

Purification and characterization of infectious myonecrosis virus of penaeid shrimp

Bonnie T. Poulos,¹ Kathy F. J. Tang,¹ Carlos R. Pantoja,¹
Jean Robert Bonami² and Donald V. Lightner¹

Correspondence

Bonnie T. Poulos
bpoulos@u.arizona.edu

¹Aquaculture Pathology Laboratory, Department of Veterinary Science and Microbiology,
The University of Arizona, 1117 E. Lowell Street, Tucson, AZ 85721, USA

²UMR 5098, CNRS/IFREMER/UM2, cc080 Place E. Bataillon, 34095 Montpellier Cedex 5,
France

The causative agent of myonecrosis affecting cultured *Penaeus vannamei* in Brazil was demonstrated to be a virus after purification of the agent from infected shrimp tissues. Purified viral particles were injected into specific pathogen-free *P. vannamei*, resulting in a disease that displayed the same characteristics as those found in the original shrimp used for purification. The virus was named infectious myonecrosis virus (IMNV). The viral particles were icosahedral in shape and 40 nm in diameter, with a buoyant density of 1.366 g ml⁻¹ in caesium chloride. The genome consisted of a single, double-stranded (dsRNA) molecule of 7560 bp. Sequencing of the viral genome revealed two non-overlapping open reading frames (ORFs). The 5' ORF (ORF 1, nt 136–4953) encoded a putative RNA-binding protein and a capsid protein. The coding region of the RNA-binding protein was located in the first half of ORF 1 and contained a dsRNA-binding motif in the first 60 aa. The second half of ORF 1 encoded a capsid protein, as determined by amino acid sequencing, with a molecular mass of 106 kDa. The 3' ORF (ORF 2, nt 5241–7451) encoded a putative RNA-dependent RNA polymerase (RdRp) with motifs characteristic of totiviruses. Phylogenetic analysis based on the RdRp clustered IMNV with *Giardia lamblia virus*, a member of the family *Totiviridae*. Based on these findings, IMNV may be a unique member of the *Totiviridae* or may represent a new dsRNA virus family that infects invertebrate hosts.

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INTRODUCTION

During 2002, shrimp growers in north-east Brazil reported a disease in cultured *Penaeus vannamei* characterized by focal to extensive necrotic areas in skeletal muscle tissues, primarily in the distal abdominal segments and the tail fan (Lightner *et al.*, 2004a, b). Often the tail muscle was white and opaque in appearance. Typically, the disease progressed slowly, with low mortality rates that persisted throughout the growing season. At harvest time, cumulative mortalities in shrimp ponds reached 70 % (Nunes *et al.*, 2004).

In mid-2003, frozen and Davidson's alcohol-formalin-acetic acid (AFA)-fixed tissues, from a farm in Brazil where the shrimp displayed gross signs of the disease, were received by the University of Arizona Aquaculture Pathology Laboratory to investigate the cause of the disease. As reported here, the disease was determined to have an infectious aetiology

and the frozen tissues were used for purification and characterization of the aetiological agent.

METHODS

Shrimp. Shrimp taxonomy used in this paper is according to Holthius (1980). Frozen *P. vannamei* showing signs of muscle necrosis were obtained from a shrimp farm in Brazil. Replicate samples of shrimp from the same grow-out ponds were fixed with Davidson's AFA for 24–48 h prior to being immersed in 70 % ethanol for subsequent histological analysis according to the procedures of Bell & Lightner (1988). Paraffin sections were stained with Mayer-Bennet haematoxylin and eosin-phloxine (H&E). Shrimp used for the laboratory experiments were specific pathogen-free (SPF) *P. vannamei* (Kona stock) obtained from the SPF breeding programme at the Oceanic Institute in Hawaii (Wyban *et al.*, 1992; Lotz, 1997). Shrimp were reared and maintained at the University of Arizona Aquaculture Pathology Laboratory utilizing the protocols described by White *et al.* (2002).

Virus purification. The procedure used for purification of the infectious agent was modified from that of Bonami *et al.* (1997). Briefly, gnathothoraces ('heads') from 25 subadult Brazilian *P. vannamei* showing signs of disease were homogenized in TN buffer [20 mM Tris/HCl (pH 7.5), 400 mM NaCl]. The suspension was

The GenBank/EMBL/DDBJ accession number for the IMNV sequence reported in this paper is AY570982.

A supplementary figure showing the complete cDNA genome sequence and translated ORFs of IMNV is available in JGV Online.

clarified three times by low-speed centrifugation. The supernatant fluid, after clarification, was centrifuged for 3 h at 205 000 *g* and the resulting pellet was resuspended in TN buffer. The suspension was treated with fumed silica (2 mg ml⁻¹; Sigma-Aldrich) for 30 min to remove lipids and centrifuged for 20 min at 12 000 *g* to separate the supernatant fluid from the silica (Neoh *et al.*, 1986). This suspension was shaken with 1 g activated charcoal and filtered on a bed of Celite 535 (Fluka Chemie). The filtrate was centrifuged for 3 h at 205 000 *g* and the resulting pellet was resuspended in TN buffer. The suspension was fractionated on a 15–40% (w/w) linear sucrose gradient layered onto a bed of 50% sucrose by using an Autodensiflow IIC (Buchler Instruments) and centrifuged for 2 h at 286 200 *g*. Gradient fractions were collected by using an Autodensiflow IIC and a Retriever II fraction collector (ISCO). The *A*₂₅₄ readings of each fraction were recorded by using a UA5 UV absorbance monitor (ISCO). Peak fractions were centrifuged for 3.5 h at 286 200 *g* and the pellets were resuspended in TN buffer. Final purification was performed by layering the peak fractions onto a 20–50% (w/w) CsCl gradient and centrifuging for at least 16 h at 135 900 *g*. Fractions were collected and washed as described above for the sucrose gradient and the final pellet was resuspended in TN buffer.

