

SECTION 2.2.

DISEASES OF CRUSTACEANS

CHAPTER 2.2.0.

GENERAL INFORMATION

The principles and methods discussed in this chapter will, of necessity, emphasise penaeid shrimp because most of the diseases currently listed (and those that are under study for possible listing) by the OIE are diseases of the penaeid shrimp (World Animal Health Organisation, 2012). The taxonomy of the penaeid shrimp was revised in 1997 (Perez Farfante & Kensley, 1997). Penaeid subgenera were raised to being full genera. However, as this change in penaeid taxonomy has not been universally accepted, the taxonomy of the penaeids as outlined by Holthuis (Holthuis, 1980) will be used in this *Aquatic Manual*.

A. SAMPLING

1. Assessing the health status of the epidemiological unit

Sampling of wild crustacean populations presents a formidable challenge when designing disease surveys. Where wild crustacean fisheries exist, there is a potential opportunity for obtaining samples. However, this represents a population of relatively healthy animals having survived to a harvestable age and size and, therefore, may under represent diseases of interest.

A general approach to surveillance and sampling is provided in the *Aquatic Animal Health Code* (Chapter 1.4 *Aquatic animal health surveillance*).

1.1. Sample material to be used for tests

Sample material depends on the disease or pathogen to be tested for and on both the size of animals and the objective of testing, i.e. diagnosis of overt disease, detection of subclinical pathogen carriers or sampling for targeted surveillance to demonstrate freedom of a specified disease. See the OIE *Guide for Aquatic Animal Health Surveillance* (Corsin *et al.*, 2009) and the individual disease chapters in this *Aquatic Manual* for specific details of sample requirements.

1.2. Specifications according to crustacean size

See the OIE *Guide for Aquatic Animal Health Surveillance* (Corsin *et al.*, 2009) and the individual disease chapters in this *Aquatic Manual* for specific details of sample requirements.

1.3. Specifications according to crustacean populations

In addition to the considerations in the OIE *Guide for Aquatic Animal Health Surveillance* (Corsin *et al.*, 2009), the following issues related to crustacean populations should be taken into account during the development of a surveillance system:

- i) Usually cultured crustacean populations are distributed into multiple holding units, which generally do not share water and may or may not share exposure to different conditions.
- ii) Crustaceans tend to stratify in the holding unit and in the water column based on health and other behavioural interactions (e.g. with diseased animals often coming to the edges or surface of ponds).
- iii) For several crustacean diseases, there are large numbers of susceptible species for which relative susceptibility is often poorly known.

- iv) Dividing or mixing crustacean populations changes the characteristics of the populations.
- v) Some wild crustacean populations are sometimes characterised by shoaling behaviour, which implies a certain degree of homogeneity within each shoal.

1.4. Specifications according to clinical status

In clinical disease episodes, carefully selected quality specimens with representative lesions should be obtained from live or moribund crustaceans. Every effort should be made to sample those specimens for diagnosis that are representative of the disease(s) that is (are) affecting the crustacean stock of interest, and that are moribund or clinically diseased. Collection of dead specimens during disease outbreaks should be avoided when possible, but recently dead samples may be suitable for some diagnostic assays. When cultured or wild crustacean stocks are presenting clinical signs of an active disease that are consistent with, or suggestive of, any one of the OIE listed crustacean diseases, care should be taken to ensure that the samples collected are preserved appropriately for the anticipated diagnostic tests (see sample preservation section for recommended methods). For the OIE listed diseases it is highly recommended that the scheduling of sampling be planned (i.e. by farm schedule, season, etc.) so that the particular life-stage(s) are sampled at a time when the pathogen of concern is most likely to be detected. This is especially important when the available diagnostic methods are dependent on simple microscopy or histological methods and do not include molecular methods. For Taura syndrome, Infectious hypodermal and haematopoietic necrosis, White spot disease, Infectious myonecrosis, necrotising hepatopancreatitis (NHP) and Yellow head disease, juveniles and subadults provide the best samples; and for crayfish plague, juveniles and adults are suitable samples.

Samples taken for molecular or antibody-based tests for OIE-listed crustacean diseases may be combined as pooled samples of no more than five specimens per pooled sample. Recently dead crayfish or other crustaceans may be suitable (depending on their condition) for certain diagnostic assays such as polymerase chain reaction (PCR), reverse-transcription (RT)-PCR, isolation and/or culture of the disease agent, bioassay, etc.).

