CHAPTER 2.2.3.

INFECTIOUS MYONECROSIS

1. Scope

Infectious myonecrosis (IMN) is a viral disease of penaeid shrimp caused by infection with infectious myonecrosis virus (IMNV) (7, 9, 12). The principal host species in which IMNV is known to cause significant disease outbreaks and mortalities is *Penaeus vannamei*. Outbreaks of IMN with sudden high mortalities may follow stressful events such as capture by cast-net, feeding, sudden changes in salinity or temperature, etc., in early juvenile, juvenile, or adult *P. vannamei* in regions where IMNV is enzootic, or in *P. vannamei* introduced from infected regions or countries. Such severely affected shrimp may have been feeding just before the onset of stress and may have a full gut, and shrimp in the acute phase of IMN disease will present focal to extensive white necrotic areas in striated (skeletal) muscles, especially in the distal abdominal segments and tail fan, which can become necrotic and reddened in some individual shrimp. Severely affected shrimp become moribund and mortalities can be instantaneously high and continue for several days.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

IMNV is a putative totivirus. Phylogenetic analysis based on its RNA-dependent RNA polymerase gene (RdRp) suggests IMNV to be most closely related to *Giardia lamblia virus*, a member of the family Totiviridae (4, 9, 12).

IMNV particles are icosahedral in shape and 40 nm in diameter, with a buoyant density of 1.366 g ml^{-1} in caesium chloride. The genome consists of a single, double-stranded (ds) RNA molecule of 7560 bp. Sequencing of the viral genome reveals two non-overlapping open reading frames (ORFs). The 59 ORF (ORF 1, nt 136–4953) encodes a putative RNA-binding protein and a capsid protein. The coding region of the RNA-binding protein is located in the first half of ORF 1 and contains a dsRNA-binding motif in the first 60 amino acids. The second half of ORF 1 encodes a capsid protein, as determined by amino acid sequencing, with a molecular mass of 106 kDa. The 39 ORF (ORF 2, nt 5241–7451) encodes a putative RNA-dependent RNA polymerase (RdRp) (12).

IMN disease is not the same disease as white tail disease of penaeid shrimp. The latter disease is a recently discovered disease with gross and histological signs that mimic IMN, but which is caused by a different type of virus (a nodavirus named *Penaeus vannamei* novavirus – PvNV) (15).

2.1.2. Survival outside the host

No data.

2.1.3. Stability of the agent (effective inactivation methods)

No data.

2.1.4. Life cycle

Not applicable.

2.2. Host factors

2.2.1. Susceptible host species

The principal host species for IMN disease is the Pacific white shrimp, *Penaeus vannamei*, in which infection by IMNV can cause significant losses in farmed populations (7, 10). Experimental IMNV infections have been reported for the Pacific blue shrimp, *P. stylirostris*, and the black tiger shrimp, *P. monodon* (14).

2.2.2. Susceptible stages of the host

Juveniles and subadults of *P. vannamei*, farmed in marine or low salinity brackish water, appear to be the most severely affected by IMN disease (7, 10, 12).

2.2.3. Species or subpopulation predilection (probability of detection)

No data.

2.2.4. Target organs and infected tissue

The principal target tissues for IMNV include the striated muscles (skeletal and less often cardiac), connective tissues, haemocytes, and the lymphoid organ parenchymal cells (7, 12, 14).

2.2.5. Persistent infection with lifelong carriers

Some members of populations of *P. vannamei* that survive IMNV infections and/or epizootics may carry the virus for life and, although not documented scientifically, are believed to pass the virus onto their progeny by vertical transmission.

2.2.6. Vectors

There are no specific data on vectors. However, because of its structure (a non-enveloped dsRNA virus) it is likely that IMNV will remain infectious in the gut and faeces of seabirds that feed on dead or moribund shrimp at farms with ongoing IMN epizootics, and be spread within and among farms by faeces or regurgitated shrimp carcasses (16).

2.2.7. Known or suspected wild aquatic animals carriers

Native wild penaeids in north-eastern Brazil are anecdotally reported as hosts.

2.3. Disease pattern

2.3.1. Transmission mechanisms

IMNV has been demonstrated to be transmitted from shrimp to shrimp by cannibalism (12). Transmission via water and vertical transmission from broodstock to progeny probably occurs. With vertical transmission, it is not known if the mode is transovarium or by contamination of the spawn eggs.

