CHAPTER 2.2.8.

INFECTION WITH WHITE SPOT SYNDROME VIRUS

1. Scope

Infection with white spot syndrome virus means infection with the pathogenic agent white spot syndrome virus (WSSV), Genus *Whispovirus*, Family *Nimaviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

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 80–120 nm in diameter and 250–380 nm in length. A flagella-like extension (appendage) may be observed at one end of the virion. WSSV has been divided into three groups: isolates originating from Thailand (WSSV-TH-96-II), isolates originating from India (WSSV-IN-07-I), and another Indian isolate (WSSV-IN-06-I). Most strains of WSSV were speculated to have originated from the Indian Ocean and then spread across the world (Zeng, 2021). Today, although various geographical isolates with genotypic variability have been identified, they are all classified as a single species (WSSV) within the genus *Whispovirus* (Lo *et al*., 2012; Wang *et al*., 2019).

2.1.2. Survival and stability in processed or stored samples

Viable WSSV was found in frozen commodity shrimp imported to Australia from Southeast Asia (McColl *et al*., 2004). The virulence of WSSV was retained for 14 months at –80°C in a filtered tissue homogenate prepared from moribund shrimp with hepatopancreas and abdomen removed (Momoyama *et al*., 1998). The virus originally collected from the haemolymph of moribund shrimp could maintain its virulence for at least 16 months at –80°C (Wu *et al*., 2002). However, WSSV might be inactivated by multiple freeze–thaw cycles due to damage the viral envelopes or nucleocapsids (Durand *et al*., 2000; Hasson *et al*., 2006).

2.1.3. Survival and stability outside the host

The agent is viable for at least 30 days at 30°C in seawater under laboratory conditions (Momoyama *et al*., 1998); and is viable in ponds for at least 3–4 days (Nakano *et al*., 1998). Laboratory emulations of drainable and non-drainable ponds suggest that the virus is no longer infective after 21 days of sun-drying or after 40 days in waterlogged pond sediment (Satheesh Kumar *et al*., 2013).

WSSV with an initial viral load of 1000 virions ml^{-1} was found to be viable for a period of 12 days in seawater of 27 ppt salinity, pH of 7.5 at 29–33°C. In shrimp pond sediment (with initial viral load of 211,500 copies g^{-1}), the virus was viable and infective up to 19 days despite sun-drying. In the case of non-drainable conditions, WSSV (753,600 copies g^{-1}) remained infective for a period of 35 days (Satheesh Kumar *et al*., 2013).

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

Of all the species that have been tested to date, no decapod (order Decapoda) crustacean from marine, brackish or freshwater sources has been reported to be refractory to infection with WSSV (Flegel, 1997; Lightner, 1996; Lo & Kou, 1998; Maeda *et al*., 2000; Stentiford *et al*., 2009).

[**Note:** an assessment of species that meet the criteria for listing as susceptible to infection with WSSV in accordance with Chapter 1.5. has not yet been completed]

2.2.2. Species with incomplete evidence for susceptibility

All life stages are potentially susceptible, from eggs to broodstock (Lightner, 1996; Venegas *et al*., 1999). WSSV genetic material has been detected in reproductive organs (Lo *et al*., 1997), but susceptibility of the gametes to WSSV infection has not been determined definitively.

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

The best life stages of crustaceans for detection of WSSV are late postlarvae (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. Distribution of the pathogen in the host

The major target tissues of WSSV are of ectodermal and mesodermal embryonic origin, especially the cuticular epithelium and subcuticular connective tissues (Momoyama *et al*., 1994; Wongteerasupaya *et al*., 1995). Although WSSV infects the underlying connective tissue in the crustacean hepatopancreas and midgut, the tubular epithelial cells of these two organs are of endodermal origin, and they do not become infected.

