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Transforming the Farm Managers into the "Family Doctors" of Their Own Ponds

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Abstract

Acute hepatopancreatic necrosis disease (*AHPND*) is caused by a virulent strain of *Vibrio parahaemolyticus* (VP_{AHPND}) not easily differentiated from other *Vibrio* spp pathogens. For the management of $VP_{AHPND,}$ an integral part of the shrimp gut and culture water ecosystems, timely responses to ecological changes in these niches is critical. For humans, companion animals, poultry and livestock, doctors or veterinarians are available to provide disease diagnosis and subsequent treatment accordingly. For shrimp, a farm manager is the best candidate to serve as the "family doctor" to prevent and/or mitigate threats from AHPND. Based on recent advances in the understanding of VP_{AHPND}, different tools have become available for AHPND management, including on-site microbiological and molecular test tools (e.g. spread plate method and insulated isothermal polymerase chain reaction (iiPCR)) for diagnosis, and ecological tools (e.g. indoor pond facilities and application of probiotics) for treatment. Working on a hand-held POCKIT™ PCR device in a format ready for on-site applications, two POCKIT™ iiPCR assays targeting different markers are available to enable identification of VP_{AHPND} in postlarvae (PL), midgut, faeces and pond water. Therefore, pond managers can be trained to use the on-site diagnostics tools and interpret test results, and to apply front-line treatments for AHPND.

Keywords: acute hepatopancreatic necrosis disease, ecological tools, ecosystem management, on-site diagnostic tools, polymerase chain reaction, probiotics, spread plate method, *Vibrio parahaemolyticus*

Introduction

Shrimp aquaculture is one of the major global agriculture industries. Emerging shrimp diseases are on the rise in recent decades largely because shrimp are often cultured at high density, exposed to environmental stress and traded globally. Efficient disease control and management practices could help minimize disease outbreaks, leading to the sustainability of the shrimp-culture industry.

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Acute hepatopancreatic necrosis disease (*AHPND*) is caused by a virulent strain of *Vibrio parahaemolyticus* (VP_{AHPND}). Without vaccines or effective treatments for shrimp diseases, the shrimp industry relies greatly on biosecurity measures to prevent the introduction of pathogens and to reduce their spread throughout the culture environment. In addition, shrimp growth and survival throughout the culture period rely heavily on good water quality, including appropriate temperature, salinity, pH, dissolved oxygen and eco-balance of microflora. A variety of *Vibrio* spp. found to cause shrimp diseases, such as *V. alginolyticus (*Liu et al. 2004), *V. harveyi, (*Karunasagar et al. 1994; Zhou et al. 2012*)* and *V. parahaemolyticus* (Vandenberghe et al. 1999) are an integral part of the ecosystems in the shrimp gut and in culture water. Therefore, reducing the effects of bacterial replication on the digestive system and/or reducing or eliminating the pathogenic bacteria could also help in the control of bacterial diseases.

In the pond water, bacteria can multiply rapidly and cause massive shrimp mortality once the environment becomes severely unbalanced (Vandenberghe et al. 1998; Saulnier et al. 2000; Jayasree et al. 2006). Fluctuations of intra- and interpopulational density can be monitored by quorum sensing (QS), allowing *Vibrio* spp. to initiate specific community-scale responses, including expressing and releasing virulence factors to launch an effective attack on the host. Therefore, in addition to measures that help strengthen shrimp health and the immune system, environmental management is one of the best interventions for the control of bacterial disease. As timely response to ecological changes occurring in the shrimp pond is critical, the pond manager is the best candidate to prevent potential threats from AHPND and to treat infected shrimp. Different aquaculture facilities have different individual eco-conditions, which can be quite complicated.

As the pond manager is the person who is most familiar with the shrimp production facility, he or she is therefore, the best candidate to be trained to know the "patient" and the diseases, how to use on-site diagnostics tools and interpret results, and how to treat the disease with front-line treatments. For bacterial shrimp disease management, an effective strategy will require the integration of variable information and technologies to achieve the following: i) identification of the pathogen; ii) identification of the source of the pathogen; iii) maintenance of the eco-balance among shrimp, algae and bacteria; and iv) avoiding uncontrollable issues. Like other diseases caused by *Vibrio* spp., AHPND cannot be managed 100 % effectively by a single approach.

The Pathogen and its Detection

Vibrio parahaemolyticus, a Gram-negative halophilic bacterium, can be found in shrimp and the farming environment, and also in marine environments worldwide. VP_{AHPND} can colonize the gastro-intestinal tract and produce toxin(s) that cause dysfunction and destruction of the hepatopancreas in *Penaeus vannamei* and *P. monodon* (Flegel 2012; Leaño and Mohan 2012; Lightner et al. 2012; Tang and Lightner 2014). In 2013, the VP_{AHPND} strain was found to contain a virulence-associated plasmid (pVA) (Han et al. 2015; Lee et al. 2015) encoding toxin 1 which is composed of subunits A and B with high homology to the 2 subunits of the insecticidal *Photorhabdus* insect-related binary toxin (PirA and PirB, respectively).

The VP_{AHPND} toxin 1 can cause damage to the hepatopancreas and induce AHPND-like clinical signs in diseased *P. monodon* (Lai et al. 2015; Lee et al. 2015). Most molecular detection methods developed for AHPND diagnosis target the toxin genes and/or other regions in pVA. At early stages, VP_{AHPND} in the environment (e.g. water) invades and establishes infection in the shrimp stomach; the pathogen can generally be detected by polymerase chain reaction (PCR) in the stomach but not in the midgut at this stage (Fig. 1). At the acute stage, VP_{AHPND} colonizes in the stomach and secretes significant amounts of toxin(s) which is transported into the hepatopancreas and cause the damage therein, and spreads to the midgut; VP_{AHPND} can be detected in both the stomach and midgut by PCR at this stage (Fig. 1).

