, *V. rotiferianus*, *V. communis*, *V. owensii*, *V. brasiliensis*, and *Vibrio* spp. The findings were supported by Amatul-Samahah et al. [44] demonstrating that all of *Vibrio* strains were positive for *tlh* (100%). Furthermore, Mohamad et al. [39] revealed that more than 50% of the *Vibrio* isolates possessed *tlh*. In addition, a study by Joshi et al. [45] also reported to be positive for *tlh* on *V. parahaemolyticus*.

The *toxR* is a transmembrane transcription regulator that regulates the expression of virulence gene in *V. cholerae* [12]. However, recent studies found that *toxR* is present in other vibrios, including *V. parahaemolyticus*, *V. fischeri*, *V. vulnificus*, *V. alginolyticus*, *V. hollisae*, *V. mimicus*, *V. fluvialis*, *V. harveyi*, *V. anguillarum*, and *Photobacterium* spp., which aligned with this study where 70% of the *Vibrio* isolates possessed *toxR* gene [17]. In addition, according to Asgarpoor et al. [46], 12 out of 70 shrimp (17.1%) were possessed for *toxR* gene. Furthermore, the occurrence of positive *toxR* in *V. parahaemolyticus* gene was approximately in line with findings identified by Abd-Elghany and Sallam [47] who discovered that 14% of shrimp samples were positive for *toxR*. Moreover, a study by Hassan et al. [48] found that 19% (38/200) of retailed shellfish samples were possessed for *toxR* gene.

According to the study's findings, 11% of the isolates possessed *pirA* and *pirB*, respectively. The AHPND-causing strain is an opportunistic marine shrimp pathogen that harbors *pirA* and *pirB* genes [49]. Originally, *pirA* and *pirB* are found in *Photorhabdus luminescens* bacteria and responsible for encoding *pirAB* toxin, which indicates the insecticidal activity against *Spodoptera exigua* larvae [50]. The *pirA* and *pirB* genes are located on the self-transmissible plasmid of AHPND causing *V. parahaemolyticus* strains [51]. A study conducted by Chonsin et al. [52] showed that 10.84% (9/83) of *V. parahaemolyticus* isolates were positive for *pirA* and *pirB*. Similarly, there is also a positive finding for *pirA* and *pirB* genes of *V. parahaemolyticus* isolates in 9.9% shrimp and 0.7% molluskan shellfish have been reported from Mekong Delta in Vietnam [53]. Contrary to the expectation, our findings showed that *V. campbellii* was dominantly possessed *pirA* and *pirB* than *V. parahaemolyticus* from the prior study. There are several factors that may contribute to the contradictory results, including species variations, regional variation, and the level of the virulence [54]. However, a study by Dong et al. [55] demonstrated the presence of *pirA* and *pirB* found in *V. campbellii*, which is consistent with our findings.

The virulence of several pathogenic bacteria has also been associated with quorum sensing, a form of bacterial cell-to-cell communication involving small signaling molecules [56]. Therefore, *luxR* is important as key regulator of virulence in pathogenic bacteria. It regulates gene expression in response to other bacteria's signals, allowing for coordinated virulence and immune evasion. Thus, *luxR* plays an important role in bacterial pathogenicity by regulating the expression of genes involved in infection and immune evasion [54]. A study by Ruwandeepika et al. [17] discovered that 48 isolates from the *Harveyi* clade carried *luxR* gene. In addition, all 63 isolates of *Harveyi* clades from Mohamad et al. [39] possessed typical virulence genes of *luxR* (100%). Both studies showed a high percentage of *luxR*. In contrast, our finding revealed a low percentage of the isolates that possessed virulence gene of *luxR* (40%) compared to the previous study. Our findings also were in close agreement reported by Deng et al. [33] where only 12.6% of the *Vibrio* strain possessed for *hlyA* gene, which among the lowest of virulence gene found in their study.

