

INFECTION WITH YELLOW HEAD VIRUS GENOTYPE 1

1. Scope

Infection with yellow head virus genotype 1 means infection with the pathogenic agent yellow head virus genotype 1 (YHV1) of the Genus *Okavirus* and Family *Roniviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

Yellow head virus genotype 1 (YHV1) is one of eight known genotypes in the yellow head complex of viruses and is the only known agent causing yellow head disease. YHV1 and other genotypes in the yellow head complex are formally classified by the International Committee on Taxonomy of Viruses as a single species (*Gill-associated virus*) in the genus *Okavirus*, Family *Roniviridae*, Order *Nidovirales* (Cowley *et al.*, 2012). Yellow head virus genotype 2 is commonly known as gill-associated virus (GAV). Four other genotypes in the complex (genotypes 3–6) occur commonly in healthy *Penaeus monodon* in East Africa, Asia and Australia and are rarely or never associated with disease (Walker *et al.*, 2001, Wijegoonawardane *et al.*, 2008a). Recently, two new YHV-complex genotypes have been reported, one designated YHV7 was detected in diseased *P. monodon* in Australia (Mohr *et al.*, 2015) and an eighth genotype was detected in *Penaeus chinensis* suspected of suffering from acute hepatopancreatic necrosis disease (Liu *et al.*, 2014). There is evidence of genetic recombination between genotypes (Wijegoonawardane *et al.*, 2009).

YHV1 forms enveloped, rod-shaped particles 40–50 nm × 150–180 nm (Chantanachookin *et al.*, 1993; Wongteerasupaya *et al.*, 1995). Envelopes are studded with prominent peplomers projecting approximately 11 nm from the surface. Nucleocapsids appear as rods (diameter 20–30 nm) and possess a helical symmetry with a periodicity of 5–7 nm. Virions comprise three structural proteins (nucleoprotein p20 and envelope glycoproteins gp64 and gp116) and a ~26 kb positive-sense single-stranded RNA genome.

2.1.2. Survival outside the host

YHV1 remains viable in aerated seawater for up to 72 hours (Flegel *et al.*, 1995b).

2.1.3. Stability of the agent (effective inactivation methods)

YHV1 can be inactivated by heating at 60°C for 15 minutes (Flegel *et al.*, 1995b). Little information is available on other inactivation methods but the virus appears to be susceptible to treatment with chlorine at 30 parts per million (0.03 mg ml⁻¹) (Flegel *et al.*, 1997).

2.1.4. Life cycle

High multiplicity YHV1 infections in cell culture have not been reported. Infection at a multiplicity of infection of 0.001 in primary cultures of lymphoid organ cells has indicated that maximum viral titres are obtained 4 days post-infection (Assavalapsakul *et al.*, 2003). Clinical signs of infection with YHV1 occur in *P. monodon* within 7–10 days of exposure. YHV1 replicates in the cytoplasm of infected cells in which long filamentous pre-nucleocapsids are abundant and virions bud into cytoplasmic vesicles in densely packed paracrystalline arrays for egress at the cytoplasmic membrane (Chantanachookin *et al.*, 1993).

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with YHV1 according to Chapter 1.5 of *Aquatic Animal Health Code (Aquatic Code)* include: Blue shrimp (*Penaeus stylirostris*), dagger blade

grass shrimp (*Palaemonetes pugio*), giant tiger prawn (*Penaeus monodon*), jinga shrimp (*Metapenaeus affinis*) and whiteleg shrimp (*Penaeus vannamei*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with yellow head virus genotype 1 according to Chapter 1.5 of the *Aquatic Code* include: Banana prawn (*Penaeus merguensis*), Carpenter prawn (*Palaemon serrifer*), kuruma prawn (*Penaeus japonicus*), northern brown shrimp (*Penaeus aztecus*), northern pink shrimp (*Penaeus duorarum*), northern white shrimp (*Penaeus setiferus*), Pacific blue prawn (*Palaemon styliiferus*), red claw crayfish (*Cherax quadricarinatus*), Sunda river prawn (*Macrobrachium sintangense*) and yellow shrimp (*Metapenaeus brevicornis*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but an active infection has not been demonstrated: Acorn barnacle (*Chelonibia patula*), blue crab (*Callinectes sapidus*), cyclopoid copepod (*Ergasilus manicatus*), gooseneck barnacle (*Octolasmis muelleri*), Gulf killifish (*Fundulus grandis*) and paste shrimp (*Acetes* sp.).

2.2.3. Susceptible stages of the host

Penaeus monodon are susceptible to YHV1 infection beyond PL15 (Khongpradit *et al.*, 1995).

2.2.4. Species or subpopulation predilection (probability of detection)

YHV1 infections are usually detected only when disease is evident and whilst they do not occur commonly in healthy *P. monodon*, infections have been detected in healthy wild populations of *P. stylirostris* (Castro-Longoria *et al.*, 2008). During outbreaks in aquaculture ponds, the YHV1 infection prevalence can be assumed to be high. Natural YHV1 infections have been detected in *P. japonicus*, *P. merguensis*, *P. setiferus*, *M. ensis*, and *P. styliiferus* (Cowley *et al.*, 2002; Flegel *et al.*, 1995a; 1995b), but there is little information available on the natural prevalence.

