WORLD ORGANISATION FOR ANIMAL HEALTH
*Protecting animals, preserving our future*

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*January and February 2022*

# REPORT OF THE MEETING OF THE OIE AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

**Virtual meeting, 24 & 27 January, 16–23 February 2022**

**PART B – Texts for Member comments and information**

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The OIE Aquatic Animal Health Standards Commission (the Aquatic Animals Commission) held its meeting electronically from 24 & 27 January and 16–23 February 2022. The list of participants is attached as **Annex 1**.

To facilitate the virtual 89th Annual General Session in May 2022, the February 2022 meeting report of the Aquatic Animals Commission will be distributed in two parts: **Part A** (available on the OIE website) provided information about the new and revised texts for the *Aquatic Code* and the *Aquatic Manual* that will be proposed for adoption at the 89th General Session; and **Part B** (herewith) provides information about other topics discussed at the Commission's February 2022 meeting including texts circulated for comments and information.

The Aquatic Animals Commission wished to thank the following Members for providing written comments on draft texts for the OIE *Aquatic Animal Health Code* (hereinafter referred to as the *Aquatic Code*) and OIE *Manual of Diagnostic Tests for Aquatic Animals* (hereinafter referred to as the *Aquatic Manual*) circulated in Part B of the Commission's February 2021 report: Chile, China (People's Rep. of), Colombia, Japan, New Caledonia, Norway, Switzerland, Thailand, United States of America (the USA) and the Member States of the European Union (the EU). The Commission also wished to acknowledge the valuable advice and contributions from numerous experts of the OIE scientific network.

The Commission reviewed all comments that were submitted on time and were supported by a rationale. The Commission made amendments to draft texts, where relevant, in the usual manner by '<u>double underline</u>' and '~~strikethrough~~'. In relevant Annexes, amendments proposed at this meeting are highlighted with a coloured background in order to distinguish them from those made previously. Due to the large number of comments, the Commission was not able to provide a detailed explanation for the reasons for accepting or not each of the comments considered, and focused its explanations on significant issues. Where amendments were of an editorial nature, no explanatory text has been provided. The Commission wished to note that not all texts proposed by Members to improve clarity were accepted; in these cases, it considered the text clear as currently written.

The Aquatic Animals Commission reminded Members that *ad hoc* Group reports can be found on the OIE Website: https://www.oie.int/en/what-we-do/standards/standards-setting-process/ad-hoc-groups/. The Commission encourages Members to consider relevant information in previous Commission and *ad hoc* Group reports when preparing comments, especially on longstanding issues.

The table of contents includes all of the agenda items presented in Part B (herewith) of the Commission's February report and includes links to relevant items within this report. Members should note that texts in **Annexes 2 to 12** are presented for Member comments, and **Annexes 13 to 14** for information.

Comments on relevant texts in this report must reach OIE Headquarters by the **<u>15 July 2022</u>** to be considered at the September 2022 meeting of the Aquatic Animals Commission.

All comments should be sent to the OIE Standards Department at: AAC.Secretariat@oie.int.

Comments should be submitted as Word files rather than pdf files because pdf files are difficult to incorporate into the Commission's working documents.

Comments should be presented in the relevant Annex, and include any amendments to the proposed text, supported by a structured rationale or by published scientific references. Proposed deletions should be indicated in '~~strikethrough~~' and proposed additions with 'double underline'. Members should not use the automatic 'track-changes' function provided by Word processing software, as such changes may be lost in the process of collating Members' submissions into working documents. Members are also requested not to reproduce the full text of a chapter as this makes it easy to miss comments while preparing the working documents.

The Aquatic Animals Commission strongly encourages Members to participate in the development of the OIE's international standards by submitting comments on this report.

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## 1. WORK PLAN OF THE AQUATIC ANIMALS COMMISSION

The Aquatic Animals Commission considered ongoing work plan items at its September 2021 meeting and the anticipated milestones for their completion. The workplan for ongoing work was circulated for comment in the Commission's September 2021 report.

The Commission convened an additional meeting in November 2021 to review and prioritise new work which was added to the workplan. Prioritisation of any new work took into account multiple factors including the impact of and expected improvement to the standards, benefit to Members, Member comments, activities of the OIE Aquatic Animal Health Strategy, OIE Headquarters' comments, and progress on the previous Commission's work plan.

The Commission noted that the progression of work plan items that were contingent on the convening of *ad hoc* Groups were anticipated to progress as planned for 2022.

The work plan of the Aquatic Animals Commission is presented as **Annex 2** for Member comments.

## 2. THE OIE *AQUATIC ANIMAL HEALTH CODE*

### 2.1. Texts for Member comment

#### 2.1.1. Safe Commodities – Articles X.X.3. for disease-specific chapters

*Background*

At its September 2020 meeting, the Aquatic Animals Commission reviewed Article X.X.3. of all disease-specific chapters to address comments that the recommended time/temperature treatments in these articles represented different levels of thermal treatment and that some were not commercially feasible as they would diminish product quality. The proposed amendments to Articles X.X.3. throughout the *Aquatic Code* have been made to specify the time/temperature treatments required to inactivate the pathogenic agent. The Commission noted that this is a change from the current commodity-based approach and was made in response to Member comments that some of the levels of thermal treatment in the current text were inconsistent or not commercially feasible as they would diminish product quality.

The Commission agreed to begin with a review of Section 9. Diseases of Crustaceans and developed an example article, Article 9.8.3. Infection with white spot syndrome virus, to demonstrate the suggested approach. The Commission noted that it was difficult to propose a uniform model Article X.X.3. because of differences in time/temperature treatments and product types listed in Article X.X.3. The Commission circulated the example article, Article 9.8.3., for comment in its September 2020 report.

At its February 2021 meeting, the Commission considered comments on the example Article 9.8.3. and applied these amendments to Article 9.X.3. in all of the disease-specific chapters in Section 9. Diseases of crustaceans, of the *Aquatic Code*. The time/temperature treatments provided in Articles 9.X.3. were amended in line with the information provided in the 'Safe commodity assessments for OIE listed aquatic animal diseases' published in 2016. The Commission also proposed specific thermal treatment requirements for meal. The revised Articles 9.X.3. were circulated for comments in the Commission's February 2021 report.

At its September 2021 meeting, the Commission reviewed comments and revised the proposed Articles 9.X.3. to improve clarity including re-ordering the aquatic animal products and circulated the revised Articles 9.X.3 for comments.

#### *2.1.1.1. Revised Articles 8.X.3. for amphibian disease-specific chapters*

**Previous Commission reports where this item was discussed:**

September 2020 (Item 4.7., page 10); February 2021 (Part B: Item 1.4., page 8); September 2021 (Item 5.1.5., page 24).

**February 2022 meeting**

As agreed at its September 2021 meeting, the Aquatic Animals Commission amended Article 8.X.3. of all amphibian disease-specific chapters to align with the approach proposed for Article 9.X.3. (crustacean) and Article 10.X.3. (fish) of disease-specific chapters.

The time/temperature treatments provided in Article 8.X.3. was based on information provided in the Safe commodity assessments for OIE listed aquatic animal diseases, published in 2016. These articles were also amended to ensure alignment with the relevant changes being proposed for adoption in Article 9.X.3 (crustacean) and Article 10.X.3. (fish).

The revised Articles 8.X.3. for amphibian disease-specific chapters are presented as **Annex 3** for Member comments.

#### *2.1.1.2. Revised Articles 11.X.3. for mollusc disease-specific chapters*

**Previous Commission reports where this item was discussed:**

September 2020 (Item 4.7., page 10); February 2021 (Part B: Item 1.4., page 8); September 2021 (Item 5.1.5., page 24).

**February 2022 meeting**

As agreed at its September 2021 meeting, the Aquatic Animals Commission amended Article 11.X.3. of all mollusc disease-specific chapters to align with the approach proposed for Article 9.X.3. (crustacean) and Article 10.X.3. (fish) of disease-specific chapters.

The time/temperature treatments provided in Article 11.X.3. was based on information provided in the Safe commodity assessments for OIE listed aquatic animal diseases, published in 2016. These articles were also amended to ensure alignment with the relevant changes being proposed for adoption in Article 9.X.3. (crustacean) and Article 10.X.3. (fish).

The Commission wished to inform Members that it has included a review of the safe commodity assessments on its work plan to ensure that the time/temperatures for inactivation of pathogenic agents is based on current scientific evidence.

The Commission noted that for some mollusc diseases, no thermally treated safe commodities are currently listed in the *Aquatic Code*. This is because no products were considered relevant for these diseases and their susceptible hosts at the time the safe commodity assessments were originally undertaken. The Commission agreed that this should be re-considered when the safe commodity assessments are reviewed.

Due to insufficient evidence on the time/temperature requirements for inactivation of some mollusc diseases, the Commission agreed to maintain the current thermal treatment requirements for hermetically sealed mollusc products in Articles 11.X.3. until the Safe Commodity Assessments can be updated to reflect any new scientific information.

The Commission also amended the time period which included a fraction of a minute to minutes and seconds to facilitate translation into different languages.

The revised Articles 11.X.3. for mollusc disease-specific chapters are presented as **Annex 4** for Member comments.

#### 2.1.2. Article 9.3.1. of Chapter 9.3. Infection with *Hepatobacter penaei* (Necrotising hepatopancreatitis)

The Aquatic Animals Commission agreed to amend Article 9.3.1. to ensure consistency with Chapter 1.3.

The revised Article 9.3.1. of Chapter 9.3. Infection with *Hepatobacter penaei* (Necrotising hepatopancreatitis), is presented as **Annex 5** for Member comments.

#### 2.1.3. Article 9.4.1. and 9.4.2. of Chapter 9.4. Infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV)

The Aquatic Animals Commission agreed to amend Article 9.4.1. to reflect an update in the taxonomic classification of infection with IHHNV and to ensure consistency with other disease-specific chapters (see Item 3.1.1.3.).

In Article 9.4.2., the Commission agreed to list crustacean species susceptible to IHHNV, in line with the convention to list susceptible species alphabetically according to common name (see Item 3.1.1.3.).

The revised Articles 9.4.1. and 9.4.2. of Chapter 9.4. Infection with infectious hypodermal and haematopoietic necrosis virus, are presented as **Annex 6** for Member comments.

### 2.2. Texts for Member information

#### 2.2.1. Emerging diseases

##### *2.2.1.1. Infection with carp edema virus (CEV)*

Comments were received from Japan.

*Background*

At its February 2020 meeting, the Aquatic Animals Commission reviewed the scientific information on infection with CEV and agreed it met the OIE definition of an 'emerging disease' and, as such, Members should report it in accordance with Article 1.1.4. of the *Aquatic Code*. At its September 2020 meeting, the Commission informed Members that it continued to monitor the situation and requested Members to report infection with CEV as an emerging disease.

At its February 2021 meeting, the Commission reviewed Member comments and any new evidence on infection with CEV. The Commission also circulated a comprehensive list of the evidence it had reviewed for Member's information and reiterated that infection with CEV meets the definition of an emerging disease.

At its September 2021 meeting, the Commission reviewed the latest scientific evidence and noted that infection with CEV continues to be reported to impact production and cause mortality events in wild and farmed populations but the severity of the impacts is unclear. The Commission reiterated that detections of infection with CEV should be reported to the OIE as an emerging disease, in accordance with Article 1.1.4. of the *Aquatic Code*.

**Previous Commission reports where this item was discussed:**

February 2020 (Item 7.3.3., page 17); September 2020 (Item 6.3., page 17); February 2021 (Part B: Item 2.2., page 11); September 2021 (Item 5.2.1.1., page 27).

**February 2022 meeting**

The Commission reviewed the latest scientific information for infection with CEV and noted the following evidence further confirmed the severity of infection with CEV in some regions of the world:

- infection with CEV was found to be the causative pathogenic agent for severe mortality in wild adult common carp (*Cyprinus carpio*) in Italy in mid-June 2020 (Marsella *et al*., 2021);

- during the outbreak and mass mortality event in the wild common carp population in Lake Swartout, Minnesota, USA, high CEV viral loads were present (Tolo *et al*., 2021);

- a survey to determine the prevalence of CEV in Germany detected CEV in 69% of common carp (*Cyprinus carpio*) populations and 41% of koi carp (a variety of *Cyprinus carpio*) populations. In addition, most fish sampled from clinically affected common carp or koi carp populations harboured high virus loads. The authors concluded that infection with CEV was highly prevalent in Germany and implied the spread of the disease through intense trading of common carp and koi carp (Ademek *et al*., 2021);

- the blood chemistry profile of CEV infected common carp revealed that CEV infection exerts complex adverse effects and results in severe metabolic disturbance due to impaired gill respiratory and excretory function (Pikula *et al*., 2021).

Findings reported in the late 1990s (Way *et al*., 2015) and in the early 2000s (Haenen *et al*., 2014) only showed the possibility of infection with CEV distribution in very limited areas of Europe and USA. The rapid spread to other countries and induced mortalities since 2009 confirms condition a) of the definition of emerging disease is met. The Commission also considered that the culture of common carp is considered traditional aquaculture in many countries and that mortalities caused by infection with CEV has aroused great concern among scientists and ornamental breeders with more reports and scientific literature published every year.

The Commission also noted that the genome of CEV published in 2021 (Mekata *et al*., 2021) will assist in the promotion of epidemiological studies, phylogenetic analysis of CEV and development of new diagnostic assays for infection with CEV in the future.

Based on this review of the latest scientific evidence, and in conjunction with information provided in previous reports of the Commission, the Commission determined that infection with CEV continues to meet the definition of an emerging disease. The Commission reminded Members that any detections of infection with CEV should be reported to the OIE as an emerging disease, in accordance with Article 1.1.4. of the *Aquatic Code*.

The Commission informed Members that a technical disease card for infection with CEV is under development and will be published on the OIE website.

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WAY, K., MAIRTIN, P., READING, A., WILLIAMS, C., ENGELSMA, M., HAENEN, O. & STONE, D. (2015). Detection of carp edema-like virus in archive DNA and tissue sampled from disease outbreaks in common carp (*Cyprinus carpio*) in the UK and the Netherlands: a link with spring carp mortality syndrome. *EAFP 17th International Conference on disease of fish and shellfish, Las Palmas de Gran Canaria 7–11 September 2015*. **Poster P-029**, p 253

#### *2.2.1.2. Infection with Entercytozoon hepatopenaei (EHP)*

Comments were received from New Caledonia.

*Background*

At its September 2021 meeting, the Aquatic Animals Commission noted that there have been reports of significant economic and production impacts related to infection with EHP, particularly in Asia, that have been ongoing for some time. It also noted that there are available diagnostic methods and evidence that EHP can be spread through trade. The Commission agreed that infection with EHP meets the definition of an emerging disease and that any detections of infection with EHP should be reported to the OIE as an emerging disease, in accordance with Article 1.1.4. of the *Aquatic Code*.

**Previous Commission report where this item was discussed:**

September 2021 (Item 5.2.1.2., page 28).

**February 2022 meeting**

The Commission noted that there was no new scientific information that would change the assessment of infection with EHP as meeting the definition of an emerging disease. The Commission reminded Members that any detections of infection with EHP should be reported to the OIE as an emerging disease, in accordance with Article 1.1.4. of the *Aquatic Code*.

The Commission informed Members that a technical disease card for infection with EHP is under development and will be published on the OIE website.

## 3. OIE *MANUAL OF DIAGNOSTIC TESTS FOR AQUATIC ANIMALS*

### 3.1. Texts for Members' comment

The Aquatic Animals Commission wished to remind Members that it has commenced the process of progressively reformatting the disease-specific chapters of the *Aquatic Manual* into a new template. As the reformatted and updated chapters have substantial changes, the Commission agreed at its meeting in September 2019 that only clean versions of the chapters would be provided in the report. Subsequent changes made to these initial revisions following Member comments would be indicated in the usual style (i.e. strikethrough for deletions and double underline for additions).

A software-generated document that compares the adopted version of a chapter and the proposed new text will be created. This comparison document will not be included in the Commission's report, but will be made available upon request from the OIE Standards Department (AAC.Secretariat@oie.int).

The Commission has commenced the process of reviewing Chapter 2.2.0. *General Information* (*diseases of crustaceans*) and will consult all crustacean disease Reference Laboratory experts in this review. The Commission noted that some changes will be necessary to the revised crustacean disease-specific chapters once the draft revision of Chapter 2.2.0 is complete to ensure complementarity.

The process of reviewing the updated and reformatted chapters has brought to light the wide variation among the chapters in the level of detail given in the description of the polymerase chain reaction (PCR) methods in Section 4.4. *Nucleic acid amplification*, and how the information is presented. The Commission decided to address this issue by developing a template for the description of PCR methods, which will include concise, uniform and generic text on nucleic acid extraction methods, cycling parameters and the controls used in the tests, while giving all the information necessary on the primer and probe sequences in table format. The proposed template will be sent to the OIE Reference Laboratories for their input, and finalised at the next meeting in September 2022. Once finalised it will be applied to the chapters.

#### 3.1.1. Section 2.2. Diseases of Crustaceans

##### *3.1.1.1. Chapter 2.2.1. Acute hepatopancreatic necrosis disease*

According to the scope of the chapter, acute hepatopancreatic necrosis disease (AHPND) means infection with strains of *Vibrio parahaemolyticus* ($Vp_{AHPND}$) that contain a ~70-kbp plasmid with genes that encode homologues of the *Photorhabdus* insect-related (Pir) toxins, PirA and PirB. The Commission is aware of reports of AHPND caused by other *Vibrio* species. At the next meeting in September 2022, the Commission will assess, in consultation with the two OIE Reference Laboratories, any published information received on non-*Vibrio parahaemolyticus* species that have been associated with AHPND.

The Aquatic Animals Commission reviewed Chapter 2.2.1. Acute hepatopancreatic necrosis disease, which had been updated by the OIE Reference Laboratory experts and reformatted using the new disease chapter template.

The main amendments include:

– updated information on the aetiological agent;

– updated sections on disease pattern, biosecurity and disease control strategies, and on specimen selection, sample collection, transportation and handling;

– updated the section on diagnostic methods including completing Table 4.1.; and

– revised definitions of suspect and confirmed case in apparently healthy and clinically affected animals; and

– completed the tables in the section on diagnostic sensitivity and specificity for diagnostic tests.

The revised Chapter 2.2.1. Acute hepatopancreatic necrosis disease, is presented as **Annex 7** for Member comments.

#### *3.1.1.2. Chapter 2.2.3. Infection with* Hepatobacter penaei *(necrotising hepatopancreatitis)*

The Aquatic Animals Commission reviewed Chapter 2.2.3. Infection with *Hepatobacter penaei* (necrotising hepatopancreatitis), which had been updated by the OIE Reference Laboratory experts and reformatted using the new disease chapter template.

The main amendments include:

– updated information on the aetiological agent;

– updated sections on agent factors, disease pattern, biosecurity and disease control strategies, and on specimen selection, sample collection, transportation and handling;

– updated the section on diagnostic methods including completing Table 4.1. and revising the section on wet mounts, on nucleic acid amplification and molecular tests;

– revised definitions of suspect and confirmed case in apparently healthy and clinically affected animals; and

– completed the tables in the section on diagnostic sensitivity and specificity for diagnostic tests.

The revised Chapter 2.2.3. Infection with *Hepatobacter penaei* (necrotising hepatopancreatitis), is presented as **Annex 8** for Member comments.

#### *3.1.1.3. Chapter 2.2.4. Infection with infectious hypodermal and haematopoietic necrosis virus*

The Aquatic Animals Commission reviewed Chapter 2.2.4. Infection with infectious hypodermal and haematopoietic necrosis virus, which had been updated by the OIE Reference Laboratory experts and reformatted using the new disease chapter template.

The main amendments include:

– updated the scope of the chapter;

– updated sections on agent factors, disease pattern, biosecurity and disease control strategies, and on specimen selection, sample collection, transportation and handling;

– updated the section on diagnostic methods including completing Table 4.1. and revising the molecular tests and bioassay; and

– revised definitions of suspect and confirmed case in apparently healthy and clinically affected animals.

The revised Chapter 2.2.4. Infection with infectious hypodermal and haematopoietic necrosis virus, is presented as **Annex 9** for Member comments.

### 3.1.2. Section 2.3. Diseases of fish

#### *3.1.2.1. Chapter 2.3.1. Infection with* Aphanomyces invadans *(epizootic ulcerative syndrome)*

*Background*

At its September 2021 meeting, the Aquatic Animals Commission reviewed the first draft of Chapter 2.3.1. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome), which had been reformatted using the new disease chapter template. The Commission decided to work further on the revision and review the revised chapter again at its February 2022 meeting.

**Previous Commission report where this item was discussed:**

September 2021 (Item 6.2.2., page 41).

**February 2022 meeting**

The Aquatic Animals Commission reviewed Chapter 2.3.1. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome), which had been updated by the OIE Reference Laboratory experts and reformatted using the new disease chapter template.

The main amendments include:

– updated the scope of the chapter;

– updated information on the aetiological agent;

– updated sections on disease pattern, biosecurity and disease control strategies, and on specimen selection, sample collection, transportation and handling;

– updated the section on diagnostic methods including completing Table 4.1. and revising the description of squash preparation methods and molecular tests; and

– revised definitions of suspect and confirmed case in apparently healthy and clinically affected animals.

The revised Chapter 2.3.1. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome), is presented as **Annex 10** for Member comments.

### *3.1.2.2. Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus*

Comments were received from China (People's Rep. of), Colombia, Switzerland, Thailand, the USA and the EU.

*Background*

The Aquatic Animals Commission reviewed Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus, which had been updated by the OIE Reference Laboratory experts and reformatted using the new disease chapter template. The revised chapter was circulated for Member comments in the Commission's September 2021 report.

**Previous Commission report where this item was discussed:**

September 2021 (Item 6.1.3., page 31).

**February 2022 meeting**

The Commission did not agree with comments requesting that Section 2.2.2. Species with incomplete evidence for susceptibility, be deleted. The section, which is included in all revised *Aquatic Manual* chapters, is considered to provide information that is useful to guide research and risk assessments.

In Section 2.2.3. Likelihood of infection by species, host life stage, population or sub-populations, the Commission did not agree to move a sentence giving the weight of the three life stages in Table 4.1. to Chapter 2.3.0. *General information* (*diseases of fish*). The Commission noted that there may be differences among life stages among the susceptible host species for different fish diseases.

For clarity, the Commission agreed to replace 'has a high case fatality rate' with 'once infected most fish succumb to the disease' in Section 2.3.1. Mortality, morbidity and prevalence.

In Section 2.4.5. Inactivation methods, the Commission agreed to add that EHNV in cell culture supernatant is resistant 'to 60°C for 15 minutes' to improve the accuracy of the sentence.

In Section 3.5.2. Preservation of samples for molecular detection, the Commission did not agree to include more information on other preservation options but instead agreed to replace the text with a cross reference to Chapter 2.3.0. Section B.2.5. Use of molecular techniques for surveillance testing, confirmatory testing and diagnosis. This is a horizontal amendment that will also be made to the template.

Likewise, in Section 3.5.3. Samples for histopathology, immunohistochemistry or *in-situ* hybridisation, the Commission agreed to replace the text with a cross reference to Chapter 2.3.0. Section B.2.2. as had been agreed as a horizontal amendment at the last meeting. The text is already included in the template.

At the last meeting in September 2021, the Commission had proposed amendments to the explanatory text for Table 4.1. OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals. At this meeting, the text was finalised and is being applied to all disease-specific chapters (see Item 3.2.2.).

In Table 4.1., the Commission added the rating '++' to all life stages for the conventional PCR purpose C. Confirmatory diagnosis of a suspect result from surveillance or presumptive diagnosis, and '1' as the level of validation.

In Section 4.2. Histopathology and cytopathology, two sentences were deleted form the last paragraph as they were electron microscopic observations and not relevant in a diagnostic context.

In Section 4.4.1. Real-time PCR, a Member asked why RNA extraction kits were mentioned given that EHN is a DNA virus. The Reference Laboratory experts advised that the kits are fit-for-purpose for extracting DNA and that the text states that they are used 'in the OIE Reference Laboratory' and that 'commercially available nucleic acid extraction kits may be used'.

Also in Section 4.4.1., a Member questioned the reference to PCR amplification of 45 cycles of 95°C for 15 seconds. The Reference Laboratory experts advised that use of 45 cycles increases the sensitivity of the test and reduces the risk of false-negative test results.

In Table 4.4.1.1. Ranavirus primer and probe sequences and Table 4.4.2.1. MCP-1 and MCp-2 primer sequences, the Commission agreed to add '(5'–3')' after 'Sequence' in the heading and to delete '5-' and '3-' from the primer sequences themselves to have a consistent approach.

In Section 4.4.2. Conventional PCR, the Commission agreed to replace 'ethidium bromide' with 'containing SYBRTM Safe (Thermo Fisher Scientific) or equivalent'. This amendment will be applied throughout the *Aquatic Manual*.

In Section 4.7., Immunohistochemistry, added the biotin-labelled secondary antibody incubation step as it had been omitted.

The revised Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus, is presented as **Annex 11** for Member comments.

### *3.1.2.3. Chapter 2.3.7. Infection with red sea bream iridoviral disease*

Comments were received from China (People's Rep. of), Colombia, Switzerland, Thailand, the USA and the EU.

*Background*

At its September 2021 meeting, the Aquatic Animals Commission reviewed Chapter 2.3.7. Infection with red sea bream iridovirus, which had been updated by the OIE Reference Laboratory experts and reformatted using the new disease chapter template.

The Commission noted that other viruses in the Genus *Megalocytivirus*, for example, infectious spleen and kidney necrosis virus (ISKNV) and turbot reddish body iridovirus (TRBIV), may also cause disease of fish. These viruses are not currently listed by the OIE and are not included within the scope of the infection with red sea bream iridovirus (RSIV) chapter. If ISKNV, TRBIV or other megalocytiviruses were to be listed, the viruses would need to be assessed against the listing criteria in Chapter 1.2. of the *Aquatic Code*. If they were found to fulfil the listing criteria, they could be proposed for listing to the OIE Assembly. In the meantime, this chapter remains focused on infection with RSIV. The revised chapter was circulated for comments in the Commission's September 2021 report.

**Previous Commission report where this item was discussed:**

September 2021 (Item 6.1.6., page 38).

**February 2022 meeting**

The Commission did not agree to add to Section 1. Scope that RSIV is one of the genotypes of infectious spleen and kidney necrosis virus (ISKNV). The Commission noted that there are many viruses, including RSIV, are not designated formally by ICTV[1], but RSIV is listed by the OIE. The Commission stressed that the aetiological agent is discussed in Section 2.1.1.

In Section 2.1.1. Aetiological agent, the Commission agreed to include pompano iridovirus (PIV) and a supporting reference as a synonym of RSIV. The Commission also agreed to editorial changes to clarify the text in this section.

In Section 2.2.1. Susceptible host species, the Commission agreed to correct the classification of the Japanese jack mackerel (*Trachurus japonicus*) by moving it from the family Haemulidae to the family Carangidae. The Commission did not agree to change '*Lateolabrax* sp.*'* to *'Lateolabrax* spp.*'* but preferred to wait for the *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases to assess the proposal.

As described for item 3.1.2.2 above, the Commission did not agree with comments requesting that Section 2.2.2. Species with incomplete evidence for susceptibility, be deleted.

1 ICTV: International Committee on the Taxonomy of Viruses

In Section 2.2.4. Distribution of the pathogen in the host, the Commission agreed to include 'other organs' to the list of organs where infected cells are observed as the pathogen characteristically causes systemic infections so most, if not all, organs can be infected.

In Section 2.3.4. Modes of transmission and life cycle, the Commission did not agree to replace 'water', the principal mode of horizontal transmission of RSIV, with 'contaminated water or cohabitation with RSIV infected fish' as the proposal did not improve the clarity of the text.

In Section 2.3.6. Geographical distribution, the Commission agreed to replace countries where RSIV have been detected with continents in accordance with the template. The Commission also agreed to delete a sentence and reference on the role of international trade in spreading the viruses as it is out of place in this section.

In Section 3.5.2. Preservation of samples for molecular detection, the Commission did not agree to include more information on other preservation options but instead agreed to replace the text with a cross reference to Chapter 2.3.0. Section B.2.5. Use of molecular techniques for surveillance testing, confirmatory testing and diagnosis. This is a horizontal amendment that will also be made to the template.

Likewise, in Section 3.5.3. Samples for histopathology, immunohistochemistry or *in-situ* hybridisation, the Commission agreed to replace the text with a cross reference to Chapter 2.3.0. Section B.2.2 as had been agreed as a horizontal amendment at the last meeting. The text is already included in the template.

At the last meeting in September 2021, the Commission had proposed amendments to the explanatory text for Table 4.1. OIE recommended diagnostic methods and their level of validation for surveillance of apparently health animals and investigation of clinically affected animals. At this meeting, the text was finalised and is being applied to all disease-specific chapters (see Item 3.2.2).

In Table 4.1., the Commission added the ratings to all life stages for histopathology, the indirect fluorescent antibody test, cell culture, and both real-time and the conventional PCR for the purpose C. Confirmatory diagnosis of a suspect result from surveillance or presumptive diagnosis. The ratings are consistent with the case definitions given in Section 6. Corroborative diagnostic criteria.

In Section 4.2. Histopathology and cytopathology, the Commission did not agree to include 'other organs' with those listed that may reveal abnormally enlarged cells in the histological section as enlarged cells are typical histopathological diagnostic change and will not appear in all infected organs.

In Section 4.3. Cell culture for isolation, the Commission agreed to delete the text stating that isolation of the viruses from freshwater fish such as gourami is difficult because it applies to ISKNV genotypes and not to RSIV genotypes.

In Section 4.4.2. Conventional PCR, the Commission noted that the primer sequences for 4-F and 4-R, which specifically amplify RSIV, had been omitted. The sequences were reinstated along with the expected sizes of the generated amplicons.

In Section 4.5. Amplicon sequencing, a Member proposed that more information be included on interpretation of the results of Sanger sequence generated from a PCR-positive sample because of the complexity caused by the sequence overlap among strains of the RSIV genotype and strains of ISKNV and TRBIV genotypes. The Commission acknowledged the complexity of RSIV, but also inconsistencies in labelling the published sequences, including those in GenBank/EMBL, with some researchers publishing sequences as ISKNV while others publishing their sequences as RSIV, leading to the requirement for phylogenetic analysis. The Commission agreed that an approach similar to that taken in Chapter 2.3.3. Infection with *Gyrodactylus salaris* – in which a Table of GenBank accession numbers for nucleotide sequences has been included – would be a useful addition

to the chapter. The Commission agreed to work further on this issue and review the draft table at its September meeting.

In Section 6.1.2. Definition of confirmed case in apparently healthy animals and Section 6.2.2. Definition of confirmed case in clinically affected animals, the Commission did not agree to delete 'with sequence consistent with RSIV' following confirmation by sequence analysis because amplified PCR products should be sequenced and analysed in comparison with the target gene.

The revised Chapter 2.3.7. Infection with red sea bream iridovirus, is presented as **Annex 12** for Member comments.

## 3.2. Texts for Members' information

### 3.2.1. Guidance document on the use of environmental DNA methods for aquatic animal disease surveillance

Comments were received from Canada, China (People's Rep. of), Colombia, Switzerland, Thailand, the UK, the USA, the EU and AU-IBAR.

*Background*

The monitoring of aquatic systems using environmental DNA (eDNA) is a rapidly advancing research field that will provide opportunities for rapid, cost-effective, non-destructive methods to screen for pathogens, especially in wild aquatic populations where sampling may be difficult or removal of animals undesirable. The Aquatic Animals Commission is aware that eDNA methods exist for detecting pathogenic agents of several listed diseases, including infection with *Xenohaliotis californiensis*, infection with *Batrachochytrium dendrobatidis*, infection with *Aphanomyces astaci* and infection with *Gyrodactylus salaris*.

The Commission agreed that as these methods are available and currently in use, it would be advisable for guidance to be provided on appropriate application and potential limitations. The Commission noted that as accurate estimates of diagnostic performance are not available for designing surveillance programmes using eDNA assays, data obtained from eDNA methods may not be suitable to support declaration of freedom from listed diseases. The Commission also noted that confirmation of infection by listed diseases could not be made using eDNA methods; however, positive results could be appropriate criteria for a suspect case.

At its February 2021 meeting, the Commission developed a discussion document outlining the benefits and limitations of eDNA detection within a diagnostic or disease surveillance context. This document is intended to guide the appropriate purposes of use and assay performance reporting required for an eDNA assay to be considered for inclusion in the *Aquatic Manual*.

At its September 2021 meeting, the Commission revised the discussion document and noted that it would be circulated for a second round of comments after which it would be published on the OIE website.

**Previous Commission reports where this item was discussed:**

February 2020 (Item 8.4.2., page 22); September 2020 (Item 6.4., page 17); February 2021 (Part B: Item 3.1., page 12); September 2021 (Item 6.1.1., page 30).

**February 2022 meeting**

A number of Members commented that eDNA methodologies need to be further developed and validated, should not be used alone to support a declaration of disease freedom or to confirm a positive case, and pose risks from false positive results. The Commission noted that all of these

comments actually support the conclusions of the document. The Commission wished to emphasise the need to carefully consider in advance the application of eDNA methods in a surveillance programme and how any positive results will be interpreted and further investigated.

The Commission reiterated that the document provides general guidance and thus did not accept to include specific information on different types of pathogen. The Commission did however agree to change 'DNA' when it is without the prefix 'e' to DNA/RNA throughout the document.

The finalised document, which gives the framework on how to approach eDNA methods, is presented as **Annex 13** for information. It will be uploaded to the Commission's webpage in the near future.

#### 3.2.2. Table 4.1. OIE recommended diagnostic methods and their level of validation for surveillance of apparently health animals and investigation of clinically affected animals

Comments were received from Colombia, Switzerland, the USA and the EU.

*Background*

Table 4.1. of the new *Aquatic Manual* chapter template includes a column for the level of validation of each test method (from 1 to 4 in accordance with Chapter 1.1.2. Principles and methods of validation of diagnostic assays for infectious diseases) in addition to rating each test against its purpose of use. The Commission recognised that having two different scoring systems for two different components of diagnostic test use and interpretation has led to some confusion. Questions about divergence between the two scores have often arisen (e.g. low level of validation but high rating for purpose of use or vice versa).

To address this issue, the Commission revised the explanatory text for Table 4.1 in the disease chapter template and requested OIE Reference Laboratories for feedback before it was included in the chapter template.

**Previous Commission report where this item was discussed:**

September 2021 (Item 6.1.7.3., page 40).

**February 2022 meeting**

The Commission reviewed the comments received from Members and Reference Laboratory experts, and amended the text accordingly. The Commission did not agree with a proposal to provide benchmarks for each test rating – the current ratings are relative and qualitative and do not need to be overly complicated. The Commission also did not agree that the rating against purpose of a test is consistent with the level of validation of that test; for example, a method may have been validated to level 3 but may be expensive, slow or diagnostic performance may be poor such that its rating might only be '+'.

The finalised document is presented as **Annex 14** for information. It has been applied to all the chapters reviewed at this meeting (the two chapters proposed for adoption and the six approved for circulation for Member comment), and will be applied to all future updated *Aquatic Manual* chapters.

### 3.3. Other Issues

#### 3.3.1. Develop a platform for communication among laboratory experts on diagnostic test performance issues

The last paragraph in the explanatory text for Table 4.1. states that OIE Reference Laboratories would welcome feedback on the diagnostic performance of recommended tests. At the 3rd meeting of the Steering Committee of the OIE Regional Collaboration Framework for Aquatic Animal Health

in Asia and the Pacific, and in communications to headquarters, Reference Laboratory experts asked how such issues could be communicated so that the OIE Reference Laboratories can provide advice and standards can be amended, if necessary, in a timely manner.

The Commission discussed this issue and considered possible mechanisms to encourage communication among OIE Reference Laboratories, and with other laboratories. The Commission agreed that this issue requires further consideration and consultation with Reference Laboratory experts. In the meantime, laboratories with information on the diagnostic performance of any tests recommended in the *Aquatic Manual* can provide their comments to the OIE Reference Laboratories or the Aquatic Animals Commission's Secretariat.

## 4. *AD HOC* GROUPS

### 4.1. *Ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases

The *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases met during November–December 2021 to conduct assessments for susceptibility of mollusc species to infection with *Marteilia refringens.*

The Aquatic Animals Commission was informed that the *ad hoc* Group did not complete their assessments of *M. refringens* due to the complexity associated with the pathogenic agent. The Commission reviewed and provided feedback on the *ad hoc* Group's interim report which outlined the work completed to date. The *ad hoc* Group is planning to meet again in June 2022 to finalise the assessments of species susceptible to *M. refringens*.

### 4.2. *Ad hoc* Group on new draft Chapters 4.X. Emergency disease preparedness and 4.Y. Disease outbreak management

The *ad hoc* Group on Emergency disease preparedness and Disease outbreak management, met during December 2021 to initiate work on the development of the new draft Chapters 4.X. Emergency disease preparedness and 4.Y. Disease outbreak management taking in consideration the article structure presented in the Aquatic Animals Commission February 2021 meeting report.

The Aquatic Animals Commission reviewed the interim report of the *ad hoc* Group and the work progressed on the draft Chapter 4.X. thus far and were informed that the *ad hoc* Group intends to meet again in March 2022 to continue the development of the draft chapters.

### 4.3. *Ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases

The Commission was informed that the *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases would be reconvened to progress its work on the applying the criteria in Chapter 1.5., for listing species as susceptible to infection with a specific pathogen to OIE listed diseases. The *ad hoc* Group plans to meet twice in 2022, to conduct assessments for susceptibility of fish species to infection with red seabream iridovirus.