However, none of the isolates were detected for the presence of *tdh*, *trh*, and *ctxA* genes. The *tdh* and *trh* genes are vital for the virulency of pathogen, as these genes encode the pore-forming proteins involved in the invasiveness of pathogen [44]. These genes were not detected in this study, most likely because these isolates were isolated from an environmental source as reported by Xie et al. [57]. This implies that there is no association between these genes and pathogenic *Vibrio* in humans, indicating a low potential risk for human health. Our findings appear to be well supported by previous studies. A finding by Chonsin et al. [52] showed that none of the tested samples contained *tdh* and *trh* genes. Similarly, Joshi et al. [45] reported that none of their *Vibrio* isolates carrying both *tdh* and *trh* genes. A study by Guardiola-Avila et al. [7] did not discover the *ctxA* gene, which is consistent with our findings. In addition, several other researchers have also discovered that this gene may not always present in the

environment [58]. Therefore, it is essential to emphasize that the strains isolated in our study, even without the *ctxA* gene, could pose a significant health risk to the population, as they are capable of causing potentially life-threatening diarrheal diseases [59].

The virulence factors may be essential for infecting toward different hosts, as *Vibrio* spp. have the capacity to infect a broad spectrum of aquatic organisms [32, 60]. While the relationship between the number of virulence genes and pathogenicity in *Vibrio* is complicated, the acquisition of atypical virulence genes may enhance the ability of the bacteria to infect specific hosts [33]. The virulency of the isolates was further assessed by conducting challenge tests with PL of *P. monodon* to determine the pathogenicity of the *Vibrio* isolates. Despite the presence of typical virulence genes in all isolates, there was considerable variation in their virulence levels. In addition, some of the isolates causing high mortality did not have any atypical virulence genes, while others causing low mortality have atypical virulence genes [17].

In the pathogenicity test, three *Vibrio* spp., including *V. parahaemolyticus* S12-3, *V. campbellii* S10-4, and *V. rotiferianus* S24-4, were selected due to the highest number of virulence genes possessed in each of the isolates. The findings suggest that virulence genes are transferred among *Vibrio* spp. and that acquiring virulence genes would increase the virulency of the *Vibrio* to hosts [33]. In aquatic environments, *Vibrio* spp. are a significant reservoir of potential virulence and bacterial DNA material exchange through HGT [61].

According to the findings, when the shrimp were challenged with *V. parahaemolyticus* S12-3, it resulted in 100% of mortality within 72 hr at 1×10^8 CFU/mL. The results obtained were similar with a study by Thakur et al. [62] in which the shrimp were challenged with *V. parahaemolyticus* that showed 100% of mortality within 72 hr. When the shrimp were challenged with *V. rotiferianus* S24-4 and *V. campbellii* S10-4 with the same concentration of 1×10^8 CFU/mL, the percent mortality observed was 11% and 2%, respectively. In comparison to Thakur et al. [62], *V. parahaemolyticus* isolated from *P. monodon* in India's West Coast had a greater LC_{50} of 5.99×10^5 CFU/mL as opposed to 1×10^5 CFU/mL in our findings. On the other hand, *V. campbellii* S10-4 in the present study had a higher LC_{50} of 1×10^{13} CFU/mL in contrast to *V. campbellii* strain as reported by Nurhafizah et al. [63] which had LC_{50} of 6.0×10^8 CFU/mL. The *V. parahaemolyticus* S12-3 has a high mortality rate and can cause death in a short time. In addition, *V. parahaemolyticus* S12-3 has the lowest LC_{50} compared to *V. rotiferianus* S24-4 and *V. campbellii* S10-4. Therefore, this suggest that the presence of virulence genes plays an important role in the pathogenicity of *Vibrio* spp. [33].

The LC_{50} values may differ due to the virulence properties of the two *Vibrio* spp., as well as the state and condition of the experimental shrimp employed. Wang et al. [64] stated that factors such as bacterial concentration, exposure period, and infection method were identified as contributing to mortality. As for example, a study conducted by Joshi et al. [45] revealed that by performed immersion challenge on shrimp with *V. parahaemolyticus* at the concentration of 10^8 CFU/mL led to 100% of mortality within 24 hr. However, our study, employing the same method, concentration, and species, demonstrated 100% of mortality at 72 hr, indicating a longer duration for mortality compared to findings by Joshi et al. [45]. Another study by Le Hong et al. [65] revealed that when the shrimp challenged with *V. campbellii* at the concentration of 10^8 CFU/mL, they exhibited mortality less than 20% at 48 hr. In contrast, our findings observed a lower mortality rate (2%) at 72 hr.

