

Reviving the Philippine shrimp industry: molecular diagnostics and therapeutics

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In 1994, the Philippines was one of the top three producers in shrimp aquaculture, reaching a peak of 91MT. However, diseases caused a rapid decline in shrimp production, down to less than 50MT in 1997, and the Philippine status in shrimp aquaculture production has dwindled to the 13th spot. Production has not yet recovered and stabilized, as these diseases still plague the industry up to this day. The strict implementation of importation laws for broodstock and better management practices helped the industry to make up for losses, and is now starting to revive. The scientific community is doing its part to hasten this process, shrimp research is of consistent interest to several research institutions providing deeper understanding regarding viral and bacterial diseases and their interactions with the host. However, the challenge remains as to how research can be translated and applied in the field, to provide the shrimp industry with science-based management strategies. This review provides a balance of both basic and applied research with the ultimate goal of reaching out to the industry through useful and beneficial knowledge together with simpler and cost-effective detection methods and alternative, effective immunostimulants.

Keywords: shrimp biotechnology, shrimp immunity, molecular detection

INTRODUCTION

Marine and freshwater shellfish, including shrimps, are a key source of protein in a continuously growing population. The increased consumption of shrimps and the limited supply from capture fisheries have led to a demand for farmed shrimps and an expansion of the shrimp industry in the Philippines. Due to the demand for high-value seafood products,

the industry has generated profits, foreign exchange earnings and an important source of livelihood. However, the expansion of the shrimp industry was accompanied by an increased incidence of economically significant diseases, particularly of viral and bacterial origin [1, 2].

Among the viruses affecting farmed shrimps in the Philippines are the white spot syndrome virus (WSSV), the taura syndrome virus (TSV) and the Laem-Singh virus (LSNV). WSSV has been used for challenge tests due to its devastating impact in shrimp populations upon

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infection, whereas TSV and LSNV have been detected in the country for the first time. The clinical signs, transmission, distribution and incidence of diseases due to these viral pathogens have been reviewed by Andrino-Felarca *et al.* [3]

Bacterial agents from the genus *Vibrio* have also caused significant morbidity and mortality in shrimps in the Philippines. The major species causing Vibriosis are *V. harveyi*, *V. fluvialis*, *V. parahaemolyticus*, *V. damsela*, and *V. vulnificus* [4]. It is worth noting that a toxic plasmid of *V. parahaemolyticus* has been found to be the causative agent for an emerging disease in shrimp, acute hepatopancreatic degenerative necrotic disease (AHPND) or commonly known as early mortality syndrome (EMS) due to mass mortality of infected shrimp within days of stocking [5].

These pathogens have posed a significant threat to the shrimp industry, causing millions of dollars of losses in production. The detection, control and prevention of shrimp diseases caused by these pathogenic agents are essential in supporting the ailing shrimp farming industry. Efficient detection methods have been provided by molecular diagnostics technology, and contribute significantly to the mitigation of production losses. A therapeutic tool for the management of shrimp diseases is gene silencing, which is based on the elucidation of the interaction between the shrimp and the viral agents. Immunostimulants have provided an effective means for increasing immunocompetency and disease resistance in shrimps, and have been widely accepted by the shrimp culture industry [6, 7].

This review paper intends to provide an insight and understanding of several aspects of shrimp research. It covers loop-mediated isothermal amplification (LAMP)-based and polymerase chain reaction (PCR)-based detection of shrimp diseases, the utilization of RNA interference for

the elucidation of gene function in relation to WSSV infection and the use of immunostimulants for shrimps.

DETECTION OF SHRIMP PATHOGENS

Viral and bacterial pathogens are the most common causes of the shrimp diseases, accounting for 60% of the economic losses in aquaculture for viral diseases and 20% for bacterial infections [8]. Early detection and constant surveillance of these pathogens remain to be one of the most effective countermeasures against shrimp diseases because it provides ample time for officials to make break-even strategies and proper aquaculture practices to mitigate production losses [9]. Because of their economic importance, rapid and simple diagnostics for the WSSV, TSV, LSNV, and species of *Vibrio* were developed and are reviewed in this paper.

Polymerase chain reaction-based methods. In recent years, molecular detection has become the most widely accepted technique in disease diagnosis because it is faster and more accurate than the traditional procedures [10]. Among these, PCR detection and its derivatives are the most reliable in detecting aquaculture pathogens [9].

