CHAPTER 2.2.5.

WHITE SPOT DISEASE

1. Scope

For the purpose of this chapter, white spot disease (WSD) is considered to be infection with white spot syndrome virus (WSSV).

2. Disease information

2.1. Agent factors

Various WSSV isolates with small genetic polymorphisms have been identified (variants). It should be realised, however, that as the *Nimaviridae* is a newly recognised family, the species concept will be subject to change after existing and new isolates have been studied in more detail.

2.1.1. Aetiological agent, agent strains

WSSV was assigned by the International Committee on Taxonomy of Viruses (ICTV) as the only member of the genus *Whispovirus* within the *Nimaviridae* family. Virions of WSSV are ovoid or ellipsoid to bacilliform in shape, have a regular symmetry, and measure 120–150 nm in diameter and 270–290 nm in length. Most notable is the thread- or flagella-like extension (appendage) at one end of the virion. Today, although various geographical isolates with genotypic variability have been identified, they are all classified as a single species (white spot syndrome virus-1) within the genus *Whispovirus* (26).

2.1.2. Survival outside the host

The agent is viable for at least 30 days at 30°C in seawater under laboratory conditions (13); and is viable in ponds for at least 3–4 days (14).

2.1.3. Stability of the agent (effective inactivation methods)

The agent is inactivated in <120 minutes at 50°C and <1 minute at 60°C (14).

2.1.4. Life cycle

In-vitro studies with primary cell culture and *in-vivo* studies with postlarvae (PL) show that the replication cycle is approximately 20 hours at 25°C.

2.2. Host factors

WSSV has an extremely wide host range. The virus can infect a wide range of aquatic crustaceans including marine, brackish and freshwater penaeids, crabs and crayfish (10).

2.2.1. Susceptible host species

All decapod (order Decapoda) crustaceans from marine and brackish or freshwater sources are susceptible host species (6. 7, 9, 10).

2.2.2. Susceptible stages of the host

All life stages are potentially susceptible, from eggs to broodstock (8, 23).

2.2.3. Species or subpopulation predilection (probability of detection)

There is a higher probability of detecting the virus in crabs than in shrimp. The best life stages of crustaceans for detection are late PL stages, juveniles and adults. Probability of detection can be increased by exposure to stressful conditions (e.g. eye-stalk ablation, spawning, moulting, changes in salinity, temperature or pH, and during plankton blooms).

2.2.4. Target organs and infected tissue

The major targets of WSSV infection are tissues of ectodermal and mesodermal embryonic origin, especially the cuticular epithelium and subcuticular connective tissues (12, 29). Although WSSV infects

the underlying connective tissue in the shrimp hepatopancreas and midgut, the hepatopancreatic tubular epithelial cells of these two organs are of endodermal origin, and they do not become infected.

2.2.5. Persistent infection with lifelong carriers

Persistent infection occurs commonly and lifelong infection has been shown (9). Viral loads during persistent infection can be extremely low and potentially undetectable by any available diagnostic test.

2.2.6. Vectors

Vectors include rotifers (31), marine molluscs, polychaete worms (25) and non-decapodal crustaceans including *Artemia salina* (2) and the copepods, as well as non-crustacean aquatic arthropods such as sea slaters (Isopoda) and Euphydradae insect larvae. All these species can accumulate high concentrations of viable WSSV, although there is no evidence of virus replication (9).

2.2.7. Known or suspected wild aquatic animal carriers

No suspected wild aquatic animal carriers have been reported.

2.3. Disease pattern

Infection sometimes causes disease and sometimes not (21), depending on factors as yet poorly understood but related to species tolerance and environmental triggers. With an appropriate infection dose to allow sufficient time before mortality, animals susceptible to disease show large numbers of virions circulating in the haemolymph (8), but this may also occur for tolerant species that show no mortality. Thus, high viral loads *per se* do not cause disease or mortality for all susceptible species.

