Latest Research on Acute Hepatopancreatic Necrosis Disease (AHPND) of Penaeid Shrimps

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ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND) is caused by unique strains of *Vibrio parahaemolyticus* (VP_{AHPND}) and *V. harveyi* that have transferrable plasmid carrying the virulent PirAB-like toxin genes. The genomes of VP_{AHPND} strains and *V. harveyi* from Thailand and Viet Nam, respectively, have been characterized by our group. The genome of VP_{AHPND} strains from Mexico, Viet Nam, and China have also been studied by other groups. We have developed a conventional polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) methods for the detection of AHPND using a primer set that targets the PirAB-like toxin genes of VP_{AHPND}. We have characterized the toxin genes of VP_{AHPND} strains and also constructed a recombinant plasmid (broad host range) carrying PirAB-like toxin genes. Non-VP_{AHPND} strain N7 which does not carry the plasmid and strain FP11 which is carrying a plasmid not coding for the toxin genes were transformed with the plasmid carrying PirAB-like toxin genes. As a result, the transformed N7 and FP11 strains became virulent and killed whiteleg shrimp (*Penaeus vannamei*) similar to or at par with the virulence of VP_{AHPND} strain. We then fed the whiteleg shrimp with commercial feed containing the formalin-killed VP_{AHPND} strain. After 2 days of feeding, all of the whiteleg shrimp died. These results clearly indicate that the PirAB-like toxin is the virulence factor of VP_{AHPND}.

We have been investigating the virulence mechanism of the PirAB-like toxin produced by VP_{AHPND} strains. First, we calculated the copy number of plasmid encoding the PirAB-like toxin genes of several VP_{AHPND} strains. The copy number of the plasmid varied, ranging from 1 to 36 copies. Interestingly, VP_{AHPND} strains carrying low copy number of plasmid were more virulent than VP_{AHPND} strains carrying high copy number of the plasmid. These results imply that the copy number of toxin genes is not an important factor responsible for the degree of virulence of the VP_{AHPND} strains. We are also studying other factors associated with the virulence of PirAB-like toxin. Likewise, we are developing prevention methods against AHPND including the use of formalin-killed cell vaccine, IgY additive in feed, and nano-bubble treatment of rearing water. This paper summarizes the current R&D on the disease.

Introduction

Acute hepatopancreatic necrosis disease (AHPND), formerly known as early mortality syndrome (EMS), is a devastating disease that has been implicated in mass mortality of cultivated penaeid shrimps in China (2009), Viet Nam (2010), Malaysia (2011), Thailand (2012), Mexico (2014), and recently in the Philippines (2015) (Tran *et al.*, 2013; Joshi *et al.*, 2014; Soto-Rodriguez *et al.*, 2015; Dabu *et al.*, 2015; dela Peña *et al.*, 2015). AHPND induces necrosis in the hepatopancreas of the infected shrimp causing them to become lethargic and anorexic. The disease was first referred to as early mortality syndrome (EMS) because it was typified by shrimp mortality occurring within 30 days after stocking shrimp postlarvae (PL) in grow-out ponds (Lightner *et al.*, 2012). However, there was confusion regarding the usage of the term EMS as mortalities during the early phase of shrimp cultivation could also be attributed to other etiologies, hence, the more precise name AHPND was adapted after the group of D.V. Lightner discovered in 2013 that unique strains

of *V. parahaemolyticus* (VP_{AHPND}) colonizing the stomach of shrimp produced toxin responsible for the sloughing of hepatopancreatic tubule epithelial cells of the hepatopancreas (Tran *et al.*, 2013). By employing an infection bioassay, i.e. through an immersion challenge using *V. parahaemolyticus* isolated from ponds with AHPND outbreak, 100% mortality was obtained in naive shrimp; as the dead shrimp exhibited sloughing of the tubule epithelial cells of the hepatopancreas, thereby satisfying Koch's postulates (Tran *et al.*, 2013). Moreover, they also documented that cell-free broth cultures of VP_{AHPND} could induce the massive sloughing of the hepatopancreatic tubule cells even in the absence of bacterial cells (Tran *et al.*, 2013). Fortunately, all VP_{AHPND} isolates tested did not possess the pathogenicity island (human pathogen markers *tdh* and *trh*) associated with human infections (Nishibuchi *et al.*, 1985; Nishibuchi *et al.*, 1989).