White spot disease, caused by WSSV, remains to be one of the most serious viral disease, due to its high mortality rate that can take place 3–10 days after post-infection [11]. Infected shrimps exhibit loss of appetite, lethargy and the characteristic white spots located on the carapace from calcified deposits, symptoms that eventually lead to mass mortality [12]. The virus is viable to a wide range of economically-important shrimp species in the Philippines, *Peneaus monodon* [13], *Peneaus vannamei* [14], and *Macrobrachium rosenbergii* [15].

In 2011, WSSV was detected from *P. monodon* samples in Bulacan [13]. The authors also

reported that incidental shrimps such as *Metapenaeus dalli* all tested negative in one-step and nested PCR, a possible display of resistance to the virus. On the same year, a partial sequence of the WSSV isolate from the Philippines was generated [14] and was subsequently used to design PCR primers. PCR detection of the disease using the designed primers showed a low incidence rate of 15% for *P. vannamei*. Continued surveillance of WSSV from June 2014 to July 2015 for both *P. monodon* and *P. vannamei* was conducted in other sites in the Philippines and showed a higher total prevalence rate of 25% [unpublished data].

The successful detection of WSSV indicated that other shrimp pathogens may be present but are left undetected and unreported in the country, which may result to the rapid spread of diseases. To prevent this, LSNV became the target for possible detection. Initial screening studies for the RNA-based virus, which causes Monodon slow growth syndrome (MSGs), was conducted between the years 1998–2007. The studies revealed that there have been no carriers of the disease in the country [16].

However, a recent study [unpublished data] showed the presence of LSNV in *P. monodon* samples from the province of Bulacan. Detection was done by observing the external symptoms of the sampled shrimps. LSNV was further detected in the shrimp samples via PCR amplification, and the sequenced the target gene exhibited a 99% homology to LSNV.

Another RNA-based virus, TSV, was also detected in shrimp farms across the country. TSV is capable of causing significant mortalities in reared *P. vannamei* three days after the onset of disease [17] and was also previously unreported in the country. In 2014 [unpublished data], TSV was detected via PCR and the resulting sequences indicated close homology to the TSV Taiwan isolate.

Aside from viral pathogens, bacteria are also major causes of mortalities in shrimp ponds. Vibriosis is a common example, caused by several species of *Vibrio* such as *V. alginolyticus* [18], *V. anguillarum* [19], *V. harveyi* [20], *V. vulnificus* [20], and *V. parahaemolyticus* [21], among others. Initial efforts for the rapid detection of species of *Vibrio* were done using PCR and microbiological methods [22]. A virulent strain of *V. parahaemolyticus* was associated with an emerging disease that causes massive mortalities around Southeast Asia and Mexico called acute hepatopancreatic necrosis disease (AHPND) [23]. The disease creates lesions in the hepatopancreas in shrimp when the *V. parahaemolyticus* plasmid with the toxin gene was present in the species [5]. In 2015, the presence of the virulent strain of *V. parahaemolyticus* was first recorded from across the country and confirmed by PCR amplification and histopathology, together with microbiological and biochemical techniques. Alarmingly, the prevalence of AHPND was found to be 24% in the Philippines [24].

Early detection is important for the mitigation and the control of the spread of these diseases. But it remains to be a problem for local shrimp farms especially for those located far from accredited testing laboratories and do not have the capital to invest in self-test detection kits. The delay in action from time lost in transportation and testing of samples can greatly reduce shrimp harvest when diseases are present. It is also expensive because PCR-based assays require expensive reagents, specialized equipment and technical personnel to perform. It is of utmost importance that the diagnosis be rapid, reliable, and can be done on-site.

Loop-mediated isothermal amplification. A novel and emerging technology called LAMP [25] was used as the platform for the developed diagnostic kit for WSSV. LAMP gained popularity in the detection of several pathogens

for the aquaculture sector such as *Edwardsiella tarda* in Japanese flounder [26], fish iridovirus [27], TSV [28] and WSSV [29]. The LAMP reaction uses a specialized polymerase, the *Bst* polymerase which has a strand displacement activity, and four sets of primers that target six specific regions in the gene of interest, making LAMP more specific and sensitive to the target sequence. The amplification proceeds in isothermal conditions for one hour. The visualization of the result is straightforward and can be performed by the naked eye. The simplicity and rapidity of LAMP makes it desirable as a diagnostic platform for farm applications.

