Sequence-optimized and targeted double-stranded RNA as a therapeutic antiviral treatment against infectious myonecrosis virus in *Litopenaeus vannamei*

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ABSTRACT: Infectious myonecrosis virus (IMNV) is a significant and emerging pathogen that has a tremendous impact on the culture of the Pacific white shrimp *Litopenaeus vannamei*. IMNV first emerged in Brazil in 2002 and subsequently spread to Indonesia, causing large economic losses in both countries. No existing therapeutic treatments or effective interventions currently exist for IMNV. RNA interference (RNAi) is an effective technique for preventing viral disease in shrimp. Here, we describe the efficacy of a double-stranded RNA (dsRNA) applied as an antiviral therapeutic following virus challenge. The antiviral molecule is an optimized dsRNA construct that targets an IMNV sequence at the 5′ end of the genome and that showed outstanding antiviral protection previously when administered prior to infection. At least 50% survival is observed with a low dose of dsRNA administered 48 h post-infection with a lethal dose of IMNV; this degree of protection was not observed when dsRNA was administered 72 h post-infection. Additionally, administration of the dsRNA antiviral resulted in a significant reduction of the viral load in the muscle of shrimp that died from disease or survived until termination of the present study, as assessed by quantitative RT-PCR. These data indicate that this optimized RNAi antiviral molecule holds promise for use as an antiviral therapeutic against IMNV.

KEY WORDS: Infectious myonecrosis virus · IMNV · RNA interence · RNAi · Double-stranded RNA · dsRNA · Therapeutic · Shrimp · *Litopenaeus vannamei*

INTRODUCTION

Infectious myonecrosis (IMN) is a severe and emerging shrimp disease of viral etiology that was first described in 2003 after a severe outbreak in northeastern Brazil that was characterized by high mortality of cultured *Litopenaeus vannamei* (Poulos

et al. 2006). The etiologic agent, infectious myonecrosis virus (IMNV), is a non-enveloped, double-stranded RNA (dsRNA) virus that is a member of the Totiviridae family. IMNV was discovered in Indonesia in 2006 (Senapin et al. 2007) and has caused tremendous impact on the shrimp industry in both Brazil and Indonesia (Lightner et al. 2012). During

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outbreaks of IMNV, mortality ranges from 40 to 70% prior to harvest, and epizootics cause a dramatic increase in feed conversion (Andrade et al. 2007). An epidemiological survey of IMNV in Brazil found that long growout periods and high stocking densities are significant risk factors that precipitate epizootics (da Silva et al. 2010). The insidious presentation of this disease, which can manifest in a gradual loss of animals over the duration of the growout period, makes IMNV an excellent target for a therapeutic agent because treatments could be initiated immediately following a diagnosis of IMNV in a pond. RNA interference (RNAi) is one possible and promising method to mitigate viral disease in shrimp (Robalino et al. 2005, 2007, Krishnan et al. 2009, Shekhar & Lu 2009, Hirono et al. 2011, Bartholomay et al. 2012). RNAi has been used to prevent shrimp disease caused by white spot syndrome virus (WSSV) (Robalino et al. 2005, Xu et al. 2007), yellowhead virus (YHV) (Tirasophon et al. 2005, 2007, Yodmuang et al. 2006), Taura syndrome virus (TSV) (Robalino et al. 2004), *Penaeus stylirostris* densovirus (PstDNV) (formerly called infectious hypodermal and hematopoietic necrosis virus, IHHNV) (Ho et al. 2011), and IMNV (Loy et al. 2012). In addition to administration prior to viral exposure, the potential for a therapeutic effect using RNAi has been tested against many different RNA viruses and DNA viruses of animals and humans, several of which are being evaluated in Phase 1 and 2 human clinical trials as antiviral therapeutics (Haasnoot et al. 2007). In shrimp, a therapeutic effect has been described against WSSV (Xu et al. 2007) and YHV (Tirasophon et al. 2007) after inoculation with dsRNA. Some evidence indicates that dsRNA administration and induction of RNAi pathways may allow for clearance of virus in shrimp naturally infected with *Penaeus monodon* densovirus (PmDV) (Attasart et al. 2011).

