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Activities of the Specialist Commissions

AQUATIC ANIMAL HEALTH STANDARDS COMMISSION

Proposed amendments to the

Aquatic Code* and the *Aquatic Manual

(90 SG/10 SC4)

TABLE OF CONTENTS

1. OVERVIEW

1.1. INTRODUCTION

Since the 89th General Session in May 2022, the Aquatic Animal Health Standards Commission (the Aquatic Animals Commission) met twice, from 14 to 21 September 2022 and from 19 January and 15 to 22 February 2023. Among its activities, the Commission progressed work on the development of new and revised texts of the *Aquatic Animal Health Code* (the *Aquatic Code*) and the *Manual of Diagnostic Tests for Aquatic Animals* (the *Aquatic Manual*), in accordance with its work plan. Details of the Aquatic Animals Commission's activities, including the texts circulated for comment, were published in the Commission's September 2022 and February 2023 meeting reports and are available on the Delegate's only website as well as the WOAH Website.

This document provides some background information for each of the new and revised texts of the *Aquatic Code* and *Aquatic Manual* that will be presented for adoption at the 90th General Session. Details of the Commission's considerations of comments received on draft texts circulated for comment were provided in the Commission's September 2022 and February 2023 reports. The Commission encourages Members to refer to these reports as well as other previous Commission reports, as relevant, for more details about the amended texts being proposed for adoption.

The annexes in this document present the proposed amendments to the *Aquatic Code* and *Aquatic Manual* that will be presented to the World Assembly of Delegates for adoption at the 90th General Session. The annex numbers used in this document align with the annex numbers provided in the Aquatic Animals Commission's February 2023 report.

In the process of drafting and reviewing these amendments, the Aquatic Animals Commission considered comments submitted by Members. The Aquatic Animals Commission also worked in close cooperation with the Terrestrial Animal Health Standards Commission and several *ad hoc* Groups.

2. *AQUATIC CODE* TEXTS THAT WILL BE PROPOSED FOR ADOPTION

2.1. ARTICLE 9.3.1. OF CHAPTER 9.3. INFECTION WITH *HEPATOBACTER PENAEI* (NECROTISING HEPATOPANCREATITIS) (ANNEX 4)

Minor editorial amendments are proposed for Article 9.3.1.

The revised article has been circulated three times, the first time was in the February 2022 Commission report.

The revised Article 9.3.1. of Chapter 9.3. Infection with *Hepatobacter penaei* (Necrotising hepatopancreatitis), **Annex 4**, is to be presented for adoption at the 90th General Session in May 2023.

2.2. ARTICLES 9.4.1. AND 9.4.2. OF CHAPTER 9.4. INFECTION WITH INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS (ANNEX 5)

An amendment to update the taxonomic classification of the virus is proposed for Article 9.4.1. and minor editorial amendments for Article 9.4.2.

The revised articles have been circulated three times, the first time was in the February 2022 Commission report.

The revised Articles 9.4.1. and 9.4.2. of Chapter 9.4. Infection with infectious hypodermal and haematopoietic necrosis virus, **Annex 5**, is to be presented for adoption at the 90th General Session in May 2023.

2.3. ARTICLE 9.5.2. OF CHAPTER 9.5. INFECTION WITH INFECTIOUS MYONECROSIS VIRUS (ANNEX 6)

Minor editorial amendments are proposed for Article 9.5.2.

The revised article has been circulated twice, the first time was in the September 2022 Commission report.

The revised Article 9.5.2. of Chapter 9.5. Infection with infectious myonecrosis virus, **Annex 6,** is to be presented for adoption at the 90th General Session in May 2023.

2.4. ARTICLE 10.9.2. OF CHAPTER 10.9. INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS (ANNEX 7)

An amended list of susceptible species is proposed for Article 10.9.2.

At the request of the Aquatic Animals Commission, the *ad hoc* Group on Susceptibility of fish species to WOAH listed diseases assessed the susceptibility of Jinsha barbel carp (*Percocypris pingi)* to infection with spring viraemia of carp virus. The *ad hoc* Group agreed that Jinsha barbel carp met the criteria to be listed as a susceptible species and it was proposed to be included in Article 10.9.2. Details of the *ad hoc* Group's assessment were provided in Annex 10 of the Commission's September 2022 report.

