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ORIGINAL ARTICLE

Protection of *Penaeus monodon* from Infection of *White spot syndrome virus* by DNA Construct Expressing Long Hairpin-RNA Against ICP11 Gene

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Abstract A plasmid construct (pICP11-LH) was designed to constitutively express long-hairpin RNA (lhRNA) against *icp11* gene, which is reportedly the most highly expressed gene of *White spot syndrome virus* (WSSV) and likely to have an important role in viral pathogenesis. The construct was used singly and in combination with other similar constructs designed against vp28 and vp19. A total of 6 treatments, T1 (pICP11-LH; 35 μg), T2 (pVP28-LH; 35 μg), T3 (pVP28-LH and pVP19-LH; 17.5 μg each), T4 (pVP28-LH and pVP19-LH; 25 μg:10 μg), T5 (pICP11-LH, pVP28-LH and pVP19-LH; 11.5 μg each) and T6 (pGFP-LH; 35 μg) were injected intramuscularly into 20 g *Penaeus monodon* specimens. The shrimp were challenged with WSSV 24 hpi and protection efficacy was measured in terms of survival and viral load 15 days after challenge. Appropriate negative and positive controls were used. T2 and T3 offered highest protection (75%) followed by T1 (67%) and T4 and T5 groups (58%), while T6 showed 25% protection. In all the target specific treatments, the viral load as estimated by single tube WSSV kit was kept in check (10–100 copies), whereas in the unimmunized challenged controls it progressed to severe infection (>10^5 copies). In spite of over 3 times higher expression of ICP11 compared to VP28, its knockdown by pICP11-LH did not offer any protective advantage over pVP28-LH, either singly or in combination. Moreover, none of the combinations bettered the protection efficacy of pVP28-LH administered alone. To investigate concerns about deleterious effect of plasmid persistence and constitutive expression on shrimp growth, a lab-scale 1 month growth study was conducted with 4 treatments T2, T3, T4 and T6, where no difference in specific growth rate was observed compared to controls.

Keywords ICP11 · WSSV · *Peneaus monodon* · Long hairpin RNA · Combinatorial RNAi

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Introduction

Since the first outbreak in 1992 in Northern Taiwan, White spot syndrome virus (Family: Whispovirus, Genus: Nimiviridae) clearly has the distinction of being the most researched shrimp virus during the last two decades due its catastrophic effect on global economy. Lately, considerable scientific effort has been directed at exploring the use of RNAi molecules for controlling this devastating virus [8, 10, 15, 28]. This strategy is particularly attractive because the rudimentary specific immune system of shrimp limits the success of conventional strategies like protein based vaccination [20, 22, 31, 32] and use of immunostimulants [3].

RNAi mediated silencing of genes of viral pathogens such as WSSV, TSV and YHV has been reported in various shrimp species [8, 15, 24, 28, 35] using synthetic siRNA, long dsRNA or lhRNA administered through injection or feed. The key to effective viral silencing by RNAi is to target genes that have a high significance in the viral life-cycle and pathogenesis. VP28 and VP19 are such important structural proteins as reported by several researchers [21, 25, 34, 36]. Recently, ICP11 has been identified as the most highly expressed protein of WSSV [26] indicating its high functional significance and it is important to evaluate its potential as a protective antigen. This is also important in the light that despite being the most highly expressed

protein, it was not reported as a major antigenic protein in numerous studies conducted since the virus was first reported in 1992. A recent study from our lab [10, 11] demonstrated the efficacy of DNA constructs capable of expressing lhRNA against WSSV capsid genes (*vp19* and *vp28*) in protecting *Penaeus monodon* from WSSV infection. The results showed VP28 to be more protective than VP19, which is why several researchers have targeted this protein for vaccine development [8, 21, 28, 31]. Here, a similar construct was designed against ICP11 to evaluate its protection efficiency on WSSV challenge.

Moreover, since Krishnan et al. [10, 11] could not obtain total protection by targeting *vp28*, it seemed likely that targeting more than one crucial viral genes simultaneously, would improve protection as it does in case of subunit vaccines. Therefore, lhRNA expressing constructs targeted against *icp11*, *vp28* and *vp19* were used in various combinations to see the effect on survival of challenged shrimp. CoRNAi therapies have been used earlier to blend inhibitors of viral gene expression in order to maximize efficacy and minimize the risk of mutational escape [5].