Previous studies on the genome sequences of *V. parahaemolyticus* strains that caused and did not cause AHPND were established to identify the virulence genes that might be involved in the pathogenicity of VP_{AHPND} strains to cultured penaeids (Kondo *et al.*, 2014; Yang *et al.*, 2014; Gomez-Gil *et al.*, 2014). These studies revealed that VP_{AHPND} strains possess a unique 69-kbp plasmid carrying the suspected genes homologous to PirAB toxin genes that encodes for the *Photorhabdus* insect-related (Pir) toxins (Lee *et al.*, 2015), indicating that the PirAB-like toxins are the virulence factors of VP_{AHPND} strains.

Characterization of the causative agent of AHPND and its virulence to penaeid shrimps

AHPND is caused by unique strains of *V. parahaemolyticus* (VP_{AHPND}) and *V. harveyi* that have a transferrable virulent plasmid carrying the PirAB-like toxin genes (Figure 1). However, non-VP_{AHPND} strains also possess the plasmid (Figure 2). To ascertain that shrimps are indeed infected with AHPND, the target region for detection employing the polymerase chain reaction (PCR) method should be the toxin region. The genomes of VP_{AHPND} strains from Thailand, Mexico, and China, as well as *V. harveyi* from Viet Nam and *V. owensii* from China have been studied by several research groups (Kondo *et al.*, 2014, 2015; Yang *et al.*, 2014, Gomez-Gil, *et al.*, 2014, Liu *et al.*, 2015). The findings indicate that spreading of the plasmid coding for the PirAB toxin genes among several bacterial species in the shrimp pond is feasible. This finding further indicates that in worst case scenarios, plasmid transfer from VP_{AHPND} to normal bacterial microbiota in the shrimp pond may inadvertently occur and could result in unwarranted outbreaks of AHPND among pond-cultivated shrimps (Figure 3).

We have developed the conventional PCR method using a primer set that targets the PirA-like toxin gene of *V. parahaemolyticus* (Tinwongger *et al.*, 2014). At present, the

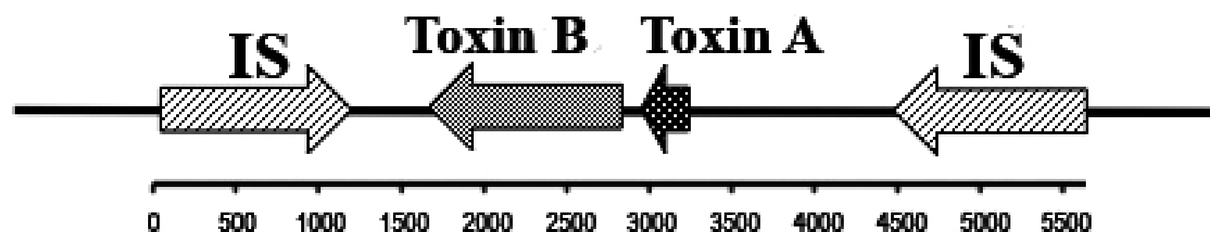

Figure 1. Structure of the toxin genes region of VP_{AHPND}.

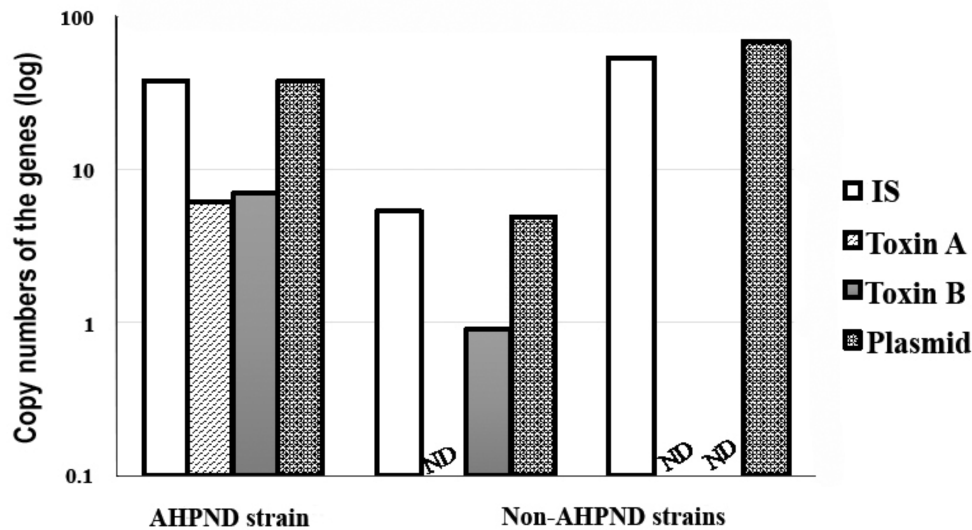

Figure 2. Relative copy number of toxin genes in VP_{AHPND} and non-VP_{AHPND} strains compared to the ToxR gene which is a single copy gene on the genome. ND: Not detected.