The findings revealed that although each of the isolates possessed the same number of the virulence factors (7/11), there were variations in their virulence level. The differences of pathogenicity were depended on species tested and on the strain characteristics. Moreover, the study observed that mortality resulting from challenge test depended on the doses and species [64]. In addition, global climate change, antibiotics, heavy metals, and nutrient pollutants have been reported to induce the expression of virulence genes and increase the pathogenicity [66]. According to Guijarro et al. [67], *V. coralliilyticus* exhibits 16-fold elevation in virulence factors related to motility, host degradation, secretion, resistance to antimicrobials, and transcriptional regulation when the temperature increased from 24 to 27°C.

During challenge test, the affected shrimp can be identified by observing both physical and behavior of shrimp. On a regular basis, there was a decrease in shrimp appetite, empty gut, and a lot of leftover feed on the basis of maintenance. Furthermore, the larvae looked weak and sluggish with the head constantly facing up and uncontrolled movements. Moreover, the shrimp tail was necrotizing, melanosis on the skin, the body of the shrimp looked pale while swimming legs, and telson and uropod were become reddish [68]. The symptoms were comparable in our study where the symptom can be observed as early as 12 hr after being challenged with *V. parahaemolyticus* S12-3. Similar clinical signs have been recorded in shrimp with vibriosis when challenged with *V. parahaemolyticus* at the concentration of 1×10^6 CFU/mL [69].

Histopathology of the hepatopancreas revealed that the hepatopancreas was in various stages of degeneration after immersion challenge test. The findings were similar to the findings by Tran et al. [24] and Rowley and Pope [70]. Based on histopathology analysis, the hepatopancreatic tubular epithelial cells have been changed and extended toward the lumen, resulting in cellular desquamation. Furthermore, there was a sloughing of hepatopancreatic tubular epithelial cells, hemocytic infiltration, and disorganized structure of hepatopancreas tubule that were characterized by massive vacuolation due to inflammatory response [71, 72]. Conversely, normal hepatopancreas showed a complete structure of hepatopancreatic tubular cells similarly with a study conducted by Nurhafizah et al. [63].

5. Conclusion

In conclusion, there were presence of multiple virulence genes in *V. parahaemolyticus*, *V. rotiferianus*, and *V. campbellii*. From the pathogenicity test, it was found

that *V. parahaemolyticus* S12-3 was the most virulent with LC_{50} 1×10^5 CFU/mL. The study was supported by histopathological findings, which were typical after *Vibrio* spp. infection. The results from this study would provide valuable insights on *Vibrio* pathogenesis and will contribute in the development of vaccines, probiotics, immunostimulants, and ecological control systems in the aquaculture industry. Therefore, this study warrants a continuous long-term monitoring of the pathogens of shrimp, such as *Vibrio* spp., since its outbreak may have devastating impact on the Malaysian shrimp export industry in particular.

Data Availability

All other data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

All procedures involving shrimp in this study were approved by the Institutional Animal Care and Use Committee, Universiti Putra Malaysia (AUP No: UPM/IACUC/AUP-R061/2022).

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Wan Omar Haifa-Haryani performed the experiment at the sampling sites and laboratory, data analysis, and contributed in manuscript writing; Mohamad Azzam-Sayuti performed data analysis and reviewed the manuscript writing; Md. Ali Amatul-Samahah and Yong Kit Chin contributed in manuscript writing; Mohd Zamri-Saad, I. Natrah, and Mohammad Noor Azmai Amal reviewed the manuscript; Salleh Annas observed the H&E slides and reviewed the manuscript; and Md Yasin Ina-Salwany conceived the idea, designed the experiments, and reviewed the manuscript. All authors share in writing this manuscript and revising it.

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Supplementary Materials

Table S1: list of virulence genes possessed by each of the *Vibrio* spp. (*Supplementary Materials*)

References

[1] FAO, *FAO Yearbook. Fishery and Aquaculture Statistics-2011*, p. 76, Food and Agriculture Organization of the United Nations, Rome, 2013.

