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Review

PirAB from *Vibrio parahaemolyticus* exhibits a binary toxin action with bactericidal activity and cytotoxicity in *Penaeus vannamei*

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Abstract: PirAB is a binary protein complex secreted by specific strains of *Vibrio parahaemolyticus* (*Vp*) that harbor the pVA1 virulence plasmid and express PirA^{Vp} and PirB^{Vp} toxins. PirAB^{Vp} causes acute hepatopancreatic necrosis disease (AHPND), a newly emergent penaeid shrimp disease that can cause 70–100% mortality and has resulted in great economic losses since its appearance. The cytotoxic effect of PirAB^{Vp} on the epithelial cell of the shrimp hepatopancreas has been extensively reported. Our studies found that the PirB^{Vp} subunit has lectin activity and recognizes mucin-like *O*-glycosidic structures in the shrimp hepatopancreas. The PirA^{Vp} subunit may have a stabilization function of the binary complex. However, we also found that *Vp* AHPND changes the water microbiota community structure and causes a significant reduction in several bacteria, especially *Neptuniibacter* spp. We propose that the PirAB^{vp} toxin could exhibit a dual role: damage the shrimp hepatopancreas and kill surrounding bacteria.

Keywords PirAB; *Vibrio parahaemolyticus*; AHPND; shrimp; microbiota change

Key Contribution: *Vp* AHPND changes the microbiota community structure of the seawater and causes cytotoxic effects on the epithelial cells of the hepatopancreas.

1. Introduction

In 2013, *Vibrio parahaemolyticus* (*Vp)* strains were first reported as the causal agent of the emerging shrimp disease, acute hepatopancreatic necrosis disease (AHPND) [1], a highly infectious, OIE-listed, enteric disease, threatening farmed shrimp [2]. This lethal disease of marine shrimp emerged in China in 2009, originally known as early mortality syndrome (EMS). It has been a devastating disease and has caused great economic losses in the shrimp industry of Asian countries ([3–5], Mexico ([6], South America [7], and the USA [8]. China, Thailand, and Mexico reported losses of $11, $8, and nearly $1 billion US dollars, respectively, due to AHPND between 2009 and 2016 [9]. Virulence of *Vp* strains *(Vp* AHPND) is due to a conjugative plasmid of approximately 70 kbp (pVA1) that expresses

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a binary PirAB toxin, homologous to the Pir toxin secreted by *Photorhabdus* spp., which is responsible for the characteristic lesions in the shrimp hepatopancreas ([1, 10, 11]. Later, other AHPND-causing *Vibrio* spp. were identified from affected shrimp, including *Vibrio harveyi*, *V. owensii*, *V. campbellii*, and *V. punensis* ([4, 12–17]. AHPND can be caused by strains of several *Vibrio* spp. because the toxin genes *pirAB*^{Vp} reside in a conjugative plasmid, allowing for horizontal transfer between bacterial species [18–20]. AHPND is a disease caused by toxigenic bacteria that produce the PirAB toxin, however, as the disease progresses, there is a secondary bacterial colonization of the damaged hepatopancreas. The disease represents a special threat to the penaeid shrimp culture due to its diverse etiology, complexity, rapid pathogenesis, and the widespread nature of this disease. In addition, to date, mechanisms of the AHPND toxigenesis are far to be understood.

In this review, we present an overview of AHPND, including the disease-associated signs and the progression of histopathology lesions, the degree of virulence of *Vp* strains, and the current knowledge of virulent pVA1 plasmid, as well as the status of the changes in the bacterial community structure caused by *Vp* AHPND and possible factors that could induce or inhibit toxin production. Finally, we present new research on putative membrane receptors and potential inhibitors of the PirAB^{V}p toxin.

2. Acute hepatopancreatic necrosis disease in penaeid shrimp

To date, AHPND continues to be the bacterial disease of greatest economic importance that affects the tiger shrimp (*Penaeus monodon*) and the Pacific white shrimp (*Penaeus vannamei*) [21]. Although the crustacean decapod´s hepatopancreas (Hp) has been the target organ of AHPND, the Australian red claw crayfish (*Cherax quadricarinatus*) is not susceptible to *Vp* AHPND in cohabitation bioassays [22]. The authors hypothesize that there are differences in the putative receptor binding sites between penaeid shrimp and crayfish, such as the PirAB binary toxin could not bind to the hepatopancreatic epithelium of the crayfish's receptor(s), which does not cause intoxication.

In the early life stages, shrimp are more susceptible to AHPND strains intoxication with a threshold infective density of > 10^4 CFU mL^{-1} [11], and there is an increased mortality rate of *P. vannamei* inoculated with *Vp* AHPND at higher salinity levels. AHPND is characterized by severe dysfunction of the shrimp Hp accompanied by clinical signs and particular histopathological changes in the acute disease stage [23]. The shrimp affected with AHPND exhibits expanded chromatophores, lethargy, anorexia, empty digestive tract, and pale to white hepatopancreas. However, these clinical signs are also commonly observed in other bacterial diseases, such as necrotizing hepatopancreatitis (NHP-B) and septic hepatopancreatic necrosis (SHPN) [24]. Hence, confirmatory diagnosis of AHPND in shrimp should include, in addition to clinical signs, i.e., the histopathological lesions observed in the acute stage of the disease, molecular detection of *pirA*^{Vp} and *pirB*^{Vp} genes coupled with bioassays. Diagnosis of AHPND through only *pirA*^{Vp} and *pirB*^{Vp} genes detection may be inadequate due to instability of these genes, as observed in strains isolated from different geographical regions [25].

2.1. Degree of virulence

The term virulence is used to determine the relative ability of a microorganism to cause disease in a susceptible host, better known as degree of pathogenicity [26]. This ability allows evaluating the virulence quantitatively; to achieve this, some mechanisms of pathogenicity are evaluated like evasion of host defense mechanisms, antibiotic resistance, severity degree of lesions, percentage of induced death, invasiveness, and toxigenic capacity. Bacterial strains also possess different degrees of virulence [27, 28], which may come from phenotypic or genotypic variations. For example, the genome of a virulent *Listeria*

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monocytogenes strain contains a large number of anti-sense RNA, responsible for its virulence, contrary to the non-pathogenic *L. monocytogenes* strains [29].

The expression of *pirA*^{Vp} and *pirB*^{Vp} genes can influence the degree of virulence [5] found low *pirA* gene expression (< 0.4 of relative expression) from *Vp* AHPND strains with moderate virulence, whereas finding high gene expression (2.1 of relative expression) from less virulent strains. As *pirA*^{Vp} and *pirB*^{Vp} genes are located in the same operon in the virulent plasmid (pVA1) [30], theoretically, both genes must be expressed constitutively, so that strains should have a similar virulence. However, it is evident that other factors are also involved. Our studies show that the copy number of pVA1 is related to the bacterial density [31]. The PirA and PirB proteins (or the PirAB^{Vp} complex) can be differentially secreted by the bacterial cells affecting their virulence for shrimp [3, 5, 32] or be affected by post-translational modifications. Through western blot was observed a wider band of PirA and PirB proteins from more virulent strains [33]. However, to date, the role of pVA1 is not clear nor that of the secreted toxins in the virulence degree of bacterial AHPND strains.

Lastly, the *Vp* AHPND strains have different lifestyles during experimental infections that could influence pathogenesis; whereas the more virulent *Vp* M0904 adhered preferably to the bottom surface of the experimental units, the less virulent *Vp* M0607 strain adhered to the bottom and remained suspended in the water column [34]. In Mexico, during the shrimp mortality events associated with *Vp* AHPND, several strains were isolated with different degrees of virulence in terms of time of death [11]. In experimental infections, using similar laboratory conditions, i.e., immersion assays with *P. vannamei* challenged at bacterial density of 10^6 CFU mL^{-1} in natural or synthetic seawater (between 8 and 35 g L^{-1}), shrimp reached 100% mortality starting at 17 h post-inoculation (p.i.) to 72 h p.i. or shrimp did not reach this mortality along the whole experiment (Table 1). Our observations with Mexican strains indicate that the shrimp size in relevant during experimental infections, small shrimp are more susceptible to AHPND than larger ones, possibly the PirAB toxin is dose-dependent.

Table 1. Pathogenicity of *Vibrio* species responsible for AHPND in penaeid shrimp.

Strain	Origin	Shrimp size (g)	Density (CFU/mL)	Histo.	First dead-100% mortality (h)	Reference
Vp 13-028A/3	Vietnam	0.5-2.0	2 × 10^6	Yes	< 24 - 48	[1]
Vp 3HP	Thailand	~ 2.0	1 × 10^6	Yes	ND - 24	[3]
Vp S02	China	~ 2.0	1 × 10^6	Yes	ND - 24	[3]
Vp 13-306D/4	Mexico	~ 2.0	ND	Yes	> 24 - 72	[6]
Vp 13-511A/1	Mexico	~ 3.0	2 × 10^6	Yes	ND - 24	[6]
Vp M0607	Mexico	0.5-1.0	7.8 × 10^6	Yes	15 – 48*	[11]
Vp M0802	Mexico	0.5-1.0	3.3 x 10^6	Yes	7 - 25	[11]
Vp M0904	Mexico	0.5-1.0	2.2 × 10^6	Yes	4 - 17	[11]
Vp 2S01	China	~ 1.0	1 × 10^6	Yes	3 - 18	[16]
Vp-BA94C2	Lat America	2.5±0.5	2 × 10^6	Yes	6 - 70	[17]
Vp D6	Thailand	3-5	1 × 10^6	ND	144 - 216	[33]
Vp D6	Thailand	0.82	5 × 10^5	ND	24 - 96	[33]
Vp GD10	China	~ 2.0	~ × 10^6	Yes	< 24 - 72	[35]
Vp 5HP	Thailand	1.8±0.2	~ × 10^6	Yes	> 24 – 96*	[36]
Vp XN89	Vietnam	1.8±0.2	~ ×10^6	Yes	> 24 – 96*	[36]

Vp 15-250/20	Lat America	1-1.5	2×10^6	Yes	< 12 – 168*	[37]
Vp 19-021-D1	Korea	1-1.5	2×10^6	Yes	< 12 – 168*	[37]
Vp 19-022-A1	Korea	1-1.5	2×10^6	Yes	< 12 – 168*	[37]
Vp C3	Thailand	2.0	2×10^5	Yes	ND - 72	[38]
Vpu-BA55	Lat America	2.5±0.5	2×10^6	Yes	8 – 70*	[17]
Vc 20130629003S01	China	~ 1.0	2×10^6	Yes	12 - 36	[16]
Vc 16-904/1	Lat America	2.0	2×10^5	Yes	ND - 72	[38]
Vc 20130629003S01	China	~ 1.0	1×10^6	Yes	3 - 24	[39]
Vc 34	Peru	1.2	~ ×× 10^6	Yes	< 24 - 120	[40]
Vc 36	Peru	1.2	~ × 10^6	Not	< 24 - 120	[40]
Vc 43	Peru	1.2	~ × 10^6	Not	< 24 - 120	[40]
Vo SH-14	China	0.5-2.0	~ × 10^6	Yes	12 - 96	[13]
Vo SH-14	China	0.5-2.0	~ × 10^6	ND	< 20 – 40*	[18]

Vp: *Vibrio parahaemolyticus*; Vpu: *Vibrio punensis*; Vc: *Vibrio campbellii*; Vo: *Vibrio owensii*; * shrimp did not reach 100% cumulative mortality; Histo: Histopathology study; Yes: Typical histopathological lesions of AHPND acute stage; ND: not determinated; Not: No histopathological lesions of AHPND acute stage; Not: No histopathology was done.

