CHAPTER 2.2.6.

INFECTION WITH *MACROBRACHIUM ROSENBERGII* NODAVIRUS (WHITE TAIL DISEASE)

1. Scope

Infection with *Macrobrachium rosenbergii* nodavirus means infection with the pathogenic agent *Macrobrachium rosenbergii* nodavirus (MrNV), Family *Nodaviridae*. The disease is commonly known as white tail disease (WTD).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The aetiological agents are two viral pathogens, namely MrNV (primary) and extra small virus (XSV) (associate) (Qian *et al.,* 2003; Romestand & Bonami, 2003). MrNV is important in disease outbreaks but the role of XSV in pathogenicity remains unclear. Strains are not yet known. MrNV belongs in the family Nodaviridae (Bonami *et al.,* 2005; King *et al.,* 2012). XSV is the first sequenced satellite virus in animals and it is also the first record of a satellite-nodavirus association (Bonami *et al.,* 2005).

2.1.2. Survival outside the host

Survival outside the host is not known, however viral inoculum prepared from tissue homogenate stored at –20°C caused 100% mortality in post-larvae (PL) of *M. rosenbergii* by immersion challenge (Qian *et al.,* 2003; Sahul Hameed *et al.,* 2004a).

2.1.3. Stability of the agent (effective inactivation methods)

Agent stability is not known. However, heat treatment at 65°C for 2 hours destroyed infectivity of MrNV and XSV in challenge experiments (Qian *et al.,* 2003).

2.1.4. Life cycle

Not known.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with MrNV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) include: giant river prawn (*Macrobrachium rosenbergii*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with MrNV according to Chapter 1.5. of the *Aquatic Code* include: white leg shrimp (*Penaeus vannamei*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results (but not active infection) have been reported in the following species: kuruma prawn (*Penaeus japonicus*), Indian white prawn (*P. indicus*), giant tiger prawn (*P. monodon*), dragonfly (*Aeshna* sp.), giant water bug (*Belostoma* sp.), beetle (*Cybister* sp.), backswimmer (*Notonecta* sp.), hairy river prawn (*Macrobrachium rude*), monsoon river prawn (*Macrobrachium malcolmsonii*), brine shrimps (*Artemia* sp.) and red claw crayfish (*Cherax quadricarinatus*).

2.2.3. Susceptible stages of the host

Larvae, PL and early juveniles are susceptible, whereas adults are resistant (Qian *et al.,* 2003; Sahul Hameed *et al.,* 2004a).

2.2.4. Species or subpopulation predilection (probability of detection)

No mortality was observed either in naturally or experimentally (MrNV/XSV) infected subadult and adult prawns. Experimental studies confirmed vertical transmission from infected broodstock to PL (Sudhakaran *et al.,* 2006a).

2.2.5. Target organs and infected tissue

MrNV and XSV are confined to gill tissue, head muscle, heart, abdominal muscle, ovaries, pleopods and tail muscle, but not the hepatopancreas or eyestalk (Sahul Hameed *et al.,* 2004a; Sri Widada *et al.,* 2003). Pleopods are a convenient source of RNA for non-destructive screening of MrNV and XSV (Sahul Hameed *et al.,* 2004a).

2.2.6. Persistent infection

Challenge experiments indicate long-term persistent infection in adults and also the possibility of transmitting MrNV from broodstock to larvae and PL (Sahul Hameed *et al.,* 2004a; Sudhakaran *et al.,* 2006a).

2.2.7. Vectors

Not known.

2.3. Disease pattern

A high prevalence of infection with MrNV has been reported in hatchery-reared larvae and PL of *M. rosenbergii*.

2.3.1. Transmission mechanisms

Transmission is vertical (trans-ovum) and horizontal by the waterborne route (Qian *et al.,* 2003; Sahul Hameed *et al.,* 2004a; Sudhakaran *et al.,* 2006a).

2.3.2. Prevalence

Prevalence is variable from 10% to 100% in hatchery, nursery and grow-out systems (Arcier *et al.,* 1999; Qian *et al.,* 2003; Sahul Hameed *et al.,* 2004a; 2004b).