2.2.5. Target organs and infected tissue

YHV1 targets tissues of ectodermal and mesodermal origin including lymphoid organ, haemocytes, haematopoietic tissue, gill lamellae and spongy connective tissue of the subcutis, gut, antennal gland, gonads, nerve tracts and ganglia (Chantanachookin *et al.*, 1993; Lightner, 1996).

2.2.6. Persistent infection

YHV1 was detected by reverse-transcription polymerase chain reaction (RT-PCR) in clinically normal wild *P. stylirostris* collected for surveillance purposes in the Gulf of California in 2003 (Castro-Longoria *et al.*, 2008). The infectious nature of the YHV1 detected was confirmed by experimental infections. There is also evidence that YHV1 can persist in survivors of experimental infection (Longyant *et al.*, 2005; 2006).

2.2.7. Vectors

There are no known vectors of YHV1.

2.3. Disease pattern

2.3.1. Transmission mechanisms

YHV1 infection can be transmitted horizontally by injection, ingestion of infected tissue, immersion in sea water containing tissue extracts filtered to be free of bacteria, or by co-habitation of naive shrimp with infected shrimp (Flegel *et al.*, 1995b; Lightner, 1996). Infection of shrimp has also been established by injection of extracts of paste prawns (*Acetes* sp.) collected from infected ponds (Flegel *et al.*, 1995a). The dynamics of how YHV1 infection spreads within aquaculture ponds have not been studied. However, the rapid accumulation of mortalities during disease outbreaks suggests that horizontal transmission occurs very effectively.

2.3.2. Prevalence

The infection prevalence of yellow head complex viruses in healthy *P. monodon* (as detected by reverse transcription-nested PCR [RT-nPCR]) can be high (50–100%) in farmed and wild populations in Australia, Asia and East Africa as well as in *LP. vannamei* farmed in Mexico (Cowley *et al.*, 2004; Sanchez-Barajas *et al.*, 2009; Walker *et al.*, 2001; Wijegoonawardane *et al.*, 2008a). The prevalence of

individual genotypes varies according to the geographical origin of the shrimp. The use of detection methods less sensitive than nested PCR (e.g. histology, immunoblot, dot-blot, *in-situ* hybridisation), is likely in most cases to result in the real infection prevalence amongst populations of shrimp being underestimated.

2.3.3. Geographical distribution

YHV1 has been reported in Chinese Taipei, Indonesia, Malaysia, the Philippines, Sri Lanka, Thailand and Vietnam (Walker *et al.*, 2001). GAV and other genotypes in the yellow head complex have been detected in healthy *P. monodon* from Australia, Chinese Taipei, India, Indonesia, Malaysia, Mozambique, the Philippines, Thailand and Vietnam (Wijegoonawardane *et al.*, 2008a). YHV1 has also been detected in *P. vannamei* in Mexico (Sanchez-Barajas *et al.*, 2009).

2.3.4. Mortality and morbidity

With *P. monodon* being farmed in ponds, disease caused by YHV1 can cause up to 100% mortality within 3–5 days of the first appearance of clinical signs (Chantanachookin *et al.*, 1993). Whilst mortalities can easily be induced by experimental exposure of *P. monodon* to YHV1 or GAV, bioassays have identified YHV1 to be far more virulent (~10⁶-fold by lethal dose [LD₅₀] 50% end-point analysis) (Oanh *et al.*, 2011). Genotypes 3, 4, 5 and 6 have not yet been associated with disease (Wijegoonawardane *et al.*, 2008a).

2.3.5. Environmental factors

Elevated virus infection levels accompanied by disease can be precipitated by physiological stress induced by sudden changes in pH or dissolved oxygen levels, or other environmental factors (Flegel *et al.*, 1997).

2.4. Control and prevention

2.4.1. Vaccination

No effective vaccination methods have been developed.

2.4.2. Chemotherapy

No effective commercial anti-viral product is yet available.

2.4.3. Immunostimulation

No scientifically confirmed reports.

2.4.4. Resistance breeding

Not reported.

2.4.5. Restocking with resistant species

All marine shrimp species farmed commercially appear to be susceptible to YHV1.

2.4.6. Blocking agents

Injection of shrimp with double-stranded (ds) RNA homologous to ORF1a/1b gene regions of YHV1 or GAV (thus targeting the genome length viral RNA) can inhibit viral replication and prevent mortalities following experimental challenge. The antiviral action of the dsRNA appears to involve the RNA interference (RNAi) pathway (Tirasophon *et al.*, 2007).

2.4.7. Disinfection of eggs and larvae

Not reported.

2.4.8. General husbandry practices

Specific pathogen free (SPF) or PCR-negative seedstock and biosecure water and culture systems may be used to reduce the risk of disease.

3. Sampling

3.1. Selection of individual specimens

For diagnosis during a disease outbreak, moribund shrimp collected from pond edges are the preferred source of material for examination. Apparently normal shrimp should also be collected from the same ponds. For

surveillance for evidence of infection in populations of apparently healthy shrimp, life stages from mysis onwards (mysis, postlarvae [PL], juveniles or adults) can provide tissue sources useful for testing.

3.2. Preservation of samples for submission

Moribund shrimp (or tissue from moribund shrimp) should be snap-frozen on-site in a dry ice and alcohol slurry and preserved frozen in dry ice, liquid nitrogen or in a –80°C freezer. Freezing at or above –20°C is unsuitable.