As Table 1 shows, some studies did not do histopathological analysis of infected shrimp to confirm the disease development and, in certain cases, histological observations do not report AHPND or do not correspond to the acute stage of AHPND lesions. The next section will describe the progression of the histopathological lesions during AHPND.

2.2. *Histopathology of AHPND*

Since the first reports [41], the pathological manifestations of AHPND have been described by histopathological examinations [1, 3, 6, 8, 11, 15, 17, 23]. Although there are currently several methods for AHPND detection (clinical signs, histopathology and molecular techniques), the histopathological analysis remains the primary method to confirm positive cases [2] and to evaluate the cytotoxic effects of the PirAB toxin [32]. AHPND causes clinical signs like lethargy, erratic swimming, empty gut, discoloration, and atrophy of Hp, and progressive tissue changes that include massive sloughing of epithelial cells of hepatopancreatic tubules [1].

Histologically, three stages (initial, acute, and terminal) have been commonly reported as part of the pathogenic course of AHPND (Figure 1) [1, 11, 23]. However, because of the shrimp's capacity to tolerate the effect of PirAB toxin, a remission stage of the disease has been observed in surviving shrimp, where the E (embryonic) cells are bio indicators for this stage [34]. In the initial stage of AHPND, there is a decrease in vacuoles of the R and B cells and elongation of the epithelial cells, which signal the cell sloughing (Figure 1a, b) in absence of the pathogenic bacteria [11]. In the acute stage, a massive sloughing of epithelial cells (R, F, and B) occurs, which accumulate in the lumen of the affected tubules (Figure 1c, d). This is the most remarkable histopathological aspect used for the clinical diagnosis of AHPND; furthermore, the mitotic activity in the E cells is absent [1, 11, 41].

In the terminal stage, the hepatopancreatic tubules' epithelium is entirely necrotic, dead cells are in different degrees of lysis within the lumen, and there is a proliferation of bacteria associated with the necrotic material, indicating a secondary infection (Figure 1e, f) [1, 41]. The inflammatory response increases over time causing melanization, hemocytic nodules and hemocytic capsules around the affected tubules, which delimit disease progression, but spread the secondary infection.

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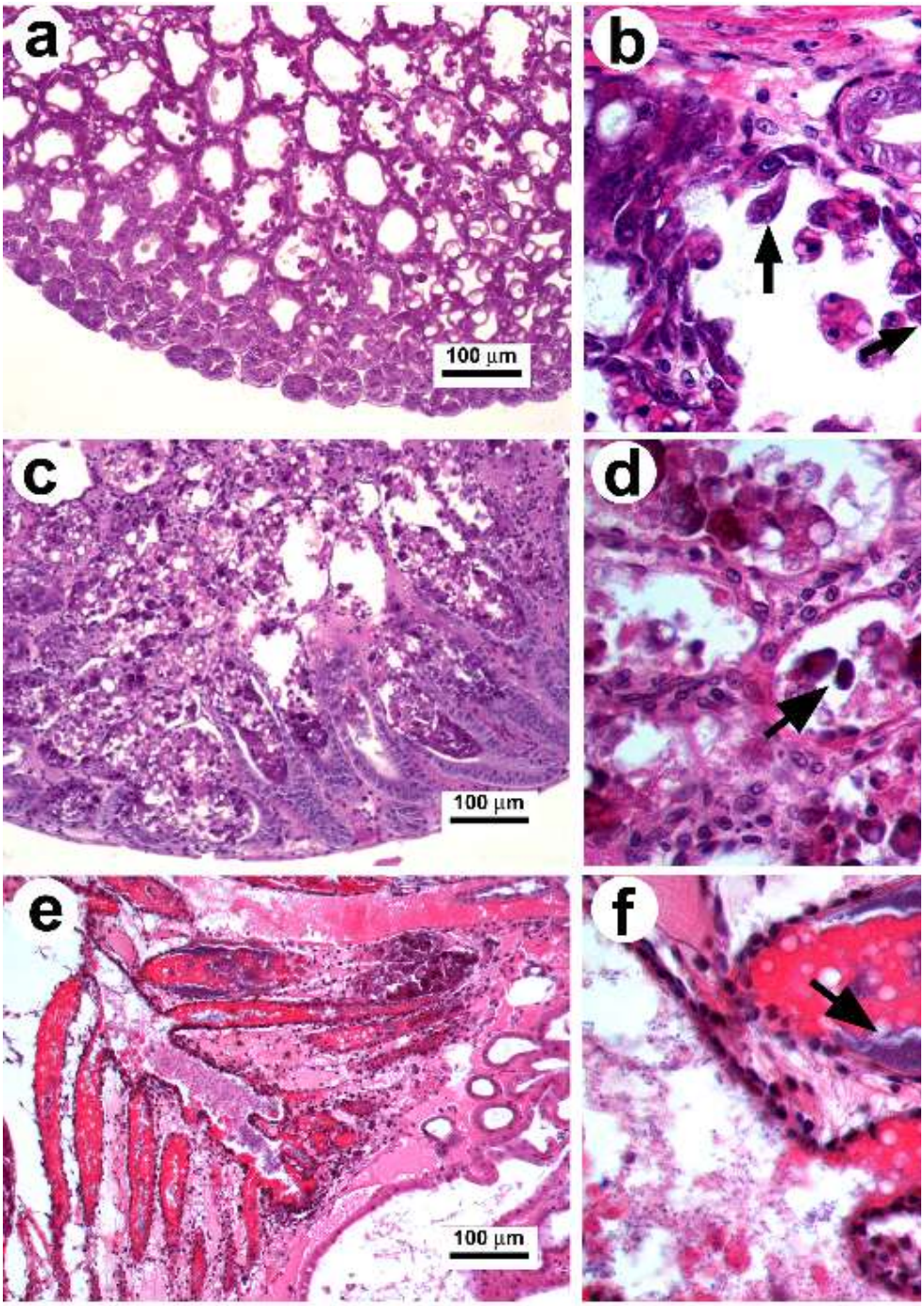

Figure 1. Photomicrograph of the *P. vannamei*'s hepatopancreas affected with AHPND. (**a, b)** Hepatopancreatic tubules in the initial stage: the tubular epithelium undergoes reduction of vacuoles and elongated cells in the lumen (arrow) without evidence of pathogenic bacteria. (**c, d)** Tissue in the acute stage: the distal region of tubules shows the E cells without mitotic activity, and the proximal and middle region of the tubular epithelium show massive sloughing of epithelial cells (arrow). (**e, f)** Tubules in terminal stage with hemocytic infiltration in the intertubular tissue, necrotic epithelium and dead cells with bacterial masses in the tubular lumen (arrow). H&E stain.

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Recently, we found that surviving shrimp after 3 d post-infection (p.i.) with *Vp* AHPND are able to develop larger melanized necrotic lesions similarly to septic hepatopancreatic necrosis (SHPN) [34, 42]. Thus, surviving shrimp can decrease the cytotoxic effect caused by the PirAB^{Vp} toxin and enter into a remission stage of the disease. This stage is characterized by the reactivation of the mitotic activity in E cells, declination of the clinical signs associated with AHPND, and a decrease of mortality [34]. In addition, the necrotic lesions associated with the terminal stage of AHPND quickly decrease over p.i. time (Figure 2a). The histopathological evidence suggests that the development of AHPND lesions, under experimental conditions, can follow three main routes from the terminal stage to disease recovery [34]. Surviving shrimp after 5 d p.i. show different histopathological conditions ([34, 42], which include recovered shrimp, displaying normal hepatopancreas without evidence of lesions (Figure 2b); shrimp with atrophied hepatopancreatic epithelium without vacuoles in R cells associated with a chronic effect (Figure 2c, d); shrimp with lesions similar to SHPN (Figure 2e), with persistence of a secondary bacterial infection, but delimited by hemocytic nodules; and, finally, shrimp displaying a combination of chronic effects and SHPN (Figure 2f).

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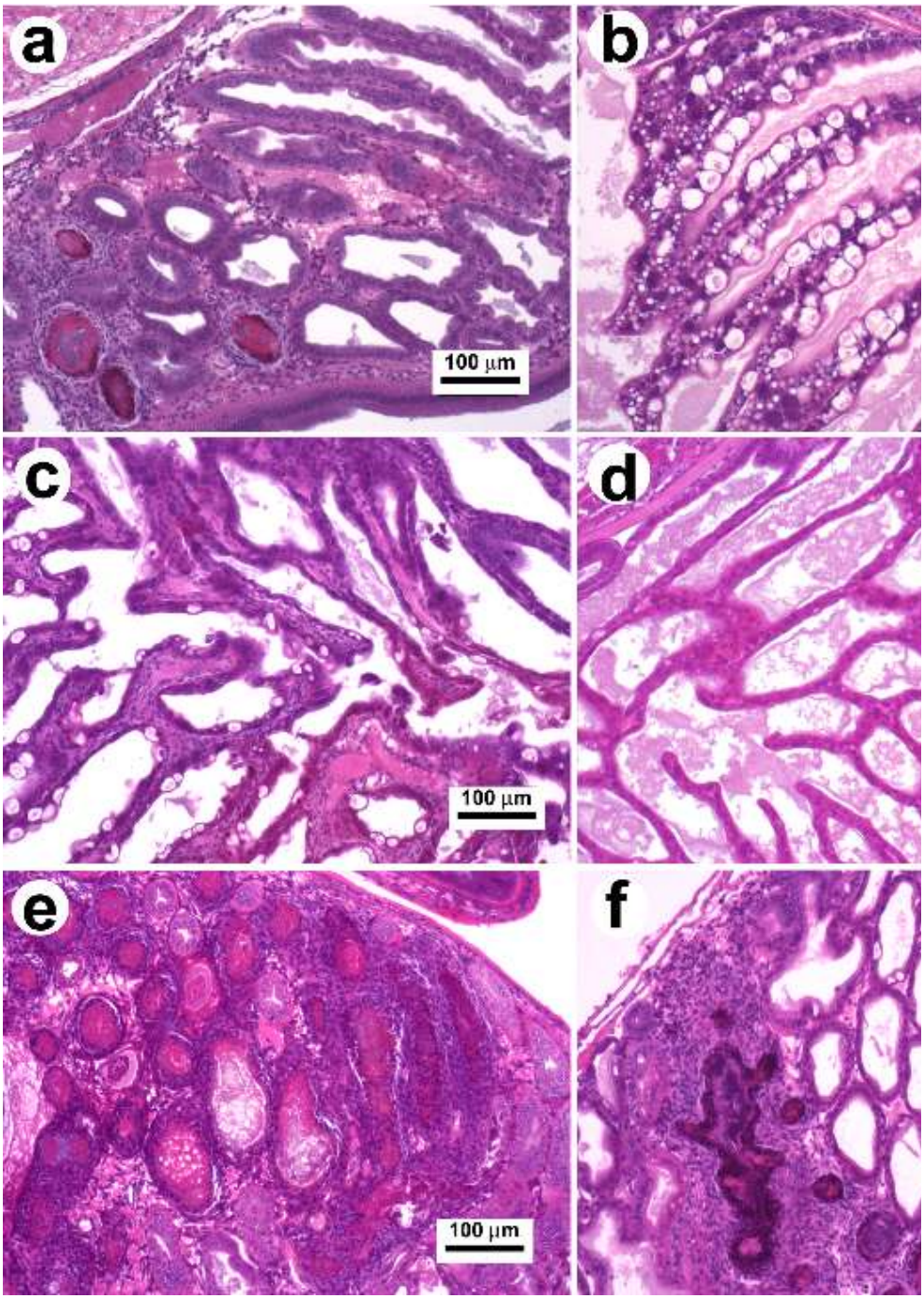