2.3.3. Geographical distribution

The disease was first reported in the French West Indies (Arcier *et al.,* 1999), later in China (People's Rep. of) (Qian *et al.,* 2003), India (Sahul Hameed *et al.,* 2004b), Chinese Taipei (Wang *et al.,* 2008), Thailand (Yoganandhan *et al*., 2006) and Australia (Owens *et al.,* 2009).

2.3.4. Mortality and morbidity

Larvae, PL and juveniles of *M. rosenbergii* are highly susceptible to infection with MrNV, which often causes high mortalities in these life stages. Mortality may reach a maximum in about 5 or 6 days after the appearance of the first gross signs. Very few PL with infection with MrNV survive beyond 15 days in an outbreak, and PL that survive may grow to market size like any other normal PL. Adults are resistant to infection with MrNV, but act as carriers (Qian *et al.,* 2003; Sahul Hameed *et al.,* 2004a).

2.3.5. Environmental factors

Not much is known about environmental factors. However, outbreaks of infection with MrNV may be induced by rapid changes in salinity, temperature and pH (Arcier *et al.,* 1999; Qian *et al.,* 2003).

2.4. Control and prevention

Information on control and prevention of infection with MrNV is limited. However, proper preventive measures, such as screening of brood stock and PL, and good management practices may help to prevent infection with MrNV in culture systems. As the life cycle of *M. rosenbergii* is completed under controlled conditions, specific pathogen free (SPF) brood stock and PL can be produced by screening using sensitive diagnostic methods such as reverse-transcription PCR (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) (Romestand & Bonami, 2003; Sri Widada *et al.,* 2003; Yoganandhan *et al*., 2005).

2.4.1. Vaccination

Not yet available.

2.4.2. Chemotherapy

No known chemotherapeutic agents reported for infection with MrNV.

2.4.3. Immunostimulation

No reports available concerning the use of immunostimulants infection with MrNV.

2.4.4. Breeding for resistance

None reported.

2.4.5. Restocking with resistant species

No report on the occurrence of resistant species.

2.4.6. Blocking agents

Not known.

2.4.7. Disinfection of eggs and larvae

Routine procedures followed for crustacean viral disease control are suggested. For example, application of formalin or iodophor helps to eliminate virus (Chen *et al.,* 1992).

2.4.8. General husbandry practices

Experimental infection confirmed the possibility of horizontal and vertical transmission of MrNV in culture systems (Qian *et al.,* 2003; Sahul Hameed *et al.,* 2004a; Sudhakaran *et al.,* 2006a). Good husbandry practices, such as proper disinfection of tanks, water and broodstock, and the use of RT-PCR negative broodstock in the hatchery grow-out ponds may be useful in the prevention of infection with MrNV in culture systems (Chen *et al.,* 1992; Sri Widada *et al.,* 2003; Sudhakaran *et al.,* 2008). There is no evidence that crop rotation either with rice or polyculture with fish prevents infection with MrNV. Some farmers have considered either mixed culture of shrimp (*P. monodon*) with *M. rosenbergii* or crop rotation of these two species as a viable alternative for their sustenance and economic viability. This situation invites the possibility of transmitting pathologically significant organisms from native to non-native hosts as observed by Sudhakaran *et al.* (2006b) and Ravi *et al.* (2009) in their studies. Based on their results, it would seem that mixed culture of *M. rosenbergii* with *P. monodon* should be avoided before adopting any preventive measures in the management of infection with MrNV.

3. Sampling

3.1. Selection of individual specimens

Infection with MrNV is indicated by the whitish coloration of abdominal and tail muscle (Arcier *et al.,* 1999; Romestand & Bonami, 2003; Sahul Hameed *et al.,* 2004b). However, this clinical sign is not specific to infection with MrNV and diagnosis is not easy, particularly in the earlier stages of infection. PL affected by infection with MrNV are more milky and opaque. Once this clinical sign appears, death usually follows; mortality rates are variable and reach up to 95%. The tissues most affected in moribund PLs/early juveniles are striated muscles of the abdomen, cephalothorax and tail. PLs with whitish muscle are suitable for diagnostic purposes (Sahul Hameed *et al.,* 2004a).