Four sets of primers (patented) highly specific for the Philippine WSSV isolates were developed [30]. The different sets of primers were tested, and the most consistent and sensitive was used for the diagnostic kit. The designed primers were further optimized [22], and compared to the conventional PCR for its analytical sensitivity and specificity.

Utilizing the optimized LAMP assay, a prevalence rate of 48% for WSSV from selected sites in the Philippines was recorded. Additionally, the assay provided faster and ten times more sensitive results than PCR. It was also noted that the formation of LAMP products is possible at temperatures lower than 60°C, and the incubation period can be reduced to 45 min with consistent results.

Despite the improvements made for the LAMP protocol, the DNA extraction remains to be time consuming and costly. To resolve this difficulty, our group formulated and developed a rapid, simple and cost-effective diagnostic kit that can be used for shrimp farm monitoring of WSSV. The developed diagnostic kit includes a simple DNA extraction kit and protocol (patent pending), LAMP premix buffers and a fabricated heat block tailor-made for LAMP applications. Using the developed diagnostic kit, WSSV was tested

across the Philippines. A prevalence rate of 88% was observed in Luzon, 63% in Visayas and 65% in Mindanao [unpublished data].

The alarming number of pathogens present in our country remains to be a constant threat to the Philippine shrimp industry. A summary of the pathogens detected in the Philippines is presented in Table 1, and the areas covered by the pathogens are shown in Fig. 1, together with their annual prevalence during the period of 2010–2016. Developing rapid and farm-ready diagnostic kits for these pathogens are essential in aiding the industry and the government in shrimp health management. Early detection and rapid diagnosis of the disease is only the first step in creating a sustainable and productive aquaculture industry, its breakthroughs will go a long way for the mitigation and prevention of diseases, as well as understanding the nature of the relationship of the pathogen to its host. In the future lies a rapid, simple and cost-effective diagnostic platform that can be used by farm and hatchery operators for timely and accurate on-site detection of pathogens.

GENE SILENCING THERAPY

The development of strategies for the prevention of shrimp disease outbreak could be facilitated by an understanding of host-virus interactions. The suppression of gene expression based on sequence homology between the dsRNA trigger and the target gene comprises RNAi together with a set of related cellular processes. It is an approach with incredible potential for therapeutic purposes, using dsRNA that targets viral sequences or homologous viral sequences *in vivo* [31]. RNAi applications for shrimp diseases has been reviewed by Maningas and Tare [32], and the review is supplemental to the current paper. From the functional analysis of transglutaminase (TGase) and clotting protein (CP) in the shrimp clotting system using gene silencing as the determining assay for the role of the two genes

Table 1. Pathogens of Interest Detected in Shrimp Via Molecular Diagnostics

Pathogen	Infected shrimp species	Molecular detection platform	Sampling sites in the Philippines	Reference
<i>Bacterial</i>				
<i>Vibrio</i> spp.	<i>P. vannamei</i>	PCR and LAMP	Pangasinan	Nicolasora <i>et al.</i> (2014)
<i>Vibrio parahaemolyticus</i> causing Acute Hepatopancreatic Necrosis Disease	<i>P. vannamei</i>	PCR	Bataan, Bulacan, Pampanga, Cebu, Bohol, General Santos, Saranggani	Dabu <i>et al.</i> (2015)
<i>Viruses</i>				
WSSV	<i>P. monodon</i>	PCR and Nested PCR	Bulacan	Alenton and Maningas (2011)
	<i>P. vannamei</i>	PCR	Batangas, Zambales, Capiz and General Santos city	Maralit <i>et al.</i> (2011)
	<i>P. vannamei</i>	LAMP	Batangas, Zambales, Capiz and General Santos city	Maralit <i>et al.</i> (2012)
	<i>P. vannamei</i>	PCR and LAMP	Bulacan, Batangas, Laoag, Iloilo, and Leyte	Nicolasora <i>et al.</i> (2014)
	<i>P. monodon</i> and <i>P. vannamei</i>	PCR and LAMP	Bulacan, Bataan, Cebu, Davao and General Santos city	(Unpublished)
TSV	<i>P. vannamei</i>	PCR	Bulacan, Batangas, Bohol and Cebu	(Unpublished)
LSNV	<i>P. monodon</i>	PCR	Bulacan, Batangas, Pangasinan and Pampanga	(Unpublished)