Although plasmid constructs are stable, economical and possible to use in shrimp due to absence of interferon response, they are known to persist for at least 2 months [16] in the shrimp post injection. In addition, lhRNA expression is from a constitutive promoter, thereby raising concerns about its possible impact on the growth of treated shrimp. Hence, the present work also included a lab-scale growth study to evaluate any deleterious effects of knockdown constructs.

Materials and Methods

In Silico Analysis of the Target Sequence

Following the guidelines of Elbashir et al. [4] and Hsieh et al. [7] the portion of sequence to be used in making the lhRNA expression construct was drawn from the WSSV *icp11* gene sequence (or wsv230, Acc. No: NC_003225.1) using E-RNAi software (www.e-rnai.dkfz.de). A BLASTn homology search was performed with the target sequence to ensure that it did not share any significant homology with other reported genes of *P. monodon* or WSSV. Also, a multiple sequence alignment between different target sequences (*icp11*, *vp19*, *vp28 and gfp*) was performed using ClustalW online software to determine their specificity and to rule out any possible off-target effects at least computationally. The lhRNA was also analyzed using RNAfold online software (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) to see that the sequence forms a perfect hairpin and there are no any other secondary structures which might affect the efficiency of the lhRNA by interfering with its hairpin formation.

Construction of lhRNA Expression Plasmid against *icp11* Gene

Genomic DNA was isolated from WSSV infected *P. monodon* specimens procured from Nellore, Andhra Pradesh, India in January, 2009. Long hairpin RNA expression construct against WSSV *icp11* gene was made following the procedure of Krishnan et al. [10, 11]. The selected portion (204 bp) of the *icp11* gene was amplified from WSSV infected shrimp genomic DNA using a linker primer pair 5′-AAAGCTAGCGCTGGTGGGG GATGATACTA-3′ and 3′-AAAAAGCTTGGCACAATCATGGGTTGAAT-5′ and cloned in sense orientation between *Nhe*I and *Hind*III sites of the pcDNA3.1(+) vector. The same fragment was amplified with the same primers having different linkers, this time for *Xho*I and *Kpn*I and was cloned in antisense orientation downstream to the sense fragment leaving a spacer region of 8–10 bp in between. The spacer was to facilitate folding of expressed transcript into a long hairpin. The clones were transformed into *E. coli* DH5α using standard protocols [17] and the positive clones were confirmed by sequencing. The resultant plasmid construct was designated as pICP11-LH.

Rearing of Experimental Animals

Penaeus monodon of 10–20 g body weight were maintained in natural sea water in 1,000 l fibreglass tanks with aeration at r.t. (27–30°C) and salinity ranging between 20 and 25 ppt. The animals were fed thrice a day with artificial pellet feed (CP, Thailand). Water quality parameters such as temperature, pH, salinity and dissolved oxygen were observed at fortnightly intervals. Prior to use, the experimental animals were screened for WSSV using Single-tube WSSV detection kit (Bangalore Genei, India) and only healthy individuals were used for the experiments.

The experiments were conducted in 100 l capacity rectangular crates filled two-thirds with seawater and thoroughly aerated. The animals were maintained in individual compartments within the crates using perforated plastic separators to avoid cannibalism. Water quality was maintained uniformly throughout the period.

In Vivo Titration of Viral Dose

The crude WSSV extract for experimental challenging of the animals was prepared by the method of Xie and Yang [33]. The viral dose was assessed to determine the dilution factor that results in 90–100% mortality of shrimp in about 10 days. Each crate with six individuals constituted one batch. The experiment was done in duplicate. Crude WSSV preparation was diluted with STE buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA; pH 8.0), 1:1 and 1:2 times and 75 µl of each dilution

and also the undiluted extract was administered to one batch of shrimp constituting 12 individuals by an intramuscular injection on the ventral side of the third abdominal segment. The fourth control batch received 75 µl of STE buffer and the mortality pattern was observed daily over 10 days.