[2] FAO, *State of Fisheries and Aquaculture in the World*, Food and Agriculture Organization of the United Nations, Rome, Italy, 2019.

[3] Y. Yano, K. Hamano, M. Satomi, I. Tsutsui, M. Ban, and D. Aue-Umneoy, "Prevalence and antimicrobial susceptibility of *Vibrio* species related to food safety isolated from shrimp cultured at inland ponds in Thailand," *Food Control*, vol. 38, pp. 30–36, 2014.

[4] X. Xu, J. Cheng, Q. Wu, J. Zhang, and T. Xie, "Prevalence, characterization, and antibiotic susceptibility of *Vibrio parahaemolyticus* isolated from retail aquatic products in North China," *BMC Microbiology*, vol. 16, no. 1, pp. 1–9, 2016.

[5] B. K. Kumar, V. K. Deekshit, J. R. M. Raj et al., "Diversity of *Vibrio parahaemolyticus* associated with disease outbreak among cultured *Litopenaeus vannamei* (Pacific white shrimp) in India," *Aquaculture*, vol. 433, pp. 247–251, 2014.

[6] T. Kumaran and T. Citarasu, "Isolation and Characterization of *Vibrio* Species from shrimp and artemia culture and evaluation of the potential virulence factor," *Intellectual Property Rights: Open Access*, vol. 4, 2016.

[7] I. Guardiola-Avila, V. Martínez-Vázquez, R. Requena-Castro et al., "Isolation and identification of *Vibrio* species in the Rio Bravo/Grande and water bodies from Reynosa, Tamaulipas," *Letters in Applied Microbiology*, vol. 67, no. 2, pp. 190–196, 2018.

[8] M. Choi, A. M. Stevens, S. A. Smith, D. P. Taylor, and D. D. Kuhn, "Strain and dose infectivity of *Vibrio parahaemolyticus*: the causative agent of early mortality syndrome in shrimp," *Aquaculture Research*, vol. 48, no. 7, pp. 3719–3727, 2017.

[9] D. Ceccarelli, N. A. Hasan, A. Huq, and R. R. Colwell, "Distribution and dynamics of epidemic and pandemic *Vibrio parahaemolyticus* virulence factors," *Frontiers in Cellular and Infection Microbiology*, vol. 3, Article ID 97, 2013.

[10] M. Schroeder, B. D. Brooks, and A. E. Brooks, "The complex relationship between virulence and antibiotic resistance," *Genes*, vol. 8, no. 1, Article ID 39, 2017.

[11] H. A. Darshanee Ruwandeepika, T. Sanjeewa Prasad Jayaweera, P. Paban Bhowmick, I. Karunasagar, P. Bossier, and T. Defoirdt, "Pathogenesis, virulence factors and virulence regulation of vibrios belonging to the Harveyi clade," *Reviews in Aquaculture*, vol. 4, no. 2, pp. 59–74, 2012.

[12] P. Sarkar, P. K. Issac, S. V. Raju, P. Elumalai, A. Arshad, and J. Arockiaraj, "Pathogenic bacterial toxins and virulence influences in cultivable fish," *Aquaculture Research*, vol. 52, no. 6, pp. 2361–2376, 2021.

[13] N. Mohamad, M. N. A. Amal, I. S. M. Yasin et al., "Vibriosis in cultured marine fishes: a review," *Aquaculture*, vol. 512, Article ID 734289, 2019.

[14] F. G. D. Menezes, M. T. Rodriguez, F. C. D. Carvalho et al., "Pathogenic *Vibrio* species isolated from estuarine environments (Ceará, Brazil)-antimicrobial resistance and virulence potential profiles," *Anais da Academia Brasileira de Ciências*, vol. 89, pp. 1175–1188, 2017.

[15] W. O. Haifa-Haryani, M. A. Amatul-Samahah, M. Azzam-Sayuti et al., "Antibiotics Resistance and Plasmid Profiling of

Vibrio spp. Isolated from Cultured Shrimp in Peninsular Malaysia," *Microorganisms*, vol. 10, no. 9, pp. 1–23, 2022.