Tissue samples for PCR screening should be preserved in 80–90% analytical/reagent-grade (absolute) ethanol. At least 10 times the volume of ethanol to tissue should be used. The use of lower grade (laboratory or industrial grade) ethanol is not recommended.

Tissue samples for histology should be sampled from fresh shrimp and preserved in Davidson's fixative. Formalin (10%) in seawater may be a useful alternative. At least 10 times the volume of fixative to tissue should be used.

Tissues for electron microscopy should be sampled from live shrimp.

For guidance on sample preservation methods for the intended test methods, see Chapter 2.2.0.

3.3. Pooling of samples

For detecting YHV1 infection in large populations of shrimp, pooling of tissue samples is acceptable for screening or surveillance of batches of mysis to PL from a hatchery tank or batches of juvenile shrimp in a pond. The total numbers of shrimp sampled, either as a single pool or as multiple smaller pools, are selected based on the infection prevalence expected and the required confidence limits of detection. Typically in populations comprising more than a 100,000 shrimp, if the prevalence of infection exceeds 5%, a total of 60 individuals tested in appropriate pool sizes will be required to detect YHV1 at a 95% confidence limit. However, definitive detection may be compromised if the YHV1 loads in the infected shrimp are very low or if tests less sensitive than two-step RT-PCR or real-time RT-PCR are employed. See also Chapter 2.2.0.

3.4. Best organs or tissues

In moribund shrimp suspected to be infected with YHV1, lymphoid organ and gill are the most suitable sample tissues. For screening or surveillance of juvenile or adult shrimp that appear grossly normal, lymphoid organ is preferred. Gills or haemolymph can be used for non-sacrificial sampling.

3.5. Samples or tissues that are not suitable

Not determined.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Shrimp from late postlarvae stages onwards can be infected experimentally with YHV1. In cultured shrimp, infection can result in mass mortality occurring, usually in early to late juvenile stages. Moribund shrimp may exhibit a bleached overall appearance and a yellowish discoloration of the cephalothorax caused by the underlying yellow hepatopancreas, which may be exceptionally soft when compared with the brown hepatopancreas of a healthy shrimp. In many cases, the total loss of a pond crop occurs within a few days of the first appearance of shrimp showing gross signs of YHV1 (Chantanachookin *et al.*, 1993). However, these disease features are not particularly distinctive, and in the absence of other more pathognomonic gross signs are not reliable even for preliminary diagnosis of YHV1.

4.1.2. Behavioural changes

Exceptionally high feeding activity followed by an abrupt cessation of feeding may occur within 2–4 days of the appearance of gross clinical signs of disease and mortality. Moribund shrimp may congregate at pond edges near the surface (Chantanachookin *et al.*, 1993).

4.2. Clinical methods

4.2.1. Gross pathology

See Section 4.1.

4.2.2. Clinical chemistry

None described.

4.2.3. Microscopic pathology

Fix the cephalothorax tissues of moribund shrimp suspected to be affected by YHV1 in Davidson's fixative, prepare tissue sections and stain with Meyer's haematoxylin and eosin (H&E) using standard histological procedures (Lightner, 1996). Examine tissues of ectodermal and mesodermal origin by light microscopy for the presence of moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusions approximately 2 µm in diameter or smaller (Chantanachookin *et al.*, 1993). Tissues of the lymphoid organ, stomach subcuticulum and gills are particularly informative.

4.2.4. Wet mounts

Fix whole shrimp or gill filaments overnight in Davidson's fixative (Lightner, 1996). After fixation, wash some gill filaments thoroughly with tap water to remove the fixative and stain with H&E (Lightner, 1996). After staining and dehydration, when the tissue is in xylene, place a gill filament on a microscope slide in a drop of xylene and, using a fine pair of needles (a stereo microscope is helpful), break off several secondary filaments. Replace the main filament in xylene where it can be stored indefinitely in a sealed vial as a permanent reference. Being careful not to let the xylene dry, tease apart the secondary filaments and remove any large fragments or particles that would thicken the mount unnecessarily. Add a drop of mounting fluid and a cover-slip and use light pressure to flatten the mount as much as possible. This procedure may also be used with thin layers of subcuticular tissue. Examine under a light microscope using a ×40 objective lens. For samples from YHV1-affected shrimp, moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusions (approximately 2 µm in diameter or smaller) will be observed (Flegel *et al.*, 1997). Evidence of such pathology should be used to support results from haemolymph smears (see below) in making a presumptive diagnosis of YHV1. As for the fixed tissues and gill filaments preserved in xylene, these whole-mount slides can be preserved as a permanent record.

If rapid results are required, the fixation step can be shortened to only 2 hours by replacing the acetic acid component of Davidson's fixative with a 50% dilution of concentrated HCl. For good fixation, this fixative should not be stored for more than a few days before use. After fixation, wash thoroughly to remove the fixative and check that the pH has returned to near neutral before staining. Do not fix for longer periods or above 25°C as this may result in excessive tissue damage that will make it difficult or impossible to identify specific pathology.