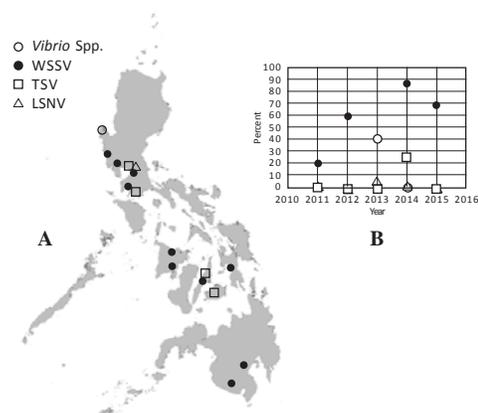


Figure 1. (A) Detected shrimp pathogen distribution in the Philippines; (B) Prevalence of shrimp pathogens per year.

[33] to the proposed molecular mechanism of the shrimp clotting system [34], succeeding studies followed a trend of uncovering host-virus interactions towards understanding and

harnessing these relationships for the prevention of disease outbreak [35].

WSSV-shrimp homologs were found to be involved in viral infectivity in kuruma shrimp, *Marsupenaeus japonicus* [36]. This finding was instrumental in several studies discussed in this review, reporting the discovered contigs involved in WSSV infection in the freshwater shrimp, *Macrobrachium rosenbergii* and the black tiger prawn, *Penaeus monodon*.

Immune defense responses are affected upon invasion of WSSV, as shown by the characterization of hemocyanin-like subunits of *M. rosenbergii*, initially known as contigs 13 and 37. Both genes were found to be upregulated during the early stages of WSSV infection [37].

Contig 23 is one of the most promising candidates for therapeutic development for

having rendered 100% survival rate at seven days post-infection when injected with c23-dsRNA and infected with WSSV, signifying its interaction with the virus for rendering its protective effect upon silencing both in *M. rosenbergii* and *P. monodon* [38]. The rest of the contigs, 20, 31 and 34 were found to be involved in the shrimp’s metabolic processes, which also responds to viral activity and triggers host responses upon infection [our unpublished data]. In addition to host genes, the knockdown of a viral gene VP9 has significantly increased shrimp survival even upon re-infection and is suspected to play a key role in viral replication [39].

A transcriptome database of genes from tiger shrimp that survived WSSV challenged was generated by Maralit [40], who also unveiled a ubiquitin conjugating enzyme-like gene that renders protective effect to WSSV challenged *M. rosenbergii* [unpublished data]. Table 2 summarizes the genes utilized by RNA interference discussed in this review.

An exciting platform has been provided by the gene silencing technology by showing the effect of knocking down a target gene on the organism. Interests on its application in shrimp is at a steady rate, Fig. 2 summarizes the authors intention to continuously join this effort, with the main goal of identifying genes suitable for

protecting the shrimps against infection thereby stabilizing shrimp production.

IMMUNOSTIMULATION FOR DISEASE RESISTANCE

Another approach that our group has adopted towards the prevention of shrimp pathogenesis is based on the natural process of immunostimulation. Immunostimulation is the introduction of molecules into the system of an organism with the intention of improving its response against infection from pathogenic causes [41–43]. An update on the use of immunostimulants in the Philippines has been published [44]. Thus, the coverage of this review focuses on the multiple studies engrossed on immunostimulation and done at the aforementioned laboratory; all these exploring the potential of recycled natural products and bacterial species found in shrimp gut (our unpublished data) as immunostimulants (Table 3).

