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Australian and New Zealand Standard Diagnostic Procedure (ANZSDP) for White Spot Syndrome Virus

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Acknowledgement of Country

We acknowledge the Traditional Custodians of Australia and their continuing connection to land and sea, waters, environment and community. We pay our respects to the Traditional Custodians of the lands we live and work on, their culture, and their Elders past and present.

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Summary

White spot syndrome virus (WSSV) is the aetiological agent of white spot disease (WSD), a contagious viral disease that affects a wide range of decapod crustaceans, especially penaeid prawn species. WSSV is an enveloped, double-stranded DNA virus classified by the International Committee on Taxonomy of Viruses as the sole member of the genus *Whispovirus*, in the family *Nimaviridae*.

First reported in Asia in 1991, WSD is still considered the most serious threat to *Penaeus monodon* and *P. vannamei* farmers in Asia. WSSV has since spread throughout most prawn culture areas in the Indo-Pacific, the Americas and the Middle East.

In aquaculture, WSD outbreaks are often characterised by high and rapid mortality of infected populations. WSSV infections in farmed prawn populations frequently result in cumulative mortalities of up to 100% within 3 to 10 days of the onset of clinical signs. However, sub-clinical infections are also known to occur. There is uncertainty about the extent of clinical disease that wild animals experience due to infection with WSSV.

Prawns suffering from WSD may display various clinical signs including lethargy, reduced food consumption and anorexia, pink to red discolouration of the body, the appearance of white spots (0.5 to 2.0 mm in diameter) on the cuticle and high mortality. However, the presence of clinical signs is variable and in farmed prawns mortality may be the only observable sign. Lesions are associated with the systematic destruction of the ectodermal and mesodermal tissues, especially the cuticular epithelium and subcuticular connective tissues.

There are no clinical signs or lesions pathognomonic for WSD. White spots on the cuticle are not observed in all cases and they can also be caused by bacterial infection or environmental conditions. A presumptive diagnosis in clinically infected animals may be made on recognition of characteristic microscopic changes in tissues, but a confirmed diagnosis of WSD requires the use of polymerase chain reaction and sequence analysis or *in-situ* hybridisation (ISH).

Status of Australia and New Zealand

White spot syndrome virus is limited to the Movement Regulated Area in south-east Queensland in Australia and is under an official control and containment program. New Zealand is considered free from WSSV. Infection with white spot syndrome virus is included in the World Organisation for Animal Health (WOAH; formerly OIE) Aquatic Code and WOAH Aquatic Manual as it is notifiable to the WOAH (WOAH 2022).

How to use this document

The ANZSDPs are diagnostic procedures and sampling techniques intended for use in Australian and New Zealand laboratories. During an outbreak investigation, they are complementary to the disease specific AQUAVETPLAN manual, which contains details of appropriate operational and management procedures. The *Aquatic Animal Diseases Significant to Australia: Identification Field Guide* (DAWE 2020) provides further information on the clinical signs, and ways to identify the disease in the field.

The objectives of an ANZSDP are to:

- standardise and harmonise test procedures to ensure consistency between laboratories using methods selected for their accuracy, sensitivity, specificity, and robustness
- provide standard methods that can be used in:
  - external proficiency testing programs
  - development of capability within a laboratory quality system.

Some ANZSDPs may include modifications to WOAH requirements where specific procedures and interpretation are necessary for Australian and New Zealand circumstances.

Table 1 Summary of aquatic disease preparedness and response planning documents in Australia

Publication	For
Aquatic field guide	Information for field identification of notifiable aquatic diseases, including exotic diseases
AQUAVETPLAN – disease manuals	Specific preparedness planning for key diseases
AQUAVETPLAN – operational procedures	Destruction/disposal/decontamination manuals for use during a disease response
AQUAVETPLAN – management manuals	Guidelines on running a disease control centre and information for enterprises to develop disease control strategies
ANZSDP – pathogen specific manuals	Diagnostic procedure information and laboratory protocols that are preferred for use in Australia
ANZSDP – general manuals	Technical information on sampling, surveillance and topics related to laboratory diagnostics

1 Diagnostic overview

1.1 Agent factors

1.1.1 Aetiological agent

White spot syndrome virus (WSSV) is the aetiological agent of white spot disease (WSD). The disease affects a wide range of decapod crustaceans, especially penaeid prawn species (WOAH 2022).

WSSV is an enveloped, double-stranded DNA virus (Chou et al. 1995; Wang et al. 1995; Wongteerasupaya et al. 1995). It is a member of the genus *Whispovirus* in the family *Nimaviridae* (Lo et al. 2011). WSSV virions are ovoid or ellipsoid to bacilliform in shape measuring 120 to 150 nm in diameter and 270 to 290 nm in length, sometimes with a flagella-like appendage at one end (Lo et al.2011).

When WSSV first appeared in multiple regions, it was given multiple names before WSSV was recognised as the generic virus name (Lightner 2011; Lo et al. 2011). Various geographical strains of WSSV with genotypic variations have been identified, but all are classified as a single species of WSSV (Nakano et al. 1994; Wongteerasupaya et al. 2003; Marks et al. 2004; Lo et al. 2011; Oakey and Smith 2018).

1.1.2 Survival and stability inside host tissues

WSSV can remain infectious in host tissues following prolonged exposure to freezing temperatures (– 20° to –70°C) (Lightner et al. 1997b; Wang et al. 1997; Wang et al. 1999a; Nunan et al. 1998; Scott-Orr et al. 2017; John et al. 2010; Reddy et al. 2010). Experimental trials conducted using infectious materials obtained from prawn carcasses suggests that WSSV can remain infective for 6 days at 25.5°C to 28.8°C (Wang et al. 2002). However, a separate study suggests the virus can remain infective for at least 14 days in prawn heads at 27°C and for at least 28 days in prawn tails at the same temperature (Prior et al. 2000).

1.1.3 Survival and stability outside the host

WSSV can remain infectious in sterile seawater for up to 30 days at 30°C and 120 days at 15°C under laboratory conditions (Momoyama et al. 1998) and for 3 to 4 days in ponds (Nakano et al. 1998). An experimental study found WSSV was viable and infectious in seawater for 12 days at 27 ppt, pH 7.5 and 30°C to 32°C (Kumar et al. 2013).