Confirmation of infectious myonecrosis viral aetiology (Rivers' postulate). To prepare the inoculum, virions purified from infected shrimp from Brazil were diluted 1:100 in 2% sterile saline. Fifteen SPF *P. vannamei* indicator shrimp (3–4 g each) were injected intramuscularly with 20 µl viral suspension into the third abdominal segment. As a negative control, another group of 15 SPF shrimp was injected intramuscularly with sterile 2% saline only. Both groups of shrimp were held at 25 parts per thousand salinity and 32 °C for the duration of the experiment (20 days). Shrimp were monitored daily for the presence of necrotic lesions in the skeletal muscle (visible as whitish, opaque areas in the abdominal muscle) and those exhibiting lesions were preserved in Davidson's AFA fixative for routine histology. As an additional confirmatory test, selected specimens displaying infectious myonecrosis virus (IMNV) lesions were subjected to *in situ* hybridization with a previously developed gene probe specific for detection of IMNV (Tang *et al.*, 2005).

Virus density. The refractive index of each 1 ml fraction from four separate CsCl gradients (prepared with DEPC-treated water) was measured by using an Abbe refractometer (Spectronic Instruments). Two measurements were taken per fraction and the results were plotted against the absorbance readings. The mean value of the refractive index of each virus peak was used to determine the buoyant density using the conversion table for aqueous CsCl solutions (Weast & Astle, 1980).

Transmission electron microscopy. Washed and resuspended peak fractions from the sucrose and CsCl gradients were adhered to collodion/carbon-coated grids and negatively stained with 2% phosphotungstic acid at pH 7.0. The grids were examined by using a Phillips CM12 transmission electron microscope. *Tobacco mosaic virus* was used on selected grids to provide a size reference for calibration purposes.

SDS-PAGE. A preparation of purified virions from CsCl was applied to an 8–16% gradient gel (Gradipore) after boiling in Laemmli buffer (Laemmli, 1970) containing 10 M urea to denature structural proteins. Following electrophoresis, the gel was stained for 1 h with 0.1% Coomassie blue (Wilson, 1983) to visualize the separated proteins. A pre-mixed low-range molecular mass marker (Roche) was electrophoresed in the same gel and the molecular mass of the proteins was estimated from measurement of electrophoretic mobilities (Weber & Osborn, 1969). Alternatively, for protein sequencing, the unstained gel was electrotransferred to a PVDF membrane (Millipore) (Matsudaira, 1987).

Protein sequencing. Proteins separated by SDS-PAGE were transferred to an Immobilon-P PVDF membrane (0.2 µm pore size; Millipore) by using 10 mM CAPS (3-cyclohexylamino-1-propane-sulfonic acid) buffer (pH 11) containing 20% methanol. The N terminus of the viral protein was sequenced for 11 cycles at the University of Arizona Laboratory for Protein Sequencing and Analyses using an ABI 477A pulsed-liquid protein sequencer (Applied Biosystems).

RNA extraction. Total RNA was extracted from infected shrimp tissue by using TRIzol reagent (Invitrogen) according to the manufacturer's directions. RNA was isolated from a preparation of purified virions by using a High Pure RNA Tissue kit (Roche) according to the manufacturer's directions. The viral RNA was eluted from the column by using DEPC-treated water.

Enzymes. Mung bean nuclease (MBN; New England Biolabs), RNase A (Sigma-Aldrich), S1 nuclease (Invitrogen) and RNase-free DNase I (Roche) were used according to the manufacturers' instructions to determine whether the genome of IMNV was single-stranded or double-stranded and whether it was composed of DNA or RNA. The nuclease digestions were performed in 10 µl volumes using 225 ng viral RNA and were incubated for 30 min at 37 °C. MBN was tested by using 2 U enzyme, S1 nuclease was tested by using 2 U enzyme and DNase I was tested by using 10 U enzyme. RNase A was tested by using 20 ng enzyme under both low-salt (10 mM) and high-salt (300 mM) conditions. Linearized plasmid DNA (200 ng per reaction) from pUC18 was used as a control for the enzymic digestions.

cDNA synthesis and cloning. RNA isolated from infected shrimp and RNA isolated from purified virions were used to prepare two cDNA libraries. RNA isolated from infected shrimp was used to synthesize cDNA, which was cloned into the pSport I plasmid (Invitrogen) as described previously (Tang *et al.*, 2005). The same methods were used to synthesize cDNA from the RNA that was isolated from purified virions except that the pUC18 plasmid was used for cloning. All clones were screened by colony PCR using M13 primers as described previously (Tang *et al.*, 2005) for the pSport I recombinant clones and using pUC18 primers (5'-TGTAACACGACGGCCAGT-3' and 5'-TCACACAGGAAACAGCTATGAC-3') for the pUC18 recombinant clones.

Nucleotide sequencing and analysis of the viral genome. Nucleotide sequencing of cDNA inserts from the two cloned libraries was performed at the University of Arizona DNA Sequencing Facility using an ABI PRISM 377 DNA Sequencer (Applied Biosystems). The nucleotide sequence was analysed by using Clone Manager 5 and Align Plus 4 software (Scientific & Educational Software). Analysis of the translated proteins was performed by using online programs. A search for open reading frames (ORFs), determination of protein molecular mass and isoelectric point (pI) determination of translated proteins were performed with the European Molecular Biology Open Software Suite program (EMBOSS; www.ch.embnet.org/EMBOSS) (Rice *et al.*, 2000). A search for significant similarity between the IMNV amino acid sequence and sequences in GenBank was performed by using BLASTP at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). A search for conserved domains was also performed at NCBI (Marchler-Bauer & Bryant, 2004). Multiple alignment among members of the family *Totiviridae* was performed by using the program HMMFAM, implemented in the Accelrys GCG software package (www.accelrys.com/products/gcg). Conserved motifs found with reverse position-specific BLAST were used as a control of the validity of alignments.