## 5. OIE REFERENCE CENTRES OR CHANGE OF EXPERTS

### 5.1. Evaluation of applications for OIE Reference Centres for aquatic animal health issues or change of experts

The Aquatic Animals Commission reviewed applications for changes of expert and recommended acceptance of the following:

*Infection with white spot disease and acute hepatopancreatic necrosis disease* (*AHPND*)

Dr Han-Ching Wang to replace Dr Grace Lo at the International Centre for Scientific Development of Shrimp Aquaculture, National Cheng Kung University (NCKU), Tainan District, New Taipei City, Chinese Taipei

*Infection with* Mikrocytos mackini

Dr Cathryn Abbot to replace Dr Gary Meyer at the Pacific Biological Station, Nanaimo, British Colombia, Canada

## 5.2. Review of the annual reports of activities in 2021 of the OIE Reference Centres

Annual reports had been received from all OIE Reference Laboratories for diseases of aquatic animals (37) and all Collaborating Centres for aquatic animal issues (3).

In accordance with the adopted Procedures for designation of OIE Reference Laboratories (the SOPs) (https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-2) and the Procedures for designation of OIE Collaborating Centres (https://www.oie.int/en/what-we-offer/expertise-network/collaborating-centres/#ui-id-2), the Aquatic Animals Commission reviewed all the reports received, noting in particular the performance of each Reference Centre with regard to fulfilling the Terms of Reference (ToR) to the benefit of OIE Members.

The Commission noted the significant contributions that had been made by Reference Centres during 2021 despite the ongoing difficulties caused by the COVID-19 pandemic and wished to thank designated experts for leading these valuable contributions to the OIE mission. The Commission expressed its on-going appreciation for the enthusiastic support and expert advice given to the OIE by the Reference Centres. In particular, the Commission is grateful for the ongoing support and essential contributions of reference laboratory experts for revision of the disease-specific chapters of the *Aquatic Manual*.

One institution that housed a number of OIE Reference Laboratories had submitted general information on the diseases it covers in each report making it difficult to determine disease-specific information in individual reports. The experts would be asked to provide disease-specific information in their reports next year.

One laboratory reported that it had lost its accreditation to ISO 17025 quality management system as they had changed their activities from aquatic to human testing as a national response to the COVID-19 pandemic. The laboratory envisages recovering the accreditation during this year. In accordance with the SOPs, the laboratory would be temporarily suspended for a period no longer than two years. It will be reinstated should it submit a certificate within the two-year period. Otherwise it will lose its designation and will need to submit a new application for evaluation once it is accredited again.

The Commission noted that very few of the laboratories were working on validating tests to the OIE standard and encourages laboratories to be more active in this area. The Commission also noted that the report template asks if laboratories are developing and validating new test methods but asks nothing about existing test methods. The Commission agreed to review the template at its next meeting in September to ensure questions are more pertinent to the important activities of the Reference Laboratories, the OIE aquatic strategy and the future of the OIE's science system.

## 5.3. Evaluation of applications for OIE Reference Laboratory for Infection with decapod iridescent virus 1

The Aquatic Animals Commission reviewed an application for an OIE Reference Laboratory for Infection with decapod iridescent virus 1 and recommended its acceptance:

OIE *Reference Laboratory for* decapod iridescent virus 1

Aquatic Medicine Laboratory, Biology Division of Animal Health Research Institute (AHRI), Council of Aquaculture, CHINESE TAIPEI
Tel.: (+886-2) 2621.2111 ext. 203; (+886-2) 762.3039
E-mail: ctu@mail.nvri.gov.tw; yplu@mail.nvri.gov.tw; Website: www.nvri.gov.tw
Designated Reference Expert: Dr Chien Tu

## 6. NEXT MEETING

The next meeting of the Aquatic Animals Commission is scheduled for 14–21 September 2022.

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…/Annexes

**MEETING OF THE OIE**
**AQUATIC ANIMAL HEALTH STANDARDS COMMISSION**

**Virtual meeting, 24 & 27 January, 16–23 February 2022**

## List of participants

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## WORK PLAN FOR THE AQUATIC ANIMALS COMMISSION

**Items proposed for adoption at the May GS 2022**

| ***Aquatic Code*** | | | |
|---|---|---|---|
| **Chapter/Subject** | **Status** | | |
| | **September 2021** | **February 2022** | **May GS 2022** |
| **User's Guide** | Reviewed amended articles and presented for comments | Review comments (1st round) | Propose for adoption |
| **Glossary definitions: 'Competent Authority', 'Veterinary Authority' and 'Aquatic Animal Health Services'** | Reviewed comments (1st round) and presented for comments | Review comments (2nd round) | Propose for adoption |
| **Glossary definitions: 'Basic biosecurity conditions', 'Biosecurity plan', 'Early detection system' and 'Passive surveillance'** | Reviewed comments (1st round) and presented for comments | Review comments (2nd round) | Propose for adoption |
| **Chapter 1.3. Listing of infection with tilapia lake virus (TiLV)** | Reassessed infection with TiLV for listing | Review comments (1st round) | Propose for adoption |
| **Chapter 1.4. Aquatic Animal Health Surveillance** | Reviewed comments (1st round) | Review comments (2nd round) | Propose for adoption |
| **Model Articles X.X.4. to X.X.8. for disease-specific chapters to address declaration of freedom from [Pathogen X]** | Reviewed comments (2nd round) | Review comments (3rd round) | Propose for adoption |
| **Safe commodities – Crustacean diseases – Articles 9.X.3.** | Reviewed comments (1st round) | Review comments (2nd round) | Propose for adoption |
| **New Chapter 9.X. Infection with decapod iridescent virus 1** | Reviewed draft chapter and presented for comments | Review Comments (1st round) | Propose for adoption |
| **Safe commodities – Fish diseases – Articles 10.X.3.** | Reviewed amended articles and presented for comments | Review comments (1st round) | Propose for adoption |
| **Chapter 10.1. Infection with epizootic haematopoietic necrosis virus** | Reviewed amended articles and presented for Member comments | Review 1st round Member comments | Propose for adoption |
| **Chapter 10.7. Infection with koi herpes virus** | Reviewed amended articles and presented for Member comments | Review 1st round Member comments | Propose for adoption |
| **Susceptible Species – Infection with abalone herpesvirus – Articles 11.1.1. and 11.1.2.** | Reviewed *ad hoc* Group report and presented the revised articles for comments | Review Comments (1st round) | Propose for adoption |
| **Susceptible Species – Infection with *Bonamia exitiosa* –Articles 11.2.1. and 11.2.2.** | Review comments (1st round) | Review comments (2nd round) | Propose for adoption |
| ***Aquatic Manual*** | | | |
| **Chapter/Subject** | **Status** | | |
| | **September 2021** | **February 2022** | **May GS 2022** |
| **Chapter 2.3.0. General information (diseases of fish)** | Update Section 2.5 and presented for comments | Review comments | Propose for adoption |
| **Chapter 2.3.4. Infection with HPR-deleted or HPR0 infectious salmon anaemia virus** | Reviewed Member comments (1st round) | Review comments (2nd round) | Propose for adoption |

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| | and presented for comment | | |
| **Chapter 2.3.6. Infection with koi herpesvirus** | Reviewed comments (1st round) and presented for comment | Review comments (2nd round) | Propose for adoption |
| **Sections 2.2.1., 2.2.2. of Chapter 2.4.1. Infection with abalone herpesvirus** | Reviewed *ad hoc* Group report and presented revised articles for comments | Review comments (1st round) | Propose for adoption |
| **Sections 2.2.1., 2.2.2. of Chapter 2.4.2. Infection with *Bonamia exitiosa*** | Reviewed comments (1st round) and presented for comments | Review comment (2nd round) | Propose for adoption |

**On-going work for adoption in 2023 or later**

| ***Aquatic Code*** | | | |
|---|---|---|---|
| **Chapter/Subject** | **Status** | | |
| | **September 2021** | **February 2022** | **September 2022** |
| **Monitor emerging diseases and consider any required actions** | On-going | | |
| **Chapter 4.X. New draft chapter on Emergency disease preparedness** | Requested an *ad hoc* Group be convened | Consider *ad hoc* Group report | AAC Review of proposed Chapter 4.X. |
| **Chapter 4.Y. New draft chapter on Disease outbreak management** | *ad hoc* Group to be convened | *ad hoc* Group to start drafting after completion of Chapter 4.X. | ad hoc Group to start drafting after completion of Chapter 4.X. |
| **Safe commodities – Amphibians – Articles 8.X.3.** | | Review amended articles and present for comments | Review Comments (1st round) |
| **Article 9.3.1. of Chapter 9.3. Infection with *Hepatobacter penaei* (Necrotising hepatopancreatitis)** | | Review amended articles and present for comments | Review Comments (1st round) |
| **Susceptible Species – Articles 9.4.1. and 9.4.2., Infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV)** | | Review amended articles and present for comments | Review Comments (1st round) |
| **Susceptible Species – Fish diseases – Articles 10.X.1. and 10.X.2. for:**<br>- Infection with Red seabream iridovirus/infectious<br>- Infection with *Aphanomyces invadans* (Epizootic ulcerative syndrome | | *Ad hoc* Group reconvened: Assessment for RSIV/ISKNV planned for April 2022 | Review *ad hoc* Group report |
| **Susceptible Species**<br>Assessment of new species for previously assessed diseases as necessary | As needed | | |
| **Chapter 10.X. Infection with tilapia lake virus (pending adoption)** | | | Review draft chapter and present for comment |
| **Susceptible species – Fish diseases – Articles 11.X.1. and 11.X.2. for:**<br>- Infection with *Marteilia refringens*<br>- Infection with *Xenohaliotis californiensis*<br>- Infection with *Perkinsus marinus*<br>- Infection with *Perkinsus olseni* | Requested *ad hoc* Group continue its assessments | Next *ad hoc* Group meeting planned for June 2022 | Review *ad hoc* Group report |

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|---|---|---|---|
| **Safe commodities – Mollusc diseases – Articles 11.X.3.** | | Review amended articles and present for comments | Review Comments (1st round) |
| ***Aquatic Manual*** | | | |
| **Chapter/Subject** | **Status** | | |
| | **September 2021** | **February 2022** | **September 2022** |
| **The use of environmental DNA methods for aquatic animal disease surveillance** | Reviewed comments (1st round) | Review Member comments, revise and upload onto OIE website | |
| **Levels of validation and the test scoring system in Table 4.1** | Reviewed comments and sent to Reference Laboratories for feedback | Review feedback from Reference Laboratories | |
| **Section 2.2. General provisions – Crustaceans** | | | Review amended Chapter and present for comments |
| **Chapter 2.2.1. Acute hepatopancreatic necrosis disease** | | Updated and reformatted and presented for comments | Review comments (1st round) |
| **Chapter 2.2.2. Infection with *Aphanomyces astaci* (Crayfish plague)** | | Updated and reformatted | Review further updated draft and present for comments |
| **Chapter 2.2.3. Infection with *Hepatobacter penaei* (necrotising hepatopancreatitis)** | | Updated and reformatted and presented for comments | Review comments (1st round) |
| **Chapter 2.2.4. Infection with infectious hypodermal and haematopoietic necrosis virus** | | Updated and reformatted and presented for comments | Review comments (1st round) |
| **Chapter 2.2.6. Infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)** | | Updated and reformatted | Review further updated draft and present for comments |
| **Chapter 2.2.X. Infection with decapod iridescent virus 1** | | | Develop draft chapter for review |
| **Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus** | Updated and reformatted and presented for comments | Review comments (1st round) | Review comments (2nd round) |
| **Chapter 2.3.1. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome)** | Updated and reformatted | Review further updated draft and present for comments | Review comments (1st round) |
| **Chapter 2.3.7. Red sea bream iridoviral disease** | Updated and reformatted and presented for comments | Review comments (1st round) | Review comments (2nd round) |
| **Chapter 2.3.X. Infection with tilapia lake virus (Pending adoption)** | | | Develop draft chapter for review |

**Newly prioritised items to commence before May 2024**

| ***Aquatic Code*** | | | |
|---|---|---|---|
| **Chapter/Subject** | **Status** | **First Priority** | **Second Priority** |
| **Safe Commodity Assessments (for all listed diseases)** | Review of any new scientific evidence to update standards if necessary | ✓ | |

| | | | |
|---|---|---|---|
| **Chapter 1.3. Diseases listed by the OIE** | Review any new diseases for listing or de-listing as necessary | On going | |
| **Chapter 4.2. Zoning and Compartmentalisation** | Re-develop chapter to focus solely on zoning | | ✓ |
| **Chapter 4.3. Application of Compartmentalisation** | Re-develop chapter to improve guidance and align with new Chapter 4.1 on biosecurity. | ✓ | |
| **Section 5. New Chapter on ornamental aquatic animals** | Development of new standards to facilitate safe trade of ornamental aquatic animals | ✓ | |
| **Section 5. New Chapter on genetic materials** | Development of new standards to facilitate safe trade of genetic materials | ✓ | |
| **Articles 9.X.1. and 9.X.2. Update lists of susceptible species** | Remaining crustacean diseases for review:<br>- Infection with *Aphanomyces astaci* (Crayfish plague)<br>- Infection with white spot syndrome virus | | Dependant on the completion of work to update the assessments of species susceptible to all fish and mollusc OIE listed diseases |
| ***Aquatic Manual*** | | | |
| **Chapter/Subject** | **Status** | **First Priority** | **Second Priority** |
| **Chapters 2.2.X. Crustacean disease specific chapters** | Update and reformat chapters using the new template (Infection with IMNV, TSV, WSSV, YHV) | ✓ | |
| **Section 2.4. General provisions – Molluscs** | Review and update the introductory chapter on mollusc diseases | | ✓ |
| **Chapters 2.4.X. Mollusc disease specific chapters** | Update and reformat chapters using the new template (all diseases) | | ✓ |

Return to Agenda

# ARTICLES 8.X.3 FOR AMPHIBIAN DISEASE-SPECIFIC CHAPTERS (TRACK CHANGES AND CLEAN VERSIONS)

## (TRACK CHANGES VERSION)

CHAPTER 8.1.

# INFECTION WITH *BATRACHOCHYTRIUM DENDROBATIDIS*

[…]

Article 8.1.3.

**Measures for the ~~I~~importation or transit of aquatic animal products for any purpose regardless of the infection with *B. dendrobatidis* status of the exporting country, zone or compartment**

~~1.~~ The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products*, *Competent Authorities* should not require any *sanitary measures* ~~conditions~~ related to *B. dendrobatidis*, regardless of the infection with *B. dendrobatidis* status of the *exporting country*, *zone* or *compartment*:~~, when authorising the importation or transit of the following *aquatic animal products* derived from a species referred to in Article 8.1.2. that are intended for any purpose and comply with Article 5.4.1.:~~

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 60°C for at least five minutes, or a time/temperature equivalent that inactivates *B. dendrobatidis*;

~~a) heat sterilised hermetically sealed amphibian products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent that has been demonstrated to inactivate *B. dendrobatidis*);~~

~~b) cooked amphibian products that have been subjected to heat treatment at 100°C for at least one minute (or any time/temperature equivalent that has been demonstrated to inactivate *B. dendrobatidis*);~~

~~c) pasteurised amphibian products that have been subjected to heat treatment at 90°C for at least ten minutes (or any time/temperature equivalent that has been demonstrated to inactivate *B. dendrobatidis*);~~

~~d)~~2) mechanically dried amphibian products that have been subjected to ~~(i.e.~~ a heat treatment sufficient to attain a core temperature of at least ~~100~~60°C for at least five~~30~~ minutes, or a~~any~~ time/temperature equivalent that ~~has been demonstrated to~~ inactivate~~s~~ *B. dendrobatidis*~~)~~;

~~e)~~3) amphibian skin leather.

~~2) When authorising the importation or transit of *aquatic animal products* derived from a species referred to in Article 8.1.2., other than those referred to in point 1 of Article 8.1.3., *Competent Authorities* should require the conditions prescribed in Articles 8.1.7. to 8.1.12. relevant to the infection with *B. dendrobatidis* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animal products* derived from a species not referred to in Article 8.1.2. but which could reasonably be expected to pose a *risk* of transmission of *B. dendrobatidis*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this analysis.~~

[…]

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**(CLEAN VERSION)**

# CHAPTER 8.1.

# INFECTION WITH *BATRACHOCHYTRIUM DENDROBATIDIS*

[…]

Article 8.1.3.

**Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *B. dendrobatidis* status of the exporting country, zone or compartment**

The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products*, *Competent Authorities* should not require any *sanitary measures* related to *B. dendrobatidis*, regardless of the infection with *B. dendrobatidis* status of the *exporting country*, *zone* or *compartment*:

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 60°C for at least five minutes, or a time/temperature equivalent that inactivates *B. dendrobatidis*;

2) mechanically dried amphibian products that have been subjected to a heat treatment sufficient to attain a core temperature of at least 60°C for at least five minutes, or a time/temperature equivalent that inactivates *B. dendrobatidis*;

3) amphibian skin leather.

[…]

**(TRACK CHANGES VERSION)**

CHAPTER 8.2.

# INFECTION WITH BATRACHOCHYTRIUM SALAMANDRIVORANS

[…]

Article 8.2.3.

**Measures for the i~~I~~mportation or transit of aquatic animal products for any purpose regardless of the infection with *B. salamandrivorans* status of the exporting country, zone or compartment**

~~1)~~ The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorizing the importation or transit of these *aquatic animal products*, *Competent Authorities* should not require any *sanitary measures* ~~conditions~~ related to *B. salamandrivorans*, regardless of the infection with *B. salamandrivorans* status of the *exporting country*, *zone* or *compartment*~~:, when authorising the importation or transit of the following *aquatic animal products* derived from a species referred to in Article 8.2.2. that are intended for any purpose and comply with Article 5.4.1.:~~

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 60°C for at least five minutes, or a time/temperature equivalent that inactivates *B. salamandrivorans*;

~~a) heat sterilised hermetically sealed amphibian products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent that has been demonstrated to inactivate *B. salamandrivorans*);~~

~~b) cooked amphibian products that have been subjected to heat treatment at 100°C for at least one minute (or any time/temperature equivalent that has been demonstrated to inactivate *B. salamandrivorans*);~~

~~c) pasteurised amphibian products that have been subjected to heat treatment at 90°C for at least ten minutes (or any time/temperature equivalent that has been demonstrated to inactivate *B. salamandrivorans*);~~

~~d)~~2) mechanically dried amphibian products that have been subjected to ~~(i.e.~~ a heat treatment sufficient to attain a core temperature of at least ~~100~~60°C for at least five~~30~~ minutes, or a~~any~~ time/temperature equivalent that ~~has been demonstrated to~~ inactivate*s* *B. salamandrivorans*;

~~e)~~3) amphibian skin leather.

~~2) When authorising the importation or transit of *aquatic animal products* derived from a species referred to in Article 8.2.2., other than those referred to in point 1 of Article 8.2.3.., *Competent Authorities* should require the conditions prescribed in Articles 8.2.7. to 8.2.12. relevant to the infection with *B. salamandrivorans* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animal products* derived from a species not referred to in Article 8.2.2. but which could reasonably be expected to pose a *risk* of transmission of *B. salamandrivorans*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this analysis.~~

[…]

**(CLEAN VERSION)**

# CHAPTER 8.2.

# INFECTION WITH *BATRACHOCHYTRIUM SALAMANDRIVORANS*

[…]

Article 8.2.3.

**Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *B. salamandrivorans* status of the exporting country, zone or compartment**

The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorizing the importation or transit of these *aquatic animal products*, *Competent Authorities* should not require any *sanitary measures* related to *B. salamandrivorans*, regardless of the infection with *B. salamandrivorans* status of the *exporting country*, *zone* or *compartment*:

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 60°C for at least five minutes, or a time/temperature equivalent that inactivates *B. salamandrivorans*;

2) mechanically dried amphibian products that have been subjected to a heat treatment sufficient to attain a core temperature of at least 60°C for at least five minutes, or a time/temperature equivalent that inactivates *B. salamandrivorans*;

3) amphibian skin leather.

[…]

**(TRACK CHANGES VERSION)**

CHAPTER 8.3.

# INFECTION WITH RANAVIRUS SPECIES

[…]

Article 8.3.3.

**Measures for the ~~i~~importation or transit of aquatic animal products for any purpose regardless of the infection with *Ranavirus* species status of the exporting country, zone or compartment**

~~1)~~ The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products*, *Competent Authorities* should not require any *sanitary measures* ~~conditions~~ related to ~~Ranavirus~~ species, regardless of the infection with *Ranavirus* species status of the *exporting country*, *zone* or *compartment*:~~, when authorising the importation or transit of the following *aquatic animal products* derived from a species referred to in Article 8.3.2. that are intended for any purpose and comply with Article 5.4.1.:~~

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 65°C for at least 30 minutes, or a time/temperature equivalent that inactivates *Ranavirus* species;

~~a) heat sterilised hermetically sealed amphibian products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent that has been demonstrated to inactivate *Ranavirus* species);~~

~~b) cooked amphibian products that have been subjected to heat treatment at 65°C for at least 30 minutes (or any time/temperature equivalent that has been demonstrated to inactivate *Ranavirus* species);~~

~~c) pasteurised amphibian products that have been subjected to heat treatment at 90°C for at least ten minutes (or any time/temperature equivalent that has been demonstrated to inactivate *Ranavirus* species);~~

~~d~~2) mechanically dried amphibian products that have been subjected to ~~(i.e.~~ a heat treatment sufficient to attain a core temperature of at least ~~100~~65°C for at least 30 minutes, or a~~any~~ time/temperature equivalent that ~~has been demonstrated to~~ inactivate~~s~~ *Ranavirus* species~~)~~.

~~2 When authorising the importation or transit of *aquatic animal products* derived from a species referred to in Article 8.3.2., other than those referred to in point 1 of Article 8.3.3., *Competent Authorities* should require the conditions prescribed in Articles 8.3.7. to 8.3.12. relevant to the infection with *Ranavirus* species status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animal products* derived from a species not referred to in Article 8.3.2. but which could reasonably be expected to pose a *risk* of transmission of *Ranavirus* species, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this analysis.~~

[…]

**(CLEAN VERSION)**

# CHAPTER 8.3.

# INFECTION WITH RANAVIRUS SPECIES

[...]

Article 8.3.3.

**Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *Ranavirus* species status of the exporting country, zone or compartment**

The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products, Competent Authorities* should not require any *sanitary measures* related to *Ranavirus* species, regardless of the infection with *Ranavirus* species status of the *exporting country*, *zone* or *compartment*:

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 65°C for at least 30 minutes, or a time/temperature equivalent that inactivates *Ranavirus* species;

2) mechanically dried amphibian products that have been subjected to a heat treatment sufficient to attain a core temperature of at least 65°C for at least 30 minutes, or a time/temperature equivalent that inactivates *Ranavirus* species.

[...]

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Return to Agenda

# ARTICLES 11.X.3 FOR MOLLUSC DISEASE-SPECIFIC CHAPTERS (TRACK CHANGES AND CLEAN VERSIONS)

## (TRACK CHANGES VERSION)

CHAPTER 11.1.

# INFECTION WITH ABALONE HERPESVIRUS

[…]

Article 11.1.3.

**Measures for the i~~I~~mportation or transit of ~~aquatic animals and~~ aquatic animal products for any purpose regardless of the infection with abalone herpesvirus status of the exporting country, zone or compartment**

~~1)~~ The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products*, *Competent Authorities* should not require any *sanitary measures*~~conditions~~ related to infection with abalone herpesvirus regardless of the infection with abalone herpesvirus status of the *exporting country*, *zone* or *compartment*~~, when authorising the importation or transit of the following *aquatic animal products* from the species referred to in Article 11.1.2. which are intended for any purpose and which comply with Article 5.4.1.~~:

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 121°C for at least 3 minutes and 36 seconds, or a time/temperature equivalent that inactivates AbHV;

~~a) heat sterilised hermetically sealed abalone products (i.e. a heat treatment at 121˚C for at least 3.6 minutes or any time/temperature equivalent);~~

~~b~~2) mechanically dried abalone products ~~(i.e.~~ that have been subjected to a heat treatment sufficient to attain a core temperature of at least ~~100~~121˚C for at least 3 minutes and 36 seconds, ~~30 minutes~~ or ~~any~~a time/temperature equivalent ~~which has been demonstrated to~~ that inactivates AbHV~~)~~.

~~2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 11.1.2., other than those referred to in point 1 of Article 11.1.3., *Competent Authorities* should require the conditions prescribed in Articles 11.1.7. to 11.1.11. relevant to the infection with abalone herpesvirus status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* of a species not covered in Article 11.1.2. but which could reasonably be expected to pose a *risk* of spread of infection with abalone herpesvirus, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this assessment.~~

[…]

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**(CLEAN VERSION)**

CHAPTER 11.1.

# INFECTION WITH ABALONE HERPESVIRUS

[…]

Article 11.1.3.

**Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with abalone herpesvirus status of the exporting country, zone or compartment**

The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products*, *Competent Authorities* should not require any *sanitary measures* related to infection with abalone herpesvirus regardless of the infection with abalone herpesvirus status of the *exporting country*, *zone* or *compartment:*

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 121°C for at least 3 minutes and 36 seconds, or a time/temperature equivalent that inactivates AbHV; and

2) mechanically dried abalone products that have been subjected to a heat treatment sufficient to attain a core temperature of at least 121˚C for at least 3 minutes and 36 seconds, or a time/temperature equivalent that inactivates AbHV.

[…]

**(TRACK CHANGES VERSION)**

CHAPTER 11.2.

# INFECTION WITH *BONAMIA EXITIOSA*

[…]

Article 11.2.3.

**Measures for the i~~I~~mportation or transit of ~~aquatic animals and~~ aquatic animal products for any purpose regardless of the infection with *B. exitiosa* status of the exporting country, zone or compartment**

~~1)~~ The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products*, *Competent Authorities* should not require any *sanitary measures*~~conditions~~ related to infection with *B. exitiosa*, regardless of the infection with *B. exitiosa* status of the *exporting country*, *zone* or *compartment:*~~, when authorising the importation or transit of the following *aquatic animals* and *aquatic animal products* from the species referred to in Article 11.2.2. which are intended for any purpose and which comply with Article 5.4.1.:~~

~~a)~~1) frozen oyster meat; and

~~b)~~2) frozen half-shell oysters.

~~2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 11.2.2., other than those referred to in point 1 of Article 11.2.3., *Competent Authorities* should require the conditions prescribed in Articles 11.2.7. to 11.2.11. relevant to the infection with *B. exitiosa* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* of a species not covered in Article 11.2.2. but which could reasonably be expected to pose a *risk* of spread of infection with *B. exitiosa*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this assessment.~~

[…]

**(CLEAN VERSION)**

# CHAPTER 11.2.

# INFECTION WITH *BONAMIA EXITIOSA*

[…]

Article 11.2.3.

**Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *B. exitiosa* status of the exporting country, zone or compartment**

The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products, Competent Authorities* should not require any *sanitary measures* related to infection with *B. exitiosa*, regardless of the infection with *B. exitiosa* status of the *exporting country*, *zone* or *compartment:*

1) frozen oyster meat; and

2) frozen half-shell oysters.

[…]

**(TRACK CHANGES VERSION)**

CHAPTER 11.3.

# INFECTION WITH *BONAMIA OSTREAE*

[…]

Article 11.3.3.

**Measures for the i~~I~~mportation or transit of ~~aquatic animals and~~ aquatic animal products for any purpose regardless of the infection with *B. ostreae* status of the exporting country, zone or compartment**

~~1)~~ The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products*, *Competent Authorities* should not require any *sanitary measures*~~conditions~~ related to infection with *B. ostreae*, regardless of the infection with *B. ostreae* status of the *exporting country*, *zone* or *compartment:*~~, when authorising the importation or transit of the following *aquatic animals* and *aquatic animal products* from the species referred to in Article 11.3.2. which are intended for any purpose and which comply with Article 5.4.1.:~~

~~a)~~1) frozen oyster meat; and

~~b)~~2) frozen half-shell oysters.

~~2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 11.2.2., other than those referred to in point 1 of Article 11.2.3., *Competent Authorities* should require the conditions prescribed in Articles 11.2.7. to 11.2.11. relevant to the infection with *B. ostreae* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* of a species not covered in Article 11.2.2. but which could reasonably be expected to pose a *risk* of spread of infection with *B. ostreae*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this assessment.~~

[…]

**(CLEAN VERSION)**

# CHAPTER 11.3.

# INFECTION WITH *BONAMIA OSTREAE*

[…]

Article 11.3.3.

**Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *B. ostreae* status of the exporting country, zone or compartment**

The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products, Competent Authorities* should not require any *sanitary measures* related to infection with *B. ostreae*, regardless of the infection with *B. ostreae* status of the *exporting country*, *zone* or *compartment:*

1) frozen oyster meat; and

2) frozen half-shell oysters.

[…]

**(TRACK CHANGES VERSION)**

CHAPTER 11.4.

# INFECTION WITH *MARTEILIA REFRINGENS*

[…]

Article 11.4.3.

**Measures for the i~~I~~mportation or transit of ~~aquatic animals and~~ aquatic animal products for any purpose regardless of the infection with *M. refringens* status of the exporting country, zone or compartment**

~~1)~~ The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products, Competent Authorities* should not require any *sanitary measures*~~conditions~~ related to infection with *M. refringens*, regardless of the infection with *M. refringens* status of the *exporting country*, *zone* or *compartment:* ~~, when authorising the importation or transit of heat sterilised hermetically sealed mollusc products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent) from the species referred to in Article 11.4.2. which are intended for any purpose and which comply with Article 5.4.1.~~

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 121°C for at least 3 minutes and 36 seconds, or a time/temperature equivalent that inactivates *M. refringens*.

~~2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 11.4.2., other than those referred to in point 1 of Article 11.4.3., *Competent Authorities* should require the conditions prescribed in Articles 11.4.7. to 11.4.11. relevant to the infection with *M. refringens* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* of a species not covered in Article 11.4.2. but which could reasonably be expected to pose a *risk* of spread of infection with *M. refringens*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1. The *Competent Authority* of the *exporting country* should be informed of the outcome of this assessment.~~

[…]

**(CLEAN VERSION)**

# CHAPTER 11.4.

# INFECTION WITH *MARTEILIA REFRINGENS*

[...]

Article 11.4.3.

**Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *M. refringens* status of the exporting country, zone or compartment**

The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products, Competent Authorities* should not require any *sanitary measures* related to infection with *M. refringens*, regardless of the infection with *M. refringens* status of the *exporting country*, *zone* or *compartment:*

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 121°C for at least three minutes and 36 seconds, or a time/temperature equivalent that inactivates *M. refringens*.

[...]

**(TRACK CHANGES VERSION)**

CHAPTER 11.5.

# INFECTION WITH *PERKINSUS MARINUS*

[…]

Article 11.4.3.

**Measures for the i~~I~~mportation or transit of ~~aquatic animals and~~ aquatic animal products for any purpose regardless of the infection with *P. marinus* status of the exporting country, zone or compartment**

~~1)~~ The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products, Competent Authorities* should not require any *sanitary measures*~~conditions~~ related to infection with *P. marinus*, regardless of the infection with *P. marinus* status of the *exporting country*, *zone* or *compartment:*~~, when authorising the importation or transit of heat sterilised hermetically sealed mollusc products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent) from the species referred to in Article 11.5.2. which are intended for any purpose and which comply with Article 5.4.1.~~

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 121°C for at least three minutes and 36 seconds, or a time/temperature equivalent that inactivates *P. marinus*.

~~2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 11.5.2., other than those referred to in point 1 of Article 11.5.3., *Competent Authorities* should require the conditions prescribed in Articles 11.5.7. to 11.5.11. relevant to the infection with *P. marinus* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* of a species not covered in Article 11.5.2. but which could reasonably be expected to pose a *risk* of spread of infection with *P. marinus*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1.The *Competent Authority* of the *exporting country* should be informed of the outcome of this assessment.~~

[…]

**(CLEAN VERSION)**

CHAPTER 11.5.

# INFECTION WITH *PERKINSUS MARINUS*

[...]

Article 11.4.3.

**Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *P. marinus* status of the exporting country, zone or compartment**

The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products, Competent Authorities* should not require any *sanitary measures* related to infection with *P. marinus*, regardless of the infection with *P. marinus* status of the *exporting country*, *zone* or *compartment:*

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 121°C for at least three minutes and 36 seconds, or a time/temperature equivalent that inactivates *P. marinus*.

[...]

**(TRACK CHANGES VERSION)**

CHAPTER 11.6.

# INFECTION WITH *PERKINSUS OLSENI*

[…]

Article 11.6.3.

**Measures for the i~~I~~mportation or transit of ~~aquatic animals and~~ aquatic animal products for any purpose regardless of the infection with *P. olseni* status of the exporting country, zone or compartment**

~~1)~~ The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products, Competent Authorities* should not require any *sanitary measures* ~~conditions~~ related to infection with *P. olseni*, regardless of the infection with *P. olseni* status of the *exporting country*, *zone* or *compartment:*~~, when authorising the importation or transit of heat sterilised hermetically sealed mollusc products (i.e. a heat treatment at 121°C for at least 3.6 minutes or any time/temperature equivalent) from the species referred to in Article 11.6.2. which are intended for any purpose and which comply with Article 5.4.1.~~

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 121°C for at least three minutes and 36 seconds minutes, or a time/temperature equivalent that inactivates *P.olseni*.

~~2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 11.6.2., other than those referred to in point 1 of Article 11.6.3., *Competent Authorities* should require the conditions prescribed in Articles 11.6.7. to 11.6.11. relevant to the infection with *P. olseni* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* of a species not covered in Article 11.6.2. but which could reasonably be expected to pose a *risk* of spread of infection with *P. olseni*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1.The *Competent Authority* of the *exporting country* should be informed of the outcome of this assessment.~~

[…]

**(CLEAN VERSION)**

CHAPTER 11.6.

# INFECTION WITH *PERKINSUS OLSENI*

[…]

Article 11.6.3.

**Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *P. olseni* status of the exporting country, zone or compartment**

The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products, Competent Authorities* should not require any *sanitary measures* related to infection with *P. olseni*, regardless of the infection with *P. olseni* status of the *exporting country*, *zone* or *compartment:*

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 121°C for at least three minutes and 36 seconds, or a time/temperature equivalent that inactivates *P.olseni*.

[…]

**(TRACK CHANGES VERSION)**

CHAPTER 11.7.

# INFECTION WITH *XENOHALIOTIS CALIFORNIENSIS*

[…]

Article 11.7.3.

**Measures for the i~~I~~mportation or transit of ~~aquatic animals and~~ aquatic animal products for any purpose regardless of the infection with *X. californiensis* status of the exporting country, zone or compartment**

~~1)~~ The ~~following~~ *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of ~~the~~ these *aquatic animal products* ~~listed below~~, *Competent Authorities* should not require any *sanitary measures*~~conditions~~ related to infection with *X. californiensis*, regardless of the infection with *X. californiensis* status of the *exporting country*, *zone* or *compartment:*~~, when authorising the importation or transit of heat sterilised hermetically sealed abalone products (i.e. a heat treatment at 121˚C for at least 3.6 minutes or any time/temperature equivalent) from the species referred to in Article 11.7.2. which are intended for any purpose and which comply with Article 5.4.1.~~

~~a~~1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 121°C for at least 3 minutes and 36 seconds, (or a time/temperature equivalent that inactivates *X. californiensis*.~~;~~

~~2) When authorising the importation or transit of *aquatic animals* and *aquatic animal products* of a species referred to in Article 11.7.2., other than those referred to in point 1 of Article 11.7.3., *Competent Authorities* should require the conditions prescribed in Articles 11.7.7. to 11.7.11. relevant to the infection with *X. californiensis* status of the *exporting country*, *zone* or *compartment*.~~

~~3) When considering the importation or transit of *aquatic animals* and *aquatic animal products* of a species not covered in Article 11.7.2. but which could reasonably be expected to pose a *risk* of spread of infection with *X. californiensis*, the *Competent Authority* should conduct a *risk analysis* in accordance with the recommendations in Chapter 2.1.The *Competent Authority* of the *exporting country* should be informed of the outcome of this assessment.~~

[…]

**(CLEAN VERSION)**

# CHAPTER 11.7.

# INFECTION WITH *XENOHALIOTIS CALIFORNIENSIS*

[…]

Article 11.7.3.

**Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with *X. californiensis* status of the exporting country, zone or compartment**

The *aquatic animal products* listed below have been assessed as meeting the criteria for safety of *aquatic animal products* in accordance with Article 5.4.1. When authorising the importation or transit of these *aquatic animal products*, *Competent Authorities* should not require any *sanitary measures* related to infection with *X. californiensis*, regardless of the infection with *X. californiensis* status of the *exporting country*, *zone* or *compartment:*

1) *aquatic animal products* that have been subjected to a heat treatment sufficient to attain a core temperature of at least 121°C for at least 3 minutes and 36 seconds, or a time/temperature equivalent that inactivates *X. californiensis*.

[…]

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# CHAPTER 9.3.

# INFECTION WITH *HEPATOBACTER PENAEI* (NECROTISING HEPATOPANCREATITIS)

Article 9.3.1.

For the purposes of the *Aquatic Code*, infection with *Hepatobacter penaei* (necrotising hepatopancreatitis) means *infection* with the *pathogenic agent Candidatus Hepatobacter penaei*, an obligate intracellular bacterium of the Order alpha-Proteobacteria. ~~The *disease* is commonly known as necrotising hepatopancreatitis.~~

Information on methods for *diagnosis* is provided in the *Aquatic Manual*.

[...]

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# CHAPTER 9.4.

# INFECTION WITH INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS

Article 9.4.1.

For the purposes of the *Aquatic Code*, infection with infectious hypodermal and haematopoietic necrosis virus means *infection* with the *pathogenic agent Decapod ~~penstyldensovirus~~penstylhamaparvovirus* 1, ~~commonly known as infectious hypodermal and haematopoietic necrosis virus (IHHNV),~~ of the Genus ~~*Penstyldensovirus*~~*Penstylhamaparvovirus* and Family *Parvoviridae*.

