Characterization of newly revealed sequences in the infectious myonecrosis virus genome in Litopenaeus vannamei

Duan S. Loy, Sijun Liu, Mark A. Mogler, J. Dustin Loy, Bradley J. Blitvich and Lyric C. Bartholomay

1Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50011, USA
2Department of Entomology, Iowa State University, Ames, IA 50011, USA
3Harrisvaccines, Inc., Ames, IA 50010, USA

Infectious myonecrosis virus (IMNV) causes significant economic losses in farmed shrimp, where associated mortality in ponds can reach 70%. To explore host/pathogen interactions, a next-generation sequencing approach using lymphoid organ tissue from IMNV-infected Litopenaeus vannamei shrimp was conducted. Preliminary sequence assembly of just the virus showed that there were at least an additional 639 bp at the 5' terminus and 23 nt at the 3' terminus as compared with the original description of the IMNV genome (7561 nt). Northern blot and reverse transcription-PCR analysis confirmed the presence of novel sequence at both ends of the genome. Using 5' RACE, an additional 4 nt were discovered; 3' RACE confirmed the presence of 22 bp rather than 23 bp of sequence. Based on these data, the IMNV genome is 8226 bp in length. dsRNA was used to trigger RNA interference (RNAi) and suppress expression of the newly revealed genome sections at the 5' end of the IMNV genome in IMNV-infected L. vannamei. An RNAi trigger targeting a 376 bp length of the 5' UTR did not improve survival of infected shrimp. In contrast, an RNAi trigger targeting a 381 bp sequence in ORF1 improved survival to 82.2% as compared with 2.2% survival in positive control animals. These studies revealed the importance of the new genome sections to produce high-titre infection, and associated disease and mortality, in infected shrimp.

INTRODUCTION

A large number of pathogens threaten the shrimp aquaculture industry with a majority of these being viral in aetiology. The total economic impact is significant, with losses in the billions of dollars (Lightner et al., 2012). The emergence of novel pathogens is inevitable because of the combined effects of high-intensity farming, and a globalized aquaculture industry that exports and imports either live or unprocessed products, and thereby rapidly disseminates pathogens worldwide. The emergence of infectious myonecrosis virus (IMNV) is thought to have occurred via international transport of live animals (Senapin et al., 2007). IMNV was first reported in Brazil in 2003 after a disease outbreak occurred in 2002 (Poulos et al., 2006) and then an outbreak occurred in Indonesia in 2006 where it caused devastating economic losses (Senapin et al., 2007). Clinically, IMNV-infected animals demonstrate lethargy and the appearance of white muscle that spreads from the sixth abdominal segment; this pathology in individuals is an indicator for IMNV-induced mortality in ponds that can reach 70% (Senapin et al., 2007).

IMNV is an unenveloped, non-segmented, dsRNA virus with icosahedral symmetry and complex protrusions at the fivefold symmetry axis that are thought to be involved in virus entry (Tang et al., 2008). IMNV is classified in the family Totiviridae based on its genomic organization and significant amino acid identity to the genome of a strain of Giardia lamblia virus, specifically in the RNA-dependent RNA polymerase (RdRp) gene (Poulos et al., 2006). The IMNV genome encodes two ORFs. ORF1 is translated
into a polyprotein that is processed into three smaller proteins, the functions of which remain undetermined, via '2A-like' cleavage sites (Nibert, 2007; Tang et al., 2008) and also encodes the major capsid protein (MCP). ORF2 encodes the RdRp. Another protein (the MCP–RdRp fusion protein) is translated by virtue of a −1 frameshift within ORF1 (Nibert, 2007; Poulos et al., 2006).

The IMNV genome was initially reported to be 7561 bp in length (GenBank accession number EF061744.2) (Poulos et al., 2006). Although the reported IMNV genome size was approximately in the size range of members of the family Totiviridae (5.0–7.0 kbp), the published IMNV sequence did not appear to have a predicted internal ribosomal entry site (IRES) in its 5′ UTR that is characteristic of viruses in this family (Garlapati & Wang, 2005). In addition, some protein bands observed from purified IMNV virions did not match the sizes of the predicted cleavage products from the ORF1 polypeptide (Tang et al. 2008).