Phylogenetic analysis. A phylogenetic tree was calculated by using the neighbour-joining method using the quartet-puzzling

program TREE-PUZZLE (www.tree-puzzle.de) (Strimmer & von Haeseler, 1996; Schmidt *et al.*, 2002). The sequences and GenBank accession numbers of the totiviruses that were used in the phylogenetic analysis are listed in Table 1.

RESULTS

Virus purification

Opaque bands were visible in both the sucrose and CsCl gradients after centrifugation. The band in sucrose was located three-quarters of the way into the gradient, slightly above the interface with the 50 % sucrose cushion. In CsCl, the band appeared as a tightly spaced doublet that was located about two-thirds of the way into the gradient. The presence of the virus was confirmed in the sucrose fractions by transmission electron microscopy (Fig. 1a). The fractions from CsCl were examined by transmission electron microscopy and very few empty particles were observed in the preparation (Fig. 1b). The particles were icosahedral in shape with no envelope or surface projections. The size of the particles was measured by using *Tobacco mosaic virus* as the calibrator and determined to be 40 nm in diameter (40.43 nm mean diameter; $n=100$; SD=1.33). The mean refractive index of the virus peak taken at 20 °C was 1.36915, which is equivalent to a density in CsCl of 1.366 g ml⁻¹.

Confirmation of IMN viral aetiology (Rivers, 1937)

Virions purified from the Brazilian shrimp were injected into SPF *P. vannamei* to determine whether the isolated virus particles induced the same lesions that were detected in the

original shrimp with myonecrosis (Fig. 2a). The first gross signs of skeletal muscle necrosis were observed in three indicator shrimp 3 days after injection with purified IMNV. By day 5 after injection, every indicator shrimp exhibited necrotic lesions in the skeletal muscle (Fig. 2b). These gross signs and lesions were identical to those observed in clinical specimens. Shrimp displaying necrotic lesions were preserved in Davidson's solution and examined after staining with H&E. Histologically, lesions were characterized by coagulative muscle necrosis, often accompanied by fluid accumulation in between muscle fibres ('oedema'), haemolytic infiltration and fibrosis (Fig. 3a). Lymphoid organ spheroids were typically seen within the lymphoid organ and were ectopic in the haemocoel and loose connective tissues (not shown). Darkly basophilic inclusion bodies were seen within the cytoplasm of muscle cells, haemocytes and connective tissue cells of some specimens (Fig. 3b). These lesions were identical to those observed in clinical specimens originating from affected shrimp farms in Brazil (Lightner *et al.*, 2004b). A positive reaction to the IMNV gene probe was observed within necrotic muscle cells, thus confirming the presence of IMNV in affected tissues (Fig. 3c). No necrotic lesions were observed in the negative-control group, either grossly or by histological analysis. Only shrimp exposed to purified IMNV demonstrated histological lesions indicative of myonecrosis.

Protein composition

Purified virions obtained from the CsCl gradient demonstrated a single major polypeptide after SDS-PAGE with a molecular mass of 106 kDa (Fig. 4a). Amino acid sequence analysis of the major capsid protein indicated the

Table 1. Members of the family *Totiviridae* used for phylogenetic analysis

Virus	Abbreviation	Genus*	GenBank accession no.
<i>Giardia lamblia</i> virus	GLV	<i>Giardiavirus</i>	NC_003555
<i>Trichomonas vaginalis</i> virus	TVV	<i>Giardiavirus</i>	NC_003824
<i>Trichomonas vaginalis</i> virus II	TVV II	<i>Giardiavirus</i>	NC_003873
<i>Trichomonas vaginalis</i> virus 3	TVV 3	<i>Giardiavirus</i>	NC_004034
<i>Ustilago maydis</i> virus H1	UmV H1	<i>Totivirus</i>	NC_003823
<i>Saccharomyces cerevisiae</i> virus L-A	ScV L-A	<i>Totivirus</i>	NC_003745
<i>Saccharomyces cerevisiae</i> virus L-BC	ScV L-BC	<i>Totivirus</i>	NC_001641
<i>Helminthosporium victoriae</i> virus 190S	HvV 190S	<i>Totivirus</i>	NC_003607
<i>Helicobasidium mompa</i> No. 1-17 virus	HmV 1-17	<i>Totivirus</i>	NC_005074
<i>Zygosaccharomyces bailii</i> virus Z	ZbV Z	<i>Totivirus</i>	NC_003874
<i>Gremmeniella abietina</i> RNA virus L1	GaRV L1	<i>Totivirus</i>	NC_003876
<i>Leishmania</i> RNA virus 1-1	LRV 1-1	<i>Leishmaniavirus</i>	NC_002063
<i>Leishmania</i> RNA virus 1-4	LRV 1-4	<i>Leishmaniavirus</i>	NC_003601
<i>Leishmania</i> RNA virus 2-1	LRV 2-1	<i>Leishmaniavirus</i>	NC_002064
<i>Eimeria brunetti</i> RNA virus 1	EbRV 1	Unassigned	NC_002701
<i>Sphaeropsis sapinea</i> RNA virus 1	SsRV 1	Unassigned	NC_001963
<i>Sphaeropsis sapinea</i> RNA virus 2	SsRV 2	Unassigned	NC_001964
<i>Magnaporthe grisea</i> virus 1	MgV 1	Unassigned	NC_006367

*Taken from <http://www.dpvweb.net/seqs/fungusviruses.php>.