Natural immunostimulants are safe for the environment and cost-effective. In addition, these can be sourced from bacteria, plant products, such as herbs and fruits, and even animal scraps, such as shells [42, 45–47]. What marks these products as viable candidates for immunostimulants is the throng of minerals and

Table 2. Target Genes Used in RNAi Studies Discussed in This Review

Target Gene	Source	Reference
Hemocyanin-like subunits	Host	Tare <i>et al.</i> (2015)
Contig 20	Host	Unpublished
Contig 23	Host	Unpublished
Contig 31	Host	Unpublished
Contig 34	Host	Unpublished
Ubiquitin conjugating enzyme-like gene	Host	Unpublished
VP9	Viral	Alenton <i>et al.</i> (2016)

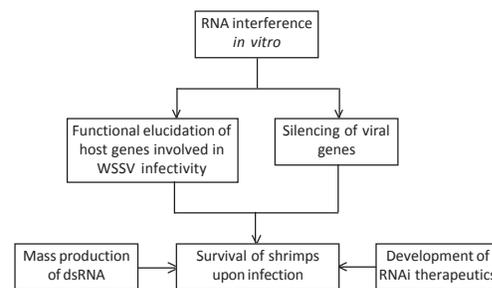


Figure 2. The conceptual framework of RNAi studies starting from *in vitro* production of dsRNA, the goal is to develop a more efficient, cost-effective, mass production of dsRNA and the eventual development of RNAi therapeutics for application in the field.

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compounds stored within their varying components. Waste plant products provide rich sources of metabolites such as carbohydrates, proteins and mineral constituents. It is also abundant in phytochemicals such as flavonoids, alkaloids, tannins and saponins which act as antioxidant and defense mechanism against free radicals that can cause debilitating disease in shrimps [48].

Several natural products derived from plant, algae, animal and bacterial sources have been explored in our laboratory for their immunostimulation potential (Table 3). To make use of the specific compounds, the raw materials were either subjected to further extraction, through either aqueous or ethanolic solutions [15, 49, 50], or introduced in their crude powdered forms [18, 51, 52]. Extracted treatments were more widely used since the crude extracts are comprised of more purified compounds such as phenols, tannins and flavonoids [48].

Herbal medicine like *Cyanodon dactylon*, *Aegle marmelos*, *Tinospora cordifolia*, *Picrorhiza kurooa*, and *Eclipta* resulted to better performance in the haematological, biochemical and immunological parameters from the immunostimulant-incorporated diet fed to WSSV-challenged *P. monodon* [49]. *Mangifera*

indica crude extract and powdered treatments against WSSV was found to yield 80% survival rates and elevated immune parameter values [our unpublished data]. *Mangifera indica* kernels have been used as immunostimulant for *Penaeus indicus* against WSSV [53]. Ethanolic and hot-water extracts from algae, (*Fucus*, *Laminaria*, and *Gracilaria*) exhibited antiviral activity and aided in the prevention of the attachment of the virus to host cells [54, 55]. Aside from algae, fruit berries also exhibit possible immunomodulatory properties. A crude ethanolic extract of a species of raspberry *Rubus coreanus* exhibited significantly increased immunity and expression of antioxidant enzyme activities in *P. vannamei* after challenged with *Vibrio alginolyticus* [56].

The method of administering immunostimulants to the shrimps revolve around three methods: feeding, immersion and injection. Of all the three methods, injection is the most invasive and has been devised in a study involving agar-carotenoid complex [unpublished data], chitin and chitosan [57], hot-water extract of *Gelidium amansi*, [58] and hot-water extract of *S. duplicatum* [59]. Apart from injection, introduction through feeding was also employed for eregosan [47], *Sargassum fusiforme* [60] and

Table 3. A Summary of the Immunomodulatory Studies Done by the Authors in This Paper

Source	Components	Extraction Method	Method of Introduction	Pathogen	Reference
<i>Plant Products</i>					
<i>Citrus microcarpa</i>	Vitamin C	Ethanol	Feeding	<i>V. alginolyticus</i>	Unpublished
<i>Curcuma longa</i>	Curcumin	Powdered	Feeding	<i>V. alginolyticus</i>	Alambra <i>et al.</i> (2012)
<i>Mangifera indica</i>	Mangiferin	Powdered	Feeding	WSSV	Unpublished
<i>Mangifera indica</i>	Mangiferin	Ethanol	Feeding	WSSV	Unpublished
<i>Musa paradisiaca</i>	Polyphenols	Powdered	Feeding	<i>V. alginolyticus</i>	Unpublished
<i>Marine Algae</i>					
<i>Gracilaria edulis</i>	Betaglucan	Aqueous	Immersion	<i>V. alginolyticus</i>	Maningas <i>et al.</i> (2013)
<i>Gracilaria edulis</i>	Betaglucan	Aqueous	Immersion	WSSV	Unpublished
<i>Sargassum polycystum</i>	Fucoidan	Ethanol	Feeding	WSSV	Arizo <i>et al.</i> (2015)
<i>Animal Sources</i>					
Shrimp Shells	Carotenoids	TOSS	Injection	<i>V. alginolyticus</i>	Unpublished
<i>Bacterial Sources</i>					
<i>Exiguobacteria</i> sp.	LPS	None	Feeding	<i>V. alginolyticus</i>	Unpublished

