

WHITE TAIL DISEASE

1. Scope

White tail disease (WTD) or white muscle disease (WMD) is defined as a viral infection caused by *Macrobrachium rosenbergii* nodavirus (MrNV) and its associate extra small virus (XSV). They cause a milky whitish appearance in larvae/postlarvae (PL)/early juveniles, and are responsible for large-scale mortalities in the freshwater prawn *M. rosenbergii*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The aetiological agents are two viral pathogens, namely *Macrobrachium rosenbergii* nodavirus (MrNV) (primary) and extra small virus (XSV) (associate) (10, 12). MrNV is important in WTD outbreaks in prawns, although the role of XSV in pathogenicity remains unclear. Strains are not yet known. MrNV belongs in the family Nodaviridae (3, 23). XSV is the first satellite virus in animals and it is also the first record of a satellite-nodavirus association (3).

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 PL of *M. rosenbergii* by immersion challenge (10, 13).

2.1.3. Stability of the agent (effective inactivation methods)

Agent stability is not known. However, heat treatment destroyed infectivity of MrNV and XSV in challenge experiments (10).

2.1.4. Life cycle

Not known.

2.2. Host factors

WTD is responsible for huge mortalities in larvae and PL of the freshwater prawn, *M. rosenbergii*, in hatcheries with subsequent economic losses to nursery systems.

2.2.1. Susceptible host species

The giant freshwater prawn, *Macrobrachium rosenbergii* (DeMan, 1879). Other proven or suspected hosts are not yet known.

2.2.2. Susceptible stages of the host

Larvae, PL and early juveniles are susceptible, whereas adults are resistant and act as carriers (10, 13).

2.2.3. 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 (18).

2.2.4. 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 (13, 16). The presence of both viruses in ovarian tissue indicates the possibility of vertical transmission of WTD from broodstock to larvae and PL. Experiments proved that pleopods would be a convenient source of RNA for non-destructive screening of MrNV and XSV without stress to the prawns (13).

2.2.5. Persistent infection with lifelong carriers

Challenge experiments indicate long-term persistent infection in adults and also the possibility of transmitting WTD from broodstock to larvae and PL (13, 18).

2.2.6. Vectors

Penaeid shrimp (*Penaeus indicus*, *P. monodon*, *P. japonicus*) (20), *Artemia* (22), and aquatic insects (*Belostoma* sp., *Aesohna* sp., *Cybister* sp., and *Notonecta* sp.) are vectors of WTD (17).

2.2.7. Known or suspected wild aquatic animal carriers

Not known.

2.3. Disease pattern

A high prevalence of WTD infection has been reported in hatchery-reared larvae and PL of *M. rosenbergii*. WTD may be transmitted both vertically and horizontally in culture systems.

2.3.1. Transmission mechanisms

Transmission is vertical (trans-ovum) and horizontal by the waterborne route (10, 13, 18).

2.3.2. Prevalence

Prevalence is variable from 10% to 100% in hatchery, nursery and grow-out systems, as well as in experimental infection by immersion challenge, and 100% mortality has been reported 5–7 days after the appearance of the first gross signs in PL in natural or experimental infection (1, 10, 13, 14).

2.3.3. Geographical distribution

The disease was first reported in the French West Indies (1), later in China (People's Rep. of) (10), India (14), Chinese Taipei (24) and Thailand (25).

2.3.4. Mortality and morbidity

Larvae, PL and juveniles of *M. rosenbergii* are highly susceptible to WTD, 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 WTD 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 WTD, but act as carriers (10, 13).

2.3.5. Environmental factors

Not much is known about environmental factors. However, outbreaks of WTD may be induced by rapid changes in salinity, temperature and pH (1, 10).

2.4. Control and prevention

No work has been carried out on control and prevention of WTD. However, proper preventive measures, such as screening of brood stock and PL, and good management practices may help to prevent WTD 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 polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) (12, 16, 26).

2.4.1. Vaccination

Not yet available.

2.4.2. Chemotherapy

No known chemotherapeutic agents reported for WTD.

2.4.3. Immunostimulation

No reports available concerning the use of immunostimulants for WTD.

2.4.4. Resistance breeding

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 (4).

2.4.8. General husbandry practices

Experimental infection confirmed the possibility of horizontal and vertical transmission of WTD in culture systems (10, 13, 18). 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 WTD in culture systems (4, 16, 17). There is no evidence of WTD prevention by crop rotation either with rice or polyculture with fish. 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.* (20) and Ravi *et al.* (11) in their studies. Based on their results, it would seem that mixed culture of *M. rosenbergii* with *P. monodon* is to be avoided before adopting any preventive measures in the management of WTD.