Gene Transfer in the Vibrio harveyi Clade

Vibrio parahaemolyticus is closely related to other members of the *V. harveyi* clade, which consists of 11 species (Sawabe et al. 2007; Cano-Gomez et al. 2011). Different genetic transfer mechanisms, including homologous recombination, transposition, conjugation or transformation help achieve genetic material exchange between different *Vibrio* spp. The virulence genes, *PirA* and *PirB*, were found within "pathogenic islands" flanked by inverted repeats of transposase genes in the pVA plasmid (Tang and Lightner 2014; Han et al. 2015b), suggesting that the virulence genes could be transferred via genetic transfer mechanisms.

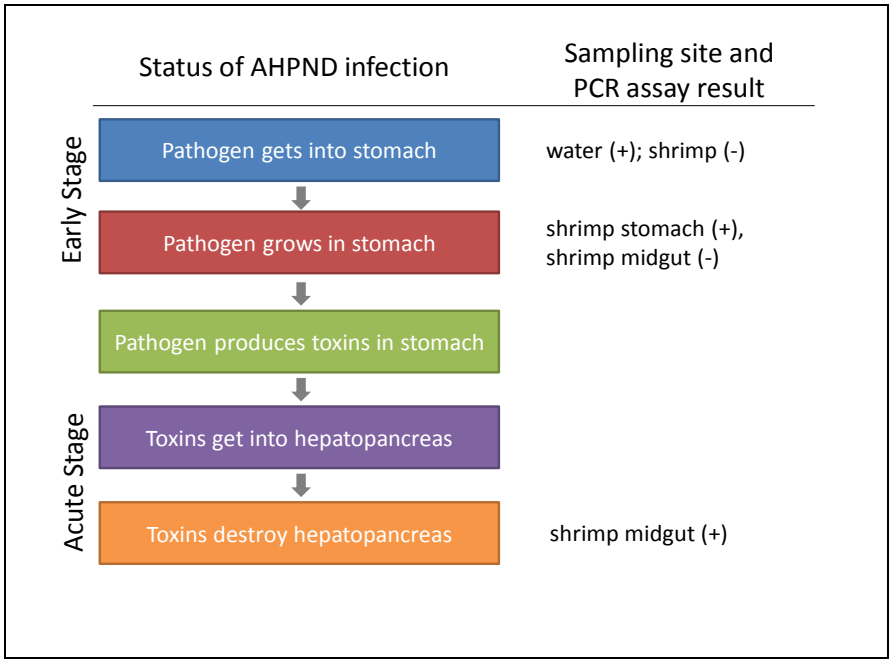


Fig. 1. Acute hepatopancreatic necrosis disease (AHPND) infection status and the corresponding polymerase chain reaction (PCR) assay results in different sampling sites. (+) = positive result, (-) = negative result.

Recent evidence indicated that the AHPND pVA plasmid can jump to other members in the *V. harveyi* clade (Busico-Salcedo and Owens 2014), and a *V. harveyi* isolate (KC13.17.5) was identified as causing AHPND in northern Viet Nam. Sequencing analysis showed that this isolate contained pVA plasmid-like sequences and the putative virulence genes (Kondo et al. 2015).

In addition, one *V. owensii* strain (SH-14) was found to cause AHPND and has a large plasmid containing the *Pir toxin* genes in a pVA-like plasmid *(*Liu et al. 2015*)*. So far, the pVA plasmid has not been found in any species outside of the *V. harveyi* clade. Since >95 % of the aetiological agents of AHPND have been identified as VP_{AHPND}, we recommend that pond managers focus on managing VP_{AHPND} for now.

Diagnostic Tools for Marine Bacterial Pathogens

Diagnosis of a disease can facilitate effective decision-making, leading to implementation of the right treatment and preventive measures. Information such as the type of pathogen (e.g. virus, bacteria, parasite or a combination of them), the severity of the disease, pathogen prevalence, pathogen titer (pathogen levels) and pathogen strain (pathogenic, partial or non-pathogenic) are all crucial for pond management. Disease diagnosis has become less challenging lately, with various diagnostics tools being available for use in a central laboratory or in the field.

Spread Plate Methods

It is a general rule in aquaculture practices that a high number of *Vibrio* spp. in the water poses a potential threat to the farmed animals (Diggles et al. 1999). It is suggested that the pathogenic *Vibrio* loads should be kept below 1 000 colony forming units (cfu).mL^{-1} in an aquaculture system (Ganesh et al. 2010). The spread plate methods can provide quantitative information on the bacterial populations. Marine agar, a non-selective medium for organisms living in high Na^+ environments, is used to obtain the total bacterial count (Fig. 2A). Thiosulfate-citrate-bile salt-sucrose (TCBS) agar, a highly selective medium for many *Vibrio* spp., can be used to estimate the numbers of *Vibrio* spp. (Fig. 2B) (Jayasinghe et al. 2006).

The combination of sucrose fermentation and bromothymol blue and thymol blue indicators in the medium leads to yellow colonies, allowing for the identification of sucrose-utilizing *Vibrio* spp. Important shrimp pathogens such as *V. parahaemolyticus* and *V. harveyi* produce greenish colonies on TCBS agar. Luminescent colony counts can help estimate the population of potentially harmful bacteria, since luminescence activity strongly indicates quorum sensing and virulence of *Vibrio* bacteria. De *Man Rogosa Sharpe* (*MRS*) agar, containing ingredients that favour growth of *Lactobacilli* and suppress competing bacteria, is often used to estimate the concentration of *Lactobacilli*.

Various microbiological indexes, I(M), have been designed to help decision-making in pond management. The optimal I(M) for each farm is different. I(M) calculation is based on the following numbers: M, total bacterial counts from marine agar plating; T, *Vibrio* spp. bacterial count from TCBS agar plating; TY, yellow colony counts from TCBS agar plating (likely avirulent *Vibrio* spp.); TG, green colony counts from TCBS agar plating (likely virulent *Vibrio* spp.). A typical I(M) example indicating good water quality is as follows: $M > 20 \times T$, $M > 10^5$, $T < 10^3$, in case $TG > TY$, $TG < 10^3$ ($TG < 5 \times 10^2$ preferred).