Evaluation of Protection by Knockdown Constructs

The pICP11-LH prepared in this study and three LH constructs prepared earlier, namely pVP28-LH, pVP19-LH and pGFP-LH [10, 11], were used singly and in combination to evaluate their protection efficiency against WSSV. A total of eight treatment groups with six animals each were set up in triplicate. The predetermined effective dosage of 35 µg [10, 11] of total plasmid concentration at a constant volume of 75 µl was administered to each of the experimental groups. In case of silencing construct cocktail, different concentration ratios (totalling to 35 µg) were tested to optimize the effective concentration of each construct in a combination (Table 1). Both positive and negative control animals were administered 75 µl of STE buffer in place of the RNAi constructs. All the treatment groups except negative control were challenged with 75 µl crude viral extract 24 h after injection of construct/s.

The efficiency of different silencing constructs in protecting shrimp from WSSV was determined both by calculating the percent survival over a period of time and semi-quantitative estimation of viral copy number. The death of animals in treatment groups was confirmed to have been caused by WSSV using Single Tube WSSV detection kit (Bangalore Genei, India) and histopathology [12]. This kit also allows semi-quantification of viral copy number with severely infected samples showing 3 bands of 941, 525 and 204 bp ($>10^5$ copies); those with moderate to high infection showing 2 bands of 525 and 204 bp (10^3–10^5 copies) and those with low infection showing one 204 bp band (10–10^2 copies).

Growth Studies on Vaccinated Shrimp

Growth studies were conducted to determine any effect of plasmid persistence and expression on the growth rate of vaccinated shrimp. Sixty animals each weighing 15–25 g were transferred to rectangular plastic crates divided into six compartments each. This set-up enabled us to collect data from the same individual at the end of the experimental period, although it might not have been an ideal condition for high growth rate. The initial weight of the animals was noted followed by acclimatisation to tank conditions for 2 days. Five treatments, T2, T3, T4, T6 and negative control (NC) detailed in Table 1 were set up in duplicate. A total of 35 µg plasmid DNA was administered to each animal in a constant volume of 75 µl as before. The control animals were given 75 µl of STE buffer. The experimental shrimp were fed ad libidum with commercial pelleted feed (CP Feed, Thailand) and monitored thrice a day to ensure healthy conditions. After 1 month, the final weight of the animals was recorded and the effect of treatments on growth was estimated using ANOVA.

Results

In Silico Analysis

The specificity of the selected 204 bp sequence of ICP11 gene was confirmed by BLASTn homology search with "highly similar" and "more dissimilar" parameters, which resulted in hits entirely from WSSV ICP11 gene of Taiwanese and Chinese isolates. The 204 bp sequence amplified from the Indian isolate has been submitted to NCBI GenBank (Acc. No. HM778020). A multiple sequence alignment was also performed to check the level of homology between different RNAi and target sequences used in this study to identify possible cross talk between unrelated sequences. It was observed that not even a single stretch of more than 4 bp identity was found between *icp11*, *vp19*, *vp28* and *gfp* sequences. Off-target effects on shrimp genes could not be taken into account as genome information is not available. The *icp11* lhRNA sequence was also analyzed for the formation of hairpin *in silico*, which showed a perfect hairpin while no other secondary structures were observed.

Table 1 Description of treatment groups

Treatment	Treatment description	Dose	Survival (%)
T1	pICP11-LH	35 µg	67+%
T2	pVP28-LH	35 µg	75+%
T3	pVP28-LH:pVP19-LH	17.5 µg:17.5 µg (1:1)	75+%
T4	pVP28-LH:pVP19-LH	25 µg:10 µg (2.5:1)	58.3+%
T5	pVP28-LH:pVP19-LH:pICP11-LH	11.6 µg each (1:1:1)	58.3+%
T6	pGFP-LH	35 µg	25%
P.C.	Positive control	75 µl of STE buffer	100%
N.C.	Negative control	75 µl of STE buffer	0%