on fucoidan [61]. Immersion strategies have also been devised and utilized [50, 58]. Between feeding and immersion, the former is more widely employed since it required a smaller amount of extracted compounds [50, 59, 62, our unpublished data], as little as 500 mg of an extract was enough to fuel the whole study [63, 49, 15, our unpublished data]. As far as efficacy and complexity is concerned, all three treatment strategies generate lessened mortalities and higher immune parameter values; however, feeding is the simplest mode of administration due to the minimal volume of extracts required and the hassle-free preparation of experimental treatments [58, our unpublished data].

Compounds of immunostimulatory functions are often exploited for their antioxidant properties or their structural similarity to pathogenic components [46, 64]. Compounds with molecular structure similar to that of pathogens are termed as pathogen-associated molecular patterns (PAMPs), and are rooted to play a crucial role in the activation of the key enzymatic reaction of the crustacean immune system, such as the proPhenoloxidase (PO) system [65]. The use of PAMPs tend to produce inverse correlation between survival and compound concentrations [our unpublished data, 15]. Higher treatment concentrations cause an increase in free radical production that induce irreversible damage to both host and foreign cells and lead to weaker survival rates despite the increased immune parameter values. Similarly, since more PO reactions are produced, an exhaustion of the immune system can be observed as indicated by the drop in immune parameter values [68].

Antioxidants have also been employed as an immunostimulant [our unpublished data, 49, 53]. Antioxidant key compounds are expected to promote increased survivability, heightened hemocyte count and increased superoxide dismutase levels. This can be related to the

damage prevention to host cells, since the enzyme reactions produced by the humoral immune response tend to release free radicals that destroy foreign cells [67].

Aside from conferring protection against infection, growth was also found to be affected by the administration of immunostimulants. Crude fucoidan was able to increase the weekly weight gain of shrimp [15, 68]. Modified feeds enhanced the feed utilization and the growth performance of treated shrimps [69–71]. In summary, materials containing these compounds prove to aid in improving the shrimp growth performance and immune response against pathogens through their effective actions in regulating enzyme reactions (Fig. 3).

Developing immunomodulatory feed additives from scrap materials paves way to providing cost-effective substitutes for commercial immunostimulants such as Macroguard™. A U.S. patent 6440466 has been granted to the composite of plant extracts of *Lantena camera*, *Aegle marmelos*, *Ocimum sanctum*, *Mimosa pudica*, *Cynodon dactylon*, *Curcuma longa*, and *Allium sativum* which was found useful as prophylactic and therapeutic agents against viral (WSSV) and bacterial disease [72].

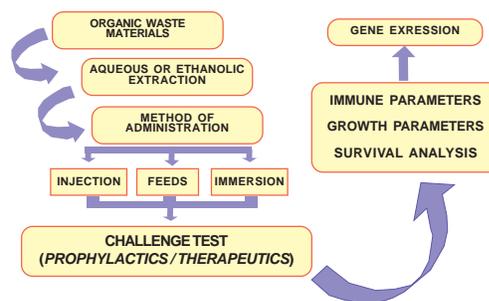


Figure 3. Immunostimulation experiments begin with the treatment preparation from organic materials and continue on to the challenge experiments wherein immune parameters, growth rate, survival analysis and even gene expression is evaluated.

Despite the advances made in the use of immunostimulants, the immune mechanism of crustaceans against viruses is yet to be defined, and more efficient natural therapeutic strategies can be developed. Endemic Philippine fruits and plants can be explored for possible efficacy as bactericidal and antiviral treatments.

CONCLUSION

The emergence of pathogens has yet to cease and thus, the struggle to develop mitigation techniques to hamper economic loss and promote shrimp production is a persistent challenge worth pursuing. The development of molecular diagnostics, RNAi technology and immunostimulants play a vital and significant role in improving shrimp health management and practices, and in reducing losses in the shrimp farming industry.

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