2.3.1. Transmission mechanisms

The infection can be transmitted vertically (trans-ovum), horizontally by consumption of infected tissue (e.g. cannibalism, predation, etc.), and by water-borne routes. Transmission of infection can occur from apparently healthy animals in the absence of disease. Dead and moribund animals can be a source of disease transmission (9).

2.3.2. Prevalence

Prevalence is highly variable, from <1% in infected wild populations to up to 100% in captive populations (9).

2.3.3. Geographical distribution

WSD has been identified from crustaceans in China (People's Rep. of), Japan, Korea (Rep. of), South-East Asia, South Asia, the Indian Continent, the Mediterranean, the Middle East, and the Americas. WSD-free zones and compartments are known within these regions (26).

2.3.4. Mortality and morbidity

All penaeid shrimp species are highly susceptible to infection, often resulting in high mortality. Crabs, crayfish, freshwater prawns, spiny lobsters and clawed lobsters are susceptible to infection, but morbidity and mortality as a consequence of infection is highly variable (9). High level infections are known in some decapods in the absence of clinical disease.

2.3.5. Environmental factors

Disease outbreaks may be induced by stressors, such as rapid changes in salinity. Water temperature has a profound effect on disease expression, with average water temperatures of below ~30°C being conducive to WSD outbreaks (24).

2.4. Control and prevention

Although the underlying mechanism remains unknown, laboratory experiments have shown that 'vaccinated' shrimp and crayfish have better survival rates after WSSV challenge. It was first shown that *Penaeus japonicus* shrimp that survived natural and experimental WSSV infections displayed resistance to subsequent challenge with WSSV (22). Later studies showed that intramuscular injection of inactivated WSSV virions or recombinant structural protein, (VP28), provided shrimp with some protection against experimental WSSV infection. Furthermore, shrimp fed with food pellets coated with inactivated bacteria over expressing VP28 showed better survival rates after WSSV challenge (28). However, although these results seemed promising, the protection was effective only when the shrimp were infected with a low dosage of WSSV. Also, the effect usually lasted for

only a few days, or in the case of crayfish, for about 20 days. Another potential means of protecting shrimp against WSSV infection is to use RNA interference (RNAi). WSSV gene-specific double-stranded (ds) RNAs produced strong anti-WSSV activity, protecting the shrimp against WSSV infection, but the same study showed that long dsRNA induced both sequence-dependent and independent anti-viral responses in shrimp (18). A more recent study even showed that oral administration of bacterially expressed VP28 dsRNA could protect shrimp against WSSV infection (19). To date, however, there are still no field trial data for either the vaccination or the RNAi approach.

2.4.1. Vaccination

No consistently effective vaccination methods have been developed.

2.4.2. Chemotherapy

No scientifically confirmed reports.

2.4.3. Immunostimulation

Several reports have shown that beta-glucan, vitamin C, seaweed extracts (fucoidan) and other immunostimulants may improve resistance to WSD (1, 3).

2.4.4. Resistance breeding

No significant improvements have been reported.

2.4.5. Restocking with resistant species

Not applicable for WSD.

2.4.6. Blocking agents

There are no efficient blocking agents that can be recommended at this time. rVP28 has an effect, but it cannot yet be used as a practical blocking agent.

2.4.7. Disinfection of eggs and larvae

For transovum transmission, disinfection of egg is likely to be effective (9), but this has not yet been confirmed in formal scientific trials.

2.4.8. General husbandry practices

A number of husbandry practices have been used successfully to manage WSD, such as avoiding stocking in the cold season, use of specific pathogen free (SPF) or polymerase chain reaction (PCR)-negative seed stocks, and use of biosecure water and culture systems (27).

3. Sampling

3.1. Selection of individual specimens

Samples of moribund shrimp or shrimp that show clinical signs (see Section 4.1.1) or exhibit behavioural changes (Section 4.1.2) should be selected for WSSV detection.

3.2. Preservation of samples for submission

See Chapter 2.2.0 for guidance on preservation of samples for the intended test method.