4.2.5. Electron microscopy/cytopathology

For transmission electron microscopy (TEM), the most suitable tissues of shrimp suspected to be infected with YHV1 infection are lymphoid organ and gills. For screening or surveillance of grossly normal shrimp, the most suitable tissue is lymphoid organ.

Stun live shrimp by immersion in iced water until just immobilised or kill by injection of fixative. Quickly dissect and remove small portions of target tissue (no larger than a few mm in diameter) and fix in a volume of 6% glutaraldehyde at least 10 times greater than the volume of tissue, held at 4°C and buffered with sodium cacodylate (Na[CH₃]₂AsO₂·3H₂O) solution (8.6 g Na cacodylate, 10 g NaCl, distilled water to make 100 ml, adjusted to pH 7 with 0.2 N HCl) or phosphate solution (0.6 g NaH₂PO₄·H₂O, 1.5 g Na₂HPO₄, 1 g NaCl, 0.5 g sucrose, distilled water to make 100 ml, adjusted to pH 7 with 0.2 N HCl). Fix for at least 24 h prior to processing. For long-term storage in fixative at 4°C, reduce glutaraldehyde to 0.5–1.0%. Processing involves post-fixation with 1% osmium tetroxide, dehydration, embedding, sectioning and staining with uranyl acetate and lead citrate according to standard TEM reagents and methods (Lightner, 1996).

In the cytoplasm of cells infected with YHV1, both nucleocapsid precursors and complete enveloped virions are observed. Nucleocapsid precursors appear as long filaments approximately 15 nm in diameter that can vary markedly in length (80–450 nm) and that can sometimes be packed densely in paracrystalline arrays. Virions appear as rod-shaped, enveloped particles 40–50 nm × 150–180 nm with rounded ends and prominent projections (8–11 nm) extending from the surface. In the cell cytoplasm, virions are commonly seen to be localised or packed densely within intracellular vesicles. Virions may

also be seen budding at the cytoplasmic membrane and in interstitial spaces. GAV virions and nucleocapsids are indistinguishable from YHV1 by TEM.

Lymphoid organ spheroids are commonly observed in healthy *P. monodon* chronically infected with YHV1 or GAV and lymphoid organ necrosis often accompanies disease (Spann *et al.*, 1997). However, spheroid formation and structural degeneration of lymphoid organ tissue also result from infection by other shrimp viruses (Lightner, 1996).

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

See Section 4.2.4.

4.3.1.1.2. Smears

See Section 4.2.5.

4.3.1.1.3. Fixed sections

See Section 4.2.3.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

Although primary shrimp cell culture methods are available, they are not recommended to isolate and identify YHV1 as a routine diagnostic method because of the high risk of them becoming contaminated with adventitious agents. No continuous cell lines suitable for YHV1 culture are yet available.

4.3.1.2.2. Antibody-based antigen detection methods

Reagents and protocols for detecting YHV1 proteins with antibodies have been published (Loh *et al.* 1998; Lu *et al.* 1994). Virions purified from haemolymph of experimentally infected shrimp have been used to produce antiserum in New Zealand white rabbits. From this antiserum, immunoglobulin (IgG) was purified using protein-G-linked columns and cross-reacting normal shrimp antigens were removed by adsorption to acetone-dried, ground shrimp muscle tissue and haemolymph. To detect YHV1 proteins by Western blotting, dilute 0.1 ml haemolymph collected from a live shrimp in an equal volume of citrate buffer and either run immediately or store at -80°C until used. Clarify 200 μl of the sample at 8000 **g** for 5 minutes and then pellet virions from the clarified supernatant by ultracentrifugation at 140,000 **g** for 5 minutes. Resuspend pellets in 100 μl 2 × loading buffer (2.5 ml 0.5 mM Tris/HCl pH 6.8, 4 ml 10% sodium dodecyl sulphate [SDS], 2 ml glycerol, 1 μl β -mercaptoethanol, 0.5 ml deionised distilled water) and heat at 95°C for 5 minutes. Load 10 μl sample and electrophorese at 200 V. Blot the gel onto a 0.1 mm pore size nitrocellulose membrane in blotting buffer (3.03 g Tris-base, 14.4 g glycine, 200 ml methanol per litre) at 100 V for 1 hour. Rinse the membrane with phosphate buffered saline (PBS pH 7.4), block in 5% skim milk (in PBS) for 1 hour, and rinse with PBS for 5 minutes. Soak the membrane in a 1/1000 dilution of the anti-YHV1 antibody (IgG) for 1 hour, rinse three times with PBS for 5 minutes, and then soak for 1 hour in a 1/2500 dilution of goat anti-rabbit IgG-horseradish-peroxidase (HRP) conjugate. Rinse membrane three times with PBS for 5 minutes and then soak in HRP substrate 3,3',5,5'-tetramethylbenzidine, until blue-purple colour develops. Stop the reaction by soaking the membrane in distilled water. All incubations should be carried out at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Use a purified viral preparation as a positive control to identify positions of the YHV1 116 kDa, 64 kDa and 20 kDa structural proteins. The Western blot YHV1 detection sensitivity is approximately 0.4 ng YHV1 protein ($\approx 10^6$ virions).