Recently, we described a sequence-optimized antiviral dsRNA (dsRNA 194-475) that targets the 5′ end of the IMNV genome and shows outstanding protection from IMNV-induced disease and mortality (Loy et al. 2012). The objective of these experiments was to test the therapeutic efficacy of this optimized antiviral during an active IMNV infection.

MATERIALS AND METHODS

SPF animal rearing

Specific pathogen-free (SPF) *Litopenaeus vannamei* postlarvae were provided by Shrimp Improvement Systems (Plantation Key, FL, USA) and reared in a biosecure animal holding facility at Iowa State University, Ames, IA, USA. Animals were placed in 1000 l tanks containing artificial seawater (Crystal Sea Marine Mix) at 25 ppt salinity, an oystershell airlift biofilter, and an activated carbon filter. The temperature was maintained at 30°C. The animals were fed a commercial growout diet until they reached 3 to 5 g in weight.

Preparation of viral inoculum

Based on a previously described methodology (Hasson et al. 1995), tissue macerate from infected animals (that tested negative for other shrimp viruses by PCR) was diluted 1:3 in TN buffer (0.02 M Tris-HCL, 0.4 M NaCl, pH 7.4) and clarified through 3 centrifugation steps: (1) $4000 \times g$ for 30 min, (2) $15\,000 \times g$ for 15 min, and (3) $25\,000 \times g$ for 60 min, after each of which the supernatant was removed. The final supernatant was diluted 1:10 in sterile 2% NaCl and passed through a 0.2 µm filter. This stock viral clarification was aliquoted and frozen at −80°C. Quantitative PCR analysis of RNA extracted following a freeze-thaw indicated 10^5 viral copy numbers µl^{-1} were present in the stock.

Determination of challenge dose

The optimum lethal dose of IMNV that resulted in infection and disease was tested by injection in SPF *Litopenaeus vannamei* weighing 8 to 10 g as described in detail previously (Loy et al. 2012). Briefly, each animal was injected with 100 µl of a 1:100 dilution of viral clarification into the third abdominal segment, a lethal dose that produces 100% mortality at 18 d post-challenge (d p.c.).

Quantitative reverse-transcription PCR

Quantitative reverse-transcription PCR (qRT-PCR) was performed using methods adapted from a previous report (Andrade et al. 2007). Briefly, RNA template was purified from 30 mg of macerated muscle tissue and homogenized using a QIAShedder column (Qiagen) followed by extraction with an RNeasy Minikit (Qiagen). RNA template was eluted from the column into 50 µl RNase-free water and stored at −80°C until RT-PCR was performed. For qRT-PCR, reaction templates were thawed and boiled at 100°C

for 5 min prior to reverse transcription. RNA template (2 µl) was mixed with 5 µl of One Step RT-PCR Master Mix (Qiagen), 1 µl Enzyme Mix, 1 µl dNTP, 0.3 µl IMNV412F (20 µM) (3′), 0.3 µl IMNV545R (20 µM), 0.3 µl IMNVP1 (10 µM), and 15.1 µl RNase-free water for a total reaction volume of 25 µl. This reaction was then run in duplicate or triplicate on a BioRad CFX96 real-time PCR detection system. Reverse transcription ran for 30 min at 48°C and 10 min at 95°C. The thermal cycling parameters included 35 cycles of 15 s at 95°C and 1 min at 60°C. The viral copy number was calculated using CFX Manager Software (Bio-Rad) using an *in vitro* transcribed RNA standard generated according to methods from a previous report (Andrade et al. 2007).

dsRNA preparation

dsRNA was prepared using the Ambion Megascript RNAi Kit following the manufacturer's instructions. Briefly, templates for *in vitro* transcription were prepared by PCR amplification from template cDNA using oligonucleotide primers with flanking T7 promoter sites (Table 1) (Loy et al. 2012). For injection into animals, dsRNA was diluted in RNase-free water to achieve a final dose of either 0.5 or 5.0 µg of dsRNA. A total volume of 50 µl containing the desired amount of dsRNA in RNAse-free water, or RNAse-free water alone as a control, was injected into the muscle of the third abdominal segment of 3 to 5 g SPF juveniles.