3.3. Pooling of samples

Samples taken for molecular or antibody-based test methods for WSD may be combined as pooled samples of no more than five specimens per pooled sample of juveniles or subadults. However, for eggs, larvae and PL, pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50 to 150 PL depending on their size/age) may be necessary to obtain sufficient sample material. See also Chapter 2.2.0.

3.4. Best organs or tissues

Tissue tropism analysis from both experimentally infected shrimp and wild-captured brooders shows that tissues originating from the ectoderm and mesoderm, especially the cuticular epithelium and subcuticular

connective tissues, as well as other target tissues (e.g. antennal gland, haematopoietic organ, etc.), are the main target tissues for WSSV. Samples of or from the pleopods, gills, haemolymph, stomach or abdominal muscle are recommended for submission (8).

For non-destructive screening by PCR, it is recommended to submit (a small piece of) gill, (a small aliquot of) haemolymph or (a small piece of) pleopod. There is also some evidence to suggest that an ablated eyestalk would be a good alternative, provided that the compound eye is removed prior to submission.

NOTE: It is important to adhere to the sampling procedure described by Lo *et al.* (8) when excising pleopods for non-destructive screening.

3.5. Samples/tissues that are not suitable

Although WSSV infects the underlying connective tissue in the shrimp hepatopancreas and midgut, the columnar epithelial cells of these two organs are of endodermal embryonic origin (8), and they are not appropriate tissues for detection. The compound eye may contain a PCR inhibitor (8) and it is therefore not suitable for PCR-based diagnosis.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

White spots embedded within the exoskeleton are the most commonly observed clinical sign. In most shrimp, these spots range from barely visible to 3 mm in diameter, and they sometimes coalesce into larger plates. However, it should be noted that environmental stress factors, such as high alkalinity, or bacterial disease can also cause white spots on the carapace of shrimp, and that moribund shrimp with WSD may in fact have few, if any, white spots. Therefore, the appearance of white spots is absolutely not a good diagnostic sign of WSSV infection. Furthermore, other crustaceans, such as most crayfish, are often reported to show no sign of white spots when infected with WSSV.

High degrees of colour variation with a predominance of reddish or pinkish discoloured shrimp are seen in diseased populations.

4.1.2. Behavioural changes

The presence of white spots does not always mean that the condition is terminal. For instance, under non-stressful conditions, infected shrimp that have white spots may survive indefinitely. However, if the shrimp also appear lethargic, if their colour changes to pink or reddish-brown, if they gather around the edges of ponds/tanks at the water surface, or if there is a rapid reduction in food consumption, then a very high mortality rate in the shrimp population can be expected within a few hours to a few days of the onset of these signs.

4.2. Clinical methods

4.2.1. Gross pathology

See Section 4.1.1 and 4.1.2. above.

4.2.2. Clinical chemistry

Haemolymph withdrawn from WSSV-infected shrimp always has a delayed (or sometimes completely absent) clotting reaction.

4.2.3. Microscopic pathology

4.2.3.1. Wet mounts

Demonstration of hypertrophied nuclei in squash preparations of the gills and/or cuticular epithelium, which may be stained or unstained.

4.2.3.2. Smears

Demonstration of WSSV aggregates in unstained smear preparations of haemolymph by dark-field microscopy.

NOTE: This is the simplest of the microscopic techniques and is recommended for people with limited expertise in WSSV. The aggregates appear as small reflective spots of 0.5 µm in diameter (11).

4.2.3.3. Fixed sections

Histological demonstration of pathognomonic inclusion bodies in target tissues.

4.2.3.4. *In situ* hybridisation

Use of WSSV-specific DNA probes with histological sections to demonstrate the presence of WSSV nuclei acid in infected cells.

4.2.3.5. Immunohistochemistry

Use of WSSV-specific antibodies with histological sections or wet mounts to demonstrate the presence of WSSV antigen in infected cells.

4.2.4. Electron microscopy/cytopathology

Demonstration of the virus in tissue sections or in semi-purified negatively stained virus preparations (e.g. from haemolymph). See Section 2.1.1 for virion morphology.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

Not reported.