2.2.5. Aquatic animal reservoirs of infection

Wild decapods known to be reservoirs of infection with WSSV include *Mysis* sp. (Huang *et al*., 1995), *Acetes* sp., *Alpheus* sp., *Callianassa* sp., *Exopalaemon* sp., *Helice* sp., *Hemigrapsus* sp. *Macrophthalmus* sp., *Macrophthel* sp., *Metaplax* sp., *Orithyia* sp., *Palaemonoidea* sp., *Scylla* sp., *Sesarma* sp., and *Stomatopoda* sp. (Desrina *et al*., 2022; He & Zhou, 1996; Lei *et al*., 2002). These species can express the disease under suitable environmental conditions. However, non-decapodal crustaceans, such as copepods (Huang *et al*., 1995), rotifers (Yan *et al*., 2004), *Balanus* sp. (Lei *et al*., 2002), *Artemia* (Li *et al*., 2004; Zhang *et al*., 2010) and *Tachypleidue* sp. (He & Zhou, 1996) may be apparently healthy carrier animals. Other marine molluscs, polychaete worms (Vijayan *et al*., 2005), as well as non-crustacean aquatic arthropods such as sea slaters (*Isopoda*), and *Euphydradae* insect larvae can mechanically carry the virus without evidence of infection (Lo & Kou, 1998).

2.2.6. Vectors

The harpacticoid copepod *Nitocra* sp. (Zhang *et al*., 2008), microalgae (Liu *et al*., 2007), and the polychaete, *Dendronereis* spp. (Peters) (Desrina *et al*., 2013; Haryadi *et al*., 2015) are vectors for WSSV.

2.3. Disease pattern

Infection with WSSV sometimes causes clinical disease (Tsai *et al*., 1999), depending on factors that are 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 (Lo *et al*., 1997), 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. Mortality, morbidity and prevalence

All penaeid shrimp species are highly susceptible to infection with WSSV, often resulting in high mortality. Crabs, crayfish, freshwater prawns, spiny lobsters and clawed lobsters are susceptible to infection with WSSV, but morbidity and mortality as a consequence of infection are highly variable (Lo & Kou, 1998). High level infections with WSSV are known in some decapods in the absence of clinical disease.

Prevalence of infection with WSSV is highly variable, from <1% in infected wild populations to up to 100% in captive populations (Lo & Kou, 1998).

2.3.2. Clinical signs, including behavioural changes

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 infection with WSSV may have few, if any, white spots. Therefore, the appearance of white spots is not a reliable diagnostic sign of infection with WSSV infection. High degrees of colour variation with a predominance of reddish or pinkish discoloured shrimp are seen in diseased populations.

WSSV infections can be subclinical or manifest as clinical disease. The penaeid shrimp in aquaculture will generally show clinical signs associated with high morbidity and mortality. Some animals may die without showing any clinical signs. Non-penaeid species (e.g. crab, lobster) generally have subclinical infections under natural conditions.

The affected animals can show lethargy, decreased or absent feed consumption and abnormal swimming behaviour – slow swimming, swimming on side, swimming near water surface and gathering around edges of rearing units (Corbel *et al*., 2001; Sahul Hameed *et al*., 1998; 2001). A very high mortality rate in the shrimp population can be expected within a few days of the onset of behavioural signs.

2.3.3 Gross pathology

In addition to the clinical and behavioural signs in Section 2.3.2. above, the following gross pathology has been reported in clinically affected penaeid shrimp: loosened attachment of the carapace with underlying cuticular epithelium (Sanchez-Paz, 2010), so that the carapace can be easily removed (Zhan *et al*., 1998); empty gastro-intestinal tract due to anorexia (Escobedo-Bonilla, 2008); delayed clotting of haemolymph (Heidarieh *et al*., 2013); excessive fouling of gills (Wu *et al*., 2013) and exoskeleton.