2.3.2. Prevalence

In regions where the virus is enzootic in farmed stocks of *P. vannamei*, the prevalence of IMNV may reach 100% (1, 10).

2.3.3. Geographical distribution

Reported from north-eastern Brazil (1, 7, 10, 12) and South-East Asia, including Java Island and Sumatra Island, Indonesia, Hainan Island, China (People's Rep. of) and southern Thailand (13).

2.3.4. Mortality and morbidity

Mortalities from IMN range from 40 to 70% in cultivated *P. vannamei*, and food conversion ratios (FCR) of infected populations increase from normal values of ~ 1.5 to 4.0 or higher (1).

2.3.5. Environmental factors

Temperature and salinity effects are considered to be likely predisposing factors to disease outbreaks, but no experimental data are available.

2.4. Control and prevention

2.4.1. Vaccination

No effective vaccines for IMNV are available.

2.4.2. Chemotherapy

No effective therapeutants have been reported for IMN.

2.4.3. Immunostimulation

No data.

2.4.4. Resistance breeding

There are anecdotal reports that some selected lines of *P. vannamei* give better survival and culture performance in farms where IMN is enzootic.

2.4.5. Restocking with resistant species

No data.

2.4.6. Blocking agents

No data.

2.4.7. Disinfection of eggs and larvae

While IMNV is believed to be transmitted vertically (transovarian transmission), there have been no published reports documenting this route of transmission. Disinfection of eggs and larvae (3) is a good management practice recommended to reduce the potential of IMNV contamination of spawned eggs and larvae produced from them.

2.4.8. General husbandry practices

Some husbandry practices have been successfully applied to the prevention of IMNV infections and IMN disease. Foremost among these has been the application of reverse-transcription polymerase chain reaction (RT-PCR) for prescreening pond-reared broodstock and/or their spawned eggs/nauplii and discarding those that test positive for the virus (1). Fallowing and restocking of affected farms or entire culture regions with IMNV-free stocks of *P. vannamei*, and the development of specific pathogen free (SPF) shrimp stocks of *P. vannamei* most suited to local culture conditions has proven to be the most successful husbandry practice for the prevention and control of other virus diseases of shrimp and should be applicable to control and prevention of IMN disease (5, 6, 8, 17).

3. Sampling

3.1. Selection of individual specimens

Suitable specimens for testing for infection by IMNV include postlarvae (PL), juveniles and adults. While IMNV may infect all life stages, infection severity, and hence virus load, may be below detection limits in spawned eggs and in the larval stages, so these life stages may not be suitable samples for IMNV detection or certification for IMN disease freedom.

3.2. Preservation of samples for submission

For routine histology or molecular assays, and guidance on preservation of samples for the intended test method see Chapter 2.2.0.

3.3. Pooling of samples

Samples taken for molecular tests may be combined as pooled samples representing no more than five specimens per pooled sample of juveniles, subadults and adults. However, for eggs, larvae and PL, pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50–150 PL depending on their size/age) may be necessary to obtain sufficient sample material (extracted nucleic acid) to run a diagnostic assay. See also Chapter 2.2.0.

3.4. Best organs or tissues

IMNV infects tissues of mesodermal origin. The principal target tissues in the acute phase of IMNV infection are the striated muscles (skeletal and less commonly cardiac muscle), connective tissues, haemocytes, and the lymphoid organ tubule parenchymal cells. In chronic infections, the lymphoid organ may be the principal target tissue.

Haemolymph or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary

3.5. Samples/tissues that are not suitable

IMNV is a systemic virus, and it does not replicate in enteric tissues (e.g. the hepatopancreas, the midgut, or its caeca). Hence, enteric tissues are inappropriate samples for detection of infection by IMNV.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Only acute-phase IMN disease can be presumptively diagnosed from clinical signs. See Section 4.2 for a description of gross clinical signs presented by shrimp with acute-phase IMN disease.

4.1.2. Behavioural changes

Only shrimp with acute-phase IMN disease present behavioural changes. Typically, severely affected shrimp become lethargic during or soon after stressful events such as capture by cast-netting, feeding, sudden changes in temperature, sudden reductions in salinity, etc.). Severely affected shrimp may have been feeding just before the onset of stress and often have a full gut.