Histopathology

Moribund shrimp that were observed during the course of the experiment were fixed in whole David-son's fixative (Bell & Lightner 1988) for 24 h and then transferred to 70% EtOH prior to embedding in paraffin. In addition, 2 surviving animals from each tank at the termination of the experiment (20 d post-infection) were anesthetized in an ice slurry and fixed in Davidson's fixative (Bell & Lightner 1988), separated into head and tail sections, embedded in paraffin, sectioned onto slides, stained with hematoxylin and eosin, and evaluated for the presence of IMNV lesions.

Shrimp bioassays

Tanks containing 200 l of synthetic seawater and an oystershell airlift biofilter were stocked with 10 SPF juveniles weighing 3 to 5 g that were allowed to acclimate for 72 h. The tanks were randomized, and 3 replicates were assigned for each treatment. Following acclimation, shrimp were challenged by injection in the third abdominal segment with the known lethal dose of IMNV. Sham challenge groups received an equivalent volume of 2% NaCl, while negative control groups received only viral diluent. After 48 h, 5.0 µg of *in vitro*-synthesized dsRNA was inoculated into animals that had been challenged with IMNV. The sham-injection animals received an equivalent dose of RNase-free water, and the negative control animals were sham challenged with an equivalent dose of sterile 2% NaCl (viral diluent). The animals were counted daily for mortality and assessed for clinical signs. Moribund animals that were found were fixed for histopathology. At termination, 2 animals from each tank were fixed and submitted for histopathological examination. Dead animals were removed throughout the experimental period and frozen at −80°C.

Dose/duration bioassay

Ten shrimp per tank were challenged with IMNV then subjected to dsRNA treatment at 24, 48, or 72 h. Sham challenge (strict negative) groups received an equivalent volume of 2% NaCl (viral diluent). The

Table 1. Oligonucleotide primer sequences for double-stranded RNA (dsRNA) synthesis (with T7 sites underlined) and quantitative RT-PCR (qRT-PCR) (IMNV412F, IMNV545R and IMNVP1)

Oligonucleotide	Sequence 5′-3′	Source
IMNV194T7F	<u>TAA TAC GAC TCA CTA TAG GG</u>A AAC CGG AGC TGA CCA CAT TCC AA	Loy et al. (2012)
IMNV275T7R	<u>TAA TAC GAC TCA CTA TAG GG</u>A CTG TGT CAC ATG TTG CTG CTT CG	Loy et al. (2012)
eGFPT7F	<u>TAA TAC GAC TCA CTA TAG GG</u>A GAA TGG TGA GCA AGG GCG AGG AGC TGT	Loy et al. (2012)
eGFPT7R	<u>TAA TAC GAC TCA CTA TAG GG</u>A GAT TAC TTG TAC AGC TCG TCC ATG CCG	Loy et al. (2012)
IMNV412F	GGA CCT ATC ATA CAT AGC GTT TGCA	Andrade et al. (2007)
IMNV545R	AAC CCA TAT CTA TTG TCG CTG GAT	Andrade et al. (2007)
IMNVP1	(6FAM) CCA CCT TTA CTT TCA ATA CTA CAT CAT CCC CGG (TAMRA)	Andrade et al. (2007)

treatment groups were subjected to injection with either 0.5 or 5.0 µg of *in vitro*-synthesized dsRNA 194-475, and the control groups consisted of animals injected either with 5.0 µg of enhanced green fluorescent protein (eGFP) dsRNA (dseGFP) or an equivalent volume of RNase-free water.

Statistical analysis

End-point analysis of mean survival was conducted using 1-way ANOVA followed by a Tukey's multiple comparison test using JMP 8 software (SAS Institute). Mean log viral copy-number data were normalized from assays run in triplicate and then compared using 1-way ANOVA followed by a Tukey's multiple comparison test using JMP 8 software (SAS Institute).