2.3.4. Modes of transmission and life cycle

Infection with WSSV can be transmitted horizontally by consumption of infected tissue (e.g. cannibalism, predation, fomites, etc.), by water-borne routes, and by other routes of transmission (e.g. via sea birds, anthropogenic movements, feeding, rotifer, copepods, etc) (Haryadi *et al*., 2015; Vanpatten *et al*., 2004; Zhang *et al*., 2006; 2008). Transmission of WSSV can occur from apparently healthy animals in the absence of disease. Dead and moribund animals can be a source of disease transmission (Lo & Kou, 1998). Microalgae could serve as a potential horizontal transmission pathway for WSSV (Liu *et al*., 2007).

True vertical transmission (intra-ovum) of WSSV to the progeny has not been demonstrated.

In-vitro studies with primary cell cultures and *in-vivo* studies with PL show that the replication cycle is approximately 20 hours at 25°C (Chang *et al*., 1996; Chen *et al*., 2011; Wang *et al*., 2000).

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 between 18 and 30°C being conducive to WSSV outbreaks (Song *et al*., 1996; Vidal *et al*., 2001). Under experimental challenge condition, WSSV-induced mortality in shrimp is reduced when the temperature increases above 32°C (Vidal *et al*., 2001).

2.3.6. Geographical distribution

Infection with WSSV has been identified from crustaceans in Asia, the Mediterranean (Stentiford & Lightner, 2011), the Middle East, Oceania (Moody *et al*., 2022) and the Americas. Zones and compartments free from infection with WSSV are known within these regions (Lo *et al*., 2012).

See WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

No consistently effective vaccination methods have been developed for infection with WSSV.

2.4.2. Chemotherapy including blocking agents

No published or validated methods.

2.4.3. Immunostimulation

Several reports have shown that beta-glucan, vitamin C, seaweed extracts (fucoidan) and other immunostimulants may improve resistance to infection with WSSV (Chang *et al*., 2003; Chotigeat *et al*., 2004).

2.4.4. Breeding resistant strains

Progress in breeding *P. vannamei* for resistance to infections with WSSV has been reported (Cuellar-Anjel *et al*., 2012; Huang *et al*., 2012).

2.4.5. Inactivation methods

Method	Treatment	Reference
Heat	55°C/90 min 70°C/5 min	Chang *et al*., 1998
	50°C/60 min 60°C/1 min 70°C/0.2 min	Nakano *et al.*, 1998
pH	pH 3/60 min pH 12/10 min	Chang *et al*., 1998; Balasubramanian *et al.,* 2006
UV	9.30×10^5 µWs/cm^2	Chang *et al*., 1998
Ozone	0.5 µg ml^{-1}/10 min	Chang *et al*., 1998
Chlorine	100 ppm/10 min	Chang *et al*., 1998; Balasubramanian *et al.,* 2006
Iodophore	100 ppm/10 min	Chang *et al*., 1998

2.4.6. Disinfection of eggs and larvae

For transovum transmission, disinfection of egg is likely to be effective (Lo & Kou, 1998), but this has not yet been confirmed in formal scientific trials.

2.4.7. General husbandry

Management practices in endemic areas principally involve the exclusion of WSSV from production populations or avoiding risk factors for development of clinical disease. Examples include avoiding stocking in the cold season, use of specific pathogen-free (SPF) or polymerase chain reaction (PCR)-negative seed stocks, use of biosecure water and culture systems (Withyachumnarnkul, 1999). Polyculture of shrimp and fish has been proposed to reduce WSSV transmission in infected populations (Wang *et al*., 2021).

3. Specimen selection, sample collection, transportation and handling

This section draws on information in Sections 2.2, 2.3 and 2.4 to identify populations, individuals and samples that are most likely to be infected.

3.1. Selection of populations and individual specimens

Samples of moribund shrimp or shrimp that show clinical signs or exhibit behavioural changes (Sections 2.3) should be selected for detection of WSSV.

3.2. Selection of 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 infection with WSSV. Samples from the pleopods, gills, haemolymph, stomach or abdominal muscle are recommended for submission (Lo *et al*., 1997).