Figure 2. Photomicrograph of the *P. vannamei*'s hepatopancreas (Hp) affected with AHPND. (**a)** Hp in the remission stage with a declination of the necrotic lesions and the presence of a secondary infection confined by melanized hemocytic nodules. (**b)** Normal tubular epithelium of recovered shrimp with abundant vacuoles in R and B cells. **(c**. **d)** Hepatopancreatic tubules in a chronic stage with atrophied epithelium, absence of vacuoles in R cells, and no evidence of bacteria. (**e)** Tubules with necrotic lesions similar to septic hepatopancreatic necrosis. (**f)** A combined lesion of (**c, d)** and (**e).** H&E stain.

The development of lesions in shrimp affected with AHPND is associated with the bacterial density, degree of strain virulence [3, 11, 23, 35], PirA and PirB toxins concentration [32], and infection time [34]. We observed a delay in the time of the acute stage lesions

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of AHPND in *P. vannamei* infected with strains *Vp* at 10^5 CFU mL^{-1}, which was dependent on the degree of virulence [34]. With the most virulent strain, the acute stage occurred in the first 4 h p.i., whereas with the less virulent strain it was 8 h later. The terminal stage was present at 24 h p.i. for both strains, but it remained until 48 h p.i. only for the most virulent strain. Shrimp displayed a marked immune response such as hemocytic infiltration, hemocytic nodules, and melanization to delimit the bacterial proliferation and damage caused by PirAB toxins. We suggest that development and persistence of the terminal stage depend on the damage degree, genetic line, and immunologic status of shrimp.

Previously was reported that a minimum concentration of 10 μg g^{-1} of PirA and PirB toxin is necessary to induce the typical lesions of AHPND [32]; below 5 μg g^{-1} toxins do not cause the disease, but that concentration can induce collapse (atrophy) of the tubular hepatopancreatic epithelium [32]. The atrophied epithelium has also been observed in shrimp under experimental infections at *Vp* AHPND doses lower than the infective threshold (< 10^4 CFU mL^{-1}) [11], with strains of low virulence [3, 11, 36, 40], and in surviving shrimp [34]. An explanation for the atrophied epithelium of surviving shrimp could be that, under experimental infection, after the acute stage of AHPND, there is a decrease in the PirAB production/secretion, which reduces the lesions caused by the disease and favors the shrimp's survival. Likewise, we suggest that the atrophied epithelium might be the combined effect of low concentration and continuous exposure to the PirAB toxin. Studies should be carried out to quantify and to know the dynamics of toxin production of *Vp* AHPND during an *in vivo* experiment.

3. Virulence plasmid pVa1

AHPND is mainly caused by *Vp* that harbors a plasmid of ~70 kbp pVA1 containing the pirAVp and pirBVp genes, which encode a delta-endotoxin responsible for the typical lesions in shrimp Hp [1, 10). The genomes of pVA1-harboring *Vp* revealed a large pan genome, high genetic diversity grouped into three main clades, and specific structural differences, in addition to the instability of the $pirAB^{Vp}$ region of the pVA1 plasmid [25]. The structural differences found in pVA1 are likely due to horizontal propagation of the plasmid to other *Vibrio* species [19], such as *V. harveyi* [4, 18], *V. campbellii* and *V. owensii* [18], and *V. punensis* [17. The above processes might cause the appearance of new pathogenic AHPND strains and a major threat for the shrimp industry. Likewise, this variability in the structural elements could eventually influence their ability to adapt to niches, growth behavior, and virulence/pathogenesis.

Recently we observed different growth of two strains of *Vp* AHPND, with distinct genetic elements and virulence during an experimental infection. The most virulent strain was adhered to the bottom surface and the moderate virulent strain was present on the bottom and suspended in the column of seawater [34]. Large variability of the plasmid copy number per bacterial cell of *Vp* AHPND strains was registered, from 7 to 121 copies, analyzed by qPCR [30, 43], and, although, it has been reported that virulence is not dependent on the copy number of $pirA^{Vp}$/$pirB^{Vp}$ genes [33], to date, no clear evidence of their role in AHPND exists. A study using a shotgun metagenomics approach to the bottom seawater during an experimental infection with juvenile shrimp (*P. vannamei*) inoculated with two *Vp* AHPND strains registered more than one copy of the pVA1 per bacterial cell (from 1.9 to 13.5 copies per bacterial cell) throughout the experimental infection time [31]. In this study, we found that the copy number of the virulent plasmid is not dependent on the degree of virulence of the *Vp* AHPND strain, but dependent on the bacterial density (Figure 3).

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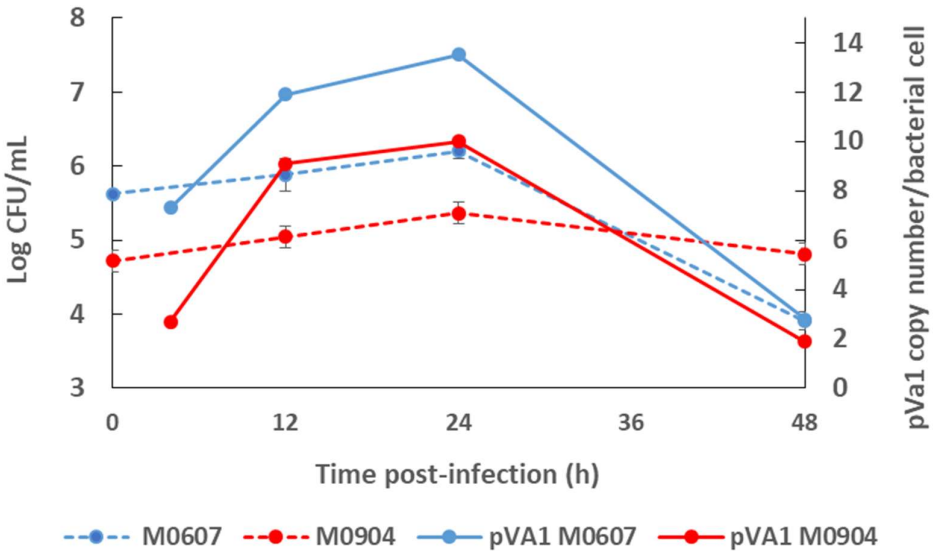

Figure 3. Bacterial density of the bottom seawater and virulent plasmid copy number of moderate virulence *Vp* strain M0607 and high virulent *Vp* strain M0904 during experimental infections at 10^5 CFU mL^{-1}.

There is a lack of studies about the variability of the plasmid copy number per bacterial cell in relation with the degree of virulence and bacterial density, and how it influences the pathogenesis of AHPND.

4. Changes in the microbiota of seawater

To date, there are few works about microbial communities present in the seawater of cultured shrimp ([44, 45]. Most studies have focused on bacterial community structures of the intestinal microbiota ([46–48] or effects of environmental factors on the microbial community in shrimp farms [49]. Proteobacteria have dominated the penaeid shrimp gut microbiota, and the microbiome is involved in the regulation of shrimp health and disease [50]. Most studies of *P. vannamei* affected with AHPND are focused on the bacterial community characterization of the Hp, stomach, intestine or sediment, using 16S rRNA amplicons [51–53]. Toxins affect the microbial communities of their host [54], but there are few studies about the effects on the surrounding microbiota of the seawater by the presence of diseased aquatic organisms [55]. The type III secretion system (T3SS) has been suggested as the mechanism for which the $PirAB^{Vp}$ toxin is secreted [32]. However, the type VI secretion system (T6SS) is involved in the virulence of human pathogenic *Vp* strains through the secretion of effector proteins, which are toxic to surrounding bacteria [56]). The T6SS1 of a *Vp* AHPND isolate was functional during the challenge of *P. vannamei* [57].

Recently, our research team studied the changes in the water microbiome of juvenile *P. vannamei* inoculated with a moderate virulent *Vp* strain (M0607) and a highly virulent *Vp* strain (M0904), using the shotgun metagenomics sequencing approach [31]. We found dominance of the Proteobacteria phylum in the water according to the bacterial community associated with AHPND [58]. At the family level, Rhodobacteraceae was the most predominant taxon that has already been detected in both healthy and diseased shrimp microbiota and in the culture water [45, 51, 58]. Oceanospirillaceae appear related to environmental conditions [45] and Vibrionaceae are significant during AHPND infection ([52, 58].

A dominance of the Neptuniibacter complex shows high genetic variation in the initial community structure [31]. *Neptuniibacter* spp. are common in seawater and associated

with farmed organisms [59]. A significant marked reduction was observed in the reads assigned to *Neptuniibacter* spp. after the inoculation of both strains, particularly with *Vp* M0904 from 4 h p.i. on, with a gradual increase at 48 h of *Pseudoalteromonas stutzeri*, *Halomonas* sp., and *Marinobacter adhaerens*.

The depletion pattern in the Neptuniibacter complex suggests that these species could be highly affected by bacterial toxins secreted from both *Vp* strains [60], specially the PirB^{Vp} subunit due to its lectin-like activity [61]. The reduction in abundance of the Neptuniibacter complex suggests that bacterial competition could be mediated by the T6SS, which regulates bacterial interactions [60, 62]. Some *Vp* AHPND strains contain active T3SS1, T6SS1, and T6SS2 [63]. We found an enrichment of the functions associated with these systems related to inoculation with *Vp* strains [31], and these functions are closely associated with bacterial pathogenesis [64]. The T6SS represents a complex secretion machinery and contributes to competitive survival or pathogenesis in many Gram-negative bacteria [57]. Three effector proteins of T6SS were detected only in inoculated treatments, mainly in the M0904 strain: a) the cytotoxin Hcp; b) the temperature-dependent protein that activates the T6SS according to certain environmental conditions [60]; and c) the antitoxin serine/threonine protein kinase [31], which is a type of immunity protein that protects the bacterial community against self-intoxication of effector proteins from T6SS [65]. The T6SS1 system is active under specific conditions of temperature (30 °C) and salinity (3% NaCl), which were maintained during experimental infections [60], so it could be functional in both *Vp* AHPND strains. This antibacterial system, found in 12 strains of *Vp* AHPND, mediates interspecies and intraspecies competition, promoting shrimp infection [63]. It is strongly suggested that both *Vp* AHPND strains could use the T6SS1 as a selective advantage during shrimp intoxication by killing surrounding bacteria.