RESULTS

A disease-challenge model was established that uses intramuscular injection of a clarified virus preparation that in previous studies resulted in 100% mortality over a 18 d period in 8 to 10 g SPF shrimp; this was used as the virus challenge dose for all subsequent experiments (Loy et al. 2012). In the first experiment, 3 to 5 g SPF *Litopenaeus vannamei* were subjected to a lethal intramuscular challenge of IMNV. After 48 h, a single 5.0 µg dose of dsRNA194-275 was administered to IMNV-infected animals. The animals that were given dsRNA194-275 dsRNA at 48 h p.c. demonstrated 50% mean survival at the termination of the study. By comparison, animals that received the same dose of dseGFP or the equivalent volume of RNase-free water at 48 h p.c. showed 100% mortality (Fig. 1). Significant differences (p = 0.007) were evident between the experimental and control groups.

Virus replication was measured from these animals as virus genome copy number using qRT-PCR on muscle tissues sampled from animals that were found dead either at 8 to 12 d p.c. or at the termination of the study. A significant reduction in virus copy number was evident between animals to which 5.0 µg dsRNA194-275 was administered (p < 0.001) (mean 5.84×10^5 and 4.87×10^4 IMNV copy numbers µl^{-1} RNA in animals that either succumbed to disease or survived until termination, respectively) compared with animals that received a 5.0 µg dose of dseGFP or RNAse-free water (5.43×10^6 and 4.29×10^7 IMNV copy numbers µl^{-1} RNA, respectively) (Fig. 1).

Dosage and temporal effects

To further characterize the observed therapeutic response, the temporal effects and dose-response factors that influence a successful IMNV therapeutic treatment were tested in a second experiment. At 24 h post-viral challenge, shrimp that received a 5.0 µg dose of dsRNA demonstrated 60% survival after treatment compared to 50% survival of the animals treated with 0.5 µg dsRNA. Control animals that
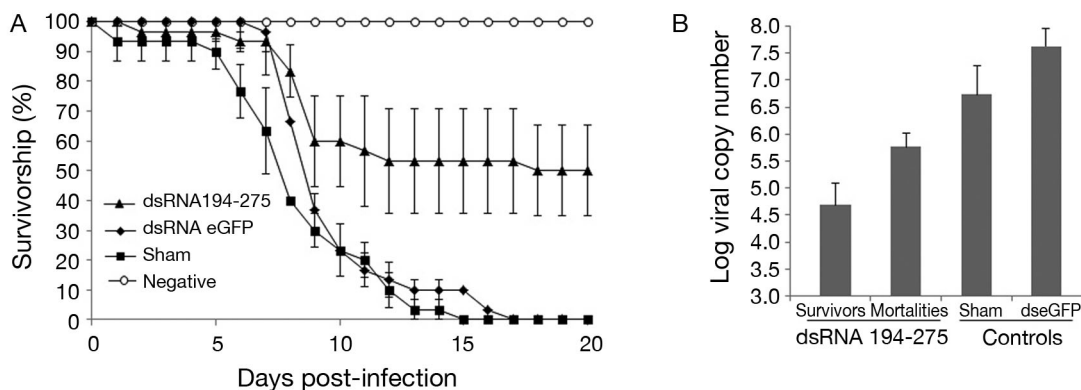

Fig. 1. *Litopenaeus vannamei*. (A) Evidence of therapeutic disease protection from an antiviral double-stranded RNA (dsRNA). Percent survivorship of shrimp (*y*-axis) over time (*x*-axis) that were infected with a lethal dose of infectious myonecrosis virus (IMNV) (Day 0), then injected with dsRNA194-275 at 48 h post-challenge (p.c.). Animals were observed for 20 d. n = 30 ind. in 3 replicate tanks of 10 ind. per treatment. (B) Log virus-genome copy number, as determined by quantitative RT-PCR and based on an RNA standard curve, in muscle tissue from IMNV-infected *L. vannamei* that were subjected to dsRNA 194-275, dsRNA eGFP, or sham inoculation. Muscle samples were collected from dead or moribund animals from Day 9 to 14 p.c. from the dsRNA 194-275 mortalities, sham, and dseGFP groups. Tissues were collected from dsRNA194-275 survivors (Survivors) that were sacrificed at Day 20 p.c. Bars represent standard error within the sample. n = 6 ind. per group