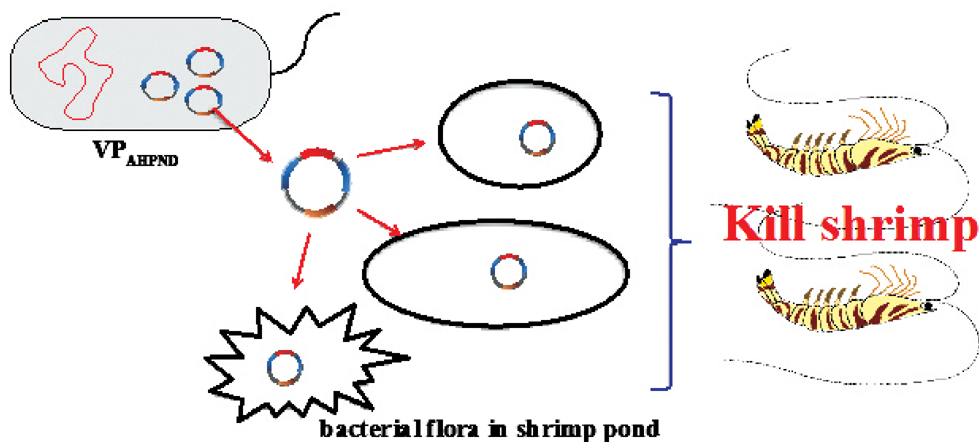

Figure 3. Hypothetical illustration of a worst case scenario of AHPND outbreak in cultured shrimps through inadvertent transfer of plasmid coding for the PirAB toxin genes from VP_{AHPND} to other species of bacterial flora in the shrimp pond.

accuracy of our diagnostic methods for AHPND is 100%. We have also developed a loop-mediated isothermal amplification (LAMP) method for the detection of VP_{AHPND} strains (Koiwai *et al.*, 2016).

We have also confirmed the virulence of PirA-like and PirB-like toxins by using transformant of non-VP_{AHPND} carrying the toxin genes in the broad host range plasmid (Tinwongger *et al.*, 2016, unpublished data). The transformant of non-VP_{AHPND} strain showed virulence to whiteleg (*P. vannamei*) and kuruma (*Marsupenaeus japonicus*) shrimps. The virulence level of this transformed strain was similar to that of VP_{AHPND} strain. In addition, we studied the virulence of a natural mutant from highly virulent VP_{AHPND} strain. Because a major part of the toxin genes of this mutant strain was naturally deleted during the course of culture in nutrient broth, our results clearly show that this strain lost its virulence to whiteleg shrimp (Tinwongger *et al.*, 2016, unpublished data). Moreover, these results indicate that the toxins are the virulence factors of VP_{AHPND} strain.

The region encoding the toxin genes is composed of approximately 6 kbp and exhibits

terminal inverted repeats of about 1.2 kbp (Tinwongger *et al.*, 2016, unpublished data). The repeats encode insertion sequence (IS). The IS encodes transposase and is identical to other reported strains of *V. parahaemolyticus* (Kamruzzaman and Nishibuchi, 2008). The non-virulent strains carrying the plasmid completely lack the toxin region, but possess an IS. Interestingly, we found that the virulent strains also possess the region lacking toxin genes but have a single IS. These results suggest that the IS might have transposase activity which is involved in deletion and/or insertion of the toxin genes (Figure 4). Importantly, these results denote that several colonies will be necessary for PCR diagnosis of AHPND especially when using bacteria grown on agar plates.

We compared the virulence of five different VP_{AHPND} strains to shrimp (Tinwongger *et al.*, 2016, unpublished data). We also studied the copy number of plasmids in these five VP_{AHPND} strains. Two of these strains have a low copy number of the plasmid and toxin genes. The other three strains have more than 30 copies of the plasmid and toxin genes. Interestingly, VP_{AHPND} strains carrying low copy number of plasmid were more virulent than VP_{AHPND} strains carrying high copy number of the plasmid. These results imply that the copy number of toxin genes is not an important factor responsible for the degree of virulence of the VP_{AHPND} strains. We further studied the secretion of toxin of these five VP_{AHPND} strains. The most virulent strain secreted the highest amount of toxin compared to other VP_{AHPND} strains, suggesting that virulence of VP_{AHPND} strains corresponded to the amount of secreted toxin.