Information on methods for *diagnosis* is provided in the *Aquatic Manual*.

Article 9.4.2.

**Scope**

The recommendations in this chapter apply to the following species that meet the criteria for listing as susceptible in accordance with Chapter 1.5.: blue shrimp (*Penaeus stylirostris*), giant tiger prawn *(Penaeus monodon)*, northern white shrimp *(Penaeus setiferus).* yellowleg shrimp *(Penaeus californiensis)*, ~~giant tiger prawn *(Penaeus monodon)*~~, ~~northern white shrimp *(Penaeus setiferus)*~~, ~~blue shrimp~~*~~(Penaeus stylirostris)~~* and whiteleg shrimp *(Penaeus vannamei)*.

[…]

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CHAPTER 2.2.1.

# ACUTE HEPATOPANCREATIC NECROSIS DISEASE

## 1. Scope

Acute hepatopancreatic necrosis disease (AHPND) means infection with strains of *Vibrio parahaemolyticus* (*Vp*$_{AHPND}$) that contain a ~70-kbp plasmid with genes that encode homologues of the *Photorhabdus* insect-related (Pir) toxins, PirA and PirB. Although there are reports of the isolation of other *Vibrio* species from clinical cases of AHPND, only *Vp*$_{AHPND}$ has been demonstrated to cause AHPND.

## 2. Disease information

### 2.1. Agent factors

#### 2.1.1. Aetiological agent

AHPND has a bacterial aetiology (Kondo *et al*., 2015; Tran *et al*., 2013). It is caused by specific virulent strains of *V. parahaemolyticus* (*Vp*$_{AHPND}$) that contain a ~70-kbp plasmid with genes that encode homologues of the *Photorhabdus* insect-related (Pir) binary toxin, PirA and PirB (Gomez-Gil *et al.,* 2014; Gomez-Jimenez *et al*., 2014; Han *et al.,* 2015a; Kondo *et al.,* 2014; Lee *et al.,* 2015; Yang *et al.,* 2014). The plasmid within *Vp*$_{AHPND}$ has been designated pVA1, and its size may vary slightly. Removal (or "curing") of pVA1 abolishes the AHPND-causing ability of *Vp*$_{AHPND}$ strains.

Within a population of *Vp*$_{AHPND}$ bacteria, natural deletion of the Pir$^{vp}$ operon may occur in a few individuals (Lee *et al.,* 2015; Tinwongger *et al.,* 2014). This deletion is due to the instability caused by the repeat sequences or transposase that flank the Pir toxin operon. When the deletion occurs, it means that a *Vp*$_{AHPND}$ strain will lose its ability to induce AHPND. However, if the Pir toxin sequence is used as a target for detection, then a colony that has this deletion will produce a negative result even though the colony was derived from an isolate of AHPND-causing *Vp*$_{AHPND}$. A recent report describes a naturally occurring deletion mutant of *Vp*$_{AHPND}$ that does not cause a clinical manifestation of AHPND (Aranguren *et al.,* 2020a).

The plasmid pVA1 also carries a cluster of genes related to conjugative transfer, which means that this plasmid is potentially able to transfer to other bacteria.

#### 2.1.2. Survival and stability in processed or stored samples

AHPND cannot be transmitted from infected samples that have been stored frozen (Tran *et al*., 2013). Some *Vibrio* species are sensitive to freezing (Muntada-Garriga *et al*., 1995; Thomson & Thacker, 1973).

#### 2.1.3. Survival and stability outside the host

*Vp*$_{AHPND}$ is expected to possess similar properties to other strains of *V. parahaemolyticus* found in seafood that have been shown to survive up to 9 and 18 days in filtered estuarine water and filtered seawater at an ambient temperature of 28 ± 2°C (Karunasagar *et al*., 1987).

For inactivation methods, see Section 2.4.5.

### 2.2. Host factors

#### 2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to AHPND according to Chapter 1.5. of the *Aquatic Code* are: giant tiger prawn (*Penaeus monodon*) 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 AHPND according to Chapter 1.5. of the *Aquatic Code* are: f[2]leshy prawn (*Penaeus chinensis*).

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In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following organisms, but an active infection has not been demonstrated: kuruma prawn (*Penaeus japonicus*).

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

Mortalities occur within 30–35 days, and as early as 10 days, of stocking shrimp ponds with postlarvae (PL) or juveniles (Joshi *et al*., 2014b; Nunan *et al.,* 2014; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013). De la Pena *et al*. (2015) reported disease outbreaks in the Philippines occurring as late as 46–96 days after pond-stocking.

#### 2.2.4. Distribution of the pathogen in the host

Gut, stomach, and hepatopancreas.

#### 2.2.5. Aquatic animal reservoirs of infection

In experimental challenges, *Macrobrachium rosenbergii* and *Cherax quadricarinatus* did not show clinical signs of the disease or histopathological changes induced by AHPND but tested positive by PCR assay. However, whether these species serve as reservoirs of infection or are resistant to AHPND needs further investigation (Powers *et al.,* 2021; Schofield *et al.,* 2020).

#### 2.2.6. Vectors

No vector is known, although as *Vibrio* spp. are ubiquitous in the marine environment, the possibility that there are vector species could be expected.

### 2.3. Disease pattern

#### 2.3.1. Mortality, morbidity and prevalence

AHPND is characterised by sudden, mass mortalities (up to 100%) usually within 30–35 days of stocking grow-out ponds with PLs or juveniles (Hong *et al.,* 2016). Older juveniles may also be affected (de la Pena *et al*., 2015).

In regions where AHPND is enzootic in farmed shrimp, evidence indicates a near 100% prevalence (Tran *et al*., 2014).

#### 2.3.2. Clinical signs, including behavioural changes

The onset of clinical signs and mortality can start as early as 10 days post-stocking. Clinical signs include a pale-to-white hepatopancreas (HP), significant atrophy of the HP, soft shells, guts with discontinuous, or no contents and black spots or streaks visible within the HP (due to melanised tubules). In addition, the HP does not squash easily between the thumb and forefinger (probably due to increased fibrous connective tissue and haemocytes) (NACA, 2014).

#### 2.3.3 Gross pathology

AHPND has three infection phases. In the acute phase, there is massive and progressive degeneration of the HP tubules from proximal to distal, with significant rounding and sloughing of the HP tubule epithelial cells into the lumen of the tubule, the HP collecting ducts and the posterior stomach and the absence of bacterial cells. In the terminal phase, the HP shows intra-tubular haemocytic inflammation and develops massive secondary bacterial infections that occur in association with the necrotic and sloughed HP tubule cells. Animals that survive an acute infection reach a chronic phase, in which they present with limited cellular changes in the hepatopancreas tubule and only a few tubules with epithelial necrosis accompanied by bacteria and inflammation. The chronic phase pathology resembles a septic hepatopancreatic necrosis (SHPN) (Aranguren *et al.,* 2020a; NACA, 2014; Nunan *et al*., 2014; Soto-Rodriguez *et al*., 2015; Tran *et al*., 2013; 2014).

#### 2.3.4. Modes of transmission and life cycle

*Vp*$_{AHPND}$ has been transmitted experimentally by immersion, feeding (*per os*) and reverse gavage (Dabu *et al.,* 2017; Joshi *et al*., 2014b; Nunan *et al.,* 2014; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013), simulating natural horizontal transmission via oral routes and co-habitation.

#### 2.3.5. Environmental factors

Water sources with low salinity (<20 ppt) seem to reduce the incidence of the disease. Peak occurrence seems to occur during the hot, dry season from April to July. Overfeeding, poor seed quality, poor water quality, poor feed quality, algal blooms or crashes are also factors that may lead to occurrences of AHPND in endemic areas (NACA, 2014).

#### 2.3.6. Geographical distribution

The disease was initially reported in Asia in 2010. It has since been reported in the Americas (2013) and Africa (2017).

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

### 2.4. Biosecurity and disease control strategies

#### 2.4.1. Vaccination

Not available.

#### 2.4.2. Chemotherapy including blocking agents

Not available.

#### 2.4.3. Immunostimulation

None known to be effective.

#### 2.4.4. Breeding resistant strains

Not available.

#### 2.4.5. Inactivation methods

Experimental studies have shown that *Vp*$_{AHPND}$ could not be transmitted via frozen infected shrimp (Tran *et al*., 2013). Similarly, other strains of *V. parahaemolyticus* are known to be sensitive to freezing, refrigeration, heating and common disinfectants (Muntada-Garriga *et al*., 1995; Thomson & Thacker, 1973).

#### 2.4.6. Disinfection of eggs and larvae

Not available.

#### 2.4.7. General husbandry

As with other infectious diseases of shrimp, established good sanitary and biosecurity practices, such as improvement of hatchery sanitary conditions and PL screening are likely to be beneficial; good broodstock management, use of high-quality post-larvae and good shrimp farm management including strict feeding rate control, appropriate stocking density etc. are all well-established practices that reduce the impact of disease, including AHPND. An AHPND-tolerant line of *P. vannamei* was recently reported, but at present (2022) no genetically improved lines are commercially available (Aranguren *et al.,* 2020b).

## 3. Specimen selection, sample collection, transportation and handling

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

### 3.1. Selection of populations and individual specimens

Samples of moribund shrimp or shrimp that show clinical signs (see Section 2.3.2) should be selected for AHPND diagnosis. It is assumed that adults (broodstock) can carry strains of *Vp*$_{AHPND}$ (Lee *et al*., 2015; Nunan *et al.,* 2014; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013). Therefore, broodstock without clinical signs may also be selected for diagnostic testing.

## 3.2. Selection of organs or tissues

Samples may be taken from gut-associated tissues and organs, such as the hepatopancreas, stomach, midgut and hindgut. In the case of valuable broodstock, non-lethal faecal samples may be collected instead, however the utility of faecal samples compared with tissue samples has not been evaluated.

## 3.3. Samples or tissues not suitable for pathogen detection

Samples other than gut-associated tissues and organs are not appropriate (NACA, 2014; Nunan *et al.,* 2014; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013).

## 3.4. Non-lethal sampling

Faecal matter may be collected from valuable broodstock for AHPND diagnosis. However, compared with tissue sampling, the relative utility of faecal samples for detecting AHPND-causing bacteria has not been evaluated.

## 3.5. Preservation of samples for submission

Samples to be submitted are (i) fresh and chilled on ice for bacterial isolation, (ii) fixed in 90% ethanol for PCR detection and (iii) preserved in Davidson's AFA fixative for histology (Joshi *et al.,* 2014a; 2014b; Lee *et al.,* 2015; Nunan *et al.,* 2104; Sirikharin *et al.,* 2015; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013).

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

### 3.5.1. Samples for pathogen isolation

High quality samples are essential for successful pathogen isolation and bioassay. Sample quality depends mainly on the time since collection and time spent in storage. Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. To avoid degradation of samples, use alternative storage methods only after consultation with the receiving laboratory.

### 3.5.2. Preservation of samples for molecular detection

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

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

Tissue samples for histopathology, immunohistochemistry or in-situ hybridization can be preserved in Davidson's AFA fixative for histology (Joshi *et al.,* 2014a; =2014b; Nunan *et al.,* 2014; Sirikharin *et al.,* 2015; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013).

### 3.5.4. Samples for other tests

Not applicable.

## 3.6. Pooling of samples

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

# 4. Diagnostic methods

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

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

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

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

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

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

| Method | A. Surveillance of apparently healthy animals | | | | B. Presumptive diagnosis of clinically affected animals | | | | C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV |
| **Wet mounts** | | | | | | | | | | | | |
| **Histopathology** | | + | + | NA | | + | + | NA | | | | |
| **Cell culture** | | | | | | | | | | | | |
| **Real-time PCR** | ++ | ++ | ++ | 1 | ++ | ++ | ++ | 1 | | | | |
| **Conventional PCR** | ++ | ++ | ++ | 2 | ++ | ++ | ++ | 2 | ++ | ++ | ++ | 2 |
| **Amplicon sequencing** | | | | | | | | | +++ | +++ | +++ | 1 |
| ***In-situ* hybridisation** | | | | | | | | | | | | |
| **Bioassay** | | | | | + | + | + | NA | + | + | + | NA |
| **LAMP** | | | | | | | | | | | | |
| **Ab-ELISA** | | | | | | | | | | | | |
| **Ag-ELISA** | | | | | | | | | | | | |
| **Other antigen detection methods[3]** | | | | | | | | | | | | |
| **Other methods[3]** | | | | | | | | | | | | |

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively; NA = Not available.

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

[3]Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

## 4.1. Wet mounts

Not applicable.

## 4.2. Histopathology and cytopathology

The disease has three distinct phases:

i) The acute phase is characterised by a massive and progressive degeneration of the HP tubules from proximal to distal, with significant rounding and sloughing of HP tubule epithelial cells into the HP tubules, HP collecting ducts and posterior stomach in the absence of bacterial cells (Nunan *et al*., 2014; Soto-Rodriguez *et al*., 2015; Tran *et al*., 2013; 2014).

ii) The terminal phase is characterised by marked intra-tubular haemocytic inflammation and development of massive secondary bacterial infections that occur in association with the necrotic and sloughed HP tubule cells (NACA, 2012; Nunan *et al*., 2014; Soto-Rodriguez *et al*., 2015; Tran *et al*., 2013; 2014).

iii) The chronic phase is characterised by only a few tubules with epithelial necrosis accompanied by bacteria and inflammation. This phase resembles a septic hepatopancreatic necrosis (SHPN) (Aranguren *et al*., 2020b).

## 4.3. Cell culture for isolation

### 4.3.1. Enrichment of samples prior to DNA extraction

Preliminary enrichment culture for detection of *Vp*$_{AHPND}$ from sub-clinical infections or environmental samples may be carried out using any suitable bacteriological medium (e.g. tryptic–soy broth or alkaline peptone water containing 2.5% NaCl supplement) incubated for 4 hours at 30°C with shaking. Then, after letting any debris settle, the bacteria in the culture broth are pelleted by centrifugation. Discarding the supernatant, DNA can be extracted from the bacterial pellet in preparation for PCR analysis.

### 4.3.2. Agent purification

*Vp*$_{AHPND}$ may be isolated in pure culture from diseased shrimp, sub-clinically infected shrimp, or environmental samples using standard microbiological media for isolation of *Vibrio* species from such sources (Lightner, 1996; Tran *et al*., 2013). Confirmation of identification of *Vp*$_{AHPND}$ may be undertaken by PCR analysis.

## 4.4. Nucleic acid amplification

PCR methods have been developed that target the Vp$_{AHPND}$ toxin genes. The AP3 method is a single-step PCR that targets the 12.7 kDa PirA$^{vp}$ gene (Sirikharin *et al*., 2015). It was validated for 100% positive and negative predictive value by testing 104 isolates of Vp$_{AHPND}$ and non-pathogenic bacteria (including other *Vibrio* and non-*Vibrio* species) that had previously been tested by bioassay (Sirikharin *et al*., 2015). Subsequently, Soto-Rodriguez *et al*. (2015), using 9 Vp$_{AHPND}$ and 11 non-pathogenic isolates of *V. parahaemolyticus* reported that the AP3 method produced the highest positive (90%) and negative (100%) predictive values of five PCR methods tested.

Single-step PCRs such as the AP3 method and others, e.g. VpPirA-284, VpPirB-392 (Han *et al*., 2015a) and TUMSAT-Vp3 (Tinwongger *et al*., 2014), have relatively low sensitivity when used for detection of Vp$_{AHPND}$ at low levels (e.g. sub-clinical infections) or in environmental samples such as sediments and biofilms. For such samples, a preliminary enrichment step (see Section 4.3.1. *Enrichment of samples prior to DNA extraction*) is recommended.

Alternatively, a nested PCR method, AP4, has been developed with a 100% positive predictive value for Vp$_{AHPND}$ using the same 104 bacterial isolates used to validate AP3 above (Dangtip *et al*., 2015), and has greater sensitivity (1 fg of DNA extracted from Vp$_{AHPND}$), allowing it to be used directly with tissue and environmental samples without an enrichment step.

In addition, real-time PCR methods, for example the Vp$_{AHPND}$-specific TaqMan real-time PCR developed by Han *et al*. (2015b), and an isothermal loop-mediated amplification protocol (LAMP) method developed by Koiwai *et al*. (2016) also have high sensitivity and can be used directly with tissue and environmental samples without an enrichment step.

A general DNA extraction method may be used to extract DNA from the stomach or hepatopancreatic tissue of putatively infected shrimp, from cultures of purified bacterial isolates or from bacterial pellets from enrichment cultures (see Section 4.3). The amount of template DNA in a 25 µl PCR reaction volume should be in the range of 0.01–1 ng of DNA when extracted from bacterial isolates (i.e. directly from a purified culture) and in the range of 10–100 ng of total DNA when extracted from shrimp tissues or from a bacterial pellet derived from an enrichment culture.

The following controls should be included in all *Vp*$_{AHPND}$ PCR assays: a) negative extraction control i.e. DNA template extracted at the same time from a known negative sample; b) DNA template from a known positive sample, such as *Vp*$_{AHPND}$-affected shrimp tissue or DNA from an *Vp*$_{AHPND}$-positive bacterial culture, or plasmid DNA that contains the target region of the specific set of primers; c) a non-template control. In addition, a further control is required to demonstrate that extracted nucleic acid is free from PCR inhibitors, for example, for shrimp tissues use of the decapod 18S rRNA PCR (Lo *et al*., 1996) or use the 16S rRNA PCR for bacteria (Weisburg *et al*., 1991).

### 4.4.1. Real-time PCR

This protocol is based on the method described by Han *et al*. (2015b). The TaqMan Fast Universal PCR Master Mix (Life Technologies) is used and extracted DNA is added to the real-time PCR mixture containing 0.3 µM of each primer and 0.1 µM probe to a final volume of 10 µl. Real-time PCR conditions consist of 20 seconds at 95°C, followed by 45 cycles of 3 seconds at 95°C and 30 seconds at 60°C. At the completion of the TaqMan real-time PCR assay, the presence of PirA DNA is demonstrated by the presence of specific amplicons, identified by software-generated characteristic amplification curves. No-template controls must have no evidence of specific amplicons.The primers and probe and target gene for the *Vp*$_{AHPND}$-specific real-time PCR are listed in Table 4.4.1.1.

***Table 4.4.1.1.*** *Primers and probe for the real-time PCR method for detection of pirA toxin gene*

| Primer/ probe name | Sequence (5'–3') | Target gene | Reference |
|---|---|---|---|
| VpPirA-F | TTG-GAC-TGT-CGA-ACC-AAA-CG | pirA | Han *et al.,* 2015b |
| VpPirA-R | GCA-CCC-CAT-TGG-TAT-TGA-ATG | | |
| VpPirA Probe | FAM-AGA-CAG-CAA-ACA-TAC-ACC-TAT-CAT-CCC-GGA-TAMRA | | |

### 4.4.2. Conventional PCR

#### One-step PCR detection of pVA1 plasmid

Two one-step PCR methods (AP1 and AP2) are described here for detection of the pVA1 plasmid in enrichment broth cultures. The primers, target gene and the size of the expected amplicons are listed in Table 4.4.2.1.

***Table 4.4.2.1.*** *PCR primers for one-step PCR detection of pVA1 plasmid*

| Method name | Primers (5'–3') | Target gene | Expected amplicon size | Reference |
|---|---|---|---|---|
| AP1 | AP1F: 5CCT-TGG-GTG-TGC-TTA-GAG-GAT-G<br>AP1R: GCA-AAC-TAT-CGC-GCA-GAA-CAC-C | *pVA1* | 700bp | Flegel & Lo (2014) |
| AP2 | AP2F: TCA-CCC-GAA-TGC-TCG-CTT-GTG-G<br>AP2R: CGT-CGC-TAC-TGT-CTA-GCT-GAA-G | *pVA1* | 700bp | Flegel & Lo (2014) |

#### Protocol for the AP1 and AP2 PCR methods

This protocol follows the method described by Flegel & Lo (2014). The PCR reaction mixture consists of 2.5 µl 10× PCR mix, 0.7 µl 50 mM $MgCl_2$, 0.4 µl 10 mM dNTPs, 0.5 µl 10 µM AP1/AP2F, 0.5 µl 10 µM AP1/AP2R, 0.2 µl Taq DNA polymerase and approximately 0.01–1 ng of template DNA in a total volume of 25 µl made up with distilled water. For PCR a denaturation step of 94°C for 5 minutes is followed by 25–30 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 60 seconds with a final extension step at 72°C for 10 minutes and then the reaction mixture can be held at 4°C (https://enaca.org/publications/health/disease-cards/ahpnd-detection-method-announcement.pdf).

**One-step PCR detection of PirA/PirB toxin genes**

Four one-step PCR methods (AP3, TUMSAT-Vp3, VpPirA-284 and VpPirB-392) are described here for detection of Pir toxin genes in enrichment broth cultures. The primers, target gene and the size of the expected amplicons are listed in Table 4.4.2.3.

***Table 4.4.2.2.*** *PCR primers for one-step PCR detection of PirA and PirB toxin genes*

| Method name | Primers (5'–3') | Target gene | Expected amplicon size | Reference |
|---|---|---|---|---|
| AP3 | AP3-F: ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC<br>AP3-R: GTG-GTA-ATA-GAT-TGT-ACA-GAA | *pir*A$^{vp}$ | 333bp | Sirikharin *et al*., 2015 |
| TUMSAT-Vp3 | TUMSAT-Vp3 F: GTG-TTG-CAT-AAT-TTT-GTG-CA<br>TUMSAT-Vp3 R: TTG-TAC-AGA-AAC-CAC-GAC-TA | *pir*A$^{vp}$ | 360bp | Tinwongger *et al*., 2014 |
| VpPirA-284 | VpPirA-284F: TGA-CTA-TTC-TCA-CGA-TTG-GAC-TG<br>VpPirA-284R: CAC-GAC-TAG-CGC-CAT-TGT-TA | *pir*A$^{vp}$ | 284bp | Han *et al.,* 2015a |
| VpPirB-392 | VpPirB-392F: TGA-TGA-AGT-GAT-GGG-TGC-TC<br>VpPirB-392R: TGT-AAG-CGC-CGT-TTA-ACT-CA | *pirB*$^{vp}$ | 392bp | Han *et al.,* 2015a |

**Protocol for the AP3 PCR method**

This protocol follows the method described by Sirikharin *et al*. (2015). The PCR reaction mixture consists of 2.5 µl 10× PCR mix, 0.7 µl 50 mM $MgCl_2$, 0.4 µl 10 mM dNTPs, 0.5 µl 10 µM AP3-F1, 0.5 µl 10 µM AP3-R1, 0.2 µl Taq DNA polymerase and approximately 100 ng of template DNA in a total volume of 25 µl made up with distilled water. For PCR a denaturation step of 94°C for 5 minutes is followed by 30 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 40 seconds with a final extension step at 72°C for 5 minutes and then the reaction mixture can be held at 4°C.

**Protocol for the VpPirA-284 and VpPirB-392 PCR methods**

This protocol follows the method described by Han *et al.* (2015a) and uses PuReTaq ready-to-go PCR beads (GE Healthcare). A 25 µl PCR reaction mixture is prepared with PuReTaq ready-to-go PCR beads. Each reaction contains 0.2 µM of each primer, 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of Taq DNA polymerase, and 1 µl of extracted DNA. For PCR a 3-minute denaturation step at 94°C is followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 7 minutes.

**Protocol for the TUMSAT-Vp3 PCR method**

This protocol follows the method described by Tinwongger *et al.* (2014). A 30 µl PCR mixture is prepared containing 1 µl DNA template, 10× PCR buffer, 0.25 mM dNTP mixture, 0.6 µM of each primer and 0.01 U Taq polymerase. PCR conditions consist of an initial preheating stage of 2 minutes at 95°C, followed by 30 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 56°C and 30 seconds extension at 72°C.

**AP4 nested PCR protocol for detection of $Vp_{AHPND}$**

This protocol follows the method described by Dangtip *et al.* (2015). The first PCR reaction mixture consists of 2.5 µl 10× PCR mix, 1.5 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 0.5 µl 10 µM AP4-F1, 0.5 µl 10 µM AP4-R1, 0.3 µl of Taq DNA pol (5 units $µl^{-1}$) and approximately 100 ng of template DNA in a total volume of 25 µl made up with distilled water. The PCR protocol is 94°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds with a final extension step at 72°C for 2 minutes and hold at 4°C.

The nested PCR reaction mixture consists of 2.5 µl 10x PCR mix, 1.5 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 0.375 µl 10 µM AP4-F2, 0.375 µl 10 µM AP4-R2, 0.3 µl Taq DNA pol (5 units $µl^{-1}$) and 2 µl of the first PCR reaction in a total volume of 25 µl. The nested PCR protocol is 94°C for 2 minutes followed by 25 cycles of 94°C for 20 seconds, 55°C for 20 seconds and 72°C for 20 seconds and hold at 4°C.

The nested PCR primers, designed using the China (People's Rep. of) isolate of AHPND bacteria (Yang *et al.,* 2014), are shown in Table 4.4.2.7. The expected amplicon sizes are 1269 bp for the outer primers (AP4-F1 and AP4-R1) and 230 bp for the inner primers (AP4-F2 and AP4-R2). At high concentrations of target DNA, additional amplicons may occur as the product of residual primer AP4-F1 pairing with AP4-R2 (357 bp) or AP4-F2 with AP4-R1 (1142 bp) in the nested step.

***Table 4.4.2.3.*** *Primers for the AP4, nested PCR method for detection of PirA and PirB toxin genes*

| Method name | Primers (5'–3') | Expected amplicon size | Reference |
|---|---|---|---|
| AP4<br>Step 1 | AP4-F1: ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC<br>AP4-R1: ACG-ATT-TCG-ACG-TTC-CCC-AA | 1269 | Dangtip *et al.,* 2015 |
| AP4<br>Step 2 | AP4-F2: TTG-AGA-ATA-CGG-GAC-GTG-GG<br>AP4-R2: GTT-AGT-CAT-GTG-AGC-ACC-TTC | 230 | |

**Analysis of conventional PCR products by agarose gel electrophoresis**

After PCR, amplicons are visualised by agarose gel electrophoresis. Twenty µl of the PCR reaction mixture, with 6× loading dye added, is loaded onto a 1.5% agarose gel and electrophoresis is carried out at 90 volts for 40 minutes. Amplicons are visualised with SYBR Safe gel stain (Invitrogen, Cat. No. 33102) according to the manufacturer's instructions. Amplicons of the expected size appropriate for the PCR methods used (Tables 4.4.2.1, 4.4.2.2 and 4.4.2.3) indicate a positive result.

### 4.4.3. Other nucleic acid amplification methods

Cruz-Flores *et al.* (2019) developed a multiplex real-time PCR-based SYBR green assay for simultaneous detection of *pirA*, *pirB*, 16S rRNA and 18S rRNA, and a duplex real-time PCR-based Taqman probe assay showing high specificity and sensitivity – limit of detection was 10 copies for both *pirA and pirB*.

A recombinase polymerase amplification assay was developed by Mai *et al*. (2021). This assay has a limit of detection of five copies of the *pirAB* gene and high specificity. A LAMP-based assay for AHPND detection developed by Koiwai *et al.* (2016) also shows high specificity and sensitivity.

## 4.5. Amplicon sequencing

The positive results obtained from conventional PCR described in 4.4.2 need to be confirmed by sequencing.

## 4.6. *In-situ* hybridisation

ISH is not currently available (December 2021).

## 4.7. Immunohistochemistry

An immunohistochemistry assay to detect AHPND was developed by Kumar *et al.,* (2019). However, the assay requires further validation.

## 4.8. Bioassay

*Vp*$_{AHPND}$ has been transmitted experimentally by immersion and by reverse gavage (Joshi *et al*., 2014b; Nunan *et al.,* 2014; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013), simulating natural horizontal transmission via oral routes and co-habitation. Thus, following isolation and purification of a bacterium that is suspected to cause AHPND, a bioassay can be performed to confirm the presence of the causative agent. The immersion procedure is carried out by immersing 15 shrimp for 15 minutes, with aeration, in a suspension (150 ml clean artificial seawater) of $2 \times 10^8$ cells of the cultured bacterium per ml. Following this initial 15-minute period, the shrimp and the inoculum are transferred to a larger tank with a volume of clean artificial seawater to make the final concentration of the bacterium $2 \times 10^6$ cells $ml^{-1}$. Shrimp are monitored at 6- to 8-hour intervals. Dead shrimp can be processed for *Vp*$_{AHPND}$ PCR and sequence analysis. Moribund or surviving shrimp are processed for histology, bacterial re-isolation, PCR and sequence analysis. A positive bioassay is indicated by the detection of characteristic histological lesions and *Vp*$_{AHPND}$ by PCR and amplicon sequence analysis.

## 4.9. Antibody- or antigen-based detection methods

An indirect enzyme-linked immunosorbent assay (I-ELISA) for AHPND detection developed by Mai *et al.* (2020) showed high sensitivity (the limit of detection was 0.008 ng $\mu l^{-1}$ for PirA$^{vp}$ and 0.008 ng $\mu l^{-1}$ for PirB$^{vp}$) and specificity.

### 4.10. Other methods

None.

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

Real-time PCR (Han *et al.,* 2015b) is recommended for demonstrating freedom from AHPND in an apparently healthy population.

## 6. Corroborative diagnostic criteria

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

The case definitions for suspect and confirmed cases have been developed to support decision-making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

### 6.1. Apparently healthy animals or animals of unknown health status[3]

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

#### 6.1.1. Definition of suspect case in apparently healthy animals

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

i) A positive result by any of the real-time or conventional PCR methods recommended in Table 4.1

ii) Histo- or cytopathological changes consistent with the presence of the pathogen or the disease

#### 6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with *Vibrio parahaemolyticus* ($Vp_{AHPND}$) is considered to be confirmed if the following criterion is met:

i) Positive results by real-time PCR and conventional PCR followed by amplicon sequence analysis.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

### 6.2 Clinically affected animals

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

#### 6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with *Vibrio parahaemolyticus* ($Vp_{AHPND}$) shall be suspected if at least one of the following criteria is met:

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

[3] For example transboundary commodities.

ii) A positive result by real-time PCR

iii) A positive result by conventional PCR

### 6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with *Vibrio parahaemolyticus* (*Vp*$_{AHPND}$) is considered to be confirmed if the following criterion is met:

i) Positive results by real-time PCR and conventional PCR followed by amplicon sequence analysis.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

## 6.3. Diagnostic sensitivity and specificity for diagnostic tests

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

### 6.3.1. For presumptive diagnosis of clinically affected animals

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| Conventional PCR | Diagnosis | Clinically diseased and apparently healthy shrimp | AHPND causing and non-causing bacterial isolates | *Penaeus vannamei* | 100 | 100 | Bioassay | Sirikharin *et al.,* 2015 |
| | | | | | | | | |
| Conventional PCR | Diagnosis | Clinically diseased and apparently healthy shrimp | AHPND causing and non-causing bacterial isolates | NA | 100[1] | 100 | Bioassay | Tinwongger *et al*., 2014 |
| Real-time PCR | Diagnosis | Clinicallly diseased animals | Hepato-pancreas | *Penaeus vannamei* | 100 | NA | Bioassay and histopathology | Han *et al.* 2015b |

DSe = diagnostic sensitivity, DSp = diagnostic specificity, NA= Not available,
PCR: = polymerase chain reaction.
[1]100% sensitivity for TUMSAT-Vp3 primer set

### 6.3.2. For surveillance of apparently healthy animals

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

DSe = diagnostic sensitivity, DSp = diagnostic specificity NA= Not available,
PCR: = polymerase chain reaction.

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* *

**NB:** There are OIE Reference Laboratories for acute hepatopancreatic necrosis disease
(see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list:
https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the OIE Reference Laboratory for any further information on
acute hepatopancreatic necrosis disease

**NB**: FIRST ADOPTED IN 2017; MOST RECENT UPDATES ADOPTED IN 2018.

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CHAPTER 2.2.3.

# INFECTION WITH *HEPATOBACTER PENAEI* (NECROTISING HEPATOPANCREATITIS)

## 1. Scope

Infection with Candidatus *Hepatobacter penaei* means infection with the pathogenic agent *H. penaei,* an obligate intracellular bacterium of the Order α-*Proteobacteria*.

## 2. Disease information

### 2.1. Agent factors

#### 2.1.1. Aetiological agent

*Hepatobacter penaei* is a pleomorphic, Gram-negative, intracytoplasmic bacterium (Nunan *et al.,* 2013). It is a member of the α-*Proteobacteria* (Frelier *et al.,* 1992; Lightner & Redman, 1994; Loy & Frelier, 1996; Loy *et al.,* 1996). More recently it has been suggested that it belongs to the *Holosporaceae* family within the *Rickettsiales* (Leyva *et al.,* 2018). The predominant form is a rod-shaped rickettsial-like organism (0.25 × 0.9 µm), whereas the helical form (0.25 × 2–3.5 µm) possesses eight flagella at the basal apex (Frelier *et al.,* 1992; Lightner & Redman, 1994; Loy & Frelier, 1996; Loy *et al.,* 1996). Genetic analysis of *H. penaei* associated with North and South American outbreaks suggests that the isolates are either identical or very closely related subspecies (Loy *et al.,* 1996). Recently analysis based on the 16S rRNA confirm the high similarity among different *H. penaei* isolates in the Americas (99–100%) (Aranguren & Dhar, 2018).

#### 2.1.2. Survival and stability in processed or stored samples

*Hepatobacter penaei*-infected tissues remain infectious after repeated cycles of freeze–thawing and after storage in 50% glycerine. *Hepatobacter penaei* frozen at –20°C to –70°C and –80°C have been shown to retain infectivity in experimental transmission trials with *Penaeus vannamei* (Crabtree *et al.*, 2006; Frelier *et al.,* 1992). Flash freezing *H. penaei* at –70°C to –80°C does not significantly affect the infectivity (Aranguren *et al*., 2010; Crabtree *et al.,* 2006).

#### 2.1.3. Survival and stability outside the host

No information available.

### 2.2. Host factors

#### 2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with *H. penaei* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) include: whiteleg shrimp (*P. 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 *H. penaei* according to Chapter 1.5. of the *Aquatic Code* include: aloha prawn (*P. marginatus*), banana prawn (*P. merguiensis*), blue shrimp (*P. stylirostris*), giant tiger prawn (*P. monodon*), northern brown shrimp (*P. aztecus*), northern pink shrimp (*P. duorarum*) and northern white shrimp (*P. setiferus*).

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: American lobster (*Homarus americanus*) (Avila-Villa *et al.,* 2012; Bekavac *et al.,* 2022).

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

Infection with *H. penaei* has been demonstrated in postlarvae, juveniles, adults and broodstock of *P. vannamei* (Aranguren *et al.*, 2006).

### 2.2.4. Distribution of the pathogen in the host

The target tissue is the hepatopancreas: infection with *H. penaei* has been reported in all hepatopancreatic cell types (Lightner 2012). *Hepatobacter penaei* is also present in the faeces (Brinez *et al.,* 2003).

### 2.2.5. Aquatic animal reservoirs of infection

Some members of *P. vannamei* populations that survive infection with *H. penaei* may carry the intracellular bacteria for life and transmit it to other populations by horizontal transmission (Aranguren *et al.,* 2006; Lightner, 2005; Morales-Covarrubias, 2010; Vincent & Lotz, 2005).

### 2.2.6. Vectors

No vectors are known in natural infections.

## 2.3. Disease pattern

### 2.3.1. Mortality, morbidity and prevalence

Infection with *H. penaei* results in the mortalities approaching 100% in *P. vannamei*, 5.6–15% in *P. duorarum*, and 5–17% in *P. aztecus* (Aguirre-Guzman *et al.,* 2010).

The prevalence was reported as 0.77% in cultured *P. vannamei* and 0.43 in cultured *P. stylirostris* in Peru (Lightner & Redman, 1994), 5–86.2% in Mexico (Ibarra-Gamez *et al.,* 2007), and 0.6–1.3% in *P. vannamei* in Belize, Brazil, Guatemala, Honduras, Mexico, Nicaragua and Venezuela (Morales-Covarrubias *et al*., 2011).

NHP-affected broodstock ponds in Colombia reported mortalities of up to 85%, while non NHP-affected broodstock ponds in the same farm experienced mortalities of 40–50% (Aranguren *et al.,* 2006)

### 2.3.2. Clinical signs, including behavioural changes

A wide range of gross signs can be used to indicate the possible presence of infection with *H. penaei*. These include lethargy, reduced food intake, atrophied hepatopancreas, anorexia and empty guts, noticeably reduced growth and poor length weight ratios ('thin tails'); soft shells and flaccid bodies; black or darkened gills; heavy surface fouling by epicomensals organisms; bacterial shell disease, including ulcerative cuticle lesions or melanised appendage erosion; and expanded chromatophores resulting in the appearance of darkened edges in uropods and pleopods. None of these signs are pathognomonic. (Lightner, 1996; Loy *et al.,* 1996).

### 2.3.3 Gross pathology

Infection with *H. penaei* often causes an acute disease with very high mortalities in young juveniles, adults and broodstock. In horizontally infected young juveniles, adult and broodstock, the incubation period and severity of the disease are somewhat size or age dependent. Gross signs are not specific, but shrimp with acute infection with *H. penaei* show a marked reduction in food consumption, followed by changes in behaviour and appearance including pale discoloration of the hepatopancreas with further size reduction.