3.2. Preservation of samples for submission

Infected larvae or PL with prominent signs of whitish muscle in the abdominal region are collected from disease outbreak areas. Samples are washed in sterile saline, transferred to sterile tubes, transported to the laboratory on dry ice and stored at –70°C until further processed (Sahul Hameed *et al.,* 2004b; Sri Widada *et al.,* 2003; Yoganandhan *et al*., 2005). Frozen samples can be used for virus isolation and detection by RT-PCR or ELISA (Romestand & Bonami, 2003). Samples for virus detection by RT-PCR can be transported to the laboratory after fixing in 70% ethanol (Sahul Hameed *et al.,* 2004b; Sri Widada *et al.,* 2003; Yoganandhan *et al*., 2005). See also Chapter 2.2.0 *General information* (on diseases of crusteaceans).

3.3. Pooling of samples

The effect of pooling on diagnostic sensitivity has not been evaluated, therefore larger shrimp should be processed and tested individually. However, samples, especially PL or specimens up to 0.5 g, can be pooled to obtain enough material for molecular testing. See also chapter 2.2.0.

3.4. Best organs or tissues

The whole PL body is preferred (Sahul Hameed *et al.,* 2004b; Sri Widada *et al.,* 2003; Yoganandhan *et al*., 2005). All the organs, except eyestalks and the hepatopancreas, of adult *M. rosenbergii* are best for screening the viruses by RT-PCR. Pleopods (swimming legs) are a convenient source of RNA for non-destructive screening of MrNV and XSV (Sahul Hameed *et al.,* 2004a).

3.5. Samples or tissues that are not suitable

Eyestalks and the hepatopancreas of adult prawns are not suitable (Sahul Hameed *et al.,* 2004a; Sri Widada *et al.,* 2003).

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Infected PL become opaque and develop a whitish appearance, particularly in the abdominal region. The whitish discolouration appears first in the second or third abdominal segment and gradually diffuses both anteriorly and posteriorly. In severe cases, degeneration of telson and uropods may occur. Mortality may reach a maximum in about 5 days after the appearance of the first gross signs.

4.1.2. Behavioural changes

PLs are highly susceptible to infection with MrNV and mortality reaches a maximum in about 5 days after the appearance of whitish discolouration. Floating exuviae (moults) in the tanks appear abnormal and resemble ‘mica flakes’ (Arcier *et al.,* 1999). The infected PL show progressive weakening of their feeding and swimming ability (Sahul Hameed *et al.,* 2004a).

4.2. Clinical methods

4.2.1. Gross pathology

Infection with MrNV and XSV, is indicated by whitish coloration of abdominal muscle; however, this clinical sign is not pathognomonic.

4.2.3. Microscopic pathology

The most affected tissue in infected PL is striated muscle of the cephalothorax, abdomen and tail. Histological features include the presence of acute Zenker’s necrosis of striated muscles, characterised by severe hyaline degeneration, necrosis and muscular lysis. Moderate oedema and abnormal open spaces among the affected muscle cells are also observed, as is the presence of large oval or irregular basophilic cytoplasmic inclusion bodies in infected muscles (Arcier *et al.,* 1999; Hsieh *et al.,* 2006). Pathognomonic oval or irregular basophilic cytoplasmic inclusion bodies are demonstrated in the target tissues by histology (Arcier *et al.,* 1999; Hsieh *et al.,* 2006).

The presence of MrNV in infected cells can be demonstrated in histological sections using a DIG-labelled DNA *in-situ* hybridisation probe specific for MrNV (Sri Widada *et al.,* 2003).

4.2.4. Wet mounts

None to date.

4.2.5. Smears

None to date.

4.2.6. Electron microscopy/cytopathology

Using transmission electron microscopy (TEM), infected cells appear necrotic, exhibiting a disorganised cytoplasm. TEM studies reveal the presence of two types of non-enveloped para-spherical virus particles of different sizes within the cytoplasm of connective cells and muscle cells. Large viral particles are five- to six-sided, with a diameter of 26–27 nm, and would be characteristic of MrNV. Smaller viral particles similar in structure (five- to six-sided), but with a diameter of 14–16 nm, would be characteristic of XSV (Qian *et al.,* 2003).

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

Genome and antibody-based diagnostic methods are available to detect MrNV/XSV (Romestand & Bonami, 2003; Sri Widada *et al.,* 2003; Yoganandhan *et al*., 2005).

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

None to date.

4.3.1.1.2. Smears

None to date.