5. Factors that could induce or inhibit toxin production

5.1. Quorum sensing

Quorum sensing (QS) is a cell-to-cell signaling mechanism in response to an increase of bacterial cell-population [66]. Bacterial QS produce, release, and recognize molecular autoinducers (AI) that bind to surface bacterial receptors, triggering signal transduction cascades that alter the expression of genes related with survival and infection factors, like sporulation, luminescence, biofilm formation, and virulence, among others [67]. The QS mechanism is widely distributed in Vibrionaceae members, being the acyl-homoserine lactones (AHLs) one of the common AIs. For example, AHLs are implicated in the signaling mechanisms that activate the production of luciferase in *V. fischeri* [66]. *V. harveyi* produces and responds to other three AIs, a) HAI-1, [N-(3- hydroxy butyryl)-homoserine lactone], an intra-species AI; b) CAI-1, [(Z)-3-aminoundec-2- en-4one] that is restricted to the *Vibrio* genera; and c) the inter-spices AI-2 ((2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate). These three AIs act in parallel to regulate over 600 target genes through complex signaling cascades ([66, 68]. The Vibrionaceae's capacity to "sensing self" and "sensing others" allows for both competition and cooperation in complex microbial communities [69].

Virulence gene expression regulated by QS has been studied extensively in *V. harveyi* and may serve as a basis for the understanding of QS mechanisms in Vp since this pathogen contains the central conserved components of the QS pathway known in *V. harveyi* [70]. For example, a LuxT homolog of *V. harveyi*, the SwrT, activates genes that encode for translocation across surfaces, swarming, and is lateral-flagellar-driven in *Vp* [71, 72]. *V. harveyi* and, presumably, *Vp* produce three types of AIs called auto inducer 2 (AI-2), harveyi auto inducer 1 (HAI1), and cholerae auto inducer 1 (CAI1), which are recognized by the surface membrane receptors LuxP/LuxQ, LuxN, and CqsS, respectively [73]. In a preliminary study, [74] showed that the production of PirAB^{Vp} binary toxin is regulated by

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the AI-2 QS process. They tested the effect of a cell-free supernatant from *V. harveyi* containing AI-2 (CFS-VH) on an AHPND-causing *Vp* strain. The AI-2-containing supernatant accelerated the production time and yield of both PirA^{Vp} and PirB^{Vp} toxins, whereas the application of the furanone [(5Z)-4-bromo5-(bromomethylene)-2(5H)-furanone] AI-2 antagonist delayed the AHPND toxin production or secretion. This study opens new perspectives on the knowledge of QS mechanisms in *Vp* and on possible treatments for the management strategies to control AHPND infection in shrimp culture. Interestingly, AI-2 is synthetized by numerous bacterial species and can facilitate inter-species cell–cell signaling [75], resulting in changes of *Vp* behavior in complex microbial communities.

5.2. Environmental factors

Bacterial adaptation and survival depend on their capacity to properly respond to changes in their internal and external environments. *Vibrio* spp. survival in marine environments follows responses to carbon and energy sources, dissolved oxygen, water pH, salinity, temperature, and starvation [76]. Environmental stress can increase horizontal gene transfer mechanisms in AHPND-causing *Vp* strains, promoting their growth [77, 78] and, therefore, increasing the risk of AHPND outbreaks and disease dispersion in tropical zones. Recently was investigated the effect of temperature shifts on the *pirA*^{Vp} and *pirB*^{Vp} genes expression of AHPND-*Vp* AAHMRU04 strain isolated from white shrimp exhibiting clinical signs of AHPND [79]. Tested bacteria were grown at 30 °C for 24 h and subsequently exposed to a set of different temperature trials for 4 days. *pirA*^{Vp} and *pirB*^{Vp} genes were induced when the temperature shifted from high (26–32°C) to lower (22-28°C) temperatures [79]. Changes in temperature due to global warming are a growing concern for aquaculture due to the risk of the increase of *Vp*-caused diseases.

The relation between salinity and AHPND in *P. vannamei* was studied by [80]. Pathogen-free shrimp cultures (5, 10, 15, and 20 g L^{-1} of NaCl) were challenged with a *Vp* AHPND broth. *Vp* AHPND caused infection in shrimps at all salinity treatments as confirmed by histological damage and the presence of *pirAB*^{Vp} toxin genes by PCR analysis. However, cumulative mortality was different, showing higher survival in shrimp maintained at lower salinity. Since *Vp* reproduces more efficiently in higher salinity environments, the production of a greater amount of PirAB^{Vp} toxin is probable, which resulted in a higher cumulative mortality in *P. vannamei* maintained at these conditions. A different behavior was observed when challenging *P. vannamei*, growing at different salinity conditions, with *Vp* AHPND-E9 [81]. In this study, mortality was higher at lower saline concentration, finding a positive correlation with the expression of *pirA*^{Vp} gene. Although more experiments are needed to determine the influence of salinity on the expression of *pirAB*^{Vp}, these experiments corroborate that the toxin can be expressed at different salinities [82] and that the management of saline concentration in shrimp culture can be an important factor to control *Vp* infectivity.

Another environmental factor studied in relation to the production of the PirAB^{vp} binary toxin is related to fluid shear and hydrodynamic forces to which *Vp* could be subjected either by natural influences or by the use of aquaculture equipment to enhance shrimp productivity, such as blowers or aerators [83]. The effect of shaking conditions on the AHPND-causing *Vp* M0904 was studied [84]. At 110 rpm constant agitation, bacteria develop cellular aggregates together with the levan (branched polymeric fructans)-containing biofilm formation and acquisition of tolerance against antimicrobial agents (kanamycin, ampicillin, rifampicin, and tetracycline), possibly due to high biofilm production. In addition, a significant decrease was observed not only in the PirA^{Vp}/PirB^{Vp} toxins production but also in the virulence of *Vp* M0904 to *Artemia* and *Macrobrachium* larvae. An increase in shaking speed to 120 rpm produced an increase in the PirA^{Vp}/PirB^{Vp} toxins production, in the virulence of *Vp* M0904 to *Artemia* and *Macrobrachium* larvae, and in the

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expression of polar flagellin (flaA), polar flagellin-specific chaperone (fliS), and chemotaxis protein (CheR). This type of study provides valuable information for understanding the behavior of *Vp* AHPND in aquaculture environments [84].

5.3. Biofilm formation

Formation of bacterial biofilms represents one of the most important survival mechanisms, attachment strategies, and host colonization of bacteria [85].This phenomenon is influenced by abiotic and biotic factors regulated by QS [86]. ToxR is an important virulence regulator implicated in the synthesis of *Vp* biofilm formation and also controls the expression of virulence factors found in human pathogenic *Vp*, including thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH), type III secretion system T3SS [87, 88]. Expression of these factors is regulated by QS through the production and response to AI-2 ([86, 89, 90]. Under these conditions, biofilm and toxin production appear to be simultaneous activities.

There is a lack of information about the relationship between biofilm formation and the production of PirAB^{Vp} binary toxin in *Vp* AHPND. The only study found is that of [84] who observed an inverse relationship between the production of biofilms and that of the PirAB^{Vp} toxin. This behavior refers to the formation of abiotic films in response to fluid shear and hydrodynamic forces. However, the regulation, growth kinetics, and characteristic of *Vp* AHPND biofilms in the host and their relation with PirAB^{Vp} toxin production are yet to be characterized.

6. Search for membrane receptors of PirA^{Vp} and PirB^{Vp}

6.1. Biological activities of the PirA^{Vp} and PirB^{Vp} subunits

Bacterial protein toxins, like PirAB^{Vp}, are molecular self-governing virulence factors that target specific host cells, triggering different damaging processes involved in the disease of the infected organism. Binding of bacterial toxins to the plasma cell surface receptors is an essential first step for shrimp intoxication. Knowing the structure of these receptors can contribute to a better understanding of the pathogen's infection mechanisms and to prevent host disease by blocking the toxin-receptor interaction using a mimetic antagonist [91].

It is known that PirA^{Vp} and PirB^{Vp} form a heterodimeric complex that binds to receptors located on the cells of the shrimp Hp [92, 93]. However, the precise nature of the toxin receptors is still not known. The first crystal structures for *Vp* PirA^{Vp}/PirB^{Vp} showed a similar structural topology to *Bacillus thuringiensis* (*Bt*) Cry insecticidal toxin [10]. This evidence suggests that PirA^{Vp}/PirB^{Vp} is a binary pore-forming toxin and that PirA^{Vp}/PirB^{Vp} possesses similar structural mechanisms of receptor binding to those of the *Bt* Cry insecticidal toxin. Structural alignment of both toxins indicates that the PirA^{Vp} subunit is similar to the lectin-like recognition domain III of *Bt* toxin whereas PirB^{Vp} would correspond to pore forming I and II domains ([10, 92, 94, 95]. In this context, the initial interaction of the PirA^{Vp}/PirB^{Vp} toxin would be through a lectin-carbohydrate recognition between PirA^{Vp} and the glycans exposed on the surface of the plasma membrane of Hp cells [92]. Structural features and molecular docking of the PirA^{Vp} subunit shows a potential sugar-binding cavity for glycans containing the GalNac molecule whereas the PirB^{Vp} subunit structure contains a C-terminal receptor domain similarily to the Cry domain II for protein-protein ligand interactions and an N-terminal consistent with other membrane pore-forming toxins, including Cry I domain [10, 95]. However, experiments conducted with recombinant proteins, rPirA^{Vp} and rPirB^{Vp}, showed that only the PirAB^{Vp} complex and the rPirB^{Vp} displayed a Mg^{2+} or Ca^{2+} independent hemagglutinating activity (HA) toward rat

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red cells, whereas rPirA^{Vp} was not able to agglutinate erythrocyte from several animal species [61].

In a first attempt to determine the putative PirB^{Vp} lectin´s sugar specificity, subsequent competition experiments were conducted using a wide battery of mono- and disaccharides and glycoproteins. GalNH2 and GlcNH2 monosaccharides were better sugar inhibitors for rPirB^{Vp} than the rest of mono and disaccharides tested. Among glycoconjugates, the fetuin glycoprotein showed the strongest rPirB^{Vp} HA inhibition capacity, whereas egg white chicken ovalbumin and heparin showed a relative inhibitory potency. With this in mind, the PirB^{Vp} subunit binds to a glycoconjugate glycan moiety containing amino sugars [61]. Further experiments conducted by the same group showed the existence of different glycan receptors for PirB^{Vp}, at least a mucin-like receptor located at the surface membrane of the cell hepatopancreas and an internal hexosaminidase glycoprotein receptor, possibly involved in toxin-causing cell damage to shrimp tissues [96]. Beta-hexosaminidase (β-*N*-acetyl hexosaminidase) is a ubiquitous lysosomal enzyme with multiple roles in protein glycosylation and synthesis and metabolism of glycoconjugates [97]. This glycoprotein plays an important role in arthropod molting and chitin degradation, and also in the defense system of *P. vannamei* against parasites ([98, 99]. Extracellular beta-hexosaminidases secreted by eukaryotes occur as dimers and possess N-glycosidically-linked glycans with oligomannosidic and complex-type glycan structures [100, 101]. The possibility that PirB^{Vp} could recognize N-linked oligosaccharides expressed by endosomal or secreted beta-hexosaminidase, allowing increased pathogenesis of *Vp* in crustaceans, cannot be excluded.