3. Sampling

3.1. Selection of individual specimens

WTD of freshwater prawns is mainly diagnosed by the whitish coloration of abdominal and tail muscle (1, 12, 14). However, this clinical sign is not specific to WTD and diagnosis is not easy, particularly in the earlier stages of infection. WTD-affected PL 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 (13).

3.2. Preservation of samples for submission

Infected larvae/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 use (14, 16, 26). Frozen samples can be used for virus isolation and detection by RT-PCR or ELISA (12). Samples for virus detection by RT-PCR can be transported to the laboratory after fixing in 70% ethanol (14, 16, 26). See also Chapter 2.2.0.

3.3. Pooling of samples

Infected larvae or PL (5 to 10 in number) can be pooled for screening tests. See also Chapter 2.2.0.

3.4. Best organs or tissues

The whole PL body is preferred (14, 16, 26). All the organs, except eyestalks and the hepatopancreas, of adult *M. rosenbergii* are best for screening the viruses by RT-PCR. Pleopods (swimming legs) would be a convenient source of RNA for non-destructive screening of *MrNV* and *XSV* without stress to the broodstock (13).

3.5. Samples/tissues that are not suitable

Eyestalks and the hepatopancreas of adult prawns are not suitable (13, 16).

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 discoloration 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 WTD and mortality reaches a maximum in about 5 days after the appearance of whitish coloration. Floating exuviae (moult) in the tanks appear abnormal and resemble 'mica flakes' (1). The infected PL show progressive weakening of their feeding and swimming ability (13).

4.2. Clinical methods

4.2.1. Gross pathology

The WTD of *M. rosenbergii*, resulting from *MtNV* and XSV infection, is mainly diagnosed by the whitish coloration of abdominal muscle. However, this clinical sign is not specific to WTD, but it is associated with high mortality rates.

4.2.2. Clinical chemistry

Not available.

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 (1, 7). Pathognomonic oval or irregular basophilic cytoplasmic inclusion bodies are demonstrated in the target tissues by histology (1, 7).

The presence of *MtNV* in infected cells can be demonstrated in histological sections using a DIG-labelled DNA *in-situ* hybridisation probe specific for *MtNV* (16).

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 *MtNV*. Smaller viral particles similar in structure (five- to six-sided), but with a diameter of 14–16 nm, would be characteristic of XSV (10).

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

Genome and antibody-based diagnostic methods are available to detect *MtNV*/XSV (12, 16, 26).

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*

MtNV/XSV can be easily propagated in the C6/36 mosquito *Aedes albopictus* cell line (19) 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 (19). Other cell lines, namely the fish SSN-1 cell line, partially support the multiplication of these viruses (6).

4.3.1.2.2. *Antibody-based antigen detection methods*

Antibody-based diagnostic methods for *MtNV* include the ELISA described by Romestand & Bonami (11) or the triple-antibody sandwich (TAS) ELISA based on a monoclonal antibody (9).

4.3.1.2.2.1. *ELISA protocol (12)*

- 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-*MtNV* 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, H₂O₂ at a 0.33% final concentration).
- vii) Stop the reaction after 15 minutes by adding 25 µl of H₂SO₄ 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 (9)*

- i) Coat ELISA plates with rabbit polyclonal antibody raised against *MtNV* 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 *MtNV* 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, H₂O₂ at a 0.33% final concentration).
- viii) Stop the reaction after 15 minutes by adding 25 µl H₂SO₄ 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 *MtNV*/*XSV* developed by Sri Widada *et al.* (16) and Sahul Hameed *et al.* (13, 14) is recommended for all situations. *MtNV* 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 (26). Nested RT-PCR (nRT-PCR) is also available and recommended for screening broodstock and seed (18).

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 *MtNV* and *XSV*. The first protocol is a one-step RT-PCR adapted from Sri Widada *et al.* (16) and Sahul Hameed *et al.* (14), and this method can be used for confirmation of *MtNV* and *XSV* in PL of prawns collected from suspected WTD outbreaks. The second protocol is a sensitive nRT-PCR protocol described by Sudhakaran *et al.* (18). 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.* (26). It can be used for the simultaneous detection of *MtNV* 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 *MtNV* or *XSV* in infected prawn PL or juveniles (14, 16, 21):

The following controls should be included in every RT-PCR assay for *MtNV* or *XSV*: a) a known *MtNV*/*XSV*-negative tissue sample; b) a known *MtNV*/*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 *MtNV* 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 *MtNV* and a 546 bp product for *XSV*. The sensitivity of the assay is approximately 2.5 fg of total RNA.

