CHAPTER 2.2.7.

YELLOW HEAD DISEASE

1. Scope

For the purpose of this chapter, yellow head disease (YHD) is considered to be infection with yellow head virus (YHV).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

Yellow head virus (genotype 1) is one of six known genotypes in the yellow head complex of viruses and is the only known agent of YHD. Gill-associated virus (GAV) is designated as genotype 2. GAV and four other known 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 (38, 41, 42). YHV and other genotypes in the yellow head complex are classified by the International Committee on Taxonomy of Viruses as a single species (Gill-associated virus) in the genus *Okavirus*, family *Roniviridae*, order *Nidovirales* (37). There is evidence of genetic recombination between genotypes (41).

YHV virions are enveloped, rod-shaped particles (40–60 nm × 150–200 nm). Envelopes are studded with prominent peplomers projecting approximately 11 nm from the surface. Nucleocapsids are helical in symmetry (diameter 20–30 nm) with a periodicity of 5–7 nm. Virions comprise three structural proteins (nucleoprotein p24 and envelope glycoproteins gp64 and gp116) and a ~26 kb positive-stranded single-stranded RNA genome (37).

2.1.2. Survival outside the host

YHV remains viable in aerated seawater for up to 72 hours (11).

2.1.3. Stability of the agent (effective inactivation methods)

YHV can be inactivated by heating at 60°C for 15 minutes (11). 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}) (9).

2.1.4. Life cycle

High multiplicity YHV infections in cell culture have not been reported. Infection at a multiplicity of infection of 0.001 in primary lymphoid organ cell culture has indicated that maximum viral titre is obtained at 4 days post-infection. Clinical signs of YHD occur in *P. monodon* within 7–10 days of exposure. YHV 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 (3, 4).

2.2. Host factors

2.2.1. Susceptible host species

YHD outbreaks have been reported only in the black tiger prawn (*P. monodon*) and the white Pacific shrimp (*P. vannamei*) (3, 5, 29). However, natural infections have also been detected in the kuruma prawn (*P. japonicus*), white banana prawn (*P. merguiensis*), Pacific blue prawn (*P. stylirostris*), white prawn (*P. setiferus*), red endeavour prawn (*Metapenaeus ensis*), mysid shrimp (*Palaemon styliferus*) and Antarctic krill (*Euphasia superba*). Other species of penaeid and palemonid shrimp and prawns and krill that have been reported to be susceptible to experimental infection include: brown tiger prawn (*P. esculentus*), brown prawn (*P. aztecus*); pink prawn, hopper and brown-spotted prawn (*P. duorarum*), greentail prawn (*Metapenaeus bennettae*), Sunda river prawn (*Macrobrachium sintangense*), barred estuarine shrimp (*Palaemon serrifer*), and the paste prawn (*Ascetes* sp.). There are variations in the susceptibility of different species to disease. Laboratory trials have shown that YHV can cause high mortality in *P. monodon, P. vannamei, P. stylirostrus, P. aztecus, P. duorarum, M. sintangense, P. styliferus* and *P. serrifer* (17, 20–23). A survey of 16 crab species collected from the vicinity of shrimp

farms in Thailand detected no evidence of either natural infection or experimental susceptibility (20). Other genotypes in the YHV complex have been detected almost exclusively in *P. monodon* to date (42).

2.2.2. Susceptible stages of the host

Penaeus monodon are susceptible to YHV infection beyond PL15 (14). Experimental infections with GAV indicate that larger (~20 g) *P. japonicus* are less susceptible to disease than smaller (~6–13 g) shrimp of the same species (31).

2.2.3. Species or sub-population predilection (probability of detection)

Amongst susceptible species of shrimp, viruses in the yellow head complex (genotypes 2–6) are only known to occur commonly (prevalence up to 100%) in healthy *P. monodon*, which appears to be the natural host (38, 41, 42). However, YHV (genotype 1) infections are usually detected only in the event of disease and do not occur commonly in healthy *P. monodon*. During disease outbreaks in ponds, the prevalence of YHV infection can be assumed to be high. Natural YHV infections have been detected in *P. japonicus, P. merguiensis, P. setiferus, M. ensis, P. styliferus* and *E. superba* (7, 10, 11), but there is little information available on the natural prevalence.