Effect of RNAi Constructs on Shrimp Survival

The efficiency of the sequence specific viral lhRNA expression constructs (singly and in combination) and an unrelated construct in preventing mortality in WSSV challenged shrimp were compared. The experimental animals administered the various constructs as given in Table 1 and the positive control group injected with STE buffer were challenged 24 h post injection with WSSV crude extract. The negative control group was not challenged. Each treatment was administered to three batches of 6 animals each. The optimum viral dose was determined in a pilot study by injecting different batches of shrimp with 1:1 and 1:2 dilutions along with an undiluted crude extract and monitoring the mortality pattern over a period of 10 days (Fig. 1). For challenge studies the viral titre in the inoculum was to be such that it caused 90–100% mortality in 10 days. That condition was met by the crude extract which caused 92% mortality on 10th day (and complete mortality by the 13th day). On this day 1:1 dilution caused 50% mortality and 1:2 dilution caused 17% mortality. Hence, the crude extract was used for challenge experiments.

The percent survival of shrimp in all the experimental groups post challenge was calculated over a period of 15 days. The positive control group animals succumbed to the infection with 100% mortality in 15 days while there was no significant mortality in the negative control group. Among all the treatment groups, both T2 and T3 had highest survival percentage (75%) with T6 (unrelated control) being the lowest (25%). The mean survival percentage in T1 group was 67%, followed by T4 and T5 where it was 58.3% (Fig. 2). Within the various combinations of silencing constructs used, pVP28-LH and pVP19-LH with a concentration ratio of 1:1 showed better protection, which is equal to the level of protection offered by VP28 alone. During the experiment, the shrimp were observed periodically and histopathological studies were done to establish that mortality was due to WSSV only. Histological sections prepared from the moribund shrimp of all treatment groups showed the characteristic inclusion bodies, typical of WSSV (Fig. 3).

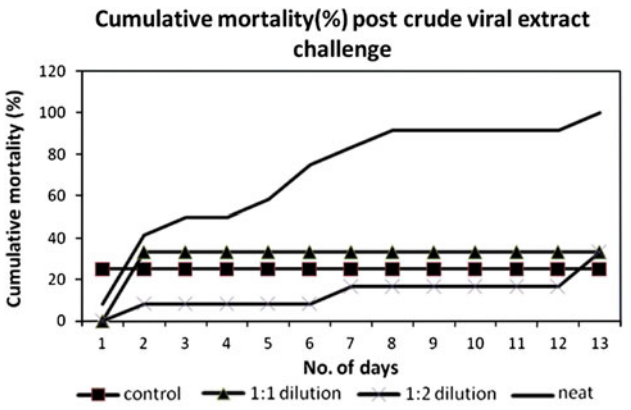

Fig. 1 Cumulative mortality caused by different dilutions of WSSV crude extract in shrimp

Effect of Knockdown Constructs on Viral Copy Number

Pleopod samples were obtained on 15th day from 3 surviving animals in each treatment group. Since all but one shrimp died on the 13th day in PC group, DNA was isolated from 3 animals collected on that day. The samples were analysed using the Single tube WSSV detection kit (Bangalore Genei, India) as per the manufacturer's

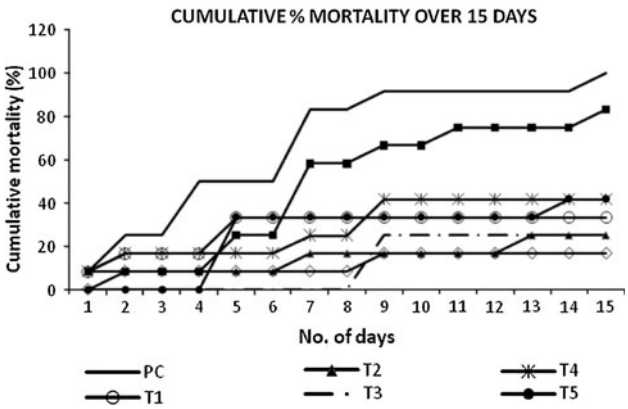

Fig. 2 Cumulative percentage mortality in different experimental groups over 15 days