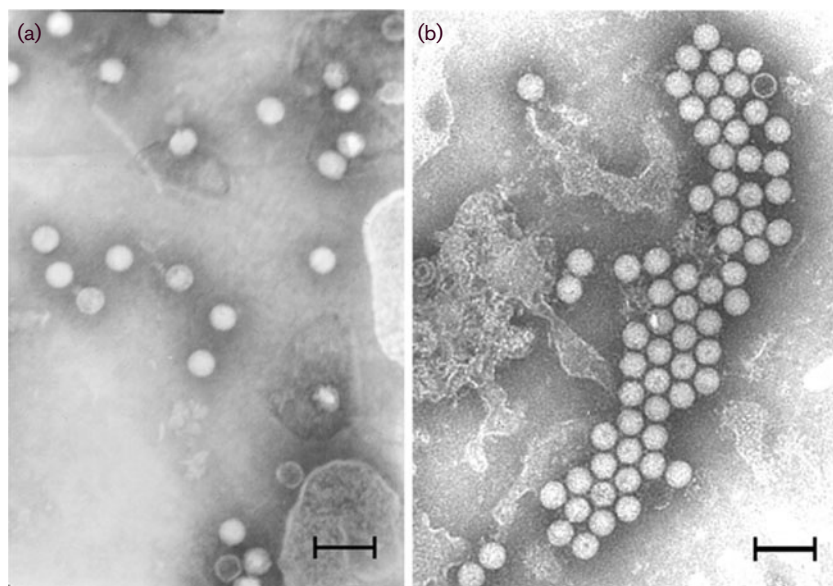


Fig. 1. Transmission electron micrographs of IMNV. (a) Sucrose-gradient fraction of virus. (b) Caesium chloride-gradient fraction of purified virus. Stained with 2% phosphotungstic acid. Bars, 100 nm.

N-terminal sequence to be IVSMENQSEID, which was found in the translated nucleotide sequence starting at nt 2248. When the sample was overloaded in the SDS-polyacrylamide gel with respect to the major polypeptide, three other protein bands with molecular masses of 149, 42 and 24 kDa were seen (Fig. 4b). It was not possible to obtain enough of these proteins for sequencing, so it was not known whether these bands were derived from the purified virus or whether they were minor contaminants from the shrimp tissues used for the purification of the virus particles.

Viral nucleic acid

Total nucleic acid was isolated from the purified virions and subjected to enzymic analysis to determine the character of the viral genome. Digestions were performed on the viral nucleic acid using DNase I, RNase A, MBN and S1 nuclease, with linear plasmid DNA as a control nucleic acid for the enzymic digestions. As shown in Fig. 5, the viral nucleic acid was digested only with RNase A under low-salt conditions and not with RNase A under high-salt conditions or with DNase I, MBN or S1 nuclease, indicating that the viral genome was composed of double-stranded RNA (dsRNA). The untreated viral nucleic acid produced a single band with an approximate size of 7 kbp in non-denaturing 1% agarose gel, indicating that the IMNV dsRNA was non-segmented.

Sequencing and analysis of the viral genome

The viral genome was sequenced initially from clones prepared by using total RNA extracted from infected Brazilian shrimp. Subsequently, the sequence was confirmed by using clones prepared from RNA that was isolated from purified virions. The base composition of the 7560 bp genome (GenBank no. AY570982 and Supplementary Figure, available in JGV Online) was found to have a high content (62 mol%) of A + U, consisting of 37% A, 25% U, 20% G and 18% C. A search for ORFs showed the presence of two

non-overlapping ORFs: ORF 1 spanning nt 136–4953 (4818 nt) in reading frame 1 and ORF 2 spanning nt 5241–7451 (2211 nt) in reading frame 3 (Fig. 6a). There were 287 nt between ORF 1 and ORF 2, the region being composed of 67 mol% A + U. The 5' untranslated region (UTR) and 3' UTR were 135 and 109 nt, respectively (Fig. 6a and Supplementary Figure). Both the 5' and 3' UTRs had high contents of A + U (64 and 62 mol%, respectively). Translation of the ORF 1 sequence predicted a polypeptide of 1606 aa, which is longer than the average major capsid protein for totiviruses (approx. 750 aa). A sequence search with BLASTP showed no matches with significant similarity in GenBank. However, a conserved domain search revealed a 60 aa dsRNA-binding motif (DSRM) in the extreme N-terminal region of ORF 1 (coding region nt 136–315) that showed 35% identity to the full-length consensus sequence (Fig. 6b). The 11 aa sequence that was determined for the major capsid protein was located within ORF 1; however, the coat protein sequence began at nt 2248, which is well into ORF 1. An initiation codon was present starting at nt 2227 immediately upstream of the start of the capsid protein, but no termination codon was found in the upstream sequence within ORF 1. Analysis of the translated coat protein (nt 2248–4953) predicted that the protein would contain 901 aa with a mass of 99.3 kDa and a pI of 5.4. By SDS-PAGE analysis, the major capsid protein was shown to have a mass of 106 kDa (Fig. 4a), which corresponded closely to the predicted mass of the protein.

Based on sequence analysis, ORF 2 (nt 5241–7451) was predicted to encode a polypeptide of 736 aa with a molecular mass of 85 kDa and a pI of 9.6. A search for the presence of conserved domains revealed a significant match with the consensus sequence of the RNA-dependent RNA polymerase (RdRp) of the family *Totiviridae* (NCBI CCD pfam05888) (Fig. 6c). The BLASTP search of translated amino acids from IMNV ORF 2 produced significant alignments