2.2.4. Target organs and infected tissue

YHV 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 (5, 15).

2.2.5. Persistent infection with lifelong carriers

GAV persists as a chronic infection in surviving *P. esculentus* for at least 50 days following experimental challenge (32). The high prevalence of infection of GAV and other yellow head complex viruses (genotypes 2–6) in all life stages of healthy *P. monodon* suggests that lifelong chronic infections occur commonly (38, 42). There is also evidence of persistence of YHV (genotype 1) in survivors of experimental infection (20, 21). Recent evidence suggests that preferential suppression of expression of envelope glycoprotein gp116 is associated with viral persistence (4).

2.2.6. Vectors

There are no known vectors of YHV.

2.2.7. Known or suspected wild aquatic animal carriers

Infection susceptibility and long-term persistence indicate the potential for a wide range of wild penaeid and palemonid shrimp to act as carriers.

2.3. Disease pattern

2.3.1. Transmission mechanisms

YHV can be transmitted horizontally by injection, ingestion of infected tissue, immersion in membrane-filtered tissue extracts, or by co-habitation with infected shrimp (11, 15). Transmission has also been demonstrated by injection of extracts of paste prawns (*Acetes* sp.) collected from infected ponds (10). For GAV, vertical transmission has been shown to occur from both male and female parents, probably by surface contamination or infection of tissue surrounding the fertilised egg (7). The dynamics of YHV infection in ponds have not been studied, but the rapid accumulation of mortalities during disease outbreaks suggests very effective horizontal transmission.

2.3.2. Prevalence

The overall prevalence of yellow head complex viruses in healthy *P. monodon* (as detected by nested polymerase chain reaction [PCR]) is very high (50–100%) in most sampled farmed and wild populations in Australia, Asia, East Africa and Mexico (6,28, 38, 42), and they are known to be present in Mexico. The prevalence of individual genotypes varies according to the geographic origin of the shrimp. The prevalence of YHV (genotype 1) may be low (>1%) in healthy wild or farmed *P. monodon,* but would be very high (approaching 100%) in disease outbreak ponds. Using other less sensitive detection methods (e.g. histology, immunoblot, dot-blot, *in-situ* hybridisation), the prevalence of infection in healthy shrimp would be found to be lower.

2.3.3. Geographical distribution

YHD has been reported in China (People's Rep. of), Chinese Taipei, India, Indonesia, Malaysia, the Philippines, Sri Lanka, Thailand and Vietnam (1, 15, 24, 39, 40, 42). 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 (42). YHV has also been detected in cultured *P. vannamei* from Mexico (28).

2.3.4. Mortality and morbidity

YHD can cause up to 100% mortality in infected *P. monodon* ponds within 3–5 days of the first appearance of clinical signs (5). GAV has been associated with mortalities of up to 80% in *P. monodon* ponds in Australia. Mortalities can be induced experimentally by exposure to YHV or GAV but YHV is far more virulent (~10^6-fold by 50% lethal dose [LD_{50}]). Other genotypes have not been associated with disease (42).

2.3.5. Environmental factors

Virus amplification and associated disease can be precipitated by physiological stress induced by sudden changes in pH or dissolved oxygen, or other environmental factors (8, 9). The much higher virulence of YHV compared with GAV and other genotypes appears to ensure that the threshold of infection required for disease is far more easily obtained.

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 commercially farmed marine shrimp species appear to be susceptible to YHV.

2.4.6. Blocking agents

Treatment of experimentally infected shrimp with double-stranded RNA homologous to YHV protease gene (ORF1a) sequences can inhibit viral replication and prevent mortalities (36). The mechanism of anti-viral action appears to involve RNA interference (RNAi).

2.4.7. Disinfection of eggs and larvae

Not reported.

2.4.8. General husbandry practices

Specific pathogen free (SPF) or PCR-negative seed stocks and biosecure water and culture systems may be used to reduce the risk of disease (16).