Previous data suggest a putative lectin-like PirB^{Vp} subunit activity [61, 96] that contrasts with the function of domains I and II proposed for the Cry toxin and also for the proposed function of PirA^{Vp} subunit because with the experiments carried out to date, it was not possible to verify that this subunit has the ability to recognize carbohydrates. In light of this new evidence, the PirA^{Vp} subunit could be playing an initial stabilizing role allowing PirB^{Vp} to bind with higher affinity to the glycan receptors located at the surface of hepatopancreas cells.

6.2. *Expression of mucin-like O-glycosidic structures in shrimp*

O-glycans are critical for the development and function of multicellular organisms. Mucin-type glycans are widely found on the cell surface and secreted glycoconjugates of invertebrates [102]. These O-glycans may serve as receptor-binding sites for a variety of pathogenic bacteria and their toxins [103]. A small unit of *P. vannamei* hemocyanin had O-glycans closely associated with its agglutination activity toward *Vibrio fluvialis, V. alginolyticus,* and *V. parahaemolyticus* ([104, 105).

A mucin-like peritrophin-like gene from fleshy shrimp (*Fenneropenaeus chinensis)* could bind Gram-negative bacteria [106], while another mucin-like peritrophin-like gene from the shrimp *Exopalaemon carinicauda* is involved in the white spot syndrome virus infection [107]. In addition, a mucin-like peritrophin is implicated in the *V. harveyi* infection in the black tiger shrimp *P. monodon* ([108]. Abiotic characteristics, such as the decrease of temperature and type of diet, increase the expression of several mucin-like proteins in *P. vannamei* [109–111]. These modifications could be related in the pathologic development of *Vibrio* infection, increasing their binding targets on the shrimp digestive system. Searching for possible receptors for the lectin-like PirB^{Vp} [96] found some correspondence with a mucin-like protein expressed in the shrimp Hp of *P. vannamei*. These studies are the beginning of a better understanding of the infection mechanisms of *Vp* in shrimp.

6.3. *Receptor on shrimp hemocytes*

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The PirAB^{Vp} toxin is known to mainly target the epithelial cells of the shrimp hepatopancreatic tubules. In addition, [112] found that the PirAB^{Vp} toxin binds to epithelial cells of the digestive tract and produces similar lesions in the midgut and hindgut regions in germ-free brine shrimp, *Artemia*. Moreover, the dysregulation of apoptosis-related genes in *Vp* AHPND-challenged *P. vannamei* hemocytes suggests that *Vp* AHPND induces apoptosis in hemocytes [113]). For the *Bt* Cry toxin, apoptosis is induced by a series of processes that start with the interaction between the Cry1A toxin and the carbohydrate moiety (surface receptor binding) of an N aminopeptidase (APN) [114]. In the transcriptome of *Vp* AHPND-challenged *P. vannamei* was identified an aminopeptidases N1 (*Lv*APN1) gene [115]. DNA sequence analysis of the *LvAPN*1 gene showed a putative C-terminal transmembrane domain and various putative N- and O-glycosylation sites. The expression of *Lv*APN1 increases in hemocytes, after challenging *P. vannamei* with either *Vp* AHPND or the partially purified *Vp* AHPND toxins. Silencing of *Lv*APN1 significantly reduced *Lv*APN1 transcription levels in the stomach, hepatopancreas, and hemocytes, and increased the survival of adult *P. vannamei* that were challenged with the partially purified *Vp* AHPND toxins. These observations suggest the putative role of *Lv*APN1 as a PirAB^{Vp} toxin receptor located in the surface of hemocytes [115]. Other putative carbohydrate receptors for the PirAB^{Vp} toxin could be located in the surface of *P. vannamei* hemocytes as these cells express a plethora of glycoconjugates. Using commercial lectins with different carbohydrate specificity was proved the presence of carbohydrate moieties containing mainly N-acetyl-glucosamine (GlcNAc) and N-acetylneuraminic acid (sialic acid) [116]. In other study, these carbohydrates were recognized by the rPirB^{Vp} subunit [61].

6.4. Search of potential inhibitors of the PirAB^{Vp} toxin.

Understanding the structural biology of PirAB^{Vp} becomes essential in finding or developing antiadhesive agents or receptor analogs that could prevent adhesion and subsequent cell entry of the toxin and inhibit its activity. In particular, it is important to decipher the role and structure features of complex carbohydrates that serve as toxin receptors. According to research by our group, the PirB subunit presents lectin activity and its adhesion can be inhibited in the presence of fucosylated glycans and by those that contain N-acetyl glucosamine [61, 96].

In addition to glycans, the study of peptides that can interact with PirAB^{Vp} also acquires importance. Computational tools like molecular docking can play an important role in the search of antiadhesive peptides or in the design of antiadhesive peptides analogs through construction of precise structural models of peptide-toxin complexes and calculation of binding free energies [117, 118]. An interesting aspect is the search for bifunctional peptides that can be used to improve shrimp growth performance and, at the same time, protect them from the PirAB^{Vp} toxin. An example are the oilseed peptides that contribute to improved shrimp health and growth performance when used as shrimp feed ingredients [119]. In-silico studies reveal six dual-target peptides from different oilseed proteins capable of intercepting the PirA^{Vp}/PirB^{Vp} complex formation. Such peptides (1139–2977 Da in mass and 10–28 residues in length), are possible candidates for the future development of peptide-based anti-AHPND agents [118].

7. Concluding Remarks and Future Perspectives

To date, the mechanism(s) of the PirAB^{Vp} that cause AHPND in penaeid shrimp is far to be known. However, although, we currently know more about the pVA1 virulent plasmid, there is a lack of studies about the variability of the plasmid copy number per bacterial cell and how it influences the pathogenesis of AHPND. According to our studies, we suggest that *Vp* AHPND strains could use the T6SS1 as a selective advantage during

shrimp intoxication by killing surrounding bacteria. Future studies on the type and activity of effector proteins of the T6SS in *Vp* AHPND during an infection process can help to design strategies to control AHPND-causing strains. Although studies have reported that environment factors, like salinity, can affect the production of toxins and shrimp survival, more experiments are needed to determine the influence of salinity on the expression of *pirAB*^{Vp} genes and the pathogenesis of AHPND. Furthermore, there is evidence that the PirA^{Vp} subunit could be playing an initial stabilizing role allowing PirB^{Vp} to bind with higher affinity to different glycan receptors, at least a mucin-like receptor, located at the surface membrane of the cell hepatopancreas, and an internal hexosaminidase glycoprotein receptor that could be involved in the PirAB^{Vp} toxin. These studies are the beginning of a better understanding of the infection mechanisms of *Vp* in shrimp.

The structural biology of PirAB^{Vp} becomes essential to find or develop antiadhesive agents or receptor analogs that could prevent adhesion and subsequent cell entry of the toxin. It is important to elucidate the role and structure features of complex carbohydrates that serve as toxin receptors. The PirB^{Vp} subunit presents lectin activity and, therefore, its adhesion can be inhibited by glycans. In this sense, research with putative glycomimetic antagonists, like fucoidan, will provide new directions for the future development of PirAB^{Vp} inhibitors.

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References

1. Tran, L.H.; Nunan, L.; Redman, R.M.; Mohney, L.L.; Pantoja, C.R.; Fitzsimmons, K.; Lightner, D.V. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Dis Aquat Org*, **2013**, *105*, 45–55.
2. OIE. 2019. *Manual of diagnostic tests for aquatic animals (2019)*. Paris, World Organisation for Animal Health.
3. Joshi, J.; Srisala, J.; Truong, V.H.; Chen, I.T.; Nuangsaeng, B.; Suthienkul, O.; Lo, C.F.; Flegel, T.W.; Sritunyalucksana, K.; Thitamadee, S. Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). *Aquaculture*, **2014**, *428*, 297–302.
4. Kondo, H.; Van, P.T.; Dang, L.T.; Hirono, I. Draft genome sequence of non-*Vibrio parahaemolyticus* acute hepatopancreatic necrosis disease strain KC13.17.5, isolated from diseased shrimp in Vietnam. *Genome Announc*, **2015**, *3*: e00978–15.
5. Feng B.; Liu H.; Wang M.; Sun X.; Pan Y. Diversity analysis of acute hepatopancreatic necrosis disease-positive *Vibrio parahaemolyticus* strains. *Aquac Fish*, **2017**, *2*, 278–285.
6. Nunan, L.; Lightner, D.; Pantoja, C.; Gomez-Jimenez, S. Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. *Dis Aquat Org*, **2014**, *111*, 81–86
7. Restrepo, L.; Bayot, B.; Betancourt, I.; Pinzón, A. Draft genome sequence of pathogenic bacteria *Vibrio parahaemolyticus* strain Ba94C2, associated with acute hepatopancreatic necrosis disease isolate from South America. *Genom Data*, **2016**, *9*,143–144.
8. Dhar, A.K.; Piamsomboon, P.; Caro, L.F.A.; Kanrar, S.; Adami Jr, R.; Juan, Y.S. First report of acute hepatopancreatic necrosis disease (AHPND) occurring in the USA. *Dis Aquat Org*, **2019**, *132(3)*, 241-247.
9. Shinn, A.P.; Pratoomyot, J.; Griffiths, D.; Trong, T.Q., Vu, N.T.; Jiravanichpaisal, J.; Briggs, M. Asian shrimp production and the economic costs of disease. *Asian Fish Sci*, **2018**, *31S*, 29–58.