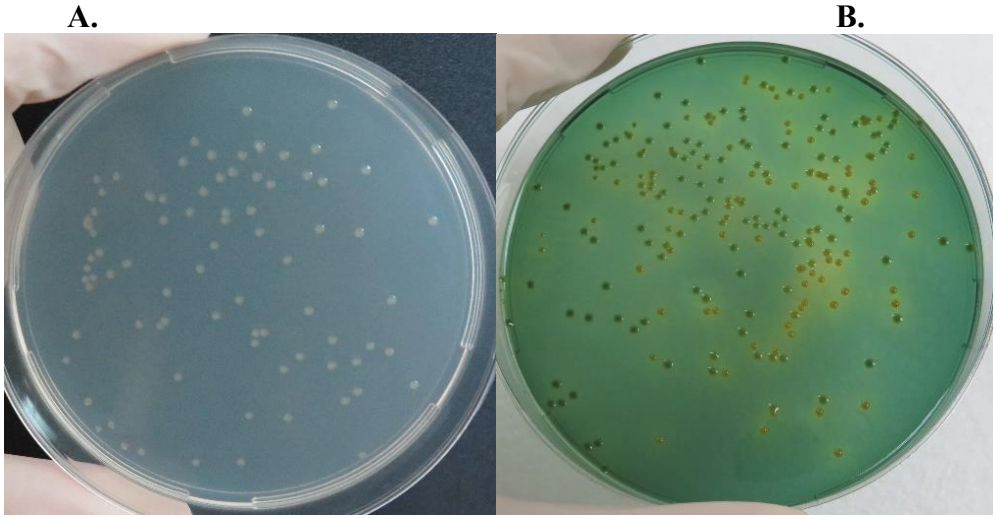


Fig. 2. Spread plate methods. Total marine bacteria and *Vibrio* spp. counts can be obtained by marine agar. (A), a non-selective medium to obtain total organisms living in high Na^+ environments, and (B) thiosulfate-citrate-bile salt-sucrose (TCBS) agar, a highly selective medium for *Vibrio* spp., where pathogenic *Vibrio* spp. such as *V. parahaemolyticus* and *V. harveyi* produce greenish colonies.

Insulated Isothermal PCR – an On-Site Molecular Detection Tool for Aquaculture Pathogens

PCR assay, with its high sensitivity and specificity, has been commonly used at various large-scale aquaculture facilities for pathogen surveillance or disease diagnosis, improving overall shrimp production in the long run. By detecting pathogens at early stages, appropriate measures can be implemented in a timely fashion to help control the spread of major shrimp pathogens, such as white-spot syndrome virus (WSSV). On-site pathogen detection tools enable farms of various scales to minimize economic losses caused by AHPND. Optimal on-site pathogen detection systems should be rapid, inexpensive, sensitive, easy to maintain and perform by anyone with minimal training, and not cross-react with shrimp DNA and any irrelevant microorganisms. The reagents should be provided in a format (such as lyophilized) that allows easy shipping and storage.

A user friendly field-deployable molecular detection method is available for on-site detection of various shrimp pathogens. The assay is based on insulated isothermal PCR (iiPCR) and works on a series of CE-IVD-marked portable devices (POCKIT™ Nucleic Acid Analyzer series, GeneReach Biotech) which can amplify target sequences, detect fluorescent signals and display simple readouts within one hour. The POCKIT™ Nucleic Acid Analyzer could run 1 to 8 samples within 55 minutes with a single programme for PCR and RT-PCR. Probe detection adds specificity and eliminates post-PCR manipulation. It could run with rechargeable battery. Dual-channel fluorescence detection allows inclusion of an internal control. User-friendly features include touch panel control and automatic data interpretation. The newly available hand-held POCKIT™ Micro Nucleic Acid Analyzer (for PCR) and POCKIT™ Micro Plus Nucleic Acid Analyzer (for both PCR and reverse transcription PCR [RT-PCR]) (GeneReach Biotech) have added flexibility to its field application and allowed shorter turnaround time. Results are obtained in around 30 min with POKIT™ Micro and 45 min with POKIT™ Micro Plus.

Both models are small (dimensions: 152 (L) x 63 (W) x 50 (H) mm), light weight (380 g), and can be charged with a micro USB Recharger (100–240V). The reliability of the iiPCR system has been shown by the fact that one WSSV iiPCR method (IQ Plus™ WSSV Kit with POCKIT™ System) was certified by the World Organisation for Animal Health (OIE) as fit for detecting WSSV in tissue of ectodermal and mesodermal origin of *P. vannamei* in 2013; that several methods on POCKIT™ have been listed in the Biodetection Technologies for First Responders: 2015 edition by Pacific Northwest National Laboratory, and that several iiPCR on POCKIT™ methods have been demonstrated to provide sensitivity and specificity equivalent to those of reference real-time PCR (qPCR) or nested PCR for the detection of a significant number of animal and human viral pathogens, including WSSV and dengue virus (Tsen et al. 2013; Tsai et al. 2014; Balasuriya et al. 2014; Wilkes et al. 2014, 2015a,b; Ambagala et al. 2015; Lung et al. 2015; Carossino et al. 2016; Chua et al. 2016; Go et al. 2016; Kuo et al. 2016; Soltan et al. 2016; Zhang et al. 2016).

Two POCKIT™ iiPCR methods targeting markers to allow identification of AHPND threat, *i.e. toxin 1* (*PirA* and *PirB*) or the AHPND pVA plasmid, are available. Postlarvae (PL), midgut, faeces and pond water are recommended for sampling. Molecular screening for the presence of the pVA plasmid can help detect a potential AHPND threat, since non-virulent strains of *V. parahaemolyticus* could be converted into virulent strains by obtaining the plasmid through different gene transfer mechanisms. The *toxin 1* gene is located in an unstable region in the pVA plasmid and could potentially be moved horizontally from one location to another location by transposases. Detection of the *toxin 1* gene in the shrimp or environment may indicate the presence of microorganisms with potential to produce the toxins that lead to disease in shrimp, alerting farmers of potential threats of AHPND.

Thus, both the pVA plasmid and *toxin 1* gene are the recommended detection targets for prevention and management of AHPND. When simultaneous performance of the two assays is not possible, it is recommended to perform the pVA plasmid test first to identify potential threat. In the case of plasmid-positive results, the *toxin 1* assay can be used to provide further evidence for the presence of the AHPND pathogen.