1.2 Host factors

1.2.1 Susceptible species

Decapod crustaceans (order Decapoda), including prawns, crabs, crayfish and lobsters from marine, brackish and freshwater sources are considered susceptible to infection with WSSV (Stentiford et al. 2009; Pradeep et al. 2012; WOAH 2022).

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

All life stages of prawns, from eggs to broodstock, are susceptible to infection with WSSV (Lightner 1998; Stentiford et al. 2009). In *P. vannamei*, susceptibility to WSSV increases after post larvae day 30, possibly due to genetic, physiological or etiological characteristics (Perez et al. 2005).

1.2.3 Reservoir hosts

A wide range of decapod crustaceans, including crabs and lobsters, are known to be reservoir hosts of WSSV (Lo et al. 1996a; Escobedo-Bonilla et al. 2008; Stentiford et al. 2009; Pradeep et al. 2012). In bioassays with *P. monodon* fed WSSV-infected crab and lobster tissues, clinical signs of WSD and mortality occurred in the prawns within 2 to 4 days (Rajendran et al. 1999).

Non-decapod species, such as copepods, rotifers, polychaete worms (such as *Marphysa gravelyi*, *Pereneis nuntia* and *Dendronereis* spp.), marine molluscs, sea slaters and aquatic insect larvae have been shown to act as mechanical carriers for WSSV (Lo et al. 1996a; Yan et al. 2004; Desrina et al. 2013; Haryadi et al. 2015; Laoaroon et al. 2005; Vijayan et al. 2005; Flegel 2006; Escobedo-Bonilla et al. 2008; Desrina et al. 2013; Haryadi et al. 2015; Wang et al. 2017). The detection of WSSV in prawn pond soil suggests that it too may act as a reservoir for the virus (Natividad et al. 2008; Kumar et al. 2013).

1.3 Disease pattern

1.3.1 Mortality, morbidity and prevalence

Prevalence of WSSV ranges from less than 1% in infected wild populations to up to 100% in farmed populations (WOAH 2022). WSD outbreaks in farmed prawns are often characterised by high morbidity followed by high and rapid mortality.

WSSV infections in farmed prawns frequently result in cumulative mortalities of up to 100% within 3 to 10 days of the onset of clinical signs (Chou et al. 1995; Inouye et al. 1994; Lightner 1996a; Wongteerasupaya et al. 1995b). However, subclinical disease has been observed, with occasional mortality only (Tsai et al. 1999).

1.3.2 Clinical signs

Prawns suffering from WSD may display various clinical signs including lethargy, reduced food consumption, anorexia, pink to red discolouration of the body, the appearance of white spots (0.5 to 2.0 mm in diameter) on the cuticle and high mortality (Momoyama et al 1994; Takahashi et al. 1994; Chou et al. 1995; Wang et al. 1995; Durand et al. 1996, Lightner 1996a). The white spots are the result of calcified deposits by the cuticular epidermis (Lightner 1996b). WSD-affected prawns often swim near the surface of ponds. However, prawns suffering from WSD may also display very few clinical signs.

Clinical signs of WSD are not pathognomonic and the characteristic white spots may be caused by bacterial infections (possibly *Bacillus subtilis*) (Wang et al. 2000) and environmental stressors (such as high alkalinity) and are often absent in WSD-affected prawns (Wang et al. 2000; Flegel 2006; Hossain et al. 2015; WOAH 2022). The presence of clinical signs is variable and in some prawns the only observable sign is mortality.

1.3.3 Pathology

WSSV-infected prawns may have a loosened attachment of the carapace with the underlying cuticular epithelium, delayed clotting of haemolymph and excessive fouling of the gills (WOAH 2022). Lesions are often associated with systemic destruction of the ectodermal and mesodermal tissues, including but not restricted to, the gills, cuticular epithelium, haemocytes, nervous tissue, antennal gland, lymphoid organ, muscle, midgut, hindgut, haemopoietic tissue and subcuticular connective tissue (Chang et al. 1996; Di Leonardo et al. 2005; Durand et al. 1996; Lo et al. 1997; Wang et al. 1995; Wang et al. 1999; Wongteerasupaya et al. 1995b).

Histological signs of infection with WSSV include hypertrophied nuclei containing eosinophilic to basophilic inclusions and marginalised chromatin in tissues of ectodermal and mesodermal origin (Chang et al. 1996; Lightner 1996b; Lightner et al. 1997b; Wongteerasupaya et al. 1995b). This is mostly seen in the cuticular epithelial cells and connective tissue cells (Lightner, 1996b). These intracellular inclusions can be seen in squash mounts of gills and subcuticular tissues.

Multifocal necrosis associated with pyknotic and karyorrhectic nuclei and tissue disorganisation become evident as the infection advances (Chang et al. 1996; Wang et al. 1997; Wongteerasupaya et al. 1995a).

1.3.4 Transmission of the virus

Infection may be clinical or sub-clinical. In active infections, clinical signs are evident within 2 to 7 days, while sub-clinical infections may continue for extended periods. The transition from sub-clinical to clinical WSD is generally short, possibly as short as a few hours (Lo and Kou 1998). Transition may be triggered by stressors such as spawning (Hsu et al. 1999) or environmental parameters including rainfall, temperature, and salinity (Karunasagar et al. 1997; Peinado-Guevara and Lopez-Meyer 2006; Korkut et al. 2018).

Horizontal transmission of WSSV can occur by ingestion of infected tissue (Chang et al. 1996; Wang et al. 1999b) or immersion in infected water (Chou et al. 1995; Kanchanaphum et al. 1998; Wang et al. 1997). Ingestion has been shown to be a more effective transmission route than immersion in viral extract or cohabitation (Soto et al. 2001; Perez et al. 2005). Dead and moribund prawns are a significant source of WSSV transmission (Lo & Kou 1998; Soto et al. 2001) as they shed viral particles into the water, and their infected tissues are ingested by other prawns. Experimentally, WSSV can also be transmitted by injection of infected inoculum (Takahashi et al. 1994; Momoyama et al. 1998; Nunan et al. 1998; Balasubramanian et al. 2006).

Vertical transmission can also occur, with WSSV DNA detected in the reproductive organs of infected prawns by PCR analysis (Lo et al. 1997) and in the eggs, nauplii and post-larvae of WSSV-positive broodstock (Hsu et al. 1999; Peng et al. 2001; Tsai et al. 1999).