4.3.1.2.3. Molecular techniques

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

Three RT-PCR protocols are described. The first is a 1-step RT-PCR adapted from Wongteerasupaya *et al.* (1997) that can be used to detect YHV1 in affected shrimp. This protocol will detect YHV1 but not GAV or any of the other genotypes currently recognised. The second is a more sensitive multiplex nested RT-PCR protocol adapted from Cowley *et al.* (2004). It can be used to differentiate YHV1 from GAV in diseased shrimp or for screening healthy carriers. The first stage or step of the multiplex nested RT-PCR (primary RT-PCR) detected YHV7 (Mohr *et al.*, 2015). Both the

RT-PCR and the nested PCR (second stage or step) detected the novel YHV genotype from China (People's Rep. of) (Liu *et al.*, 2014). The test is available in a suitably modified form from a commercial source (YHV/GAV IQ2000, GeneReach Biotechnology Corp., Chinese Taipei). However, this kit is not currently listed as having completed the OIE's formal process for validating and certifying commercial tests (a list of certified test kits and manufacturers is available on the OIE website¹). The third is a sensitive multiplex nested RT-PCR protocol described by Wijegoonawardane *et al.* (2008b). This test can be used for screening shrimp for any of the seven genotypes of the yellow head complex of viruses, but will not discriminate between genotypes. Assignment of genotype can be achieved by nucleotide sequence analysis of the RT-PCR product. It is not known whether this assay will detect the YHV genotype recently detected in China (People's Rep. of).

Sample preparation: For juvenile or adult shrimp, lymphoid organ, gill tissue or haemolymph may be used to prepare total RNA. Fresh tissue is preferred. Lymphoid organ and gill tissue preserved in 80% to 95% analytical-grade ethanol or stored frozen at -70°C are also suitable for total RNA preparation. Disrupt 10–20 mg lymphoid organ or gill tissue or 50 μl haemolymph in 500 μl Trizol^{TM2} reagent and extract total RNA according to the product manual. Resuspend RNA in 25 μl water treated with DEPC (diethyl-pyrocabonate)-, heat at 55°C for 10 minutes, cool on ice and use immediately or store at -70°C until required. RNA yield will vary depending on the type and freshness of tissues, quality of the preservative used, and the length of time tissue has been preserved. However, RNA yields from fresh tissues would be expected to vary from 0.2 to 2.0 $\mu\text{g } \mu\text{l}^{-1}$ and about half these amounts from alcohol-preserved tissues. Tissues can also be homogenised by bead beating and extracted using commercially available kits (e.g. QIAmp Viral RNA Mini Kit) (Mohr *et al.*, 2015).

From a nursery tank or hatchery tank containing 100,000 PL or more, sample approximately 1000 PL from each of 5 different points. Pool the samples in a basin, gently swirl the water and then select samples of live PL that collect at the centre of the basin. Choose numbers of PL to be pooled and tested according to the assumed or infection prevalence. Homogenise tissue samples in an appropriate volume of TrizolTM reagent and extract RNA according to the product manual. Based on the standard TrizolTM extraction procedure, tissue masses equivalent to 25–30 \times PL5, 15 \times PL10 and 5 \times PL15 are accommodated and produce high quality total RNA free of protein contamination.

For each set of RNA samples to be tested, DEPC-treated water and extracts known to contain YHV1 RNA and/or GAV RNA (as appropriate to the test) should be included as negative and positive controls, respectively.

Protocol 1: RT-PCR for specific detection of YHV1 in diseased shrimp

The protocol in use at the OIE Reference Laboratory, based on Mohr *et al.* (2015) is as follows: Template (2 μl) is added to 23 μl reaction mixture containing 12.5 μl 2x reaction mix, 1 μl Superscript III RT/Platinum Taq mix (Invitrogen), 180 nM of each primer and molecular grade water. After 1 cycle of 50°C for 30 minutes and 94°C for 2 minutes, PCR amplification consists of 40 cycles of 94°C for 30 seconds, 58°C for 45 seconds, 68°C for 45 seconds, followed by at 68°C for 7 minutes. Alongside a suitable DNA ladder, apply a 20 μl aliquot of the PCR to a 1.5% agarose/TAE (Tris-acetate-EDTA [ethylene diamine tetra-acetic acid]) gel containing SYBR-safe and following electrophoresis, detect the 135 bp DNA band expected for YHV using a blue-light transilluminator.

The sensitivity of the PCR is approximately 0.01 pg of purified YHV1 RNA ($\approx 10^3$ genomes).

PCR primer sequences:

10F: 5'-CCG-CTA-ATT-TCA-AAA-ACT-ACG-3'
144R: 5'-AAG-GTG-TTA-TGT-CGA-GGA-AGT-3'

Protocol 2: Nested RT-PCR for differential detection of YHV1 and GAV in healthy or diseased shrimp

The protocol in use at the OIE Reference Laboratory, based on Mohr *et al.* (2015) is as follows: For the primary PCR, 2 μl template is added to 23 μl reaction mixture containing 12.5 μl 2x reaction mix, 1 μl Superscript III RT/Platinum Taq mix (Invitrogen), 180 nM of each GY1 and GY4 primer and molecular grade water. After 1 cycle of 50°C for 30 minutes and 94°C for 2 minutes, PCR amplification consists of 35 cycles of 95°C for 30 seconds, 66°C for 30 seconds, and 68°C for 45 seconds, followed by final extension at 68°C for 7 minutes. For the second, nested PCR step, prepare a 25 μl reaction mixture containing 2 μl of the first step PCR product, 12.5 μl HotStarTaq Master Mix (Qiagen), 360 nM