Both the AHPND pVA plasmid or *toxin 1* gene iiPCR assays can detect their targets sensitively and specifically. They had detection endpoints equivalent to that of qPCR assays (10^{-3}) using a serial dilution of a virulent VP_{AHPND} strain (data not shown). Both reactions did not react with 18 non-VP_{AHPND} strains from the People's Republic of China, Mexico, Thailand, Taiwan POC, the United States of America or Viet Nam, including 11 laboratory isolates (Table 1), indicating excellent target specificity. Side-by-side comparison of *toxin 1* iiPCR with qPCR using field samples (n=18) showed that eight qPCR-positive samples were all iiPCR positive and ten qPCR-negative samples were all iiPCR negative. Similarly, 11 pVA plasmid qPCR-positive samples were all pVA plasmid iiPCR positive and seven qPCR-negative samples were all iiPCR negative). These results demonstrate that both iiPCR assays have excellent agreement with qPCR to detect the AHPND virulence markers in samples.

Furthermore, easy and simple nucleic acid extraction methods are available to work with the hand-held PCR detector on site. For example, the Grind-N-Go (GeneReach Biotech) is an easy manual DNA extraction method with all reagents provided in a single cartridge; DNA extraction could be completed within five minutes, including hands-on time of about only two minutes. No device, electricity or other consumables are needed.

Table 1. Results of real-time PCR (qPCR) and insulated isothermal polymerase chain reaction (iiPCR) assays for acute hepatopancreatic necrosis disease (AHPND) showing non-detection of non-pathogenic *Vibrio parahaemolyticus* and *V. harveyi* (– = no detection).

No.	Organism	Sample Name[1]	qPCR		iiPCR	
			Plasmid	***Toxin 1***	**Plasmid**	***Toxin 1***
1	*V. parahaemolyticus*	BCRC 10806/ATCC 17802	–	–	–	–
2	*V. parahaemolyticus*	BCRC 12863/ATCC 17803	–	–	–	–
3	*V. parahaemolyticus*	BCRC 12864/ATCC 27519	–	–	–	–
4	*V. parahaemolyticus*	BCRC 12865/ATCC 27969	–	–	–	–
5	*V. parahaemolyticus*	BCRC 12959	–	–	–	–
6	*V. parahaemolyticus*	BCRC 12963	–	–	–	–
7	*V. parahaemolyticus*	BCRC12968	–	–	–	–
8	*V. parahaemolyticus*	BCRC 13025	–	–	–	–
9	*V. harveyi*	BCRC 12907/ATCC 14126	–	–	–	–
10	*V. harveyi*	BCRC 13812/ATCC 25919	–	–	–	–
11	*V. harveyi*	BCRC 14141/ATCC 35084	–	–	–	–

[1]BCRC = Bioresource Collection and Research Center, Hsinchu, Taiwan POC; ATCC = American Type Culture Collection, Rockville, MD, USA.

Evaluation of AHPND Status Based on Diagnostic Results

In general, bacteria are difficult to eliminate and may have long-term impacts on shrimp farms once they are introduced into a facility. Multiple strategies are available to help pond managers deal with shrimp diseases at different stages: (i) pathogen eradication/suppression, (ii) pathogen neutralization, (iii) alleviation of host clinical signs and recovery, (4) protection of shrimp from pathogens and (5) optimal ecosystem establishment and maintenance. Screening and management of potential VP_{AHPND} threats in shrimp, water, live feeds and other sources is critical. To follow VP_{AHPND} threats in the environment and shrimp tissues, PCR testing can be used to categorize AHPND infection status into five stages (i.e. safe, alert, early stage, infection and outbreak), and I(M) can be used to follow bacterial populations (Table 4). On-site detection tools for VP_{AHPND} should make the implementation of these important measures possible at shrimp culture facilities of different scales at any locations.

Particular strategies are available to help combat AHPND at different stages. At early stages, VP_{AHPND} is absent in water (negative PCR results), and bacterial population in the ecosystem can be monitored by I(M) tests. Whereas at infection stages (positive PCR results in water and stomach samples), *Vibrio* spp. in both water and shrimp can be repressed and/or killed with tools that can help manipulate the bacterial composition in the waterbody and optimize the

ecosystem for shrimp growth. Various probiotics, toxin antidotes and natural formulae (e.g. plant extracts, enzymes, minerals) are now available for different approaches to AHPND management. An example of such a protocol is "A Solution" (ScienChain Biotech, Tainan, Taiwan POC), which includes probiotics (e.g. EMS-proof, Gut-Well, Bac-Up® and Bottom-Up) and natural formulae. The following section provides some information on the use of several management strategies based on commercial products developed by ScienChain Biotech for use against AHPND.

Pond Ecosystem Management

A relatively high level of microbial heterogeneity in the culture system often helps to reduce the vulnerability of farmed animals to opportunistic colonization of bacteria (Olafsen 2001). "Bad" bacteria in the pond can compete with shrimp for food and oxygen, causing stress and disease (Moriarty 1997). *Vibrio* spp. are often the dominant bacteria found in shrimp ponds and have been the major shrimp pathogens (Lightner 1993; Chatterjee and Haldar 2012). A higher proportion of total *Vibrio* in grow-out ponds implies that microbial heterogeneity in the ponds is low. Eradication of bacterial pathogens from the pond environments has proven to be very difficult. Therefore, the best way to manage bacterial diseases of shrimp would be to facilitate microbiota balances that favour shrimp growth and prevent the propagation of pathogenic bacteria in the environment, including the digestive tracts of shrimp. Most pathogenic *Vibrio* spp. are opportunistic pathogens, and careful system management, including close monitoring for the presence of potential pathogens and for ecological conditions has been found to help curb outbreaks of vibriosis (Sung et al. 2001; Phuoc et al. 2008).