4.3.1.1. Microscopic methods

See Section 4.2.3 above.

4.3.1.1.1. Wet mounts

See Section 4.2.4 above.

4.3.1.1.2. Smears

See Section 4.2.5 above.

4.3.1.1.3. Fixed sections

See Section 4.2.3 above.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Bioassay method

If SPF shrimp are available, the following bioassay method is based on Nunan *et al.* (16) and Durand *et al.* (5), is suitable for WSSV diagnosis.

i) For bioassay, remove the pleopods from shrimp suspected of WSSV infection and homogenise in TN buffer (0.02 M Tris/HCl, 0.4 M NaCl, pH 7.4).

ii) Following centrifugation at 1000 *g* for 10 minutes, dilute the supernatant fluid 1/10 with 2% NaCl and filter (0.2 µm filter).

iii) Inject 0.2 ml of inoculum into the dorso-lateral aspect of the fourth abdominal segment of indicator shrimp (e.g. SPF *Penaeus vannamei* at the juvenile stage), injecting between the tergal plates into the muscle of the third abdominal segment.

iv) Examine moribund shrimp grossly or by using the methods described above. If at 3–5 days after inoculation there are still no moribund shrimp and all test results are negative, then it is safe to conclude that the bioassay results are negative.

4.3.1.2.2. Cell culture/artificial media

WSSV can be isolated from primary cultures of lymphoid or ovary cells, However, it is NOT recommended to use cell culture as a routine isolation method because of: 1) the high risk of contamination, and, 2) the composition of the medium varies depending on the tissue type, host species and experimental purpose; that is, to date there is no standard or recognised medium that can

be recommended. As primary cell culture is so difficult to initiate and maintain for virus isolation purposes, bioassay should be the primary means for virus propagation

4.3.1.2.3. Antibody-based antigen detection methods

Both polyclonal and monoclonal antibodies raised against either the virus or a recombinant viral structural protein have been used in various immunological assays including western blot analysis, immunodot assay, indirect fluorescent antibody test (IFAT), immunohistochemistry (IHC) or enzyme-linked immunosorbent assay (ELISA) to detect WSSV (17, 20, 32). Antibody-based methods can be fast, convenient and applicable to field use, but as they have only about the same sensitivity as 1-step PCR, they are recommended only to confirm acute WSD.

4.3.1.2.4. Molecular techniques

4.3.1.2.4.1 Polymerase chain reaction (PCR)

The protocol described here is from Lo *et al.* (8), and is recommended for all situations where WSSV diagnosis is required. A positive result in the first step of this standard protocol implies a serious WSSV infection, whereas, when a positive result is obtained in the second amplification step only, a latent or carrier-state infection is indicated. Alternative assays have also been described, but are not recommended unless they have first been compared with the protocol described here.

PCR commercial kits are available for WSSV diagnosis and are acceptable provided they have been validated as fit for such purpose. Please consult the OIE Register for kits that have been certified by the OIE (http://www.oie.int/vcda/eng/en_vcda_registre.htm).

DNA extraction

i) Collect 100–200 mg shrimp tissue (pleopod of live juvenile to subadult shrimp, postlarvae 11 upwards [PL11 up] with removed heads, or whole PL10, or use 100 µl haemolymph) in a 1.5 ml microfuge tube with 600 µl lysis solution (100 mM NaCl, 10 mM Tris/HCl, pH 8, 25 mM EDTA [ethylene diamine tetra-acetic acid], 0.5% SLS [sodium N-laurylsarcosinate] or 2% SDS [sodium dodecyl sulphate], and 0.5 mg ml^{-1} proteinase K added just before use).

ii) Using a disposable stick, homogenise the tissue in the tube thoroughly.

iii) After homogenisation, incubate at 65°C for 1 hour.

iv) Add 5 M NaCl to a final concentration of 0.7 M. Next, slowly add 1/10 volume of N-cetyl N,N,Ntrimethylammonium bromide (CTAB)/NaCl solution (10% CTAB in 0.7 M NaCl) and mix thoroughly.