received 5.0 µg dseGFP demonstrated 30% survival, and RNase-free water-injected animals showed 0% survival (Fig. 2). Animals treated 48 h p.c. demonstrated 90% survival in the high-dose treatment group and 50% survival in the low-dose group, with 0% survival in groups receiving dseGFP and 20% survival in the RNase-free water-injected group (Fig. 2). At 72 h p.c., all experimental and control groups showed 0% survival (Fig. 2).

Gross lesions

Shrimp in each treatment group were examined for gross lesions daily. Presence of gross lesions was evident as single or multiple foci of opacity within the tail muscle. All individuals in all challenged groups developed focal or multi-focal muscle tissue opacity by Day 5 p.c. Several individuals treated with dsRNA194-275 in the 48 h p.c. had muscle tail opacity resolve by 12 d p.c. (Fig. 3). Lesions in untreated

animals, regardless of time point, did not resolve, and these animals succumbed to death.

Histopathology

Acute histopathologic lesions were seen only in infected shrimp and were characterized by coagulative necrosis and fragmentation of muscle bundles with edema prominent within and between affected muscle bundles. Infiltration of hemocytes was mostly limited to small clusters scattered along the connective tissue septa that separate groups of muscles. Chronic lesions were characterized by partial or total loss of myofibers with condensation/contraction of the fibrous connective tissue components. These lesions correspond with characteristic histopathological changes typically seen with IMNV infection (Poulos et al. 2006, Loy et al. 2012).

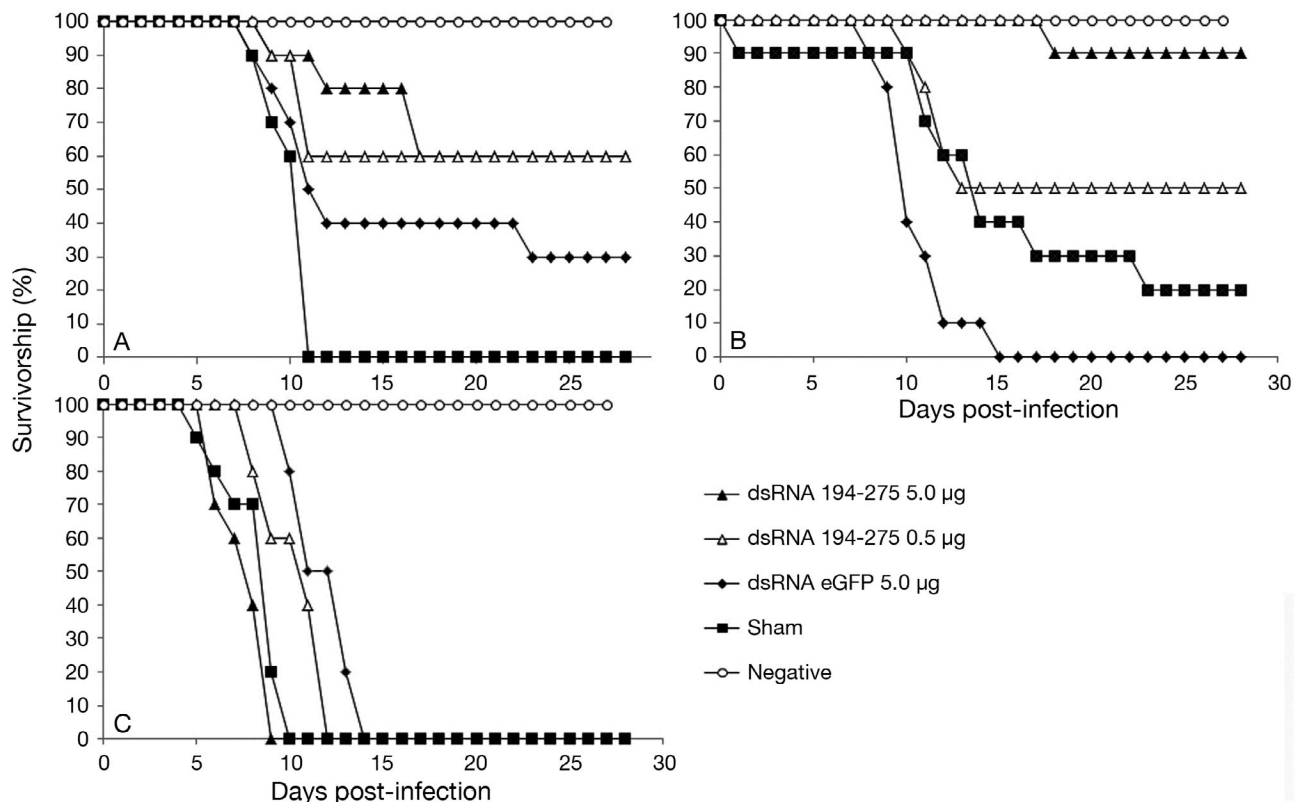Moribund shrimp that were found in tanks over the course of the study were sampled from RNase-free