For example, the pathogenic *Vibrio* loads should be kept under control (below 1 000 $cfu.mL^{-1}$) in aquaculture systems (Ganesh et al. 2010). In intensive farming, utilization of high amounts of organic manure, inorganic fertilizer, high stocking density, feed waste, faecal matter, algal bloom and human interference should be closely monitored, as they could lead to higher loads of pathogenic *Vibrio* spp. (Lloberra et al. 1991; Moriarty 1997; Heenatigala and Fernando 2016). High salinity, alkaline pH conditions, and high levels of sulfide, ammonia and dissolved oxygen (DO) in the pond appear to favour the growth of *Vibrio* spp. The upper limit of ammonia is < 0.5 $mg.L^{-1}$ (Matias et al. 2002). Ammonium concentrations has been associated with the susceptibility of shrimp to *Vibrio* spp. (Liu and Chen 2004). According to farmers' experiences, (high DO (6–8 ppm) appears to increase the tolerance of shrimp to *Vibrio* infection. In addition, reduced water exchange in closed-water systems can help reduce the probability of pathogen introduction.

Probiotics

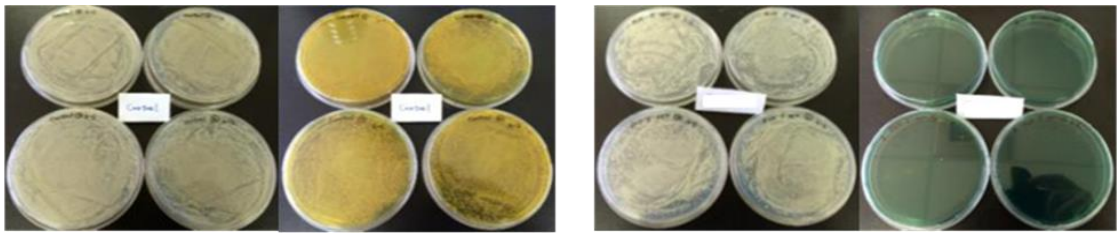
Dealing with the microflora in the ponds is difficult, as *Vibrio* spp. are always the dominant microorganisms in seawater. The most common tool for microbial control used to be antibiotics (Havenaar et al. 1992); however, with disadvantages such as alteration of microbial communities and the generation of drug-resistant bacterial strains, the use of antibiotics in water systems has been discouraged (Zanetti et al. 2001; Hansa et al. 2015). For instance, in Mexico, VP_{AHPND} strains have been found to be resistant to oxytetracycline and tetracycline (Han et al. 2015a). Different strategies such as quorum sensing disruption in the ecosystem are needed for the control of bacterial disease. Application of probiotics has been shown to improve the ecosystem in the shrimp culture facility (Pattukumar et al. 2010). Probiotics can improve ecological conditions in the culture water, remove biodebris, inhibit or compete with potential *Vibrio* pathogens for nutrients and space, colonize the gastro-intestinal tract, enhance shrimp immune systems, and provide shrimp with additional growth-promoting biocompounds. The probiotics with promising health benefit to shrimp include bacteria (e.g. *Lactobacillus* spp., *Bacillus* spp., *V. alginolyticus* and *Nitrobacter* spp.) and yeasts (e.g. *Saccharomyces cerevisiae, S. exiguous* and *Phaffia rhodozyma*) (Cruz et al. 2012). Successful strategies involving probiotics should include: (i) inclusion of organisms capable of surviving in the shrimp culture environment, (ii) inclusion of organisms producing specific anti-*Vibrio* bacteriocin to help suppress or kill *Vibrio* spp., (iii) inclusion of organisms that can be provided in a high stock concentration (e.g. 100:1 dilution) and (iv) optimization of ecological factors suitable for shrimp growth (e.g. higher DO levels). Probiotics can be applied through feed, water or a combination of feed and water in shrimp culture.

Examples of AHPND Management Strategies Using Commercially Available Ecological Tools

Reduction of Vibrio spp.

One recent example of a commercial product that was successfully used to reduce *Vibrio* spp. on brine shrimp (*Artemia* sp.) is Germ-Out, which contains natural plant extracts. The benefits of Germ-Out have been demonstrated in trials in commercial hatcheries and grow-out farms. In one case, when used at 1:10 000 x dilution (e.g. 20 mL in 200 L of seawater with 1 lb of brine shrimp eggs) at 6–10 h before hatching, Germ-Out suppressed *Vibrio* spp. only and did not affect the total marine bacterial population (including probiotics) or the hatching rates of brine shrimp (~100 %) (Fig. 3). EMS-Proof is another probiotics formula able to suppress the growth of *V. parahaemolyticus* (including VP_{AHPND}) and *V. harveyi* (fluorescent *Vibrio*). Daily feed supplementation with Germ-Out and water treatment with EMS-Proof during the grow-out phase can help to reduce the population of fluorescent *Vibrio* spp. In another trial, shrimp were fed with feed sprayed with Germ-Out (100 mL 1 000-fold diluted Germ-Out on 1 kg feed); and the pond water was also sprayed with EMS-Proof. The control group was fed with feed without Germ-Out and kept in water without EMS-Proof treatment. Both green colony and yellow colony counts on TCBS agar plate were greatly reduced with the application of EMS-Proof plus Germ-Out in two ponds (Fig. 4A). In another test, the survival rate of artificially AHPND-infected *P. vannamei* (n=30) receiving Germ-Out treatment was 77 %, greatly higher than that of the control group (0 %) (Fig. 4B).

	Marine Agar (cfu.mL^{-1})		TCBS Agar (cfu.mL^{-1})	
Germ-Out	>300	>300	1	4
	>300	>300	0	1
Control	>300	>300	>700	>700



Control		Germ-Out	
Marine Agar	TCBS Agar	Marine Agar	TCBS Agar

Fig. 3. Germ-Out treatment helped suppress *Vibrio* spp. population in *Artemia* sp. Total bacteria counts (marine agar) and *Vibrio* spp. counts (thiosulfate-citrate-bile salt-sucrose (TCBS) agar) in brine shrimp receiving Germ-Out treatment or control group receiving no treatment were determined. The numbers and plates are shown in the upper and lower panels, respectively. CFU, colony forming unit.