The revised article has been circulated twice, the first time was in the September 2022 Commission report.

The revised Article 10.9.2. of Chapter 10.9. Infection with spring viraemia of carp virus, **Annex 7,** is to be presented for adoption at the 90th General Session in May 2023.

2.5. NEW CHAPTER 10.X. INFECTION WITH TILAPIA LAKE VIRUS (ANNEX 8)

A new disease-specific chapter for infection with tilapia lake virus, is proposed for inclusion in the *Aquatic Code*. This chapter has been developed following the addition of infection with tilapia lake virus to WOAH listed diseases in 2022.

This chapter has been harmonised with other disease-specific chapters.

The draft text has been circulated twice, the first time in the September 2022 Commission report.

A new Chapter 10.X. Infection with tilapia lake virus, **Annex 8**, is to be presented for adoption at the 90th General Session in May 2023.

2.6. ARTICLE 11.2.2. OF CHAPTER 11.2. INFECTION WITH *BONAMIA EXITIOSA* (ANNEX 9)

Amendments to the taxonomy of some susceptible species are proposed for Article 11.2.2.

The revised article has been circulated twice, the first time in September 2022 Commission report.

The revised Article 11.2.2. of Chapter 11.2. Infection with *Bonamia exitiosa,* **Annex 9,** is to be presented for adoption at the 90th General Session in May 2023.

2.7. ARTICLE 11.3.2. OF CHAPTER 11.3. INFECTION WITH *BONAMIA OSTREAE* (ANNEX 10)

Amendments to the taxonomy of some susceptible species are proposed for Article 11.2.2.

The revised article has been circulated twice, the first time in September 2022 Commission report.

The revised Article 11.2.2. of Chapter 11.2. Infection with *Bonamia ostreae,* **Annex 10,** is to be presented for adoption at the 90th General Session in May 2023.

2.8. ARTICLES 11.4.1. AND 11.4.2. OF CHAPTER 11.4. INFECTION WITH *MARTEILIA REFRINGENS* (ANNEX 11)

Amendments to update the taxonomic classification are proposed for Article 11.4.1. and amendments to the list of susceptible species is proposed for Article 11.4.2.

The *ad hoc* Group on Susceptibility of mollusc species to WOAH listed diseases December 2022 report, provides details of the assessments undertaken to determine the proposed list of susceptible species.

The revised articles have been circulated twice, the first time in the September 2022 Commission report.

The revised Articles 11.4.1. and 11.4.2. of Chapter 11.4. Infection with *Marteilia refringens,* **Annex 11,** are to be presented for adoption at the 90th General Session in May 2023.

2.9. MODEL ARTICLES 11.X.9. – 11.X.14. FOR MOLLUSC DISEASE-SPECIFIC CHAPTERS (ANNEX 12)

Moderate amendments to ensure harmonisation among mollusc disease-specific chapters in the *Aquatic Code* are presented as Model Articles 11.X.9. - 11.X.14. These amendments are aligned with other disease-specific chapters, as relevant.

The Model articles have been circulated twice, the first time in the September 2022 Commission report.

The Model Articles 11.X.9. - 11.X.14. for the mollusc disease-specific chapters, **Annex 12,** are to be presented for adoption at the 90th General Session in May 2023.

3. *AQUATIC MANUAL* TEXTS PROPOSED FOR ADOPTION

3.1. CHAPTER 2.2.1. ACUTE HEPATOPANCREATIC NECROSIS DISEASE (ANNEX 22)

Comprehensive amendments to update Chapter 2.2.1. and reformatting to align with the new disease chapter template are proposed.

WOAH Reference Laboratory experts proposed amendments to update the chapter and align with the new disease chapter template.

The revised chapter has been circulated three times, the first time in the February 2022 Commission report.

The revised Chapter 2.2.1. Acute hepatopancreatic necrosis disease, **Annex 22,** is to be presented for adoption at the 90th General Session in May 2023.

3.2. CHAPTER 2.2.3. INFECTION WITH *HEPATOBACTER PENAEI* (NECROTISING HEPATOPANCREATITIS) (ANNEX 23)

Comprehensive amendments to update Chapter 2.2.3. and reformatting to align with the new disease chapter template are proposed.