4.2. Clinical methods

4.2.1. Gross pathology

Shrimp in the acute phase of IMN disease present focal to extensive white necrotic areas in striated (skeletal) muscles, especially in the distal abdominal segments and tail fan, which can become necrotic and reddened in some individual shrimp. These signs may have a sudden onset following stresses (e.g. capture by cast-net, feeding, and sudden changes in temperature or salinity). Such severely affected shrimp may have been feeding just before the onset of stress and may have a full gut. Severely affected shrimp become moribund and mortalities can be instantaneously high and continue for several days.

Exposing the paired lymphoid organs (LO) by simple dissection will show that they are hypertrophied to 3–4 times their normal size (7, 12).

4.2.2. Clinical chemistry

Not applicable.

4.2.3. Microscopic pathology

IMN disease in the acute and chronic phases can be tentatively diagnosed using histological methods (2, 7, 12). However, the lesions in striated muscles and LO are not pathognomonic for IMN. White tail disease of penaeid shrimp, caused by the nodavirus PvNV, mimics IMN (15). Hence, diagnostic information from other sources (e.g. history, gross signs, morbidity, mortality, or RT-PCR findings) may be required to confirm a diagnosis of IMN.

By histopathology, using routine haematoxylin–eosin (H&E) stained paraffin sections (2), shrimp with acute-phase IMN present myonecrosis with characteristic coagulative necrosis of striated (skeletal) muscle fibres, often with marked oedema among affected muscle fibres. Some shrimp may present a mix of acute and older lesions. In these shrimp, the affected muscle fibres appear to progress from presenting coagulative necrosis to presenting liquefactive necrosis, which is accompanied by moderate infiltration and accumulation of haemocytes. In the most advanced lesions, haemocytes and inflamed muscle fibres are replaced by a loose matrix of fibrocytes and connective tissue fibres that are interspersed with haemocytes and foci of (presumed) regenerating muscle fibres (7, 12).

Significant hypertrophy of the LO due to accumulations of lymphoid organ spheroids (LOS) is a highly consistent lesion in shrimp with acute or chronic-phase IMN lesions. Often, many ectopic LOS are found in other tissues not near the main body of the LO. Common locations for ectopic LOS include the haemocoelom in the gills, heart, near the antennal gland tubules, and ventral nerve cord (7, 12).

4.2.4. Wet mounts

Stained or unstained tissue squashes of affected skeletal muscle or of the LO may show abnormalities. Tissue squashes of skeletal muscle when examined with phase or reduced light microscopy may show loss of the normal striations. Fragmentation of muscle fibres may also be apparent. Squashes of the LO may show the presence of significant accumulations of spherical masses of cells (LOS) amongst normal LO tubules.

4.2.5 Smears

Not applicable.

4.2.6 Electron microscopy/cytopathology

Not applicable for diagnostic purposes.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

See Section 4.2.4.

4.3.1.1.2. Smears

Not applicable.

4.3.1.1.3. Fixed sections

See Section 4.2.3.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

None reported to date.

4.3.1.2.2. Antibody-based antigen detection methods

None reported to date.

4.3.1.2.3. Molecular techniques

Published methods are available for the molecular detection of IMNV by *in-situ* hybridisation (ISH), nested RT-PCR and real-time RT-PCR (1, 11, 14). A nested RT-PCR kit for detection of the virus is commercially available from GeneReach Biotechnology Corp.[1] (Chinese Taipei). As the sequence information of the IMNV genome used for the development of these tests is unique to IMNV, the specificity of these tests is 100%.

As the sensitivity of the nested and real-time RT-PCR is greater than any other available method and approaches ten viral genome copies, the sensitivity for these tests as the gold standard for IMNV should be set at 100% (1, 11).

4.3.1.2.3.1. DNA probe for ISH detection of IMNV

A cDNA library was constructed from RNA extracted from purified IMNV. From one clone, designated as IMNV-317, the cDNA was labelled with digoxigenin-11-dUTP and used as a gene probe for ISH. The probe was found to be highly specific for IMNV (100% specificity as its sequence does not match that of any known penaeid shrimp viruses) (14).

The cDNA probe is prepared from clone IMNV-317 by PCR labelling with digoxigenin-11-dUTP (DIG) (14). The primers used in the labelling reaction are IMNV993F (5'-AAC-ACA-AAA-TCT-GCC-AGC-AA-3') and IMNV993R (5'-CCC-AAC-CAC-CCA-AAT-TCA-TA-3') and they generate a 993 bp amplicon. Following PCR, the 993 bp DIG-labelled DNA probe is precipitated with ethanol, re-suspended in water and stored at –20°C until use (14). The ISH procedure with this probe follows the methods outlined for detection of IMNV by Tang *et al.* (14).