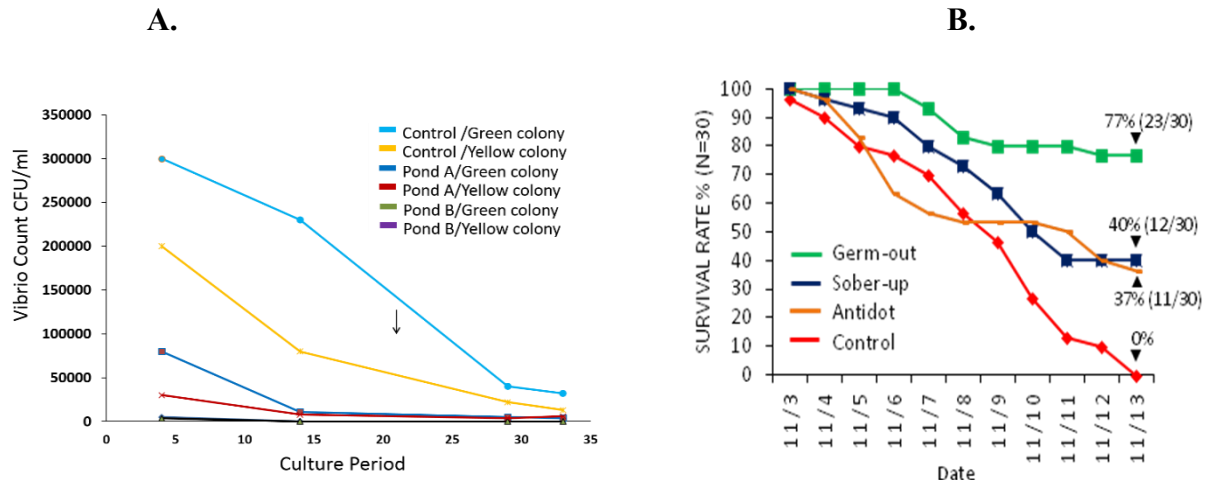


Fig. 4. (A) EMS-Proof with Germ-Out suppressed *Vibrio* spp. and increased survival rates of VP_{AHPND}-infected *Penaeus vannamei* in two ponds. EMS-Proof was activated and sprayed into the water. Germ-Out was sprayed onto shrimp feed at a 1:10 000 ratio. The control group was fed with feed without Germ-Out and the water was not treated by EMS-Proof. Green and yellow colonies on thiosulfate-citrate-bile salt-sucrose (TCBS) agar were determined. The arrow indicates the time point of control pond starting probiotics treatment. (B) Increased survival rates in artificially AHPND-infected *P. vannamei* (n=30) treated with Germ-out, Sober-up or Antidot®. The control received no treatment.

Neutralization or Removal of Toxin

The virulence of VP_{AHPND} is caused mainly by *toxin 1* (Han et al. 2015b; Sirikharin et al. 2015). Neutralization of toxin by antibody and probiotics can successfully reduce the virulence of pathogens (Sleator 2010; Paton et al. 2015). Sober-Up, a novel natural mineral mixture with

affinity for *toxin 1*, was demonstrated to absorb *toxin 1* efficiently in the shrimp stomach (Fig. 5A). This product also absorbs other algal toxins and mycotoxins. Antidot® is another formula containing secondary metabolites of *Lactobacillus* spp. and purified enzymes that can digest *toxin 1* (Fig. 5B), algal toxins and mycotoxins in the shrimp stomach. These products are best applied when positive AHPND PCR results are obtained in shrimp tissue samples. In one example, *P. vannamei* was artificially infected with VP_{AHPND} and subsequently fed with Sober-Up (final dosage 2 %; n=30) and Antidot® (final dosage 1 %) in shrimp feed. These shrimp had higher survival rates (~40 %), as compared to the control group (final dosage 0 %, n=30) (Figure 4B).

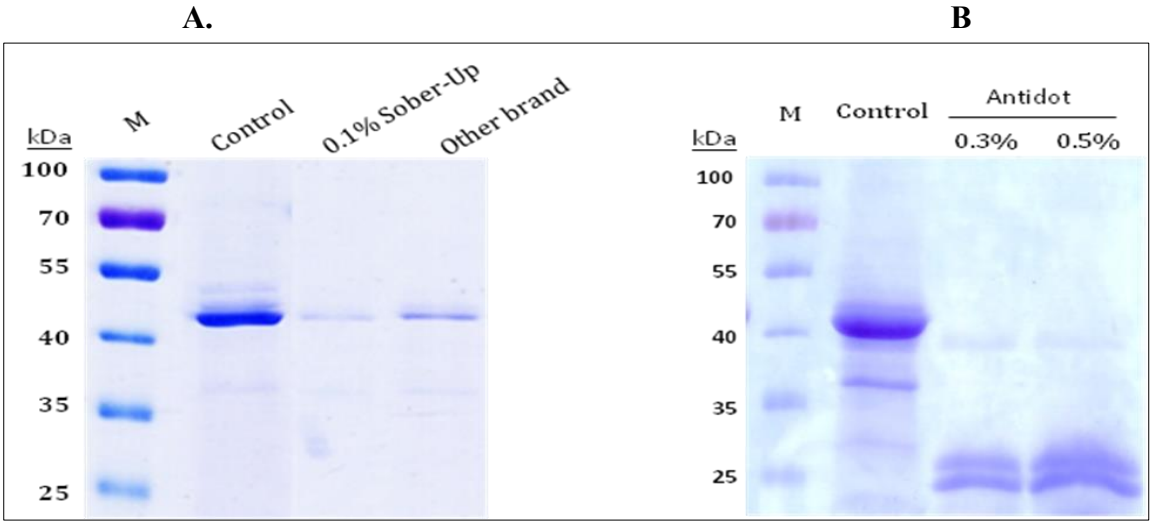


Fig. 5. *Toxin 1* was reduced by Sober-Up and Antidot®. After *toxin 1* was treated with 0.1 % Sober-Up (A), or 0.3 % or 0.5% Antidot® (B), residual *toxin 1* was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE analysis). Control, *toxin 1* protein without any treatment. (B) The same amount of *toxin 1* protein was treated with 0.3 % and 0.5 % Antidot®. The residual *toxin 1* protein was analyzed by SDS-PAGE.