To explore shrimp/virus interactions, we undertook a next-generation sequencing (NGS) approach using lymphoid organ tissues. The penaeid shrimp lymphoid organ is located at the anterior end of the shrimp on the rostral/ventral side of hepatopancreas. The function of the lymphoid organ is, in part, removal of pathogens from haemolymph during circulation. Furthermore, the lymphoid organ is a common site for virus replication and associated pathology (e.g. spheroids) to occur (Assavalapsakul et al., 2006; Escobedo-Bonilla et al., 2007). Lymphoid organ tissues express a significant number of immune-related genes in normal and infected shrimp (Pongsomboon et al., 2008). It follows that this is a lead tissue in which to explore IMNV RNA production and breakdown during an active IMNV infection. Using an NGS approach to study mRNA production in the lymphoid organ, we identified a substantial amount of virus genome sequence that extended beyond the original genome description. This additional sequence appeared to include sequence that is key to virus replication.

RESULTS AND DISCUSSION

IMNV sequencing analysis

A total of 43 567 120 trimmed 50 bp reads were obtained from IMNV-infected L. vannamei and used for sequence assembly. To identify IMNV sequence, each of the contig sets was aligned to the existing IMNV sequences. Two IMNV contigs were identified: one 8044 bp in length and another of 8223 bp. Both contigs were significantly longer in size than that of the originally published IMNV sequence (7561 nt, Genbank accession number EF061744.1). A sequence alignment revealed that 662 bp of new, putative IMNV sequence was not within the limits of the known sequence, but fell primarily upstream of the 5′ end (639 bp) and, to a more limited extent, at the 3′ end (23 bp) of the genome (Fig. S1, available in the online Supplementary Material).

New 5′ end and known IMNV sequences have an equal depth of sequencing

RNA sequencing reads that represented IMNV sequences were subjected to de novo assembly and mapped across the length of the genome (Fig. 1). Sequence coverage of the new sequences and the previously published sequences is listed in Table 1. Although there were significant variations in the sequencing depth at specific nucleotide locations in the IMNV genome (Fig. 1), the mean sequencing coverage between the new and known sequences (Table 1) was similar, suggesting that the new sequences were equally represented in the cDNA library.

Confirmation of presence of sequence in IMNV Indonesia and Brazil

Deep-sequencing results indicated that there were at least an additional 639 nt at the 5′ end and 23 nt at the 3′ end when compared with the previously published IMNV genome sequences. Several sets of primers were designed to confirm these novel IMNV sequences in isolates from Indonesia and Brazil. Four sets of primers were used to confirm the 5′ end by reverse transcription (RT)-PCR, including IMNV26F, IMNV312F, IMNV643R and IMNV1016R (Table S1). Previously published primers (95F and 475R) that amplified the known genome sequence were used as positive controls (Table S1). RT-PCR amplicons were subjected to gel electrophoresis and sequence length was confirmed (Fig. S2, lane 1–4). The largest amplicon (990 bp, Fig. S2, lane 4) was cloned and sequenced, and had 95 % nucleotide identity compared with published sequences. These primer pairs also were tested with IMNV Brazil and RT-PCR results were identical to those for IMNV Indonesia (Fig. S2, lanes 5–8), and sequenced PCR products had 100 and 99 % nucleotide identity to IMNV Brazil and Indonesia, respectively.

Two reverse primers (IMNV8204R1 and IMNV8204R2) were designed to anneal the newly discovered sequence, in combination with forward primers designed to previously known sequence at the 3′ terminus (Table S1). The novel sequence was confirmed in both IMNV strains using these primers (Fig. S3). These data did not prove that the sequence at the 3′ end of the genome strain is identical in the two strains, but amplification did indicate that additional sequence at the 3′ terminus is indeed incorporated into IMNV virions.

IMNV genome size determination and Northern blot analysis

Gel electrophoresis was used to further confirm the size of the IMNV genome. A distinct band was evident at ~8–9 kb (Fig. 2). Total RNA from purified IMNV virus
and non-infected shrimp was subjected to Northern analysis with RNA probes that were generated to anneal to the old sequence and new sequence. Again, a band of $\sim 8$–$9$ kb was detected in extracted RNA from IMNV-infected shrimp using probes designed to the original and new IMNV sequence (Fig. 3). These results further confirmed that the novel sequence discovered by deep sequencing existed in IMNV viral genome.