Development of prevention methods against AHPND

With regard to the development of pragmatic, effective, and economically-sound prevention and control methods against AHPND, we have been investigating the potential use of VP_{AHPND} formalin-killed cells (FKC) as vaccine immunogen, IgY additive in feed, nano-bubble technology, phage therapy, and the isolation and characterization of toxin receptors in the host.

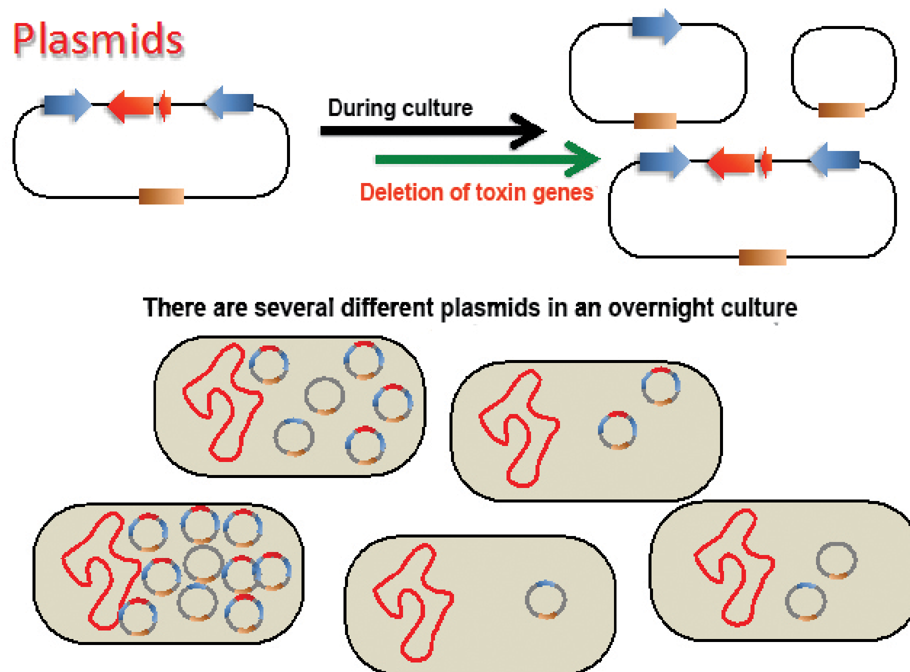

Figure 4. Insertion sequence-mediated production of several types of AHPND plasmid during culture of VP_{AHPND}.

Formalin-killed cell vaccine trials

Shrimps do not have a known adaptive immune system. However, there have been several reports showing the efficacy of vaccines targeted for infectious diseases of shrimp. Based on these reports, it was suggested that shrimp may have an unknown and unique adaptive immune system which may be completely different from the vertebrate's adaptive immune system.

We tested the efficacy of the formalin-killed cell (FKC)-VP_{AHPND} vaccine using whiteleg shrimps weighing 5 to 7 g. VP_{AHPND} was cultured in a broth at 30°C for 18 hours. After incubation, cultured VP_{AHPND} was treated with formalin for 24 hours at 4°C. When FKC-VP_{AHPND} was fed to shrimp, many shrimps died within few days post-feeding. This result suggests that the VP_{AHPND} toxin was stable in formalin and heating at 60°C. We conducted bath vaccinations using 3 different formalin-killed cells (FKC)-VP_{AHPND} strains (TUMSAT-N1, A1 and FP1) as vaccine immunogen. We added 200 mL of the FKC-VP_{AHPND} to 20 L of sea water. Then shrimp were immersed in FKC-VP_{AHPND}-seawater mixture for 2 hrs. After the bath vaccination, shrimp were transferred to another tank for periodic observation. Representative result of the VP_{AHPND} challenge test conducted at post-bath vaccination with the FKC-VP_{AHPND} is shown in Figure 5. One of the FKC-VP_{AHPND} vaccines, i.e. TUMSAT-FP1, conferred good protection in shrimp experimentally challenged with VP_{AHPND}. This result indicates that not all VP_{AHPND} strains have protective antigens against the homologous VP_{AHPND} strains. In another experiment, we used small shrimps with a mean body weight of 0.8 g. FKC-VP_{AHPND} vaccine did not confer protection to small shrimp experimentally challenged with VP_{AHPND}, indicating that the immune system of small shrimp may not have developed yet.