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

MrNV/XSV can be easily propagated in the C6/36 mosquito *Aedes albopictus* cell line (Sudhakaran *et al.,* 2007a) and this cell line can be cultured easily in Leibovitz L-15 medium containing 100 International Units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 2.5 µg ml^{-1} fungizone supplemented with 10% fetal bovine serum at 28°C (Sudhakaran *et al.,* 2007a). The C6/36 cell line was found to be useful for propagation of these viruses, and viral replication was confirmed by RT-PCR, acridine orange staining, infectivity studies and electron microscopy. A specific cytopathic effect was not observed in MrNV-infected cell lines, but multiple vacuolations were observed. Other cell lines, namely the fish SSN-1 cell line, partially support the multiplication of these viruses (Hernandez-Herrera *et al.,* 2007).

4.3.1.2.2. Antibody-based antigen detection methods

Antibody-based diagnostic methods for MrNV include the ELISA described by Romestand & Bonami (2003) or the triple-antibody sandwich (TAS) ELISA based on a monoclonal antibody (Qian *et al.,* 2006).

4.3.1.2.2.1. ELISA protocol (Romestand & Bonami, 2003)

i) Homogenise infected or healthy PL samples in 0.5 ml phosphate-buffered saline (PBS) and centrifuge at 10,000 ***g*** for 15 minutes. Collect and store the supernatant at –20°C for diagnostic purposes.

ii) Coat ELISA plates with 50 µl per well sample supernatant and incubate overnight at 4°C.

iii) Block with 250 µl 1% bovine serum albumin (BSA) in PBS for 1 hour at 37°C.

iv) Add 50 µl IgG anti-MrNV with 1% BSA and incubate for 2 hours at room temperature.

v) Add 50 µl of an anti-mouse IgG conjugated to peroxidase at 0.4 µg ml^{-1} and incubate for 1 hour at room temperature.

vi) Add 50 µl orthophenylene diamine chromogen at 0.4 mg ml^{-1} in substrate buffer (citric acid 0.1 M, sodium acetate 0.1 M, pH 5.4, H_2O_2 at a 0.33% final concentration).

vii) Stop the reaction after 15 minutes by adding 25 µl of H_2SO_4 to each well.

viii) Measure OD (optical density) at 492 nm with an ELISA plate reader.

NOTE: two rinses with PBS should be performed between each step described above.

4.3.1.2.2.2. TAS-ELISA protocol (Qian et al., 2006)

i) Coat ELISA plates with rabbit polyclonal antibody raised against MrNV and incubate for 2 hours at 37°C and keep at 4°C before use.

ii) Block with 250 µl 1% BSA in PBS for 1 hour at 37°C.

iii) Homogenise infected or healthy PL samples in 0.5 ml PBS and centrifuge at 10,000 ***g*** for 15 minutes. Collect and store the supernatant at –20°C for diagnostic purposes.

iv) Add 100 µl of sample to each well and incubate overnight at 4°C.

v) Add 50 µl of a monoclonal antibody raised against MrNV with 1% BSA and incubate for 2 hours at room temperature.

vi) Add 50 µl of an anti-mouse IgG conjugated to peroxidase at 0.4 µg ml^{-1} and incubate for 1 hour at room temperature.

vii) Add 50 µl orthophenylene diamine chromogen at 0.4 mg ml^{-1} in substrate buffer (citric acid 0.1 M, sodium acetate 0.1 M, pH 5.4, H_2O_2 at a 0.33% final concentration).

viii) Stop the reaction after 15 minutes by adding 25 µl H_2SO_4 to each well.

ix) Measure OD (optical density) at 492 nm with an ELISA plate reader.

NOTE: two rinses with PBS should be performed between each step described above.

4.3.1.2.3. Molecular techniques

4.3.1.2.3.1. Reverse-transcription polymerase chain reaction (RT-PCR)

The protocol for the RT-PCR for detection of MrNV/XSV developed by Sri Widada *et al.* (2003) and Sahul Hameed *et al.* (2004a; 2004b) is recommended for all situations. MrNV and XSV can be detected by RT-PCR separately using a specific set of primers or these two viruses can be detected simultaneously using a single-tube one-step multiplex RT-PCR (Yoganandhan *et al*., 2005). Nested RT-PCR (RT-nPCR) is also available and recommended for screening broodstock and seed (Sudhakaran *et al.,* 2006a).