2. General processing of samples

2.1. Macroscopic examination

See disease-specific chapters in this *Aquatic Manual*.

2.2. Virological examination

2.2.1. Transportation and antibiotic treatment of samples

Not applicable.

2.2.2. Virus extraction

See disease-specific chapters in this *Aquatic Manual*.

2.2.3. Treatment to neutralise enzootic viruses

Not applicable.

2.3. Bacteriological examination

Not applicable for currently listed diseases except in the case of NHP. As NHP has not been cultured and because of its very small size, bacteriological examination may be limited to Gram staining.

2.4. Parasitic examination

Not applicable for currently listed diseases.

2.5. Fungal and other protists examination

See Chapter 2.2.2 *Infection with Aphanomyces astaci (Crayfish plague)*, and Chapter 2.3.1 *Infection with Aphanomyces invadans (epizootic ulcerative syndrome)*.

B. MATERIALS AND BIOLOGICAL PRODUCTS REQUIRED FOR THE ISOLATION AND IDENTIFICATION OF CRUSTACEAN PATHOGENS

1. Crustacean viruses

1.1. Crustacean cell lines

Not applicable. There are currently no confirmed or documented crustacean cell lines.

1.2. Culture media

Not applicable.

1.3. Virus positive controls and antigen preparation

1.3.1. Virus nomenclature

In general, the virus nomenclature used in the disease-specific chapters follows the most recent taxonomy for viruses as given in the Report of the Committee on Taxonomy of Viruses (Fauquet *et al.*, 2005). Also provided in the disease-specific chapters are the disease and virus names that are in common use by the shrimp/prawn farming industries, as well as the more common synonyms that have been used or are in current use.

1.3.2. Virus production

As no cell lines (crustacean, arthropod, or vertebrate) are known that can be used to produce crustacean viruses, infection of known susceptible host species (which are free of infection by the agent in question) is the preferred method for virus production for experimental purposes.

1.3.3. Virus preservation and storage

Infectivity of all of the known crustacean viruses can be preserved by freezing infected whole crustaceans or infected target tissues at -20°C for short-term storage, or at -80°C or lower for long-term storage.

2. Crustacean bacteria

2.1. Culture media

Not applicable for currently listed diseases.

2.2. Storage of cultures

Not applicable for currently listed diseases.

3. Crustacean parasites

3.1. Culture media

Not applicable for currently listed diseases.

3.2. Storage of cultures

Not applicable for currently listed diseases.

4. Crustacean fungus

4.1. Culture media

See chapter 2.2.2 and chapter 2.3.1.

4.2. Storage of cultures

See chapter 2.2.2.

5. Techniques

The methods available for diagnosis of crustacean diseases include the traditional methods of morphological pathology (direct light microscopy, histopathology, and electron microscopy), bioassay methods with susceptible indicator hosts, and molecular methods (gene probes and PCR). While tissue culture is considered to be a standard tool in medical, veterinary, and fish diagnostic laboratories, it has yet to be developed as a usable, routine diagnostic tool for crustacean pathogens. Clinical chemistry has not become a routinely used diagnostic tool by crustacean pathologists.

At the time of writing this section of the *Aquatic Manual*, the available diagnostic methods that may be selected for diagnosis of the OIE listed crustacean diseases or detection of their aetiological agents are based on:

- i) Gross and clinical signs.
- ii) Direct bright-field, phase-contrast or dark-field microscopy with whole stained or unstained tissue wet-mounts, tissue squashes, and impression smears; and wet-mounts of faecal strands.
- iii) Histology of fixed specimens.
- iv) Bioassays of suspect or subclinical carriers using a highly susceptible host (life stage or species) as the indicator for the presence of the pathogen.
- v) Transmission or scanning electron microscopy.
- vi) Antibody-based tests for pathogen detection using immune sera polyclonal antibodies (PABs) or monoclonal antibodies (MAbs).
- vii) Molecular methods (including sequencing where appropriate for strain determination):
 - DNA probes in dot-blot hybridisation assays directly with fresh tissue samples or with extracted DNA;
 - DNA probes or RNA probes for in-situ hybridisation (ISH) assays with histological sections of fixed tissues;
 - Standard and real-time PCR and RT-PCR for direct assay with fresh tissue samples or with extracted DNA or RNA.