Fig. 2. *Litopenaeus vannamei*. Effects of dose and timing on infectious myonecrosis virus (IMNV) treatment using an antiviral double-stranded RNA (dsRNA). Survival (displayed as percentage [*y*-axis] over time [*x*-axis]) of shrimp challenged by injection with IMNV and treated with dsRNA 194-275 at (A) 24 h post-challenge (p.c.), (B) 48 h p.c., and (C) 72 h p.c. Sham animals received an equivalent dose of RNase-free water, and negative-control animals were sham challenged with an equivalent dose of sterile 2% NaCl. n = 10 ind. per treatment
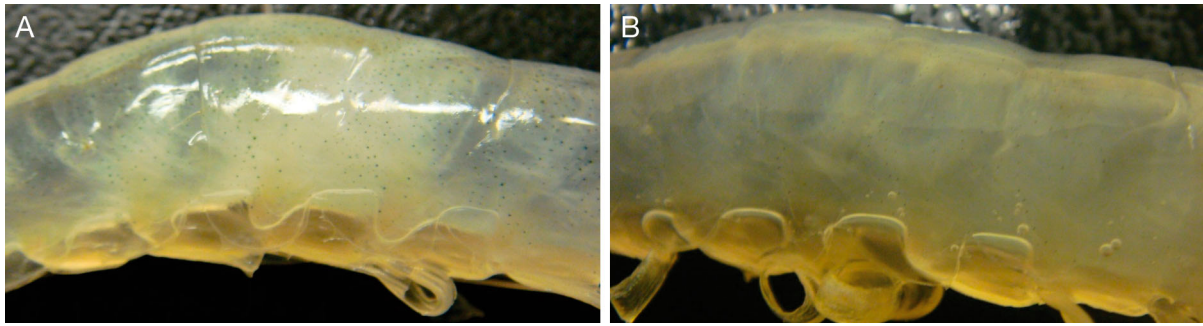
Fig. 3. Representative images of gross lesions from an individual shrimp abdomen. (A) Focal to multifocal muscle opacities were observed 4 d post-challenge (p.c.) (2 d post-dsRNA194-275 administration). (B) Gross lesions resolved by 12 d p.c. (10 d post-dsRNA administration)

water-injected controls (7 animals), eGFP dsRNA controls (3 animals), and animals treated with dsRNA194-275 (4 animals). Additionally, 2 animals from each tank were sampled from the surviving animals at the termination of the study (9 animals). Histopathological lesions in moribund animals of the animals from all groups were characterized predominantly by extensive fibrosis, occasionally with concurrent acute coagulative necrosis. Animals treated with dsRNA194-275 that survived until termination of the present study had chronic resolving lesions that varied from extensive fibrosis (2 animals) to scattered small foci of fibrosis (3 animals) or no significant lesions present (3 animals). No acute lesions were seen in any of these surviving treated animals.