WOAH Reference Laboratory experts proposed amendments to update the chapter and align with the new disease chapter template.

The revised chapter has been circulated three times, the first time in the February 2022 Commission report.

The revised Chapter 2.2.3. Infection with *Hepatobacter penaei* (necrotising hepatopancreatitis), **Annex 23,** is to be presented for adoption at the 90th General Session in May 2023.

3.3. CHAPTER 2.2.4. INFECTION WITH INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS (ANNEX 24)

Comprehensive amendments to update Chapter 2.2.4. and reformatting to align with the new disease chapter template are proposed.

WOAH Reference Laboratory experts proposed amendments to update the chapter and align with the new disease chapter template.

The revised chapter has been circulated three times, the first time in the February 2022 Commission report.

The revised Chapter 2.2.4. Infection with infectious hypodermal and haematopoietic necrosis virus, **Annex 24,** is to be presented for adoption at the 90th General Session in May 2023.

3.4. CHAPTER 2.2.5. INFECTION WITH INFECTIOUS MYONECROSIS VIRUS (ANNEX 25)

Comprehensive amendments to update Chapter 2.2.5. and reformatting to align with the new disease chapter template are proposed.

The revised chapter has been circulated twice, the first time in the September 2022 Commission report.

The revised Chapter 2.2.5. Infection with infectious myonecrosis virus, **Annex 25,** is to be presented for adoption at the 90th General Session in May 2023.

3.5. CHAPTER 2.2.7. INFECTION WITH TAURA SYNDROME VIRUS (ANNEX 26)

Comprehensive amendments to update Chapter 2.2.7. and reformatting to align with the new disease chapter template are proposed.

WOAH Reference Laboratory experts proposed amendments to update the chapter and align with the new disease chapter template.

The revised chapter has been circulated twice, the first time in the September 2022 Commission report.

The revised Chapter 2.2.7. Infection with taura syndrome virus, **Annex 26,** is to be presented for adoption at the 90th General Session in May 2023.

3.6. CHAPTER 2.2.8. INFECTION WITH WHITE SPOT SYNDROME VIRUS (ANNEX 27)

Comprehensive amendments to update Chapter 2.2.8. and reformatting to align with the new disease chapter template are proposed.

WOAH Reference Laboratory experts proposed amendments to update the chapter and align with the new disease chapter template.

The revised chapter has been circulated twice, the first time in the September 2022 Commission report.

The revised Chapter 2.2.8. Infection with white spot syndrome virus, **Annex 27**, is to be presented for adoption at the 90th General Session in May 2023.

3.7. CHAPTER 2.3.1. INFECTION WITH *APHANOMYCES INVADANS* (EPIZOOTIC ULCERATIVE SYNDROME) (ANNEX 28)

Comprehensive amendments to update Chapter 2.3.1. and reformatting to align with the new disease chapter template are proposed.

The revised chapter has been circulated three times, the first time in the February 2022 Commission report.

The revised Chapter 2.3.1. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome), **Annex 28**, is to be presented for adoption at the 90th General Session in May 2023.

3.8. CHAPTER 2.3.2. INFECTION WITH EPIZOOTIC HAEMATOPOIETIC NECROSIS VIRUS (ANNEX 29)

Comprehensive amendments to update Chapter 2.3.2. and reformatting to align with the new disease chapter template are proposed.

WOAH Reference Laboratory experts proposed amendments to update the chapter and align with the new disease chapter template.

The revised chapter has been circulated four times, the first time in the September 2021 Commission report.

The revised Chapter 2.3.2. Infection with epizootic haematopoietic necrosis virus, **Annex 29,** is to be presented for adoption at the 90th General Session in May 2023.

3.9. SECTION 2.2.1. OF CHAPTER 2.3.9. INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS (ANNEX 30)

An amended list of susceptible species is proposed for Section 2.2.1. of Chapter 2.3.9. that reflects proposed amendments to Article 10.9.2 of the *Aquatic Code*.

The revised section has been circulated twice, the first time was in the September 2022 Commission report.