### 2.3.4. Modes of transmission and life cycle

Horizontal transmission of *H. penaei* can be through cannibalism or by contaminated water (Aranguren *et al.,* 2006; 2010; Frelier *et al.,* 1993; Gracia-Valenzuela *et al*., 2011; Vincent *et al*., 2004). *Hepatobacter penaei* in faeces shed into pond water has also been suggested as a source of contamination (Aranguren *et al.,* 2006; Briñez *et al.,* 2003; Morales-Covarrubias *et al*., 2006). *Hepatobacter penaei*-positive broodstock females produce postlarvae that were also *H. penaei*-positive, which suggests that a transmission from broodstock to progeny can occurs (Aranguren *et al.,* 2006).

### 2.3.5. Environmental factors

The occurrence of infection with *H. penaei* in farms may increase during long periods of high temperatures (>29°C) and high salinity (20–38 ppt) (Morales-Covarrubias, 2010). In the months when temperatures are high during the day and low at night, high prevalence and mortality (>20%) are observed (Morales-Covarrubias, 2010).

### 2.3.6. Geographical distribution

*Hepatobacter penaei* appears to have a Western Hemisphere distribution in both wild and cultured penaeid shrimp (Aguirre-Guzman *et al.,* 2010; Del Rio-Rodriguez *et al.,* 2006). In the Western Hemisphere, *H. penaei* is commonly found in cultured penaeid shrimp in the Americas (Aranguren *et al.,* 2010; Frelier *et al.,* 1992; Ibarra-Gamez *et al.,* 2007; Morales-Covarrubias, 2010; Morales-Covarrubias *et al.*, 2011). *Hepatobacter penaei*, was introduced into Africa from North America via movement of infected *P.vannamei* broodstock, however NHP was later eradicated by fallowing (Lightner *et al.,* 2012).

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

## 2.4. Biosecurity and disease control strategies

Early detection (initial phase) of clinical infection with *H. penaei* is important for successful treatment because of the potential for cannibalism to amplify and transmit the disease. Shrimp starvation and cannibalism of infected shrimp, and positive conditions for *H. penaei* enaei multiplication, are important factors for the spread of *H. penaei* in *P. vannamei*. Preventive measures include raking, tilling, and removing sediments from the bottom of the ponds, prolonged drying (through exposure to sunlight) of ponds and water distribution canals for several weeks, disinfection of fishing gear and other farm equipment using calcium hypochlorite, extensive liming of ponds and the use of ponds liners. The use of specific pathogen-free (SPF) broodstock is an effective preventive measure. NHP, particularly in the initial phase, can be treated by using antibiotics in medicated feeds. *Hepatobacter penaei* is sensitive to oxytetracycline (Lightner & Redman, 1994).

### 2.4.1. Vaccination

No scientifically confirmed reports.

### 2.4.2. Chemotherapy including blocking agents

No scientifically confirmed reports.

### 2.4.3. Immunostimulation

No scientifically confirmed reports.

### 2.4.4. Breeding resistant strains

One population from Latin America that has been selected for several generations for resistance to Taura syndrome virus in the presence of infection with *H. penaei*, seems to be more resistant to NHP disease than the Kona line under experimental conditions (Aranguren *et al.,* 2010)

### 2.4.5. Inactivation methods

The use of hydrated lime ($Ca(OH)_2$) to treat the bottom of ponds during pond preparation before stocking can help reduce infection with *H. penaei*.

### 2.4.6. Disinfection of eggs and larvae

Disinfection of eggs and larvae is a good management practice and is recommended for its potential to reduce *H. penaei* contamination of spawned eggs and larvae (and contamination by other disease agents).

### 2.4.7. General husbandry

The prevalence and severity of infection with *H. penaei* may be increased by rearing shrimp in relatively crowded or stressful conditions. Some husbandry practices have been successfully applied to the prevention of infection with *H. penaei*. Among these has been the application of PCR to pre-screening of wild or pond-reared broodstock.

# 3. Specimen selection, sample collection, transportation and handling

## 3.1. Selection of populations and individual specimens

Suitable specimens for testing for infection with *H. penaei* are the following life stages: postlarvae (PL), juveniles and adults.

## 3.2. Selection of organs or tissues

*Hepatobacter penaei* infects most enteric tissue. The principal target tissue for *H. penaei* is the hepatopancreas (Lightner, 2012).

## 3.3. Samples or tissues not suitable for pathogen detection

*Hepatobacter penaei* does not replicate in the midgut, caeca, connective tissue cells, the gills, haematopoietic nodules and haemocytes, ventral nerve cord and ganglia, antennal gland tubule epithelial cells, and lymphoid organ parenchymal cells. Samples of pleopods or haemolymph are not recommended for *H. penaei* detection by PCR.

## 3.4. Non-lethal sampling

Faeces may be collected and used for testing (usually by PCR, when non-lethal testing of valuable broodstock is necessary (Brinez *et al.,* 2003; Frelier *et al.,* 1993; Lightner, 1996). Faeces samples have not been validated to the same level as hepatopancreas samples.

## 3.5. Preservation of samples for submission

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

### 3.5.1. Samples for pathogen isolation

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

### 3.5.2. Preservation of samples for molecular detection

Tissue samples of hepatopancreas or faeces for PCR testing should be preserved in 70–95% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.

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

Standard sample collection, preservation and processing methods for histological techniques can be found in Section 5.3 of Chapter 2.2.0.

### 3.5.4. Samples for other tests

No scientifically confirmed reports.

## 3.6. Pooling of samples

Pooling of samples from more than one individual animal for a given purpose should only be recommended where robust supporting data on diagnostic sensitivity and diagnostic specificity have been evaluated and found to be suitable. The effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, therefore, larger shrimp should be processed and tested individually. Small life stages such as PL or specimens up to 0.5 g can be pooled to obtain the minimum amount of material for *H. penaei* molecular detection.

# 4. Diagnostic methods

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

**Ratings for purposes of use.** For each recommended assay a qualitative rating for the purpose of use is provided. The ratings are determined based on multiple performance and operational factors relevant to application of an assay for a defined purpose. These factors include appropriate diagnostic performance characteristics, level of

assay validation, availability cost, timeliness, and sample throughput and operability. For a specific purpose of use, assays are rated as:

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

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

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

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

| Method | A. Surveillance of apparently healthy animals | | | | B. Presumptive diagnosis of clinically affected animals | | | | C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV |
| **Wet mounts** | | | | | | + | + | NA | | | | |
| **Histopathology** | | | | | | ++ | ++ | NA | | | | |
| **Cell culture** | | | | | | | | | | | | |
| **Real-time PCR** | ++ | +++ | +++ | 1 | ++ | +++ | +++ | 1 | ++ | ++ | ++ | 1 |
| **Conventional PCR** | ++ | +++ | +++ | 1 | ++ | +++ | +++ | 1 | ++ | +++ | +++ | 1 |
| **Amplicon sequencing** | | | | | | | | | +++ | +++ | +++ | 1 |
| ***In-situ* hybridisation** | | | | | + | ++ | ++ | NA | + | ++ | ++ | NA |
| **Bioassay** | | | | | + | + | + | NA | + | + | + | NA |
| **LAMP** | | | | | | | | | | | | |
| **Ab-ELISA** | | | | | | | | | | | | |
| **Ag-ELISA** | | | | | | | | | | | | |
| **Other antigen detection methods[3]** | | | | | | | | | | | | |
| **Other methods[3]** | | | | | | | | | | | | |

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); NA = not available; PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.

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

[3]Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

## 4.1. Wet mounts

Wet mount squash examination of hepatopancreas tissue is generally conducted to detect presumptive infection with *H. penaei*. The hepatopancreas may be atrophied and have any of the following characteristics: soft and watery; fluid filled centre; pale colour with or without black stripes (melanised tubules). Hepatopancreatic tubules show deformity at the distal portion; multifocal melanisation initially at the distal portion of the tubule and, later on, in the medial and proximal portion; reduced or absence of lipid droplets (Lightner, 2012).

## 4.2. Histopathology and cytopathology

Histological methods can be useful for indicating acute and chronic infection with *H. penaei*.

Initial infection with *H. penaei* is difficult to diagnose using routine H&E histological methods. Therefore molecular methods are recommended for initial *H. penaei* detection (e.g. by PCR or application of *H. penaei*-specific DNA probes or *in-situ* hybridisation [ISH] of histological sections).

Acute infection with *H. penaei* is characterised by atrophied hepatopancreas with moderate atrophy of the tubule epithelia, presence of bacterial cells and infiltrating haemocytes involving one or more of the tubules (multifocal encapsulations). Hypertrophic cells, individual epithelial cells, appeared to be separated from adjacent cells, undergo necrosis and desquamation into the tubular lumen. The tubular epithelial cell lipid content is variable.

The transitional phase of infection with *H. penaei* is characterised by haemocytic inflammation of the intertubular spaces in response to necrosis, cytolysis, and sloughing of hepatopancreas tubule epithelial cells. The hepatopancreas tubule epithelium is markedly atrophied, resulting in the formation of large oedematous (fluid filled or 'watery') areas in the hepatopancreas. Tubule epithelial cells within multifocal encapsulation are typically atrophied and reduced from simple columnar to cuboidal morphology. They contain little or no stored lipid vacuoles, markedly reduced or no secretory vacuoles and masses of bacteria. At this phase haemocyte nodules are observed in the presence of masses of bacteria in the centre of the nodule.

In the chronic phase of infection with *H. penaei*, tubular lesions, multifocal encapsulation and oedematous areas decline in abundance and severity and are replaced by infiltration and accumulation of haemocytes at the sites of necrosis. There are areas with fibrosis, few melanised and necrotic tubules and very low presence of hypertrophied cells with masses of bacteria in the cytoplasm and low numbers of haemocyte nodules.

## 4.3. Cell culture for isolation

*Hepatobacter penaei* has not been grown *in vitro*. No crustacean cell lines exist (Vincent & Lotz, 2007).

## 4.4. Nucleic acid amplification

PCR methods including PCR and real-time PCR have been developed that target several *H. penaei* genes including 16S rRNA and Flg E genes (Aranguren & Dhar, 2018; Aranguren *et al.,* 2010; Loy *et al*., 1996).

*DNA extraction*

A general DNA extraction method may be used to extract DNA from the hepatopancreatic tissue of putatively infected shrimp. The amount of template DNA in a 10–25 µl PCR reaction volume should be in the range of 10–100 ng of total DNA

### 4.4.1. Real-time PCR

Real-time PCR methods for detection of *H. penaei* have the advantages of speed, specificity and sensitivity. The sensitivity of real-time PCR is ~100 copies of the target sequence from the *H. penaei* genome (Aranguren & Dhar, 2018; Aranguren *et al.,* 2010; Vincent & Lotz, 2005).

**Protocol 1**

The real-time PCR method using TaqMan chemistry described below for *H. penaei* based on the 16S rRNA gene generally follows the method used in Aranguren *et al* (2010).

i) The PCR primers and TaqMan probe are selected from the 16S, rRNA gene of *H. penaei* (GenBank U65509) (Loy & Frelier*,* 1996). The primers and TaqMan probe were designed by the Primer Express software version 2.0 (Applied Biosystems). The upstream (NHP1300F) and downstream (NHP1366R) primer sequences are: 5'-CGT-TCA-CGG-GCC-TTG-TACAC-3' and 5'-GCT-CAT-CGC-CTT-AAA-GAA-AAG-ATA-A-3', respectively. The TaqMan probe NHP: 5'-CCG-CCC-GTC-AAG-CCA-TGG-AA-3', which corresponds to the region from nucleotides 1321–1340, is synthesised and labelled with fluorescent dyes 6-carboxyfluorescein (FAM) on the 5' and N,N,N,Ntetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end.

ii) *The real-time PCR reaction mixture contains:* TaqMan One-step real-time PCR SuperMix (Quanta, Biosciences), 0.3 µM of each primer, 0.1 µM of TaqMan probe, 5–50 ng DNA, and water in a reaction volume of 25 µl. For optimal results, the reaction mixture should be vortexed and mixed well.

iii) Amplification is performed with the master cycler Realplex 2.0 (Eppendorf). The cycling consists of initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. After each cycle, the levels of fluorescence are measured.

iv) It is necessary to include a 'no template control' in each reaction run. This is to rule out the presence of fluorescence contaminants in the reaction mixture and also to rule out reagent contamination with the specific target of the assay. A positive control should also be included, and this can be plasmid DNA containing the target sequence, purified bacteria, or DNA extracted from *H. penaei*-infected hepatopancreas.

**Protocol 2**

Another real-time PCR method using TaqMan chemistry described below for *H. penaei* is based on the flagella gene (flagella hook protein, flgE) (Aranguren *&* Dhar 2018).

i) The PCR primers and TaqMan probe were selected from the Flg E gene of *H. penaei* (GenBank JQAJ01000001.1) (Aranguren & Dhar, 2018). The primers and TaqMan probe were designed by the Primer Express software version 3.0 (Applied Biosystems). The upstream (NHP FlgE3qF) and downstream (FlgE3qR) primer sequences are: 5'-AAC-ACC-CTG-TCT-CCC-CAA-TTC-3'; and 5'-CCA-GCC-TTG-GAC-AAA-CAC-CTT-3', respectively. The TaqMan probe NHP: 5'-CGC-CCC-AAA-GCA-TGC-CGC-3', is synthesised and labelled with fluorescent dyes 6-carboxyfluorescein (FAM) on the 5' and N,N,N,Ntetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end.

ii) *The real-time PCR reaction mixture contains:* The amplification reactions were conducted as follows: 0.5 µM of each primer, 0.1 µM TaqMan probe, 1× TaqMan Fast Virus 1-Step Master Mix (Life Technologies), 5–50 ng DNA template and HPLC water in a reaction volume of 10 µl. For optimal results, the reaction mixture should be vortexed and mixed well.

iii) The real-time PCR profile consists of 20 seconds at 95ºC followed by 40 cycles of 1 second at 95ºC and 20 seconds at 60ºC. Amplification detection and data analysis for real-time PCR assays are carried out with the StepOnePlus real-time PCR system (Life Technologies).

iv) It is necessary to include a 'no template control' in each reaction run. This is to rule out the presence of fluorescence contaminants in the reaction mixture and also to rule out reagent contamination with the specific target of the assay. A positive control should also be included, and this can be plasmid DNA containing the target sequence, or DNA extracted from *H. penaei*-infected hepatopancreas.

### 4.4.2. Conventional PCR

Hepatopancreas may be assayed for *H. penaei* using PCR. Two different PCR methods have been developed for *H. penaei* detection using 16S rRNA gene and Flg E gene separately.

**Protocol 1**

The PCR based on 16S rRNA is based on Aranguren *et al.* (2010). Primers designated as NHPF2: 5'-CGT-TGG-AGG-TTC-GTC-CTT-CAGT-3' and NHPR2: 5'-GCC-ATG-AGG-ACC-TGA-CAT-CAT-C-3', amplify a 379-base pair (bp) fragment corresponding to the 16S rRNA of *H. penaei*. The PCR method outlined below generally follows the method described in Aranguren *et al.* (2010).

i) The following controls should be included when performing the PCR assay a) known *H. penaei* negative tissue sample; b) a known *H. penaei* positive sample (hepatopancreas); and c) a 'no template' control.

ii) The PuReTaq™ Ready-To-Go PCR Bead (RTG beads, GE Healthcare) is used for all amplification reactions described here.

iii) The optimised PCR conditions (5–50 ng DNA) (final concentrations in 25 µl total volume) for detection of *H. penaei* in shrimp hepatopancreas samples are: primers (0.2 µM each), dNTPs (200 µM each), Taq polymerase (0.1 U $\mu l^{-1}$), magnesium chloride (1.5 mM) in 10 mM Tris-HCl, pH 9.0, 50 mM KCl.

iv) The cycling parameters are: Step 1: 95°C for 5 minutes, 1 cycle; Step 2: 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, 35 cycles; Step 3: 60°C for 1 minute, 72°C for 2 minutes, 1 cycle; 4°C infinite hold.

**Protocol 2**

The PCR based on flagella gene (flagella hook protein, flgE) is based on Aranguren & Dhar (2018). Primers designated as NHP FlgE 1143F (5'-AGG CAA ACA AAC CCT TG-3') and and the NHP FlgE 1475R (5'- GCG TTG GGA AAG TT-3') amplify a 333-base pair (bp) fragment corresponding to the Flg E of *H. penaei*.

i) The following controls should be included when performing the PCR assay a) known *H. penaei* negative tissue sample; b) a known *H. penaei* positive sample (hepatopancreas); and c) a 'no template' control.

ii) The PuReTaq™ Ready-To-Go PCR Bead (RTG beads, GE Healthcare) is used for all amplification reactions described here.

iii) The optimised PCR conditions (5–50 ng DNA) (final concentrations in 25 µl total volume) for detection of *H. penaei* in shrimp hepatopancreas samples are: primers (0.2 µM each), dNTPs (200 µM each), Taq polymerase (0.1 U $\mu l^{-1}$), magnesium chloride (1.5 mM) in 10 mM Tris-HCl, pH 9.0, 50 mM KCl.

iv) The cycling parameters are: initial denaturation at 95ºC for 5 minutes followed by 35 cycles of 95ºC for 30 seconds, 62ºC for 30 seconds, and 72ºC for 30 seconds, and a final extension at 72ºC for 5 minutes followed by 4°C infinite hold.

Note: The conditions should be optimised for each thermal cycler using known positive controls.

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

### 4.4.3. Other nucleic acid amplification methods

None.

## 4.5. Amplicon sequencing

PCR products may be cloned and sequenced or sequenced directly when necessary to confirm infection with *H. penaei* or to identify false positives or nonspecific amplification (Aranguren *et al.,* 2010; Aranguren & Dhar, 2018; Vincent & Lotz, 2005).

## 4.6. *In-situ* hybridisation

The ISH method of Loy & Frelier (1996) and Lightner (1996) provides greater diagnostic sensitivity than do more traditional methods for *H. penaei* detection and diagnosis of infection that employ classical histological methods (Lightner, 1996; Morales-Covarrubias, 2010). The ISH assay of routine histological sections of acute, transition and chronic phase lesions in hepatopancreas with a specific DIG-labelled DNA probe to *H. penaei* 16S rRNA provides a definitive diagnosis of infection with *H. penaei* (Lightner, 1996; Loy & Frelier, 1996; Morales-Covarrubias *et al.,* 2006). Pathognomonic *H. penaei* positive lesions display prominent blue to blue-black areas in the cytoplasm of affected cells when reacted with the DNA probes. (See Chapter 2.2.4 *Infection with infectious hypodermal and haematopoietic necrosis virus* for details of the ISH method, and Chapter 2.2.0 Section B.5.3.ii for detailed information on the use of Davidson's AFA fixative.)

## 4.7. Immunohistochemistry

Immunohistochemistry (IHC) tests using monoclonal antibodies (MAbs) to *H. penaei*, according to the methods described in Bradley-Dunlop *et al.* (2004), are available for *H. penaei* detection.

### 4.8. Bioassay

Confirmation of infection with *H. penaei* may be accomplished by bioassay of suspect animals with SPF juvenile *P. vannamei* serving as the indicator of the intracellular bacteria (Aranguren *et al.,* 2010; Lightner, 2005). Oral protocols may be used. The oral method is relatively simple to perform and is accomplished by feeding chopped hepatopancreas of suspect shrimp to SPF juvenile *P. vannamei* in small tanks. The use of a negative control tank of indicator shrimp, which receive only a normal feed, is required. When the hepatopancreas feeding (*per os*) protocol is used to bioassay for *H. penaei*, *positive* indicator shrimp (by gross signs and histopathology) are typically apparent within 3–4 days of initial exposure, and significant mortalities occur by 3–8 days after initial exposure. The negative control shrimp must remain negative (for at least 10–15 days) for gross or histological signs of infection with *H. penaei* and unusual mortalities.

### 4.9. Antibody- or antigen-based detection methods

Serological tests are not applicable because shrimp are invertebrate animals that do not produce specific antibodies that could be used to demonstrate infection by or prior exposure to *H. penaei*.

### 4.10. Other methods

No scientifically confirmed reports.

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

Real-time PCR are the recommended test for surveillance to demonstrate freedom from infection with *H. penaei* in apparently healthy populations as described in Section 4.4.1 and 4.4.2.

## 6. Corroborative diagnostic criteria

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

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

### 6.1. Apparently healthy animals or animals of unknown health status[4]

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

#### 6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with *H. penaei* shall be suspected if at least one of the following criteria is met:

i) Positive result by real-time PCR

ii) Positive result by conventional PCR

#### 6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with *H. penaei* is considered to be confirmed if at least one of the following criteria is met:

4 For example transboundary commodities.

i) Positive result by two different probe-based real-time PCR tests targeting different region of the *H. penaei* genome

ii) Positive result by real-time PCR and conventional PCR targeting different region of the *H. penaei* genome and amplicon sequencing

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

## 6.2. Clinically affected animals

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

### 6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with *H. penaei* shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs consistent with *H. penaei* infection

ii) Histopathology consistent with *H. penaei* infection

iii) Positive result by real-time PCR

iv) Positive result by conventional PCR

v) Positive result by *in-situ* hybridisation

### 6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with *H. penaei* is considered to be confirmed if at least at least one of the following criteria is met:

i) A positive result by two different probe-based real-time PCR tests targeting different regions of the *H. penaei* genome

ii) A positive result by real-time PCR and conventional PCR targeting different regions of the *H. penaei* genome and amplicon sequencing

iii) Histopathology consistent with *H. penaei* and positive in-situ hybridisation test

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

## 6.3. Diagnostic sensitivity and specificity for diagnostic tests

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

### 6.3.1. For presumptive diagnosis of clinically affected animals

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study,
PCR: = polymerase chain reaction, ND = Not determined.

### 6.3.2. For surveillance of apparently healthy animals

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

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

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*
* *

**NB:** There is an OIE Reference Laboratory for infection with *Hepatobacter penaei* (necrotising hepatopancreatitis)
(see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: 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 *Hepatobacter penaei* (necrotising hepatopancreatitis).

**NB:** FIRST ADOPTED IN 2012; MOST RECENT UPDATES ADOPTED IN 2017.

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CHAPTER 2.2.4.

# INFECTION WITH INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS

## 1. Scope

Infection with infectious hypodermal and haematopoietic necrosis virus means infection with the pathogenic agent infectious hypodermal and haematopoietic necrosis virus (IHHNV), Family *Parvoviridae,* subfamily *Hamaparvovirinae,* Genus ***Penstylhamaparvovirus*** with IHHNV (*Decapod penstylhamaparvovirus 1)* as the Type species (Penez *et al.,* 2020).

## 2. Disease information

### 2.1. Agent factors

#### 2.1.1. Aetiological agent

IHHNV is the smallest of the known penaeid shrimp viruses. The virion is a 20–22 nm, non-enveloped icosahedron, with a density of 1.40 g $ml^{-1}$ in CsCl that contains linear single-stranded DNA with an estimated size of 3.9 kb, and has a capsid with four polypeptides of molecular weight 74, 47, 39, and 37.5 kD (Bonami *et al.,* 1990; Nunan *et al.,* 2000; GenBank NC_002190).

At least two distinct genotypes of IHHNV have been identified (Tang *et al.,* 2003): Type 1 is from the Americas and East Asia (principally the Philippines) and Type 2 is from South-East Asia. These genotypes are infectious to *Penaeus vannamei* and *P. monodon*. Two sequences homologous to part of the IHHNV genome are found embedded in the genome of penaeids. These were initially described as Type 3A from East Africa, India and Australia, and Type 3B from the western Indo-Pacific region including Madagascar, Mauritius and Tanzania (Tang & Lightner, 2006; Tang *et al*., 2007). Tissues containing the IHHNV-homologous sequences (also known as endogenous viral elements; Taengchaiyaphum *et al*., 2021) in the *P. monodon* genome are not infectious to susceptible host species (Lightner *et al*., 2009; Tang & Lightner, 2006; Tang *et al*., 2007).

#### 2.1.2. Survival and stability in processed or stored samples

IHHNV is believed to be the most stable virus of the known penaeid shrimp viruses. Infected tissues remain infectious after repeated cycles of freeze–thawing and after storage in 50% glycerine (Lightner, 1996; Lightner *et al.,* 1987; Lightner *et al.,* 2009).

#### 2.1.3. Survival and stability outside the host

No data.

For inactivation methods, see Section 2.4.5.

### 2.2. Host factors

#### 2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with IHHNV according to Chapter 1.5 of *Aquatic Animal Health Code* (*Aquatic Code*) are: yellowleg shrimp (*Penaeus californiensis*), giant tiger prawn (*P. monodon*), northern white shrimp (*P. setiferus*), blue shrimp (*P. stylirostris*), and white leg shrimp (*P. 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 IHHNV according to Chapter 1.5 of the *Aquatic Code* are: northern brown shrimp (*Penaeus aztecus*). Evidence is lacking for this species to either confirm that the identity of the pathogenic agent is IHHNV,

transmission mimics natural pathways of infection, or presence of the pathogenic agent constitutes an infection.

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following organisms, but an active infection has not been demonstrated: giant river prawn (*Macrobrachium rosenbergii)*, northern pink shrimp (*Penaeus duorarum*), western white shrimp (*P. occidentalis*), *kuruma prawn* (*P. japonicus*), green tiger prawn (*P. semisulcatus*), *Hemigrapsus penicillatus,* Argentine stiletto shrimp (*Artemesia longinaris*), Cuata swimcrab (*Callinectes arcuatus),* Mazatlan sole (*Achirus mazatlanus*), yellowfin mojarra (*Gerres cinereus*), tilapias (*Oreochromis* sp.), Pacific piquitinga (*Lile stolifera*) and blackfin snook (*Centropomus medius*).

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

IHHNV has been detected in all life stages (i.e. eggs, larvae, postlarvae, juveniles and adults) of *P. vannamei.* Nauplii produced from infected broodstock have a high prevalence of infection with IHHNV (Motte *et al.,* 2003).

### 2.2.4. Distribution of the pathogen in the host

IHHNV targets gills, haematopoietic nodules and haemocytes, ventral nerve cord and ganglia, antennal gland tubule epithelial cells, lymphoid organ, parenchymal cells, connective tissue cells and ovaries (Chayaburakul, 2005; Lightner, 1996; Lightner & Redman, 1998).

### 2.2.5. Aquatic animal reservoirs of infection

Some members of *P. stylirostris* and *P. vannamei* populations that survive IHHNV infection may carry the virus subclinically and infect their progeny or other populations by vertical and horizontal transmission (Bell & Lightner, 1984; Lightner, 1996; Motte *et al*., 2003).

### 2.2.6. Vectors

IHHNV was found in wild crabs (*Hemigrapsus penicillatus*, *Neohelice granulata*), but there were no clinical signs. Adults of *Macrobrachium rosenbergii* are carriers of IHHNV without apparent signs. Although the mussel *Mytilus edulis* is an important reservoir of IHHNV (Wei *et al.,* 2017), its capacity to transmit virus is unknown.

## 2.3. Disease pattern

### 2.3.1. Mortality, morbidity and prevalence

The effects of infection with IHHNV varies among shrimp species and populations, where infections can be either acute or chronic. For example, in unselected populations of *P. stylirostris*, infection with IHHNV results in acute, usually catastrophic, disease with mortalities approaching 100%. In contrast, in populations of *P. vannamei*, some selected lines of *P. stylirostris*, and some populations of *P. monodon*, infection with IHHNV results in a more subtle, chronic disease, runt-deformity syndrome (RDS), in which high mortalities are unusual, but where growth suppression and cuticular deformities are common (Kalagayan *et al*., 1991; Sellars *et al.,* 2019).

Infection with IHHNV interferes with normal egg, larval, and postlarval development. When broodstock are used from wild or farmed stocks where the disease is enzootic, hatching success of eggs may be reduced, and survival and culture performance of the larval and postlarval stages lowered (Motte *et al.,* 2003).

In the past, stocks of *P. stylirostris*, juveniles, subadults, and adults showed persistently high mortality rates due to infection with IHHNV. However, selected lines of *P. stylirostris* do not show mortality and appear to be tolerant to this virus. *Penaeus vannamei* and *P. monodon* stocks infected with IHHNV show poor and highly disparate growth and cuticular deformities, particularly bent rostrums and deformed sixth abdominal segments (Jagadeesan *et al.,* 2019; Sellars *et al.,* 2019).

In regions where the virus is enzootic in wild stocks, the prevalence of IHHNV has been found in various surveys to range from 0 to 100%. Some reported mean values for IHHNV prevalence in wild stocks are: 26% and 46% in *P. stylirostris* in the lower and upper Gulf of California, respectively (Pantoja *et al.,* 1999); 100% and 57%, respectively, in adult female and adult male *P. stylirostris* from the mid-region of the Gulf of California (Morales-Covarrubias *et al.,* 1999); 28% in wild *P. vannamei* collected from the Pacific coast of Panama (Nunan *et al.,* 2001); from 51 to 63% in *P. vannamei* collected from the Pacific coasts of Ecuador, Colombia and Panama (Motte *et al.,* 2003), and from 6 to 63% in *P. vannamei*

broodstock and 49.5% in post-larvae from Mexico (Fernando *et al*., 2016). In farms where IHHNV is present, its prevalence can range from very low to 100%, but high prevalence is typical (Aly *et al*., 2021; Chayaburakul *et al.,* 2004; Lightner, 1996; Lightner *et al.,* 1983).

### 2.3.2. Clinical signs, including behavioural changes

Certain cuticular deformities, specifically a deformed rostrum bent to the left or right, which may be presented by *P. vannamei* and *P. stylirostris* with RDS, are indicative of infection with IHHNV (see Section 2.3.3 *Gross pathology: Infection with IHHNV in* Penaeus vannamei). However, this clinical sign is not always apparent in shrimp populations chronically infected with IHHNV.

In acute disease, *P. stylirostris* may present behavioural changes (see Section 2.3.3 *Gross pathology: Infection with IHHNV in* Penaeus stylirostris) but with RDS, no consistent behavioural changes have been reported for affected shrimp.

### 2.3.3. Gross pathology

*Infection with IHHNV in* Penaeus stylirostris

Infection with IHHNV may result in acute disease with very high mortalities in juveniles. Vertically infected larvae and early postlarvae do not become diseased, but in approximately 35-day-old or older juveniles, gross signs of the disease may be observed, followed by mass mortalities. In horizontally infected juveniles, the incubation period and severity of the disease is somewhat size- or age-dependent, with young juveniles always being the most severely affected. Infected adults seldom show signs of the disease or mortalities (Bell & Lightner, 1984; 1987; Lightner, 1996; Lightner *et al.,* 1983). Gross signs are non-specific, but juvenile *P. stylirostris* with acute infection with IHHNV show a marked reduction in food consumption, followed by changes in behaviour and appearance. Shrimp of this species infected with IHHNV have been observed to rise slowly in culture tanks to the water surface, where they become motionless and then roll-over and slowly sink (ventral side up) to the tank bottom. Shrimp exhibiting this behaviour may repeat the process for several hours until they become too weak to continue, or until they are attacked and cannibalised by their healthier siblings. *Penaeus stylirostris* at this stage of infection often have white or buff-coloured spots (which differ in appearance and location from the white spots that sometimes occur in shrimp with WSSV infections) in the cuticular epidermis, especially at the junction of the tergal plates of the abdomen, giving such shrimp a mottled appearance. This mottling later fades in moribund *P. stylirostris* and individuals become more bluish. In *P. stylirostris* and *P. monodon* with terminal-phase infection with IHHNV, moribund shrimp are often distinctly bluish in colour, with opaque abdominal musculature (Lightner *et al.,* 1983).

*Infection with IHHNV in* Penaeus vannamei

RDS, a chronic form of infection with IHHNV, occurs in *P. vannamei.* The severity and prevalence of RDS in infected populations of juvenile or older *P. vannamei* may be related to infection during the larval or early postlarval stages. RDS has also been reported in cultured stocks of *P. stylirostris* and *P. monodon*. Juvenile shrimp with RDS may display a bent (45° to 90° bend to left or right) or otherwise deformed rostrum, a deformed sixth abdominal segment, wrinkled antennal flagella, cuticular roughness, 'bubble-heads', and other cuticular deformities. Populations of juvenile shrimp with RDS display disparate growth with a wide distribution of sizes and many smaller than expected ('runted') shrimp. The coefficient of variation (CV = the standard deviation divided by the mean of different size groups within a population) for populations with RDS is typically greater than 30% and may approach 90%, while populations of juvenile *P. vannamei* and *P. stylirostris* free from infection with IHHNV (and thus RDS-free) usually show CVs of 10–30% (Lightner, 1996; Primavera & Quinitio, 2000).

### 2.3.4. Modes of transmission and life cycle

Transmission of IHHNV can be by horizontal or vertical. Horizontal transmission has been demonstrated by cannibalism or by contaminated water (Lightner, 1996; Lightner *et al.,* 1983), and vertical transmission via infected eggs (Motte *et al.,* 2003).

### 2.3.5. Environmental factors

The replication rate of IHHNV at high water temperatures was significantly reduced in a study in which viral replication was compared in *P. vannamei* experimentally infected and held at 24°C and 32°C. After a suitable incubation period, shrimp held at 32°C had approximately $10^2$ times lower viral load than shrimp held at 24°C (Montgomery-Brock *et al*., 2007).

### 2.3.6. Geographical distribution

Infection with IHHNV has been reported from cultured shrimp in most of the major shrimp-culturing regions of the world including Asia, Oceania, North and South America and the Middle East.

IHHNV homologous sequences have been found within the genome of *P. monodon* from East Africa, Australia, and the western Indo-Pacific region (Tang & Lightner, 2006; Tang *et al.*, 2007). These sequences do not represent viral DNA (refer Section 2.1.1 *Aetiological agent*).

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

## 2.4. Biosecurity and disease control strategies

### 2.4.1. Vaccination

None available.

### 2.4.2. Chemotherapy including blocking agents

No scientifically confirmed reports.

### 2.4.3. Immunostimulation

No scientifically confirmed reports.

### 2.4.4. Breeding resistant strains

Selected stocks of *P. stylirostris* that are resistant to infection with IHHNV have been developed and these have had some successful application in shrimp farms (Lightner, 1996). However, lines of *P. stylirostris* bred for resistance to infection with IHHNV (Tang *et al.,* 2000) do not have increased resistance to other diseases, such as white spot syndrome virus (WSSV), so their use has been limited. In some stocks a genetic basis for IHHNV susceptibility in *P. vannamei* has been reported (Alcivar-Warren *et al.,* 1997).

### 2.4.5. Inactivation methods

IHHNV is a stable shrimp virus; infected tissues remain infectious after repeated cycles of freeze–thawing and after storage in 50% glycerine (Lightner, 1996; Lightner *et al.,* 2009).

### 2.4.6. Disinfection of eggs and larvae

IHHNV is transmitted vertically by the transovarian route (Motte *et al.,* 2003). Disinfection of eggs and larvae is good management practice (Chen *et al.,* 1992) that may reduce IHHNV contamination of spawned eggs and larvae but is not effective for preventing transovarian transmission of IHHNV (Motte *et al.,* 2003).

### 2.4.7. General husbandry

Some husbandry practices have been successful in preventing the spread of IHHNV. Among these has been the application of PCR pre-screening of wild or pond-reared broodstock or their spawned eggs/nauplii and discarding those that test positive for the virus (Motte *et al.,* 2003), as well as the development of specific pathogen-free (SPF) shrimp stocks of *P. vannamei* and *P. stylirostris* (Lightner, 2005). The latter has proven to be the most successful husbandry practice for the prevention and control of infection with IHHNV (Lightner, 2005).

# 3. Specimen selection, sample collection, transportation and handling

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

## 3.1. Selection of populations and individual specimens

Infection with IHHNV may be below detection limits in spawned eggs and larval stages, so these life stages are not suitable for surveillance to demonstrate freedom from infection with IHHNV.

## 3.2. Selection of organs or tissues

IHHNV infects tissues of ectodermal and mesodermal origin. The principal target tissues for IHHNV include connective tissue cells, the gills, haematopoietic nodules and haemocytes, ventral nerve cord and ganglia, antennal gland tubule epithelial cells, and lymphoid organ parenchymal cells (Lightner, 1996; Lightner & Redman, 1998). Hence, whole shrimp (e.g. larvae or postlarvae) or tissue samples containing the aforementioned target tissues are suitable for most tests using molecular methods.

## 3.3. Samples or tissues not suitable for pathogen detection

Enteric tissues (e.g. the hepatopancreas, the midgut or its caeca) are inappropriate samples for detection of IHHNV (Lightner, 1996; Lightner & Redman, 1998). Shrimp eyes contain PCR inhibitors and their use should be avoided.

## 3.4. Non-lethal sampling

Haemolymph or excised pleopods may be collected and used for testing when non-lethal testing of valuable broodstock is necessary (Lightner, 1996; Lightner & Redman, 1998).

## 3.5. Preservation of samples for submission

For routine histology or molecular assays, and guidance on preservation of samples for the intended test method see Chapter 2.2.0.

### 3.5.1. Samples for pathogen isolation

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

### 3.5.2. Preservation of samples for molecular detection

Standard sample collection, preservation and processing methods for histological techniques can be found in Section B.2.5 of Chapter 2.2.0 *General information* (diseases of crustaceans).

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

Standard sample collection, preservation and processing methods for histological techniques can be found in Section B.2.2 of Chapter 2.2.0 *General information* (diseases of crustaceans).

### 3.5.4. Samples for other tests

Not relevant.