Protection of the Shrimp Gastro-intestinal Tract by Probiotics

Gut-Well, a *Lactobacillus*-based probiotics with anti-*Vibrio* bioactivity, contains *Lactobacillus* spp. that can survive in a wide range of salinity and temperature. This product requires a 24 to 48-hr activation period before it is applied in feed at a ratio of 10 g per 20 kg feed in 2 liters of water for PL, or 10 g per 100 kg feed for grow-out shrimp.

Protection of Shrimp by a Combination of Tools

The complete rearing cycle of shrimp culture is a long process that includes the broodstock, hatchery production, nursery and grow-out stages. A combination of the approaches mentioned above is often required to have a successful operation. One trial based on the "A Solution" (ScienChain Biotech) including the use of probiotics (i.e. EMS-Proof, Gut-Well, Bac-Up® and Bottom-Up) and natural formulae was carried out at a nursery in Viet Nam rearing *P. vannamei*. Lower total *Vibrio* counts (TCBS plating) and more stable total marine bacterial counts (marine agar plating) were found in both PL and water treated with "A Solution", compared to the untreated group (Fig. 6).

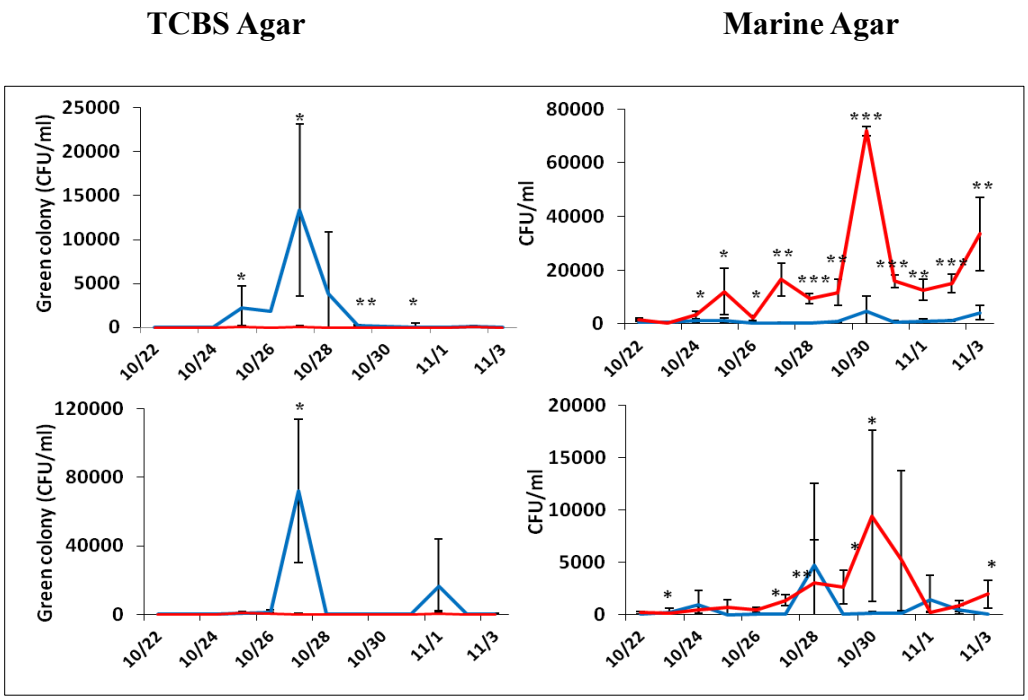
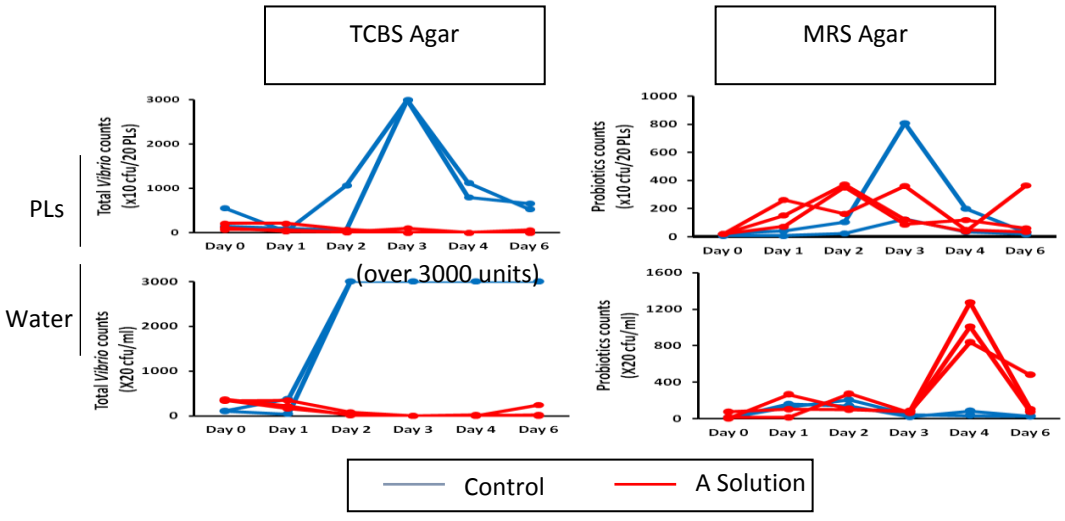


Fig. 6. "A Solution" treatment improved microbial compositions at a nursery farm in Viet Nam rearing *Penaeus vannamei*. Postlarvae (PL) and water treated with (red line) or without (blue line) "A Solution" were analyzed over a period of two weeks for their *Vibrio* counts on thiosulfate-citrate-bile salt-sucrose (TCBS) agar plate and total marine bacterial counts on marine agar plate. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

In addition, the percentages of PL with empty or disordered midguts were significantly lower in PL artificially infected with VP_{AHPND} and receiving the treatment on day 3 postinfection (data now shown). A field test with "A Solution" was also performed at a nursery farm in Panama in the summer of 2016, immediately after fluorescent *Vibrio* numeration on TCBS agar plating and results of on-site POCKIT *Vibrio harveyi* iiPCR-positive detection were obtained. A combination of *Vibrio*-controlling strategies in the "A Solution" was applied, including PCR diagnosis, *Vibrio* suppression, shrimp health maintenance and ecosystem balancing. After the "A Solution" treatment, MRS plating showed that total *Lactobacillus* spp. counts in PL and the waterbodies were elevated (Fig. 7A); and TCBS plating showed that the numbers of fluorescent *Vibrio* spp. in PL and pond water dropped significantly, compared to those found before these intervention measures (Fig. 7B).