The revised Section 2.2.1. of Chapter 2.3.9. Infection with spring viraemia of carp virus, **Annex 30,** is to be presented for adoption at the 90th General Session in May 2023.

3.10. SECTIONS 2.2.1. AND 2.2.2. OF CHAPTER 2.4.2. INFECTION WITH *BONAMIA EXITIOSA* (ANNEX 31)

Amendments to the taxonomy of some susceptible species are proposed in Sections 2.2.1. and 2.2.2. of Chapter 2.4.2.

The revised section has been circulated twice, the first time in September 2022 Commission report.

The revised Sections 2.2.1. and 2.2.2. of Chapter 2.4.2. Infection with *Bonamia exitiosa,* **Annex 31**, is to be presented for adoption at the 90th General Session in May 2023.

3.11. SECTIONS 2.2.1. AND 2.2.2. OF CHAPTER 2.4.3. INFECTION WITH *BONAMIA OSTREAE* (ANNEX 32)

Amendments to the taxonomy of some susceptible species are proposed in Sections 2.2.1. and 2.2.2. of Chapter 2.4.3.

The revised section has been circulated twice, the first time in September 2022 Commission report.

The revised Sections 2.2.1. and 2.2.2. of Chapter 2.4.3. Infection with *Bonamia ostreae,* **Annex 32,** is to be presented for adoption at the 90th General Session in May 2023.

3.12. SECTIONS 2.2.1. AND 2.2.2. OF CHAPTER 2.4.4. INFECTION WITH *MARTEILIA REFRINGENS* (ANNEX 33)

Amendments to the list of susceptible species in Sections 2.2.1. and 2.2.2. of Chapter 2.4.4. are proposed.

The *ad hoc* Group on Susceptibility of mollusc species to WOAH listed diseases December 2022 report, provides details of the assessments undertaken to determine the proposed list of susceptible species.

The revised section has been circulated twice, the first time in the September 2022 Commission report.

The revised Sections 2.2.1. and 2.2.2. of Chapter 2.4.4. Infection with *Marteilia refringens,* **Annex 33,** is to be presented for adoption at the 90th General Session in May 2023.

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…/…Annexes

Annex 4. Item 7.1. – Article 9.3.1. of Chapter 9.3. Infection with NHP

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*~~ Hepatobacter penaei, an obligate intracellular bacterium of the Family Holosporaceae of the Order Rickettsiales~~alpha-Proteobacteria~~. ~~The *disease* is commonly known as necrotising hepatopancreatitis.~~

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

[…]

Annex 5. Item 7.2. – Articles 9.4.1. and 9.4.2. of Chapter 9.4. Infection with IHHNV

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*) and yellowleg shrimp (*Penaeus californiensis*).

[…]

Annex 6. Item 7.3. – Article 9.5.2. of Chapter 9.5. Infection with IMNV

CHAPTER 9.5.

INFECTION WITH INFECTIOUS MYONECROSIS VIRUS

[…]

Article 9.5.2.

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.: ~~brown tiger prawn *(Penaeus esculentus),*~~ banana prawn (*Penaeus merguiensis*), brown tiger prawn (*Penaeus esculentus*) and whiteleg shrimp (*Penaeus vannamei*).

[…]

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Annex 7. Item 7.4. – Article 10.9.2. of Chapter 10.9. Infection with SVCV

CHAPTER 10.9.

INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

[…]

Article 10.9.2.

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.:

Family	Scientific name	Common name
Cyprinidae	*Abramis brama*	~~B~~bream
	Aristichthys nobilis	~~B~~bighead carp
	Carassius auratus	~~G~~goldfish
	Ctenopharyngodon idella	~~G~~grass carp
	Cyprinus carpio	~~C~~common carp (all varieties and subspecies)
	Danio rerio	~~Z~~zebrafish
	Notemigonus crysoleucas	~~G~~golden shiner
	Pimephales promelas	~~F~~fathead minnow
	Percocypris pingi	Jinsha barbel ~~bass~~ carp
	Rutilus kutum	Caspian white fish
	Rutilus rutilus	~~R~~roach
Siluridae	*Silurus glanis*	Wels catfish

[…]

Annex 8. Item 7.5. – Chapter 10.X. Infection with TiLV

CHAPTER 10.X.