## 3.6. Pooling of samples

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

# 4. Diagnostic methods

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

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

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

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

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

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

| Method | A. Surveillance of apparently healthy animals | | | | B. Presumptive diagnosis of clinically affected animals | | | | C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV |
| **Wet mounts** | | | | | | | | | | | | |
| **Histopathology** | | | | | | ++ | ++ | NA | | ++ | ++ | NA |
| **Cell culture** | | | | | | | | | | | | |
| **Real-time PCR** | ++ | +++ | +++ | 1 | ++ | +++ | +++ | 1 | ++ | ++ | ++ | 1 |
| **Conventional PCR** | + | ++ | ++ | 1 | ++ | ++ | ++ | 1 | ++ | ++ | ++ | 1 |
| **Amplicon sequencing** | | | | | | | | | +++ | +++ | +++ | 1 |
| ***In-situ* hybridisation** | | | | | | + | + | 1 | | ++ | ++ | 1 |
| **Bioassay** | | | | | | | | | | | | |
| **LAMP** | | | | | | | | | | | | |
| **Ab-ELISA** | | | | | | | | | | | | |
| **Ag-ELISA** | | | | | | | | | | | | |
| **Other antigen detection methods[3]** | | | | | | | | | | | | |
| **Other methods[3]** | | | | | | | | | | | | |

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); NA = not available; PCR = polymerase chain reaction;
LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.
[3]Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

## 4.1. Wet mounts

No reliable methods have been developed for direct microscopic pathology.

## 4.2. Histopathology and cytopathology

Presumptive acute infections in *P. stylirostris* can be readily diagnosed using routine haematoxylin and eosin (H&E) stained sections whereas chronic infection are much more difficult to diagnose using these staining methods. For diagnosis of chronic infections and confirmation of acute infections however, the use of molecular methods is required for IHHNV detection (e.g. by PCR or application of IHHNV-specific DNA probes to dot-blot hybridisation tests or *in-situ* hybridisation [ISH] of histological sections).

Histological demonstration of prominent intranuclear, Cowdry type A inclusion bodies provides a provisional diagnosis of infection with IHHNV. These characteristic IHHNV inclusion bodies are eosinophilic and often haloed (with H&E stains of tissues preserved with fixatives that contain acetic acid, such as Davidson's AFA and Bouin's solution) (Bell & Lightner, 1988; Lightner, 1996), intranuclear inclusion bodies within chromatin-marginated, hypertrophied nuclei of cells in tissues of ectodermal (epidermis, hypodermal epithelium of fore- and hindgut, nerve cord and nerve ganglia) and mesodermal origin (haematopoietic organs, antennal gland, gonads, lymphoid organ, and connective tissue). Intranuclear inclusion bodies caused by infection with IHHNV may be easily confused with developing intranuclear inclusion bodies caused by WSSV infection. ISH assay (see Section 4.6 In-situ *hybridisation*) of such sections with a DNA probe specific to IHHNV provides a definitive diagnosis of infection with IHHNV (Lightner, 1996a; 2011; Lightner & Redman, 1998a).

The use of Davidson's fixative (containing 33% ethyl alcohol [95%], 22% formalin [approximately 37% formaldehyde], 11.5% glacial acetic acid and 33.5% distilled or tap water) is highly recommended for all routine histological studies of shrimp (Bell & Lightner, 1988; Lightner, 1996a). To obtain the best results, dead shrimp should not be used. Only live, moribund, or compromised shrimp should be selected for fixation and histological examination. Selected shrimp are killed by injection of fixative directly into the hepatopancreas; the cuticle over the cephalothorax and abdomen just lateral to the dorsal midline is opened with fine-pointed surgical scissors to enhance fixative penetration (the abdomen may be removed and discarded), the whole shrimp (or cephalothorax) is immersed in fixative for 24 to 48 hours, and then transferred to 70% ethyl alcohol for storage. After transfer to 70% ethyl alcohol, fixed specimens may be transported by wrapping in cloth or a paper towel saturated with 70% ethyl alcohol and packed in leak-proof plastic bags.

## 4.3. Cell culture for isolation

IHHNV has not been grown *in vitro*. No crustacean cell lines exist.

## 4.4. Nucleic acid amplification

There are multiple geographical variants of IHHNV, some of which are not detected by all of the available methods. Two primer sets, 392F/R and 389F/R, are the most suitable for detecting all the known genetic variants of IHHNV (Tang *et al*., 2000; 2007). However, these tests also detect non-infectious endogenous viral elements (EVE) within the *P. monodon* genome (previously known as types 3A and 3B), which are inserted into the genome of certain stocks of *P. monodon* from the western Indo-Pacific, East Africa, Australia and India (Saksmerprome *et al.,* 2011; Tang & Lightner, 2006; Tang *et al.,* 2007). As these PCR methods may result in positive test results in uninfected *P. monodon,* positive results should be confirmed by a method that detects IHHNV but not the IHHNV-related EVEs.

PCR primers have been developed that can detect the IHHNV sequence but do not amplify IHHNV-related EVEs present in the *P. monodon* stocks from Africa, Australia (Tang *et al.,* 2007), or Thailand (Saksmerprome *et al.,* 2011). Primer set 309F/R amplifies only a genomic segment of IHHNV types 1 and 2 (the infectious forms of IHHNV), but not the non-infectious EVEs within the *P. monodon* genome (Tang & Lightner, 2006; Tang *et al.,* 2007). Primer set MG831F/R reacts only with non-infectious EVEs within the *P. monodon* genome (Tang *et al.,* 2007). Hence, confirmation of unexpected positive or negative PCR results for IHHNV with a second primer set, or use of another diagnostic method (i.e. histology, bioassay, ISH) is highly recommended.

### 4.4.1. Real-time PCR

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

Real-time PCR methods have been developed for the detection of IHHNV (Dhar *et al.,* 2001; Tang & Lightner, 2001). A highly sensitive SYBR Green real-time PCR targeting a segment of the IHHNV genome that is less susceptible to endogenisation was developed (Encinas-Garcia *et al.,* 2015). More recently, a TaqMan real-time assay capable of differentiating endogenous virus element from infectious form of IHHNV in *P. monodon* has been reported (Cowley *et al.,* 2018). The real-time PCR method using TaqMan chemistry described below for IHHNV generally follows the method used in Tang & Lightner (2001).

i) The PCR primers and TaqMan probe are selected from a region of the IHHNV genomic sequence (GenBank AF218266) that encodes for a non-structural protein. The upstream (IHHNV1608F) and downstream (IHHNV1688R) primer sequences are: 5'-TAC-TCC-GGA-CAC-CCA-ACC-A-3' and 5'-GGC-TCT-GGC-AGC-AAA-GGT-AA-3', respectively. The TaqMan probe 5'-ACC-AGA-CAT-AGA-GCT-ACA-ATC-CTC-GCC-TAT-TTG-3'), is synthesised and labelled with FAM on the 5' end and TAMRA on the 3' end.

ii) Preparation of DNA template: DNA extracted from tissues or haemolymph that was preserved in 95% ethanol and then dried. A control consisting of tissues or haemolymph from known negative animals should be included during the DNA extraction step. The DNA can be extracted by a variety of methods. Commercial DNA extraction kits include QIAamp DNA Mini Kit (Qiagen), MagMax™ Nucleic Acid kits (Life Technologies), or Maxwell® 16 Cell LEV DNA Purification Kit (Promega), or DNazol (Life Technologies). Spectrophotometric readings of the final DNA will indicate the purity of the DNA and the amount of total DNA extracted from the sample.

iii) The real-time PCR reaction mixture contains: TaqMan Fast virus 1-step Master Mix (Life Technologies, or commercially-available equivalent reagents), 0.3 µM of each primers, 0.15 µM of TaqMan probe, 5–50 ng DNA, and water in a reaction volume of 20 µl. For optimal results, the reaction mixture should be vortexed and mixed well.

iv) The cycling profile is: initial denaturation of 20 seconds at 95°C, followed by 40 cycles of denaturation at 95°C for 1 second and annealing/extension at 60°C for 20 seconds.

### 4.4.2. Conventional PCR

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

Several one-step PCR methods (Krabsetsve *et al.,* 2004; Nunan *at al.,* 2000; Shike *et al.,* 2000; Tang *et al.,* 2000; 2007), and a number of commercial PCR kits are available for IHHNV detection. Nested methods are also available.

***Table 4.4.2.1. Recommended primer sets for one-step PCR detection of IHHNV***

| Primer | Product | Sequence (5′-3′) | G+C%/Temp. | GenBank & References | Specificity |
|---|---|---|---|---|---|
| 389F | 389 bp | CGG-AAC-ACA-ACC-CGA-CTT-TA | 50%/72°C | AF218266 | All genetic variants of IHHNV |
| 389R | | GGC-CAA-GAC-CAA-AAT-ACG-AA | 45%/71°C | (Tang *et al.,* 2007) | and IHHNV-related EVEs |
| 77012F | 356 bp | ATC-GGT-GCA-CTA-CTC-GGA | 50%/68°C | AF218266 | Not given in the reference |
| 77353R | | TCG-TAC-TGG-CTG-TTC-ATC | 55%/63°C | (Nunan *et al.,* 2000) | |
| 392F | 392 bp | GGG-CGA-ACC-AGA-ATC-ACT-TA | 50%/68°C | AF218266 | All genetic variants of IHHNV and IHHNV-related EVEs |
| 392R | | ATC-CGG-AGG-AAT-CTG-ATG-TG | 50%/71°C | (Tang *et al.,* 2000) | |
| 309F | 309 bp | TCC-AAC-ACT-TAG-TCA-AAA-CCA-A | 36%/68°C | AF218266 | IHHNV but not IHHNV-related EVEs |
| 309R | | TGT-CTG-CTA-CGA-TGA-TTA-TCC-A | 40%/69°C | (Tang *et al.,* 2007) | |
| MG831F | 831 bp | TTG-GGG-ATG-CAG-CAA-TAT-CT | 45%/58°C | DQ228358 | IHHNV-related EVEs but not IHHNV |
| MG831R | | GTC-CAT-CCA-CTG-ATC-GGA-CT | 55%/62°C | (Tang *et al.,* 2007) | |

NOTE: Primers 389F/R and 392F/R described above are from the nonstructural protein-coding region of the IHHNV genome. Primers 77012F/77353R are from a region in between the nonstructural and capsid protein-coding region of the genome. In the event that results are ambiguous using the 389F/R 'universal' primer set, it is recommended to use primers from a different region of the genome for confirmatory testing. In this case, that would mean using primers 77012F/77353R or the 392F/R primer sets and follow up with sequencing of PCR amplicons for confirmation.

General PCR method for IHHNV: the PCR method described below for IHHNV generally follows the methods outlined in Tang *et al.* (2007) and Nunan *et al.* (2000). However, recent minor modifications including the sources of the reagents and the use of an automated DNA extraction instrument are acceptable. The modifications include DNA extraction method, choice of primers (Table 4.4.2.1), and the volume of reaction. These slightly modified methods have been validated in accordance with Chapter 1.1.2 *Principles and methods of validation of diagnostic assays for infectious diseases* and do not affect the diagnostic performance of the assay.

i) Use as a template, the extraction of DNA templates is the same as that described above. Use 1–5 µl of extracted DNA as a template per 25 µl reaction volume.

ii) The following controls should be included in every PCR assay for IHHNV: (a) DNA from a known negative tissue sample; (b) DNA from a known positive sample (either from tissue or haemolymph or from a plasmid clone that contains the fragment that the specific set of primers amplifies; and (c) a 'no template' control.

iii) Use as primers, primers 389F and 389R, which elicit a band 389 bp in size from IHHNV-infected material, or primers 77012F and 77353R, which elicit a band 356 bp in size from IHHNV-infected material. Prepare primers at 10 µM in distilled water.

iv) If PuReTaq™ Ready-To-Go PCR Beads (GE Healthcare) are used, the PCR profile involves a 3–5 minutes at 95°C to denature DNA followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and final extension at 72°C for 5 minutes.

v) Prepare a 'Master Mix' consisting of water and primers.

vi) For a 25 µl reaction mix, add 24 µl Master Mix to each tube and then add 1 µl of the DNA template to be tested.

vii) Vortex each tube, spin quickly to bring down all liquid. Insert tubes into the thermal cycler and start the PCR program.

viii) After PCR, run 6–10 µl of the sample in a 1.5% agarose gel (containing SYBR™ Safe (Thermo Fisher Scientific) or equivalent to stain the DNA). Look for the 389 bp band (if using primers 389F and 389R) or for the 356 bp band (if using primers 77012F and 77353R). Bands are not always seen, as it is necessary to have at least 10 ng DNA $\mu l^{-1}$ to see DNA in a gel. A direct sequencing of amplified products can be performed through gel extraction of a PCR band with correct size and the sequencing primer(s) used for amplification to confirm the presence of IHHNV.

### 4.4.3. Other nucleic acid amplification methods

Loop-mediated isothermal amplification (LAMP) assays and real-time isothermal recombinase polymerase amplification (RPA) assay are available to detect and confirm IHHNV infection (Arunrut *et al*., 2011; Sun *et al*., 2006; Xia *et al.*, 2015), however, they are currently not recommended as they are not sufficiently validated.

## 4.5. Amplicon sequencing

PCR products may be directly sequenced or cloned and sequenced when necessary to confirm infection with IHHNV, to identify false positives or nonspecific amplification, or to distinguish the amplified products from the infectious form of the virus and demonstrate the presence of the non-infectious IHHNV-related EVEs in the host genome (Tang & Lighter, 2006).

## 4.6. *In-situ* hybridisation

Direct detection methods using DNA probes specific for IHHNV are available in dot-blot and ISH formats. The ISH method uses a DIG-labelled DNA probe for IHHNV and generally follows the methods outlined in Mari *et al*. (1993) and Lightner (1996).

Gene probe and PCR methods provide greater diagnostic specificity and sensitivity than traditional techniques that employ classic histological approaches. Furthermore, these methods have the added advantage of being applicable to non-lethal testing of valuable broodstock shrimp. A haemolymph sample may be taken with a tuberculin syringe, or an appendage (a pleopod for example) may be biopsied (Bell *et al.*, 1990), and used as the sample for a dot-blot hybridisation test.

### 4.7. Immunohistochemistry

Not relevant.

### 4.8. Bioassay

If SPF shrimp are available, the following bioassay method is based on Tang *et al.* (2000), is suitable for IHHNV diagnosis.

i) For bioassay, feed the minced shrimp tissue suspected of being infected with IHHNV to the indicator shrimp species (e.g. SPF *P. vannamei* and *P. stylirostris* at the PLs or juvenile stage) at 10% of their body weight twice daily for 1 days.

ii) For the following, the indicator shrimp were maintained on a pelletised ration.

iii) Examine moribund shrimp grossly or by using the methods described above. There may be no apparent mortality during the experimental period.

iv) If at 30 days after feeding there are still no moribund shrimp and all test results are negative, then it is safe to conclude that the bioassay results are negative.

Known IHHNV positive and negative control groups should be included in the bioassay.

### 4.9. Antibody- or antigen-based detection methods

None has been successfully developed.

### 4.10. Other methods

Not available.

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

Conventional PCR and/or real-time PCR are the recommended test for surveillance to demonstrate freedom from infection with IHHNV in apparently healthy populations as described in Sections 4.4.1 and 4.4.2.

## 6. Corroborative diagnostic criteria

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

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

## 6.1. Apparently healthy animals or animals of unknown health status[5]

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

### 6.1.1. Definition of suspect case in apparently healthy animals

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

i) Positive result by conventional PCR

ii) Positive result by real-time PCR

### 6.1.2. Definition of confirmed case in apparently healthy animals

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

i) Positive result by real-time PCR and conventional PCR targeting non-overlapping regions of the viral genome and amplicon sequencing

ii) Histopathology consistent with IHHNV infection coupled with *in-situ* hybridisation and detection of IHHNV by real-time PCR

iii) Histopathology consistent with IHHNV infection coupled with *in-situ* hybridisation and detection of IHHNV by conventional PCR and amplicon sequencing

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

## 6.2 Clinically affected animals

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

### 6.2.1. Definition of suspect case in clinically affected animals

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

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

ii) Positive result by conventional PCR

iii) Positive result by real-time PCR

iv) Histopathology consistent with IHHNV infection

v) Positive result by *in-situ* hybridisation

### 6.2.2. Definition of confirmed case in clinically affected animals

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

i) Positive result by real-time PCR and conventional PCR targeting a non-overlapping region of the genome and amplicon sequencing

ii) Histopathological changes characteristic of infection with IHHNV with a positive result by *in-situ* hybridisation and detection of IHHNV by real-time PCR

5 For example transboundary commodities.

iii) Histopathological changes characteristic of infection with IHHNV or positive result by *in-situ* hybridisation and detection of IHHNV by conventional PCR and amplicon sequencing

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

## 6.3. Diagnostic sensitivity and specificity for diagnostic tests [under study]

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

### 6.3.1. For presumptive diagnosis of clinically affected animals

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

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

### 6.3.2. For surveillance of apparently healthy animals

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

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

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* *

**NB:** There are OIE Reference Laboratories for infection with infectious hypodermal and haematopoietic necrosis virus (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/ ).
Please contact the OIE Reference Laboratories for any further information on infection with infectious hypodermal and haematopoietic necrosis virus

**NB:** FIRST ADOPTED IN 1995 AS INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS; MOST RECENT UPDATES ADOPTED IN 2018.

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CHAPTER 2.3.1.

# INFECTION WITH *APHANOMYCES INVADANS* (EPIZOOTIC ULCERATIVE SYNDROME)

## 1. Scope

Infection with *Aphanomyces invadans* means all infections caused by the oomycete fungus *A. invadans* of the Genus *Aphanomyces* and Family *Leptolegniaceae.*

## 2. Disease information

### 2.1. Agent factors

#### 2.1.1. Aetiological agent

Infection with *A. invadans* is most commonly known as epizootic ulcerative syndrome (EUS) (Food and Agriculture Organization of the United Nations [FAO], 1986). It has also been known as red spot disease (RSD) (Mckenzie & Hall, 1976), mycotic granulomatosis (MG) (Egusa & Masuda, 1971; Hanjavanit, 1997) and ulcerative mycosis (UM) (Noga & Dykstra, 1986). The disease is caused by the oomycete *Aphanomyces invadans.*

Infection with *Aphanomyces invadans* is a seasonal epizootic condition of great importance in wild and farmed freshwater and estuarine fish. It is clinically characterised by the presence of invasive, non-septate hyphae in skeletal muscle, usually leading to a granulomatous response. Infections with *A. invadans* have spread widely since the first outbreak in 1971 in Japan and to date a high degree of genetic homogeneity is observed for this species based on publicly available genome sequences (Dieguez-Uribeondo *et al.,* 2009; European Food Safety Authority [EFSA] 2011a; Huchzermeyer *et al.,* 2018; Iberahim *et al.,* 2018; Lilley *et al.,* 2003). Other pathogenic viruses (mostly rhabdoviruses), bacteria (mainly *Aeromonas hydrophila*), fungi, oomycetes and parasites are routinely co-isolated from *A. invadans*-infected fish (Iberahim *et al.,* 2018).

*Aphanomyces invadans* is within a group of organisms commonly known as the water moulds. Although long-regarded as fungi because of their characteristic filamentous growth, this group, the Oomycota, is not considered a member of the Eumycota (true fungi) but is classified with diatoms and brown algae in a group called the Heterokonta or Stramenopiles within the Kingdom Chromista (Cavalier-Smith & Chao 2006; Tsui *et al.,* 2009). Junior synonyms of *A. invadans* include *Aphanomyces piscicida* and *Aphanomyces invaderis.*

#### 2.1.2. Survival and stability in processed or stored samples

There is limited published data on the stability of the pathogen in host tissues. it is not clear whether the pathogen continues to grow for some time following the death of the host (Oidtmann, 2012).

*Aphanomyces invadans* cultures can be maintained for extended periods in glucose phosphate broth (6 weeks at 10°C), agar slopes and sodium phosphate buffer (over 6 months at 20°C) (Lilley *et al.,* 1998).

#### 2.1.3. Survival and stability outside the host

How *A. invadans* survives outside the host is unclear (Oidtmann, 2012). It is assumed that the motile zoospores, which are released from an infected fish, will encyst when unsuccessful in finding a suitable substrate to grow on (Oidtmann, 2012). Encysted zoospores of *A. invadans* are capable of releasing a new zoospore generation instead of germinating in a process called repeated zoospore emergence (Dieguez-Uribeondo *et al.,* 2009). There is no suitable method to recover or isolate the encysted zoospore from affected fish ponds (Afzali *et al.,* 2013). How long the encysted spore can survive in water or on a non-fish substrate is unclear. In an *in-vitro* experiment, the encysted zoospore survived for at least 19 days (Lilley *et al.*, 2001).

For inactivation methods, see Section 2.4.5.

## 2.2. Host factors

### 2.2.1. Susceptible host species

***Table 2.1.*** *Fish species susceptible to infection with* Aphanomyces invadans

| Family | Scientific name | Common Name |
|---|---|---|
| Alestidae | *Brycinus lateralis* | striped robber |
| | *Hydrocynus vittatus* | tigerfish |
| | *Micralestes acutidens* | silver robber |
| Ambassidae | *Ambassis agassizii* | chanda perch |
| Apogonidae | *Glossamia aprion* | mouth almighty |
| Ariidae | *Arius sp.* | fork-tailed catfish |
| Belonidae | *Strongylura kreffti* | long tom |
| Centrarchidae | *Lepomis macrochirus* | bluegill |
| | *Micropterus salmoides* | largemouth black bass |
| Channidae | *Channa marulius* | great snakehead fish |
| | *Channa striatus* | striped snakehead |
| Cichlidae | *Coptodon rendalli* | redbreast tilapia |
| | *Oreochromis andersoni* | three-spoted tilapia |
| | *Oreochromis machrochir* | greenhead tilapia |
| | *Sargochromis carlottae* | rainbow bream |
| | *Sargochromis codringtonii* | green bream |
| | *Sargochromis giardi* | pink bream |
| | *Serranochromis angusticeps* | thinface largemouth |
| | *Serranochromis robustus* | Nembwe |
| | *Tilapia sparrmanii* | banded tilapia |
| Clariidae | *Clarias gariepinus* | sharptooth African catfish |
| | *Clarias ngamensis* | blunt-toothed African catfish |
| | *Clarius batrachus* | walking catfish |
| Clupeidae | *Alosa sapidissima* | American shad |
| | *Brevoortia tyrannus* | Atlantic menhaden |
| | *Nematalosa erebi* | bony bream |
| Cyprinidae | *Barbus paludinosus* | straightfin barb |
| | *Barbus poechii* | dashtail barb |
| | *Barbus thamalakanensis* | Thamalakane barb |
| | *Barbus unitaeniatus* | longbeard barb |
| | *Carassius auratus* | goldfish |
| | *Catla catla* | catla |
| | *Cirrhinus mrigala* | mrigal |
| | *Esomus sp.* | flying barb |
| | *Labeo cylindricus* | red-eye labeo |
| | *Labeo lunatus* | upper Zambezi labeo |
| | *Labeo rohita* | rohu |
| | *Puntius gonionotus* | silver barb |
| | *Puntius sophore* | pool barb |
| | *Rohtee sp.* | keti-Bangladeshi |
| Eleotridae | *Oxyeleotris lineolatus* | sleepy cod |
| | *Oxyeleotris marmoratus* | marble goby |

| Family | Scientific name | Common Name |
|---|---|---|
| Gobiidae | *Glossogobius giuris* | bar-eyed goby |
| | *Glossogobius sp.* | goby |
| | *Tridentiger obscures obscures* | dusky tripletooth goby |
| Helostomatidae | *Helostoma temmincki* | kissing gourami |
| Hepsetidae | *Hepsetus odoe* | African pike |
| Ictaluridae | *Ameiurus melas* | black bullhead |
| | *Ameiurus nebulosus* | black bullhead |
| | *Amniataba percoides* | striped grunter |
| | *Ictalurus punctatus* | channel catfish |
| Kurtidae | *Kurtus gulliveri* | nursery fish |
| Latidae | *Lates calcarifer* | barramundi or sea bass |
| Lutjanidae | *Lutjanus argentimaculatus* | mangrove jack |
| Melanotaeniidae | *Melanotaenia splendida* | rainbow fish |
| Mormyridae | *Marcusenius macrolepidotus* | bulldog |
| | *Petrocephalus catostoma* | churchill |
| Mugilidae | *Mugilidae (Mugil spp.; Liza spp.)* | mullets |
| | *Mugil cephalus* | grey mullet or striped mullet |
| | *Mugil curema* | white mullet |
| | *Myxus petardi* | mullet |
| Osmeroidei | *Plecoglossus altivelis* | ayu |
| Osphronemidae | *Colisa lalia* | dwarf gourami |
| | *Osphronemus goramy* | giant gourami |
| | *Trichogaster pectoralis* | snakeskin gourami |
| | *Trichogaster trichopterus* | three-spot gourami |
| Osteoglossidae | *Scleropages jardini* | saratoga |
| Percichthyidae | *Maccullochella ikei* | freshwater cod |
| | *Maccullochella peelii* | Murray cod |
| | *Macquaria ambigua* | golden perch |
| | *Macquaria novemaculeata* | Australian bass |
| Platycephalidae | *Platycephalus fuscus* | dusky flathead |
| Psettodidae | *Psettodes sp.* | spiny turbot |
| Salmonidae | *Oncorhynchus mykiss* | rainbow trout |
| Scatophagidae | *Scatophagus argus* | spotted scat |
| | *Selenotoca multifasciata* | striped scat |
| Schilbeidae | *Schilbe intermedius* | silver catfish |
| | *Schilbe mystus* | African butter catfish |
| Sciaenidae | *Bairdiella chrysoura* | drums or croakers |
| | *Pogonias cromis* | black drum |
| Sillaginae | *Sillago ciliata* | sand whiting |
| Siluridae | *Silurus glanis* | *wells catfish* |
| Soleidae | *Aseraggodes macleayanus* | narrow banded sole |
| Sparidae | *Acanthopagrus australis* | yellowfin sea bream |
| | *Acanthopagrus berda* | black bream |
| | *Archosargus probatocephalus* | sheepshead |
| Synbranchidae | *Fluta alba* | swamp eel |
| Terapontidae | *Anabas testudineus* | climbing perch |
| | *Bidyanus bidyanus* | silver perch |
| | *Leiopotherapon unicolor* | spangled perch |
| | *Scortum barcoo* | Barcoo Grunter |

| Family | Scientific name | Common Name |
|---|---|---|
| | *Therapon sp.* | therapon |
| Toxotidae | *Toxotes chatareus* | common archer fish |
| | *Toxotes lorentzi* | primitive acher fish |

#### 2.2.2. Species with incomplete evidence for susceptibility [under study]

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with *A. invadans* according to Chapter 1.5 of the *Aquatic Code* are: [under study]

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

Subadult and adult fish are usually described as the susceptible life stages to natural outbreaks of EUS (FAO, 2009). However, there are reports of infection with *A. invadans* being found in early life stages (fish fry or fish larvae) (Baldock *et al.,* 2005; EFSA 2011a). While the size of the fish does not determine an EUS outbreak (Cruz-Lacierda & Shariff, 1995), younger fish seem to be more prone to EUS compared with adult fish (Gomo *et al.,* 2016; Pagrut *et al.,* 2017).

An experimental injection of *A. invadans* into the yearling life stage of Indian major carp, catla, rohu and mrigal, revealed resistance to *A. invadans* (Pradhan *et al.,* 2007), even though they are naturally susceptible species. Experimental infections demonstrated that goldfish are susceptible (Hatai *et al.,* 1977; 1994), but common carp (Wada *et al.,* 1996), Nile tilapia (Khan *et al*., 1998) and European eel, (Oidtmann *et al*., 2008) are considered resistant.

#### 2.2.4. Distribution of the pathogen in the host

During the course of an infection with *A. invadans*, the free-swimming zoospore attaches to the skin of a fish host, encysts and germinates to develop hyphae invading and ramifying through host tissues (Kiryu *et al.,* 2003; Lilley *et al.,* 1998). The hyphal invasion and associated pathology are not confined to the region of dermal ulcers. The hyphae readily invade the body cavity and produce mycotic granulomas in all the visceral organs (Vishwanath *et al.,* 1998). In fish either suspected or confirmed to be infected with *Aphanomyces invadans,* hyphae have also been observed in kidney, liver, spleen, pancreatic tissue, gut, parietal peritoneum, swim bladder, gonads, spinal cord, meninges, vertebrae, inter-muscular bones, the mouth region, and the orbits (Chinabut & Roberts, 1999; Vishwanath *et al.,* 1998; Wada *et al.,* 1996).

#### 2.2.5. Aquatic animal reservoirs of infection

There is no information to indicate that fish can be lifelong carriers of *A. invadans*. Generally, most infected fish die during an outbreak. Although some fish with mild or moderate infections could recover, they are unlikely to be lifelong carriers of *A. invadans*.

#### 2.2.6. Vectors

No data available.

### 2.3. Disease pattern

#### 2.3.1. Mortality, morbidity and prevalence

The prevalence of infection with *A. invadans* in the wild and in aquaculture farms may be high (20–90%), in endemic areas with high levels of mortality observed. Mortality patterns appear to be seasonal and can vary substantially (Herbert *et al.,* 2019).

#### 2.3.2. Clinical signs, including behavioural changes

Fish usually develop red spots or small-to-large ulcerative lesions on the body. The occurrence of skin lesions and ultimately mortality varies according to fish species. Fish presenting with lesions are usually weak, appear darker in colour, have a reduced appetite, are immobile and may float at the surface of the water. Generally infected fish are encountered in shallow water and present a retarded ability to escape capture occasionally followed by short lived bouts of hyperactivity characterised by jerky movements (Huchzermeyer *et al.,* 2018; Iberahim *et al.,* 2018).

### 2.3.3 Gross pathology

Early-stage lesions or mildly infected fish are characterised by red spots observed on the lateral body surface, head, operculum or caudal peduncle of the infected fish. Scales of infected fish are often protruding or lost. In severe cases, swollen haemorrhagic areas, massive inflammation and large deep ulcers exposing the underlying necrotic muscle tissue are observed (Huchzermeyer *et al.,* 2018; Iberahim *et al.,* 2018). In advanced stages of the disease, the severity of the disease results in death of the fish (Hawke *et al.,* 2003; Iberahim *et al.,* 2018)

### 2.3.4. Modes of transmission and life cycle

*Aphanomyces invadans* has an aseptate fungal-like mycelia structure. This oomycete has two typical zoospore forms. The primary zoospore consists of round cells that develop inside the sporangium. The primary zoospore is released to the tip of the sporangium where it forms a spore cluster. It quickly transforms into the secondary zoospore, which is a reniform, laterally biflagellate cell and can swim freely in the water. The secondary zoospore remains motile for a period that depends on the environmental conditions and presence of the fish host or substratum. Typically, the zoospore encysts and germinates to produce new hyphae, although further tertiary generations of zoospores may be released from cysts (repeated zoospore emergence or polyplanetism) (Lilley *et al.,* 1998). The *A. invadans* zoospores can be horizontally transmitted from one fish to another through the water supply. It is believed that only the secondary zoospores or free-swimming stage zoospores are capable of attaching to the damaged skin of fish and germinating into hyphae. If the secondary zoospores cannot find the susceptible species or encounter unfavourable conditions, they can encyst in the pond environment. The cysts may wait for conditions that favour their transformation into tertiary generations of zoospores that are also in the free-swimming stage. The encysting property of *A. invadans* may play an important role in the cycle of outbreaks in endemic areas.

### 2.3.5. Environmental factors

Under natural conditions, infection with *A. invadans* has been reported at water temperatures in the range 10–33°C (Bondad-Reantaso et al. 1992; Hawke et al. 2003) often associated with massive rainfall (Bondad-Reantaso *et al.,* 1992). These conditions favour sporulation of *A. invadans* (Lumanlan-Mayo *et al*., 1997), and temperatures of 17–19°C have been shown to delay the inflammatory response of fish to oomycete infection (Catap & Munday, 1998, Chinabut *et al*., 1995). In some countries, outbreaks occur in wild fish first and then spread to fish ponds. Normally, a bath infection of *A. invadans* in healthy susceptible fish species does not result in clinical signs of disease. The presence of other pathogens (viruses, bacteria or ectoparasites, skin damage, water temperature (between 18 and 22 °C), low pH (6.0–7.0) and low oxygen concentration in the water have all been hypothesised as predisposing factors for infection or factors influencing the expression of the disease (Oidtmann, 2012; Iberahim *et al.,* 2018).

Movements of live ornamental fish from countries from which infection with *A. invadans* is confirmed may spread the disease as was the case with the outbreak in Sri Lanka (Balasuriya, 1994). Flooding also caused the spread of infection with *A. invadans* in Bangladesh and Pakistan (Lilley *et al.,* 1998). Once an outbreak occurs in rivers/canals, the disease can spread downstream as well as upstream where the susceptible fish species exist.

*Aphanomyces invadans* grows best at 20–30°C; it does not grow *in-vitro* at 37°C. Water salinity over 2 parts per thousand (ppt) can stop spread of the agent. Under laboratory conditions the optimal growth temperature range for *A. invadans* is 19-22°C, while under natural conditions *A. invadans* seems to be more robust (Hawke *et al.,* 2003)

### 2.3.6. Geographical distribution

Infection with *A. invadans* was first reported in farmed freshwater ayu (*Plecoglossus altivelis*) in Asia in 1971 (Egusa & Masuda, 1971). It was later reported in estuarine fish, particularly grey mullet (*Mugil cephalus*) in eastern Australia in 1972 (Fraser *et al*., 1992; Mckenzie & Hall, 1976). Infection with *A. invadans* has extended its range into South-East and South Asia, and into West Asia (Lilley *et al*., 1998; Tonguthai, 1985). Outbreaks of ulcerative disease in menhaden (*Brevoortia tyrannus*) in North America had the same aetiological agent as the disease observed in Asia (Blazer *et al*., 1999; Lilley *et al*., 1997a; Vandersea *et al*., 2006). The first confirmed outbreaks of infection with *A. invadans* on the African continent occurred in 2007, and were connected to the Zambezi-Chobe river system (Andrew *et al.,* 2008; FAO, 2009; Huchzermeyer & Van der Waal, 2012; McHugh *et al.,* 2014). In 2010 and 2011, infection with *A. invadans* appeared in wild freshwater fish in Southern Africa and in wild brown bullhead fish in North America. Infection with *A. invadans* has been reported from more than 20 countries in four continents: North America, Southern Africa, Asia and Australia.

For recent information on distribution at the country level consult the OIE WAHIS interface (https://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home/index/newlang/en).

## 2.4. Biosecurity and disease control strategies

### 2.4.1. Vaccination

There is no protective vaccine available.

### 2.4.2. Chemotherapy including blocking agents

There is no effective treatment for *A. invadans*-infected fish in the wild and in aquaculture ponds.

### 2.4.3. Immunostimulation

Experimentally infected snakehead fish fed a vitamin-supplemented feed exhibited clinical signs of infection with *A. invadans* but had higher survival than controls (Miles *et al.*, 2001).

### 2.4.4. Breeding resistant strains

No data available.

### 2.4.5. Inactivation methods

To minimise fish losses in infected fish ponds water exchange should be stopped and lime or hydrated lime and/or salt should be applied (Lilley *et al.,* 1998). Preparing fish ponds by sun-drying and liming are effective disinfection methods for *A. invadans* (EFSA 2011b; Kumar *et al.,* 2020; Oidtmann, 2012). Similar to other oomycetes or water moulds, general disinfection chemicals effectively destroy *A. invadans* that might contaminate farms, fish ponds or fishing gear (Iberahim *et al.,* 2018).

### 2.4.6. Disinfection of eggs and larvae

Routine disinfection of fish eggs and larvae against water moulds is effective against *A. invadans*. It should be noted that there is no report of the presence of *A. invadans* in fish eggs or larvae.

### 2.4.7. General husbandry

Control of *A. invadans* in natural waters is probably impossible. In outbreaks occurring in small, closed water bodies or fish ponds, treating water with agricultural limes and improving water quality, together with removal of infected fish, is often effective in reducing mortalities and controlling the disease. Preventing entry of water from *A. invadans*-infected water bodies into fish ponds can prevents spread of the disease into farms. Sodium chloride or salt and agricultural lime are safe and effective chemicals for treating or preventing the spread of *A. invadans*.

# 3. Specimen selection, sample collection, transportation and handling

## 3.1. Selection of populations and individual specimens

Scoop net, cast net or seine net represent the best choices for catching diseased fish in natural waters or in fish ponds (FAO 2009).

Fish with characteristic EUS-like lesions should be sampled from affected populations

## 3.2. Selection of organs or tissues

The motile zoospore plays an important role in the spread of the disease. Once the motile spore attaches to the skin of the fish, the spore will germinate under suitable conditions and its hyphae will invade the fish skin, muscular tissue and reach the internal organs. Fish skeletal muscle is the target organ and exhibits major clinical signs of infection with *A. invadans* with mycotic granulomas (Iberahim *et al.,* 2018). Samples should not be taken from the middle of large lesions as these are likely to be devoid of visible and viable hyphae. Instead, samples should be taken from the leading edge of the infected area or lesion and where possible, multiple samples should be taken from an infected individual to obtain viable hyphae. Fungal hyphae can be seen in tissue squash mounts and histological sections at the leading edge of the infected area. Attempting to culture *A. invadans* from severe ulcers is often constrained because of contaminating bacteria, but still should be attempted. PCR on tissue taken from the leading edge of the ulcer also should be attempted.

### 3.3. Samples or tissues not suitable for pathogen detection

Samples should not be taken from the middle of large lesions as these are likely to be devoid of visible and viable hyphae.

### 3.4. Non-lethal sampling

None available.

### 3.5. Preservation of samples for submission

Fish specimens should be transported to the laboratory live or in ice-cooled boxes for further diagnosis. Samples must not be frozen since the fungus is killed by freezing. Fish collected from remote areas should be anesthetised and can be fixed in normal 10% formalin or 10% phosphate-buffered formalin for at least 1–2 days. The fixed specimens are then transferred to double-layer plastic bags with formalin-moistened tissue paper.

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0 *General information* (*diseases of fish*).

#### 3.5.1. Samples for pathogen isolation

The success of pathogen isolation depends strongly on the quality of samples (time since collection and time in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. To avoid degradation of samples use alternative storage methods only after consultation with the receiving laboratory. Multiple samples should be taken from each lesion to increase the chances of obtaining viable hyphae.