4.3.1.2.3.2. RT-PCR for detection of IMNV

A nested RT-PCR method was developed for the detection of IMNV using two primer sets that produce 328 and 139 bp amplicons. The first step of the nested assay was found to detect as little as 100 copies of the IMNV genome, whereas the nested second step detected 10 copies (11). The method is summarised below:

1 Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this *Aquatic Manual*.

RNA templates:

1. Frozen or ethanol-fixed tissue (pleopods, cephalothorax, muscle)
2. Haemolymph (weaker reactions than with other tissues)

RT-PCR reaction mixture (Applied Biosystems rTth Enzyme and 5 × EZ Buffer #N808-0178):

Reagent	25 µl reaction	Final concentration
DD H_2O	6.5 µl	–
5 × EZ Buffer	5.0 µl	1 ×
dNTP mix (10mM each)	3.0 µl	300 µM each
Primer F (100 ng μl^{-1})	1.0 µl	0.62 µM
Primer R (100 ng μl^{-1})	1.0 µl	0.62 µM
$Mn(Oac)_2$ (25 mM)	2.5 µl	2.5 mM
rTth Enzyme (2.5 U μl^{-1})	1.0 µl	0.1 U μl^{-1}
Template[1]	1–5 µl	1–50 ng total RNA

[1]Template must be boiled for 3 minutes and put on ice just prior to adding to reaction mix. RT-PCR cycling parameters:

Primers	Temperature (°C)	Time	No. cycles	Amplicon
4587F/4914R	60, 95	30 minutes, 2 minutes	1	328 bp
	95, 60	45 seconds, 45 seconds	39	
	60	7 minutes	1	

Nested PCR reaction (Amersham Biosciences pure Taq Ready To Go Beads #27-9558-01):

Reagent	25 µl reaction	Final concentration
DD H_2O	22.5 µl	–
Primer NF (100 ng μl^{-1})	1.0 µl	0.465 µM
Primer NR (100 ng μl^{-1})	1.0 µl	0.465 µM
Template[2]	0.5 µl	–

[2]Template for the nested reaction is the product from the first step reaction

Nested PCR cycling parameters:

Primers	Temperature (°C)	Time	No. cycles	Amplicon
4725 NF/4863 NR	95	2 minutes	1	139 bp
	95, 65, 72	30 seconds, 30 seconds, 30 seconds	39	
	72	2 minutes	1	

Primer sequences:

Primer	Sequence (5′ to 3′)	Amplicon Size	Ref.
4587F	CGA-CGC-TGC-TAA-CCA-TAC-AA	328 bp	(6)
4914R	ACT-CGG-CTG-TTC-GAT-CAA-GT		
4725 NF	GGC-ACA-TGC-TCA-GAG-ACA	139 bp	
4863 NR	AGC-GCT-GAG-TCC-AGT-CTT-G		

4.3.1.2.3.3. Quantitative (real-time) RT-PCR for detection of IMNV

A real-time quantitative RT-PCR method was developed for the detection and quantification of IMNV in shrimp tissue. The method can detect as few as 10 IMNV copies per µl RNA (1). The method as published (1) is summarised below.

Primer Express software (Applied Biosystems) is used to design primers and a TaqMan probe from the ORF 1 region of the IMNV genome (GenBank accession no. AY570982 (1, 12). Primers IMNV412F (5'-GGA-CCT-ATC-ATA-CAT-AGC-GTT-TGC-A-3') and IMNV545R (5'-AAC-CCA-TAT-CTA-TTG-TCG-CTG-GAT-3') produce a fragment of 134 bp. The TaqMan probe, IMNVp1 (5'-6FAM-CCA-CCT-TTA-CTT-TCA-ATA-CTA-CAT-CAT-CCC-CGG-TAMRA-3'), which corresponds to the nucleotides 467–500, is synthesised and labelled with fluorescent dyes 5-carboxyfluoroscein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end. The IMNV genome fragments are amplified using an ABI GeneAmp 5700 sequence detection system with TaqMan One-Step RT-PCR master mix (Applied Biosystems). Prior to the real-time RT-PCR assay, extracted RNA is boiled at 100°C for 5 minutes to denature the dsRNA and it is then placed on ice. The reaction mixture contains 1 µl of RNA sample, 12.5 µl of TaqMan Master mix (2×), 0.625 µl of Multiscribe mix (40×), 300 nM each of the forward and reverse primers, 200 nM TaqMan probe in a final volume of 25 µl. The RT-PCR cycle parameters are: 30 minutes at 48°C and 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The viral copy number of the samples is determined using Gene Amp 5700 sequence detection software.