3.3. Samples or tissues not suitable for pathogen detection

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 (Lo *et al*., 1997) and are not appropriate tissues for detection. The compound eye may contain a PCR inhibitor (Lo *et al*., 1997) and is therefore not suitable for PCR-based diagnosis.

3.4. Non-lethal sampling

Gill, haemolymph or pleopod are suitable tissues for non-lethal sampling and screening by PCR.

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.2.0 *General information* (diseases of crustaceans).

3.5.1. Samples for pathogen isolation

The results of bioassay depend strongly on the quality of samples (time since collection and time in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. To avoid degradation of samples, use alternative storage methods only after consultation with the receiving laboratory.

3.5.2. Preservation of samples for molecular detection

Tissue samples for PCR testing should be preserved in 90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.

Standard sample collection, preservation and processing methods for histological techniques can be found in Section B.5.5. of Chapter 2.2.0 *General information* (diseases of crustaceans).

3.5.3. Samples for histopathology, immunohistochemistry or in-situ hybridisation

Standard sample collection, preservation and processing methods for histological techniques can be found in Chapter 2.2.0 *General information* (diseases of crustaceans).

3.5.4. Samples for other tests

Not applicable.

3.6. Pooling of samples

Pooling of samples from more than one individual animal for a given purpose should only be recommended where robust supporting data on diagnostic sensitivity and diagnostic specificity have been evaluated and found to be suitable. The effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, therefore larger specimens should be processed and tested individually. Small life stages can be pooled to obtain the minimum amount of material for virus isolation or molecular detection.

4. Diagnostic methods

The methods currently available for pathogen detection that can be used in i) surveillance of apparently healthy animals, ii) presumptive diagnosis in clinically affected animals and iii) confirmatory diagnostic purposes are listed in Table 4.1. by animal life stage.

Ratings for purposes of use. For each recommended assay a qualitative rating for the purpose of use is provided. The ratings are determined based on multiple performance and operational factors relevant to application of an assay for a defined purpose. These factors include appropriate diagnostic performance characteristics, level of assay validation, availability cost, timeliness, and sample throughput and operability. For a specific purpose of use, assays are rated as:

+++ =	Methods are most suitable with desirable performance and operational characteristics.
++ =	Methods are suitable with acceptable performance and operational characteristics under most circumstances.
+ =	Methods are suitable, but performance or operational characteristics may limit application under some circumstances.
Shaded boxes =	Not appropriate for this purpose.

Validation stage. The validation stage corresponds to the assay development and validation pathway in chapter 1.1.2. The validation stage is specific to each purpose of use. Where available, information on the diagnostic performance of recommended assays is provided in Section 6.3.

WOAH Reference Laboratories welcome feedback on diagnostic performance of recommended assays, in particular PCR methods. Of particular interest are any factors affecting expected assay sensitivity (e.g. tissue components inhibiting amplification) or expected specificity (e.g. failure to detect particular genotypes, detection of homologous sequences within the host genome). These issues should be communicated to the WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. WOAH recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts												
Histopathology					+	+	+	1				
Cell culture												
Real-time PCR	+++	+++	+++	4	+++	+++	+++	4	+++	+++	+++	4
Conventional PCR	++	++	++	2	++	++	++	2				
Conventional PCR followed by amplicon sequencing									+++	+++	+++	2
***In-situ* hybridisation**					+	+	+	1	+	+	+	1
Bioassay					+	+	+	1				
LAMP	++	++	++	1	++	++	++	1	+	+	+	1
Ab-ELISA					+	+	+	1				
Ag-ELISA					+	+	+	1				
Other antigen detection methods					+	+	+	1				
Other methods												

LV = level of validation, refers to the stage of validation in the WOAH Pathway (chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively; IFAT = indirect fluorescent antibody test.

[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.

Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

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

T-E staining

A T-E staining solution may be prepared from Trypan blue 0.6%, Eosin Y 0.2%, NaCl 0.5%, phenol 0.5%, and glycerol 20% (Huang & Yu, 1995) and used as follows:

i) Place a piece of diseased tissue (e.g. a piece of gill or stomach epithelium without the cuticle) on a slide and mince with a scalpel.

ii) Add 1–2 drops of the T-E staining solution to the minced tissue, mix and allow to stain for 3–5 minutes.

iii) Lay a cover glass over the stained tissue and cover with several pieces of absorbent paper. Use a thumb to squash the mince into a single layer of cells.

If the sample was taken from a heavily infected shrimp, hypertrophied nuclei and intranuclear eosinophilic or vacuolation-like inclusion bodies can be observed using light microscopy (400–1000× magnification).

4.2. Histopathology and cytopathology

Smears

Demonstration of aggregates of WSSV virions 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 diagnosing infection with WSSV. The aggregates appear as small reflective spots of 0.5 μm in diameter (Momoyama *et al*., 1995).

Fixed sections

Histological changes commonly reported in susceptible species include: Hypertrophied nuclei with marginated chromatin material in virus-infected cells; eosinophilic to pale basophilic (with haematoxylin & eosin stain) stained intranuclear viral inclusions within hypertrophied nuclei and multifocal necrosis associated with pyknotic and karyorrhectic nuclei in affected tissues of ectodermal and mesodermal origin. The infection with infectious hypodermal and hematopoietic necrosis virus, another DNA virus, produces similar inclusions that need to be differentiated from those of WSSV.

4.3. Cell culture for isolation

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.

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 5.5 Use of molecular and antibody-based techniques for confirmatory testing and diagnosis of Chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate.

Extraction of nucleic acids

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

4.4.1. Real-time PCR

The real-time PCR methods described by Durand & Lightner (2002) and Sritunyalucksana *et al*. (2006) are described here as modified and validated by Moody *et al*., (2022).

Pathogen/ Target	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1: Durand & Lightner, 2002[1]; GenBank Accession No.: NC_003225			
WSSV/ Capsid protein	Fwd WSS1011F: TGG-TCC-CGT-CCT-CAT-CTC-AG Rev WSS1079R: GCT-GCC-TTG-CCG-GAA-ATT-A Probe: 6FAM-AGC-CAT-GAA-GAA-TGC-CGT-CTA-TCA-CAC-A-TAMRA	900 nM 900 nM 250 nM	50°C/2 min, 95°C/10 min, then 45 cycles of: 94°C/15 sec and 60°C/1 min
Method 2: Sritunyalucksana, 2006[1]; GenBank Accession No.: AF440570			
WSSV/ Capsid protein	Fwd CSIRO WSSV-F: CCG-ACG-CCA-AGG-GAA-CT Rev CSIRO WSSV-R: TTC-AGA-TTC-GTT-ACC-GTT-TCC-A Probe: 6FAM-CGC-TTC-AGC-CAT-GCC-AGC-CG-TAMRA	900 nM 900 nM 250 nM	50°C/2 min, 95°C/10 min, then 45 cycles of: 94°C/15 sec and 60°C/1 min

[1]Method described here as modified and validated by Moody *et al.*, 2022

4.4.2. Conventional PCR

Pathogen/ Target	Primer (5'–3')	Concentration	Cycling parameters
Method 1: Lo *et al.*, 1996a; GenBank Accession No.: AF440570; amplicon size: 1447/941 bp			
WSSV	Primary Fwd 146F1: ACT-ACT-AAC-TTC-AGC-CTA-TCTAG Rev 146R1: TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A	100 pmol 100 pmol	39 cycles of 94°C/1 min, 55°C/1 min, 72°C/2 min
	Nested Fwd 146F2: GTA-ACT-GCC-CCT-TCC-ATC-TCC-A Rev 146R2: TAC-GGC-AGC-TGC-TGC-ACC-TTG	100 pmol 100 pmol	39 cycles of 94°C/1 min, 55°C/1 min, 72°C/2 min

Commercial PCR kits are available. Please consult the WOAH Register for kits that have been certified by WOAH (https://www.woah.org/en/what-we-offer/veterinary-products/#ui-id-5).