   NOTE: In addition to the CTAB extraction method described here, commercial extraction kits are often used as part of normal surveillance activities.

v) Incubate at 65°C for 10 minutes, and then, at room temperature, add an equal volume of chloroform/isoamyl alcohol (24/1) and mix gently. Centrifuge at 13,000 *g* for 5 minutes and then transfer the aqueous solution (upper layer) to a fresh 1.5 ml tube and add an equal volume of phenol.

vi) Mix gently and centrifuge at 13,000 *g* for 5 minutes. Collect the upper layer solution and repeat the phenol extraction process once or twice.

vii) Transfer the final upper layer to a new tube, mix gently with two volumes of chloroform/isoamyl alcohol (24/1) and centrifuge at 13,000 *g* for 5 minutes.

viii) Transfer the upper layer to a new tube and precipitate DNA by adding two volumes of 95% or absolute ethanol followed by standing at –20°C for 30 minutes or –80° C for 15 minutes.

ix) Centrifuge at 13,000 *g* for 30 minutes and discard the ethanol. Wash the DNA pellet with 70% ethanol, dry and resuspend in 100 µl sterilised double-distilled water at 65°C for 15 minutes.

x) Use 1 µl of this DNA solution for one PCR reaction.

   Note: the following nested PCR procedures are well established and provide reliable diagnostic results under the specified conditions. Care should be taken, however, to ensure that DNA samples are prepared from the recommended organs, and that the PCR temperature is accurately applied (particularly for annealing, the recommended temperature is 62°C). To prevent the possibility of false positive results, it is important to adhere to the specified procedures, especially when they are used to test new candidate hosts such as *Cherax quadricarinatus* (4), as well as *Procambarus clarkii* (red swamp crayfish) and *Procambarus zonangulus* (Southern white river crayfish). For diagnosed incidences of WSSV in a new host or in a previously free zone, DNA sequencing should be used to confirm the positive results.

First-step PCR reaction

i)   Add 1 µl DNA template solution (containing about 0.1–0.3 µg DNA) to a PCR tube containing 100 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, 100 pmol of each primer, 2 units of heat-stable DNA polymerase).

ii)   The outer primer sequences are 146F1, 5'-ACT-ACT-AAC-TTC-AGC-CTA-TCTAG-3' and 146R1, 5'-TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A-3'.

iii)   The PCR profile is one cycle of 94°C for 4 minutes, 55°C for 1 minute, and 72°C for 2 minutes, followed by 39 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes and a final 5-minute extension at 72°C. The WSSV-specific amplicon from this reaction is 1447 bp. The sensitivity is approximately 20,000 copies of a plasmid template.

Second step of the (nested) PCR reaction

This second step is necessary for the detection of WSSV in shrimp at the carrier stage.

i)   Add 10 µl of the first-step PCR reaction product to 90 µl of a PCR cocktail with the same composition as above except that it contains the second (inner) primer pair: 146F2 (5'-GTA-ACT-GCC-CCT-TCC-ATC-TCC-A-3') and 146R2 (5'-TAC-GGC-AGC-TGC-TGC-ACC-TTG-T-3').

ii)   Use the same PCR amplification protocol as above. The WSSV-specific amplicon from this reaction is 941 bp. The overall sensitivity of both steps is approximately 20 copies of a WSSV plasmid template.

iii)   To visualise, electrophorese 10 µl PCR reaction products on 1% agarose gels containing ethidium bromide at a concentration of 0.5 µg ml^{-1}.

iv)   Decapod-specific primers (143F 5'-TGC-CTT-ATC-AGCTNT-CGA-TTG-TAG-3' and 145R 5'-TTC-AGN-TTT-GCA-ACC-ATA-CTT-CCC-3' yielding an 848 bp amplicon; N represents G, A, T, or C) should be used in control reactions to verify the quality of the extracted DNA and the integrity of the PCR reaction. In the penaeid shrimp *P. aztecus,* the PCR product generated by this decapod-specific primer pair corresponds to nucleotide sequence 352–1200 of the 18s rRNA (42). The decapod 18s RNA sequence is highly conserved and produces a similar sized PCR product in almost all decapods. A positive control (WSSV DNA template) and negative controls (no template and shrimp DNA template) should be included in every assay.