#### 3.5.2. Preservation of samples for molecular detection

Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1.

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

Standard methods for histopathology can be found in Chapter 2.3.0.

#### 3.5.4. Samples for other tests

None

### 3.6. Pooling of samples

Pooling of samples from more than one individual animal for a given purpose should only be recommended where supporting data on diagnostic sensitivity and diagnostic specificity are available. However, smaller life stages (e.g. fry) can be pooled to provide a minimum amount of material for testing.

## 4. Diagnostic methods

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

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

+++ = Methods are most suitable with desirable performance and operational characteristics.

++ = Methods are suitable with acceptable performance and operational characteristics under most circumstances.

+ = Methods are suitable, but performance or operational characteristics may limit application under some circumstances.

Shaded boxes = Not appropriate for this purpose.

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

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

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

| Method | A. Surveillance of apparently healthy animals | | | | B. Presumptive diagnosis of clinically affected animals | | | | C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV |
| **Squash mounts** | | | | | + | + | + | 1 | + | + | + | 1 |
| **Histopathology** | | | | | ++ | ++ | ++ | 1 | ++ | ++ | ++ | 1 |
| **Cytopathology** | | | | | | | | | | | | |
| **Cell or artificial media culture** | | | | | ++ | ++ | ++ | 1 | + | + | + | 1 |
| **Real-time PCR** | | | | | | | | | | | | |
| **Conventional PCR** | | | | | ++ | ++ | ++ | 1 | ++ | ++ | ++ | 1 |
| **Amplicon sequencing[4]** | | | | | | | | | +++ | +++ | +++ | 1 |
| ***In-situ* hybridisation** | | | | | | | | | ++ | ++ | ++ | 1 |
| **Bioassay** | | | | | | | | | | | | |
| **LAMP** | | | | | | | | | | | | |
| **Ab ELISA** | | | | | | | | | | | | |
| **Ag ELISA** | | | | | | | | | | | | |
| **Other antigen detection methods[3]** | | | | | | | | | | | | |
| **Other method[3]** | | | | | | | | | | | | |

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

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

[3]Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

Diagnosis of infection with *A. invadans* in clinically affected fish may be achieved by histopathology, oomycete isolation or polymerase chain reaction amplification.

## 4.1. Squash mounts

*Aphanomyces invadans* can be detected using microscopic examination of squash preparations prepared as follows:

i) Remove ulcer surface using a sharp scalpel blade.

ii) Cut the muscular tissue at the edge of the ulcer.

iii) Place the pieces of tissue on a cutting board then make thin slices using a sharp scalpel blade.

iv) Place the thinly sliced tissue between two glass slides and squeeze gently with fingers.

v) Remove one of the glass slides and cover the tissue with a cover-slip. View under a light microscope to find the nonseptate hyphae structure of *A. invadans* (12–25 µm in diameter).

## 4.2. Histopathology and cytopathology

*Aphanomyces invadans* can be detected using microscopic examination of fixed sections, prepared as follows:

i) Sample only live or moribund specimens of fish with clinical lesions.

ii) Take samples of skin/muscle (<1 $cm^3$), including the leading edge of the lesion and the surrounding tissue.

iii) Fix the tissues immediately in 10% formalin. The amount of formalin should be 10 times the volume of the tissue to be fixed.

### 4.2.1. Histological procedure

Standard methods for processing are provided in chapter 2.3.0. H&E and general fungus stains (e.g. Grocott's stain) will demonstrate typical granulomas and invasive hyphae.

### 4.2.2 Histopathological changes

Early lesions are caused by erythematous dermatitis with no obvious oomycete involvement. *Aphanomyces invadans* hyphae are observed growing in skeletal muscle as the lesions progress from a mild chronic active dermatitis to a severe locally extensive necrotising granulomatous dermatitis with severe floccular degeneration of the muscle. The oomycete elicits a strong inflammatory response and granulomas are formed around the penetrating hyphae.

## 4.3. Cell culture for isolation

### 4.3.1. Isolation of *Aphanomyces invadans* from internal tissues

The following are two methods of isolation of *A. invadans* adapted from Lilley *et al.* (1998) and Willoughby & Roberts (1994).

Method 1: Moderate, pale, raised, dermal lesions are most suitable for oomycete isolation attempts. Remove the scales around the periphery of the lesion and sear the underlying skin with a red-hot spatula so as to sterilise the surface. Using a sterile scalpel blade and sterile fine-pointed forceps, cut through the stratum compactum underlying the seared area and, by cutting horizontally and reflecting superficial tissues, expose the underlying muscle. Ensure the instruments do not make contact with the contaminated external surface and thereby contaminate the underlying muscle. Using aseptic techniques, carefully excise pieces of affected muscle, approximately 2 $mm^3$, and place on a Petri dish containing glucose/peptone (GP) agar (see Table 4.1) with penicillin G (100 units $ml^{-1}$) and streptomycin (100 µg $ml^{-1}$). Seal plates, incubate at room temperature or at 25°C and examine daily. Repeatedly transfer emerging hyphal tips on to fresh plates of GP agar with antibiotics until cultures are free of contamination.

Method 2: Lesions located on the flank or tail of fish <20 cm in length can be sampled by cutting the fish in two using a sterile scalpel and slicing a cross-section through the fish at the edge of the lesion. Flame the scalpel until red-hot and use this to sterilise the exposed surface of the muscle. Use a small-bladed sterile scalpel to cut out a circular block of muscle (2–4 $mm^3$) from beneath the lesion and place it in a Petri dish of GP medium (see Table 4.1) with 100 units $ml^{-1}$ penicillin G and 100 µg $ml^{-1}$ streptomycin. Instruments should not contact the contaminated external surface of the fish. Incubate inoculated medium at approximately 25°C and examine under a microscope (preferably an inverted microscope) within 12 hours. Repeatedly transfer emerging hyphal tips to plates of GP medium with 12 g $litre^{-1}$ technical agar, 100 units $ml^{-1}$ penicillin G and 100 µg $ml^{-1}$ streptomycin until axenic cultures are obtained. The oomycete isolate can also be maintained at 25°C on GY agar (see Table 4.1) and transferred to a fresh GY agar tube once every 1–2 weeks (Hatai & Egusa, 1979).

### 4.3.2. Identification of *Aphanomyces invadans*

*Aphanomyces invadans* does not produce any sexual structures and should thus not be diagnosed by morphological criteria alone. However, the oomycete can be identified to the genus level by inducing sporogenesis and demonstrating typical asexual characteristics of *Aphanomyces* spp., as described in Lilley *et al.,* 1998. *Aphanomyces invadans* is characteristically slow-growing in culture and fails to grow at 37°C on GPY agar (Table 4.1). Detailed temperature–growth profiles are given in Lilley & Roberts (1997). *A. invadans* can be identified by polymerase chain reaction (PCR) amplification of the rDNA of *A. invadans*.

### 4.3.3. Inducing sporulation in *Aphanomyces invadans* cultures

The induction of asexual reproductive structures is necessary for identifying oomycete cultures as members of the genus *Aphanomyces*. To induce sporulation, place an agar plug (3–4 mm in diameter) of actively growing mycelium in a Petri dish containing GPY broth and incubate for 4 days at approximately 20°C. Wash the nutrient agar out of the resulting mat by sequential transfer through five Petri dishes containing autoclaved pond water (Table 4.1), and leave overnight at 20°C in autoclaved pond water. After about 12 hours, the formation of achlyoid clusters of primary cysts and the release of motile secondary zoospores should be apparent under the microscope.

***Table 4.1.*** *Media for isolation, growth and sporulation of* Aphanomyces invadans *cultures*

| GP (glucose/peptone) medium | GPY (glucose/peptone/ yeast) broth | GPY agar | GY agar | Autoclaved pond water |
|---|---|---|---|---|
| 3 g $litre^{-1}$ glucose<br>1 g $litre^{-1}$ peptone<br>0.128 g $litre^{-1}$ $MgSO_4.7H_2O$<br>0.014 g $litre^{-1}$ $KH_2PO_4$<br>0.029 g $litre^{-1}$ $CaCl_2.2H_2O$<br>2.4 mg $litre^{-1}$ $FeCl_3.6H_2O$<br>1.8 mg $litre^{-1}$ $MnCl_2.4H_2O$<br>3.9 mg $litre^{-1}$ $CuSO_4.5H_2O$<br>0.4 mg $litre^{-1}$ $ZnSO_4.7H_2O$ | GP broth +<br>0.5 g $litre^{-1}$ yeast extract | GPY broth +<br>12 g $litre^{-1}$ technical agar | 1% glucose,<br>0.25% yeast extract,<br>1.5% agar | Sample pond/lake water known to support oomycete growth. Filter through Whatman 541 filter paper. Combine one part pond water with two parts distilled water and autoclave. pH to 6–7. |

#### Agent purification

Maintaining *A. invadans* in the axenic culture is necessary. As it is characteristically slow-growing, it easily becomes contaminated with other micro-organisms, such as bacteria and other fast-growing oomycetes and fungi. Attempts to purify or isolate *A. invadans* from contaminated cultures usually fail.

## 4.4. Nucleic acid amplification

### 4.4.1. Real-time PCR

No real-time PCR methods for detecting A. invadans in fish tissues are available.

### 4.4.2. Conventional PCR

#### DNA preparation from *A. invadans* isolate

DNA is extracted from an actively growing colony of *A. invadans* culture in GY broth at about 4 days or when young mycelia reach 0.5–1.0 cm in diameter. The mycelia are transferred to sterile 100-mm Petri dishes, washed twice with PBS and then placed on tissue paper for liquid removal. Hyphal tips (~50–

250 mg) are excised with a sterile scalpel blade and transferred to a 1.5 ml microcentrifuge tube for DNA extraction. Commercial DNA extraction kits have been used successfully (Phadee *et al.*, 2004b; Vandersea *et al.*, 2006).

**DNA preparation from *A. invadans* -infected tissue**

Small pieces of *A. invadans*-infected tissue (25–50 mg) are suitable for DNA extractions (Phadee *et al.*, 2004a).

**Diagnostic PCR technique**

Three published techniques are specific to *A. invadans*.

*Method 1*

The species-specific forward primer site is located near the 3' end of the SSU (small subunit) gene and a species-specific reverse primer site is located in the ITS1 region for Ainvad-2F (5'-TCA-TTG-TGA-GTG-AAA-CGG-TG-3') and Ainvad-ITSR1 (5'-GGC-TAA-GGT-TTC-AGT-ATG-TAG-3'). The PCR mixture contained 25 pM of each primer, 2.5 mM each deoxynucleoside triphosphate, 0.5 U of Platinum *Taq* DNA polymerase and 20 ng of genomic DNA (either from an *Aphanomyces* isolate or from infected tissue) for a total volume of 50 µl. DNA is amplified in a thermocycle machine under the following cycle conditions: 2 minutes at 95°C; 35 cycles, each consisting of 30 seconds at 95°C, 45 seconds at 56°C, 2.5 minutes at 72°C; and a final extension of 5 minutes at 72°C. The PCR product is analysed by agarose gel electrophoresis and the target product is 234 bp (Vandersea *et al.*, 2006).

*Method 2*

The species-specific primer sites are located in the ITS1 and ITS2 regions. The forward primer is ITS11 (5'-GCC-GAA-GTT-TCG-CAA-GAA-AC-3') and the reverse is ITS23 (5'-CGT-ATA-GAC-ACA-AGC-ACA-CCA-3'). The PCR mixture contains 0.5 µM of each primer, 0.2 mM each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.6 U of *Taq* DNA polymerase and 20 ng of genomic DNA (from an *Aphanomyces* isolate) for a total volume of 25 µl. The DNA is amplified under the following cycle conditions: 5 minutes at 94°C; 25 cycles, each consisting of 30 seconds at 94°C, 30 seconds at 65°C, 1 minute at 72°C; and a final extension of 5 minutes at 72°C. The PCR product is analysed by agarose gel electrophoresis and the target product is 550 bp. PCR amplification using the DNA template from the infected tissue is similar to the above protocol except that 5 ng of the DNA template is used for 35 cycles (Phadee *et al.*, 2004b).

*Method 3*

The species-specific primer sites are located in the ITS1 and ITS2 regions. The forward primer is BO73 (5'-CTT-GTG-CTG-AGC-TCA-CAC-TC-3') and the reverse is BO639 (5'-ACA-CCA-GAT-TAC-ACT-ATC-TC-3'). The PCR mixture contains 0.6 µM of each primer, 0.2 mM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.625 units of Taq DNA polymerase, and approximately 5 ng of genomic DNA (or 2.5 µl of DNA template extracted from 25 mg of infected tissue and suspended in 100 µl buffer) in a 50 µl reaction volume (Oidtmann *et al.*, 2008). The DNA is amplified under the following cycle conditions: 96°C for 5 minutes; 35 cycles of 1 minute at 96°C, 1 minute at 58°C and 1 minute at 72°C; followed by a final extension at 72°C for 5 minutes (Oidtmann, pers. comm.). The PCR product is analysed by agarose gel electrophoresis and the target product is 564 bp.

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

### 4.4.3. Other nucleic acid amplification methods

None

## 4.5. Amplicon sequencing

Nucleotide sequencing of all conventional PCR amplicons (Section 4.4.2) is recommended as one of the final steps for confirmatory diagnosis. *Aphanomyces invadans*-specific sequences will share a high degree of nucleotide similarity to one of the published reference sequences for *A. invadans* (Genbank accession AF396684)

### 4.6. *In-situ* hybridisation

A fluorescent peptide nucleic acid *in-situ* hybridisation (FISH) technique has demonstrated a high specificity for *A. invadans.* The technique can directly detect the mycelia-like structure of the oomycete in thinly sliced tissues of affected organs of susceptible fish. The fluorescein (FLU) probe designed to hybridise the small subunit of the rRNA *A. invadans* (bp 621 to 635; GenBank acc. AF396684) is 5'-FLU-GTA-CTG-ACA-TTT-CGT-3' or Ainv-FLU3.

The *A. invadans*-affected tissue is fixed and hybridised as soon as possible after the fish are collected to minimise RNA degradation. Tissue (~20 mg) is dissected from the periphery of the lesions with sterile scalpel blades and placed in individual wells of a 24-well microtitre plate. One ml ethanol-saline fixative (44 ml of 95% ethanol, 10 ml of deionised $H_2O$, and 6 ml of 25 × SET buffer [3.75 M NaCl, 25 mM EDTA (ethylene diamine tetra-acetic acid), 0.5 M Tris/HCl, pH 7.8]) containing 3% polyoxyethyl-enesorbitan monolaurate (Tween 20) is added to enhance tissue permeabilisation. The microtitre plate is gently agitated at room temperature on an orbital shaker (30 rpm) for 1.5 hours. The fixed tissues are rinsed (twice for 15 minutes each time) with 0.5 ml of hybridisation buffer (5 × SET, 0.1% [v/v] Igepal-CA630 and 25 µg $ml^{-1}$ poly[A]) containing 3% Tween 20. The hybridisation buffer is removed, and the tissues are resuspended in 0.5 ml of hybridisation buffer containing 3% Tween 20 and 100 nM Ainv-FLU3 probe. "No-probe" control specimens are incubated with 0.5 ml of hybridisation buffer/3% Tween 20. All tissues are incubated at 60°C for 1 hour in the dark. Following incubation, the tissues are rinsed twice with 1 ml of pre-warmed (60°C) 5 × SET buffer containing 3% Tween 20 to remove residual probe. The tissue specimens are mounted onto poly-L-lysine-coated microscope slides. One drop of the light anti-fade solution is placed on the specimens, which are then overlaid with a cover-slip. Analyses are performed by light and epifluorescence microscopy. The camera and microscope settings for epifluorescent analyses are held constant so that comparative analyses of relative fluorescence intensity can be made between probed and non-probed specimens. The fluorescent oomycete hyphae appear as green fluorescence against the dark tissue background. The above detailed protocols are published by Vandersea *et al*. (2006). Using the FISH technique, *A. invadans* can be visualised very well in thinly sliced tissue compared with freshly squashed tissue.

### 4.7. Immunohistochemistry

None

### 4.8. Bioassay

Fish can be experimentally infected by intramuscular injection of 0.1 ml suspension of 100+ motile zoospores into fish susceptible to infection with *A. invadans* at 20°C. Histological growth of aseptate hyphae, 12–25 µm in diameter, should be demonstrated in the muscle of fish sampled after 7 days, and typical mycotic granulomas should be demonstrated in the muscle of fish sampled after 10–14 days.

### 4.9. Antibody or antigen detection methods

Polyclonal antibodies against *A. invadans* or *Aphanomyces* saprophyte showed cross-reactivity to each other using protein gel electrophoresis and Western blot analysis and immunohistochemistry. (Lilley *et al.*, 1997b). However, a specific monoclonal antibody against *A. invadans* developed later was found to have high specificity and high sensitivity to *A. invadans* using immunofluorescence. This monoclonal antibody could detect *A. invadans* hyphae at the early stage of infection (Miles *et al.*, 2003).

A monoclonal antibody-based flow-through immunoassay was developed by Adil *et al*. (2013). This assay was found to have high analytical (0.007mg $ml^{-1}$) and diagnostic specificity comparable to PCR.

### 4.10. Other methods

Serological methods for detection and identification of *A. invadans* in diseased specimens are not practical. If necessary, the monoclonal antibody offers a better specificity and sensitivity than polyclonal antibody for serological detection or identification of *A. invadans* in diseased specimens or in pathogen isolates.

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

The test for targeted surveillance to declare freedom from infection with *A. invadans* is examination of target populations for gross signs of infection with *A. invadans*. Surveys should be conducted during seasons that favour clinical manifestation of infection with *A. invadans* or when water temperatures are in the range 18–25°C.

Using the gross sign test for targeted surveillance, a large sample of the fish population should be examined live with a sample size sufficient to meet survey design assumptions as described in Chapter 1.4 of the Aquatic Code.

If fish show gross signs consistent with infection with *A. invadans*, they should be categorised as suspect fish, and the location/farm/compartment/zone should be considered suspect. Suspect specimens should be further tested using the methods listed under presumptive diagnosis followed by confirmative diagnosis as described in the Table 4.1.

## 6. Corroborative diagnostic criteria

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

The case definitions for suspect and confirmed cases have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent.

### 6.1. Apparently healthy animals or animals of unknown health status[6]

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

#### 6.1.1. Definition of suspect case in apparently healthy populations

The presence of infection with *A. invadans* shall be suspected if at least one of the following criteria is met:

i) Observation of clinical signs consistent with infection with *A. invadans*

ii) A positive result obtained by any of the diagnostic techniques described in Section 4.

#### 6.1.2. Definition of confirmed case in apparently healthy populations

The presence of infection with *A. invadans* is considered to be confirmed if one or more of the following criteria is met:

i) Histopathology consistent with infection with *A. invadans* and positive result by PCR and amplicon sequencing

ii) Histopathological changes consistent with infection with *A. invadans* and positive result for *in-situ* hybridisation

iii) Artificial media culture and positive result by PCR and sequencing of the amplicon

### 6.2 Clinically affected animals

#### 6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with *A. invadans* shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs associated with infection with *A. invadans* as described in this chapter, with or without elevated mortality

ii) Positive result by a recommended molecular detection test

iii) Histological changes consistent with infection with *A. invadans*

iv) Visual observation (direct or by microscopy) of *A. invadans*

v) Culture and isolation of *A. invadans*

#### 6.2.2. Definition of confirmed case in clinically affected animals

6 For example transboundary commodities.

The presence of infection with *A. invadans* is considered to be confirmed if one or more of the following criteria is met:

i) Visualisation of hyphae under squash mounts and a positive result by PCR and sequencing of the amplicon

ii) Histopathological changes consistent with infection with *A. invadans* and a positive result by PCR and sequencing of the amplicon

iii) Histopathological changes consistent with infection with *A. invadans* and positive result for *in-situ* hybridisation

iv) Artificial media culture and a positive result by PCR and sequencing of the amplicon

v) Positive result for *in-situ* hybridisation and a positive result by PCR and sequencing of the amplicon

## 6.3. Diagnostic sensitivity and specificity for diagnostic tests [under study]

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *A. invadans* is provided in Table 6.3.1. (**note**: no data are currently available). This information can be used for the design of surveys for infection with *A. invadans*, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data is only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2 and the information is available within published diagnostic accuracy studies.

### 6.3.1. For presumptive diagnosis of clinically affected animals

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

- DSe: = diagnostic sensitivity, DSp = diagnostic specificity, qPCR: = real-time polymerase chain reaction.

### 6.3.2. For surveillance of apparently healthy animals

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

- DSe: = diagnostic sensitivity, DSp = diagnostic specificity, qPCR: = real-time polymerase chain reaction.

## 7. References


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**NB:** There is currently (2022) no OIE Reference Laboratories for infection with *Aphanomyces invadans* (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

**NB:** FIRST ADOPTED IN 1995 AS EPIZOOTIC ULCERATIVE SYNDROME; MOST RECENT UPDATES ADOPTED IN 2013.

Return to Agenda

CHAPTER 2.3.2.

# INFECTION WITH EPIZOOTIC HAEMATOPOIETIC NECROSIS VIRUS

## 1. Scope

Infection with epizootic haematopoietic necrosis virus means infection with the pathogenic agent *epizootic haematopoietic necrosis virus* (EHNV) of the Genus *Ranavirus* of the Family *Iridoviridae*.

## 2. Disease information

### 2.1. Agent factors

#### 2.1.1. Aetiological agent

EHNV is a species of the genus *Ranavirus* in the Family *Iridoviridae* (Chinchar *et al.,* 2005). In addition to fish, ranaviruses have been isolated from healthy or diseased frogs, salamanders and reptiles in America, Europe and Australia (Chinchar*,* 2002; Drury *et al.,* 2002; Fijan *et al.,* 1991; Hyatt *et al.*, 2002; Speare & Smith, 1992; Whittington *et al*., 2010; Wolf *et al*., 1968; Zupanovic *et al*., 1998). Ranaviruses have large (150–180 nm), icosahedral virions, a double-stranded DNA genome 150–170 kb, and replicate in both the nucleus and cytoplasm with cytoplasmic assembly (Chinchar *et al.,* 2005).

Since the recognition of disease due to EHNV in Australia in 1986, similar systemic necrotising iridovirus syndromes have been reported in farmed fish. These include catfish (*Ictalurus melas*) in France (European catfish virus, ECV) (Pozet *et al*., 1992), sheatfish (*Silurus glanis*) in Germany (European sheatfish virus, ESV) (Ahne *et al.,* 1989; 1990), turbot (*Scophthalmus maximus*) in Denmark (Bloch & Larsen, 1993), and cod (*Gadus morhua*) in Denmark (Cod iridovirus, CodV) (Ariel *et al.,* 2010). EHNV, ECV, ESV, and CodV share >98% nucleotide identity across concatenated sequences across the RNR-α, DNApol, RNR-β, RNAse II and MCP gene regions (Ariel *et al*., 2010).

EHNV and ECV can be differentiated using genomic analysis (Ahne *et al.,* 1998; Holopainen *et al*., 2009; Hyatt *et al.*, 2000; Mao *et al*., 1996; 1997; Marsh *et al*., 2002). This enables epidemiological separation of disease events in finfish in Australia (EHNV) and Europe (ECV), and differentiation of these from ranavirus occurrences in amphibians.

#### 2.1.2. Survival and stability in processed or stored samples

EHNV can persist in frozen fish tissues for more than 2 years (Langdon, 1989) and frozen fish carcases for at least a year (Whittington *et al*., 1996).

#### 2.1.3. Survival and stability outside the host

EHNV is resistant to drying and remained infective for 97 days at 15°C and 300 days at 4°C in water (Langdon, 1989). For these reasons, it is presumed that EHNV would persist for months to years on a fish farm in water and sediment, as well as on plants and equipment.

For inactivation methods, see Section 2.4.5.

### 2.2. Host factors

#### 2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with EHNV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are:

| Family | Scientific name | Common name |
|---|---|---|
| Esocidae | *Esox lucius* | Northern pike |
| Galaxiidae | *Galaxias olidus* | Mountain galaxias |
| Ictaluridae | *Ameiurus melas* | Black bullhead |
| Melanotaeniidae | *Melanotaenia fluviatilis* | Crimson spotted rainbow fish |
| Percidae | *Perca fluviatilis* | European perch |
| | *Sander lucioperca* | Pike-perch |
| Percichthyidae | *Macquaria australasica* | Macquarie perch |
| Poeciliidae | *Gambusia holbrooki* | Eastern mosquito fish |
| | *Gambusia affinis* | Mosquito fish |
| Salmonidae | *Oncorhynchus mykiss* | Rainbow trout |
| Terapontidae | *Bidyanus bidyanus* | Silver perch |

### 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 EHNV according to Chapter 1.5 of the *Aquatic Code* are: none known.

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following organisms, but an active infection has not been demonstrated: Atlantic salmon (*Salmo salar*), freshwater catfish (*Tandanus tandanus*), golden perch (*Macquaria ambigua*), Murray cod (*Maccullochella peelii*) and purple spotted gudgeon (*Mogurnda adspersa*).

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

Natural infections and disease have been limited to European perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) in Australia. The disease is more severe in European perch and in juveniles compared with adult fish (Whittington *et al.,* 2010). There are no descriptions of infection of eggs or early life stages of any other fish species.

For the purposes of Table 4.1, larvae and fry up to approximately 5 g in weight may be considered to be early life stages, fingerlings and grower fish up to 500 g may be considered to be juveniles, and fish above 500 g may be considered to be adults.

### 2.2.4. Distribution of the pathogen in the host

Target organs and tissues infected with the virus are kidney, spleen and liver. It is not known if EHNV can be detected in gonadal tissues, ovarian fluid or milt or whether these tissues are suitable for surveillance of broodstock.

### 2.2.5. Aquatic animal reservoirs of infection

*Rainbow trout:* The high case fatality rate and low prevalence of infection with EHNV in natural infections in rainbow trout means that the recruitment rate of carriers is likely to be very low (<2%) (Whittington *et al.,* 1994). EHNV has been detected in growout fish but histopathological lesions consistent with infection with EHNV indicated an active infection rather than a carrier state (Whittington *et al.,* 1999). Anti-EHNV serum antibodies were not detected in fingerlings during or after an outbreak but were detected in a low proportion of growout fish, hence, it is uncertain whether these were survivors of the outbreak (Whittington *et al.,* 1994; 1999). There are data for European stocks of rainbow trout in experimental infections where potential carriers were identified (Ariel & Bang Jensen, 2009).

*European perch:* EHNV was isolated from 2 of 40 apparently healthy adult European perch during epizootics in juveniles in Victoria, Australia (Langdon & Humphrey, 1987), but as the incubation period extends for up to 28 days (Whittington & Reddacliff, 1995), these fish may have been in the preclinical phase.

### 2.2.6. Vectors

Birds are potential vectors for EHNV, it being carried in the gut, on feathers, feet and the bill (Whittington *et al.,* 1996).

## 2.3. Disease pattern

### 2.3.1. Mortality, morbidity and prevalence

*Rainbow trout:* It appears that under natural farm conditions EHNV is poorly infective but once infected, most fish succumb to the disease ~~has a high case fatality rate~~. Infection with EHNV may be present on a farm without causing suspicion because the mortality rate may not rise above the usual background rate. Infection with EHNV has most often been reported in young fingerlings <125 mm fork length with daily mortality of less than 0.2% and total mortality of up to 4%. However, rainbow trout of all ages may be susceptible, although infection has not yet been seen in broodstock (Whittington *et al.*, 1994; 1999). There is a low direct economic impact because of the low mortality rate. Differences in susceptibility between European and Australian stocks of rainbow trout may exist (Ariel & Bang Jensen, 2009).

*European perch:* There is a very high rate of infection and mortality in natural outbreaks that, over time, leads to loss in wild fish populations (Langdon & Humphrey, 1987; Langdon *et al.,* 1986; Whittington *et al.*, 1996). Experimental bath inoculation with as few as 0.08 $TCID_{50}$ $ml^{-1}$ was lethal, and doses too low to be detected by virus isolation in BF-2 cells were fatal by intraperitoneal inoculation (Whittington & Reddacliff, 1995). Differences in susceptibility between European and Australian stocks of European perch may exist (Ariel & Bang Jensen, 2009).

### 2.3.2. Clinical signs, including behavioural changes

Moribund fish may have loss of equilibrium, flared opercula and may be dark in colour (Reddacliff & Whittington, 1996). Clinical signs are usually more obvious in fingerlings and juvenile fish than adults of both rainbow trout and European perch. There may be clinical evidence of poor husbandry practices, such as overcrowding and suboptimal water quality, manifesting as skin, fin and gill lesions (Reddacliff & Whittington, 1996).

### 2.3.3 Gross pathology

There may be no gross lesions in affected fish. A small proportion of fish may have enlargement of kidney, liver or spleen. There may be focal white to yellow lesions in the liver corresponding to areas of necrosis (Reddacliff & Whittington, 1996).

### 2.3.4. Modes of transmission and life cycle

*Rainbow trout:* EHNV has spread between rainbow trout farms by transfer of infected fingerlings and probably transport water (Langdon *et al.,* 1988; Whittington *et al.*, 1994; 1999). The low prevalence of infection in rainbow trout means that active infection can easily go unrecognised in a population and be spread by trading fish. There are no data on possible vertical transmission of EHNV on or within ova, and disinfection protocols for ova have not been evaluated. EHNV has not yet been isolated from ovarian tissues or from broodstock. Annual recurrence in farmed rainbow trout may be due to reinfection of successive batches of fish or from wild European perch present in the same catchment.

*European perch:* The occurrence of infection with EHNV in European perch in widely separated river systems and impoundments suggested that EHNV was spread by translocation of live fish or bait by recreational fishers (Whittington *et al.,* 2010).

The route of infection is unknown. European perch and rainbow trout are susceptible to immersion exposure. The virus infects a range of cell types including hepatocytes, haematopoietic cells and endothelial cells in many organs (Reddacliff & Whittington, 1996). Virus is shed into water from infected tissues and carcasses as they disintegrate.

### 2.3.5. Environmental factors

*Rainbow trout:* Outbreaks appear to be related to poor husbandry, particularly overcrowding, inadequate water flow and fouling of tanks with feed. Damage to skin may provide a route of entry for EHNV. Outbreaks have been seen on farms at water temperatures ranging from 11 to 20°C (Whittington *et al.*, 1994; 1999). The incubation period after intraperitoneal inoculation was 3–10 days at 19–21°C compared with 14–32 days at 8–10°C (Whittington & Reddacliff, 1995).

*European perch:* Natural epizootics of infection with EHNV affecting juvenile and adult European perch occur mostly in summer (Langdon & Humphrey, 1987; Langdon *et al.,* 1986; Whittington *et al.*, 1994). It has been assumed that the disease in juvenile fish is related to the annual appearance of large numbers of non-immune young fish and their subsequent exposure to the virus while schooling in shallow waters; adults are uncommonly involved in these outbreaks. It is possible that environmental temperature is the trigger for outbreaks as juvenile fish feed in warm shallow waters on planktonic fauna, whereas adults

feed on benthic invertebrates and larger prey in deeper cooler water (Whittington & Reddacliff, 1995). Experimentally, the incubation period ranged from 10 to 28 days at 12–18°C compared with 10–11 days at 19–21°C, and adult perch were refractory to infection at temperatures below 12°C (Whittington & Reddacliff, 1995). European stocks of European perch also displayed temperature-dependent susceptibility (Ariel & Bang Jensen, 2009).

#### 2.3.6. Geographical distribution

Infection with EHNV has been reported from rainbow trout farms within two river catchments in New South Wales, Australia (Whittington *et al*., 2010). Infection with EHNV is endemic in south-eastern Australia, with a discontinuous distribution (Whittington *et al*., 2010).

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

### 2.4. Biosecurity and disease control strategies

Not available.

#### 2.4.1. Vaccination

None available.

#### 2.4.2. Chemotherapy including blocking agents

None available.

#### 2.4.3. Immunostimulation

None available.

#### 2.4.4. Breeding resistant strains

There has been no formal breeding programme for resistant strains of susceptible species. However, experimental trials using a bath exposure have shown that European perch from water bodies in New South Wales, Australia with previous EHNV infections showed lower mortality compared with European perch from neighbouring and distant water bodies in Australia that have no previous history of EHNV (Becker *et al.,* 2016).

#### 2.4.5. Inactivation methods

EHNV is susceptible to 70% ethanol, 200 mg litre$^{-1}$ sodium hypochlorite or heating to 60°C for 15 minutes (Langdon, 1989). Data for the inactivation of amphibian ranavirus may also be relevant: 150 mg/litre chlorhexidine and 200 mg/litre potassium peroxymonosulphate were effective after 1 minute contact time (Bryan *et al*., 2009). If it is first dried, EHNV in cell culture supernatant is resistant to heating to 60°C for 15 minutes (Whittington *et al.*, 2010).

#### 2.4.6. Disinfection of eggs and larvae

Not tested.

#### 2.4.7. General husbandry

Disease control in rainbow trout at the farm level relies on reducing the impact of infection by maintaining low stocking rates and adequate water quality. Investigations on one rainbow trout farm indicated that ponds with high stocking rates and low water flow, and thus poorer water quality, may result in higher levels of clinical disease compared with ponds on the same farm with lower stocking rates and higher water flow (Whittington *et al.,* 1994). The mechanism of protection may be through maintenance of healthy integument (Whittington *et al.,* 1994).

## 3. Specimen selection, sample collection, transportation and handling

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

## 3.1. Selection of populations and individual specimens

Clinical inspections should be carried out during a period when water temperature is conducive to development of clinical disease (see Section 2.3.5). All production units (ponds, tanks, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. For the purposes of disease surveillance, fish to be sampled are selected as follows:

i) The most susceptible species (e.g. rainbow trout and European perch) should be sampled preferentially. Other susceptible species listed in Section 2.2.1 should be sampled proportionally.

ii) Risk-based criteria should be employed to preferentially sample lots or populations with a history of abnormal mortality, potential exposure events or where there is evidence of poor water quality or husbandry. If more than one water source is used for fish production, fish from all water sources should be included in the sample.

iii) If weak, abnormally behaving or freshly dead fish are present, such fish should be selected. If such fish are not present, the fish selected should include normal appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample.

For disease outbreak investigations, moribund fish or fish exhibiting clinical signs of infection with EHNV should be collected. Ideally fish should be collected while alive, however recently dead fish can also be selected for diagnostic testing. It should be noted however, that there will be a significant risk of contamination with environmental bacteria if the animals have been dead for some time.

## 3.2. Selection of organs or tissues

Liver, anterior kidney and spleen from individual fish are pooled (Jaramillo *et al.,* 2012).

## 3.3. Samples or tissues not suitable for pathogen detection

Inappropriate tissues include gonads, gonadal fluids, milt and ova, ~~since~~ because there is no evidence of reproductive tract infection.

## 3.4. Non-lethal sampling

~~No~~ Non-lethal samples (blood, fin, gill, integument or mucous) are unsuitable for testing ~~EHNV~~.

## 3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0.

### 3.5.1. Samples for pathogen isolation

For recommendations on transporting samples for virus isolation to the laboratory, see Section B.2.4 of Chapter 2.3.0 *General information* (*diseases of fish*).

### 3.5.2. Preservation of samples for molecular detection

~~Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.~~ Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.2.5 of Chapter 2.3.0. *General information* (*diseases of fish*).

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

~~Tissue samples for histopathology should be fixed immediately after collection in 10% neutral buffered formalin. The recommended ratio of fixative to tissue is 10:1.~~ Standard sample collection, preservation and processing methods for histological techniques can be found in Section 2.2 of Chapter 2.3.0 *General information* (diseases of fish).

### 3.5.4. Samples for other tests

Not recommended for routine diagnostic testing.

### 3.6. Pooling of samples

Pooling of samples from more than one individual animal for a given purpose should only be recommended where robust supporting data on diagnostic sensitivity and diagnostic specificity have been evaluated and found to be suitable. If the effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, larger fish should be processed and tested individually. Small life stages such as fry or specimens can be pooled to provide the minimum amount of material needed for testing. If pooling is used, it is recommended to pool organ pieces from a maximum of five fish.

## 4. Diagnostic methods

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

~~The designations used in the Table indicate:~~

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

~~Key:~~

| | |
|---|---|
| +++ = | ~~Most suitable~~ Methods ~~—~~are most suitable with desirable performance and operational characteristics. |
| ++ = | ~~Suitable~~ Method~~(s)~~ are suitable with acceptable performance and operational characteristics under most circumstances. |
| + = | ~~Less suitable~~ Methods ~~–~~ are suitable, but performance or operational characteristics may ~~significantly~~ limit application under some circumstances. |
| Shaded boxes = | Not appropriate for this purpose. |

~~The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.~~

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

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

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

| Method | A. Surveillance of apparently healthy animals | | | | B. Presumptive diagnosis of clinically affected animals | | | | C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV |
| **Wet mounts** | | | | | | | | | | | | |
| **Histopathology** | | | | | ++ | ++ | ++ | 1 | | | | |
| **Cytopathology** | | | | | | | | | | | | |
| **Cell culture** | +++ | +++ | +++ | 2 | +++ | +++ | +++ | 2 | ++ | ++ | ++ | 2 |
| **Immunohistochemistry** | | | | | + | + | + | 1 | | | | |
| **Real-time PCR** | +++ | +++ | +++ | 2 | +++ | +++ | +++ | 2 | ++ | ++ | ++ | 2 |
| **Conventional PCR** | + | + | + | 1 | ++ | ++ | ++ | 1 | ++ | ++ | ++ | 1 |
| **Amplicon sequencing** | | | | | | | | | +++ | +++ | +++ | ~~3~~ 1 |
| ***In-situ* hybridisation** | | | | | | | | | | | | |
| **Bioassy** | | | | | | | | | | | | |
| **LAMP** | | | | | | | | | | | | |
| **Ab-ELISA** | | | + | 1 | | | | | | | | |
| **Ag-ELISA** | + | + | + | 1 | + | + | + | 1 | | | | |
| **Other antigen detection methods[3]** | | | | | | | | | | | | |
| **Other method[3]** | | | | | | | | | | | | |

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.
[3]Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

## 4.1. Wet mounts

Not applicable.