**5’ and 3’ RACE PCRs to confirm the presence of additional nucleotides in the IMNV genome**

To validate the new IMNV sequence identified by NGS, 5’ and 3’ RACE PCRs were performed. Fourteen PCR products generated from 5’ RACE of cDNA from IMNV Indonesia were sequenced; sequence analysis confirmed the presence of 639 bp identified by NGS and added an additional 4 bp upstream. These additional 4 bp were present both in IMNV Indonesia and Brazil. All told, 5’ RACE PCR confirmed that the IMNV sequences previously published and submitted to GenBank (accession numbers NC_007915.2, AY570982.2 and EF061744.1; 7561 bp) were truncated at the 5’ terminus by 643 bp.

As the IMNV genome is not polyadenylated, a synthetic poly(A) tail was added to the 3’ end and then 3’ RACE experiments were performed to confirm the presence of additional sequence at the 3’ terminus of the genome.

**Table 1. Comparison of sequencing coverage between the new (current) IMNV genome sequence produced by Illumina sequencing and previously published IMNV sequences (see also Fig. 1)**

<table>
<thead>
<tr>
<th>IMNV sequence</th>
<th>Sequencing coverage (fold)</th>
<th>Positive strand</th>
<th>Negative strand</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current IMNV genome (8223 bp)</td>
<td></td>
<td>29.39</td>
<td>26.21</td>
<td>55.60</td>
</tr>
<tr>
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<td></td>
<td>26.37</td>
<td>21.48</td>
<td>47.85</td>
</tr>
<tr>
<td>Original IMNV genome (7561 bp)</td>
<td></td>
<td>29.73</td>
<td>26.67</td>
<td>56.40</td>
</tr>
</tbody>
</table>

**Fig. 1.** Sequencing coverage of IMNV genomic sequences. Illumina reads from IMNV-infected shrimp that represent IMNV sequence were mapped to the assembled IMNV sequence using a custom-designed Perl program. Sequencing coverage was calculated according to the numbers of times each base mapped to the IMNV genome. New sequence is delineated with a vertical line at the position where the original 7561 bp sequence started or stopped at the 5’ and 3’ ends, respectively (see also Figs S1 and S4).
Ten PCR products were generated and sequenced using 3′ RACE for IMNV Brazil. These experiments confirmed that an additional 22 bp were present at the distal 3′ end, supporting the findings from the NGS analysis. We were unable to generate any positive clones in the 3′ RACE experiments performed with IMNV Indonesia. However, we were able to amplify and sequence the 3′ end of IMNV Indonesia using IMNV7852F as the forward primer and IMNV8210R2 as the reverse primer; the sequence of the latter primer corresponded to the distal 22 bp of the 3′ UTR of IMNV Brazil (Table S1). These data indicated that an additional sequence was present at the 3′ end of the genome in both virus strains. Therefore, using 5′ and 3′ RACE, we confirmed the presence of the nucleotide sequences identified by NGS at the 5′ and 3′ ends of the IMNV genome, and revealed the presence of an additional 4 nt at the distal 5′ end. The full-length 8226 bp genomic sequence of IMNV was submitted to GenBank (accession numbers KF836757 and KJ556923).

**Analysis of the newly identified sequence at the 5′ end of the IMNV genome**

The IMNV sequence is non-segmented and encodes two predicted ORFs. To clarify whether the newly revealed 639 nt at the 5′ end of the genome encoded protein sequences, we translated the total length of the IMNV genome in silico in all six frames (Fig. S4). The predicted translation analysis showed that the longer IMNV genome encoded two predicted ORFs. The longer genome produced a predicted ORF1 in frame 1 that comprised 1719 aa, which was 114 aa larger than the previously published predicted product of ORF1 (1605 aa). The predicted start codon of the new ORF1 was 342 nt upstream of the documented start codon. Fig. S1 shows the new N-terminal sequence and alignment of the new N-terminal sequences and the published sequences of the IMNV ORF1. The ORF2 (frame 3) is almost identical to the previously predicted ORF2 protein sequence. With a new understanding of the coding region, the secondary RNA structure of the 5′ UTR sequence was predicted...
using RNAfold. The resulting predicted 5′ UTR RNA structure was then compared with known IRES structures (using IRESite and IRSS). There was no significant homology with known viral IRES elements; however, the IRES elements of viruses in the family *Tobirviridae* do not always fall strictly in the 5′ UTR, and are characterized by multiple complex secondary RNA structures and multiple, non-initiating AUG codons (Garlapati & Wang, 2005). The predicted 5′ UTR structure of IMNV contains several stem–loop structures and multiple AUG codons.