1.3.5 Environmental and management factors

The introduction of WSSV into new areas is most often attributed to the movement of live broodstock and post-larvae (Takahashi et al, 1994; Lightner et al., 1997b; Stentiford et al, 2009).

The importation of frozen prawns is considered a route of introduction through the reprocessing of imported prawns at processing plants and release of untreated wastes into coastal water and landfills, the use of imported prawns as bait by fishermen and the use of imported prawns as food for the maintenance of aquaculture ponds (Humphrey 1995). Several studies have shown that WSSV remains viable and infectious in frozen prawns, and WSSV-infected prawns are thought to be responsible for the introduction of WSSV into the USA and possibly Europe (Lightner et al, 1997a; Lightner et al., 1997b; Nunan et al, 1998; Durand et al., 2000; Reville et al, 2005; Hasson et al, 2006; Reddy, et al, 2010; Bateman 2014).

Other possible pathways include prawn eating gulls and seabirds (Lightner, 1997b) and natural dispersion of either wild infected prawns or carrier hosts (Galaviz-Silva et al, 2004; Tang et al, 2013).

1.3.6 Geographical distribution

Outbreaks of disease caused by infection with WSSV were first reported in the People's Republic of China, Taiwan and Japan between 1991 and 1993 (Chou et al, 1995; Inouye et al., 1994; Momoyama et al, 1994; Takahashi et al, 1994; Escobedo-Bonilla et al, 2008). Subsequently, it has spread

throughout Asia (Flegel, 2006), the Americas (Escobedo-Bonilla, 2016; Lightner, 2011), the Mediterranean (Stentiford & Lightner, 2011), the Middle East (Yap, 2011) and Africa (Le Groumellec, 2012). Most recently, WSSV was detected during an outbreak in south-east Queensland, Australia. WSSV is under an official control and containment program (Queensland Department of Agriculture and Fisheries, 2021).

1.4 Differential diagnosis

In addition to WSSV, other exotic viruses should be included on the differential diagnostic list for unexplained mass mortalities in penaeid species farmed in Australia. The exotic pathogens that should be included in initial screening are yellow head virus genotype 1 (YHV1), Taura syndrome virus (TSV), Decapod iridescent virus 1 (DIV1) and Infectious myonecrosis virus (IMNV). The endemic pathogen to be included is gill-associated virus (GAV, or yellowhead genotype 2 (YHV2)). Further information on diseases with a similar presentation to WSD is contained in the Aquatic Animal Diseases Significant to Australia: Identification Field Guide (DAWE 2020) and the WSSV AQUAVETPLAN (Table 1).

1.5 Guidance on safety and biosecurity requirements

The WSSV restricted area in south-east Queensland is under an official control and eradication program (Queensland Department of Agriculture and Fisheries 2021). Outside the restricted area of Australia, WSSV is considered exotic and WSSV is exotic to New Zealand. Appropriate laboratory containment is required to minimise the biosecurity risk of spreading outside of the laboratory environment, and regulatory requirements involving handling of infectious materials or amplification of infectious agents must be met. Laboratory activities involving amplification of unfixed decapod material, suspected or confirmed to be infected with WSSV in a laboratory facility should only be handled at Physical Containment Level (PC) 3.

1.6 Sampling

1.6.1 Selection of populations and individual specimens

To confirm WSSV in diseased prawns, the sampler must collect at least:

- 100 representative larval to post-larval stage prawns, or
- 10 representative juvenile to adult-stage prawns.

Samples must be submitted to the relevant state or territory veterinary diagnostic laboratory. The selected prawns should have signs consistent with infection with WSSV and numbers should be sufficient for laboratory testing for each epidemiological unit (WOAH 2022).

For surveillance to demonstrate freedom, surveys should be designed in accordance with Chapter 1.4 of the WOAH Aquatic Code (WOAH 2022).

1.6.2 Suitable tissue samples for DNA extraction

Wherever possible, material for testing should be collected from live or moribund prawns, to minimise degradation of the tissue and viral DNA. Preferred tissues are the gill, pleopod or cuticular epithelium (WOAH 2022). However, muscle tissue or swab samples from muscle tissue are also suitable (Department of Agriculture and Water Resources 2017). Whole larvae or post-larvae will also suffice.

1.6.3 Samples or tissues that are not suitable for pathogen detection

Prawn eyes, eye stalks and internal organs in the head, in particular the hepatopancreas, should be avoided as they are known to contain PCR inhibitors.

1.6.4 Preservation of samples for submission

Where possible, live or moribund prawns should be anaesthetised by a brief period of chilling (not freezing) before being sampled. For histological analysis, fixed tissues are required. For molecular testing, fresh, frozen or ethanol-fixed tissues can be submitted. If there is any delay in the submission of fresh samples, they may be frozen and stored at −20°C or lower.

For histological examination, Davidsons Fixative (Appendix A: Reagents and kits) should be used. For larvae and postlarvae, immerse live animals directly in a minimum of 10 volumes of fixative. For juvenile and adult prawns the head should be removed and injected at multiple sites with fixative then placed in a minimum of 10 volumes of fixative. If ISH is required, the Davidsons Fixative should be replaced with 70% ethanol after 48 hours.

For molecular testing, 70 to 80% (v/v) ethanol (analytical grade ethanol, not technical grade) should be used. For larvae and post-larvae, immerse live animals directly in a minimum of 10 volumes of fixative. For live juvenile and adult prawns, dissect either gill tissue (whole filaments) or pleopods and immediately place the samples into a minimum of 10 volumes of fixative. After 24 hours fixation, most of the ethanol can be poured off, and the tissues are kept at ambient temperature for submission.

1.6.5 Pooling of samples

For the detection of WSSV in post-larvae, a pooled sample of whole post-larvae should be used to obtain sufficient material for nucleic acid extraction and molecular testing. For the detection of WSSV in samples from larger life stages, pools of samples from up to 5 individual animals may be used if the impact of pooling on diagnostic sensitivity has been determined for the purpose of use.

2 Diagnostic methods

In a suspected outbreak of WSD that is affecting any species of crustacean, PCR should be used initially as it provides the most rapid turnaround required to make a presumptive diagnosis. The standard approach is to screen with real-time PCR (qPCR) with confirmation of positives by conventional PCR and sequence analysis of amplicons. This is generally possible with clinically affected prawns but may not always be possible with apparently healthy prawns, due to the higher C_T values obtained. Depending on samples collected, PCR testing may also be undertaken concurrently with histology and ISH. A definitive association can then be made between the presence of WSSV and observed histopathological tissue changes characteristic of WSD.