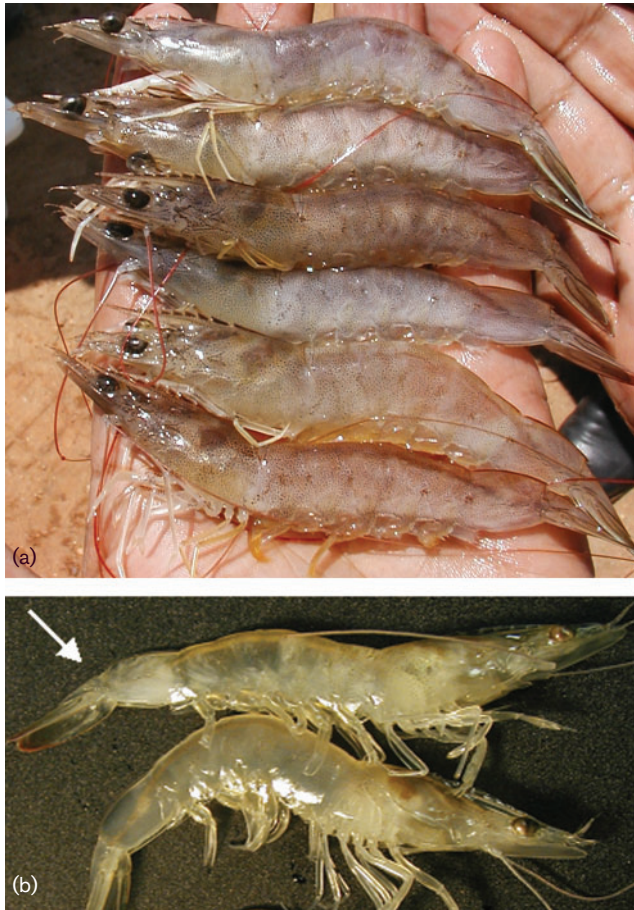


Fig. 2. Gross signs of IMNV disease. (a) Farmed *P. vannamei* shrimp from a natural outbreak, exhibiting various degrees of skeletal muscle necrosis, visible as an opaque, whitish discoloration of the abdomen. (b) Experimental SPF *P. vannamei* shrimp injected with purified IMN virions. The shrimp on the upper portion of the photograph displays evident signs of muscle necrosis along the abdomen, with particular emphasis on the sixth abdominal segment (arrow). The shrimp in the lower portion of the photograph is a normal shrimp, from the negative-control tank, injected with sterile 2% saline only. Note the normal, almost-translucid appearance of the abdomen.

(>21% sequence identity) with RdRp from members of the family *Totiviridae*, including *Giardia lamblia* virus (GLV) ($E=4 \times 10^{-7}$), *Helicobasidium mompa* No. 1-17 virus (HmV 1-17) ($E=2 \times 10^{-4}$), *Eimeria brunetti* RNA virus 1 (EbRV 1) ($E=0.094$), *Sphaeropsis sapinea* RNA virus 1 (SsRV 1) ($E=0.33$), *Trichomonas vaginalis* virus (TVV) ($E=0.34$), *Helminthosporium victoriae* virus (HvV) 190S ($E=0.75$) and *Gremmeniella abietina* RNA virus L1 (GaRV L1) ($E=0.82$). The Expect (E) value in a BLAST search is a measure of the probability that a match is due to randomness (Altschul & Erickson, 1985). The analysis indicated that ORF 2 may encode an RdRp that is related closely to the RdRp from the family *Totiviridae*, genus *Giardiavirus*. Furthermore, eight motifs characteristic of totivirus RdRp

(Bruenn, 1993) were also found in the RdRp of IMNV (Figs 6c and 7a). Among them, motifs 6 and 7 appeared to be most conserved and showed 100% identity to the consensus sequence. This supports the finding that motif 6 (GDD) is universal to RNA polymerase (Jablonski *et al.*, 1991). Three motifs, 2, 4 and 8, had moderate identity (>40%); motifs 1, 4 and 5 were the least conserved, especially 1 and 5, which had three to four gaps when aligned with the consensus motif.

Phylogenetic analysis

Phylogenetic analysis based on the RdRp sequence indicated that IMNV clustered with GLV, a totivirus belonging to the genus *Giardiavirus*, with a bootstrap value of 83% (Fig. 7b). Three isolates of TVV (genus *Giardiavirus*) were grouped strongly, with a bootstrap value of 97%. Three isolates of *Leishmania* RNA virus (LRV) (genus *Leishmania-virus*) also clustered together, with a bootstrap value of 98%. For the genus *Totivirus*, two *Saccharomyces cerevisiae* virus (ScV) isolates clustered with a bootstrap value of 93%, but did not group with other members of the same genus (*Ustilago maydis* virus H1, *Zygosaccharomyces bailii* virus Z, HmV 1-17 and HvV 190S). A group of six viruses, including four unclassified members and two members of the genus *Totivirus*, clustered with a bootstrap value of 51%. This grouping was similar to a phylogenetic tree generated by Tuomivirta & Hantula (2003).

DISCUSSION

The research presented here demonstrates that the cause of myonecrosis in *P. vannamei* from Brazil in 2003 is an infectious agent. Previous bioassay studies performed at this laboratory, where tissue homogenates prepared from Brazilian shrimp were used to pass the disease on to SPF *P. vannamei*, indicated an infectious agent as the most probable cause of the disease (unpublished results). The experiments described here demonstrate that the aetiological agent is a 40 nm virus that possesses icosahedral symmetry, has a buoyant density of 1.366 g ml^{-1} in CsCl and contains a monopartite dsRNA genome of 7560 bp. The virus has a major capsid protein with a molecular mass of 106 kDa. When virions purified from the original tissue were injected into SPF *P. vannamei*, the indicator shrimp exhibited the signs and lesions associated with the disease, thus completing Rivers' postulate for demonstration of a viral aetiology (Rivers, 1937). Based on this evidence, the disease has been named infectious myonecrosis and the aetiological agent of the disease has been designated infectious myonecrosis virus (IMNV).