INFECTION WITH TILAPIA LAKE VIRUS

Article 10.X.1.

For the purposes of the *Aquatic Code*, infection with tilapia lake virus (TiLV) means *infection* with the *pathogenic agent Tilapia tilapinevirus*, of the Genus *Tilapinevirus* and the Family *Amnoonviridae*.

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

Article 10.X.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 tilapia (*Oreochromis aureus*), Malaysian red hybrid tilapia (*Oreochromis niloticus* x *Oreochromis mossambicus*), Mango tilapia (*Sarotherodon galilaeus)*, Mozambique tilapia (*Oreochromis mossambicus*), Nile tilapia (*Oreochromis niloticus*), redbelly tilapia (*Tilapia zilli*), tinfoil barb (*Barbonymus schwanenfeldii*), Tvarnun simon (*Tristramella simonis*) and blue-nile tilapia hybrid (*Oreochromis niloticus* X *Oreochromis aureus*)] (under study).

Article 10.X.3.

Measures for the importation or transit of aquatic animal products for any purpose regardless of the infection with TiLV 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 TiLV, regardless of the infection with ~~TILV~~TiLV 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 ~~56~~60°C for at least ~~five~~120 minutes, or a time/temperature equivalent that inactivates TiLV;

2) fish meal that has been subjected to a heat treatment sufficient to attain a core temperature of at least ~~56~~60°C for at least ~~five~~120 minutes, or a time/temperature equivalent that inactivates TiLV] ~~(under study)~~;

3) fish oil;

4) fish skin leather.

Article 10.X.4.

Requirements for self-declaration of freedom from infection with TiLV

A Member Country may make a self-declaration of freedom from infection with TiLV~~TILV~~ for the entire country, a *zone* or a *compartment* in accordance with the provisions of Articles 10.X.5. to 10.X.8., as relevant. The self-declaration of freedom must be made in accordance with other relevant requirements of the *Aquatic Code,* including that the Member Country meet the following conditions:

1) complies with the provisions of Chapter 3.1.; and

2) uses appropriate methods of *diagnosis*, as recommended in the *Aquatic Manual*; and

3) meets all requirements of Chapter 1.4. that are relevant to the self-declaration of freedom.

Article 10.X.5.

Country free from infection with TiLV~~TILV~~

If a country shares water bodies with other countries, it can only make a self-declaration of freedom from infection with TiLV~~TILV~~ if all shared water bodies are within countries or *zones* declared free from infection with TiLV~~TILV~~ (see Article 10.X.6.).

As described in Article 1.4.~~X~~4., a Member Country may make a self-declaration of freedom from infection with TiLV~~TILV~~ for its entire *territory* if it can demonstrate that:

1) none of the *susceptible species* referred to in Article 10.X.2. are present and *basic biosecurity conditions* have been continuously met for at least the last [six] months;

OR

2) there has been no occurrence of infection with TiLV~~TILV~~ for at least the last [ten] years, and:

   a) the Member Country can demonstrate that conditions are conducive to the clinical expression of infection with TiLV~~TILV~~, as described in the corresponding chapter of the *Aquatic Manual*; and

   b) *basic biosecurity conditions* as described in Chapter 1.4. have been continuously met for at least the last [ten] years;

OR

3) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last [two] years without detection of TiLV~~TILV~~, and *basic biosecurity conditions* have been continuously met and have been in place for at least [one] year prior to commencement of *targeted surveillance*;

OR

4) it previously made a self-declaration of freedom from infection with TILV and subsequently lost its free status due to the detection of TiLV~~TILV~~ but the following conditions have been met:

   a) on detection of TiLV~~TILV~~, the affected area was declared an *infected zone* and a *protection zone* was established; and

   b) infected populations within the *infected zone* have been killed and disposed of by means that minimise the likelihood of further transmission of TiLV~~TILV~~, and the appropriate *disinfection* procedures (as described in Chapter 4.4.) have been completed followed by fallowing as described in Chapter 4.7.; and

   c) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since eradication of infection with TiLV~~TILV~~; and

   d) *targeted surveillance*, as described in Chapter 1.4., has been in place for:

      i) at least the last [two] years in wild ~~or~~ and farmed *susceptible species* without detection of TiLV~~TILV~~; or

      ii) at least the last [one] year without detection of TiLV~~TILV~~ if affected *aquaculture establishments* were not epidemiologically connected to wild populations of *susceptible species*.