Table 2 shows recommended diagnostic methods and their level of validation for surveillance of healthy animals and investigation of clinically affected animals. 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 in Table 2 are rated as:

- Optimal – Methods are most suitable with desirable performance and operational characteristics.
- Acceptable – Methods are suitable with acceptable performance and operational characteristics under most circumstances.
- Limited – Methods are suitable, but performance or operational characteristics may limit application under some circumstances.
- n/a – Not appropriate for this purpose.

Table 2 Recommended diagnostic methods and their level of validation for surveillance of healthy animals and investigation of clinically affected animals

Method	Surveillance of apparently healthy animals				Presumptive diagnosis of clinically affected animals				Confirmatory diagnosis of a suspect result from surveillance or presumptive diagnosis [a]			
	Early life stages [b]	Juveniles [b]	Adults	LV	Early life stages [b]	Juveniles [b]	Adults	LV	Early life stages [b]	Juveniles [b]	Adults	LV
Histopathology [c]	n/a	n/a	Limited	2	Acceptable	Optimal	Optimal	3	n/a	n/a	n/a	n/a
Real-time PCR	Acceptable	Optimal	Optimal	4	Acceptable	Optimal	Optimal	4	Acceptable	Optimal	Optimal	4
Conventional PCR	Acceptable	Acceptable	Acceptable	2	Acceptable	Acceptable	Acceptable	2	n/a	n/a	n/a	n/a
Conventional PCR followed by amplicon sequencing	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	Optimal	Optimal	Optimal	2
ISH	n/a	n/a	n/a	n/a	Limited	Acceptable	Acceptable	1	Limited	Acceptable	Acceptable	1
Bioassay	n/a	n/a	n/a	n/a	Limited	Acceptable	Acceptable	1	n/a	n/a	n/a	n/a
Lateral flow assay	n/a	n/a	n/a	n/a	Limited	Limited	Limited	1	n/a	n/a	n/a	n/a

Note: **LV** Level of validation refers to the stage of validation in the WOAH Pathway (chapter 1.1.2). **PCR** Polymerase chain reaction. qPCR = real-time PCR. **a** For confirmatory diagnoses, methods need to be carried out in combination in accordance with Section 3. **b** Early and juvenile life stages refer to larvae and post-larvae. **c** Histopathology can be validated if the results from different operators have been statistically compared.

2.1 General considerations

Virus isolation on cell lines is not available for WSSV. Detection has instead focused primarily on histology and molecular methods.

Histopathology and transmission electron microscopy (TEM) (Takahashi et al. 1994; Chou et al. 1995; Wongteerasupaya et al. 1995b) have been used to detect WSSV along with dot blot hybridisation (Edgerton, 2004), *in situ* hybridisation (ISH) (Chang et al. 1996; Durand et al. 1996; Lo et al. 1997; Wongteerasupaya et al. 1996) and loop mediated isothermal amplification (LAMP) (Mekata et al. 2009; Srisuvan et al. 2013). Various antibody-based tests using either monoclonal or polyclonal antibodies against WSSV have been developed for WSSV detection (Nadala & Loh 2000; Poulos et al. 2001; Anil et al. 2002; You et al. 2002; Yoganandhan et al. 2004; Sithigorngul et al. 2006). Molecular methods such as qPCR (Durand & Lightner 2002; Sritunyalucksana et al. 2006; Mendoza-Cano & Sánchez-Paz 2013) and conventional PCR (Lo et al. 1996a; Lo et al. 1996b; Lo et al. 1997; Maeda et al. 1997) are commonly used for WSSV screening and confirmatory testing.

2.2 Histopathology

2.2.1 Samples for histopathology

Fixation

For larvae and small post-larvae, live animals can be immersed directly into Davidson's fixative solution (Appendix A) and left for 24 to 72 hours. For larger post-larvae and very small juveniles, incise the cuticle with a needle before fixing. After fixation for 24 to 72 hours, transfer to 70% (v/v) ethanol and transport at ambient temperature. Davidson's fixative is a highly acidic fixative that decalcifies the shell to facilitate tissue processing and sectioning. Conventional fixative solutions, such as 10% neutral buffered formalin, do not decalcify or penetrate the shell, which results in poor fixation and difficulty in cutting sections. Precautions must be taken to avoid skin and eye contact with Davidson's fixative solution.

For juvenile and adult prawns, after chilling or anaesthesia, inject Davidson's fixative ensuring that the hepatopancreas is injected liberally first, and that the whole specimen is thoroughly injected thereafter with 5 to 10% of its body weight of fixative. If this is done properly, the whole body will turn red. Next, using a small pair of pointed scissors, the cuticle only should be cut along the mid-lateral side of the animal, starting at the sixth abdominal segment and moving up to the beginning of the cephalothorax, at which point the scissors should be angled in to meet the base of the rostrum. Then the whole prawn should be placed in 10 volumes of Davidson's fixative for 24 hours (up to 72 hours for larger prawns), after which it should be transferred to 70% (v/v)ethanol.

Where diagnosis is based on histology, tissues may be fixed for an unlimited time without loss of quality. However, specimens for ISH should be transferred from Davidson's fixative to 70% (v/v) ethanol after 24 to 48 hours or they will be unsuitable for ISH.

Following fixation, the specimens are embedded in paraffin wax and processed and stained with Meyer's haematoxylin and eosin (H&E) using standard methods. Inclusion bodies in target tissues are suggestive of infection with WSSV.

Sample number

For most crustaceans, the recommended minimum number of specimens that should be collected for diagnosis is 100 for larval stages, 50 for post-larval stages and 10 for juvenile and adult stages, with preference for individuals with patent signs of disease and/or gross lesions. The laboratory receiving the samples should be consulted if there is any doubt regarding the number of specimens to be submitted.