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 purposes (evidence of infection) in populations of apparently healthy shrimp, most life stages (nauplii, postlarvae [PL], juveniles or adults) are suitable for sampling.

3.2. Preservation of samples for submission

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

Samples for molecular screening by PCR should be preserved in a minimum three-fold excess of 90% analytical reagent grade (absolute) ethanol. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. Commercial RNA preservatives (e.g. RNAlater) may also be used.

Samples for histology should be preserved in Davidson's fixative. Formalin (10%) in seawater may be a useful alternative (13).

Samples for electron microscopy should be processed from live shrimp.

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

3.3. Pooling of samples

Since there is a risk of contamination, pooling of samples is usually recommended only for the screening or surveillance of batches of nauplii or PL, or juvenile shrimp in a pond. Pool size should be determined by the expected prevalence and required confidence limits of detection. Typically, the prevalence of infection in a positive batch of seed or in a pond will exceed 5%, requiring a pool size of 60 for detection of YHV at a confidence limit of 95%. However, this may be compromised if the levels of infection in individual shrimp are very low or if less sensitive tests are employed. See also Chapter 2.2.0.

3.4. Best organs or tissues

The most suitable tissues of moribund shrimp suspected of YHV infection are those of the lymphoid organ and gills. For screening or surveillance of grossly normal shrimp, the most suitable tissues are the lymphoid organ (preferred), gills or haemolymph of juvenile or adult shrimp, or whole nauplii or postlarvae.

3.5. Samples/tissues that are not suitable

Not determined.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

YHV can infect cultured shrimp from late PL stages onwards, but mass mortality usually occurs 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 normal shrimp. In many cases, total crop loss occurs within a few days of the first appearance of shrimp showing gross signs of YHD (5, 18). While cessation of feeding, congregation at pond edges and a generally bleached appearance are always seen in YHD outbreaks, these disease features are not particularly distinctive for YHD. The other more pathognomonic gross signs are not always seen and thus they are not reliable, even for preliminary diagnosis of YHD. Gross signs of GAV disease include swimming near the surface and at the pond edges, cessation of feeding, a reddening of body and appendages, and pink to yellow coloration of the gills (30). However, as these signs occur commonly in diseased shrimp, they are not considered pathognomonic for GAV disease. Shrimp chronically infected with YHV or GAV display normal appearance and behaviour.

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 (5, 18).

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 moribund shrimp from a suspected YHV outbreak in Davidson's fixative and process for preparation of standard H&E-stained tissue sections (2, 15). Examine the sections 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 in tissues of ectodermal and mesodermal origin (5). Tissues of the lymphoid organ, stomach subcuticulum and gills are particularly useful.

4.2.4. Wet mounts

Fix whole shrimp or gill filaments in Davidson's fixative (15) overnight. After fixation, wash some gill filaments thoroughly with tap water to remove the fixative, and stain with Meyer's haematoxylin and eosin (H&E) (15). 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 as a permanent reference in a sealed vial. Being careful not to let the xylene dry, tease apart the secondary filaments on a microscope slide and remove any large fragments or particles that would thicken the mount unnecessarily. Finally, add a drop of mounting fluid and a cover-slip. 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 YHD outbreak samples, moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusions (approximately 2 µm in diameter or smaller) will be observed (9). This evidence should be used together with the results from haemolymph smears (see below) in making a presumptive diagnosis of a YHD outbreak. As for the fixed tissues and the filaments in xylene, these whole-mount slides can be kept as a permanent record.

If rapid results are required, the fixation step can be shortened to only 2 hours by changing the acetic acid portion of the Davidson's fixative formula to 50% concentrated HCl. For best results, 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 interpretation difficult or impossible.