In a previously published analysis of the translation productions of IMNV ORF1, two ‘2A-like’ peptide cleavage motifs were described (Nibert, 2007), along with a cleavage site that cleaves the C terminus of ORF1 to release the MCP. These three cleavage motifs hydrolyse the ORF1 polyprotein into four predicted products: the N-terminal peptide 1 (10 kDa), peptide 2 (31 kDa), predicted peptide 3 (36 kDa) and the C-terminal 99 kDa MCP (Fig. S4). ORF2 (frame 3) is translated by −1 frameshifting of ORF1 and produces a 196 kDa MCP–RdRp fusion (Nibert, 2007; Poulos et al., 2006; Tang et al., 2008). Indeed, Tang et al. (2008) showed that when purified IMNV virions were separated by SDS-PAGE and stained, five clear protein bands were evident. Two bands at 99 and 196 kDa corresponded to MCP and MCP–RdRp, respectively. The remaining three bands were between 25 and 40 kDa (Tang et al., 2008). A 10 kDa peptide (the predicted size of peptide 1 of ORF1) was never observed. The newly revealed genome sequence explained this discrepancy because it extended the N terminus of peptide 1 to 207 aa, with a predicted molecular mass of 23 kDa. Therefore, it is likely that the 25 kDa protein observed previously was the full-length peptide 1, and the other two bands represented peptides 2 (31 kDa) and peptide 3 (36 kDa) (Tang et al., 2008).

**dsRNA targeting novel IMNV genome sections provides protection against infectious myonecrosis disease**

In order to begin to test the biological relevance of the novel sequences at the 5′ terminus of IMNV, two dsRNAs (designated dsRNA26-401 and dsRNA233-613; Fig. S4) were designed and compared with dsRNA95-475, which targeted the previously known coding region of peptide 1. dsRNA95-475 provided significant disease protection [81.67 % survival against IMNV at 30 days post-infection (p.i.)] when animals were subjected to the dsRNA prior to infection (Loy et al., 2012). For comparative purposes, dsRNAs to the novel IMNV sequence were designed to have a similar length to dsRNA95-475. Shrimp were injected with 5 μg per shrimp (100 μl) of either dsRNA26-401, dsRNA233-613, dsRNA95-475, dsEGFP or 2 % NaCl (sham inoculation), held for 48 h and then infected with IMNV (1:100 dilution) by injection at 48 h. Negative control shrimp were injected with 2 % NaCl (virus diluent) rather than virus. In three separate trials, significant mortality was observed in shrimp exposed to dsRNA26-401, dsEGFP or sham inoculation with 3.3, 2.2 and 2.2 % mean survival, respectively. In contrast, shrimp that received dsRNA233-613 and dsRNA95-475 showed similar protection against IMNV with mean survival of 82.2 and 97.8 %, respectively, at 14 days p.i. (Fig. 4). Mean survival for dsRNA233-613 and 95-475 was significantly different from the other groups (dsEGFP, dsRNA26-401 and positive control) (*P*<0.001) using one-way ANOVA followed by Tukey’s multiple comparison test. All of the negative control animals (non-challenged) survived during the experiments. Therefore, only dsRNA233-613 showed protection against IMNV and the protection efficacy was comparable with dsRNA95-475. This differential effect of RNAi triggers on IMNV virus load and virus-induced pathology is in keeping with a previous study wherein we showed that dsRNA targeting in ORF1 within the peptide-1-encoding region provided maximum protection from morbidity and mortality (Loy et al., 2012). dsRNA233-613 does encompass a region of the predicted ORF1 and dsRNA26-401 falls exclusively in the 5′ UTR (Fig. S4). ORF1 contains a dsRNA-binding protein domain; in a previous study, a dsRNA (dsRNA95-475) that also targeted the peptide-1-coding region was the most effective antiviral dsRNA tested, as compared with dsRNAs that targeted the MCP and RdRP (Loy et al., 2012).