10. Lee, C.T.; Chen, I.T.; Yang, Y.T.; Ko, T.P.; Huang, Y.T.; Huang, J.Y.; Huang, M.F.; Lin, S.J.; Chen, C.Y.; Lin, S.S.; Lightner, D.V.; Wang, H.C.; Wang, A.H.; Wang, H.C.; Hor, L.I.; Lo C.F. The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. *Pro Natl Acad Sci*, **2015**, *112*, 10798–10803.
11. Soto-Rodriguez, S.A.; Gomez-Gil, B.; Lozano-Olvera, R.; Betancourt-Lozano, M.; Morales-Covarrubias, M.S. Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease (AHPND) of cultured shrimp (*Litopenaeus vannamei*) in northwestern Mexico. *Appl Environ Microbiol*, **2015**, *81*, 1689–1699.
12. Liu, L.; Xiao, J.; Xia, X.; Pan, Y.; Yan, S.; Wang, Y. Draft genome sequence of *Vibrio owensii* strain SH-14, which causes shrimp acute hepatopancreatic necrosis disease. *Genome Announc.*, **2015**, *3*, e01395–15.
13. Liu, L.; Xiao, J.; Zhang, M.; Zhu, W.; Xia, X.; Dai, W.; Pan, Y.; Yan, S.; Wang, Y. A *Vibrio owensii* strain as the causative agent of AHPND in cultured shrimp, *Litopenaeus vannamei. J Invert Pathol*, **2018**, *153*, 156–164
14. Ahn, Y.S.; Piamsomboond, P.; Tang, K.F.J.; Han, J.E.; Kim, J.H. Complete genome sequence of AHPND-causing *Vibrio campbellii* LA16-V1 isolated from *Penaeus vannamei* cultured in a Latin American country. *Gene Announ*, **2017**, *5*, e01011–17.
15. Dong, X.; Wang, H.; Xie, G.; Zou, P.; Guo, C.; Liang, Y.; Huang, J. An isolate of Vibrio *campbellii* carrying the pirVP gene causes acute hepatopancreatic necrosis disease. *Emerg Microbiol Infect*, **2017a**, *6(1),* 1-3.
16. Dong, X.; Wang, H.; Zou, P.; Chen, J.; Liu, Z.; Wang, X.; Huang, J. Complete genome sequence of *Vibrio campbellii* strain 20130629003S01 isolated from shrimp with acute hepatopancreatic necrosis disease. *Gut Pathog*, **2017**, *9*, 31–35.
17. Restrepo, L.; Bayot, B.; Arciniegas, S.; Bajaña, L.; Betancourt, I.; Panchana, F.; Reyes Muñoz, A. PirVP genes causing AHPND identified in a new *Vibrio* species (*Vibrio punensis*) within the commensal *Orientalis* clade. *Sci Rep*, **2018**, *8*:13080.
18. Xiao, J.; Liu, L.; Ke, Y.; Li, X.; Liu, Y.; Pan, Y.; Yan, S.; Wang, Y. Shrimp AHPND-causing plasmids encoding the PirAB toxins as mediated by pirAB-Tn903 are prevalent in various *Vibrio* species. *Sci Rep*, **2017**, *7*, 42177
19. Dong, X.; Chen, J.; Song, J.; Wang, H.; Wang, W.; Ren, Y.; Guo, C.; Wang, X.; Tang, K.F.J.; Huang, J. Evidence of the horizontal transfer of pVA1-type plasmid from AHPND-causing *V. campbellii* to non-AHPND *V. owensii*. *Aquaculture*, **2019**, *503*, 396-402.
20. Dong, X.; Song, J.; Chen, J.; Bi, D.; Wang, W.; Ren, Y.; Wang, H.; Wang, G.; Tang, K.F.J.; Wang, X.; Huang, J. Conjugative transfer of the pVA1-type plasmid carrying the *pirABvp* genes results in the formation of new AHPND-causing *Vibrio*. *Front Cell Infect Microbiol*, **2019**, *9*, 195–205.
21. FAO. Report of the FAO/MARD technical workshop on early mortality syndrome (EMS) or acute hepatopancreatic necrosis syndrome (AHPNS) of cultured shrimp (under TCP/VIE/3304), Hanoi, Vietnam, 25 to 27 June 2013. FAO Fisheries and Aquaculture report no. 1053. Rome, Italy, 2013.
22. Powers, Q.; Caro, L. F. A.; Fitzsimmons, K. M.; McLain, J. E.; Dhar, A. K. Crayfish (*Cherax quadricarinatus*) susceptibility to acute hepatopancreatic necrosis disease (AHPND). *J Invert Pathol*, **2021**, 107554.
23. Soto-Rodriguez, S.A.; Gomez-Gil, B.; Lozano-Olvera, R.; Bolan-Mejía, C.; Aguilar-Rendon, K.G.; Enciso-Ibarra, J. Pathological, genomic and phenotypical characterization of *Vibrio parahaemolyticus*, causative agent of acute hepatopancreatic necrosis disease (AHPND) in Mexico. *Asian Fish Sci*, **2018**, *31*, 102–111.
24. Lightner, D.V., 1996. A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. World Aquaculture Society. Baton Rouge, LA. USA.
25. González-Gómez, J.P.; Soto-Rodriguez, S.; López-Cuevas, O.; Castro-del Campo, N.; Chaidez, C.; Gomez-Gil, B. Phylogenomic Analysis Supports Two Possible Origins for Latin American Strains of *Vibrio parahaemolyticus* Associated with Acute Hepatopancreatic Necrosis Disease (AHPND). *Curr Microbiol*, **2020**, *77*, 3851–3860.
26. Salyer, A.; Whitt, D. (2002). *Bacterial Pathogenesis, a Molecular Approach.* (ASS press, Ed.) (Second Ed.). EUA.

27. Donnenberg, M.S. Pathogenic strategies of enteric bacteria. *Nature*, **2000**, *406*, 768-774
28. Casadevall, A.; Pirofski, L. Host-pathogen interactions: the attributes of virulence. *J Infec Dis*, **2001**, *184(3)*, 337–344.
29. Wurtzel, O.; Sesto, N.; Mellin, J. R.; Karunker, I.; Edelheit, S.; Bécavin, C.; Archambaud, C.; Cossart, P.; Sorek, R. Comparative transcriptomics of pathogenic and non-pathogenic *Listeria* species. *Mol Sys Biol*, **2012**, *8(1)*, 1–14.
30. Han, J.E.; Tang, K.F.J.; Tran, L.H.; Lightner, D.V. *Photorhabdus* insect-related (Pir) toxin-like genes in a plasmid of *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. *Dis Aquat Org*, **2015**, *113*, 33–40.
31. Aguilar-Rendón, K.G.; Soto-Rodriguez, S.A.; Gomez-Gil, B.; Lozano-Olvera, R.; Yáñez-Rivera, B. Water microbiome dynamics of Pacific white shrimp *Penaeus vannamei* infected with *Vibrio parahaemolyticus* strains responsible for acute hepatopancreatic necrosis disease, Aquaculture in press.
32. Sirikharin, R.; Taengchaiyaphum, S.; Sanguanrut, P.; Chi, T.D.; Mavichak, R.; Proespraiwong, P.; Nuangsaeng, B.; Thitamadee, S.; Flegel, T.W.; Sritunyalucksana, K. Characterization and PCR detection of binary, Pir-Like toxins from *Vibrio parahaemolyticus* isolates that cause acute hepatopancreatic necrosis disease (AHPND) in shrimp. *PLoS One*, **2015**, *10(5)*, e0126987.
33. Tinwongger, S.; Nochiri, Y.; Thawonsuwan, J.; Nozaki, R.; Kondo, H.; Awasthi, S.P.; Hinenoya, A.; Yamasaki, S.; Hirono, I. Virulence of acute hepatopancreatic necrosis disease PirAB-like relies on secreted proteins not on gene copy number. *J Appl Microbiol*, **2016**, *121(6)*, 1755–1765.
34. Aguilar-Rendón, K.G.; Lozano-Olvera, R.; Yáñez-Rivera, B.; Soto-Rodriguez, S.A. Bacteriological and histopathological analysis of *Penaeus vannamei* experimentally infected with Vibrio parahaemolyticus-AHPND strains. *Dis Aquat Org*, **2020**, *140*, 167-177.
35. Hong, X.P.; Xu, D.; Zhuo, Y.; Liu, H.Q.; Lu, L.Q. Identification and pathogenicity of *Vibrio* parahaemolyticus isolates and immune responses of *Penaeus* (Litopenaeus) *vannamei* (Boone). *J Fish Dis*, **2016**, *39(9)*, 1085–1097.
36. Phiwsaiya, K.; Charoensapsri, W.; Taengphu, S.; Dong, H.T.; Sangsuriya, P.; Nguyen, G.T.T.; Pham, H.Q.; Amparyup, P.; Sritunyalucksana, K.; Taengchaiyaphum, S.; Chaivisuthangkura, P.; Longyant, S.; Sithigorngul, P.; Senapin, S. A natural *Vibrio parahaemolyticus* ΔpirAVppirBVp+ mutant kills shrimp but produces neither PirVp toxins nor acute hepatopancreatic necrosis disease lesions. *Appl Environ Microbiol*, **2017**, *83*, e00680-17.
37. Han, J. E.; Choi, S. K.; Han, S. H.; Lee, S. C.; Jeon, H. J.; Lee, C.; Lee, K. J. Genomic and histopathological characteristics of *Vibrio parahaemolyticus* isolated from an acute hepatopancreatic necrosis disease outbreak in Pacific white shrimp (*Penaeus vannamei*) cultured in Korea. *Aquaculture*, **2020**, *524*, 735284.
38. Han, J.E.; Tang, K.F.J.; Aranguren, L.F.; Piamsomboon, P. Characterization and pathogenicity of acute hepatopancreatic necrosis disease natural mutants, pirAB$_{vp}$(-) *Vibrio parahaemolyticus*, and pirAB$_{vp}$ (+) *Vibrio campbellii* strains. *Aquaculture*, **2017**, *470*, 84–90.
39. Dong, X., Wang, H., Xie, G., Zou, P., Guo, C., Liang, Y., & Huang, J. An isolate of Vibrio campbellii carrying the pirVP gene causes acute hepatopancreatic necrosis disease. *Emerg Microbes Infect*, **2017**, *6(1)*, 1-3.
40. Vicente, A.; Taengphu, S.; Hung, A.L.; Mora, C.M.; Dong, H.T.; Senapin, S. Detection of *Vibrio campbellii* and *V. parahaemolyticus* carrying full-length pirABVp but only *V. campbellii* produces PirVp toxins. *Aquaculture*, **2020**, *519*, 734708.
41. Lightner, D.V.; Redman, R.M.; Pantoja, C.R.; Noble, B.L.; Tran, L.H. Early mortality syndrome affects shrimp in Asia. *Glob Aquacult Advocate*, **2012,** *201*, 40.
42. Caro, L.F.A.; Mai, H.N.; Noble, B.; Dhar, A.K. Acute hepatopancreatic necrosis disease (*VP*AHPND), a chronic disease in shrimp (*Penaeus vannamei*) population raised in latin America. *J Invertebr Pathol*, **2020**, *174*, 107424.
43. Han, J.E.; Tang, K.F.J.; Lightner, D.V. Genotyping of virulence plasmid from *Vibrio parahaemolyticus* isolates causing acute hepatopancreatic necrosis disease in shrimp. *Dis Aquat Org*, **2015**, *115*, 245–251.