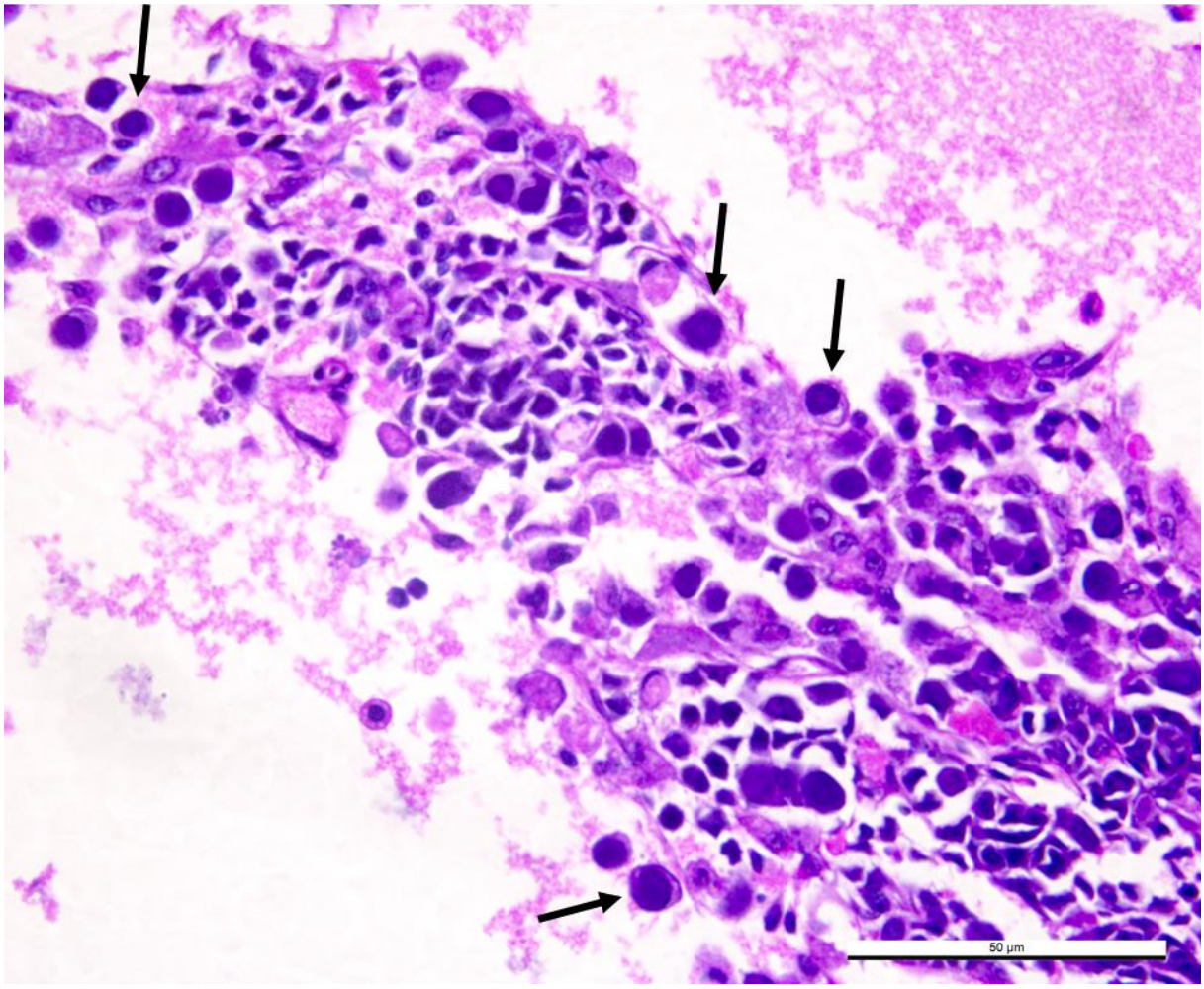
Examination

Examine tissue sections stained with H&E using standard techniques (Bell & Lightner 1988; Lightner 1996) by light microscopy for the presence of moderate to high numbers of WSSV infected cells in tissues of ectodermal and mesodermal origin. These cells typically display hypertrophied nuclei with eosinophilic to basophilic central inclusions (

) surrounded by marginated chromatin. Subcuticular tissues of the stomach, cephalothorax or gill are the most appropriate tissues for detecting histopathology changes characteristic of WSD (Wongteerasupaya et al. 1995).

Necrotic changes can also be seen in the antennal gland epithelium, lymphoid organ sheath cells and haematopoietic tissues, and in fixed phagocytes of the heart. Infected cells typically display hypertrophied nuclei containing a single intranuclear inclusion. In early stages of WSSV infection, nuclear inclusions are eosinophilic and (as an artefact of tissue preservation in Davidson's fixative) are separated by a clear halo from the marginated chromatin. Such eosinophilic or Cowdry type A intranuclear inclusions are characteristic of infections caused by many viruses of both vertebrates and invertebrates and appear as amorphous structures surrounded by clear halos beneath the nuclear membrane. Later in infection, inclusions stain lightly to darkly basophilic and can enlarge to fill the entire nucleus (Lightner 1996; WOAH 2022). This feature can be used to distinguish infection caused by WSSV from that caused by Infectious hypodermal hematopoietic necrosis virus (IHHNV), in which only Cowdry type A inclusion bodies are formed.

Figure 1 WSSV-infected *Penaeus monodon* showing necrosis of haematopoietic tissue with inclusion bodies (arrows)



2.3 *In situ* hybridisation

In situ hybridisation (ISH) uses a digoxigenin (DIG)-labelled RNA probe to detect WSSV in paraffin sections. Commercial WSSV kits are available and may be more suitable for a laboratory that is not undertaking ISH for WSSV.

If WSSV is present in the tissue section, the hybridisation process will allow the DIG-labelled RNA probe, developed by *in vitro* transcription, to bind to the complementary WSSV DNA and form a stable DNA-RNA hybrid. When an anti-DIG antibody, conjugated to alkaline phosphatase, is applied to the section and a substrate is applied, a colour reaction is observed.

The detailed protocol for WSSV ISH testing, based on the protocol reported by Nunan and Lightner (1997) is described in the WOAH Aquatic Manual for infection with white spot syndrome virus (WOAH 2022).

The Australian Centre for Disease Preparedness (ACDP) has ISH and immunohistochemistry capability, but the methods need optimising and validating so are not described here.

2.4 Transmission electron microscopy

The most suitable tissues for TEM are subcuticular tissues, gills and pleopods. These tissues should have been screened by histology or rapid-stain tissue squashes and showed signs of hypertrophied

nuclei and Cowdry type-A inclusions, or marginated chromatin surrounding a basophilic inclusion body.