4.4.2. Conventional PCR

Commercial PCR kits are available. Please consult the WOAH Register for kits that have been certified by WOAH (https://www.woah.org/en/what-we-offer/veterinary-products/#ui-id-5).

4.4.3. Loop-mediated isothermal amplification (LAMP) method

The protocol described here is from Kono *et al*. (2004). The LAMP method is sensitive and rapid, and it amplifies the target nucleic acids under isothermal conditions, therefore needing no sophisticated machine for thermal cycling.

DNA extraction

DNA extraction could be performed according to the protocol described in Section 4.4.2 *Conventional PCR* or by other suitable methods or by commercial kits.

LAMP reaction

i) Add DNA to a tube to set up a 25 µl reaction mixture (20 mM Tris/HCl, pH 8.8, 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8M Betaine, 1.4 mM of each dNTP, 40 pmol of WSSV-FIP and -BIP primers, 5 pmol of WSSV-F3 and -B3 primers).

ii) The primer sequences are WSSV-FIP: 5'-GGG-TCG-TCG-AAT-GTT-GCC-CAT-TTT-GCC-TAC-GCA-CCA-ATC-TGT-G-3', WSSV-BIP: 5'-AAA-GGA-CAA-TCC-CTC-TCC-TGC-GTT-TTA-GAA-CGG-AAG-AAA-CTG-CC-TT-3', WSSV-F3: ACG-GAC-GGA-GGA-CCC-AAA-TCG-A-3', WSSV-B3: 5'-GCC-TCT-GCA-ACA-TCC-TTT-CC-3'.

iii) Heat the mixture at 50°C for 5 minutes and at 95°C for 5 minutes, then chill on ice, and add 1 µl (8 U) of Bst DNA polymerase.

iv) Incubate the mixture at 65°C for 60 minutes, and then terminate the reaction at 80°C for 10 minutes.

v) To visualise, electrophorese 2 µl LAMP reaction products on 2% agarose gels containing ethidium bromide at a concentration of 0.5 µg ml^{-1}. This reaction produces WSSV-specific LAMP products with multiple bands of various sizes from approximately 200 bp to the loading well.

Reliable LAMP commercial kits may be an alternative for WSSV diagnosis.

4.5. Amplicon sequencing

The size of the PCR amplicon should be verified, for example by agarose gel electrophoresis. Both DNA strands of the PCR product must be sequenced and analysed in comparison with reference sequences.

4.6. In-situ hybridisation

Use of WSSV-specific DNA probes with histological sections is useful to demonstrate the presence of WSSV nucleic acid in infected cells (Nunan & Lightner, 1997). See Chapter 2.2.0 Section 5.5.4 for general comments on in-situ hybridisation.

4.7. Immunohistochemistry

See Section 4.9.

4.8. Bioassay

If SPF shrimp are available, the bioassay method based on Nunan *et al*. (1998) and Durand *et al*. (2000), is suitable for WSSV diagnosis.

4.9. 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 (Huang *et al*., 1995; Poulos *et al*., 2001; Sithigorngul *et al*., 2006; Yoganandhan *et al*., 2004).

4.10. Other methods

Lateral flow tests are commercially available but their performance needs to be evaluated before they can be recommended.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Real-time PCR is the recommended test for surveillance to demonstrate freedom of infection with WSSV in apparently healthy populations as described in Section 4.4.1.