[16] J. E. Han, K. F. J. Tang, L. H. Tran, and D. V. Lightner, "Photohabdus insect-related (Pir) toxin-like genes in a plasmid of *V. parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp," *Disease of Aquatic Organisms*, vol. 113, pp. 33–40, 2015.

[17] H. A. Ruwandeepika, T. Defoirdt, P. P. Bhowmick, M. Shekar, P. Bossier, and I. Karunasagar, "Presence of typical and atypical virulence genes in *vibrio* isolates belonging to the *Harveyi* clade," *Journal of Applied Microbiology*, vol. 109, no. 3, pp. 888–899, 2010.

[18] F. Bai, L. Pang, Z. Qi, J. Chen, B. Austin, and X. H. Zhang, "Distribution of five *vibrio* virulence-related genes among *Vibrio harveyi* isolates," *Journal of General and Applied Microbiology*, vol. 54, no. 1, pp. 71–78, 2008.

[19] V. Saravanan, H. Sanath Kumar, I. Karunasagar, and I. Karunasagar, "Putative virulence genes of *Vibrio cholerae* from seafoods and the coastal environment of Southwest India," *International Journal of Food Microbiology*, vol. 119, no. 3, pp. 329–333, 2007.

[20] V. Letchumanan, P. Pusparajah, L. T. H. Tan, W. F. Yin, L. H. Lee, and K. G. Chan, "Occurrence and antibiotic resistance of *Vibrio parahaemolyticus* from shellfish in Selangor, Malaysia," *Frontiers in Microbiology*, vol. 6, Article ID 1417, 2015.

[21] L. A. Sechi, I. Duprè, and A. Deriu, "Distribution of *V. cholerae* virulence genes among different *Vibrio* species isolated in Sardinia, Italy," *Journal of Applied Microbiology*, vol. 88, no. 3, pp. 475–481, 2000.

[22] A. K. Bej, D. P. Patterson, C. W. Brasher, M. C. L. Vickery, D. D. Jones, and C. A. Kaysner, "Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tlh*, *tdh* and *trh*," *Journal of Microbiological Methods*, vol. 36, no. 3, pp. 215–225, 1999.

[23] I. Karunasagar, R. Pai, G. R. Malathi, and I. Karunasagar, "Mass mortality of Penaeus monodon larvae due to antibiotic-resistant *Vibrio harveyi*," *Aquaculture*, vol. 128, no. 3-4, pp. 203–209, 1994.

[24] L. Tran, L. Nunan, R. M. Redman et al., "Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp," *Diseases of Aquatic Organisms*, vol. 105, pp. 45–55, 2013.

[25] C. Hernández-Cabanyero, E. Carrascosa, S. Jiménez, and B. Fouz, "Exploring the effect of functional diets containing phytobiotic compounds in whiteleg shrimp health: resistance to acute hepatopancreatic necrotic disease caused by *Vibrio parahaemolyticus*," *Animals*, vol. 13, no. 8, Article ID 1354, 2023.

[26] H. Cabanillas-Beltran, E. LLausas-Magaña, R. Romero et al., "Outbreak of gastroenteritis caused by the pandemic *Vibrio parahaemolyticus* O3: K6 in Mexico," *Fems Microbiology Letters*, vol. 265, no. 1, pp. 76–80, 2006.

[27] K. Kumar, K. P. Prasad, G. Tripathi et al., "Isolation, identification, and pathogenicity of a virulent *Aeromonas jandaei* associated with mortality of farmed *Pangasianodon hypophthalmus*, in India," *Israeli Journal of Aquaculture*, vol. 67, pp. 1–7, 2015.

[28] S. Muthukrishnan, T. Defoirdt, M. Y. Ina-Salwany et al., "Vibrio parahaemolyticus and Vibrio harveyi causing Acute Hepatopancreatic Necrosis Disease (AHPND) in Penaeus vannamei (Boone, 1931) isolated from Malaysian shrimp ponds," *Aquaculture*, vol. 511, Article ID 734227, 2019.