Previous work conducted in this laboratory developed a molecular probe for the virus that was used to demonstrate the presence of the agent in fixed tissue sections by *in situ* hybridization (Tang *et al.*, 2005). The probe reacted to the histological lesions in muscle and to the lymphoid organ spheroids in the original tissue obtained from Brazilian shrimp culture facilities, as well as in tissues from the

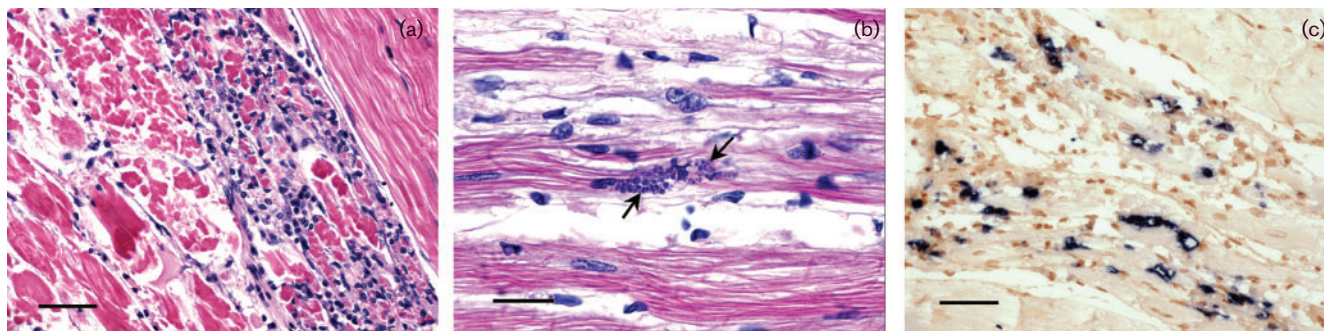


Fig. 3. Myonecrosis due to infection of *P. vannamei* with purified virions. (a) Coagulative necrosis of skeletal muscle accompanied by haemocytic infiltration and fibrosis. As a reference, normal skeletal muscle can be observed in the upper right corner. H&E stain; bar, 50 μ m. (b) Perinuclear pale basophilic to dark basophilic inclusion bodies are evident in this group of muscle cells (arrows point at some examples). H&E stain; bar, 20 μ m. (c) *In situ* hybridization of skeletal muscle tissue using a digoxigenin-labelled IMNV probe. A black precipitate is present in areas where the probe has hybridized with target IMNV. Bismarck brown counterstain; bar, 50 μ m.

infectivity experiment described in this study. The probe did not react with tissues from SPF control shrimp. All evidence to date indicates that the shrimp from Brazil in 2002 and 2003 that were displaying gross and histological signs of myonecrosis were infected with IMNV. The *in situ* hybridization results also showed that the probe reacted in the cytoplasm of infected cells, indicating that this is the most likely cellular compartment in which the virus replicates. Infection with IMNV results in a slowly progressing disease that may be influenced by conditions of temperature and salinity. It is interesting to note that the histology of IMNV-infected tissue shows marked inflammatory responses near the sites of lesion development that may be indicative of non-specific activation of the immune response by dsRNA molecules, a phenomenon recently demonstrated to occur in shrimp (Robalino *et al.*, 2004).

In the studies described here, the genome of IMNV was shown to consist of a single segment of dsRNA, which places it among a small group of dsRNA viruses that primarily infect fungi and protozoa. The International Committee for the Taxonomy of Viruses currently recognizes eight distinct families of dsRNA viruses, with only two of those families possessing non-segmented genomes (Mertens, 2004). From analysis of the physical characteristics of the virus (particle size, buoyant density, size of the major capsid protein,

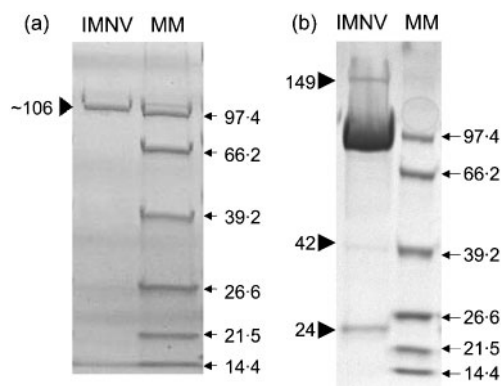


Fig. 4. Coomassie blue-stained SDS-PAGE of IMNV. The molecular masses of the proteins are indicated in kDa. (a) Virus preparation after purification on a caesium chloride gradient. (b) Lane IMNV was overloaded with respect to the major capsid protein. Minor bands were evident under these conditions. MM, Pre-mixed molecular mass markers.

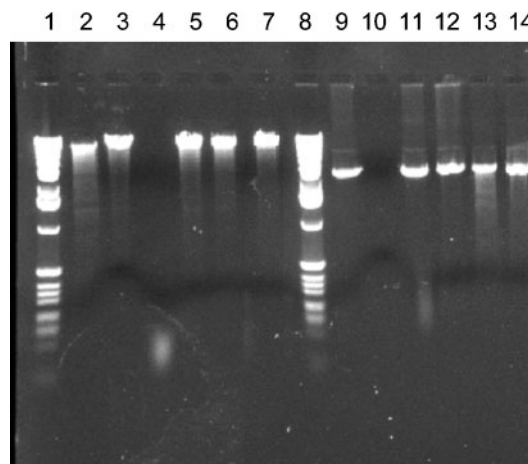


Fig. 5. Agarose gel showing the nucleic acid from IMNV and from control plasmid after digestion with various nucleases. Lanes 1 and 8, 1 kbp DNA ladder; lanes 2-7, IMNV nucleic acid (225 ng); lanes 9-14, plasmid DNA (200 ng). Lanes 2 and 9 received no treatment; lanes 3 and 10 were treated with DNase I (10 U); lanes 4 and 11 were treated with RNase A (20 ng) under low-salt (10 mM) conditions; lanes 5 and 12 were treated with RNase A (20 ng) under high-salt (300 mM) conditions; lanes 6 and 13 were treated with MBN (2 U); lanes 7 and 14 were treated with S1 nuclease (2 U).