## 4.2. Histopathology and cytopathology

*Light microscopy:* routine methods can be used for tissue fixation, such as in 10% buffered neutral formalin, paraffin embedding, preparation of 4–10 µm sections and staining with H&E to demonstrate tissue necrosis and basophilic intracytoplasmic inclusion bodies. These inclusion bodies are indicative but not confirmatory for infection with EHNV. Formalin-fixed paraffin-embedded sections can also be stained using an immunoperoxidase method (see below) to identify EHNV antigen associated with necrotic lesions.

Acute focal, multifocal or locally extensive coagulative or liquefactive necrosis of liver, haematopoietic kidney and spleen are commonly seen in routine haematoxylin and eosin (H&E)-stained sections of formalin-fixed material. A small number of basophilic intracytoplasmic inclusion bodies may be seen, particularly in areas immediately surrounding necrotic areas in the liver and kidney. Necrotic lesions may also be seen in heart, pancreas, gastrointestinal tract, gill and pseudobranch (Reddacliff & Whittington, 1996).

Affected tissues (e.g. kidney, liver and spleen) contain cells exhibiting necrosis. Cells contain conspicuous cytoplasmic inclusions that are rarefied areas of the cytoplasm in which the viruses are assembled. ~~Within the cytoplasm, aggregates (paracrystalline arrays) of large (175 nm ± 6 nm) nonenveloped icosahedral viruses are apparent; single viruses are also present. Complete viruses (containing electron-dense cores) bud/egress from the infected cells through the plasma membrane.~~ The nuclei of infected cells are frequently located peripherally and are distorted in shape.

## 4.3. Cell culture for isolation

### 4.3.1. Preparation of fish tissues for virus isolation

A simple method for preparation of fish tissues for cell culture and ELISA has been validated (Whittington & Steiner, 1993) (see sampling Section 3).

i) Freeze tubes containing tissues at –80°C until needed.

ii) Add 0.5 ml of homogenising medium (minimal essential medium Eagle, with Earle's salts with glutamine] [MEM] with 200 International Units [IU] $ml^{-1}$ penicillin, 200 µg $ml^{-1}$ streptomycin and 4 µg $ml^{-1}$ amphotericin B) to each tube. Grind tissue to a fine mulch with a sterile fitted pestle.

iii) Add another 0.5 ml of homogenising medium to each tube and mix with a pestle.

iv) Add three sterile glass beads to each tube (3 mm diameter) and close the lid of the tube.

v) Vortex the suspension vigorously for 20–30 seconds and place at 4°C for 2 hours.

vi) Vortex the suspension again as above and centrifuge for 10 minutes at 2500 ***g*** in a benchtop microcentrifuge.

vii) Transfer the supernatant, now called clarified tissue homogenate, to a fresh sterile tube. Homogenates may be frozen at –80°C until required for virus isolation and ELISA.

### 4.3.2. Cell culture~~/artificial media~~

EHNV ~~grows~~ replicates well in many fish cell lines including BF-2 (bluegill fry ATCC CCL 91), FHM (fathead minnow; ATCC CCL 42), EPC (*epithelioma papulosum cyprini* [Cinkova *et al.,* 2010]), and CHSE-214 (Chinook salmon embryo cell line; ATCC CRL 1681) at temperatures ranging from 15 to 22°C (Crane *et al.,* 2005). Incubation temperatures of 20°C or 24°C result in higher titres than 15°C; 22°C and BF-2 EPC or CHSE-214 cells are recommended to maximise titres, which might be important for the detection of low numbers of viruses in fish tissues (Ariel *et al.,* 2009). BF-2 cells are preferred by the OIE Reference Laboratory with an incubation temperature of 22°C. The procedure for BF-2 cells is provided below. A procedure for CHSE-214 cells is provided under immunoperoxidase staining below (Section 4.7). The identity of viruses in cell culture is determined by immunostaining, ELISA, immuno-electron microscopy, PCR or other methods.

### 4.3.3. Cell culture technical procedure

*Samples:* tissue homogenates.

Cells are cultured (in flasks, tubes or multi-well plates) with growth medium (MEM + 10% fetal ~~calf~~ bovine serum [F~~C~~BS] with 100 IU $ml^{-1}$ penicillin, 100 µg $ml^{-1}$ streptomycin and 2 µg $ml^{-1}$ amphotericin B). The cells are incubated until almost confluent at 22°C, which can take up to 4 days depending on the seeding rate. Medium is changed to a maintenance medium (MEM with 2% F~~C~~BS and 100 IU $ml^{-1}$ penicillin, 100 µg $ml^{-1}$ streptomycin and 2 µg $ml^{-1}$ amphotericin B) on the day of inoculation. A 1/10 dilution using homogenising medium is made of single or pooled homogenates. Each culture is inoculated with 100 µl of sample per ml of culture medium. This represents a final 1/100 dilution of a 0.1 mg $ml^{-1}$ tissue homogenate. A further 1/10 dilution is made representing a final 1/1000 dilution, and two cultures are inoculated. No adsorption step is used. As an alternative, two to three cultures can be inoculated directly with 10 µl undiluted homogenate per ml of culture medium. Note that a high rate of cell toxicity or contamination often accompanies the use of a large undiluted inoculum. The cultures are incubated at 22°C in an incubator for 6 days. Cultures are read at days 3 and ~~day~~ 6. Cultures are passed at least once to detect samples with low levels of virus. On day 6, the primary cultures (P1) are frozen overnight at –20°C, thawed, gently mixed and then the culture supernatant is inoculated onto fresh cells as before (P2), i.e. 100 µl P1 supernatant per ml culture medium. Remaining P1 supernatants are transferred to sterile 5 ml tubes and placed at 4°C for testing by ELISA or PCR or another means to confirm the cause of cytopathic effect (CPE) as EHNV. P2 is incubated as above, and a third pass is conducted if necessary.

### 4.3.4. Interpretation of results

CPE is well developed and consists of focal lysis surrounded by rounded granular cells. This change extends rapidly to involve the entire monolayer, which detaches and disintegrates. Cell cultures can be tested for EHNV DNA using real-time PCR and conventional PCR with sequence analysis as described in Section 4.4. Antigen can be detected using immunocytochemistry in cell cultures with polyclonal antibodies and protocol available from the reference laboratory.

Cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

## 4.4. Nucleic acid amplification

Although several conventional PCR or quantitative real-time PCR methods have been described (Jaramillo *et al.,* 2012; Pallister *et al,* 2007; Stilwell *et al.,* 2018) none has been validated according to OIE guidelines for primary detection of EHNV. However, identification of ranavirus at genus and species level is possible using several published PCR strategies. Samples can be screened by real-time PCR, but as the assays described are not specific for EHNV, identification of EHNV by conventional PCR and amplicon sequencing must be undertaken on any samples screening positive by real-time PCR. For testing by conventional PCR, two PCR assays using MCP primers are used with amplicon sequencing required to differentiate EHNV from ECV, FV3 and BIV (Marsh *et al*., 2002). Alternatively, PCR of the DNA polymerase gene and neurofilament triplet H1-like protein genes can be used (Holopainen *et al*., 2011) (this method is not described in this chapter).

*Samples:* virus from cell culture or direct analysis of tissue homogenate.

### 4.4.1. Real-time PCR

Tissue samples can be homogenised by manual pestle grinding or by bead beating (Rimmer *et al.,* 2012). Commercially available nucleic acid extraction kits (e.g. spin columns, magnetic beads) may be used to extract DNA directly from tissues and from tissue homogenates and cell culture supernatants. Depending on the number of samples to be tested, in the OIE Reference Laboratory, nucleic acids are extracted with either the QIAamp Viral RNA Mini Kit (Qiagen) or MagMAX-96 Viral RNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions. A negative extraction control, consisting of extraction reagents only, is included when test samples are extracted.

The ranavirus real-time screening protocol in use at the OIE Reference Laboratory, based on Pallister *et al.,* 2007 is as follows; Template (2 µl) is added to 23 µl reaction mixture containing 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM for each primer, 250 nM for probe, and molecular grade water. After 1 cycle of 50°C for 2 minutes and 95 °C for 10 minutes, PCR amplification consists of 45 cycles of 95°C for 15 seconds, 60°C for 60 seconds.

Alternative real-time PCR assays can be used according to published protocols for detection of the major capsid protein gene sequence of EHNV and other ranaviruses. The assay described by Jaramillo *et al.*

(2012) uses SYBR Green detection chemistry and the assay described by Stilwell *et al.* (2018) was designed to detect multiple ranavirus species using hydrolysis probe detection chemistry.

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

***Table 4.4.1.1.*** *Ranavirus primer and probe sequences*

| Primer | Sequence (5'–3') | Reference |
|---|---|---|
| RANA CON F | 5'-CTC-ATC-GTT-CTG-GCC-ATC-A-3' | Pallister *et al.,* 2007 |
| RANA CON R | 5'-TCC-CAT-CGA-GCC-GTT-CA-3' | |
| **Probe** | | |
| RANA CON Pr | 5'-6**FAM**-CAC-AAC-ATT-ATC-CGC-ATC-**MGB**-3' | |
| **Primer** | | |
| C1096 | GAC-TGA-CCA-ACG-CCA-GCC-TTA-ACG | Jaramillo *et al.,* 2012 |
| C1097 | GCG-GTG-GTG-TAC-CCA-GAG-TTG-TCG | |
| **Primer** | | |
| RanaF1 | CCA-GCC-TGG-TGT-ACG-AAA-ACA | Stilwell *et al.,* 2018 |
| RanaR1 | ACT-GGG-ATG-GAG-GTG-GCA-TA | |
| **Probe** | | |
| RanaP1 | 6FAM-TGG-GAG-TCG-AGT-ACT-AC-MGB | |

### 4.4.2. Conventional PCR

*PCR and restriction endonuclease analysis* (*REA*): *technical procedure*

Amplified product from PCR assay MCP-1 digested with PflM I enables differentiation of EHNV and BIV from FV3 and ECV. Amplified product from PCR assay MCP-2 digested with Hinc II, Acc I and Fnu4H I (individually) enables differentiation of EHNV and BIV from each other and from FV3 and ECV.

*Preparation of reagents*

EHNV-purified DNA and BIV-purified DNA PCR control reagents are supplied by the reference laboratory in freeze-dried form. Reconstitute using 0.5 ml of Tris-EDTA (TE) buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and allow the vial to stand at RT for 2 minutes. Mix the vial very gently. For routine use, as a PCR control, it is recommended that working stocks be prepared as a 1/10 dilution in TE buffer (pH 8.0). Aliquots of 250 µl should be stored at –20°C. Each aliquot is sufficient for at least 50 reactions (1 to 5 µl added to cocktail) and has a minimum shelf life of 6 months from date of diluting.

Primers M151 and M152 (MCP-1, 321 bp), M153 and M154 (MCP-2, 625 bp) are supplied in working strength (100 ng $\mu l^{-1}$) and should be stored at –20°C. Primers can also be ordered from commercial suppliers. For primer sequences, refer to Table 4.4.2.1.

***Table 4.4.2.1.*** *MCP-1 and MCP-2 primer sequences*

| PCR assay | Primer | Sequence (5′–3′) | Product size | Gene location |
|---|---|---|---|---|
| MCP-1 | M151 | AAC-CCG-GCT-TTC-GGG-CAG-CA | 321 bp | 266–586 |
| | M152 | CGG-GGC-GGG-GTT-GAT-GAG-AT | | |
| MCP-2 | M153 | ATG-ACC-GTC-GCC-CTC-ATC-AC | 625 bp | 842–1466 |
| | M154 | CCA-TCG-AGC-CGT-TCA-TGA-TG | | |

*PCR cocktail*

Amplification reactions in a final volume of 50 µl (including 5 µl DNA sample) contain 2.5 µl (250 ng) of each working primer, 200 µM of each of the nucleotides dATP, dTTP, dGTP and dCTP, 5 µl of 10 × PCR buffer (66.6 mM Tris/HCl, 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1.65 mg $ml^{-1}$ BSA, 10 mM beta-mercaptoethanol) and 2 U Taq polymerase. Instructions on preparation of 10 × PCR buffer are included in Table 4.4.2.2.

***Table 4.4.2.2.*** *10 × PCR buffer preparation*

| Ingredients | Amount | Final concentration in 50 µl PCR mix |
|---|---|---|
| Tris | 4.050 g | 66.6 mM |
| Ammonium sulphate | 1.100 g | 16.6 mM |
| BSA (albumin bovine fraction V fatty acid free) | 0.825 g | 1.65 mg $ml^{-1}$ |
| Magnesium chloride | 1.25 ml | 2.5 mM |
| TE buffer (sterile) | 50 ml | |

NOTE: alternative commercial buffers may also be used.

Two negative controls are included, one comprising PCR cocktail only and the second containing 5 µl TE buffer.

The MCP-1 and MCP-2 reactions have the following profile: 1 cycle of denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 1 minute; a final extension of 72°C for 5 minutes, and cooling to 4°C.

NOTE: the annealing temperature may be increased to 60 or 62°C to reduce nonspecific amplification when the assay is used to test fish tissues.

PCR results are assessed by electrophoresis in 2% agarose gels stained with ~~ethidium bromide~~ containing SYBRTM Safe (Thermo Fisher Scientific) or equivalent. EHNV PCR control DNA (1/10 working stock) should give a result similar in intensity to the 10–3 band in both cases.

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

### 4.4.3. Other nucleic acid amplification methods

Not applicable.

## 4.5. Amplicon sequencing

Amplicons generated using the MCP-1 and/or MCP-2 primers sets can be sequenced. Amplicons should be gel-purified and sequenced using both the forward and reverse primer. Consensus sequence, generated after analysis of the quality of the sequence chromatograms, can then be compared to reference sequences, for example by BlastN search of the NCBI database.

## 4.6. *In-situ* hybridisation

Not applicable

## 4.7. Immunohistochemistry

*Immunohistochemistry* (*immunoperoxidase stain*)

Samples: formalin-fixed paraffin-embedded tissue sections.

*Technical procedure*

The following protocol is intended for the qualitative demonstration of EHNV antigens in formalin-fixed paraffin-embedded tissue sections (Reddacliff & Whittington, 1996). It assumes that antigens may have become cross linked and therefore includes a protease digestion step that may be omitted if unfixed

samples are examined. A commercial kit (DAKO® LSAB K0679) with peroxidase-labelled streptavidin and a mixture of biotinylated anti-rabbit/anti-mouse/anti-goat immunoglobulins as link antibodies is used for staining. Other commercially supplied reagents are also used. For convenience these are also supplied by DAKO[7]. The primary affinity purified ~~rabbit~~ anti-EHNV antibody (Lot No. M708) is supplied freeze-dried by the OIE Reference Laboratory.

i) Cut 5 µm sections and mount on SuperFrost® Plus G/Edge slides (Menzel-Glaser, HD Scientific Cat. No. HD 041300 72P3). Mark around the section with a diamond pencil to limit the spread of reagents.

ii) Deparaffinise the section:

Preheat slides in a 60°C incubator for 30 minutes.

Place slides in a xylene bath and incubate for 5 minutes. Repeat once. Note that xylene replacements can be used without deleterious effects.

Tap off excess liquid and place slides in absolute ethanol for 3 minutes. Repeat once.

Tap off excess liquid and place slides in 95% ethanol for 3 minutes. Repeat once.

Tap off excess liquid and place slides in distilled or deionised water for 30 seconds.

iii) Expose antigens using a protease treatment. Flood slide with proteinase K (5–7 µg $ml^{-1}$) and incubate for 20 minutes (ready-to-use solution, DakoCytomation Cat. No. S3020). Rinse slide by immersing three times in water. Place in a PBST bath for 5 minutes (PBS pH 7.2, 0.05% [v/v] Tween 20). Tap off the excess wash solution and carefully wipe around the section.

iv) Perform the immunostaining reaction using the Universal DAKO LSAB®+ Kit, Peroxidase (DakoCytomation Cat No. K0679). Ensuring the tissue section is completely covered, add the following reagents to the slide. Avoid drying out.

v) 3% hydrogen peroxide: cover the section and incubate for 5 minutes. Rinse gently with PBST and place in a fresh wash bath.

vi) Primary antibody (affinity purified rabbit anti-EHNV 1:/1500 Lot No. M708) and negative control reagent (non-immune rabbit serum at a dilution of 1/1500) on a second slide. Cover the section and incubate for 15 minutes. Rinse slides.

vii) Biotin-labelled secondary link antibody: ~~Link:~~ cover the section and incubate for 15 minutes. Rinse slides.

viii) Streptavidin peroxidase: cover the section and incubate for 15 minutes. Rinse slides.

ix) Substrate–chromogen solution: cover the section and incubate for 5 minutes. Rinse slides gently with distilled water.

x) Counterstain by placing slides in a bath of DAKO® Mayer's Haematoxylin for 1 minute (Lillie's Modification, Cat. No. S3309). Rinse gently with distilled water. Immerse 10 times into a water bath. Place in distilled or deionised water for 2 minutes.

xi) Mount and cover-slip samples with an aqueous-based mounting medium (DAKO® Faramount Aqueous Mounting Medium Cat. No. S3025).

*Interpretation of results*

EHNV antigen appears as a brown stain in the areas surrounding degenerate and necrotic areas in parenchymal areas. There should be no staining with negative control rabbit serum on the same section.

Availability of test and reagents: antibody reagents and test protocols are available from the OIE Reference Laboratory.

## 4.8. Bioassay

Not applicable.

## 4.9. Antibody- or antigen-based detection methods

7 Dako Cytomation California Inc., 6392 via Real, Carpinteria, CA 93013, USA, Tel.: (+1-805) 566 6655, Fax: (+1-805) 566 6688; Dako Cytomation Pty Ltd, Unit 4, 13 Lord Street, Botany, NSW 2019, Australia, Fax: (+61-2) 9316 4773; Visit www.dakocytomation.com for links to other countries.

An antigen ELISA for detection of EHNV and an EHNV antibody detection ELISA have been described (Whittington & Steiner, 1993). The same antibodies are suitable for immunohistochemistry on fixed tissues and for detection of ranavirus antigen in cell culture. Reagents and protocols are available from the reference laboratory. It should be noted that polyclonal antibodies used in all related methods (immunoperoxidase, antigen-capture ELISA and immunoelectron microscopy) cross-react with all known ranaviruses except Santee Cooper ranaviruses (Ahne *et al.,* 1998; Cinkova *et al.,* 2010; Hedrick *et al*., 1992; Hyatt *et al.*, 2000).

### 4.10. Other methods

Neutralising antibodies have not been detected in fish or mammals exposed to EHNV. Indirect ELISA for detection of antibodies induced following exposure to EHNV has been described for rainbow trout and European perch (Whittington *et al.*, 1994; 1999; Whittington & Reddacliff, 1995). The sensitivity and specificity of these assays in relation to a standard test are not known and interpretation of results is difficult. Protocols and specific anti-immunoglobulin reagents required to conduct these tests are available from the reference laboratory.

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

Real-time PCR is the most appropriate method of screening healthy fish populations for EHNV; however, the available methods are not specific for EHNV. Any real-time PCR positive samples should be tested by conventional PCR and sequence analysis to distinguish ranaviruses.

## 6. Corroborative diagnostic criteria

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

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

### 6.1. Apparently healthy animals or animals of unknown health status[8]

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

#### 6.1.1. Definition of suspect case in apparently healthy animals

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

i) Positive result for EHNV based on virus isolation in cell cultures;

ii) Positive real-time or conventional PCR result;

iii) Positive EHNV antigen ELISA.

#### 6.1.2. Definition of confirmed case in apparently healthy animals

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

i) EHNV-typical CPE in cell culture followed by identification of EHNV by conventional PCR and sequence analysis of the amplicon;

8 For example transboundary commodities.

ii) A positive result in tissue samples by real-time PCR and identification of EHNV by conventional PCR and sequence analysis of the amplicon.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

## 6.2 Clinically affected animals

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

### 6.2.1. Definition of suspect case in clinically affected animals

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

i) Histopathology consistent with EHNV;

ii) EHNV-typical CPE in cell cultures;

iii) Positive real-time or conventional PCR result.

### 6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with EHNV is considered to be confirmed if, in addition to the criteria in Section 6.2.1, at least one of the following criteria is met:

i) EHNV-typical CPE in cell culture followed by identification of EHNV by conventional PCR and sequence analysis of the amplicon;

ii) A positive result in tissue samples by real-time PCR and identification of EHNV by conventional PCR and sequence analysis of the amplicon.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

## 6.3. Diagnostic sensitivity and specificity for diagnostic tests

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

### 6.3.1. For presumptive diagnosis of clinically affected animals

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| Real-time PCR | Diagnosis | Clinically diseased fish (multiple species) from disease outbreaks and experimental infections | Pool of kidney, liver and spleen from individual fish | European perch (*Perca fluviatilis*), river blackfish (*Gadopsis marmoratus*), golden perch (*Macquaria ambigua*), trout cod (*Maccullochella macquariensis*), freshwater catfish (*Tandanus tandanus*), Macquarie perch (*Macquaria australasica*) rainbow trout (*Oncorhynchus mykiss*) | 94.3%* (n= 105) | 100% (n= 441) | Virus isolation in BF-2 cell culture | Jaramillo *et al.,* (2012) |
| Real-time PCR | Diagnosis | Clinically diseased fish (multiple species) from | Pool of kidney, liver and spleen | European perch (*Perca fluviatilis*), river blackfish (*Gadopsis marmoratus*), golden perch (*Macquaria* | 95%* (n=106) | 100% (n=80) | Virus isolation in BF-2 cell culture | Stilwell *et al.,* 2018 |

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| | | disease outbreaks and experimental infections | from individual fish | *ambigua*), trout cod (*Maccullochella macquariensis*), freshwater catfish (*Tandanus tandanus*), Macquarie perch (*Macquaria australasica*) rainbow trout (*Oncorhynchus mykiss*) | | | | |

DSe: = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study;
PCR: = polymerase chain reaction. Note: these assays detect multiple ranaviruses in addition to EHNV that infect amphibian hosts. *A positive result requires characterisation using sequencing to confirm that the result indicates the presence of EHNV.

### 6.3.2. For surveillance of apparently healthy animals: not available

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

DSe: = diagnostic sensitivity, DSp = diagnostic specificity, qPCR: = real-time polymerase chain reaction.

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*
* *

**NB:** There is an OIE Reference Laboratory for infection with epizootic haematopoietic necrosis virus (EHNV) (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: 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 EHNV.
The OIE Reference Laboratory can supply purified EHNV DNA, heat killed EHNV antigen and polyclonal antibodies against EHNV together with technical methods.
A fee is charged for the reagents to cover the costs of operating the laboratory.

**NB:** FIRST ADOPTED IN 1995 AS EPIZOOTIC HAEMATOPOIETIC NECROSIS; MOST RECENT UPDATES ADOPTED IN 2018.

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CHAPTER 2.3.7.

# INFECTION WITH RED SEA BREAM IRIDOVIRUS

## 1. Scope

Infection with red sea bream iridovirus is considered to be infection with the pathogenic agent red sea bream iridovirus (RSIV) of the genus *Megalocytivirus*, Family *Iridoviridae*.

## 2. Disease information

### 2.1. Agent factors

#### 2.1.1. Aetiological agent

The pathogen is an icosahedral virion 140−200 nm in diameter consisting of a central electron-dense core (120 nm) and an electron translucent zone (Inouye *et al.,* 1992) with a double-stranded DNA genome of approximately 110 kbp ~~in length~~ (Kawato *et al.,* 2017a). The viral genome has a G+C ~~V~~ content of 53–55%, containing about 120 potential open reading frames (ORFs).

Phylogenetic analyses using major capsid protein (MCP) and ATPase genes shows that the viruses causing the similar clinical signs can be divided into three different genotypes: RSIV, infectious spleen and kidney necrosis virus (ISKNV) (He *et al.,* 2000; 2001), and turbot reddish body iridovirus (TRBIV) genotype (Shi *et al.,* 2004; 2010).

The aetiological agent of infection with RSIV is RSIV (Inouye *et al.,* 1992; Jeong *et al.,* 2003) and other strains belonging in the RSIV genotype (Go *et al.,* 2016; Koda *et al.,* 2018; Kurita & Nakajima, 2012). Similar diseases with the characteristic, enlarged basophilic cells within infected organs, typical of infections with megalocytiviruses, classified into the ISKNV genotype and TRBIV genotypes are excluded from this chapter. Scale drop disease virus is another virus in the genus *Megalocytivirus* causing different clinical signs in Asian seabass, *Lates calcarifer* (Groof *et al.,* 2015). RSIV genotypes are differentiated from ISKNV and TRBIV genotypes based on nucleotide sequence analysis which is required for confirmatory diagnosis. ~~Scale drop disease virus is another virus in the genus *Megalocytivirus* causing different clinical signs in Asian seabass, *Lates calcarifer* (Groof *et al.,* 2015)~~.

RSIV was first found in red sea bream, *Pagrus major,* from which the virus name (RSIV) is derived (Inouye *et al.,* 1992). As RSIV has a broad host range as shown in Section 2.2.1. *Susceptible host species*, many viruses that can be classified into the RSIV genotype are synonyms of RSIV and defined to be the aetiological agents in this chapter, e.g. rock bream iridovirus (RBIV) (Do *et al.,* 2004; Jung & Oh 2000), Taiwan grouper iridovirus (TGIV) (Chou *et al.,* 1998), large yellow croaker iridovirus (LYCIV) (Chen *et al.,* 2003), orange-spotted grouper iridovirus (OSGIV) (Lu *et al.,* 2005), spotted knifejaw iridovirus (SKIV) (Dong *et al.,* 2010), ~~and~~ giant seaperch iridovirus (GSIV) (Wen & Hong, 2016) and pompano iridovirus (PIV) (Lopez-Porras *et al.,* 2018).

#### 2.1.2. Survival and stability inside the host tissues

Unknown

#### 2.1.3. Survival and stability outside the host

Unknown

For inactivation methods, see Section 2.4.5.

### 2.2. Host factors

#### 2.2.1. Susceptible host species

~~In the case of infection RSIV:~~ Species that fulfil the criteria for listing as susceptible to infection with RSIV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are:

| Family | Scientific name | Common name |
|---|---|---|
| Carangidae | *Pseudocaranx dentex* | striped jack |
| | *Seriola dumerili* | greater amberjack |
| | *Seriola lalandi* | yellowtail amberjack |
| | *Seriola lalandi* × *Seriola quinqueradiata* | hybrid of yellowtail amberjack and Japanese amberjack |
| | *Seriola quinqueradiata* | Japanese amberjack |
| | *Trachinotus blochii* | snubnose pompano |
| | *Trachurus japonicus* | Japanese jack mackerel |
| Centrarchidae | *Micropterus salmoides* | largemouth bass |
| Centropomidae | *Lates calcarifer* | barramundi or sea bass |
| Haemulidae | *Parapristipoma trilineatum* | chicken grunt |
| | *Plectorhinchus cinctus* | crescent sweetlips |
| | ~~*Trachurus japonicus*~~ | ~~Japanese jack mackerel~~ |
| Kyphosidae | *Girella punctata* | largescale blackfish |
| Lateolabracidae | *Lateolabrax* ~~*japonicas*~~ *japonicus* | Japanese sea perch |
| | *Lateolabrax* sp. | |
| Lethrinidae | *Lethrinus haematopterus* | Chinese emperor |
| | *Lethrinus nebulosus* | spangled emperor |
| Moronidae | *Morone saxatilis* × *M. chrysops* | hybrid of striped sea bass and white bass |
| Oplegnathidae | *Oplegnathus fasciatus* | Japanese parrotfish |
| Paralichthyidae | *Paralichthys olivaceus* | bastard halibut |
| Pleuronectidae | *Verasper variegatus* | spotted halibut |
| Rachycentridae | *Rachycentron canadum* | cobia |
| Sciaenidae | *Pseudosciaena crocea* | croceine croaker |
| Scombridae | *Scomber japonicus* | chub mackerel |
| | *Scomberomorus niphonius* | Japanese Spanish mackerel |
| | *Thunnus thynnus* | northern bluefin tuna |
| Sebastidae | *Sebastes schlegeli* | rockfish |
| Serranidae | *Epinephelus akaara* | Hong Kong grouper |
| | *Epinephelus awoara* | yellow grouper |
| | *Epinephelus bruneus* | longtooth grouper |
| | *Epinephelus coioides* | orange-spotted grouper |
| | *Epinephelus fuscoguttatus* | brown-marbled grouper |
| | *Epinephelus lanceolatus* | giant grouper |
| | *Epinephelus malabaricus* | Malabar grouper |
| | *Epinephelus septemfasciatus* | convict grouper |
| | *Epinephelus tauvina* | greasy grouper |
| | *Oplegnathus punctatus* | spotted knifejaw |
| Sparidae | *Acanthopagrus latus* | yellowfin sea bream |
| | *Acanthopagrus schlegeli* | black porgy |
| | *Evynnis japonica* | crimson sea bream |
| | *Pagrus major* | red sea bream |
| Tetraodontidae | *Takifugu rubripes* | torafugu |

#### 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 (RSIV) according to Chapter 1.5 of the *Aquatic Code* are: Under study.

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

Juvenile through to adult stages are susceptible; however, the susceptibility of juveniles is generally higher than adults. Fish belonging to the genus *Oplegnathus* may be more susceptible than others.

#### 2.2.4. Distribution of the pathogen in the host

Infected cells are observed in the spleen, kidney, heart, liver, intestine ~~and~~ gill and other organs.

#### 2.2.5. Aquatic animal reservoirs of infection

Unknown

#### 2.2.6. Vectors

Unknown

### 2.3. Disease pattern

#### 2.3.1. Mortality, morbidity and prevalence

Depending on host fish species, fish size, fish age, water temperature, and other culture conditions, mortality rates range between 0% and 100%. Morbidity is unknown.

#### 2.3.2. Clinical signs, including behavioural changes

Affected fish become lethargic and show abnormal and conspicuous respiratory movements.

#### 2.3.3. Gross pathology

Fish exhibit severe anaemia, petechiae in the gills, and enlargement of the spleen and kidney.

#### 2.3.4. Modes of transmission and life cycle

The principal mode of transmission of RSIV is horizontal via the water. Vertical transmission of RSIV has not yet been investigated.

#### 2.3.5. Environmental factors

Outbreaks have been seen mostly in the summer season at water temperatures of 25°C and above.

#### 2.3.6. Geographical distribution

The first outbreak was recorded in marine cultured red sea bream in ~~Japan~~ Asia in 1990. From then on, further outbreaks and infections have been reported in many marine fish and freshwater fish in many countries. ~~The international trade of ornamental fish has contributed significantly to the spread of megalocytiviruses (Johan & Zainathan, 2020).~~

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

### 2.4. Biosecurity and disease control strategies

#### 2.4.1. Vaccination

Effectiveness of a vaccine consisting of formalin-inactivated supernatant from RSIV-infected GF cell culture has been confirmed experimentally and in field trials (Nakajima *et al.,* 1997; 1999). Currently, the formalin-inactivated vaccine for infection with RSIV is commercially available for red sea bream (*Pagrus major*), striped jack (*Pseudocaranx dentex*), Malabar grouper (*Epinephelus malabaricus*), orange-spotted grouper (*Epinephelus coioides*) and other fish species belonging to the genus *Seriola* in Japan. Protection of fish belonging to the genus *Oplegnathus* by vaccination is difficult.

#### 2.4.2. Chemotherapy including blocking agents

Not available.

#### 2.4.3. Immunostimulation

Not applicable.

#### 2.4.4. Breeding resistant strains

An RSIV-resistant strain of red sea bream (*Pagrus major*) has been developed using marker-assisted selection combined with DNA-based family selection (Sawayama *et al.*, 2019).

#### 2.4.5. Inactivation methods

RSIV is inactivated at 56°C for 30 minutes and by treatment with either ether, chloroform or formalin (0.1%), and by exposure to pH 3.0. The virus is stable in tissue at –80°C and at pH 7.0 and pH 11.0 (Nakajima & Sorimachi, 1994).

#### 2.4.6. Disinfection of eggs and larvae

Unknown

#### 2.4.7. General husbandry

Not available.

## 3. Specimen selection, sample collection, transportation and handling

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

### 3.1. Selection of populations and individual specimens

Clinical inspections should be carried out during a period when water temperature is conducive to development of clinical disease (see Section 2.3.5). All production units (ponds, tanks, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. For the purposes of disease surveillance, fish to be sampled are selected as follows:

i) The most susceptible species should be sampled preferentially (see Section 2.2.3). Other susceptible species listed in Section 2.2.1 should be sampled proportionally.

ii) Risk-based criteria should be employed to preferentially sample lots or populations with a history of abnormal mortality, potential exposure events or where there is evidence of poor water quality or husbandry. If more than one water source is used for fish production, fish from all water sources should be included in the sample.

iii) If weak, abnormally behaving or freshly dead fish are present, such fish should be selected. If such fish are not present (e.g. during surveillance of apparently healthy populations), the fish selected should include normal appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample. Smaller fish may be more appropriate because infection with RSIV can cause higher mortality in juvenile or yearling fish. However, adult fish are also susceptible to RSIV infection as the viral genome has been detected from apparently healthy broodstock. Infection with RSIV has not been reported in hatchery fish.

For disease outbreak investigations, moribund fish or fish exhibiting clinical signs of infection with RSIV should be collected. Ideally fish should be collected while alive, however recently dead fish can also be selected for diagnostic testing. It should be noted, however, that there will be a significant risk of contamination with environmental bacteria if the animals have been dead for some time.

### 3.2. Selection of organs or tissues

Although gill and visceral organs such as spleen, heart, kidney, liver and intestine can be used, it is recommended to sample spleen or kidney tissues; spleen is the most appropriate organ for the preparation of imprints for use in the IFAT. For surveillance of apparently healthy populations, spleen or kidney should be sampled.

## 3.3. Samples or tissues not suitable for pathogen detection

Fish carcasses showing advanced signs of tissue decomposition are not suitable for testing by any method.

Use of inappropriate fixatives (where required), poor sample quality, inappropriate tissues and lack of information provided with the submission may render samples unsuitable for testing.

## 3.4. Non-lethal sampling

Not available.

## 3.5. Preservation of samples for submission

Store fish samples at 4°C for use within 24 hours (or at –80°C for longer periods [up to a few years] for the purposes of molecular detection methods).

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0.

### 3.5.1. Samples for pathogen isolation

The success of pathogen isolation depends strongly on the quality of samples (which will be affected by time since collection and time in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. To avoid degradation of samples, use alternate storage methods only after consultation with the receiving laboratory.

### 3.5.2. Preservation of samples for molecular detection

~~Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.~~ Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.2.5 of Chapter 2.3.0. *General information* (*diseases of fish*)

### 3.5.3. Fixed samples for histopathology, immunohistochemistry or *in-situ* hybridisation

~~Tissue samples for histopathology should be fixed immediately after collection. The recommended ratio of fixative to tissue is 10:1.~~ Standard sample collection, preservation and processing methods for histological techniques can be found in Section 2.2 of Chapter 2.3.0 *General information* (*diseases of fish*).

### 3.5.4. Samples for other tests

Not applicable.

## 3.6. Pooling of samples

Pooling of samples from more than one individual animal for a given purpose should only be recommended where robust supporting data on diagnostic sensitivity and diagnostic specificity have been evaluated and found to be suitable. If the effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, larger fish should be processed and tested individually. Small life stages such as fry or specimens can be pooled to provide the minimum amount of material needed for testing. If pooling is used, it is recommended to pool organ pieces from a maximum of five fish.

# 4. Diagnostic methods

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

~~The designations used in the Table indicate:~~

**Ratings ~~against~~ for purposes of use.** For each recommended assay a qualitative rating ~~against~~ for the purpose of use is provided. The ratings are determined based on multiple performance and operational factors relevant to

application of an assay for a defined purpose. These factors include appropriate diagnostic performance characteristics, level of assay validation, ~~successful application by diagnostic laboratories,~~ availability cost, timeliness, and sample throughput and operability. For a specific purpose of use, assays are rated as:

~~Key:~~

| | |
|---|---|
| +++ = | ~~Most suitable~~ Methods ~~—~~ are most suitable with desirable performance and operational characteristics. |
| ++ = | ~~Suitable~~ Method~~(s)~~ are suitable with acceptable performance and operational characteristics under most circumstances. |
| + = | ~~Less suitable~~ Methods ~~—~~ are suitable, but performance or operational characteristics may ~~significantly~~ limit application under some circumstances. |
| Shaded boxes = | Not appropriate for this purpose. |

~~The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.~~

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

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

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

| Method | A. Surveillance of apparently healthy animals | | | | B. Presumptive diagnosis of clinically affected animals | | | | C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV | Early life stages[2] | Juveniles[2] | Adults | LV |
| **Tissue imprints** | + | + | + | 1 | + | + | + | 1 | | | | |
| **Histopathology** | + | + | + | 1 | ++ | ++ | ++ | 1 | ++ | ++ | ++ | 1 |
| **IFAT ~~or ICC~~** | + | + | + | 1 | ++ | ++ | ++ | 1 | ++ | ++ | ++ | 1 |
| **Cytopathology** | | | | | | | | | | | | |
| **Cell culture** | + | + | + | 1 | ++ | ++ | ++ | 1 | ++ | ++ | ++ | 1 |
| **Real-time PCR** | ++ | ++ | ++ | 2 | ++ | ++ | ++ | 2 | ++ | ++ | ++ | 2 |
| **Conventional PCR[3]** | ++ | ++ | ++ | 2 | ++ | ++ | ++ | 2 | +++ | +++ | +++ | 2 |
| **Amplicon sequencing** | | | | | | | | | +++ | +++ | +++ | NA |
| ***In-situ* hybridisation** | | | | | | | | | | | | |
| **Bioassay** | | | | | | | | | | | | |
| **LAMP** | | | | | | | | | | | | |
| **Ab-ELISA** | | | | | | | | | | | | |
| **Ag-ELISA** | | | | | | | | | | | | |
| **Other antigen detection methods[4]** | | | | | | | | | | | | |
| **Other serological method[4]** | | | | | | | | | | | | |

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2), NA = not available; IFAT = Indirect fluorescent antibody test. ICC = Immunocytochemistry PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively;
[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.
[3]Conventional PCR alone does not meet the case definition of a confirmed case but must be followed by amplicon sequencing. [4]Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

## 4.1. Tissue imprints

Samples to be tested include tissue imprints of spleen from affected fish. Use a tissue imprint of spleen from known uninfected fish as a negative control and if possible, use a tissue imprint of spleen from confirmed RSIV-infected fish as a positive control.

i) Bleed the fish thoroughly.

ii) Make spleen imprints on cleaned glass microscope slides.

iii) Store the spleen pieces at 4°C together with the other organs that may be required for virus isolation or PCR tests later.

iv) Allow the imprints to air-dry for 20 minutes.

v) Fix the imprints with cold acetone.

vi) Stain with Giemsa or Diff-Quik.

vii) Mount the microscope slides with cover-slips using a drop of mounting fluid.

viii) Examine under light microscopy using ×40–100 magnification.