There is a paucity of antibody-based diagnostic tests available for the pathogens that cause crustacean diseases. As crustaceans do not produce antibodies, antibody-based diagnostic tests are limited in their application to pathogen detection. While a number of antibody-based diagnostic methods have been developed and are described in the literature, these were developed with mouse or rabbit antibodies generated to specific disease agents purified from infected hosts. As crustacean viruses cannot be routinely cultured *in vitro* (i.e. produced in tissue culture), purified virus from infected hosts has traditionally been used to produce antibody. Recently, recombinant viral structural proteins have been produced from viral sequence information and these proteins have been used to produce antibodies. The absence of crustacean cell culture methods has severely limited the development and availability of this diagnostic tool until recently. The recent application of MAb technologies to this problem has begun to provide a few antibody-based tests. MAbs are available for the agents of several of the OIE listed crustacean diseases (WSSV, TSV, YHV, and IHNV). Antibody-based diagnostic kits/reagents for TSV, WSSV, and YHV infections are currently available from commercial sources.

Molecular methods have been developed and some methods are in widespread use for the detection of many of the viral, bacterial, fungal and protozoan pathogens of the penaeid shrimp (or certain other decapod crustaceans). Nucleic acid-based detection methods are readily available from the literature and some are available in kit form from commercial sources for the OIE listed pathogens TSV, WSSV, and YHV/GAV, IHNV, MBV, BP, and IMNV. PCR or RT-PCR methods are available for all of these viruses and are in routine use by certain sectors of the crustacean aquaculture industry. For the agents of other OIE listed diseases, specific DNA probes tagged with non-radioactive labels are either reported in the literature or are available commercially for application in dot-blot formats with haemolymph or tissue extracts or for use with routine histological sections using ISH.

Samples taken for molecular or antibody-based tests for OIE-listed crustacean diseases may be combined as pooled samples of no more than five specimens per pooled sample of juveniles, subadults and adults. However,

for eggs, larvae and postlarvae pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50 to 150 postlarvae depending on their size/age) may be necessary to obtain sufficient sample material (antigen for antibody-based tests or extracted nucleic acid for molecular tests to run a diagnostic assay).

5.1. Antibody-based tests

5.1.1. Production of rabbit antisera and polyclonal antibodies to crustacean viruses

See disease-specific chapters in this *Aquatic Manual*.

5.1.2. Antisera to crustacean bacteria

Not applicable for currently listed diseases.

5.1.3. Processing and storage of immune sera

See disease-specific chapters in this *Aquatic Manual*.

5.1.4. Mouse monoclonal antibodies to crustacean viruses and bacteria

See disease-specific chapters in this *Aquatic Manual*.

5.2. Direct microscopy

Samples for direct microscopic examination should be examined as soon as possible after collection. Use live specimens whenever possible, or use fresh, chilled, or 10% buffered formalin-fixed specimens when live specimens are not practical. If an adequate field laboratory is available, it should be used to process and examine samples near the site of collection.

5.3. Histological techniques

Collect shrimp (or other decapod crustaceans) by whatever means are available with a minimum of handling stress. Either fix the shrimp (or other decapod crustaceans) tank- or pond-side or transport to the laboratory via a well oxygenated water-filled utensil. Supply adequate aeration to the container if the shrimp (or other decapod crustaceans) are to be left for a short period of time before actual fixation. For the study of presumptively diseased shrimp, (or other decapod crustaceans) select those animals that are moribund, discoloured, displaying abnormal behaviour, or otherwise abnormal, except when sampling is aimed at assessing disease occurrence (e.g. estimation of disease prevalence) for which the preferred selection method is probability sampling.

- i) Have ready an adequate supply of fixative

A general rule is that a minimum of ten volumes of fixative should be used for one volume of tissue sample (i.e. a 10 g sample of shrimp [or other decapod crustacean] would require 100 ml of fixative).

- ii) Davidson's AFA (alcohol, formalin, acetic acid) fixative

Davidson's AFA fixative is recommended for most histological applications. The fixative is rapid, reduces autolytic changes in decapod crustaceans (i.e. especially in the penaeid shrimp, *Macrobrachium rosenbergii*, spiny lobsters of the genus *Panilurus*, and other farmed decapod crustaceans in tropical and subtropical regions), and its acidic content decalcifies the cuticle. The formulation for Davidson's AFA is (for 1 litre):

330 ml 95% ethyl alcohol

220 ml 100% formalin* (a saturated 37–39% aqueous solution of formaldehyde gas)

115 ml glacial acetic acid**

335 ml tap water (for marine crustaceans, sea water may be substituted)

Store the fixative in glass or plastic bottles with secure caps at room temperature.