6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1.) or in the presence of clinical signs (Section 6.2.) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for suspect and confirmed cases have been developed to support decision-making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. If a Competent Authority does not have the capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAH Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status[1]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Hydrographical proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with WSSV shall be suspected if at least one of the following criteria is met:

i) Positive result by conventional PCR

ii) Positive result by real-time PCR

iii) Positive result by LAMP method

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with WSSV is considered to be confirmed if at least one of the following criteria is met:

i) Positive results by real-time PCR and conventional PCR followed by amplicon sequencing

ii) Positive results by LAMP and conventional PCR method followed by amplicon sequencing

6.2. Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however they may narrow the range of possible diagnoses.

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with WSSV shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs consistent with the disease as described in this chapter, with or without elevated mortality

ii) Histopathology consistent with WSSV infection

iii) Positive result by conventional PCR

iv) Positive result by real-time PCR

v) Positive result by LAMP method

vi) Positive result by *in-situ* hybridisation

1 For example transboundary commodities.

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with WSSV is considered to be confirmed if at least at least one of the following criteria is met:

i) Positive results by real-time PCR and conventional PCR followed by amplicon sequencing

ii) Positive results by LAMP and conventional PCR method followed by amplicon sequencing

iii) Positive results by *in-situ* hybridisation and detection of WSSV by real-time PCR

iv) Positive results by *in-situ* hybridisation and detection of WSSV by conventional PCR followed by amplicon sequencing

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with WSSV are provided in Tables 6.3.1. and 6.3.2. This information can be used for the design of surveys for infection with WSSV, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data are only presented where tests are validated to at least level 2 of the validation pathway described in Chapter 1.1.2. and the information is available within published diagnostic accuracy studies.

6.3.1. For presumptive diagnosis of clinically affected animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
Real-time PCR (Durand & Lightner, 2002)	Diagnosis	Clinically diseased shrimp from farms	Gill, pleopod	*Penaeus monodon*	100% (*n*=71)	100% (*n*=71)	Real-time PCR	Moody *et al.,* 2022
Real-time PCR (Sritunyalucksana *et al.,* 2006)	Diagnosis	Clinically diseased shrimp from farms	Gill, pleopod	*Penaeus monodon*	100% (*n*=71)	100% (*n*=71)	Real-time PCR	Moody *et al.,* 2022

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study, PCR: = polymerase chain reaction.
*The nested PCR (Lo *et al.,* 1996a) is linked to false positives for WSSV when they are used to test species of *Cherax quadricarinatus* (Claydon *et al.,* 2004).

6.3.2. For surveillance of apparently healthy animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
Real-time PCR (Durand & Lightner, 2002)	Surveillance in apparently healthy animals	Wild populations of crustaceans	Gill, pleopod	*Penaeus merguiensis, P. esculentus, P. plebejus, Metapenaeus endeavouri, M. bennettae*	76.8% (*n*=1591)	99.7% (*n*=1591)	Bayesian latent class analysis	Moody *et al.,* 2022
Real-time PCR (Sritunyalucksana *et al*., 2006)	Surveillance in apparently healthy animals	Wild populations of crustaceans	Gill, pleopod	*Penaeus merguiensis, P. esculentus, P. plebejus, Metapenaeus endeavouri, M. bennettae*	82.9% (*n*=1591)	99.7% (*n*=1591)	Bayesian latent class analysis	Moody *et al.,* 2022

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
Two real-time PCR methods in parallel (Sritunyalucksana *et al.*, 2006 and Durand & Lightner, 2002)	Surveillance in apparently healthy animals	Wild populations of crustaceans	Gill, pleopod	*Penaeus merguiensis, P. esculentus, P. plebejus, Metapenaeus endeavouri, M. bennettae*	98.3% (*n*=1591)	99.4% (*n*=1591)	Bayesian latent class analysis	Moody *et al.*, 2022

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study, PCR: = polymerase chain reaction.

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NB: There are WOAH Reference Laboratories for infection with white spot syndrome virus (please consult the WOAH web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on infection with white spot syndrome virus

NB: First adopted in 1997 as white spot disease. Most recent updates adopted in 2023.