4.2.5. Smears

For moribund shrimp from YHD outbreaks, haemolymph smears are not useful because haemocytes are usually depleted in advanced stages of the disease. Haemolymph samples should be collected from grossly normal shrimp from a suspect pond where moribund shrimp have also been collected. Draw the haemolymph into a syringe containing two volumes of either 25% formalin or Davidson's fixative in which the acetic acid of the formula has been replaced by either water or formalin. Mix thoroughly, ignore clots in the syringe, place a drop on a microscope slide, smear and then air-dry before staining with H&E or other standard blood smear stains. Dehydrate, add mounting fluid and a cover-slip. Examine under a light microscope using a ×40 objective lens. For YHD outbreak samples, some of the smears will show moderate to large numbers of haemocytes with karyorrhectic or pyknotic nuclei (25). It is important that the slides with these nuclei show no evidence of concomitant bacterial infection, as bacterial infections may cause similar changes in haemocytes. When making a presumptive diagnosis of a YHD outbreak, the results from haemolymph smears should be used together with the results from rapidly stained whole mounts (see above) or stained tissue sections.

4.2.6. Electron microscopy/cytopathology

Lymphoid organ spheroids are commonly observed in healthy *P. monodon* chronically infected with YHV or GAV and lymphoid organ necrosis often accompanies disease (30, 33). However, spheroid formation and degeneration of lymphoid organ tissue also occur during infection with other shrimp viruses (15, 27).

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.

4.3.1.1.4. Transmission electron microscopy (TEM)

For TEM, the most suitable tissues of moribund shrimp suspected of YHV infection are those of the lymphoid organ and gills. For screening or surveillance of grossly normal shrimp, the most suitable tissue is from the 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 at least 10 volumes of 6% glutaraldehyde held at 4°C and buffered with sodium cacodylate ($Na[CH_3]_2AsO_2 \cdot 3H_2O$) 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 $NaH_2PO_4 \cdot H_2O$, 1.5 g Na_2HPO_4, 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 hours 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 methods. The reagents used for this procedure have been described elsewhere (15).

In YHV-infected cells, both nucleocapsid precursors and complete enveloped virions are observed. Nucleocapsid precursors are long filaments approximately 15 nm in diameter and of variable length (80–450 nm) that occur in the cytoplasm, sometimes densely packed in paracrystalline arrays. Virions are rod-shaped, enveloped particles (40–60 nm × 150–200 nm) with rounded ends and prominent projections (8–11 nm) extending from the surface. Virions are commonly seen in the cytoplasm of infected cells and in association with intracellular vesicles. Virions may also be seen budding at the cytoplasmic membrane and in interstitial spaces. GAV virions and nucleocapsids are indistinguishable from YHV by TEM.

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 for YHV isolation/identification because of the high risk of contamination with adventitious agents. No continuous cell lines suitable for YHV culture are yet available.

4.3.1.2.2. Antibody-based antigen detection methods

4.3.1.2.2.1 Immunoblot detection

Prepare reagents and carry out assays according to the protocols of Lu *et al.* (22) and Loh *et al.* (19). This includes purification of YHV virions from laboratory-infected shrimp, generation of immunoglobulins (Igs) in New Zealand white rabbits, purification of the IgG using recombinant bacterial protein-G columns and removal of cross-reacting normal shrimp antigens by adsorption on to acetone-dried, ground shrimp muscle tissue and haemolymph. For assay, remove 0.1 ml of haemolymph from live shrimp specimens and dilute in an equal volume of citrate buffer for immediate use or storage at –80°C until used. For Western blotting, use 200 µl of the sample, clarify at 8,000 ***g*** for 5 minutes and then pellet the supernatant 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 beta-mercaptoethanol, and 0.5 ml deionised distilled water) and heat at 95°C for 5 minutes. Load a 10 µl subsample on to 5% SDS/polyacrylamide gel, and conduct electrophoresis at 200 V. Blot the gel on to a nitrocellulose membrane (pore size, 0.1 mm) in blotting buffer (3.03 g Tris base, 14.4 g glycine, and 200 ml methanol per litre) at 100 V for 1 hour. Rinse the membrane with phosphate buffered saline (PBS), pH 7.4, soak in 5% skim milk (in PBS) for 1 hour, and rinse with PBS for 5 minutes. Next, treat the membrane with a 1/1000 dilution of the primary antibody (IgG) for 1 hour, rinse three times with PBS for 5 minutes, and then treat with a 1/2500 dilution of goat anti-rabbit IgG-horseradish-peroxidase conjugate for 1 hour. Rinse again three times with PBS for 5 minutes and then treat with the substrate, 3,3',5,5'-tetramethylbenzidine, until a bluish/purple colour develops. Stop the reaction by soaking the membrane in distilled water. All incubations should be carried out at 25°C ± 2°C. Use a purified viral preparation as a positive control and identify 2–4 major protein bands characteristic of YHV at 116, 64 and 20 kDa. The sensitivity is 0.4 ng of YHV protein (≈ 10^6 YHV virions).