A.



B.

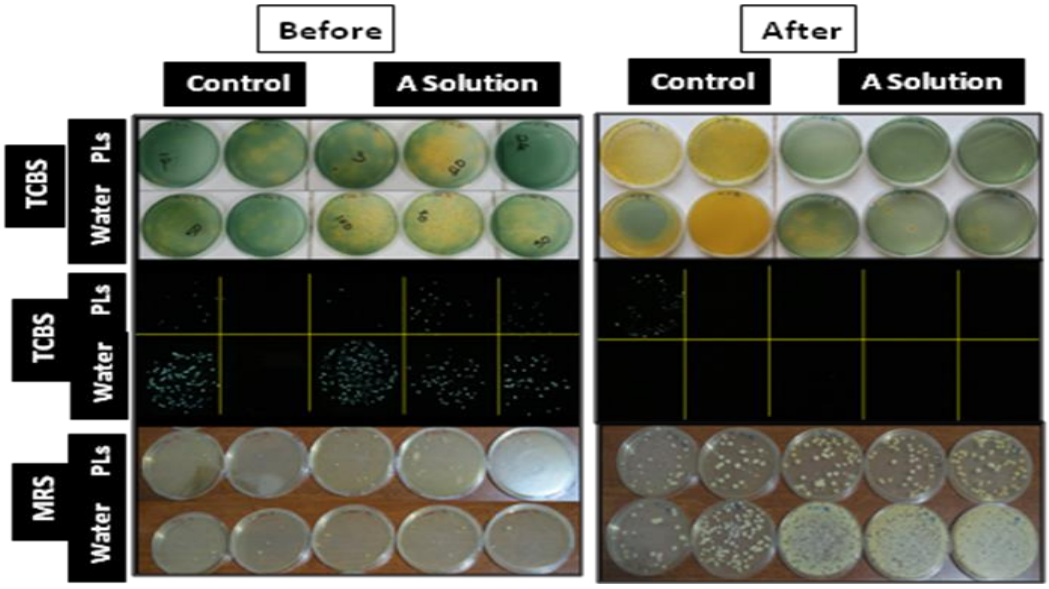


Fig. 7. "A Solution" treatment on a *V. harveyi* insulated isothermal polymerase chain reaction (iiPCR)-positive nursery farm with high fluorescent *Vibrio* counts in Panama in the summer of 2016. (A) The *Vibrio* counts on thiosulfate-citrate-bile salt-sucrose (TCBS) agar plates and probiotic counts on De *Man Rogosa Sharpe* (*MRS*) agar plates were determined in both postlarvae (PL) and water in a pond treated with "A Solution" treatment (red line) and one without treatment (blue line). (B) PL and pond water before (left panel) and after (right panel) the "A Solution" treatment were assayed for concentrations of *Vibrio* spp. on TCBS agar, luminescent *Vibrio* spp. on TCBS agar, and lactobacilli on MRS agar. The control groups received no "A Solution" treatment.

Advantages of Indoor Ponds in a Cold Climate

Basic biosecurity measures include the use of specific pathogen-free (SPF) shrimp stocks and seeds; disinfection of facilities, equipment, water and workers; coverage of culture tanks with plastic sheets to prevent pathogen introduction into hatcheries; and maintenance of optimal environmental conditions, including water, feed and stocking density in grow-out ponds throughout the culture cycle. In well-controlled environments, shrimp can be produced and supplied to the market during seasons when prices are high. Recently, a new operational concept for shrimp culture has become popular in the northern People's Republic of China.

It involves a natural biosecurity system built upon a greenhouse facility, providing a super-intensive farm with well-controlled biosecurity to keep pathogens out. In one case (Fig. 8), locally produced live shrimp from this system reached prices of US$ 5–8.kg^{-1} during the winter of 2015. However, this type of facility is extremely expensive to construct, costing about US$ 1 to 1.5 million per hectare, not including the land. In this case, stocking density was about 500–700.m^{-2} and harvest size averaged about 20 g. Eighty tonnes.ha^{-1}.crop^{-1} were harvested, resulting in 240 tonnes.ha^{-1}.yr^{-1} (valued at US$ 1.2 to 2 million per year). In winter, the temperature outside (-20 ^{o}C) can destroy the potential carriers and hosts of shrimp viruses, as well as non-viral pathogens such as VP_{AHPND} and *Enterocytozoon hepatopenaei* (*EHP*), minimizing the risk of horizontal disease transmission and providing a natural biosecurity barrier. Low water exchange also helps to minimize the risk of pathogen introduction from intake water.

Other biosecurity measures included the use of plastic sheets and bird nets on top of the facility, use of PL derived from SPF broodstock, (including freedom from the VP_{AHPND}), and well-trained pond managers who are familiar with on-site diagnostic tools and AHPND treatment options and protocols. A few AHPND outbreaks were suspected, and in these cases, the pond managers were able to make an appropriate diagnosis and apply suitable control strategies.

Fig. 8. Green houses for shrimp farming in northern People's Republic of China – a growing operation concept. The facility can provide a well-controlled, natural biosecurity barrier.

Conclusion

In shrimp culture, the "patient" is the infected pond instead of the shrimp, while the "doctor" is the pond manager. The "doctor" should understand both the disease and the "patient" very well. The "doctor" should be equipped with a comprehensive set of on-site diagnostic tools, including a hand-held PCR for molecular diagnosis and agar plating for microbiological diagnosis (TCBS plates for *Vibrio* spp., marine agar plates for total marine bacteria, and MRS plates for *Lactobacillus* spp.). The "doctor" should be provided with complete and effective treatment tools, including agents that stop or kill the pathogens, relieve clinical signs and help shrimp recover from diseases, protect them from infection and/or provide better environment for their growth.

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