[29] M. S. Morales-Covarrubias, J. Cuéllar-Anjel, A. Varela-Mejías, and C. ElizondoOvares, "Shrimp bacterial infections in Latin America: a review," *Asian Fisheries Science*, vol. 31S, pp. 76–87, 2018.

[30] F. Khamesipour, E. Noshadi, M. Moradi, and M. Raissy, "Detection of *Vibrio* spp. in shrimp from aquaculture sites in Iran using polymerase chain reaction (PCR)," *Aquaculture, Aquarium, Conservation & Legislation International Journal of the Bioflux Society*, vol. 7, pp. 1–7, 2014.

[31] V. Letchumanan, K. G. Chan, and L. H. Lee, "*Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques," *Frontiers in Microbiology*, vol. 5, Article ID 705, 2014.

[32] M. Y. Ina-Salwany, N. Al-saari, A. Mohamad et al., "Vibriosis in fish: a review on disease development and prevention," *Journal of Aquatic Animal Health*, vol. 31, no. 1, pp. 3–22, 2019.

[33] Y. Deng, L. Xu, H. Chen et al., "Prevalence, virulence genes, and antimicrobial resistance of *vibrio* species isolated from diseased marine fish in South China," *Scientific Reports*, vol. 10, no. 1, Article ID 14329, 2020.

[34] S. M. Soucy, J. Huang, and J. P. Gogarten, "Horizontal gene transfer: building the web of life," *Nature Reviews Genetics*, vol. 16, no. 8, pp. 472–482, 2015.

[35] M. Sun, M. Ye, J. Wu et al., "Positive relationship detected between soil bioaccessible organic pollutants and antibiotic resistance genes at dairy farms in Nanjing, Eastern China," *Environmental Pollution*, vol. 206, pp. 421–428, 2015.

[36] H. W. Hu, J. T. Wang, J. Li et al., "Field-based evidence for copper contamination induced changes of antibiotic resistance in agricultural soils," *Environmental Microbiology*, vol. 18, no. 11, pp. 3896–3909, 2016.

[37] J. E. M. Watts, H. J. Schreier, L. Lanska, and M. S. Hale, "The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions," *Marine Drugs*, vol. 15, no. 6, Article ID 158, 2017.

[38] M. H. Kogut and S. E. LaPatra, "Gross lesion assessment as a tool for evaluating virulence in fish pathogens," *Reviews in Aquaculture*, vol. 1, no. 2, pp. 71–79, 2009.

[39] N. Mohamad, M. N. A. Amal, M. Z. Saad et al., "Virulence-associated genes and antibiotic resistance patterns of *Vibrio* spp. isolated from cultured marine fishes in Malaysia," *BMC Veterinary Research*, vol. 15, Article ID 176, 2019.

[40] A. S. Alqahtani, O. O. Lee, and W. Chen, "The *chiA* gene, a chitinase gene, in Photobacterium damselae subsp. piscicida and its potential role in pathogenesis," *Fish & Shellfish Immunology*, vol. 60, pp. 7–13, 2017.

[41] D. Zhang, D.-H. Xu, J. Qiu, C. R. Rasmussen-Ivey, M. R. Liles, and B. H. Beck, "Chitin degradation and utilization by virulent *Aeromonas hydrophila* strain ML10-51K," *Archives of Microbiology*, vol. 199, no. 4, pp. 573–579, 2017.

[42] Q. Chen, Q. Yan, K. Wang, Z. Zhuang, and X. Wang, "Portal of entry for pathogenic *Vibrio alginolyticus* into large yellow croaker *Pseudosciaena crocea*, and characteristics of bacterial adhesion to mucus," *Diseases of Aquatic Organisms*, vol. 80, no. 3, pp. 181–188, 2008.

[43] X. H. Wang, X. H. Zhang, Y. B. Zhong, B. G. Sun, and J. X. Chen, "Genes encoding the *V. harveyi* haemolysin (*vhh*)/thermolabile haemolysin (*tlh*) are widespread in vibrios," *Acta Microbiologica Sinica*, vol. 47, pp. 874–881, 2007.