1 <https://www.oie.int/en/what-we-offer/veterinary-products/#ui-id-5>

2 Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this *Aquatic Manual*.

of each primer GY2, Y3 and G6, and molecular grade water. PCR amplification consists of 1 cycle of 95°C for 15 minutes followed by 35 cycles of 95°C for 30 seconds, 66°C for 30 seconds, and 72°C for 45 seconds, followed by final extension at 72°C for 7 minutes. Alongside a suitable DNA ladder, apply a 20 µl aliquot of the PCR to a 1.5% agarose/TAE (Tris-acetate-EDTA) gel containing SYBR-safe and following electrophoresis, detect amplicons using a blue-light transilluminator.

If the viral load is sufficiently high, a 794 bp DNA will be amplified from either GAV or YHV1 in the first PCR step. In the second PCR step, a 277 bp product indicates detection of YHV and a 406 bp product indicates detection of GAV. The presence of both 406 bp and 277 bp products indicates a dual infection with GAV and YHV1. The detection sensitivity of the second-step PCR is ~1000-fold greater than the first-step PCR and GAV or YHV1 RNA can be detected to a limit of 10 fg lymphoid organ total RNA. The nested PCR can be run as two separate assays specific for YHV1 or GAV by omitting either the G6 or Y3 primer, respectively. The primer contains a mismatch for GAV but is specific for YHV1. For GAV, the 7th base from left (T) is substituted for C so that the primer sequence for GAV should be 5'-CAT-CTG-CCC-AGA-AGG-CGT-CTA-TGA-3', according to the sequence data of the GAV genome (database accession numbers, NC_010306.1 and AF227196.2).

The sequences of RT-PCR primers generic for GAV and YHV (GY) or specific for GAV (G) or YHV (Y) are as follows:

GY1: 5'-GAC-ATC-ACT-CCA-GAC-AAC-ATC-TG-3'
 GY2: 5'-CAT-CTG-TCC-AGA-AGG-CGT-CTA-TGA-3'
 GY4: 5'-GTG-AAG-TCC-ATG-TGT-GTG-AGA-CG-3'
 GY5: 5'-GAG-CTG-GAA-TTC-AGT-GAG-AGA-ACA-3'
 Y3: 5'-ACG-CTC-TGT-GAC-AAG-CAT-GAA-GTT-3'
 G6: 5'-GTA-GTA-GAG-ACG-AGT-GAC-ACC-TAT-3'

NB: Due to reported problems with primer specificity for some emerging strains, all PCR products generated using protocol 2 should be sequenced to confirm the virus genotype.

Protocol 3: Nested RT-PCR for detection of all currently characterised genotypes in the yellow head complex YHV1 to YHV7.

The protocol in use at the OIE Reference Laboratory, based on Mohr *et al.* (2015) is as follows: For the primary PCR, 2 µl template is added to 23 µl reaction mixture containing 12.5 µl 2x reaction mix, 1 µl Superscript III RT/Platinum Taq mix (Invitrogen), 180 nM of each YC-F1ab and YC-R1ab primer pools and molecular grade water. After 1 cycle of 50°C for 30 minutes and 94°C for 2 minutes, PCR amplification consists of 1 cycle of 95°C for 15 minutes followed by 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 68°C for 45 seconds, followed by final extension at 68°C for 7 minutes. For the second, nested PCR step, prepare a 25 µl reaction mixture containing 2 µl of the first step PCR product, 12.5 µl HotStarTaq Master Mix (Qiagen), 180 nM of each YV-F2ab and YC-R2ab primer pool, and molecular grade water. PCR amplification consists of 1 cycle of 95°C for 15 minutes followed by 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 45 seconds, followed by final extension at 72°C for 7 minutes. Alongside a suitable DNA ladder, apply a 20 µl aliquot of the PCR to a 1.5% agarose/TAE (Tris-acetate-EDTA) gel containing SYBR-safe and following electrophoresis, detect amplicons using a blue-light transilluminator.

If the viral load is sufficiently high, a 358 bp DNA is amplified in the first PCR step. The second (nested) PCR step amplifies a 146 bp product. Further assignment of genotype (if required) is possible by nucleotide sequence analysis of either PCR product followed by comparison with sequences of the known genotypes by multiple sequence alignment and phylogenetic analysis. The detection sensitivity limits of the first PCR step and nested PCR step are 2,500 and 2.5 RNA templates, respectively.

PCR primer sequences (each primer comprises a pool of equal quantities of two related oligonucleotide sequences):

YC-F1ab pool: 5'-ATC-GTC-GTC-AGC-TAC-CGC-AAT-ACT-GC-3'
 5'-ATC-GTC-GTC-AGY-TAY-CGT-AAC-ACC-GC-3'
 YC-R1ab pool: 5'-TCT-TCR-CGT-GTG-AAC-ACY-TTC-TTR-GC-3'
 5'-TCT-GCG-TGG-GTG-AAC-ACC-TTC-TTG-GC-3'
 YC-F2ab pool: 5'-CGC-TTC-CAA-TGT-ATC-TGY-ATG-CAC-CA-3'
 5'-CGC-TTY-CAR-TGT-ATC-TGC-ATG-CAC-CA-3'
 YC-R2ab pool: 5'-RTC-DGT-GTA-CAT-GTT-TGA-GAG-TTT-GTT-3'
 5'-GTC-AGT-GTA-CAT-ATT-GGA-GAG-TTT-RTT-3'
 Mixed base codes: R(AG), Y(CT), M(AC), K(GT), S(GC), W(AT), H(ACT), B(GCT), V(AGC), D(AGT), N(AGCT).