Total RNA extraction

i) Collect 50 mg of PL or 100 mg of an organ piece (gill tissue, abdominal muscle, tail muscle or pleopods) from adult prawns and homogenate in 300 µl TN buffer (20 mM Tris/HCl, 0.4 M NaCl, pH 7.4).

ii) Centrifuge the homogenate at 12,000 ***g*** for 15 minutes at room temperature and collect the supernatant.

iii) Take 150 µl of supernatant and add 1 ml TRIzol. Mix thoroughly and incubate for 5 minutes at room temperature.

iv) After 5 minutes, add 200 µl chloroform to the sample, mix well and centrifuge at 12,000 ***g*** for 15 minutes at room temperature.

v) Collect the aqueous phase and transfer to a fresh tube, and precipitate RNA by mixing with 500 µl isopropanol.

vi) Incubate the sample for 10 minutes at room temperature and centrifuge at 12,000 ***g*** for 10 minutes at 4°C.

vii) Dissolve the RNA pellet in 50 µl of TE buffer (10 mM Tris/HCl, 1 mM EDTA [ethylene diamine tetra-acetic acid], pH 7.5) after a wash with 75% ethyl alcohol.

viii) Quantify the RNA by measuring the absorbance at 260 nm using UV spectrophotometer and check the purity by measuring the ratio of OD_{260nm}/OD_{280nm}.

RT-PCR protocol

Three RT-PCR methods are described to detect MrNV and XSV. The first protocol is a one-step RT-PCR adapted from Sri Widada *et al.* (2003) and Sahul Hameed *et al.* (2004b), and this method can be used for confirmation of MrNV and XSV in PL of prawns collected from suspected WTD outbreaks. The second protocol is a sensitive RT-nPCR protocol described by Sudhakaran *et al.* (2006a). This test can be used for screening healthy PL, juveniles and broodstock for viruses. The third protocol is a multiplex RT-PCR procedure adapted from Yoganandhan *et al.* (2005). It can be used for the

simultaneous detection of MrNV and XSV in disease outbreaks or for screening seeds and broodstock. In all the protocols described here, a commercial RT-PCR kit allowing reverse transcription and amplification in a single reaction tube is used.

Protocol 1: RT-PCR for specific detection of MrNV or XSV in infected prawn PL or juveniles (Sahul Hameed *et al.,* 2004b; Sri Widada *et al.,* 2003; Sudhakaran *et al.,* 2007b):

The following controls should be included in every RT-PCR assay for MrNV or XSV: a) a known MrNV/XSV-negative tissue sample; b) a known MrNV/XSV-positive sample (tissue or purified virus); and c) a 'no-template' control.

For RT-PCR, a commercial RT-PCR kit is used. The reaction is performed in 50 µl RT-PCR buffer containing 20 pmol of each primer specific to MrNV or XSV and RNA template (10–100 ng), using the following cycles: RT at 52°C for 30 minutes; denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 40 seconds, annealing at 55°C for 40 seconds, and elongation at 68°C for 1 minute, ending with an additional elongation step for 10 minutes at 68°C. Analyse the RT-PCR products by electrophoresis on a 1% agarose gel stain with ethidium bromide and a suitable DNA ladder marker and detect using an ultraviolet transilluminator.

A positive reaction will be indicated by a 425 bp product for MrNV and a 546 bp product for XSV. The sensitivity of the assay is approximately 2.5 fg of total RNA.

PCR primer sequences for MrNV (annealing temperature 55°C; product size 425 bp):

Forward: 5'-GCG-TTA-TAG-ATG-GCA-CAA-GG-3'
Reverse: 5'-AGC-TGT-GAA-ACT-TCC-ACT-GG-3'

PCR primer sequences for XSV (annealing temperature 55°C; product size 546 bp):

Forward: 5'-CGC-GGA-TCC-GAT-GAA-TAA-GCG-CAT-TAA-TAA-3'
Reverse: 5'-CCG-GAA-TTC-CGT-TAC-TGT-TCG-GAG-TCC-CAA-3'

Protocol 2: The RT-nPCR for detection of MrNV and XSV (Sudhakaran *et al.,* 2006a)

The RT-nPCR is more sensitive and useful for screening seed and broodstock (Sudhakaran *et al.,* 2006a):