* Do not use previously made 10% formalin to prepare Davidson's AFA because the formalin content of the Davidson's AFA will be inadequate to provide satisfactory fixation.

** Do not substitute other acids, such as HCl, for acetic acid. Histological sections prepared from HCl–Davidson's solution are not suitable for routine haematoxylin and eosin histological staining.

iii) Fixation procedures with Davidson's AFA

- *For larvae and postlarvae that are too small to be easily injected with fixative using a tuberculin syringe:* Using a fine mesh screen or a Pasteur pipette, select and collect specimens. Immerse shrimp (or other decapod crustaceans) selected for sampling directly in the fixative. Fix for 12–24 hours in fixative and then transfer to 70% ethyl alcohol for storage.
- *For larger postlarvae and very small juveniles that are too small to be injected:* Select and collect specimens. Use a needle or fine-pointed forceps to incise the cuticle and immediately immerse shrimp (or other decapod crustaceans) selected for sampling directly into the fixative. Fix for 12–24 hours in fixative and then transfer to 50–70% ethyl alcohol for storage.
- *For larger postlarvae, juveniles, and adults:* Inject fixative (use 5–10% volume: weight) via needle and syringe (needle gauge dependent on shrimp [or other decapod crustacean] size, i.e. 27 gauge needle for postlarvae and small juveniles) into the living shrimp (or other decapod crustaceans).

The hepatopancreas (HP) should be injected first and at two or more sites, with a volume of fixative sufficient to change the HP to a white to orange colour (when Davidson's AFA is used); then inject fixative into adjacent regions of the cephalothorax, into the anterior abdominal region, and into the posterior abdominal region.

The fixative should be divided between the different regions, with the cephalothoracic region, specifically the HP, receiving a larger share than the abdominal region.

A good guide to insure adequate fixation is to inject an equivalent of 5–10% of the shrimp's (or other crustacean's) body weight; all signs of life should rapidly cease, and visible colour change should occur in the injected areas.

Immediately following injection, slit the cuticle with dissecting scissors, from the sixth abdominal segment to the base of the rostrum, being particularly careful not to cut deeply into the underlying tissue. The incision in the cephalothoracic region should be just lateral to the dorsal midline, while that in the abdominal region should be approximately mid-lateral.

- *For shrimp (and most other crustaceans) larger than ~12 g:* After injection of fixative, the body should then be transversely bisected, at least once, just posterior to the abdomen/cephalothorax junction, and (optional) again mid-abdominally.
- *For very large crustaceans (e.g. lobsters, crabs, adult penaeids, adult *Macrobrachium rosenbergii*, some species and life stages of crabs, etc.):* The organs of interest may be excised after injection of fixative. Completion of fixation of these tissue samples is then handled as outlined previously.

Following injection, incisions and bisection/trisection, or excision of key organs, immerse the specimen in the fixative (use 10:1 fixative:tissue ratio).

Allow fixation to proceed at room temperature for 24–72 hours depending on the size of crustacean being preserved. Longer fixation times in Davidson's AFA may be used to thoroughly decalcify the shell of crabs, lobsters, crayfish, etc.

Following fixation, the specimens should be transferred to 70% ethyl alcohol, where they can be stored for an indefinite period.

Record a complete history of the specimens at the time of collection: gross observations on the condition of the crustacean, species, age, weight, source (wild, or if cultured, pond or tank number, stock number, etc.), and any other pertinent information that may be needed at a later time.

The label should stay with the specimens in the same container during fixation, storage and transport to the laboratory. Always use a No. 2 soft-lead pencil on water-resistant paper (plastic paper is recommended; never use ink or marking pens as the ink is dissolved by alcohol).

iv) Transport and shipment of preserved samples

As large volumes of alcohol should not be posted or shipped, the following methods are recommended: Remove the specimens from the 70% ethyl alcohol. For larvae, postlarvae, or small juveniles, use leak-proof, screw-cap plastic vials if available; if glass vials must be used, pack to prevent breakage. For larger specimens, wrap samples with white paper towels to completely cover (do not use raw or processed cotton). Place towel-wrapped specimens in a sealable plastic bag and saturate with 70% ethyl alcohol. Insert the label and seal the bag. Place the bag within a second sealable bag. Multiple small sealable bags can again be placed within a sturdy, crush-proof appropriately labelled container for shipment (for details see *Aquatic Code Chapter 5.10 Measures concerning international transport of aquatic animal pathogens and pathological material*).