**Novel IMNV dsRNA inhibition of IMNV replication**

Virus load was analysed by quantitative real-time (qRT)-PCR on muscle tissues from dead animals in dsRNA26-401, dsEGFP and positive control groups at 9–14 days p.i., and the surviving animals from the dsRNA233-613 and dsRNA95-475 groups at 14 days p.i. The results showed that dsRNA95-475 and dsRNA233-613 inhibited virus replication [mean $4.47 \times 10^7$ and $2.90 \times 10^7$ virus copies (μl RNA)$^{-1}$, respectively] and virus load in these animals was significantly different from that in the positive control group (mean $1.1 \times 10^8$ virus copies (μl RNA)$^{-1}$) (*P*=0.013 and *P*=0.016, respectively). By contrast, dsRNA26-401 and dsEGFP-exposed shrimp did not show reduction of virus (mean $4.77 \times 10^7$ and $7.72 \times 10^7$ virus copies (μl RNA)$^{-1}$, respectively) (Fig. S5). This was further evidence that dsRNA233-613 suppressed virus replication during IMNV infection.

In conclusion, new genome sections of IMNV were discovered using deep sequencing of the lymphoid organ from infected *L. vannamei*. Based on these data, and 5′ and 3′ RACE, the genome sequence of IMNV is 8226 bp in length, as compared with the original understanding of a 7561 bp genome. This new sequence represents sections that expand the predicted coding region of ORF1 by 114 aa and add an additional 23 nt at the 3′ end. The presence of these genome sections was confirmed by RT-PCR, RACE PCR and Northern blotting. RNAi triggers were developed to specifically target the new 5′-most sequence, including one trigger that falls on sequence that represents just the 5′ UTR, and one that encompasses some of the 5′ UTR and some of the predicted coding region of ORF1, peptide 1. The latter of these provided significant
protection from IMNV challenge manifest as significantly higher survival, decreased pathology and a reduction in viral replication compared with the RNAi trigger that targets just the 5’ UTR. This discovery provides an opportunity to further understand the function of the peptides encoded at the N terminus of ORF1, as well as novel targets for RNA-based vaccines and antivirals.

**METHODS**

**Shrimp culture.** Specific-pathogen-free (SPF) *L. vannamei* were acquired from Shrimp Improvement Systems and maintained in a SPF, biosecure facility at Iowa State University. Post-larvae were placed in 1,000 litre fiberglass tanks filled with artificial sea water. Water conditions were maintained at 25–27 °C, 30–35 parts per thousand salinity, with constant airstone aeration. Each tank was maintained with a carbon filter and an oyster shell airlift biofilter. Water quality was measured according to ammonia and nitrite levels (Nitriver3; Hach); temperature and salinity were measured daily.

**IMNV Indonesia-infected lymphoid organs.** IMNV-infected tissues used for this study were collected during an IMNV outbreak in Indonesia. The virus inoculum was prepared as described previously (Loy et al. 2012). Six shrimp (15–20 g) were infected by intramuscular injection with IMNV (strain Indonesia). Virus stock was prepared and diluted in 1 : 100 2 % NaCl [~2.11 × 10^3 viral copies (µl RNA)^−1] and filter-sterilized. Control shrimp were injected with 2 % NaCl (viral diluent), as reported by Loy et al. (2012).

Lymphoid organ tissues were collected from IMNV-infected animals at 5 days p.i. or from normal control shrimp at the same time. Gross organ characteristics were confirmed via light microscopy on a dissecting microscope. All lymphoid organs were preserved in RNALater RNA Stabilization Reagent (QIAGEN) and stored at −80°C. Samples were then transferred to 750 µl TRIzol LS (Invitrogen) and processed for RNA extraction. Residual DNA was removed from the extracted RNA using a TURBO DNA-free kit (Ambion). RNA integrity was determined using an Agilent 2100 Bioanalyzer. Total RNA from five or six individual animals was pooled and diluted in nuclease-free water to achieve a concentration of 0.1 µg ml−1. cDNA libraries were prepared from pooled RNA samples using a TruSeq RNA Sample Prep kit (Illumina) following the manufacturer’s instructions.