DISCUSSION

These studies describe the first use of RNAi to treat disease in shrimp with an active IMNV infection. A previous report from our lab demonstrated that a specific dsRNA (dsRNA194-275) confers robust and long-term resistance to IMNV-induced disease and mortality when administered prior to virus infection (Loy et al. 2012). The present experiments demonstrate that the same dsRNA can also be used to therapeutically treat animals with pre-existing viral infections. This represents a significant step toward developing an antiviral therapeutic for infected animals as part of a disease-control program. Such a protocol could help to eliminate or reduce IMNV virus loads and disease burdens in infected ponds.

To determine threshold therapeutic doses of dsRNA and simultaneously characterize the temporal activity of the RNAi response, shrimp were subjected to 2 doses of dsRNA (0.5 or 5.0 µg). Survival was increased, compared to the controls, at either dose when the animals were treated with dsRNA194-275

at 48 h post-IMNV infection. Therefore, low doses of this antiviral dsRNA provide protection in animals when administered early in the disease process. This holds true when the dsRNA is delivered prior to infection as a preventative; even a dose of 20 ng protected animals from succumbing to IMNV disease (Loy et al. 2012). In contrast, in a therapeutic context, when dsRNA194-275 was administered 72 h post-challenge, no survival-enhancing effect was observed. This may be due to a significant amount of viral replication taking place between 48 and 72 h, such that the amount of virus replication that has occurred exceeds the capacity of an RNAi antiviral effect. Previous studies of the potential therapeutic effect of dsRNA on YHV or PstDNV disease revealed such an effect only with higher doses and shorter intervals between exposure and treatment. For example, a 25.0 µg dose of dsRNA provided protection 3 or 12 h after YHV infection (Tirasophon et al. 2007), and a 5.0 to 12.5 µg dose of dsRNA was protective 24 h post-PstDNV infection (Ho et al. 2011). No experimental studies have examined the replication dynamics of IMNV in shrimp, but the data presented here suggest that a large amount of virus replication occurs between 48 and 72 h post-infection, after which time the survival-enhancing effect of dsRNA treatment is reduced.

The temporal threshold of the efficacy of dsRNA194-275 here may also be explained by the target of this dsRNA, a putative cleavage protein product called 'Protein 1' that contains a dsRNA-binding motif (Poulos et al. 2006). This protein may be involved in suppression of host cellular RNAi responses or innate immunity during early virus replication. Administration of dsRNA-targeting Protein 1 induces highly significant protection with low doses, and thus, it is proposed that this protein plays a critical role in pathogenesis. Proteins from insect viruses such as *Drosophila* C virus (DCV) and Flock

House virus (FHV) have shown dsRNA-binding activity and inhibition of the host antiviral RNAi machinery (Li et al. 2002, van Rij et al. 2006). If this putative dsRNA-binding protein indeed suppresses an element of the RNAi pathway and the protein was expressed to a high degree in a large number of infected cells, the ability for the host to elicit an appropriate antiviral RNAi response would be impeded. Further studies of the role of this dsRNA-binding protein and the role it may play in immune suppression and in IMNV pathogenesis are warranted.

We observed a significant therapeutic effect in *Litopenaeus vannamei* from an optimized IMNV-specific dsRNA provided within 48 h of exposure to a lethal dose of virus. Furthermore, a small dose (0.5 µg) of this antiviral dsRNA prevented mortality in animals with an active, early stage infection with IMNV. RT-PCR diagnostic methods are available and currently being implemented on many farm laboratories or pond sites to enable rapid turnaround for a definitive diagnosis (Andrade & Lightner 2009, Puthawibool et al. 2009). If feasible delivery methods for dsRNA in shrimp ponds become available, a therapeutic treatment of this antiviral could mitigate of IMN disease losses in virus-infected animals.

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