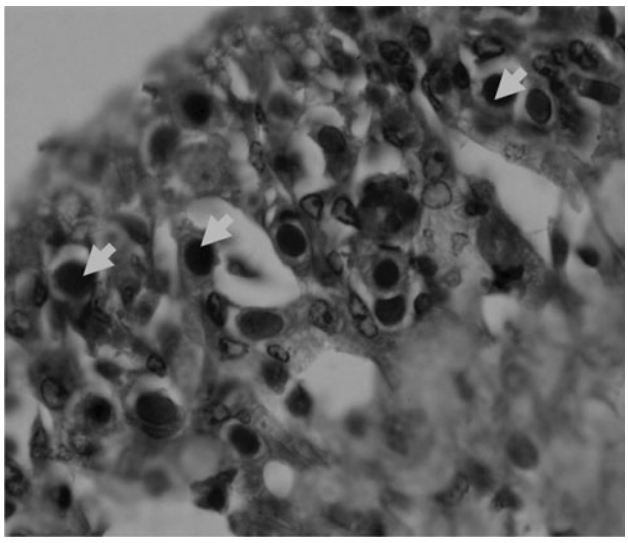

Fig. 3 Histological section of moribund shrimp ectoderm showing hypertrophied nuclei and basophilic inclusions characteristic of WSSV. ×100 (H&E)

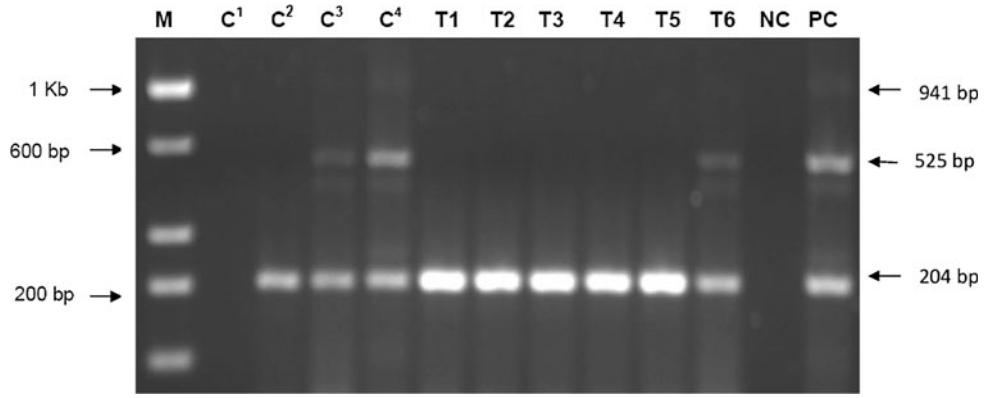

Fig. 4 Determination of viral copy number in different experimental groups. *Lane M*: molecular size marker (STWSSV kit); *lane C1*: negative control (kit); *lane C2*: mild infection (kit); *lane C3*: moderate infection (kit); *lane C4*: severe infection (kit); *lanes T1 to T5*: mild infection in samples of T1 to T5 groups; *lane T6*: moderate infection in T6 group; *lane NC*: no infection in negative control; *lane PC*: severe infection in positive control

instructions to determine the viral copy number in the animals [4]. All specific antiviral treatments (T1 to T5) were observed to keep the viral copy number in check at 10–100 copies 15 days after challenge (Fig. 4). In T6 samples the infection progressed to moderate (10^3–10^4 copies) while it was severe ($>10^5$ copies) in the positive controls as expected. The negative controls showed no infection. All the 3 individuals from each treatment showed the same PCR profile.

Effect of RNAi Treatment on Growth

Treatments T2, T3, T4 and T6 as detailed in Table 1 were set up in duplicate to observe the effect of lhRNA and the presence of silencing construct on growth of immunized shrimp. The control animals were administered 75 µl of STE buffer. Initial weight was recorded from the shrimp immunized with 35 µg of silencing construct(s) in a 75 µl volume and allowed to grow with routine feeding and cleaning. After a month of experimental period, the final weight of the animals was recorded and the specific growth rate calculated. There was a marginal increase in weight and no significant difference in the specific growth rate was observed among different treatments and control ($P \leq 0.05$), indicating that the treatments had no adverse effect on growth (Fig. 5).