5.4. Transmission or scanning electron microscopy

Electron microscopy (EM – transmission or scanning) is a valuable research tool for the study of disease in crustaceans. However, EM methods are only available as confirmatory methods for some diseases and are not routinely used for diagnosis of the diseases listed by the OIE. Hence, the application of EM is limited to specific purposes (see disease-specific chapters in this *Aquatic Manual*).

5.5. Use of molecular and antibody-based techniques for confirmatory testing and diagnosis

5.5.1. Sample preparation and types

Samples selected for nucleic acid-based or antibody-based diagnostic tests should be handled and packaged (in new plastic sample bags or bottles) with great care to minimise the potential for cross contamination among the sample set taken from different (wild or farmed) stocks, tanks, ponds, farms, etc. New plastic sample bags or bottles must be used. A water-resistant label, with the appropriate data filled out in No. 2 pencil, should be placed within each package or container for each sample set.

Some suitable methods for preservation and transport of samples taken for molecular or antibody-based tests are:

- i) *Live specimens*: these may be processed in the field or shipped to the diagnostic laboratory for testing.
- ii) *Haemolymph*: this tissue is the preferred sample for certain molecular and antibody-based diagnostic tests (see disease-specific chapters). Samples may be collected by needle and syringe through cardiac puncture, from the haemocoel (i.e. the ventral sinus in penaeids), or from a severed appendage, and immediately transferred to a tube that is half full with ~90–95% ethanol or suitable preservative (e.g. RNALater®) to preserve the sample.
- iii) *Iced or chilled specimens*: these are for specimens that can be transported to the laboratory for testing within 24 hours. Pack samples in sample bags surrounded by an adequate quantity of wet ice around the bagged samples in a Styrofoam™¹-insulated box and ship to the laboratory.
- iv) *Frozen whole specimens*: select live specimens according to the criteria listed in disease-specific chapters in this *Aquatic Manual*, quick freeze in the field using crushed dry-ice, or freeze in the field laboratories using a mechanical freezer at –20°C or lower temperature. Prepare and insert the label into the container with the samples, pack samples with an adequate quantity of dry-ice in a Styrofoam™-insulated box, and ship to the laboratory.
- v) *Alcohol-preserved samples*: in regions where the storage and shipment of frozen samples is problematic, 90–95% ethanol may be used to preserve, store, and transport certain types of samples for molecular tests. Alcohol preserved samples are generally not suitable for antibody-based tests. Whole crustaceans (any life stage provided the specimen is no larger than 2–3 g), excised tissues (i.e. pleopods) from large crustaceans, or haemolymph may be preserved in 90–95% ethanol, and then packed for shipment according to the methods described in Section 5.3, paragraph iv (see chapter 5.10 of the *Aquatic Code* for additional details on the international transport of such samples).

5.5.2. Preservation of RNA and DNA in tissues

For routine diagnostic testing by PCR, RT-PCR or for dot-blot tests with DNA probes, samples must be prepared to preserve the pathogen's nucleic acid. For most purposes, preservation of samples in alcohol (70–100%) is the preferred method for subsequent molecular tests. Other products (e.g. RNALater, various lysis buffers, etc.) are commercially available for the same purpose and information on these products is given in disease-specific chapters in this *Aquatic Manual*, where applicable.

5.5.3. DNA extraction

See disease-specific chapters in this *Aquatic Manual*.

5.5.4. RNA extraction

See disease-specific chapters in this *Aquatic Manual*.

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*.

5.5.5. Preparation of slides for *in-situ* hybridisation

See disease-specific chapters in this *Aquatic Manual*.

6. Additional information to be collected

See disease-specific chapters in this *Aquatic Manual* for recommendations on any additional information that may be required or that may assist the diagnostic laboratory in determining the most appropriate test(s) to be run for submitted samples.

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NB: FIRST ADOPTED IN 2000. MOST RECENT UPDATES ADOPTED IN 2012.