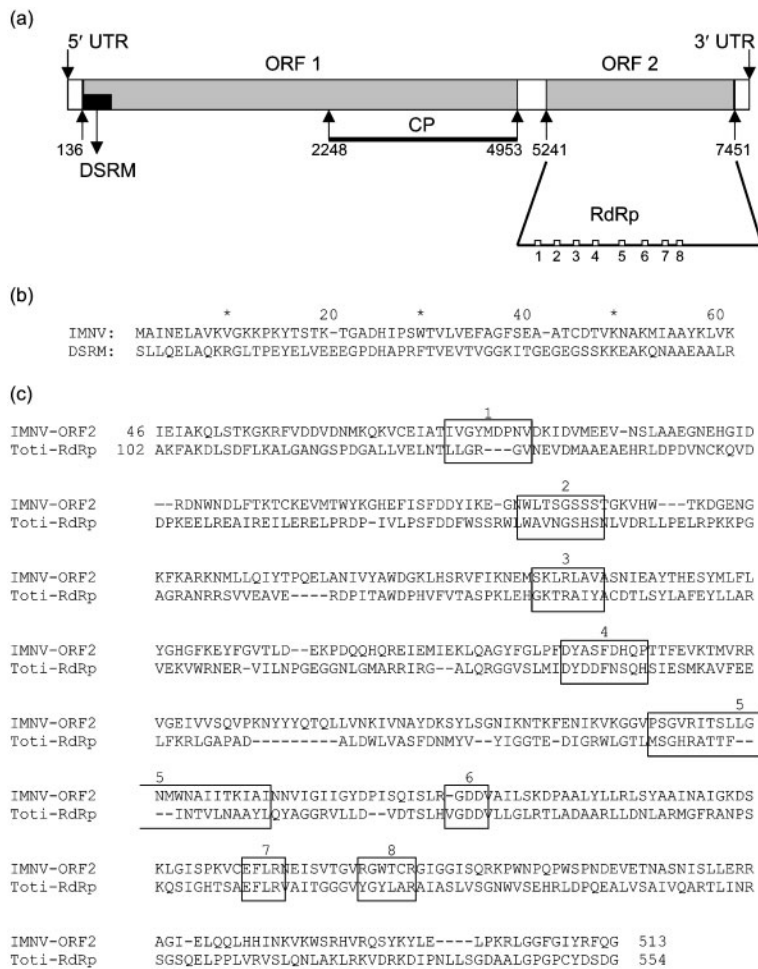


Fig. 6. Organization and conserved regions of the IMNV genome. (a) Genome organization showing the 5' and 3' UTRs, DSRM, ORFs, the coat protein (CP) and the RdRp. Numbers below the arrows indicate nucleotide positions in the genome. (b) Alignment of the DSRM of IMNV to the consensus DSRM motif of the translated regions using the one-letter amino acid code. (c) Alignment of the putative RdRp of IMNV (IMNV-ORF2; aa 46–513) to the consensus sequence of totivirus RNA polymerase (Toti-RdRp; pfam05888, aa 102–554).

monopartite dsRNA genome) and the properties of the dsRNA genome (size, presence of two ORFs, 5' proximal ORF encoding the major capsid protein, 3' proximal ORF encoding an RNA polymerase with homology to the RdRp of totiviruses), this virus appears to be related closely to the family *Totiviridae* and is most similar in size and genetic make-up to members of the genus *Giardiavirus* within that family (Fauquet *et al.*, 2005). There are, however, several important ways in which IMNV differs from totiviruses: the host range of totiviruses includes only fungal and protozoan hosts; the site of virus replication, at least for GLV in the genus *Giardiavirus*, is in the nucleus; the two ORFs are generally overlapping; and a fusion protein consisting of the major capsid protein and the RNA polymerase is often evident. GLV, for example, is reported to replicate in the nucleus of infected protozoan cells and to produce a fusion protein during replication (Wang & Wang, 1986; Wang *et al.*, 1993), whereas IMNV appears to replicate in the cytoplasm of shrimp muscle cells and shows no conclusive evidence for a fusion protein. Edgerton *et al.* (1994) reported on a putative totivirus infecting an invertebrate crustacean host, the freshwater crayfish, *Cherax quadricarinatus*. Although the authors named the virus *Cherax giardiavirus-like virus*, the virus was never isolated or characterized

sufficiently to determine whether the agent was related closely to members of the family *Totiviridae*. Another putative totivirus was isolated from an invertebrate insect host, the green stinkbug, *Nezara viridula* (Williamson & von Wechmar, 1992). The purified virus displayed physical characteristics, including buoyant density and a single segment of dsRNA, that were similar to those of members of the family *Totiviridae*. To date, neither of these invertebrate viruses has been classified officially (Fauquet *et al.*, 2005).

The totivirus capsid protein is usually encoded at the beginning of ORF 1. This is different for IMNV, where the start of the coding region for the major capsid protein was about halfway into ORF 1. The first 60 aa of ORF 1 showed homology (35 % sequence identity) to the nearly full-length (65 aa) consensus sequence of a DSRM, suggesting that the first half of IMNV ORF 1 may encode an RNA-binding protein (St Johnston *et al.*, 1992). The DSRM is highly specific for dsRNA and has been found in a variety of RNA-binding proteins such as RNA helicase, RNase III and Staufen protein (St Johnston *et al.*, 1992; Chang & Ramos, 2005). The DSRM has not been reported in totiviruses, but has been found in several other RNA viruses, including the genus *Coltivirus*, *Acyrtosiphon pisum virus* and *Drosophila*