[44] M. A. Amatul-Samahah, S. Muthukrishnan, W. H. H. W. Omar, N. F. M. Ikhsan, and M. Y. Ina-Salwany, "*Vibrio* sp. associated with acute hepatopancreatic necrosis disease (AHPND) found in penaeid shrimp pond from east cost of Peninsular Malaysia," *Journal of Environmental Biology*, vol. 41, pp. 1160–1170, 2020.

[45] J. Joshi, J. Srisala, and V. H. Truong, "Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND)," *Aquaculture*, vol. 428-429, pp. 297–302, 2014.

[46] D. Asgarpoor, F. Haghi, and H. Zeighami, "Detection and molecular characterization of *Vibrio Parahaemolyticus* in shrimp samples," *The Open Biotechnology Journal*, vol. 12, no. 1, pp. 46–50, 2018.

[47] S. Abd-Elghany and K. Sallam, "Occurrence and molecular identification of *Vibrio parahaemolyticus* in retail shellfish in Mansoura, Egypt," *Food Control*, vol. 33, pp. 399–405, 2013.

[48] Z. H. Hassan, J. T. Zwartkruis-Nahuis, and E. de Boer, "Occurrence of Vibrio parahaemolyticus in retailed seafood in The Netherlands," *International Food Research Journal*, vol. 19, pp. 39–43, 2012.

[49] C.-T. Lee, I.-T. Chen, Y.-T. Yang et al., "The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 34, pp. 10798–10803, 2015.

[50] Y. Li, X. Hu, X. Zhang et al., "Photorhabdus luminescens PirAB-fusion protein exhibits both cytotoxicity and insecticidal activity," *FEMS Microbiology Letters*, vol. 356, no. 1, pp. 23–31, 2014.

[51] C. Theethakaew, S. Nakamura, D. Motooka et al., "Plasmid dynamics in Vibrio parahaemolyticus strains related to shrimp Acute Hepatopancreatic Necrosis Syndrome (AHPNS)," *Infection, Genetics and Evolution*, vol. 51, pp. 211–218, 2017.

[52] K. Chonsin, A. Siriphap, K. Changkaew, P. Noisumdaeng, and O. Suthienkul, "Virulence genes of *Vibrio parahaemolyticus* isolated from acute hepatopancreatic necrosis disease associated with shrimp and grow-out pond water," *The Journal of Health Sciences*, vol. 28, pp. 18–27, 2021.

[53] T. T. Hong To, H. Yanagawa, N. Khanh Thuan et al., "Prevalence of *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease of shrimp in shrimp, molluscan shellfish and water samples in the Mekong Delta, Vietnam," *Biology*, vol. 9, no. 10, Article ID 312, 2020.

[54] P. Rattanama, K. Srinitiwarawong, J. R. Thompson, R. Pomwised, K. Supamattaya, and V. Vuddhakul, "Shrimp pathogenicity, hemolysis, and the presence of hemolysin and TTSS genes in *Vibrio harveyi* isolated from Thailand," *Diseases of Aquatic Organisms*, vol. 86, no. 2, pp. 113–122, 2009.

[55] X. Dong, H. Wang, G. Xie et al., "An isolate of *Vibrio campbellii* carrying the pir^{VP} gene causes acute hepatopancreatic necrosis disease," *Emerging Microbes & Infections*, vol. 6, no. 1, Article ID e2, 2017.

[56] T. Defoirdt, "Virulence mechanisms of bacterial aquaculture pathogens and antivirulence therapy for aquaculture," *Reviews in Aquaculture*, vol. 6, no. 2, pp. 100–114, 2014.

[57] T. Xie, Q. Wu, J. Zhang, X. Xu, and J. Cheng, "Comparison of *Vibrio parahaemolyticus* isolates from aquatic products and clinical by antibiotic susceptibility, virulence, and molecular characterisation," *Food Control*, vol. 71, pp. 315–321, 2017.

[58] O. Traore, O. Martikainen, A. Siitonen, A. S. Traore, N. Barro, and K. Haukka, "Occurrence of *Vibrio cholerae* in fish and water from a reservoir and a neighboring channel in Ouagadougou, Burkina Faso," *Journal of Infection in Developing Countries*, vol. 8, pp. 1334–1338, 2014.