For the RT-nPCR, the first step of the RT-PCR, as described in protocol 1, should be performed with external primers and the nPCR should be carried out using an RT-PCR product as a template. For RT-nPCR, add 2 ml RT-PCR product to a PCR tube containing 20 µl of reaction mixture (10 mM Tris/HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 µM of each dNTP, 20 pmol of each internal primer, 1.25 units of heat-stable DNA polymerase). The RT-nPCR protocol for both viruses comprise an initial 95°C for 10 minutes, followed by 30 cycles of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C with a final extension at 72°C for 5 minutes. Analyse the RT-nPCR products by electrophoresis on a 1% agarose gel, stain with ethidium bromide and a suitable DNA ladder marker, and detect using an ultraviolet transilluminator.

If the viral load is sufficiently high, a 425 bp product will be amplified for MrNV and 546 bp product for XSV in the first PCR step. In the nPCR step, a 205 bp product indicates detection of MrNV and a 236 bp product indicates detection of XSV. The detection sensitivity of the RT-nPCR is ~1000-fold greater than the one-step RT-PCR.

The sequence of external primers for MrNV and XSV is given in protocol 1 and the sequence of internal primers is given below:

The sequence of internal primers for MrNV (annealing temperature 55°C; product size 205 bp):

Forward: 5'-GAT-GAC-CCC-AAC-GTT-ATC-CT-3'
Reverse: 5'-GTG-TAG-TCA-CTT-GCA-AGA-GG-3'

The sequence of internal primers for XSV (annealing temperature 55°C; product size 236 bp):

Forward: 5'-ACA-TTG-GCG-GTT-GGG-TCA-TA-3'
Reverse: 5'-GTG-CCT-GTT-GCT-GAA-ATA-CC-3'

Protocol 3: multiplex RT-PCR assay for simultaneous detection of MrNV and XSV (Yoganandhan *et al*., 2005).

To avoid the necessity of carrying out two separate RT-PCR reactions, a modified method for simultaneous detection of MrNV and XSV in a single-tube, one-step multiplex RT-PCR assay can be performed. The reaction is performed in 50 µl RT-PCR buffer containing 20 pmol of each primer specific to MrNV and XSV, and RNA template (10–100 ng), using the following cycles: RT at 52°C for 30 minutes; denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 40 seconds, annealing at 55°C for 40 seconds, and elongation at 68°C for 1 minute, ending with an additional elongation step for 10 minutes at 68°C. Analyse the RT-PCR products by electrophoresis on a 1% agarose gel, stain with ethidium bromide and a suitable DNA ladder marker, and detect using an ultraviolet transilluminator.

If MrNV and XSV are present in the sample, a 681 bp product for MrNV and 500 bp product for XSV will be amplified. The presence of both 681 bp and 500 bp products indicates the presence of MrNV and XSV. The detection sensitivity of the multiplex RT-PCR assay is approximately 25 fg of total RNA.

PCR primer sequences for MrNV (annealing temperature 55°C; product size 681 bp):

Forward: 5'-GAT-ACA-GAT-CCA-CTA-GAT-GAC-C-3'
Reverse: 5'-GAC-GAT-AGC-TCT-GAT-AAT-CC-3'

PCR primer sequences for XSV (annealing temperature 55°C; product size 500 bp):

Forward: 5'-GGA-GAA-CCA-TGA-GAT-CAC-G-3'
Reverse: 5'-CTG-CTC-ATT-ACT-GTT-CGG-AGT-C-3'

Protocol 4: Real-time RT-PCR assay

Real-time RT-PCR assay can be performed to quantify the MrNV/XSV in the infected samples using the SYBR Green dye based on the method described by Hernandez-Herrera *et al*. (2007) and Zhang *et al*. (2006).

i) Extraction of total RNA from the samples as per the procedure mentioned above.

ii) Incubate the RNA samples at 37°C for 1 hour in RT mixture (150 ng of total RNA, 8 U μl^{-1} M-MLV RT in buffer, 20 ng μl^{-1} hexaprimers and 0.2 mM dNTP) to obtain total cDNA and quantify the amount of cDNA by measuring the absorbance at 260 nm.

iii) Perform real-time RT-PCR using real-time PCR mixture (1 µl of cDNA [10 ng], 6 µl of sterile water, 0.5 µl of each primer specific to MrNV and XSV [25 µM concentration] and 2 µl of reaction mixture containing Fast Start *Taq* polymerase, dNTP mix, SYBR Green, 10 mM $MgCl_2$ and 1 µl dye solution).

iv) The PCR programme consists of initial *Taq* polymerase activation for 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, 5 seconds at 60°C and 10 seconds at 72°C. Melting temperatures will be measured by returning to 70°C for 30 seconds and gradual heating to 95°C in 10 minutes. The negative control reactions should contain water in place of cDNA template in each run to ensure the absence of viruses.

v) The number of viral cDNA copies in the sample will be determined using Light Cycler fit point method.