IgY: Immunoglobulin in egg yolk

IgY is a specific antibody of birds especially present in their eggs. When a female chicken was immunized with recombinant VP_{AHPND} toxins, it produced eggs containing high titers of IgY specific for VP_{AHPND} toxins. Furthermore, we used the egg yolk extract as feed additive and successfully demonstrated its efficacy in conferring protection in shrimp experimentally challenged with VP_{AHPND} strain. In addition,

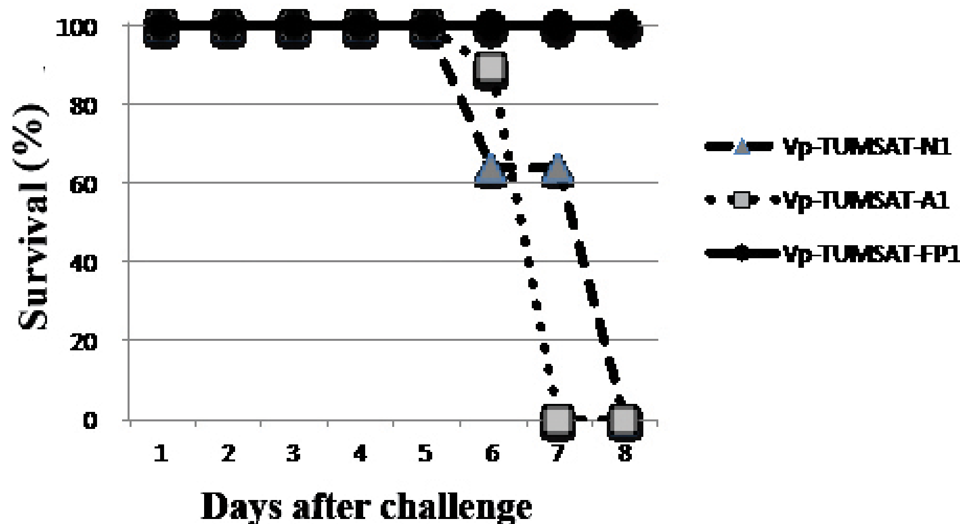

Figure 5. Survival rates (%) of bath-vaccinated (formalin-killed VP_{AHPND} strains) whiteleg shrimp (*Penaeus vannamei*) challenged with a virulent VP_{AHPND} strain.

our data clearly indicate the potential of incorporating IgY in feed as a practical strategy to prevent AHPND outbreaks in shrimp most particularly during the early stage (first 20-30 days) of culture.

Receptors of toxins

Proteins with high homology to VP_{AHPND} toxins in other bacteria have been well characterized. These toxins bind to some receptors of host insects causing some damages to the host's digestive system. However, several reports revealed that toxin-resistant individuals exist in nature. Such toxin-resistant individuals have a mutated receptor for the specified toxins. It is therefore seemingly evident that in nature, there are certain individual shrimps that are resistant to the VP_{AHPND} toxin. To find an AHPND-resistant individual and/or family, it is prudent to identify their receptors for VP_{AHPND} toxin in shrimp. We are currently conducting some experiments focusing on the receptors for VP_{AHPND} toxin in penaeid shrimps by using next generation sequencing and immunological methods using anti-toxin antibody.

Nano-bubble water

Treatment with ozone-nano-bubble water could reduce mortality of shrimps infected with VP_{AHPND} strain. In addition, ozone-nano-bubble water treatment of shrimp could confer protection against WSSV infection. However, we still have to conduct more experiments to thoroughly elucidate the efficacy of nano-bubble water technology for the prevention and/or treatment of microbial infections in penaeid shrimps. Likewise, its practical application in grow-out cultivation ponds needs to be looked into.

Way forward

Our completed and ongoing studies aim to generate information geared at preventing and controlling AHPND in cultivated shrimps. Experiments focusing on the virulence mechanisms of the VP_{AHPND} toxin and effects on the immune responses of shrimp are being carried out. Experiments aimed at elucidating the receptors for VP_{AHPND} toxin in the host's cells are likewise being conducted. Notably, we have observed that a low percentage of shrimp could survive after exposure to VP_{AHPND} toxin, indicating that these surviving shrimp might be resistant to AHPND. However, more data need to be generated to substantiate this speculation.

Hepatopancreatic microsporidiosis (HPM) caused by the microsporidian parasite *Enterocytozoon hepatopenaei* (EHP) has also been recently recognized as an economically important parasitic disease of cultured shrimps. However, information on HPM-EHP is scarce. We have already started analyzing the genome of EHP. We believe that the data that will be generated from our ongoing EHP genome analysis will be pivotal in the establishment of accurate diagnostic methods needed in the formulation of effective, pragmatic, and economically-sound approaches against HPM in cultured penaeids.

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