PCR primer sequences for *MtNV* (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 nRT-PCR is more sensitive and useful for screening seed and broodstock (18):

For the nRT-PCR, 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 nRT-PCR, 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 MgCl₂, 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 nRT-PCR 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 nRT-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 the viral load is sufficiently high, a 425 bp DNA will be amplified for *MtNV* and 546 bp DNA for *XSV* in the first PCR step. In the nPCR step, a 205 bp product indicates detection of *MtNV* and a 236 bp product indicates detection of *XSV*. The detection sensitivity of the nRT-PCR is ~1000-fold greater than the one-step RT-PCR.

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

The sequence of internal primers for *MtNV* (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 *MtNV* and *XSV* (26).

To avoid the necessity of carrying out two separate RT-PCR reactions, a modified method for simultaneous detection of *MtNV* 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 *MtNV* 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 *MtNV* and *XSV* are present in the sample, a 681 bp DNA for *MtNV* and 500 bp DNA for *XSV* will be amplified. The presence of both 681 bp and 500 bp products indicates the presence of *MtNV* and *XSV*. The detection sensitivity of the multiplex RT-PCR assay is approximately 25 fg of total RNA.

PCR primer sequences for *MtNV* (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: quantitative RT-PCR assay

Quantitative RT-PCR (RT-qPCR) assay can be performed to quantify the *MNV*/*XSV* in the infected samples using the SYBR green dye based on the method described by Hernandez-Herrera *et al.* and Zhang *et al.* (6, 27).

- 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 RT-qPCR using q-PCR mixture (1 μl of cDNA [10 ng], 6 μl of sterile water, 0.5 μl of each primer specific to *MNV* 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 *MNV* (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 (16, 28)

- i) Fix infected PL in neutral-buffered, modified Davidson's fixative without acetic acid (RNA friendly fixative) (5).
- ii) Embed the tissues in paraffin according to standard procedures (2) 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 $\mu\text{g ml}^{-1}$ 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 \times Denhart's solution, 4 \times 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 *MNV*.
- ix) Wash the slides at 37°C for 10 minutes with 1 \times SSC, for 10 minutes with 0.5 \times 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 MgCl₂).
- 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 2x 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 (8)

Pillai *et al.* (8) have applied loop-mediated isothermal amplification (LAMP) for rapid diagnosis of *MtNV* and *XSV* in the freshwater prawn. A set of four primers, two outer and two inner, have been designed separately for detection of *MtNV* and *XSV*. In addition, a pair of loop primers specific to *MtNV* and *XSV* has been used to accelerate LAMP reaction.

- i) Extraction of total RNA from the samples as per the procedure mentioned above.
- iii) 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 MgSO₄, 8 U of Bst DNA polymerase along with 1x 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.

4.3.1.2.3.4. Sequencing

For confirmation of suspected new hosts of *MtNV/XSV*, the DNA fragment amplified from the PCR should be sequenced according to standard protocols (15).

4.3.1.2.4. Agent purification

MtNV and *XSV* can be purified according to the protocol described by Bonami *et al.* (3). The detailed procedure for viral purification is given below:

- i) Collect sufficient quantity of infected PL and homogenate 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 WTD 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
DNA probes – <i>in situ</i>	c	b	b	c	a	a
PCR	a	a	a	a	a	a
Sequence	d	d	d	a	d	a

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

6. Test(s) recommended for targeted surveillance to declare freedom from white tail disease

The method for targeted surveillance to declare freedom from WTD is nRT-PCR.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

Appearance of whitish muscle associated with mortality is a suspected case of WTD. It usually affects larval, PL and juvenile stages of *M. rosenbergii* and may appear as a cessation of feeding, reduced swimming activity and whitish coloration of the abdominal and tail muscles. Mortality reaches a maximum of up to 95% at 5 days after the appearance of the whitish coloration. Corroborative diagnostic criteria are summarised in Section 4.2 above.

7.2. Definition of confirmed case

Suspect cases should first be checked by RT-PCR and confirmed by nRT-PCR, sequencing, TEM and DNA probes.

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NB: There is an OIE Reference Laboratory for White tail disease (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).