PCR primer sequences for MrNV (annealing temperature 60°C; product size 211 bp):

Forward: 5'-AGG-ATC-CAC-TAA-GAA-CGT-GG-3'
Reverse: 5'-CAC-GGT-CAC-AAT-CCT-TGC-G-3'

PCR primer sequences for XSV (annealing temperature 58°C; product size 68 bp):

Forward: 5'-AGC-CAC-ACT-CTC-GCA-TCT-GA-3'
Reverse: 5'-CTC-CAG-CAA-AGT-GCG-ATA-CG-3'

4.3.1.2.3.2. *In-situ* hybridisation method (*Sri Widada et al., 2003; Zsikla et al., 2004*)

i) Fix infected PL in neutral-buffered, modified Davidson's fixative without acetic acid (RNA friendly fixative) (Hasson *et al.,* 1997).

ii) Embed the tissues in paraffin according to standard procedures (Bell & Lightner, 1988) and cut into 7 µm sections. Place sections on to positively charged microscope slides.

iii) Dry the slides in an oven at 60°C. Remove paraffin and rehydrate through an ethanol series to water.

iv) Incubate the sections twice for 5 minutes with diethylpyrocarbonate (DEPC)-treated Tris/HCl (0.2 M, pH 7.4) and 10 minutes with DEPC-treated Tris/HCl containing 100 mM glycine.

v) Treat the sections for 5 minutes at 37°C with TE buffer (10 mM Tris/HCl, 5 mm EDTA, pH 8.0) containing 10 µg ml^{-1} RNAse-free proteinase K.

vi) Post-fix the sections with DEPC-treated PBS containing 4% formaldehyde for 5 minutes.

vii) The sections are acetylated for 10 minutes with 0.1 M triethanolamine (TEA) buffer, pH 8, containing 0.25% (v/v) acetic anhydride.

viii) After dehydration, incubate the slides at 42°C for 16 hours in a humid chamber with hybridisation buffer containing 40% deionised formamide, 10% dextran sulphate, 1× Denhart's solution, 4× SSC (standard saline citrate), 10 mM dithiothreitol (DTT), 1 mg ml^{-1} yeast tRNA, 1 mg ml^{-1} denatured and sheared salmon sperm DNA and 40 ng ml^{-1} denatured digoxigenin-labelled DNA probe specific to MrNV.

ix) Wash the slides at 37°C for 10 minutes with 1 × SSC, for 10 minutes with 0.5 × SSC and for 5 minutes twice with buffer III (100 mM Tris/HCl [pH 7.5], 150 mM NaCl).

x) Incubate for 20 minutes in buffer IV (buffer III, 1% normal goat serum) at room temperature.

xi) Incubate the slides for 1 hour in a humid chamber with buffer III containing 1% normal goat serum and 0.1% sheep anti-DIG alkaline phosphatase.

xii) Wash the slides successively for 10 minutes three times with buffer III and for 5 minutes twice with buffer V (100 mM Tris/HCl [pH 9.5], 100 mM NaCl, 50 mM $MgCl_2$).

xiii) Develop the reaction by incubating the slides in buffer V containing NBT and BCIP in a dark and humid chamber for a minimum of 2 hours or overnight. Stop the reaction by incubating the slides in buffer III 2× for 15 minutes.

xiv) Counterstain the slides with 1% Brown Bismarck, mount with a cover-slip and examine with a bright field microscope.

xv) Positive hybridisation appears as a dark blue to black precipitate against the yellow to brown counterstain.