4.3.1.2.3. Molecular techniques

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

Three RT-PCR methods are described. The first protocol is a 1-step RT-PCR adapted from Wongteerasupaya *et al.* (44) that can be used for confirmation of YHV in shrimp collected from

suspected YHD outbreaks. This protocol will detect only YHV and not GAV or other genotypes. The second protocol is a more sensitive multiplex nested RT-PCR procedure adapted from Cowley *et al.* (6). It can be used for differential detection of YHV and GAV in disease outbreak shrimp or for screening of healthy carriers. This test will not detect all known genotypes in the yellow head complex, and genotype 3 may react as GAV. The test is available in a suitably modified form from a commercial source (Farming IntelliGene Technology Corporation, Chinese Taipei). The OIE has a formal process for validating and certifying commercial tests. A list of certified test kits and manufacturers is available on the OIE website. The third protocol is a sensitive multiplex RT-nested PCR procedure provided by Wijegoonawardane *et al*. (43). This test can be used for screening healthy shrimp for viruses in the yellow head complex. It will detect all six currently known genotypes (including YHV and GAV), but will not discriminate between genotypes. Assignment of genotype can be achieved by nucleotide sequence analysis of the RT-PCR product.

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 95% analytical-grade ethanol or RNA*later* (various manufacturers), 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™[1] reagent and extract total RNA according to the product manual. Resuspend RNA in 25 µl DEPC (diethyl-pyrocarbonate)-treated water, heat at 55°C for 10 minutes, cool on ice and use immediately or store at –70°C until required. Ideally, a 1/200 dilution (i.e. 2.5 µl RNA in 500 µl DEPC-treated water) should be prepared, and absorbances A_{260} nm and A_{280} nm (a UV spectrophotometer is required) should be determined to quantify and check the quality of the RNA. RNA yield varies with the type and freshness of tissues as well as the quality of the preservative used and the length of preservation. However, approximate RNA yields from fresh tissues vary from 0.2 to 2.0 µg μl^{-1} and alcohol-preserved tissues yield approximately 0.1–1 µg μl^{-1}.

From a nursery or hatchery tank containing 100,000 PL or more, sample approximately 1000 PL from each of five different points. Pool the samples in a basin, gently swirl the water in the basin and then select an assay sample from live PL that collect at the centre of the basin. Choose the sample number according to the assumed or target prevalence. Homogenise the sample in an appropriate volume of Trizol reagent and extract RNA according to the product manual.

For each set of RNA samples to be tested, DEPC-treated water and extracts known to contain YHV 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 YHV in diseased shrimp

Mix 2 µl RNA in 20 µl of PCR buffer (10 mM Tris/HCl, pH 8.3, 50 mM KCl) containing 2.5 U of M-MLV (Moloney murine leukaemia virus) reverse transcriptase, 1.0 U ribonuclease inhibitor, 0.75 µM antisense primer (144R, below), 1 mM each of dATP, dTTP, dCTP, and dGTP, and 5 mM $MgCl_2$, and incubate at 42°C for 15 minutes to synthesise cDNA. Next, incubate the mixture at 100°C for 5 minutes to inactivate the reverse transcriptase and allow the mixture to cool to 5°C. Add the PCR mixture (10 mM Tris/HCl, pH 8.3, 50 mM KCl) containing 2.5 U *Taq* DNA polymerase, 2 mM $MgCl_2$ and 0.75 µM of sense primer (10F, below) to give a final volume of 100 µl. Overlay the tubes with 100 µl of mineral oil and conduct PCR amplification for 40 cycles at 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, and finishing at 72°C for 10 minutes. Apply 20µl of the amplified PCR product to a 2% agarose/TAE (Tris-acetate-EDTA [ethylene diamine tetra-acetic acid]) gels containing 0.5 µg ml^{-1} ethidium bromide alongside a suitable DNA ladder and detect using a UV transilluminator.