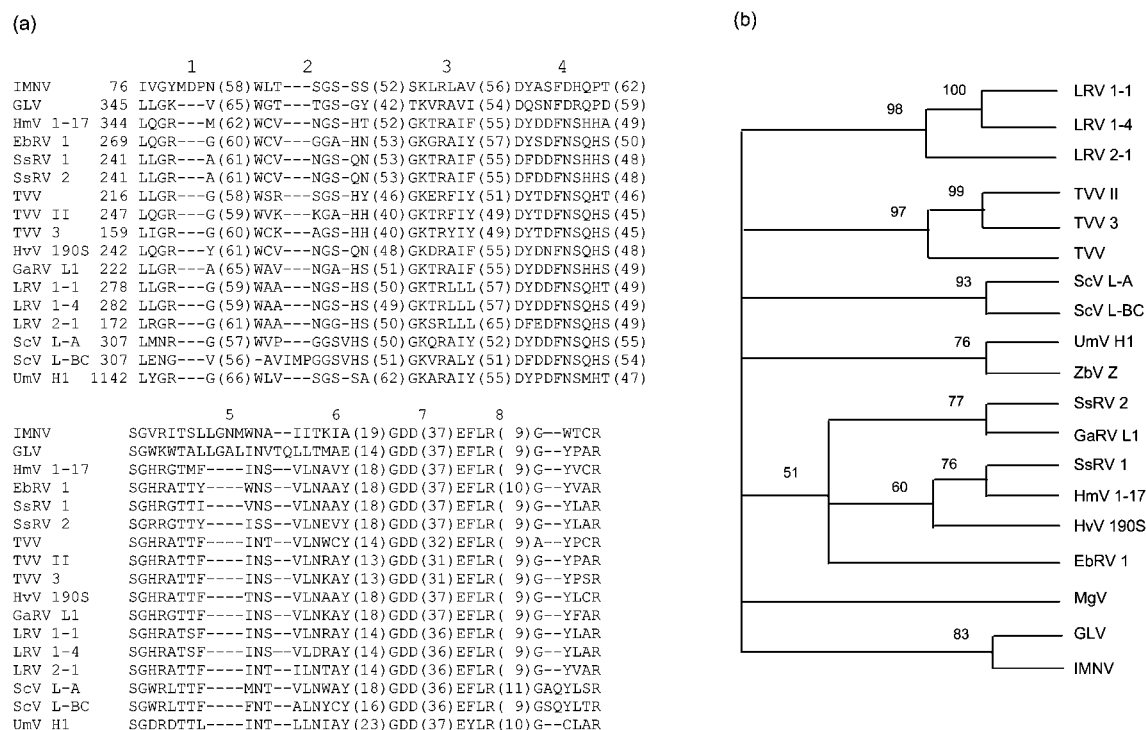


Fig. 7. Protein alignments and phylogenetic tree based on the RdRp region of IMNV. (a) Alignment of the IMNV putative RdRp with the RNA polymerases of 16 members of the family *Totiviridae*. Numbers above the sequences indicate the RdRp motifs and numbers in parentheses indicate the number of amino acids separating the motifs. Virus abbreviations are listed in Table 1. (b) An unrooted tree generated by the neighbour-joining method using the quartet-puzzling program TREE-PUZZLE; numbers indicate percentages of bootstrap support from 1000 replicates.

C virus, although the function of the protein was not determined (van der Wilk *et al.*, 1997; Attoui *et al.*, 1998; Johnson & Christian, 1998). In IMNV, both the putative RNA-binding protein and the capsid protein were translated from the ORF 1 sequence, suggesting the formation of a fusion protein during translation, which should then be cleaved by a protease and the capsid proteins subsequently assembled into a virion. However, the predicted fusion protein consisting of 1606 aa with a molecular mass of 179 kDa was not detected in the SDS-polyacrylamide gel stained with Coomassie blue, indicating either that this protein is not incorporated into the viral particle or that the quantity was too low to be detected under the conditions used.

IMNV ORF 2 was predicted to encode a 736 aa protein with a molecular mass of 85 kDa that showed homology to RNA polymerase and demonstrated significant alignments with the eight RdRp motifs characteristic of totiviruses. However, with few exceptions, totiviruses have been found to encode a fusion protein from overlapping ORFs consisting of the major capsid protein and the RdRp, which is cleaved prior to virus assembly. A fusion protein was not conclusively detected in IMNV, although it could have been in too low a quantity to be detected. Some of the mechanisms for expression of totivirus RdRp as a fusion protein from overlapping ORFs are a -1 ribosomal frame shift as seen with

GLV (Wang *et al.*, 1993), ScV L-A (Dinman *et al.*, 1991) and TVV II (Bessarab *et al.*, 2000) and a $+1$ ribosomal frame shift as proposed for LRV 1-1 and LRV 1-4 (Stuart *et al.*, 1992; Kim *et al.*, 2005). In the case of LRV 2-1, which has non-overlapping ORFs, ribosomal hopping was proposed for translation of a fusion protein (Scheffter *et al.*, 1995). As the two ORFs in IMNV are not overlapping, a translational frame-shift mechanism would not serve to explain production of a fusion protein. ORF 1 and ORF 2 of IMNV are separated by a stretch of 287 nt, so it seems unlikely that ribosome hopping could occur, as this is several times longer than the distance of 50 nt reported for the translation of gene 60 topoisomerase in T4 bacteriophage (Farabaugh, 1996). Recently, Garlapati & Wang (2005) demonstrated the presence of an internal ribosome entry site in the initial coding region of the GLV capsid protein, which constitutes the first report of such an RNA editing function during virus replication in monopartite dsRNA viruses. Determination of whether a capsid-RdRp fusion protein occurs in IMNV and the mechanism that produces such a protein await further analysis.

The 5' and 3' UTRs of IMNV were found to have high contents of A + U. An AU-rich 5' UTR on the plus strand is a general feature of dsRNA viruses and is the region where the dsRNA separates during virus replication as the polymerase

enters (Bruenn, 2002). An AU-rich 3' UTR may also be related to the separation of dsRNA so that transcription can take place. For dsRNA viruses, replication and transcription are performed within the viral particle by the same RNA polymerase.

The alignments and phylogenetic analysis of IMNV RdRp clustered the virus with members of the family *Totiviridae*, but in other respects, IMNV appears to be distinct from this virus family. Further analysis will be required to determine whether IMNV is a novel member of the *Totiviridae* or whether IMNV is a member of a novel family of dsRNA viruses that infect invertebrate hosts.

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