4.3.1.2.3.3. Loop-mediated isothermal amplification (Haridas et al., 2010; Pillai et al., 2006; Puthawibool et al., 2010)

Haridas *et al*. (2010) and Pillai *et al.* (2006) have applied loop-mediated isothermal amplification (LAMP) for rapid diagnosis of MrNV and XSV in the freshwater prawn. A set of four primers, two outer and two inner, have been designed separately for detection of MrNV and XSV. In addition, a pair of loop primers specific to MrNV and XSV has been used to accelerate LAMP reaction.

i) Extraction of total RNA from the samples as per the procedure mentioned above.

ii) Carry out the RT-LAMP reaction in the reaction mixture (2 µM each of inner primers FIP and BIP, 0.2 µM each of outer primers F3 and B3, 1400 µM of dNTP mix, 0.6 M betaine, 6 mM $MgSO_4$, 8 U of Bst DNA polymerase along with 1× of the supplied buffer, 0.125 U of AMV RTase and the specified amount of template RNA in a final volume of 25 µl) at 55, 60, 63 and 65°C for 1 each, followed by heat inactivation at 80°C for 2 minutes to terminate the reaction. Uninfected samples and reaction mix without template serve as the negative controls.

iii) Analyse the LAMP products by electrophoresis on a 2% agarose gel, stain with ethidium bromide and a suitable DNA ladder marker, and detect using an ultraviolet transilluminator.

iv) Without use of agarose electrophoresis, amplification of DNA can be detected by addition 1.0 µl of 10^{-1} diluted SYBR Green to the reaction mixture and observe the colour change.

4.3.1.2.3.4. Sequencing

For confirmation of suspected new hosts of MrNV/XSV, the DNA fragment amplified from the PCR should be sequenced according to standard protocols (Sambrook & Russell, 2001).

4.3.1.2.4. Agent purification

MrNV and XSV can be purified according to the protocol described by Bonami *et al.* (2005). The detailed procedure for viral purification is given below:

i) Collect sufficient quantity of infected PL and homogenise in PBS buffer (pH 7.4) using a tissue blender.

ii) Centrifuge at 10,000 ***g*** for 25 minutes at 4°C. Collect supernatant and centrifuge again at 160,000 ***g*** for 4 hours at 4°C.

iii) Suspend the pellet in PBS and extract two or three times with freon (1,1,2-trichloro-2,2,1-trifluoroethane).

iv) Collect the aqueous layer and centrifuge at 160,000 ***g*** for 4 hours at 4°C.

v) Suspend the pellet in TN buffer and separate the two viruses with a 15–30% (w/v in PBS) sucrose gradient, followed by a CsCl gradient.

vi) Examine the purity of the viruses by TEM using collodion-carbon-coated grids, negatively stained with 2% PTA (phosphotungstic acid), pH 7.0.

4.3.2. Serological methods

None developed

5. Rating of tests against purpose of use

The methods currently available for targeted surveillance and diagnosis of infection with MrNV 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	c	c	d	c	d
Bioassay	d	c	d	d	c	c
Direct LM	d	c	c	d	c	c
Histopathology	d	c	c	c	b	b
Transmission EM	d	d	d	d	d	a
Antibody-based assays	d	c	d	d	b	b
In-situ DNA probes	c	b	b	c	a	a
Real-time RT-PCR, RT-PCR	a	a	a	a	a	a
Sequence	d	d	d	a	d	a

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; RT-PCR = reverse transcription polymerase chain reaction.

6. Test(s) recommended for targeted surveillance to declare freedom from infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)

The method for targeted surveillance to declare freedom from infection with MrNV is RT-nPCR.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Infection with MrNV is suspected if two or more of the following criteria are met:

i) clinical signs consistent with infection with MrNV

or

ii) histopathology consistent with infection with MrNV

or

iii) a positive result by RT-PCR.

or

iv) a positive result by real-time RT-PCR.

7.2. Definition of confirmed case

Infection with MrNV is considered to be confirmed if two or more of the following criteria are met:

i) histopathology consistent with infection with MrNV

ii) ISH positive result in target tissues.

iii) RT-PCR (followed by sequencing),

iv) Real-time RT-PCR.

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NB: There is an OIE Reference Laboratory for infection with *Macrobrachium rosenbergii* nodavirus (white tail disease) (see Table at the end of this *Aquatic Manual* or consult the OIE web site: https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the OIE Reference Laboratories for any further information on infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)

NB: FIRST ADOPTED IN 2009. MOST RECENT UPDATES ADOPTED IN 2017.