4.3.1.2.4. *In-situ* hybridisation

The protocol of Tang *et al.* (2002) described is suitable for detecting YHV1 or GAV (Tang & Lightner, 1999). To preserve viral RNA accessibility, fix tissues sampled from live shrimp in neutral-buffered, modified Davidson's fixative without acetic acid (RF-fixative) (Hasson *et al.*, 1997). To achieve good tissue preservation whilst also preserving RNA accessibility, normal Davidson's fixative can be used as long as the fixation time is limited to 24 hours (maximum of 48 hours). Process the fixed tissue using standard histological methods and prepare 4 µm thick sections on Superfrost Plus slides (Fisher Scientific, Pennsylvania, USA). Prior to hybridisation, incubate sections at 65°C for 45 minutes, remove paraffin with Hemo-De (Fisher Scientific, Pennsylvania, USA), and rehydrate through a reducing ethanol concentration series to water. Digest sections with proteinase K (100 µg ml⁻¹, in 50 mM Tris/HCl pH 7.4, 10 mM NaCl, 1 mM EDTA) for 15 minutes at 37°C, followed by post-fixation in 0.4% formaldehyde for 5 minutes. Rinse in 2 × SSC (standard saline citrate), then pre-hybridise with 500 µl pre-hybridisation solution (4 × SSC, 50% formamide, 1 × Denhardt's, 0.25 mg ml⁻¹ yeast RNA, 0.5 mg ml⁻¹ sheared salmon sperm DNA, 5% dextran sulphate) at 42°C for 30 minutes. For hybridisation, overlay the sections with 250 µl hybridisation solution containing a digoxigenin-labelled DNA probe (20–40 ng ml⁻¹) at 42°C overnight. The next day, wash the sections as follows: 2 × SSC once for 30 minutes at room temperature; 1 × SSC twice for 5 minutes at 37°C; 0.5 × SSC twice for 5 minutes at 37°C. Incubate the sections with sheep anti-digoxigenin-alkaline phosphatase conjugate (Roche) at 37°C for 30 minutes. Wash with 0.1 M Tris/HCl pH 7.5, 0.15 M NaCl twice for 10 minutes at room temperature and rinse with 0.1 M Tris/HCl pH 9.5, 0.1 M NaCl. Incubate with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate in the dark for 1–2 hours for colour development. Counterstain with Bismarck Brown Y (0.5%), dehydrate through a series of ethanol and Hemo-De, add Permount (Fisher Scientific, Pennsylvania, USA) and cover with a cover-slip. YHV-infected cells give a blue to purple-black colour against the brown counter stain. Include positive controls of YHV-infected tissue and negative controls of uninfected shrimp tissue. The digoxigenin-labelled DNA probe can be prepared by PCR labelling using the following primers:

YHV1051F: 5'-ACA-TCT-GTC-CAG-AAG-GCG-TC-3'
 YHV1051R: 5'-GGG-GGT-GTA-GAG-GGA-GAG-AG-3'

4.3.1.2.5 *Agent purification*

A YHV1 purification method based on density gradient ultracentrifugation is described (Wongteersupaya *et al.* 1995). Approximately 250 healthy juvenile *P. monodon* (or *P. vannamei*) shrimp (approximately 10 g) should ideally be used as a source of virus for purification. After acclimatising for several days in 1500 litre tanks (approximately 80 shrimp/tank) at a salinity of 3.5 parts per thousand (mg ml⁻¹), inoculate each shrimp intramuscularly with 100 µl of a 1/100 gill extract suspension prepared from YHV-infected shrimp. At 2 days post-infection, harvest moribund shrimp showing typical signs of YHV1. Use a syringe to draw haemolymph from the sinuses at the base of the walking legs and mix carefully on ice with the same volume of lobster haemolymph medium (LHM) (486 mM NaCl, 15 mM CaCl₂, 10 mM KCl, 5 mM MgCl₂, 0.5 mM Na₂HPO₄, 8.1 mM MgSO₄, 36 mM NaHCO₃, 0.05% dextrose in Minimal Eagle's Medium, adjusted pH 7.6 with 1 N NaOH). Centrifuge the mixture at 480 **g** for 30 minutes at 4°C to remove cellular debris. Ultracentrifuge the supernatant at 100,000 **g** for 1 hour at 4°C. Discard the supernatant and gently resuspend the pellet overnight at 4°C in 1 ml LHM. Layer this suspension over a continuous gradient of 20–40% Urografin and ultracentrifuge at 100,000 **g** for 1 hour at 4°C. After centrifugation, collect the viral band by using a Pasteur pipette and dilute with NTE buffer (0.02 M EDTA, 0.2 M NaCl, 0.2 M Tris/HCl [pH 7.4]) to a final volume of 12 ml. Ultracentrifuge the suspension at 100,000 **g** for 1 hour at 4°C and resuspend the pellet (purified virus) in 100 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA [pH 7.4]) and store in 20 µl aliquots at –80°C until required.