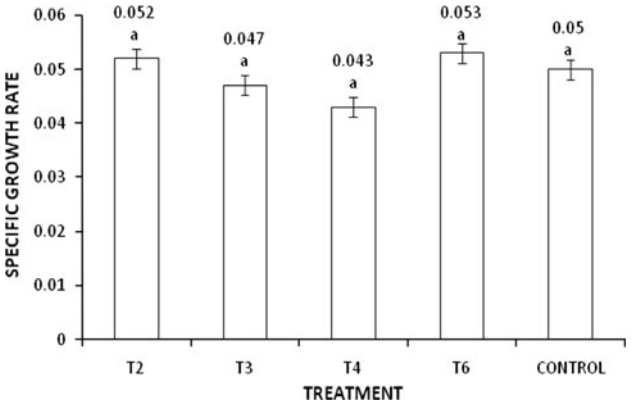

Fig. 5 Specific growth rate of *P. monodon* over a period of 1 month in different experimental groups injected with RNAi vaccines

Discussion

It is generally accepted that those viral proteins that play a crucial role in establishment and propagation of infection are good targets for vaccine development. VP28, which is a major envelop protein of WSSV, has proved to be one such target and many researchers have reported high efficacies of RNAi molecules targeted against this gene [10, 11, 14, 18, 19, 29, 30]. *Icp11* gene has been shown to be expressed over three times more than *vp*28 by Wang et al. [26], who used microarray and EST screening techniques. Though ICP11 is a late gene [26] ICP11 protein could be detected in Western blots as early as 18 h post infection (hpi) due to high levels of expression, whereas VP28 could not be detected until 48 hpi. Although Western blotting could not detect the presence of ICP11 in the WSSV virion, indicating its non-structural nature [26], its high expression in infected organs of *P. monodon* suggests an important role in the pathogenesis of the virus. An indication of what this role might be has also been provided by Wang et al. [27], who have shown that ICP11 that co-localizes in nucleus and cytoplasm [26] binds to histone proteins H2A, H2B and H3 and interferes with nucleosome assembly. Their findings also indicate that ICP11 inhibits repair of double strand breaks in host DNA and can promote apoptosis when expressed in mammalian HeLa cells. This action of ICP11, coupled with the suggestion that this could be a late gene [26] seems to indicate that this protein could be

playing a role in causing cell lysis of the host shrimp cells and thereby contributing to the spread of infection. Its high expression corresponds to the highly expressed histone proteins, which it seems to sequester. With this understanding the *icp11* gene was selected for knockdown to evaluate the protection this would provide against WSSV challenge. As is evident by the results, high expression does not necessarily qualify a protein as an effective protective antigen, and the survival of animals in the treatment group injected with pICP11-LH alone was not the best observed in this study. It is however possible that higher transcript level of ICP11 could not be effectively silenced by the pICP11-LH dose used in the study. In fact, dose dependent silencing of ICP11 transcript can be estimated by Real time PCR in future studies. The only other construct tried singly pVP28-LH gave a significantly higher protection. In an earlier study, Krishnan et al. [10, 11] reported the same survival percentage (75%) with this construct, while it was 69% with pVP19-LH and 25% pGFP-LH, when administered singly. It may be noted that the protection offered by pICP-LH in this study is similar to that given by pVP-19 earlier [10, 11].

The *icp11* sequence used in this study is a 249*nt* long ORF coding for an 82 amino acid protein of 9.2 kD reported by Wang et al. [26]. The lhRNA designed against this target gene spans almost its entire length (82%) of the ORF. The 204 bp sequence amplified from the Indian isolate (infected shrimp collected from Nellore, Andhra Pradesh in January 2009) had 99% homology with the 4 GenBank entries of Taiwanese and Chinese isolates. It seems that *icp11* too is highly conserved like the *vp28* gene for which several sequences have been reported from various countries. The lhRNA against *vp28*, *vp19* and *gfp* genes was 326, 186 and 359 bp long, covering about 53, 51 and 48% of the respective ORFs. Thus it is noteworthy that even though lhRNA against *icp11* targeted 82% of the ORF, it did not improve survival on WSSV challenge. The VP19-LH, VP28-LH and VPGHP-LH have been shown to silence the respective genes both in vitro and in vivo using RT-PCR. The protein expression was also knocked down as observed by In-Vision His-tag In-Gel staining [9].