4.3.1.2.4.2 DNA sequencing of PCR products

For confirmation of suspected new hosts of WSSV, the DNA fragment amplified from the two-step nested diagnostic PCR should be sequenced. The cloning and sequencing protocols described here are according to Claydon *et al.* (4).

Note: to save time and money, it is acceptable to sequence the PCR amplicon directly. If a positive result is obtained, then go to step iv below. In the event that only band[s] of unexpected size are obtained, then the sample should be tested again using the cloning and sequencing procedures described below.

i)   Excise the DNA fragments selected for further analysis from the agarose gels and purify them using any of the commercially available PCR clean up kits.

ii)   Ligate amplicons into vector plasmid and clone the construct.

iii)   Use suitable primers to amplify the inserted amplican, and then subject the amplified product to DNA sequencing.

iv)   Compare the sequences obtained with available databases using the Basic Local Alignment Search Tool (BLAST) to determine approximate phylogenetic affiliations.

4.3.1.2.4.3 In-situ *hybridisation (ISH) method*

The protocol described here is based on that developed by Nunan & Lightner (15)

i)   Fix moribund shrimp with Davidson's AFA fixative for 24–48 hours.

ii)   Embed the tissues in paraffin and cut into 5 µm sections. Place sections on to positively charged microscope slides.

iii)   Heat the slide on a hot plate at 65°C for 30 minutes.

iv) Deparaffinise, rehydrate and then treat for 2–30 minutes (depending on tissue type) with 100 µg ml^{-1} proteinase K in Tris/NaCl/EDTA (TNE) buffer at 37°C.

v) Post-fix the slides by chilling in pre-cooled 0.4% formaldehyde for 5 minutes at 4°C and wash the slides in 2 × standard saline citrate (SSC; 1 × SSC = 150 mM NaCl, 15 mM tri-sodium citrate, pH 7.0) at room temperature.

vi) Pre-hybridise the slides with pre-hybridisation solution (50% formamide, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 5 × SSC, 1 mM EDTA, 50 mM Tris/HCl, pH 8) for 30 minutes at 42°C.

vii) Follow with hybridisation with the 1447 bp WSSV-specific PCR amplicon (see Section 4.3.1.2.3.1 "First-step PCR reaction" above) that has been labelled with digoxigenin. It is recommended that the probe be labelled by incorporating DIG-dNTP by the PCR method. Optimum concentration should be determined by testing and adjusting until a high specific signal is obtained against a low background.

viii) For hybridisation, boil the probe for 10 minutes and immediately place on ice. Dilute the probe to 30–50 ng ml^{-1} in pre-hybridisation solution and apply 500 µl to each slide.

ix) Put the slide on a hotplate at 85–95°C for 6–10 minutes (make sure that it does not reach boiling point), quench slides on ice for 5 minutes and then transfer to a humid chamber for 16–20 hours at 42°C.

x) After hybridisation, wash the slides twice for 15 minutes each time with 2 × SSC at room temperature, twice for 5 minutes with 1 × SSC at 37°C, and twice for 5 minutes with 0.5 × SSC at 37°C.

xi) For hybridisation detection, wash slides with maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 5 minutes at room temperature.

xii) Block the slides with blocking solution (2% normal goat serum and 0.3% Triton X-100 in maleic acid buffer) for 30 minutes at 37°C.

xiii) Add 250 µl anti-DIG alkaline phosphatase (AP)-conjugated antibody solution (1 µl ml^{-1} anti-DIG/AP-Fab fragment in maleic acid buffer containing 1% normal goat serum and 0.3% Triton X-100) to each slide, and incubate at 37°C for 30 minutes.

xiv) Wash the slides twice with maleic acid buffer for 10 minutes each and once with detection buffer (100 mM Tris/HCl, 100 mM NaCl, pH 9.5) at room temperature.