44. Cardona, E.; Gueguen, Y.; Magré, K.; Lorgeoux, B.; Piquemal, D.; Pierrat, F.; Noguier, F. Saulnier, D. Bacterial community characterization of water and intestine of the shrimp *Litopenaeus stylirostris* in a biofloc system. *BMC Microbiol*, **2016**, *16*, 1-9.
45. Md Zoqratt, M.; Eng, W.W.H.; Thai, B.T.; Austin, C.M.; Gan, H.M. Microbiome analysis of Pacific white shrimp gut and rearing water from Malaysia and Vietnam: implications for aquaculture research and management. *PeerJ*, **2018**, *6*, e5826.
46. Rungrassamee, W.; Klanchui, A.; Chaiyapechara, S.; Maibunkaew, S.; Tangphatsornruang, S.; Jiravanichpaisal, P.; Karoonuthaisiri, N. Bacterial population in intestines of the black tiger shrimp (*Penaeus monodon*) under different growth stages. *PloS One*, **2013**, *8*, 60802.
47. Zhang. M.; Sun, Y.; Chen, K.; Yu, N.; Zhou, Z.; Chen, L.; Du, Z.; Li, E. Characterization of the intestinal microbiota in Pacific white shrimp, *Litopenaeus vannamei*, fed diets with different lipid sources. *Aquaculture*, **2014**, *434*, 449–455.
48. Tzeng, T.D., Pao, Y.Y., Chen, P., Weng, F., Jean, W.D., Wang, D. Effects of host phylogeny and habitats on gut microbiomes of oriental river prawn (*Macrobrachium nipponense*). *PLoS One*, **2015,** *10(7)*, e0132860.
49. Zhang, H.; Sun, Z.; Liu, B.; Xuan, Y.; Jiang, M.; Pan, Y.; Zhang, Y. Dynamic changes of microbial communities in *Litopenaeus vannamei* cultures and the effects of environmental factors. *Aquaculture*, **2016**, *455*, 97–108.
50. Holt, C.C.; Bass, D.; Stentiford, G.D.; van der Giezen, M. Understanding the role of the shrimp gut microbiome in health and disease. *J Invertebr Pathol*, **2020**,107387.
51. Chen, W.Y.; Ng, T.H.; Wu, J.H.; Chen, J.W.; Wang, H.C. Microbiome dynamics in a shrimp grow-out pond with possible outbreak of acute hepatopancreatic necrosis disease. *Sci Rep*, **2017**, *7*, 9395.
52. Yao, Z.; Yang, K.; Huang, L.; Huang, X.; Qiuqian, L.; Wang, K.; Zhang, D. Disease outbreak accompanies the dispersive structure of shrimp gut bacterial community with a simple core microbiota. *AMB Express*, **2018**, *8*, 1–10.
53. Yang, Q.; Dong, X.; Xie, G.; Fu, S.; Zou, P.; Sun, J.; Wang, Y.; Huang, J. Comparative genomic analysis unravels the transmission pattern and intra-species divergence of acute hepatopancreatic necrosis disease (AHPND)-causing *Vibrio parahaemolyticus* strains. *Mol Genet Genomics*, **2019**, *294*, 1007–1022.
54. Kohl, K.D.; Dearing, M.D. The woodrat gut microbiota as an experimental system for understanding microbial metabolism of dietary toxins. *Front Microbiol*, **2016**, *7*, 1165.
55. Xiong, J.; Zhu, J.; Dai, W.; Dong, C.; Qiu, Q.; Li, C. Integrating gut microbiota immaturity and disease discriminatory taxa to diagnose the initiation and severity of shrimp disease. *Environ Microbiol*, **2017**, *19*, 1490–1501.
56. Yu, Y.; Yang, H.; Li, J.; Zhang, P.; Wu, B., Zhu, B.; Zhang, Y.; Fang, W. Putative type VI secretion systems of *Vibrio parahaemolyticus* contribute to adhesion to cultured cell monolayers. *Arch Microbiol*, **2012**, *194*, 827–835.
57. Lien, Y.W.; Lai, E.M. Type VI secretion effectors: methodologies and biology. *Front Cell Infect Microbiol*, **2017,** *15*, 7:254.
58. Pinoargote, G., Flores, G., Cooper, K., Ravishankar, S. Effects on survival and bacterial community composition of the aquaculture water and gastrointestinal tract of shrimp (*Litopenaeus vannamei*) exposed to probiotic treatments after an induced infection of acute hepatopancreatic necrosis disease. Aquaculture, **2018**, *49*, 3270–3288.
59. Diéguez, A.L.; Balboa, S.; Magnesen, T.; Romalde, J.L. Neptuniibacter pectenicola sp. nov. and *Neptuniibacter marinus* sp. nov., two novel species isolated from a Great scallop (*Pecten maximus*) hatchery in Norway and amended description of the genus *Neptuniibacter*. *Syst App. Microbiol*, **2017**, *40*, 80–85.
60. Salomon, D.; Gonzalez, H.; Updegraff, B.L.; Orth, K. *Vibrio parahaemolyticus* Type VI secretion system 1 is activated in marine conditions to target bacteria, and is differentially regulated from system 2. *PLoS One,* **2013**, *8(4)*, e61086.
61. Victorio-De Los Santos, M.; Vibanco-Pérez, N.; Soto-Rodriguez, S.; Pereyra, A.; Zenteno, E.; Cano-Sánchez, P. The B Subunit of PirAB(vp) Toxin Secreted from *Vibrio parahaemolyticus* Causing AHPND Is an Amino Sugar Specific Lectin. *Pathogens*, **2020**, *9*, 182.

62. Fu, S., Wang, L., Tian, H., Wei, D., Liu, Y. Pathogenicity and genomic characterization of *Vibrio parahaemolyticus* strain PB1937 causing shrimp acute hepatopancreatic necrosis disease in China. *Ann Microbiol*, **2018**, *68*, 175–184.
63. Li, P.; Kinch, L.N.; Ray, A.; Dalia, A.B.; Cong, Q.; Nunan, L.M.; Camilli, A.; Grishin, N.V.; Salomon, D.; Orth, K. Acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* strains maintain an antibacterial type VI secretion system with versatile effector repertoires. *Appl Environ Microbiol*, **2017,** *83*; 3:e00737–17.
64. Johnson, D.I., 2018. Bacterial virulence factors. In: Johnson DI (ed.). Bacterial Pathogens and Their Virulence Factors. Springer International Publishing. 1–38.
65. Russell, A.B.; Singh, P.; Brittnacher, M.; Bui, N.K.; Hood, R.D.; Carl, M.A.; Agnello, D.M.; Schwarz, S.; Goodlett, D.R.; Vollmer, W.; Mougous, J. A widespread bacterial type VI secretion effector superfamily identified using a heuristic approach. *Cell Host Microbe*, **2012**, *11*, 538–549.
66. Papenfort, K.; Bassler, B.L. Quorum sensing signal-response systems in Gram-negative bacteria. *Nat Rev Microbiol,* **2016**, *14*, 576–588.
67. Rutherford, S.T.; Bassler, B.L. Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb Perspect Med,* **2012**, *2(11)*, a012427.
68. Federle, M.J.; Bassler, B.L. Interspecies communication in bacteria. *J Clin Invest*, **2003**, *112*, 1291-1299.
69. Wellington, S.; Greenberg, E.P.; Winans, S.C.; Blackwell, H.; Venturi, V. Quorum sensing signal selectivity and the potential for interspecies cross talk. *mBio,* **2019**, *10(2)*, e00146–00119.
70. Makino, K.; Oshima, K.; Kurokawa, K.; Yokoyama, K.; Uda, T.; Tagomori, K.; Iijima, Y.; Najima, M.; Nakano, M.; Yamashita, A.; Kubota, Y.; Kimura, S.; Yasunaga, T.; Honda, T.; Shinagawa, H.; Hattori, M.; Iida, T. Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V cholerae*. *Lancet*, **2003**, *361*, 743–749.
71. Jaques, S.; McCarter, L.L. Three new regulators of swarming in *Vibrio parahaemolyticus*. *J Bacteriol*, **2006**, *188*, 2625–2635.
72. Eickhoff, M.J.; Fei, C.; Huang, X.; Bassler, B.L. LuxT controls specific quorum-sensing-regulated behaviors in *Vibrionaceae* spp. via repression of qrr1, encoding a small regulatory RNA. *PLoS Genet*, **2021**, *17(4)*, e1009336.
73. Zhang, Y.; Qiu, Y.; Tan, Y.; Guo, Z.; Yang, R.; Zhou, D. Transcriptional regulation of opaR, qrr2-4 and aphA by the master quorum-sensing regulator OpaR in *Vibrio parahaemolyticus*. *PLoS One*, **2012**, *7*, e34622–e34622.
74. Pumkaew, M.; Taengchaiyaphum, S.; Powtongsook, S.; Pungrasmi, W.; Sritunyalucksana, K. Production of acute hepatopancreatic necrosis disease toxin is affected by addition of cell-free supernatant prepared from Al-2-producing *Vibrio harveyi* mutant. *J World Aquac Soc,* **2019**, *50*.
75. Federle, M.J. Autoinducer-2-based chemical communication in bacteria: complexities of interspecies signaling. *Contrib Microbiol,* **2009**, *16*, 18-32.
76. Takemura, A.F.; Chien, D.M.; Polz, M.F. Associations and dynamics of Vibrionaceae in the environment, from the genus to the population level. *Front Microbiol*, **2014**, *5*, 38.
77. Williams, S.L.; Jensen, R.V.; Kuhn, D.D.; Stevens, A.M. Analyzing the metabolic capabilities of a *Vibrio parahaemolyticus* strain that causes Early Mortality Syndrome in shrimp. *Aquaculture,* **2017**, *476*, 44–48.
78. Fu, S.; Wei, D.; Yang, Q.; Xie, G.; Pang, B.; Wang, Y.; Lan, R.; Wang, Q.; Dong, X.; Zhang, X.; et al. Horizontal plasmid transfer promotes the dissemination of Asian acute hepatopancreatic necrosis disease and provides a novel mechanism for genetic exchange and environmental adaptation. *mSystems,* **2020**, *5*, e00799-00719.
79. Pragthong, P.; Chirapongsatonkul, N. Temperature-dependent expression of virulence genes in *Vibrio parahaemolyticus* AHPND strain (VpAHPND). *Int J Agric Technol,* **2020,** *16(5),* 1185–1198.
80. Schofield, P.J.; Noble, B.L.; Caro, L.F.A.; Mai, H.N.; Padilla, T.J.; Millabas, J.; Dhar, A.K. Pathogenicity of Acute Hepatopancreatic Necrosis Disease (AHPND) on the freshwater

prawn, *Macrobrachium rosenbergii*, and Pacific White Shrimp, *Penaeus vannamei*, at various salinities. *Aquac Res,* **2021**, *52*, 1480–1489.