Tissues are fixed in a 10:1 fixative to tissue volume of 6% glutaraldehyde at 4°C and buffered with sodium cacodylate or phosphate to pH 7.0, for at least 24 hours. For long-term storage, glutaraldehyde concentration is reduced to 1%. The tissues are then fixed in 1% osmium tetroxide and stained with uranyl acetate and lead citrate.

WSSV virions are rod-shaped to elliptical with a trilaminar envelope and measure approximately 80 to 120 nm × 250 to 380 nm and often characterised by a flagella-like protrusion from the envelope (WOAH 2022).

2.5 Molecular methods

2.5.1 Quality control aspects

Molecular testing for pathogens on Australia's National List of Reportable Diseases of Aquatic Animals must be operated under a quality management program accredited and audited according to the international standard ISO/IEC 17025. A quality management program will include initial evaluation of kits and reagents; validation of testing methods (Table 2); ongoing internal evaluation through mandatory use of appropriate quality control samples; and performance monitoring through external quality assessment or proficiency testing programs.

Quality control samples (positive, negative and reagent controls) must be specified in the laboratory's protocols and included in every run. The data generated must be recorded to monitor run-to-run performance.

2.5.2 DNA extraction method

Fresh and ethanol-fixed tissues can be homogenised (e.g. bead-beating tubes) directly in lysis or extraction buffer or digested in extraction buffer, provided with commercially available DNA extraction kits. Commercially available extraction kits (e.g. spin columns and magnetic bead platforms) should be validated or undergo equivalence testing with current validated extraction procedures, according to the laboratory's quality system requirements, including if extraction kit chemistries change.

2.6 Polymerase Chain Reaction (PCR)

In general, qPCR methods have high sensitivity and specificity, and are most appropriate for high-throughput molecular testing. Following adequate validation (Table 2), they can be used for direct detection of viral nucleic acids. qPCR can be used in surveillance of apparently healthy populations, as well as in the diagnosis of clinically affected animals, although conventional PCR and sequence analysis are required for confirmatory diagnosis.

Due to the highly sensitive nature of PCR tests, highly developed technical skills, use of appropriate quality controls (e.g. positive, negative extraction and no template controls) and separate work areas (e.g. mastermix preparation, nucleic acid extraction, template addition and amplification) are required to avoid cross-contamination and production of false-positive results.

2.7 Real-time PCR methods

2.7.1 WSSV qPCR assays

qPCR assay components (e.g. platforms, internal controls, master mixes and probe chemistries, cycling parameters etc.) may vary between laboratories; however in all cases, these methods should

be accredited to ISO 17025, or equivalent, and demonstrated to be comparable to the assays described in this ANZSDP.

The TaqMan qPCR method combines amplification of target DNA by PCR with the simultaneous detection of the target by a specific labelled probe with a reporter fluorophore. The 2 WSSV qPCR methods described herein are designed with WSSV DNA sequence-specific primers and probes that are based on conserved genome regions coding for the WSSV capsid protein. The probe further increases the specificity of the method because, in addition to the primers, this probe must also bind to the target DNA sequence.

The 2 WSSV TaqMan qPCR methods described in this procedure are both recognised by the Australian Government Department of Agriculture, Fisheries and Forestry as suitable for the detection of WSSV DNA for biosecurity risk management The diagnostic sensitivity and diagnostic specificity of the assays differ depending on the purpose of the test (e.g. detection of WSSV in clinically diseased prawns or in apparently healthy prawns) and are described in Table 7. The CSIRO WSSV qPCR uses the primers and probe described by East et al. (2004) and Sritunyalucksana et al. (2006), and the OIE WSSV qPCR uses the primers and probe described by Durand and Lightner (2002) outlined in the World Organisation for Animal Health (OIE) *Manual of Diagnostic tests for Aquatic Animals* (WOAH 2022). Primers and probes are described in Table 3.

Table 3 Primer and probe sequences for the CSIRO and OIE WSSV qPCR assays

Assay	Primer/probe	Sequence
CSIRO WSSV qPCR	CSIRO WSSV-F	5’- CCG ACG CCA AGG GAA CT -3’
	CSIRO WSSV-R	5’- TTC AGA TTC GTT ACC GTT TCC A -3’
	CSIRO WSSV probe	5’- 6FAM CGC TTC AGC CAT GCC AGC CG TAMRA -3’
OIE WSSV qPCR	OIE WSSV 1011F	5'- TGG TCC CGT CCT CAT CTC AG -3'
	OIE WSSV 1079R	5'- GCT GCC TTG CCG GAA ATT A -3'
	OIE WSSV Probe	5'- 6FAM AGC CAT GAA GAA TGC CGT CTA TCA CAC A TAMRA -3'

As implemented at ACDP (Moody et al, 2022), both assays use the following reaction and cycling conditions: each 25 µl reaction mix contains 2 µl extracted nucleic acid template, 12.5 µl TaqMan Universal PCR Master Mix (Life Technologies), a final concentration of 900 nM for each primer, 250 nM for the probe and molecular-grade water. PCR amplifications are programmed as follows: 1 cycle of 50°C for 2 minutes, 1 cycle of 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A threshold of 0.1 is used to determine C_T value. The qPCR assays are performed in a 7500 Fast Real-Time PCR System or QuantStudio 5 Real-Time PCR System (Life Technologies) and analysed with the 7500 or QuantStudio design and analysis software, respectively.

The following negative and positive controls must be included in each qPCR run:

- A negative extraction control: this control is a blank sample or WSSV-negative tissue run through the extraction process, to confirm that the reagents and equipment used for the DNA extraction are free from contamination with WSSV DNA.

- A no template control: this control contains all the components of the qPCR reaction mixture, but no template DNA, to confirm that the reagent and equipment used for the qPCR are free from contamination with WSSV DNA.
- At least one positive control for WSSV; this control consists of DNA extracted from prawn tissues confirmed to be infected by WSSV, or a plasmid or synthetic DNA containing the target WSSV DNA fragment, to confirm all the reactions components were included in the mastermix and cycling conditions were as described.

For the assay and test results to be valid the following criteria must be fulfilled: All negative controls must have no evidence of typical amplification curves. Each positive control must yield a typical amplification curve and mean CT values within the acceptable range according to quality control data accumulated by the NATA accredited diagnostic laboratory.