A presumptive positive result is indicated by the presence of abnormally enlarged cells. Negative control slides should not exhibit any abnormally enlarged cells. If enlarged cells are observed in the test samples, identification procedures PCR followed by amplicon sequencing must be undertaken immediately.

## 4.2. Histopathology and cytopathology

Examination of histological sections from diseased fish may reveal abnormally enlarged cells from the spleen, heart, kidney, liver, intestine or gill. These enlarged cells react to anti-RSIV MAb M10 (4.9.1.) using an immunohistochemistry test (Bermudez *et al.,* 2018). However, this method is not ~~yet fully~~ validated.

## 4.3. Cell culture for isolation

Cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

Isolation of RSIV (and ISKNV) is undertaken using the Grunt fin (GF) cell line[9] or SKF-9 cell line (Kawato *et al.,* 2017b)~~; isolation of the viruses from freshwater fish such as gourami is difficult~~. Spleen and/or kidney tissues from diseased fish are suitable samples. Cells should be grown in Eagle's basal medium (BME) for GF cell line and Hank's minimum essential medium (HMEM) for SKF-9 cell line, supplemented with 10% fetal bovine serum (FBS) at 25°C in a temperature-controlled incubator. A virus isolate to be used as positive control can be obtained from the OIE Reference Laboratory for RSIV. Use uninfected cells as negative control. Following development of viral cytopathic effect (CPE), virus identification would be undertaken using conventional PCR and sequencing. SKF-9 cell line can be obtained from the OIE Reference Laboratory for RSIV.

### 4.3.1. Virus isolation in cell cultures

#### 4.3.1.1. Inoculation of cell monolayers

i) Cell cultures (GF or SKF-9) maintained at 25°C and passaged at 7–14 day intervals should be used for virus isolation to ensure virus susceptibility. Prepare cell monolayers in 25 $cm^2$ flask, 6-well, 24-well, or 96-well plates according to the purpose and sample size on the day before sample inoculation.

ii) Following the virus isolation procedure described in Chapter 2.3.0 *General information* (on diseases of fish), Section A.2.3.2, make an additional tenfold dilution of the 1/10 spleen homogenate supernatants and transfer an appropriate volume of each of the two dilutions onto the cell monolayers. To avoid cytotoxic effect (CTE), final concentration of the organ in the cultured medium should be less than 1% w/v.

9 European Collection of Authenticated Cell Cultures (ECACC) Catalogue No. 88010601; www.phe-culturecollections.org.uk/products/celllines/generalcell/detail.jsp?refId=88010601&collection=ecacc_gc

iii) Without withdrawing the inoculum, incubate at 25°C.

#### 4.3.1.2. Monitoring incubation

i) Follow the course of infection in positive controls and other inoculated cell cultures by daily microscopic examination at ×40–100 magnification for 10 days. The use of a phase-contrast microscope is recommended.

ii) If CPE appears in those cell cultures inoculated with dilutions of the test homogenates, identification procedures by PCR followed by amplicon sequence analysis must be undertaken immediately.

iii) If no CPE develops in the inoculated cultures (despite normal progression of CPE in the positive controls cultures) after 10 days incubation, the inoculated cultures should be subcultured and incubated for a further 7 days. Should the virus control fail to develop CPE, the process should be repeated with fresh susceptible cells and new batches of samples.

#### 4.3.1.3. Subcultivation procedure

i) Collect aliquots of cell culture medium from all monolayers inoculated with dilutions of each supernatant of the test homogenates.

ii) Inoculate cell monolayers as described above (Section 4.3.1.1 Inoculation of cell monolayers, steps i and ii).

iii) Incubate and monitor as described above (Section 4.3.1.1 Inoculation of cell monolayers, steps ii and iii and Section 4.3.1.2 Monitoring incubation steps i and ii).

If no CPE occurs, the test may be declared negative.

## 4.4. Nucleic acid amplification

See Chapter 2.3.0 *General information* (on diseases of fish), Section B.2.5 for information on the use of molecular techniques for virus identification. Both real-time PCR and conventional PCR tests are available for RSIV identification. Samples to be tested include spleen from affected fish or supernatants from cell cultures that have developed CPE. The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control. Use extracted DNA from the spleen and kidney of uninfected fish or extracted DNA from the supernatant of an uninfected cell culture as the negative control. Use extracted DNA from the spleen of confirmed RSIV-infected fish or extracted DNA from the supernatant of an infected cell culture or Viral DNA or plasmid in which target sequence is inserted as the positive control. Select controls depending on the kinds of samples to be tested.

Tissue samples can be homogenised by manual pestle grinding or by bead-beating. Commercially available nucleic acid extraction kits may be used to extract DNA directly from tissues, from tissue homogenates and cell culture supernatants according to the manufacturer's instructions. A negative extraction control, consisting of extraction reagents only, is included when test samples are extracted. Use a pre-confirmed RSIV-affected organ or supernatant from RSIV-infected cell cultures as positive controls. Use organs from healthy fish or supernatants from non-infected cell cultures as negative controls

N.B. Viral DNA or a plasmid in which the PCR target sequence is inserted that can be used as the positive control can be obtained from the OIE Reference Laboratory for RSIV.

### 4.4.1. Real-time PCR

Several real-time PCR assays available for detection of RSIV have been evaluated (Kawato *et al*., 2021). Two probe-based real-time PCR assays, designated as the Mohr et al. assay (Mohr *et al*., 2015) and the Cummins assay, were deemed equivalent to each other and superior to the other tests evaluated in this study. The primer sets and probes of each of these two assays are designed to detect a major capsid protein (MCP) gene sequence and are as follows:

i) Mohr real-time PCR

RSIV RT F: 5'-TGA-CCA-GCG-AGT-TCC-TTG-ACT-T-3'

RSIV RT R: 5'-CAT-AGT-CTG-ACC-GTT-GGT-GAT-ACC-3'

RSIV Probe: 5'-FAM-AAC-GCC-TGC-ATG-ATG-CCT-GGC-TAMRA-3'

ii) Cummins real-time PCR

AFDL Megalo F: 5'-GGC-GAC-TAC-CTC-ATT-AAT-GTG-3'

AFDL Megalo R: 5'-CAC-CAG-GTC-GTT-AAA-TGA-CA-3'

AFDL Megalo Pr: 5'-FAM-CTG-CGT-GTT-AAG-ATC-CCC-TCC-A-TAMRA-3'

The protocol in use at the OIE Reference Laboratory for RSIV is as follows: Template (2 µl) is added to 23 µl reaction mixture containing 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM for each primer, 250 nM for probe, and molecular grade water. After 1 cycle of 50°C for 2 minutes and 95°C for 10 minutes, amplification consists of 45 cycles of 95°C for 15 seconds, 60°C for 60 seconds.

The detection sensitivity limits of both real-time PCRs are approximately 1-10 copies/µl template DNA which is higher than that of conventional PCR. However, since the real-time PCRs have cross-reactivity to the ISKNV and TRBIV genotypes, conventional PCR followed by amplicon sequence analysis (see Section 4.5.) is required for confirmatory diagnosis.

### 4.4.2. Conventional PCR

The conventional PCR primer set consisting of the forward primer 1-F (5'-CTC-AAA-CAC-TCT-GGC-TCA-TC-3') and reverse primer 1-R (5'-GCA-CCA-ACA-CAT-CTC-CTA-TC-3') is used for amplification of a 570 base region of the genome sequence across two ORFs (Kurita *et al.,* 1998). Primer set 4-F (5'-CGG-GGG-CAA-TGA-CGA-CTA-CA-3') and 4-R (5'-CCG-CCT-GTG-CCT-TTT-CTG-GA-3') also has adequate sensitivity for RSIV, and generates an amplicon of 568 bases, but it cannot be used to amplify ISKNV DNA (Kurita et al, 1998). The reactivity of these primer sets against TRBIV has not yet been confirmed

The protocol in use at the OIE Reference Laboratory for RSIV, based on Kurita *et al.*, (1998), is as follows: Template (1 µl) is added to 19 µl reaction mixture containing 2 µl 10× reaction buffer, 1.6 µl dNTP mixture (2.5 mM each), 0.2 µl *TaKaRa ExTaq* HS (5 U/µl) (TaKaRa), 1 µM each primer, and molecular grade water. After 1 cycle of 94°C for 2 minutes, PCR amplification consists of 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by final extension at 72°C for 2 minutes. Amplified DNA (567 or 570 bp) is analysed by agarose gel electrophoresis using a 1.5% agarose/TAE (Tris-acetate-EDTA) gel containing SYBR™ Safe (Thermo Fisher Scientific) or equivalent.

The detection sensitivity limit of the 1-F/1-R PCR is approximately 10-100 copies/µl template DNA. However, the primer set 1-F and 1-R is confirmed to amplify both RSIV and ISKNV DNA, and hence, amplicon sequencing is required for confirmatory diagnosis. The cross reactivity of these primer sets against TRBIV has not yet been validated.

### 4.4.3. Other nucleic acid amplification methods

Not applicable

## 4.5. Amplicon sequencing ~~of the amplicon~~

The primer set 1-F and 1-R can amplify both RSIV and ISKNV DNA and sequencing of the amplicon is required for virus identification. Amplicons should be ~~gel-~~purified and sequenced using both the forward and reverse primer. Consensus sequence, generated after analysis of the quality of the sequence chromatograms, can then be compared to reference sequences, for example by BlastN search of the NCBI database.

## 4.6. *In-situ* hybridisation

Not applicable

## 4.7. Immunohistochemistry

Not applicable

## 4.8. Bioassay

Not applicable

## 4.9. Antibody-based or antigen detection methods

### 4.9.1. Antibody-based antigen detection methods: indirect fluorescent antibody test (IFAT) ~~or immunocytochemistry (ICC)~~

Reagent and protocols for detecting RSIV proteins with a monoclonal antibody (MAb) M10 have been published (Kawato *et al.*, 2017b; 2020; Nakajima & Sorimachi, 1995). The MAb M10-reactive epitope has been demonstrated to be a 7 amino acid sequence (EYDCPEY) of a non-structural protein encoded by the laminin-type epidermal growth factor-like domain gene in RSIV and ISKNV (Takano *et al.,* 2019). MAb M10 detects both RSIV- and ISKNV-infected cells (Kawato *et al.*, 2020) but it does not detect ranaviruses (Nakajima & Sorimachi, 1995). The reactivity of MAb against TRBIV has not yet been confirmed. MAb M10 can be obtained from the OIE Reference Laboratory for RSIV.

Samples to be tested include tissue imprints of spleen from affected fish. Use a tissue imprint of spleen from uninfected fish as a negative control and if possible, use a tissue imprint of spleen from confirmed RSIV-infected fish as a positive control. Similarly, IFAT can be conducted directly after virus isolation in cell culture. Samples to be taken for testing include acetone-fixed infected cell monolayers that have developed CPE. Use an uninfected cell monolayer as a negative control and if possible, use a confirmed RSIV-infected cell monolayer as a positive control. The protocol for tissue imprints is as follows and can be adapted for IFAT on cell cultures.

i) Bleed the fish thoroughly.

ii) Make spleen imprints on cleaned glass microscope slides.

iii) Store the spleen pieces at 4°C together with the other organs that may be required for virus isolation.

iv) Allow the imprints to air-dry for 20 minutes.

v) Fix the imprints with cold acetone.

vi) Prepare a diluted solution of MAb M10 in PBS (1/100)

vii) Treat the imprints with the MAb M10 solution for 30 minutes at 37°C in a humid chamber.

viii) Rinse three times with PBS.

ix) Incubate the imprints for 30 minutes at 37°C in a humid chamber with a solution of a specific anti-mouse FITC-conjugated antibody prepared according to the supplier's instructions.

x) Rinse three times with PBS.

xi) Mount the microscope slides with cover-slips using glycerol saline prior to microscopic observation.

xii) Examine using a fluorescence microscope. Positive and negative controls must be found to give the expected results prior to any other observation.

A positive result is indicated by the presence of abnormally enlarged cells with strong fluorescence. Negative control slides should not exhibit any strong fluorescence.

If the test is positive, the fish from which the samples were obtained is considered infected with RSIV or ISKNV.

Alternatively, a peroxide-conjugated second antibody could be used rather than fluorescence conjugate.

## 4.10. Other methods

RSIV cannot be identified by neutralisation tests as the antisera generated by the immunisation of rabbits have few neutralising antibodies.

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

As indicated in Table 4.1, real-time PCR is the most appropriate method of screening healthy fish populations for RSIV; however, the available methods are not specific for RSIV. Any real-time positive samples should be tested by conventional PCR followed by amplicon sequence analysis to distinguish megalocytiviruses.

## 6. Corroborative diagnostic criteria

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

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE reference laboratory.

### 6.1. Apparently healthy animals or animals of unknown health status[10]

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

#### 6.1.1. Definition of suspect case in apparently healthy animals

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

i) Positive result by real-time or conventional PCR

ii) ~~Cyto- or~~ Histopathological changes consistent with infection with RSIV ~~infection or disease~~

iii) Cytopathic effect in cell culture

iv) Positive result from IFAT ~~or ICC~~

#### 6.1.2. Definition of confirmed case in apparently healthy animals

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

i) Positive result by a recommended molecular or antigen detection test with confirmation by conventional PCR and sequence analysis, with sequence consistent with RSIV

ii) ~~Cyto- or~~ Histopathological changes consistent with the presence of infection with RSIV ~~the pathogen or the disease with confirmation by~~ and conventional PCR and sequence analysis, with sequence consistent with RSIV

iii) Cytopathic effect in cell culture with ~~confirmation~~ identification of RSIV by conventional PCR and sequence analysis~~, with sequence consistent with RSIV~~

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods. Positive test results will result in notification to the OIE.

### 6.2 Clinically affected animals

10 For example transboundary commodities.

Clinical signs (see Section 2.3.2) are not pathognomonic for a single disease; however they may narrow the range of possible diagnoses.

### 6.2.1. Definition of suspect case in clinically affected animals

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

i) Presence of gross pathology or clinical signs associated with infection with RSIV ~~disease~~ as described in this chapter, with or without elevated mortality

ii) ~~Cyto- or~~ Histopathological changes consistent with the presence of ~~the pathogen or the disease~~ infection with RSIV

iii) Positive result from IFAT ~~or ICC~~

iv) Cytopathic effect typical for RSIV infection in cell culture

v) Positive result by real-time PCR or conventional PCR

### 6.2.2. Definition of confirmed case in clinically affected animals

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

i) Positive result by a recommended antigen detection test ~~with confirmation by~~ and a positive result by conventional PCR ~~and sequence analysis,~~ with sequence consistent with RSIV

ii) Cyto- or histopathological changes consistent with ~~the presence of the pathogen or the disease with~~infection with RSIV and ~~confirmation~~ a positive result by conventional PCR ~~and sequence analysis,~~ with sequence consistent with RSIV

iii) Cytopathic effect in cell culture with ~~confirmation~~ identification of RSIV by conventional PCR ~~and sequence analysis,~~ with sequence consistent with RSIV

iv) Positive result by real-time PCR ~~test~~ and positive result by ~~with confirmation by~~ conventional PCR ~~and sequence analysis,~~ with sequence consistent with RSIV

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods. Positive test results will result in notification to the OIE.

## 6.3. Diagnostic sensitivity and specificity for diagnostic tests [under study]

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

### 6.3.1. For presumptive diagnosis of clinically affected animals

| Test type | | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |

DSe: = diagnostic sensitivity, DSp = diagnostic specificity.

### 6.3.2. For surveillance of apparently healthy animals

| Test type | Test purpose | Source populations | Tissue or sample types | Species | DSe (*n*) | DSp (*n*) | Reference test | Citation |
|---|---|---|---|---|---|---|---|---|

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

DSe: = diagnostic sensitivity, DSp = diagnostic specificity.

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**NB:** There is an OIE Reference Laboratory for red sea bream iridoviral disease (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: 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 red sea bream iridoviral disease.

**NB:** FIRST ADOPTED IN 2000); MOST RECENT UPDATES ADOPTED IN 20XX.

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# THE USE OF ENVIRONMENTAL DNA METHODS FOR DETECTION OF OIE LISTED AQUATIC ANIMAL DISEASES

A discussion paper developed by the OIE Aquatic Animal Health Standards Commission (Aquatic Animals Commission) for Member comments.

Version: February 2022

## 1. Summary

The monitoring of aquatic systems using environmental DNA (eDNA) is a rapidly advancing research field that will provide opportunities for cost-effective, non-destructive methods to screen for pathogenic agents, including those of wild aquatic populations where samples may be difficult or undesirable to obtain.

The Aquatic Animals Commission is aware that eDNA methods are being applied for detecting the causative agents of several OIE listed diseases. As these methods are available and currently in use, the Commission has agreed that it would be advisable for guidance to be provided on appropriate application of eDNA methods and potential limitations.

The Commission notes that, as accurate estimates of diagnostic performance are not available for designing surveillance programmes using eDNA assays, data obtained from eDNA methods are not suitable to support declarations of freedom from listed diseases. Confirmation of infection with listed diseases could also not be made using eDNA methods because a positive result does not demonstrate that a susceptible host animal(s) is infected.

Positive eDNA results could, however, provide evidence amounting to suspicion of infection such as the presence of the pathogen DNA/RNA in the sample. This application of eDNA methods may be particularly useful for the monitoring of high-value or rare animals as an alternative to collection of tissue samples. It has a potential role in early detection of disease incursion in wild populations or under circumstances when infection is not likely to result in observable clinical signs. However, following suspicion, based on positive eDNA, samples obtained directly from aquatic animals need to be tested as described in the relevant disease-specific chapters of the *Manual of Diagnostic Tests for Aquatic Animals* (*Aquatic Manual*) to confirm or exclude the case.

The application of eDNA methods for a given purpose should be considered carefully. Methods should be chosen with consideration given to all relevant factors including the surveillance objective, the target pathogen, the reliability of the method, and the environment to be sampled. It is important that the implications of positive results be considered in advance of applying an eDNA method as any positive results may require that surveys involving direct sampling and testing of susceptible animal be conducted to confirm or exclude a suspect case. eDNA methods will not be an appropriate choice for many aquatic animal disease surveillance purposes.

This document is intended to explore the potential use of eDNA methods with respect to the standards of the OIE *Aquatic Animal Health Code* (*Aquatic Code*) and *Aquatic Manual* and to outline benefits and limitations.

The use of an eDNA method for the detection of *Gyrodactylus salaris* has been included in *Aquatic Manual* Chapter 2.3.3 Infection with *Gyrodactylus salaris*[11]. The inclusion of this method conforms with the conclusions of this discussion paper.

## 2. Definitions for eDNA

Numerous definitions for eDNA exist (e.g. Bass *et al*., 2015; Diaz-Ferguson & Moyer, 2014; Thomsen & Willerslev, 2015). Most definitions regard eDNA as detectable short DNA/RNA fragments from a living

11 https://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/2.3.03_G_salaris.pdf

organism derived from cellular components or fluids secreted into the abiotic components of surrounding environment (i.e. water, air, sediments).

For the purposes of this document, we define eDNA as: "nucleic acids of pathogenic agents extracted from 'true' environmental samples (such as water, soil, sediment, biofilm)". Direct host-derived material such as faeces, sloughed cells and mucous, are excluded from this definition. Once extracted from the environmental sample, target eDNA fragments can be detected using a variety of molecular methods (Diaz-Ferguson & Moyer, 2014). Furthermore, eDNA can be sequenced directly as metagenetic libraries or after PCR amplification of specific target gene regions (Bass *et al*., 2015).

The actual performance of eDNA based detection depends on the sample collection and processing methodology (e.g. volume filtered, presence and removal of PCR inhibitors), biological processes (e.g. rates of shedding, temporal variation) and abiotic factors (analyte degradation, hydrodynamic factors). It is important to evaluate these factors empirically so that the results can be properly interpreted. It is only with a clear understanding of how these factors influence the probability of pathogenic agent detection that eDNA-based detection can be used reliably in a variety of settings (Brunner, 2020).

## 3. Objectives

This paper considers i) the benefits and ii) limitations of eDNA pathogenic agent detection methods, iii) validation of eDNA methods, iv) the conditions for inclusion of an eDNA method in a disease-specific chapter of the *Aquatic Manual* and v) use of eDNA evidence as diagnostic criteria.

## 4. Review of published eDNA methods for the detection of aquatic animal pathogenic agents

A literature review was undertaken to assess the application of eDNA methods for the detection and study of pathogens and parasites of aquatic animals. Thirty-three publications reporting the use of eDNA to detect thirteen OIE listed pathogenic agents were identified (see Appendix 1, Table 1 for details). Methods have been developed for the detection of the causative agents of OIE listed pathogenic agents of amphibians, crustaceans, fish and molluscs. The majority of publications concern the detection of the listed pathogenic agents in wild aquatic animal populations, notably infection with *Aphanomyces astaci*, infection with *Batrachochytrium dendrobatidis*, infection with *B. salamandrivorans*, infection with *Ranavirus* species, infection with *G. salaris*.

A further thirteen publications were found that targeted other specific pathogenic agents (e.g. *Mikrocytos mackini*), groups of pathogenic agents (e.g. of ornamental fish) or applied eDNA methods to broader areas of study (e.g. water-borne transmission of viruses) (see Appendix 1, Table 2 for details).

## 5. Benefits of eDNA methods for the detection of aquatic animal pathogenic agents

eDNA detection is a promising tool that can be used to complement direct sampling of aquatic animals for surveillance. eDNA methods offer some benefits compared to direct sampling and testing of aquatic animals, including, but not limited, to the following:

1. eDNA methods do not require intrusive or lethal sampling of aquatic animal hosts. They may be particularly useful for rare or valuable aquatic animals, or difficult to collect wild animals (e.g. Rusch *et al*., 2018).

2. eDNA methods do not require handling of animals, avoiding the stress associated with obtaining non-destructive tissue samples (Brunner, 2020).

3. Sample collection and sample processing time and associated costs may be reduced substantially compared to collection and processing of individual animal samples (Rusch *et al*., 2018).

4. As environmental samples may contain analyte from the entire, or a large percentage of a target captive population, many fewer samples may be required to detect a pathogenic agent (compared to individual animal samples), even when diagnostic sensitivity of the eDNA method is low (Brunner, 2020).

5. The same environmental sample can be analysed for the presence of host species (e.g. see Rusch *et al*., 2018) and multiple pathogens.

6. eDNA methods could be used for assessment of potential introduction pathways where sampling of hosts is not possible (e.g. ballast water).

## 6. Limitations of eDNA methods

Limitations to the application of eDNA based pathogenic agent detection include, but are not limited to, the following:

1. Very little target pathogen DNA/RNA may be available in the environmental sample due to dilution in the environment and degradation of nucleic acids. This may negatively impact the sensitivity of the method (Brunner, 2020).

2. The concentration of target DNA/RNA in an environmental sample will vary due a range of factors such as host density, prevalence and intensity of infection, sampling method (e.g. for water volume sampled, filter pore size, storage conditions) and environmental conditions (e.g. amount of organic matter). Sensitivity of eDNA methods may, therefore, vary more between localities, surveys undertaken at different time points and target taxa than direct sampling and testing of animal tissues (Brunner, 2020).

3. There are formal frameworks to assess diagnostic performance of tests using animal-derived samples, but these have not been developed for eDNA methods. This means that the design of surveys to demonstrate freedom from infection using eDNA methods is problematic.

4. A positive detection of target pathogen DNA/RNA in an environmental sample may be more likely to result from a source of contamination not representative of viable pathogen (e.g. inactivated pathogen from heat treated products) compared with animal-derived samples. Similarly, it may not indicate infection of a host animal with the target pathogenic agent.

## 7. Validation of eDNA methods

There is an increasing likelihood that disease management decisions will be made based on results from eDNA studies. It is thus imperative that data generated by eDNA studies is reliable, defendable and executed with high quality assurance standards (Klymus *et al*., 2019). Empirical validation of eDNA-based pathogen detection should focus on understanding the causes and consequences of variation in test characteristics across sampling conditions and needs to take into consideration a clear understanding of what is being sampled/assayed for in the case of each pathogen of interest.

Chapter 1.1.2. of the *Aquatic Manual* describes the principles and methods of validation of diagnostic assays for infectious diseases. The recommendations of this chapter are intended for diagnostic testing of animal-derived samples; however, the principles and many of the methods are applicable to eDNA methods. It is recommended that the general principles and methods of Chapter 1.1.2. be applied to the validation of eDNA detection methods for OIE listed diseases. It should be noted that the process of sample collection, the concentration of target DNA, the DNA extraction, the sensitivity and other performance (indicators) should be emphasised and validated.

Design and reporting standards are available for diagnostic accuracy studies for methods utilising aquatic animal-derived samples (e.g. Laurin *et al*., 2018). Many of the design and reporting considerations are also applicable to eDNA methods and it is recommended that these standards be applied for eDNA diagnostic accuracy studies.

Additional to the guidance described above, design and reporting considerations have been published specifically for eDNA methods (e.g. Doyle & Uthicke, 2020; Goldberg *et al*., 2016; Klymus *et al*., 2019). Many of these studies report on considerations for detection of macro-organisms rather than pathogenic agents; however, the considerations are generally relevant for eDNA detection methods for pathogenic agents. This guidance will be of particular use for the field collection, processing and preservation of eDNA samples.

## 8. Minimum requirements for inclusion of an eDNA method in the *Aquatic Manual*

It is recognised that the validation pathway described in Chapter 1.1.2. of the *Aquatic Manual* and the design and reporting standards described by Laurin *et al*., 2018 (see above) are not met by many diagnostic methods currently included in the *Aquatic Manual*. Indeed, many assays included in the *Aquatic Manual* may be validated only to level 1 or 2 of the validation pathway described in Chapter 1.1.2. of the *Aquatic Manual*.

For this reason, the Commission proposes that the following minimum reporting requirements be met for an eDNA method to be considered for inclusion in the *Aquatic Manual* [Adapted from Goldberg *et al*., (2016)]:

1. The intended purpose or application of the assay or protocol needs to be clearly defined (note that appropriate purposes of use for eDNA methods in the context of OIE standards are discussed further in section 9).

2. Description of sample collection methods and precautions taken to eliminate contamination, including collection volume, container material, negative controls, number of replicates and sampling locations/depth.

3. Description of the methods used to concentrate the target DNA/RNA (precipitation/filtration), filter type (if applicable) and filtering location (e.g. in the field).

4. Description of sample preservation and storage (method, temperature, duration).

5. Description of the DNA/RNA extraction process including protocol adjustments, contamination precautions, negative controls, and internal positive controls.

6. Description of the molecular detection method and optimisation according to (Bustin *et al*., 2009). Furthermore, assays should be validated (Level 1) in an environmental matrix according to its purpose of use.

## 9. Potential application of eDNA detection methods in the disease-specific chapters of the *Aquatic Manual*

The disease-specific chapters of the *Aquatic Manual* recommend tests to identify suspect cases and to confirm suspicion for apparently healthy (or those of unknown health status) and clinically affected animals. Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographic proximity to, or movement of aquatic animals or aquatic animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate freedom.

The following points describe the suitability of evidence from eDNA detection methods for inclusion as case definition criteria in section 6 of the disease-specific chapters of the *Aquatic Manual.*

### a) Apparently healthy animals

#### i) Definition of suspect case in a population of apparently healthy animals

*Suitable as a criterion*. A positive result obtained from an eDNA method recommended in the *Aquatic Manual* is considered to provide adequate evidence to be included as a criterion for a suspect case when known susceptible species exist in the environment from which the sample was taken.

#### ii) Definition of confirmed case in apparently healthy animals

*Not suitable as a criterion.* A positive result obtained from an eDNA method recommended in the *Aquatic Manual* is not considered to provide appropriate evidence to confirm a case in apparently healthy animals. Methods utilising animal-derived samples are considered more appropriate for criteria to confirm a case. Evidence to confirm a case in apparently healthy animals must meet the requirements of Section 6.1.2. of the relevant disease-specific chapter of the *Aquatic Manual*. eDNA evidence will not be included as a criterion within this section.

**b) Clinically affected animals**

**i) Definition of a suspect case in clinically affected animals**

*Suitable as a criterion.* Taking an environmental sample to investigate the cause of disease in a population of clinically affected animals is not generally recommended as samples from clinically affected animals are more likely to lead to pathogenic agent detection and are more suitable for disease investigation. However, under some circumstances, an eDNA method may detect a pathogenic agent and lead to the recognition of previously unobserved or unassociated clinical signs of disease. In these circumstances, a positive result obtained from an eDNA method recommended in the *Aquatic Manual* is considered to provide adequate evidence to be included as a criterion for a suspect case.

**ii) Definition of confirmed case**

*Not suitable as a criterion.* A positive result from an eDNA method recommended in the *Aquatic Manual* would not be included as a criterion for the confirmation of a pathogenic agent in clinically affected animals (or apparently healthy animals, see point 9.a.ii above). Any positive eDNA test would require further investigation involving the collection and testing of animal tissues as stipulated in the relevant disease-specific chapter of the *Aquatic Manual*. Evidence to confirm a case in clinically affected animals must meet the requirements of Section 6.2.2. of the relevant disease-specific chapter of the *Aquatic Manual*. eDNA evidence will not be included as a criterion within this section.

## 10. Discussion

The key limitations of eDNA is the lack of validation and diagnostic performance data, meaning that negative results cannot be used to demonstrate disease freedom and positive results always require confirmation using animal samples (Brunner, 2020). Nevertheless, there are circumstances where the advantages of environmental, over animal, sampling means that eDNA approaches can be usefully integrated into a surveillance programme.

A country or zone claiming freedom from a specified pathogenic agent(s) are required to have in place an early detection system for disease incursion. Farmer reporting of morbidity and mortality is a key component of an early detection system. Farmed populations can act as sentinels for wild populations only if they are epidemiologically connected (i.e. through shared water). Otherwise, active surveillance in wild populations is required as morbidity or mortality is unlikely to be reported (especially as dead or dying animals are likely to be quickly scavenged or predated). Animal sampling of wild populations can present considerable logistical challenges, especially if populations are remote, sparse or if low numbers make destructive sampling undesirable. eDNA based pathogenic agent detection methods overcome many of the challenges of sampling wild aquatic animals (Kamoroff & Goldberg, 2017; Trebitz *et al*., 2017).

Infection with some listed pathogenic agents, under certain conditions or in some host species, will not invariably cause detectable clinical signs. Early detection systems that rely on observations by farmers (or others) of mortality or morbidity are ineffective in these circumstances and active surveillance would be required. Sampling farmed animals on a frequent basis, and at a level to detect a low prevalence, presents considerable logistical challenges and the cost is likely to be unacceptable. eDNA methods can offer a viable alternative (Trujillo-Gonzalez *et al*., 2019a) for active surveillance for pathogens which may not reliably cause observable clinical signs. They have the additional advantage that the sample will contain analyte from a large percentage, if not the entire, captive population. Thus relatively few environmental, compared with animal samples, are needed (provided sufficient DNA/RNA can be extracted).

## 11. Conclusions

1. eDNA methods may have utility for enhancing passive surveillance systems for early detection; particularly in circumstances where conditions are not conducive to clinical expression of disease, or populations are not under sufficient observation to detect clinical disease should it occur.

2. eDNA methods may have utility for rare, valuable or difficult to collect wild aquatic animals, where direct sampling of animals is undesirable or cost prohibitive. They may also provide cost advantages for disease monitoring programs in production environments.

3. There are currently no frameworks to allow evaluation of diagnostic performance of eDNA methods in a manner similar to animal-derived samples. For this reason, evidence from eDNA detection methods cannot be used as evidence for self-declaration of freedom from disease.

4. eDNA methods will be considered for inclusion in disease-specific chapters of the *Aquatic Manual*, if minimum disease and reporting standards as described in this paper are met.

5. Positive results from an eDNA method that has been included in the *Aquatic Manual* will be considered as an appropriate criterion for a suspect case of a disease.

6. The application of eDNA methods for a given purpose should be considered carefully with respect to the pathogen to be detected, the environment to be sampled, the reliability of the method and the implications of positive results that may require surveys of susceptible animal populations to confirm or exclude a suspect case.

7. Positive results from an eDNA methods that has been included in the *Aquatic Manual* will not be considered as an appropriate criterion for a confirmed case of a disease in either apparently healthy or clinically affected animals.

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**Appendix 1. Publications describing eDNA methods for aquatic animal pathogenic agents**

**Table 1.** Published applications of eDNA methods for the detection of OIE listed pathogenic agents of aquatic animals

| OIE LISTED DISEASE | PUBLICATION |
|---|---|
| **Amphibian diseases** | |
| Infection with *Batrachochytrium dendrobatidis* | Brannelly *et al*., 2020; Julian *et al*., 2019; Kamoroff & Goldberg, 2017; Mosher *et al*., 2017; Pierson & Horner, 2016; Walker *et al*., 2007 |
| Infection with *Batrachochytrium salamandrivorans* | Brunner, 2020; Spitzen-van der Sluijs *et al*., 2020 |
| Infection with Ranavirus species | Hall *et al*., 2016; Julian *et al*., 2019; Miaud *et al*., 2019; Pierson & Horner, 2016; Vilaca *et al*., 2020 |
| **Fish diseases** | |
| Infection with *Gyrodactylus salaris* | Fossoy *et al*., 2020; Rusch *et al*., 2018; |
| Infection with HPR-deleted or HPRO infectious salmon anaemia virus | Gregory *et al*., 2009 |
| Infection with koi herpesvirus | Haramoto *et al*., 2007; Honjo *et al*., 2010; 2012 |
| Infection with salmonid alphavirus | Bernhardt *et al*., 2020; Weli *et al*., 2021 |
| **Crustacean diseases** | |
| Acute hepatopancreatic necrosis disease | Kongrueng *et al*., 2015 |
| Infection with *Aphanomyces astaci* (crayfish plague) | Robinson *et al*., 2018; Rusch *et al*, 2020; Strand *et al*., 2011; 2014; Vralstad *et al*., 2016; Wittwer *et al*., 2018a; 2018b |
| Infection with white spot syndrome virus | Natividad *et al*., 2008; Quang *et al*., 2009 |
| **Mollusc diseases** | |
| Infection with *Bonamia ostreae* | Jorgensen *et al*., 2020 |
| Infection with *Perkinsus marinus* | Audemard *et al*., 2004 |
| Infection with *Xenohaliotis californiensis* | Lafferty & Ben-Horin, 2013 |

**Table 2.** Published eDNA studies of pathogenic agents of aquatic animals not listed by the OIE

| SUBJECT | PUBLICATION |
|---|---|
| Ornamental fish parasite detection | Trujillo-Gonzalez *et al*., 2019b; 2019a |
| Parasitology | Bass *et al*., 2015 |
| Protozoan parasite outbreaks in fish farms | Bastos Gomes *et al*. 2017; 2019 |
| Disease transmission in open water Salmon cages | Salama & Rabe, 2013 |
| Emerging aquatic parasites | Sana *et al*., 2018 |
| Pathogenic microbes in bait | Mahon *et al*., 2018 |
| Waterborne virus detection | Oidtmann *et al*., 2018 |
| *Halioticida noduliformans* in lobsters | Holt *et al*., 2018 |
| *Microcytos mackini* | Polinski *et al*., 2017 |
| Trematode parasite *Ribieroia ondatrae* | Huver *et al*., 2015 |
| *Schistosoma* species | Alzaylaee *et al*., 2020 |

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***Aquatic Manual* disease chapters Table 4.1. *OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals:* current Key for Table 4.1 with suggested edits tracked)**

## 4. Diagnostic methods

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

~~The designations used in the Table indicate:~~

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

~~Key:~~

| | |
|---|---|
| +++ = | ~~Most suitable~~ Methods ~~—~~ are most suitable with desirable performance and operational characteristics. |
| ++ = | ~~Suitable~~ Method~~(s)~~ are suitable with acceptable performance and operational characteristics under most circumstances. |
| + = | ~~Less suitable~~ Methods ~~—~~ are suitable but performance or operational characteristics may ~~significantly~~ limit application under some circumstances. |
| Shaded boxes = | Not appropriate for this purpose. |

~~The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.~~

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

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

[…]

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