The combinatorial RNAi approach has mainly been aimed at getting around viral escape by point mutation, especially in cases where si/shRNAs are used to target single genes as reviewed by Grimm and Kay [5]. Use of ds/lhRNA itself is a strategy to generate several siRNAs thereby preventing viral escape. Sarathi et al. [18] used bacterially synthesized 615 bp long dsRNA specific to WSSV VP28 gene and reported a 100% survival in WSSV-challenged shrimp injected with VP28-dsRNA. Kim et al. [8] targeted WSSV VP28, VP281 and protein kinase genes using long dsRNA of 489, 511 and 502 bp, respectively and obtained 100, 80 and 93.3% survival on WSSV challenge. In our case however, the combinatorial RNAi approach is analogous to a subunit vaccine where several protective antigens are pooled to generate a more effective and broad based immunity against the pathogen. LhRNAs have not been tried in combination earlier and this study was meant to explore the possibility of increasing the survival percentage by this means. However, this was not observed for any of the combinations tried.

Since there are concerns of saturating the endogenous RNAi pathway [1, 2, 6, 11], the final dose was restricted to 35 μg in all cases as was earlier standardized by Krishnan et al. [10]. Since T3 (pVP28-LH:pVP19-LH = 1:1) was as protective as T2 (pVP28-LH alone), it is difficult to say why T4 (pVP28-LH:pVP19-LH = 2.5:1) should've provided significantly lower protection. A larger field study may further confirm these differences in survival. The combination of all the three constructs in equal ratio (T5) too failed to better the survival provided by pVP28-LH alone. However, a semi-quantitative measurement of the viral load in all groups showed that all the specific treatments (T1-T5) could hold the viral infection at 10–100 copy numbers. Perhaps absolute viral copy number estimation by Real Time would have revealed finer differences between treatments in tune with survival data, and that could not be included in this study.

The unrelated GFP-LH construct could ensure 25% survival of challenged animals, which was significantly more than the unimmunized challenged controls. Protection by unrelated sequences has been observed earlier and is generally attributed to triggering of non-specific immunity in the host or off-target effects of the unrelated sequence [8, 15, 23, 28]. In case of organisms for which full genome sequences are available it is possible to take off-target effects into account while designing RNAi molecules, but this could not be done for *P. monodon*.

Although use of plasmid constructs expressing RNAi molecules as vaccines addresses issues of stability and economics in shrimp aquaculture, its persistence and constitutive expression raises concerns of any deleterious effects on shrimp growth. The lab-scale growth trials conducted for 4 selected treatments show no such effect and the growth rates remained similar to the un-injected control group after a period of 1 month. Similar observation was recorded by Lu and Sun [13] who developed a transgenic shrimp expressing AS-RNA against TSV coat protein gene. They reported that there was no statistically significant difference in the growth rate of transgenic shrimp as compared to normal control animals, as measured by body weight. However, field trials for growth will be needed to confirm these findings. So far the plasmid construct has been shown to persist in the shrimp body for at least 2 months. Krishnan et al. [10] also amplified the plasmid sequences from shrimp 1 month after injection, as

also confirmed in this study (results not shown). A longer duration study will show whether it actually persists through the entire culture period that lasts about 6 months. Since, *P. monodon* does not mature in normal culture conditions the effect of this plasmid on fertility and fecundity related parameters is not a major concern. However, since injection of the vaccine is not convenient for field application and the requisite plasmid dose is considerably high, different routes of administration and doses will need to be standardized before the vaccine is directly applicable.

We would like to conclude that regardless of its being a highly expressed protein, ICP11 knockdown was not as effective in protecting the shrimp against WSSV as VP28. Plasmid constructs expressing lhRNAs when used in combination could not provide better survival against WSSV challenge although they could contain the viral infection at low severity as effectively. The persistence of the plasmid and its constitutive expression did not affect the specific growth rate of treated individuals in a lab-scale study. Since pVP28-LH could provide the highest protection observed in this study, it can be developed into a stable, economical option for protection against WSSV in shrimp aquaculture.

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