In the meantime, the part of the country outside the infected zone and protection zones ~~part or all of the country, apart from the *infected* and *protection zones,*~~ may be declared a free *zone* in accordance with Article 1.4.4. provided ~~that such a part meets the conditions in point 2 of Article 10.X.6.~~points 4. a) to c) have been achieved.

Article 10.X.6.

Zone free from infection with TiLV~~TILV~~

If a *zone* extends over the *territory* of more than one country, it can only be declared a *zone* free from infection with TiLV~~TILV~~ if all of the relevant *Competent Authorities* confirm that all relevant conditions have been met.

As described in Article 1.4.X., a Member Country may make a self-declaration of freedom from infection with TiLV~~TILV~~ for a *zone* within its *territory* if it can demonstrate that:

1) none of the *susceptible species* referred to in Article 10.X.2. are present and *basic biosecurity conditions* have been continuously met for at least the last [six] months;

OR

2) there has been no occurrence of infection with TiLV~~TILV~~ for at least the last [ten] years, and:

   a) the Member Country can demonstrate that conditions are conducive to the clinical expression of infection with TiLV~~TILV~~, as described in Article 1.4.8. of Chapter 1.4.; and

   b) *basic biosecurity conditions* as described in Chapter 1.4. have been continuously met for the *zone* for at least the last [ten] years;

OR

3) *targeted surveillance*, as described in Chapter 1.4., has been in place in the *zone* for at least the last [two] years without detection of TiLV~~TILV~~, and *basic biosecurity conditions* have been continuously met and have been in place for at least [one] year prior to commencement of *targeted surveillance*;

OR

4) it previously made a self-declaration of freedom for a *zone* from infection with TILV and subsequently lost its free status due to the detection of TiLV~~TILV~~ in the *zone* but the following conditions have been met:

   a) on detection of TiLV~~TILV~~, the affected area was declared an *infected zone* and a *protection zone* was established; and

   b) infected populations within the *infected zone* have been killed and disposed of by means that minimise the likelihood of further transmission of TiLV~~TILV~~, and the appropriate *disinfection* procedures (as described in Chapter 4.4.) have been completed followed by fallowing as described in Chapter 4.7.; and

   c) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since eradication of infection with TiLV~~TILV~~; and

   d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last [two] years without detection of TiLV~~TILV~~.

In the meantime, a part of the *zone* outside the *infected zone* and *protection zone* may be declared a new free *zone* in accordance with Article 1.4.4. provided points 4. a) to c) have been achieved.

Article 10.X.7.

Compartment free from infection with TiLV~~TILV~~

As described in Article 1.4.X., a Member Country may make a self-declaration of freedom from infection with TiLV~~TILV~~ for a *compartment* within its *territory* if it can demonstrate that:

1) *targeted surveillance*, as described in Chapter 1.4., has been in place in the *compartment* for at least the last [one] year without detection of TiLV~~TILV~~, and *basic biosecurity conditions* have been continuously met and have been in place for at least [one] year prior to commencement of *targeted surveillance*;

OR

2) it previously made a self-declaration of freedom for a *compartment* from infection with TILV and subsequently lost its free status due to the detection of TiLV~~TILV~~ in the *compartment* but the following conditions have been met:

a) all *aquatic animals* within the *compartment* have been killed and disposed of by means that minimise the likelihood of further transmission of TiLV~~TILV~~, the appropriate *disinfection* procedures (as described in Chapter 4.4.) have been completed, and the *compartment* has been fallowed as described in Chapter 4.7.; and

b) previously existing *basic biosecurity conditions*, including the *compartment biosecurity plan*, have been reviewed and modified as necessary and have continuously been in place from the time of restocking with *aquatic animals* from an approved pathogen free source in accordance with the requirements of Articles 10.X.9. and 10.X.10. as appropriate; and

c) ~~*targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last [one] year~~ one survey for infection with TiLV has been completed at least [six months] after restocking (as described in Article 1.4.14.) without detection of the pathogen~~TILV~~.