xv) Add 500 µl development solution (prepare immediately before use by adding 45 µl NBT salt solution [75 mg ml^{-1} in 70% dimethyformamide], 35 µl 5-bromo-4-chloro-3-indoyl phosphate, toluidinum salt [X-phosphate] solution [50 mg ml^{-1} in dimethylformamide] and 1 ml 10% PVA to 9 ml of detection buffer) to each slide and incubate in the dark in a humid chamber for 1–3 hours.

xvi) Stop the reaction by washing the slides in TE buffer (10 mM Tri/HCl, 1 mM EDTA, pH 8.0) for 15 minutes at room temperature. Wash the slides in distilled water for ten dips, counterstain the slides in 0.5% aqueous Bismarck Brown Y for approximately 5 minutes and then rinse with water. Wet mount using aqueous mounting media for observation immediately or dehydrate the slides and mount with mounting media for long-term preservation.

xvii) Mount the slides with cover-slips and examine with a bright field microscope. Positive hybridisation appears as a dark blue to black precipitate against the yellow to brown counterstain.

4.3.1.2.5. Agent purification

The WSSV virion can be purified on ice as described previously with slight modifications (30). Briefly, collect five or six moribund crayfish or shrimp (20–25 g each) at 3 days to 1 week post-infection. Homogenise all tissues excluding the hepatopancreas for 2 minutes using a mechanical homogeniser in 1200 ml TNE buffer (50 mM Tris/HCl, 400 mM NaCl, 5 mM EDTA, pH 8.5) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 1 mM Na$_2$S$_2$O$_5$). Centrifuge at 3500 **g** for 5 minutes. Save the supernatant and rehomogenise the pellet in 1,200 ml TNE buffer. Filter the pooled supernatant through a nylon net (400 mesh) and centrifuge at 30,000 **g** for 30 minutes. Discard the supernatant and carefully rinse out the upper loose layer (pink) of the pellet using a Pasteur pipette. Resuspend the lower compact layer (grey) in 10 ml TM buffer (50 mM Tris/HCl, 10 mM MgCl$_2$, pH 7.5). Pool the crude virus suspension and centrifuge at 3000 **g** for 5 minutes. Centrifuge the supernatant again at 30,000 **g** for 20 minutes. Remove the supernatant and pink loose layer and resuspend the white pellet in 1.2 ml TM buffer containing 0.1% NaN3. Transfer to a 1.5-ml Eppendorf tube. Centrifuge the suspension three to five times at 650 **g** for 5 minutes each time to remove pink impurities. Finally, store the milk-like pure virus suspension at 4°C until use.

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 WSSV 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	d	d	c	b
Direct LM	d	d	c	c	c	c
Histopathology	d	c	c	c	a	a
Transmission EM	d	d	d	d	d	a
Antibody-based assays	d	d	c	c	a	b
DNA probes – *in situ*	d	d	c	c	a	a
PCR	d	b	a	a	a	a
Sequence	d	d	d	d	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 spot disease

Two-step PCR and sequencing are the recommended methods for declaring freedom, only for juveniles and adults and possibly PLs. Two-step PCR negative results are required. Where a two-step PCR positive result cannot be confirmed by sequencing, this also counts as a negative result.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

For juvenile and adult shrimp: gross signs of WSD (See Sections 4.1.1 and 4.1.2. above).

For shrimp at any life stage (*larva to adult*): mortality.

For shrimp and crab at any life stage (*larva to adult*): hypertrophied nuclei in squash preparations of gill and/or cuticular epithelium; unusual aggregates in haemolymph by dark-field microscopy; inclusion bodies in histological sections in target tissues.

7.2. Definition of confirmed case

Suspect cases should first be checked by PCR. If in a previously WSSV-free country/zone/compartment, PCR results are positive, they should be confirmed by sequencing. Histopathology, probes and electron microscopy also can be used to confirm the case.

8. References

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NB: There is an OIE Reference Laboratory for White spot 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)