A positive reaction will be indicated by a 135 bp product. The sensitivity of the assay is approximately 0.01 pg of purified YHV RNA (≈ 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'

1 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*.

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

For cDNA synthesis, 2 µl RNA (ideally 1.0 µg total RNA, if quantified) and 0.7 µl 50 pmol μl^{-1} primer GY5 to a total to 6 µl in DEPC-treated water, incubate at 70°C for 10 minutes and chill on ice. Add 2 µl Superscript II buffer × 5 (250 mM Tris/HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 1 µl 100 mM DTT and 0.5 µl 10 mM dNTP stock mixture (i.e. 10 mM dATP, 10 mM dTTP, 10 mM dCTP, 10 mM dGTP) and mix gently. Preheat to 42°C for 2 minutes, add 0.5 µl 200 U μl^{-1} reverse transcriptase and incubate at 42°C for 1 hour. Then heat the reaction at 70°C for 10 minutes, chill on ice and spin briefly in a microcentrifuge to collect the contents of the tube. For the first PCR step, prepare a 50 µl reaction mixture containing 1 × *Taq* buffer (10 mM Tris/HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 35 pmol of each primer GY1 and GY4, 200 µM each of dATP, dTTP, dCTP and dGTP and 2.5 U *Taq* polymerase in a 0.5 ml thin-walled tube. Overlay the reaction mixture with 50 µl liquid paraffin, heat at 85°C for 2–3 minutes and then add 1 µl cDNA. Conduct PCR amplification using 35 cycles at 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. For the second PCR step, prepare a 50 µl reaction mixture containing 2 µl of the first step PCR product, 1 × *Taq* buffer (above), 1.5 mM $MgCl_2$, 35 pmol of each primer GY2, Y3 and G6, 200 µM each of dATP, dTTP, dCTP and dGTP and 2.5 U *Taq* polymerase in a 0.5 ml thin-walled tube overlaid with liquid paraffin. Conduct PCR using amplification conditions as described above. Apply 10 µl of the amplified PCR product to 2% agarose/TAE gels containing 0.5 µg ml^{-1} ethidium bromide alongside a suitable DNA ladder and detect using a UV transilluminator.

If the viral load is sufficiently high, a 794 bp DNA will be amplified from either GAV or YHV 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 YHV. The detection sensitivity of the second-step PCR is ~1000-fold greater than the first-step PCR and GAV or YHV RNA can be detected to a limit of 10 fg lymphoid organ total RNA.

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'

Protocol 3: Nested *RT*-PCR for detection of all currently known genotypes in the yellow head complex (including YHV and GAV)