4.3.1.2.6 *Bioassay*

The bioassay procedure is based on that described by Spann *et al.* (1997), but similar procedures have been described by several other authors (Lu *et al.*, 1994). The bioassay should be conducted in susceptible shrimp (see Section 2.2 above) ideally that have been certified as SPF and have been obtained from a biosecure breeding facility. Alternatively, susceptible wild or farmed shrimp to be used for bioassay should be screened by nested RT-PCR using RNA extracted from tissue or haemolymph to confirm the absence of pre-existing chronic infections with YHV complex or related viruses. Throughout the procedure, shrimp should be maintained under optimal conditions for survival of the species in laboratory tank systems.

Collect moribund shrimp from a YHV1-affected ponds or shrimp suspected of being carriers of infection and maintain at 4°C or on ice. Remove and discard the tail and appendages. If necessary, the whole shrimp or the retained cephalothorax may be snap-frozen and stored at –80°C or in liquid nitrogen until required. Thaw stored samples rapidly in a 37°C water bath within two snap-seal plastic bags and then maintain at 4°C or on ice during all procedures. Remove the carapace and calciferous

mouth-parts. Suspend the remaining tissues in six volumes of TN buffer (0.02 M Tris/HCl, pH 7.4, 0.4 M NaCl) and homogenise in a tissue grinder to form a smooth suspension. Clarify the homogenate at 1300 **g** for 20 minutes at 4°C. Remove the supernatant fluid below the lipid layer and pass through a 0.45 µm filter. Maintain the filtrate at 4°C for immediate use or snap-freeze and store in aliquots at –80°C or in liquid nitrogen. Thaw the filtrate rapidly at 37°C and maintain on ice prior to use.

Inject at least 12 juvenile (1–5 g) shrimp of a known susceptible species (*P. monodon*, *P. esculentus*, *P. japonicus*, *P. merguensis*, *P. vannamei*, *P. stylirostris*), with 5 µl of filtrate per gram body weight into the second abdominal segment using a 26-gauge needle. Inject two equivalent groups of at least 12 shrimp with TN buffer and a filtered tissue extract prepared from uninfected shrimp. One additional group of at least 12 shrimp should be injected last with a known and calibrated positive control inoculum from shrimp infected with YHV1. Maintain each group of shrimp in a separate covered tank with a separate water supply for the duration of the bioassay. Ensure no inadvertent transfer of water between tanks by good laboratory practice. Observe the shrimp and record mortalities for at least 21 days or until the test and positive control groups reach 100% mortality. Collect at least one moribund shrimp from each of the four groups for examination by histology, TEM, *in situ* nucleic acid hybridisation, and PCR or Western-blot analysis to confirm the presence of YHV1 in the sample (refer to the Sections above for test procedures).

NOTE: shrimp to be tested that are suspected of being carriers of low level chronic infections may produce an inoculum containing a very low dose of virus. In bioassay, such an inoculum may not necessarily cause mortalities, gross signs of disease or histology characteristic of a lethal infection. In this event, molecular tests (PCR or ISH) or TEM must be applied to the bioassay shrimp.

4.3.2. Serological methods

Not applicable.

5. Rating of tests against purpose of use

The methods currently available for targeted surveillance and diagnosis of YHV1 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	d	d	a	d
Histopathology	d	d	c	c	a	d
Transmission EM	d	d	c	c	d	b
Antibody-based assays	d	d	c	c	a	b

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
<i>In-situ</i> DNA probes	d	d	c	c	b	a
PCR	a	a	a	a	a	a
Sequence	a	a	a	a	d	a

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

6. Test(s) recommended for targeted surveillance to declare freedom from infection with yellow head virus genotype 1

Nested RT-PCR (Section 4.3.1.2.3.1; Protocol 3) followed by confirmatory sequencing of the amplified PCR product is the prescribed method for declaring freedom. Two-step PCR negative results are required.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

A suspect case of infection with YHV1 is defined as a disease outbreak in marine shrimp with rapidly accumulating mortalities (up to 100%) in the early to late juvenile stages, which may be preceded by cessation of feeding and congregation of shrimp at pond edges. Moribund shrimp may exhibit a bleached overall appearance and a yellowish discoloration of the cephalothorax caused by the underlying yellow hepatopancreas. Histological examination of fixed lymphoid organ tissues should reveal moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusions (approximately 2 µm in diameter or smaller).

7.2. Definition of confirmed case

YHV1 may be confirmed by the detection of high levels of disseminated infection in tissues of ectodermal and mesodermal origin by *in situ* hybridisation in conjunction with the detection of amplified products of the prescribed size using discriminatory RT-PCR assays and sequencing, as described in Section 4.3 of this chapter.

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NB: There is an OIE Reference Laboratory for infection with yellow head virus genotype 1 (see Table at the end of this *Aquatic Manual* or consult the OIE web site: <https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>). Please contact the OIE Reference Laboratories for any further information on infection with yellow head virus genotype 1

NB: FIRST ADOPTED IN 1995 AS YELLOWHEAD DISEASE. MOST RECENT UPDATES ADOPTED IN 2019.