**Illumina sequencing, sequence assembly and sequencing data processing.** cDNA libraries were subjected to 50 nt (single-end)
sequencing using the HiSeq 2000 platform (Illumina). Raw sequencing reads were obtained and trimmed to remove adaptor sequences, empty reads and low-quality sequences using the fastX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). These reads were assembled using Velvet (version 1.2.08) (Zerbino & Birney, 2008). The performance of Velvet assemblies is determined by hash length (k) and coverage cut-off (c). An iterative process was used to select the optimum k and c values to produce the best assembly (Liu et al., 2011). The parameters used for assembly of contigs were k=31 and multiple c (c=2–10). To identify contigs derived from IMNV sequences, the resulting contig sets (≥100 nt) were aligned against IMNV genomic sequences by running BLAST (Altschul et al., 1990). To determine IMNV sequencing coverage, transcriptomic reads were clustered using the fastX toolkit and clustered reads were subsequently mapped to the IMNV genome using a custom-designed Perl program. The Perl script was designed to only allow mapping once to the reference sequence, with no mismatched residues allowed. The number of reads that mapped to each nucleotide position was used to calculate coverage (fold) of each nucleotide.

A biological sequence alignment editor (BioEdit version 7.1.11; http://www.mbio.ncsu.edu/bioedit/page2.html) was used as a platform for basic sequence manipulations of IMNV (e.g. sequence alignment, protein translation, ORF prediction, etc.). ClustalW2 was used for multiple sequence alignment (Larkin et al., 2007).

Identification of sequences at the 5’ and 3’ ends of the IMNV genome by RT-PCR. Oligonucleotide primers were designed to amplify novel IMNV sequence at both the 5’ and 3’ end of the genome (Table S1). Total RNA was extracted from shrimp inoculated with the Indonesia or Brazil strains of IMNV using TRIzol LS (Invitrogen). A one-step RT-PCR kit (QIAGEN) was used with the genome (Table S1). Total RNA was extracted from shrimp inoculated with Indonesia or Brazil strains of IMNV using TRIzol LS (Invitrogen). A one-step RT-PCR kit (QIAGEN) was used with the following amplification parameters: 50°C for 30 min, 95°C for 15 min and 35 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 45 s, followed by a final elongation at 72°C for 5 min. RT-PCR products were cloned into the pCR 2.1 vector (TOPO TA Cloning kit; Invitrogen) and transformed into Escherichia coli (One Shot TOP10; Invitrogen). Cells were grown on Luria–Bertani agar containing kanamycin (50 µg ml⁻¹). A subset of colonies was screened for the presence of the desired insert by PCR, and these inserts were then purified and sequenced with vector-specific primers (M13F and M13R) using a 3730xl DNA sequencer (Applied Biosystems) at the Iowa State University DNA facility.

RACE PCR

5’ RACE PCR. To identify the nucleotide sequences at the 5’ end of the IMNV genome sequence, 5’ RACE was conducted. Total RNA (1 µg) was reverse transcribed using the appropriate primer (452R for IMNV Indonesia and 163R for IMNV Brazil) and the resulting cDNAs were purified using a Purelink Quick PCR Purification kit (Invitrogen). Oligo(dC) tails were added to the 3’ end of 2 µg purified cDNA using 15 U terminal deoxynucleotidyltransferase (Invitrogen), 1 mM dCTP and 50 µg BSA ml⁻¹ in 1 x tailing buffer (100 mM potassium cacodylate, pH 7.2, 2 mM CoCl₂ and 0.2 mM DTT). Tailing reactions were performed at 37°C for 30 min and heat inactivated at 65°C for 10 min. C-tailed products were purified then PCR amplified using a consensus forward primer specific to the C-tailed termini (C tail F) and one of several reverse primers specific to the IMNV cDNA sequences (428R for IMNV Indonesia and 243R for IMNV Brazil). The resulting products were cloned and transformed into competent E. coli cells. Selected colonies were screened by PCR using IMNV-specific primers (M13F and M13R) and IMNV-specific primers (273F, 428R for IMNV Indonesia and 51F, 243R for IMNV Brazil). Select amplicons were purified (QIAquick PCR purification kit; QIAGEN) and sequenced with an IMNV-specific reverse primer (374R for IMNV Indonesia and 304R for IMNV Brazil).