81. López-Cervantes, G.; Álvarez-Ruiz, P.; Luna-Suárez, S.; Luna-González, A.; Esparza-Leal, H.M.; Castro-Martínez, C.; Gámez-Jiménez, C.; Soto-Alcalá, J. Temperature and salinity modulate virulence and PirA gene expression of *Vibrio parahaemolyticus*, the causative agent of AHPND. *Aquac Int*, **2021**, *29*, 743–756.
82. Soto-Rodriguez, S.; Lozano Olvera, R.; Palacios-Gonzalez, D.; Bolan-Mejía, M.; Aguilar-Rendon, K. Characterization and growth conditions of *Vibrio parahaemolyticus* strains with different virulence degrees that cause acute hepatopancreatic necrosis disease in *Litopenaeus vannamei*. *J World Aquac Soc,* **2019**, *50*, 1–14.
83. Sultana, T.; Haque, M.; Salam, M.; Alam, M. Effect of aeration on growth and production of fish in intensive aquaculture system in earthen ponds. *J Bangladesh Agric Univ,* **2017**, *15*, 113–122.
84. Kumar, V.; Roy, S.; Baruah, K.; Van Haver, D.; Impens, F.; Bossier, P. Environmental conditions steer phenotypic switching in acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus*, affecting PirAVP/PirBVP toxins production. *Environ Microbiol*, **2020**, *22*, 4212–4230.
85. Flemming, H.-C.; Wuertz, S. Bacteria and archaea on Earth and their abundance in biofilms. *Nat Rev Microb*, **2019**, *17*, 247-260.
86. Mizan, M.F.; Jahid, I.K.; Kim, M.; Lee, K.H.; Kim, T.J.; Ha, S.D. Variability in biofilm formation correlates with hydrophobicity and quorum sensing among *Vibrio parahaemolyticus* isolates from food contact surfaces and the distribution of the genes involved in biofilm formation. *Biofouling,* **2016**, *32*, 497–509.
87. Zhang, Y.; Hu, L.; Osei-Adjei, G.; Zhang, Y.; Yang, W.; Yin, Z.; Lu, R.; Sheng, X.; Yang, R.; Huang, X.; et al. Autoregulation of ToxR and Its Regulatory Actions on Major Virulence Gene Loci in *Vibrio parahaemolyticus*. *Front Cell Infect Microbiol,* **2018b**, *8*, 291–291.
88. Yildiz, F.H.; Visick, K.L. Vibrio biofilms: so much the same yet so different. *Trends Microbiol*, **2009**, 17*,* 109–118.
89. Henke, J.M.; Bassler, B.L. Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. *J Bacteriol,* **2004**, *186*, 3794–3805.
90. Zhu, J.; Miller, M.B.; Vance, R.E.; Dziejman, M.; Bassler, B.L.; Mekalanos, J.J. Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci,* **2002**, *99*, 3129–3134.
91. Sharon, N. Carbohydrates as future anti-adhesion drugs for infectious diseases. *Biochim Biophys Acta Gen Subj,* **2006**, *1760 (4)*, 527–537.
92. Lin, S.J.; Hsu, K.C.; Wang, H.C. Structural insights into the cytotoxic mechanism of *Vibrio parahaemolyticus* PirAvp and PirBvp toxins. *Mar Drugs*, **2017,** *15(12),* 373.
93. Lin, S.J.; Chen, Y.F.; Hsu, K.C.; Chen, Y.L.; Ko, T.P.; Lo, C.F.; Wang, H.C.; Wang, H.C. Structural insights to the heterotetrameric interaction between the *Vibrio parahaemolyticus* PirAvp and PirBvp toxins and activation of the cry-like pore-forming domain. *Toxins*, **2019**, *11(4),* 233.
94. Sengupta, A.; Sarkar, A.; Priya, P.; Ghosh Dastidar, S.; Das, S. New insight to structure-function relationship of GalNAc mediated primary interaction between insecticidal Cry1Ac toxin and HaALP receptor of *Helicoverpa armigera*. *PLoS One,* **2013,** *8(10),* e78249–e78249.
95. Kitami, M.; Kadotani, T.; Nakanishi, K.; Atsumi, S.; Higurashi, S.; Ishizaka, T.; Watanabe, A.; Sato, R. *Bacillus thuringiensis* cry toxins bund specifically to various proteins via domain III, which had a galactose-binding domain-like fold. *Biosci Biotechnol Biochem*, **2011**, *75*, 305–312.
96. De Los Santos, M.V.; Sánchez-Salgado, J.L.; Pereyra, A.; Zenteno, E.; Vibanco-Pérez, N.; Ramos-Clamont Montfort, G.; Soto-Rodriguez, S.A. The *Vibrio parahaemolyticus* subunit toxin PirB(vp) recognizes glycoproteins on the epithelium of the *Penaeus vannamei* hepatopancreas. *Comp Biochem Physiol B, Biochem Mol Biol*, **2021**, *257*, 110673.
97. Fantus, I.G.; Goldberg, H.J.; Whiteside, C.I. The hexosamine biosynthesis pathway. 1st ed.; Humana Press, Totowa, New Jersey, EE.UU: **2006**; pp. 117-133.

98. Xie, X.-L.; Huang, Q.-S.; Wang, Y.; Ke, C.-H.; Chen, Q.-X. Modification and Modificatory Kinetics of the Active Center of Prawn β-N-Acetyl-D-glucosaminidase. *J Biomol Struct Dyn*, **2009**, *26*, 781–786.

99. Song, Y.; Evenseth, L.M.; Iguchi, T.; Tollefsen, K.E. Release of chitobiase as an indicator of potential molting disruption in juvenile *Daphnia magna* exposed to the ecdysone receptor agonist 20-hydroxyecdysone. *J Toxicol Enviro Health Part A*, **2017**, *80*, 954–962.

100. Ettrich, R.; Kopecký, V., Jr.; Hofbauerová, K.; Baumruk, V.; Novák, P.; Pompach, P.; Man, P.; Plíhal, O.; Kutý, M.; Kulik, N.; Sklenář, J.; Rysľavá, H.; Křen, V.; Bezouška, K. Structure of the dimeric N-glycosylated form of fungal beta-N-acetyl hexosaminidase revealed by computer modeling, vibrational spectroscopy, and biochemical studies. *BMC Struct Biol*, **2007**, *7*, 32-32.

101. Weitz, G.; Proia, R.L. Analysis of the glycosylation and phosphorylation of the alpha-subunit of the lysosomal enzyme, beta- hexosaminidase A, by site-directed mutagenesis. *J Biol Chem*, **1992**, *267*, 10039–10044.

102. Zhu, F.; Li, D.; Chen, K. Structures and functions of invertebrate glycosylation. *Open Biol*, **2019**, *9*, 180232–180232.

103. Erlandson, M.A.; Toprak, U.; Hegedus, D.D. Role of the peritrophic matrix in insect-pathogen interactions. *J Insect Physiol*, **2019**, *117*, 103894.

104. Zhang, Z.; Wang, F.; Chen, C.; Zheng, Z.; Aweya, J.J.; Zhang, Y. Glycosylation of hemocyanin in *Litopenaeus vannamei* is an antibacterial response feature. *Immunol Let*, **2017**, *192*, 42–47.

105. Zhang, Z.; Li, R.; Aweya, J.J.; Wang, F.; Zhong, M.; Zhang, Y. Identification and characterization of glycosylation sites on *Litopenaeus vannamei* hemocyanin. *FEBS Letters*, **2019**, *593*, 820–830.

106. Du, X.-J.; Wang, J.-X.; Liu, N.; Zhao, X.-F.; Li, F.; Xiang, J. Identification and molecular characterization of a peritrophin-like protein from fleshy prawn (*Fenneropenaeus chinensis*). *Mol Immunol*, **2006,** *43*, 1633-44.

107. Wang, L.; Li, F.; Xiang, J., A new shrimp peritrophin-like gene from *Exopalaemon carinicauda* involved in white spot syndrome virus (WSSV) infection. *Fish Shellfish Immunol*, **2013,** *35*, 840–846.

108. Soonthornchai, W.; Rungrassamee, W.; Karoonuthaisiri, N.; Jarayabhand, P.; Klinbunga, S.; Söderhäll, K.; Jiravanichpaisal, P. Expression of immune-related genes in the digestive organ of shrimp, *Penaeus monodon*, after an oral infection by *Vibrio harveyi*. *Dev Comp Immunol*, **2010,** *34(1)*, 19–28.

109. Duan, Y.; Wang, Y.; Liu, Q.; Dong, H.; Li, H.; Xiong, D.; Zhang, J. Changes in the intestine microbial, digestion and immunity of *Litopenaeus vannamei* in response to dietary resistant starch. *Sci Rep*, **2019**, *9*, 6464.

110. Duan, Y.; Yun, W.; Ding, X.; Xiong, D.; Zhang, J. Response of intestine microbiota, digestion, and immunity in Pacific white shrimp *Litopenaeus vannamei* to dietary succinate. *Aquaculture*, **2019**, *517*, 734762.

111. Wang, Z.; Zhou, J.; Li, J.; Zou, J.; Fan, L. The immune defense response of Pacific white shrimp (*Litopenaeus vannamei*) to temperature fluctuation. *Fish Shellfish Immunol*, **2020**, *103*, 103–110.

112. Kumar, V.; Nguyen, D.V.; Baruah, K.; Bossier, P. Probing the mechanism of VPAHPND extracellular proteins toxicity purified from *Vibrio parahaemolyticus* AHPND strain in germ-free *Artemia* test system. *Aquaculture*, **2019**, *504*, 414–419.

113. Zheng, Z.; Wang, F.; Aweya, J.J.; Li, R.; Yao, D.; Zhong, M.; Li, S.; Zhang, Y. Comparative transcriptomic analysis of shrimp hemocytes in response to acute hepatopancreas necrosis disease (AHPND) causing *Vibrio parahaemolyticus* infection. *Fish Shellfish Immunol*, **2018**, *74*, 10–18.

114. Soberón, M.; Pardo, L.; Muñóz-Garay, C.; Sánchez, J.; Gómez, I.; Porta, H.; Bravo, A. Pore formation by Cry toxins. *Advances Exp Med Biol*, **2010**, *677*, 127–142.

115. Luangtrakul, W.; Boonchuen, P.; Jaree, P.; Kumar, R.; Wang, H.-C.; Somboonwiwat, K. Cytotoxicity of *Vibrio parahaemolyticus* AHPND toxin on shrimp hemocytes, a newly identified target tissue, involves binding of toxin to aminopeptidase N1 receptor. *Plos Pathog*, **2021**, *17*, e1009463.

116. Estrada, N.; Velázquez, E.; Rodríguez-Jaramillo, C.; Ascencio, F. Carbohydrate moieties and cytoenzymatic characterization of hemocytes in white leg shrimp *Litopenaeus vannamei*. *Int J Cell Biol*, **2016**, 9032181.

117. Kuyucak, S.; Norton, R. S. Computational approaches for designing potent and selective analogs of peptide toxins as novel therapeutics. *Future Med Chem*, **2014,** *6*(15), 1645–1658. doi:10.4155/fmc.14.98

118. Ong, J.H.; Wong, W.L.; Wong, F.C.; Chai, T.T. Targeting PirAvp and PirBvp toxins of *Vibrio parahaemolyticus* with oilseed peptides: an *in silico* approach. *Antibiotics*, **2021**, *10*(10), 1211.

119. Shao, J.; Zhao, W.; Han, S.; Chen, Y.; Wang, B.; Wang, L. Partial replacement of fishmeal by fermented soybean meal in diets for juvenile white shrimp (*Litopenaeus vannamei*). *Aquaculture Nutrition*, **2019,** *25*(1), 145–153.