To generate an RNA standard for the real-time RT-PCR assay, the primers IMNV218F and IMNV682R (5'-GCT-GGA-CTG-TAT-TGG-TTG-AG-3' and 5'-AAC-CAA-GTT-CTT-CTT-CTC-CAG-TT-3', respectively) are used to generate an amplicon of 464 bp from the IMNV genome. The RT-PCR product is cleaned using a QIAquick PCR Purification kit (Qiagen). The fragments are then ligated to pGEM-T Easy Vector and transformed into *Escherichia coli* JM109 cells (Promega). A recombinant plasmid, pIMNV-1, is confirmed by sequencing. Then, pIMNV-1 is linearised by *Pst*I digestion and used as the template for an *in-vitro* transcription with T7 RNA polymerase. A total of 1 µg of plasmid is used in a 50 µl reaction at 37°C for 2 hours, followed by DNase I digestion at 37°C for 30 minutes. The synthesis of the RNA standard is confirmed by electrophoresis in a 1.5% agarose gel containing ethidium bromide. The RNA standard is cleaned using a Qiaquick PCR purification kit, quantified by a spectrophotometer, and stored at –70°C.

4.3.1.2.4. Agent purification

IMNV has been purified from infected shrimp tissue by density gradient centrifugation (12). However, purification of the agent from infected tissue is not a recommended method for diagnostic purposes.

4.3.2. Serological methods

Not applicable because shrimp are invertebrate animals which do not produce specific antibodies that could be used to demonstrate infection by or prior exposure to IMNV.

5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of Infectious myonecrosis are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	c	c	c	d
Bioassay	d	d	c	c	c	c
Direct LM	d	d	c	c	c	c
Histopathology	d	d	b	b	a	b

***Table 5.1.* (*cont.*)** *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Transmission EM	d	d	d	d	d	d
Antibody-based assays	d	d	d	d	d	d
DNA probes (ISH)	d	d	a	a	a	a
PCR	a	a	a	a	a	a

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; PCR = polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from infectious myonecrosis

As indicated in Table 5.1, RT-PCR (Section 4.3.1.2.3.2) is the recommended method for targeted surveillance for reasons of availability, utility, and diagnostic specificity and sensitivity.

When investigating acute mortality episodes as part of a targeted surveillance programme, histological demonstration of characteristic IMNV-induced lesions in the striated muscles and the extreme hypertrophy of the LO caused by LOS formation (with or without confirmation by ISH with IMNV-specific DNA probes) is a suitable method (Table 5.1). The occurrence of significant mortality distinguishes IMN from penaeid white tail disease caused by PvNV, in which the gross signs and histopathology mimics IMN disease (15).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Sudden high mortalities, usually following stressful events such as capture by cast-net, feeding, sudden changes in salinity or temperature, etc., in early juvenile, juvenile, or adult *P. vannamei* in regions where IMNV is enzootic or where introduction of *P. vannamei* from infected regions or countries has occurred. Such severely affected shrimp may have been feeding just before the onset of stress and may have a full gut, and shrimp in the acute phase of IMN disease will present focal to extensive white necrotic areas in striated (skeletal) muscles, especially in the distal abdominal segments and tail fan, which can become necrotic and reddened in some individual shrimp. Severely affected shrimp become moribund and mortalities can be instantaneously high and continue for several days. Exposing the paired LO by simple dissection will show that they are hypertrophied to 3–4 times their normal size.

7.2. Definition of confirmed case

Any combination of a molecular (PCR or ISH) test and a morphological (histology) test using at least two of the following three methods (with positive results):

- Histological demonstration of diagnostic acute, transition or chronic-phase IMNV lesions in the striated muscles and/or the LO.
- ISH positive (with a IMNV-specific cDNA probe) signal to IMNV-type lesions in striated necrotic muscle fibres or to distinctive LOS in the lymphoid organs of shrimp with transition or chronic-phase IMNV infections in histological sections.
- RT-PCR positive results for IMNV.

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NB: There is an OIE Reference Laboratory for Infectious myonecrosis (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).