For cDNA synthesis, mix 2 µl RNA (ideally 1.0 µg total RNA, if quantified), 50 ng random hexamer primers and 1.0 µl 10 mM dNTP and make up to a total volume of 14 µl in sterile DEPC-treated water, incubate at 65°C for 5 minutes and chill on ice. Add 4.0 µl Superscript III buffer × 5, 1.0 µl 100 mM DTT, 1.0 µl 40 U μl^{-1} RNaseOUT™ (Invitrogen) and 1.0 µl 200 U μl^{-1} reverse transcriptase and mix gently. Incubate at 25°C for 5 minutes and then at 42°C for 55 minutes, stop the reaction by heating at 70°C for 15 minutes, chill on ice and spin briefly in a microcentrifuge to collect the contents of the tube. For the first PCR step, add 1 µl cDNA to a total 25 µl reaction mixture containing 1 × *Taq* buffer (10 mM Tris/HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1.5 µl 25 mM $MgCl_2$, 0.35 µl primer mix containing 25 pmol μl^{-1} of each primer pool (see below) YC-F1ab and YC-R1ab, 0.5 µl 10 mM dNTP mix and 0.25 µl 5 U μl^{-1} *Taq* DNA polymerase. Conduct PCR amplification using denaturation at 95°C for 1 minute followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 40 seconds, followed by a final extension at 72°C for 7 minute. For the second PCR step, use 1 µl of the first PCR product in the reaction mixture as prepared above but substituting primer pools YC-F2ab and YC-R2ab. Conduct PCR amplification using denaturation at 95°C for 1 minute followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, followed by a final extension at 72°C for 7 minute. Apply 8 µl of the amplified PCR product to 2% agarose/TAE gels containing 0.5 µg ml^{-1} ethidium bromide alongside a suitable DNA ladder and detect using a UV 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. The detection of these products indicates detection of one of the six genotypes in the yellow head complex. 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 step PCR and nested PCR 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).

4.3.1.2.3. In-situ *hybridisation*

The protocol of Tang & Lightner (34) is described here. The method is suitable for detection of YHV or GAV (35). To preserve the viral RNA, fix live shrimp with neutral-buffered, modified Davidson's fixative without acetic acid (RF-fixative) (12). Process the fixed shrimp 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 an ethanol 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 formaldehyde (0.4%) 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 m^{-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 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 diagnostic 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'

4.3.1.2.3 Agent purification

The agent purification method is based on that described by Wongteersupaya *et al.* (45). Approximately 250 healthy juvenile *P. monodon* shrimp (approximately 10 g) should ideally be used as a source of virus for purification. After acclimatising for several days in 1,500 L 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 suspension of infected gill extract. At 2 days post-infection, harvest moribund shrimp showing typical signs of YHD. Draw haemolymph by syringe 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 $CaCl_2$, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM Na_2HPO_4 8.1 mM $MgSO_4$, 36 mM $NaHCO_3$, 0.05% dextrose in Minimal Eagle's Medium, adjusted pH 7.6 with 1N NaOH). Centrifuge the mixture at 480 ***g*** for 30 minutes at 4°C to remove cellular debris. After centrifugation, discard the pellet and further centrifuge the supernatant fluid at 100,000 ***g*** for 1 hour at 4°C. Discard the supernatant fraction and gently resuspend the pellet at 4°C overnight in 1 ml of 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 further 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 of TE buffer (10 mM Tris/HCl, 1 mM EDTA [pH 7.4]) and store in 20 µl aliquots at –80°C until required.

4.3.1.2.4 Bioassay

The bioassay procedure is based on that described by Spann *et al.* (30) but similar procedures have been described by several other authors (22, 26, 40). Bioassay should be conducted in susceptible

shrimp (see Section 2.2 above) 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 reverse-transcription (RT)-PCR on haemolymph samples to confirm the absence of pre-existing chronic infections with YHV, GAV or related viruses. Shrimp should be maintained throughout the procedure under optimal conditions for survival of the species in laboratory culture.

Collect moribund shrimp from a disease outbreak 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 twelve juvenile (1–5 g) shrimp of a known susceptible species (*P. monodon*, *P. esculentus*, *P. japonicus*, *P. merguiensis*, *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 YHV or GAV (as required). 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 YHV or GAV (as required) in the sample (refer to the Sections below 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 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 YHD 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

***Table 5.1.* (*cont.*)** *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Transmission EM	d	d	c	c	d	b
Antibody-based assays	d	d	c	c	a	b
DNA probes – *in situ*	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 yellow head disease

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. The very rare case when a two-step PCR positive result cannot be confirmed by sequencing is also considered to be a negative result. As genetic recombination between genotypes can occur, the presence of any genotype is considered to be evidence of the presence of YHD.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

A suspect case of YHD 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

YHD 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. As low-level chronic infections with yellow head complex viruses are common in some regions, detection of the presence of virus is not, in itself, evidence of aetiology.

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NB: There is an OIE Reference Laboratory for Yellow head disease (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int)