2.7.2 Interpretation of WSSV qPCR results

A positive WSSV qPCR result indicates the presence of a WSSV DNA fragment (targeted by the specific qPCR method applied) in the sample tested. A negative WSSV qPCR result indicates that there is no target WSSV DNA detected in the sample tested.

Using either the CSIRO WSSV qPCR or OIE WSSV qPCR method, the following rules must be applied for interpreting of results.

For initial testing

- A sample that does not yield a typical amplification curve within 45 cycles is ‘negative’ for WSSV.
- A sample that generates a typical amplification curve which has crossed the threshold before 40 cycles is ‘positive’ for WSSV.
- A sample that generates a typical amplification curve crossing the threshold between 40 and 45 cycles, or a sample that generates a typical amplification curve within 45 cycles but does not cross the threshold, is ‘suspect positive’ for WSSV. ‘Suspect positive’ samples must be retested within the same laboratory using one of the 2 qPCR methods described in this procedure.
- If the result of retesting is positive or suspect positive, the sample is reported as ‘positive’, ‘suspect positive’ or ‘indeterminate’ according to laboratory reporting terminology and jurisdictional requirements.
- If retesting of a suspect positive produces a negative result, the sample should remain classified as suspect positive and be referred for confirmatory testing at ACDP.

For confirmatory testing at ACDP

- A sample that does not yield a typical amplification curve within 45 cycles is ‘negative’ for WSSV.
- A sample that generates a typical amplification curve within 45 cycles is ‘positive’ for WSSV.

As WSSV is listed on Australia’s National List of Reportable Diseases of Aquatic Animals, any positive or suspect detection of WSSV must immediately be reported to the relevant authority (i.e. state or territory Chief Veterinary Officer and/or Director of Fisheries) and referred to ACDP for confirmatory testing.

2.7.3 Shrimp EF1 Housekeeping-gene qPCR

A qPCR housekeeping gene assay, targeting the penaeid elongation factor 1-α/β (EF1) genes, the CSIRO Shrimp EF1 qPCR, has been developed by Cowley et al. (2018). This assay can be used to

confirm nucleic acids were extracted and are free of PCR inhibitors. Primer and probe sequence are described in Table 4.

Table 4 Primer and probe sequences for the CSIRO Shrimp EF1 qPCR

Assay	Primer/Probe	Sequence
CSIRO Shrimp EF1 qPCR	Shrimp-EF1qF1	5'- GGC CGT GTG GAG ACT GGT AT -3'
	Shrimp-EF1qR1	5'- CGT GGT GCA TCT CCA CAG A-3'
	Shrimp-EF1qPr1	5'- **6FAM** CTG AAG CCA GGT ATG GTT GTC AAC TTT GCC **TAMRA** -3'

As implemented at ACDP, the reaction, cycling conditions and results interpretation are as for the WSSV-specific qPCR assays. The CSIRO Shrimp EF1 qPCR will detect *Penaeus spp*., *Litopenaeus* spp. and *Melicertus latisulcatus*, but will not detect DNA extracted from *Metapenaeus* spp., *Macrobrachium* spp., *Paratya australiensis*, *Pleoticus muelleri* and other non-penaeid decapods (e.g. crabs, crayfish). For most of these species, the OIE Decapod PCR described in 2.8.3 can be used.

2.8 Conventional PCR methods

2.8.1 OIE WSSV nested PCR

The OIE nested PCR is based on the primer sequences described by Lo et al. (1996b) and amplifies a 1447 base pair (bp) sequence of viral genomic DNA in the first reaction and an internal fragment of 941 bp in the nested reaction. Primers are described in Table 5. Reaction products are visualised by agarose gel electrophoresis.

Table 5 Primers used in the OIE WSSV nPCR

Primer	Sequence	Amplicon size
WSSV 146F1	5’-ACT ACT AAC TTC AGC CTA TCT AG-3’	1447 bp
WSSV 146R1	5’-TAA TGC GGG TGT AAT GTT CTT ACG A-3’	1447 bp
WSSV 146F2	5’-GTA ACT GCC CCT TCC ATC TCC A-3’	941 bp
WSSV 146R2	5’-TAC GGC AGC TGC TGC ACC TTG T-3’	941 bp

- As implemented at ACDP, the OIE WSSV nPCR assay uses the following reaction and cycling conditions: each primary PCR reaction mix of 25 µl is made up of 2 µl extracted nucleic acid, 12.5 µl HotStarTaq Master Mix (Qiagen) and 360 nM of each primer.
- PCR amplifications are programmed as follows: 1 cycle of 95°C for 15 minutes followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds.
- After a final extension at 72°C for 7 minutes reactions are held at 4°C.
- For the nested PCR, 2 µl of the primary PCR is added to the nested PCR master mix, which is made as described using the nested primers, with PCR amplification using the same cycling conditions as for the primary PCR.

For the assay and test results to be valid, the following criteria must be fulfilled:

- The no template control and negative extraction control must have no evidence of specific amplicons.
- The positive control must yield a specific amplicon of 1447 bp for the primary PCR and 941 bp for the nested PCR.

- Test samples with amplicons of the expected size of 1447 bp for the primary PCR and/or 941 bp for the nested PCR are considered test positive. Sequencing of amplicons is performed as described in section2.8.2.

As WSSV is listed on Australia's National List of Reportable Diseases of Aquatic Animals, any positive or suspect detection of WSSV must immediately be reported to the relevant authority (i.e. state or territory Chief Veterinary Officer and/or Director of Fisheries) and referred to ACDP for confirmatory testing.

2.8.2 DNA sequencing of PCR products

If conventional WSSV PCR is utilised as a criterion for a confirmed case (see section 4), the amplicons from the primary or nested PCR should be sequenced. Amplicons of the expected size, visualised by agarose gel electrophoresis, should be excised from the gel and purified using a commercially available agarose gel DNA extraction kits. The purified and quantified amplicons should be sequenced using both the forward (WSSV 146F1 and/or WSSV 146F2) and reverse (WSSV 146R1 and/or WSSV 146R2) primers and chromatograms analysed, consensus sequence generated and comparison with reference sequences available from NCBI GenBank, undertaken using commercially available sequence analysis software.