[59] S. Kabore', P. Cecchi, T. Mosser et al., "Occurrence of *Vibrio cholerae* in water reservoirs of Burkina Faso," *Research in Microbiology*, vol. 169, pp. 1–10, 2018.

[60] J. M. Janda, A. E. Newton, and C. A. Vibriosis Bopp, *Clinics in Laboratory Medicine*, vol. 35, pp. 273–288, 2015.

[61] I. H. Igbinosa, E. O. Igbinosa, and A. I. Okoh, "Detection of antibiotic resistance, virulence gene determinants and biofilm formation in *Aeromonas* species isolated from cattle," *Environmental Science and Pollution Research*, vol. 22, pp. 17596–17605, 2015.

[62] A. Thakur, R. B. Vaidya, and S. A. Suryawanshi, "Pathogenicity and antibiotic susceptibility of *Vibrio* species isolated from moribund shrimps," *Indian Journal of Marine Sciences*, vol. 32, pp. 71–75, 2003.

[63] W. W. I. Nurhafizah, L. Leong, L. Abdul Razzak et al., "Virulence properties and pathogenicity of multidrug-resistant *Vibrio harveyi* associated with luminescent vibriosis in Pacific white shrimp, *Penaeus vannamei*," *Journal of Invertebrate Pathology*, vol. 186, Article ID 107594, 2021.

[64] R. Wang, Y. Zhong, X. Gu, J. Yuan, A. F. Saeed, and S. Wang, "The pathogenesis, detection, and prevention of *Vibrio parahaemolyticus*," *Frontiers in Microbiology*, vol. 6, Article ID 144, 2015.

[65] P. Le Hong, M. Corteel, C. T. Nguyen et al., "Effect of dose and challenge routes of *Vibrio* spp. on co-infection with white spot syndrome virus in Penaeus vannamei," *Aquaculture*, vol. 290, no. 1-2, pp. 61–68, 2009.

[66] Z. Huang, M. A. Anokyewaa, J. Wang, J. Jian, and Y. Lu, "Pathogenicity and antibiotic resistance analysis of *Vibrio* species found in coastal water at mainly beach of Shenzhen, China," *Frontiers in Marine Science*, vol. 9, Article ID 980593, 2022.

[67] J. Guijarro, D. Cascales, A. Garcia Torrico, M. Garcia, and J. Mendez, "Temperature-dependent expression of virulence genes in fish-pathogenic bacteria," *Frontiers in Microbiology*, vol. 6, Article ID 700, 2015.

[68] I. N. Mulyadi and W. Iba, "Efficacy of seaweed (*Sargassum* sp.) extract to prevent vibriosis in white shrimp (*Litopenaeus vannamei*) juvenile," *International Journal of Zoological Research*, vol. 16, pp. 1–11, 2020.

[69] S. A. Soto-Rodriguez, B. Gomez-Gil, R. Lozano-Olvera, M. Betancourt-Lozano, and M. S. Morales-Covarrubias, "Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in northwestern Mexico," *Applied and Environmental Microbiology*, vol. 81, no. 5, pp. 1689–1699, 2015.

[70] A. F. Rowley and E. C. Pope, "Vaccines and crustacean aquaculture: a mechanistic exploration," *Aquaculture*, vol. 334–337, pp. 1–11, 2012.

[71] R. Sirikharin, S. Taengchaiyaphum, P. Sanguanrut et al., "Characterization and PCR detection of binary, pir-like toxins from *Vibrio parahaemolyticus* isolates that cause acute hepatopancreatic necrosis disease (AHPND) in shrimp," *PLoS One*, vol. 10, no. 5, Article ID e0126987, 2015.

[72] S. A. Soto-Rodriguez, B. Gomez-Gil, R. Lozano-Olvera, C. Bolanmejia, K. G. Aguilar-Rendon, and J. Enciso-Ibarra, "Pathological, genomic and phenotypical characterization of *Vibrio parahaemolyticus*, causative agent of acute hepatopancreatic necrosis disease (AHPND) in Mexico," *Asian Fisheries Science*, vol. 31, pp. 102–111, 2018.