3’ RACE PCR. To identify the nucleotide sequences at the 3’ end of the IMNV genome, synthetic poly(A) tails were added to 10 µg IMNV RNA using 6 U poly(A) polymerase A (Ambion) in 1 x reaction buffer (40 mM Tris/HCl, pH 8.0, 10 mM MgCl₂, 2.5 mM MnCl₂, 250 mM NaCl, 50 µg BSA ml⁻¹ and 1 mM ATP). Tailing reactions were incubated at 37°C for 1 h and then placed at −20°C for 2 min to inactivate the enzyme. Reaction products were purified by ethanol precipitation and poly(A)-tail-enriched RNAs were reverse transcribed using a reverse primer specific to the poly(A) tail (3’ RT). Resulting cDNAs were then PCR amplified using the appropriate primers (IMNV7852F and 3’Rev). PCR products from the 3’ RACE were also cloned and screened with gene specific primers (IMNV7852F and 3’Rev) and plasmid primers (M13F and M13R), and then sequenced with 7945F for both IMNV strains.

dsRNA synthesis. Two dsRNAs, each ~381 bp in length, were generated in the 639 bp of newly discovered sequence at the 5’ end of the IMNV genome. Primers for dsRNA synthesis were designed to amplify from positions 26 to 401 (IMNV26T7F and IMNV401T7R) and 233 to 613 (IMNV233T7F and IMNV613T7F) (Table S1, Fig. S4). The aforementioned dsRNAs were nearly identical in size to dsRNA95-475 (with the nucleotide range dictated by the position on the original IMNV genome sequence), which had proven protective effect in suppressing IMNV replication. dsRNA95-475 of IMNV (IMNV original sequences was generated according to a previous study (Loy et al., 2012). dsRNA was synthesized using the Ambion Megascript RNAi kit according to the manufacturer’s instructions. Briefly, a PCR was run containing primers with 7 sequences added to the 5’ terminus of primers (Table S1). Complementary DNA was generated following the manufacturer’s instructions for ThermoScript RT (Invitrogen) using total RNA extracted from IMNV-infected shrimp and forward primer IMNV26F. PCR was performed using PCR master mix (PuReTaq Ready-To-Go PCR Beads; GE Healthcare), cDNA template and the primers containing T7 extensions that were designed to specific regions of the new IMNV genome. PCR products were purified using a QIAquick PCR purification kit (QIAGEN), concentrated using a vacuum concentrator and 1 µg was added to an in vitro transcription reaction. The synthesis reaction was incubated at 37°C for 12–16 h, and then treated with RNase and DNase I. The formation of dsRNA was confirmed by 1 % agarose gel electrophoresis and quantified by spectrophotometry (NanoDrop 2000; Thermo Scientific). The dsRNAs were stored in −20°C until use and were then diluted in RNA-free water to produce the desired concentration.

IMNV Brazil viral isolation. An IMNV isolate from Brazil was kindly provided by the Aquaculture Pathology Laboratory at the University of Arizona. Virus inoculum was clarified directly from frozen infected samples as described previously (Loy et al., 2012). Shrimp (7–10 g) were injected intramuscularly (100 µl) with 1:100 dilution of IMNV Brazil [~7.2 x 10^5 virus copies (µl RNA)^−1] (Loy et al., 2012). Clinical signs of lethargy and white muscle were observed at 3 days p.i. Moribund shrimp with gross lesions were collected for RNA extraction using a QIAGEN RNeasy Mini kit according to the manufacturer’s instructions. The RNA from shrimp infected with IMNV Brazil was tested by RT-PCR and sequence length was confirmed on a 1 % agarose gel.