2.8.3 OIE Decapod PCR

The OIE Decapod PCR (Lo et al. 1996a) targets the decapod 18S rRNA gene to generate an amplicon of 848 bp. This assay can be used to verify the integrity of extracted nucleic acid and absence of PCR inhibitors. Primers are described in Table 6.

Table 6 Primers used in the OIE Decapod PCR

Primer	Sequence	Amplicon size
143F	5'-TGC CTT ATC AGC TN*T CGA TTG TAG-3'	848 bp
145R	5'-TTC AGN* TTT GCA ACC ATA CTT CCC-3'	848 bp

Note: N* = G, A, T or C

As implemented at ACDP, the reaction and cycling conditions are the same as those for the OIE WSSV nPCR.

For the assay and test results to be valid the following criteria must be fulfilled:

- The no template control and negative extraction control must have no evidence of specific amplicons.
- The positive control must yield a specific amplicon of 848 bp.
- Test samples with amplicons of the expected size of 848 bp are considered test positive.

The OIE Decapod PCR will generate amplicons from DNA extracted from prawns, crabs, crayfish and stomatopods but not from *Metapenaeus* spp.

2.9 Immunological assays

Both polyclonal and monoclonal antibodies have been produced to detect various WSSV proteins, and the WOAH Aquatic Manual summarises antibody-based tests that can be used to diagnose infection with WSSV (WOAH 2022). These detection methods have a WSSV detection sensitivity much lower than conventional PCR (Chaivisuthangkura et al. 2010).

Immunoassay lateral flow tests are commercially available internationally and can provide relatively rapid pond-side detection of WSSV proteins in clinical samples. These tests are targeted more towards management to prevent farm mortalities in regions where WSD is endemic. Approval for use of such test-kits rests within individual jurisdictions. As these methods are not in routine use, they are not described in this ANZSDP.

2.10 Bioassays

When used in isolation, a bioassay is insufficient for definitive WSSV diagnosis as the clinical sample might contain other pathogenic viruses. Therefore, other tests must be used in conjunction with a bioassay to confirm WSSV as the cause of morbidity and/or mortality events suspected to involve WSD. Bioassay protocols for WSSV have been published (Durand et al. 2000; McColl et al. 2004; Rajendran et al. 1999. Wesche et al. 2021).

2.11 Tests recommended for surveillance to demonstrate freedom in apparently healthy populations

qPCR is the recommended method for declaring freedom from WSSV (WOAH 2022). Diagnostic sensitivity (DSe) and diagnostic specificity (DSp) for the 2 qPCR assays described in this ANZSDP are in Table 7.

Table 7 Diagnostic performance of tests recommended for surveillance

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe [95% PI] (n)	DSp [95% PI] (n)	Citation
CSIRO WSSV qPCR	Diagnosis	Clinically diseased farmed prawns	Gill, pleopod	Penaeus monodon	100 (n=71)	100 (n=71)	Moody et al (2022)
OIE WSSV qPCR	Diagnosis	Clinically diseased farmed prawns	Gill, pleopod	Penaeus monodon	100 (n=71)	100 (n=71)	Moody et al (2022)
CSIRO WSSV qPCR	Surveillance in apparently healthy animals	Wild Australian prawns	Pleopods	Various	82.9% [75.0-90.2] (n=1591)	99.7% [98.6-99.99] (n=1591)	Moody et al (2022)
OIE WSSV qPCR	Surveillance in apparently healthy animals	Wild Australian prawns	Pleopods	Various	76.8% [68.9-84.9] (n=1591)	99.7% [98.7-99.99] (n=1591)	Moody et al (2022)
The two qPCR methods in parallel	Surveillance in apparently healthy animals	Wild Australian prawns	Pleopods	Various	98.3% [91.6–99.99] (n=1591)	99.4% [97.9–99.99] (n=1591)	Moody et al (2022)

DSe diagnostic sensitivity, **DSp** diagnostic specificity, **PI** probability interval, **n** number of samples used

3 Corroborative diagnostic criteria

The following case definitions for a suspect and confirmed case have been developed to support decision making. Confirmation of a suspect case in an area previously considered free from WSSV will be considered by the Aquatic Consultative Committee for Emergency Animal Diseases and will include consideration of laboratory test results and other relevant information related to the event. For a summary of aquatic disease preparedness and response planning documents please refer to Table 1.

3.1 Case definition

3.1.1 Suspect case

Infection with WSSV is suspected if at least one of the following criteria is met:

- Clinical signs consistent with infection with WSSV.
- Histopathology consistent with infection with WSSV.
- Positive conventional PCR result.
- Positive qPCR result.
- Positive result by bioassay.
- Positive result by a lateral flow assay.

Suspect detection of WSSV in farmed or wild crustacean populations previously considered free from infection will initiate the alert phase of an emergency response. Further testing and the collection of additional samples (as necessary) should be expedited to confirm or exclude the criteria of a confirmed case (see Section 3.1.2).

3.1.2 Confirmed case

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

- Positive qPCR results and positive conventional PCR results with sequence analysis consistent with WSSV.
- Positive results by real-time PCR and positive results by in-situ hybridisation.
- Positive results by in-situ hybridisation and positive results by conventional PCR with sequence analysis consistent with WSSV.

Results must be carefully assessed to ensure that they are unlikely to be the result of contamination (e.g. detection of non-viable WSSV DNA in feed).

Confirmation of a suspected case of WSSV infection based on a preliminary molecular diagnostic method (e.g. real-time PCR), requires sequence analysis of amplicons generated by conventional PCR. Results should correlate with the pathology of the animal when diseased animals are being examined. Sub-clinical infection may occur when an animal does not show pathological signs of WSD but gives a positive result by PCR.

qPCR detections in animals with low intensity infections (e.g. in wild populations) may not be able to be confirmed by conventional PCR and sequencing due to insufficient target nucleic acid. In these situations, attempts to meet the criteria of a confirmed case should be made by additional sampling

of the suspect population. If repeat sampling fails to provide sufficient material, an additional qPCR method can be used to confirm the case, but multiple animals must test positive on both assays.

4 Appendix A: Reagents and kits

Appendix 1 Ingredients for Davidson's fixative solution

Ingredient	Amount
40% formaldehyde	220 mL
95% ethanol	330 mL
glacial acetic acid	115 mL
distilled water	335 mL

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