Determination of novel dsRNA disease protection efficacy in vivo. Shrimp (3–5 g) were divided into five groups of 10 shrimp with three replicates in each group and 10 shrimp for negative controls. Tanks were maintained at 29–30°C. Shrimp were injected intramuscularly with 5 µg per shrimp (100 µl) of dsRNA95-475, dsRNA26-401, dsRNA233-613 and dsEGFP or 2 % NaCl, and shrimp were injected at 48 h p.i. with 2 % NaCl (negative control group) or IMNV 1:100 dilution of IMNV Indonesia that reached 95–100 % mortality within 2 weeks [ ~2.11 x 10^5 virus copies (µl RNA)^−1] (Loy...
Shrimp were fed and mortality observed twice per day. Tissue samples from moribund or dead shrimp were stored in −80°C for further quantification of virus copy number. qRT-PCR. qRT-PCR for assay virus load was adapted from a previously described method (Andrade et al., 2007). RNA was extracted using a RNeasy Mini kit (QIAGEN) and qRT-PCR was performed with conditions from a previous report (Loy et al., 2012). Each reaction was run in duplicate on a Bio-Rad CFX96 Real-Time PCR Detection System using a standard as described previously (Andrade et al., 2007; Loy et al., 2012).

IMNV purification. IMNV was purified using a combination of methods adapted from Mello et al. (2011) and Poulos & Lightner (2006). Briefly, 100 shrimp (7–10 g) were infected with a 1 : 10 dilution of IMNV Indonesia (Loy et al., 2012). Moribund shrimp were collected during a 2 week course of infection to obtain ∼500 g abdominal muscle tissue from acutely infected animals. Heads were collected during a 2 week course of infection to obtain IMNV Indonesia (Loy et al., 2006). Briefly, 100 shrimp (7–10 g) were infected with a 1 : 100 dilution of IMNV Indonesia (Loy et al., 2012). IMNV was purified using a combination of IMNV purification. Tissue samples from moribund or dead shrimp were stored in 10 % polyethylene glycol (w/v) and 4 % NaCl (w/v), stirred at 4°C for 2 h. The supernatant was removed and centrifuged again to remove the tissue debris. The supernatant was collected and precipitated by adding 10 % polyethylene glycol (w/v) and 4 % NaCl (w/v), stirred at 4°C for 1 h, and centrifuged at 8000 g for 20 min at 4°C (Mello et al., 2011). The pellet was then resuspended and loaded onto 10–40 % sucrose gradient. The virus pellet was removed from the ultracentrifuge tube observed in the bottom of the ultracentrifuge tube at the 40 % sucrose gradient. The virus pellet was removed from the ultracentrifuge tube and diluted in the same buffer to be stored at −80°C. The presence of virus was confirmed by RNA extraction and RT-PCR. Purified virus was also injected intramuscularly into 7–10 g shrimp to confirm the virulence and presence of virus in the preparation.

Northern blot analysis. Northern blot analysis was performed using methods adapted from Brown et al. (2001) and the manufacturer’s instructions (NorthernMax-Gly; Life Technologies). Total RNA (5 µg) was resolved on a 1 % agarose glyoxal gel for 1 h at 100 V, alongside a biotinylated RNA molecular mass marker (Millennium Marker; Ambion). The gel was observed for the presence of RNA via ethidium bromide staining in a UV light box prior to transfer. Then the gel was nicked with 500 kJ UV light and transferred to a positively charged membrane (Whatman Nytran Supercharge TurboBlotter; Sigma Aldrich) using a passive down hill transfer technique for 1 h. Membranes were UV cross-linked at 1200 J·m and hybridized overnight in ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion) at 68°C with biotinylated RNA probes. Biotinylated RNA probes, including a probe to positions 95 (sense) and 475 (antisense), and to the new sequences at positions 233 (sense) and 613 (antisense) (Table S1), were generated using a BrightStar Psoralen-Biotin Nonspecific Labeling kit (Ambion). PCR templates with T7 sequence at the 5‘ ends were used to generate in vitro transcripts (T7 RNA polymerase; Promega). RNA transcripts were then labelled with biotin according to the manufacturer’s instruction (BrightStar Biotin-Psoralen kit). The hybridized membranes were developed following the manufacturer’s instruction and the signal was detected by chemiluminescence using a BrightStar BioDetect kit (Ambion).

Statistical analysis. Survival and qRT-PCR data were analysed using one-way ANOVA followed by Tukey’s multiple comparison test (P < 0.05) using JMP Pro 10.0.0 software (SAS Institute). Survival data and viral copy number were expressed as the mean ± SEM. Mean survival data were tested at the termination of experiment on day 14 p.i. and the mean log viral copy number was compared between groups.

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