Article 10.X.8.

Maintenance of free status

A country, *zone* or *compartment* that is declared free from infection with TiLV~~TILV~~ following the provisions of Articles 10.X.4. to 10.X.7. (as relevant) may maintain its status as free from infection with TiLV~~TILV~~ provided that the requirements described in Article 1.4.15. are continuously maintained.

Article 10.X.9.

Importation of aquatic animals or aquatic animal products from a country, zone or compartment declared free from infection with TiLV~~TILV~~

When importing *aquatic animals* of a species referred to in Article 10.X.2., or *aquatic animal products* derived thereof, from a country, *zone* or *compartment* declared free from infection with TiLV~~TILV~~, the *Competent Authority* of the *importing country* should require that the consignment be accompanied by an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country*. The *international aquatic animal health certificate* should state that, on the basis of the procedures described in Articles 10.X.5., 10.X.6. or 10.X.7. (as applicable) and 10.X.8., the place of production of the *aquatic animals* or *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with TiLV~~TILV~~.

The *international aquatic animal health certificate* should be in accordance with the Model Certificate in Chapter 5.11. This article does not apply to *aquatic animal products* listed in Article 10.X.3.

Article 10.X.10.

Importation of aquatic animals for aquaculture from a country, zone or compartment not declared free from infection with TiLV~~TILV~~

When importing, for *aquaculture*, *aquatic animals* of a species referred to in Article 10.X.2. from a country, *zone* or *compartment* not declared free from infection with TiLV~~TILV~~, the *Competent Authority* of the *importing country* should assess the *risk* in accordance with Chapter 2.1. and consider the *risk* mitigation measures in points 1 and 2 below.

1) If the intention is to grow out and harvest the imported *aquatic animals*, consider applying the following:

   a) the direct delivery to and lifelong holding of the imported *aquatic animals* in a *quarantine* facility; and

   b) before leaving *quarantine* (either in the original facility or following biosecure transport to another *quarantine* facility) the *aquatic animals* are killed and processed into one or more of the *aquatic animal products* referred to in Article 10.X.3. or other products authorised by the *Competent Authority*; and

   c) the treatment of all transport water, equipment, effluent and waste materials to inactivate TiLV~~TILV~~ in accordance with Chapters 4.4., 4.8. and 5.5.

OR

2) If the intention is to establish a new stock for *aquaculture*, consider applying the following:

   a) In the *exporting country*:

i) identify potential source populations and evaluate their *aquatic animal* health records;

ii) test source populations in accordance with Chapter 1.4. and select a founder population (F-0) of *aquatic animals* with a high health status for infection with TiLV~~TILV~~.

b) In the importing *country*:

i) import the F-0 population into a *quarantine* facility;

ii) test the F-0 population for TiLV~~TILV~~ in accordance with Chapter 1.4. to determine their suitability as broodstock;

iii) produce a first generation (F-1) population in *quarantine*;

iv) culture the F-1 population in *quarantine* for a duration sufficient for, and under conditions that are conducive to, the clinical expression of infection with TiLV~~TILV~~, and sample and test for TiLV~~TILV~~ in accordance with Chapter 1.4. of the *Aquatic Code* and Chapter X.X.6. of the *Aquatic Manual*;

v) if TiLV~~TILV~~ is not detected in the F-1 population, it may be defined as free from infection with TiLV~~TILV~~ and may be released from *quarantine*;

vi) if TiLV~~TILV~~ is detected in the F-1 population, those animals should not be released from *quarantine* and should be killed and disposed of in a biosecure manner in accordance with Chapter 4.~~7~~8.

Article 10.X.11.

Importation of aquatic animals or aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from infection with TiLV~~TILV~~

When importing, for processing for human consumption, *aquatic animals* of a species referred to in Article 10.X.2., or *aquatic animal products* derived thereof, from a country, *zone* or *compartment* not declared free from infection with TILV, the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities until processing into one of the products referred to in Article 10.X.3. or in point 1 of Article 10.X.14., or other products authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport are treated to ensure inactivation of TiLV~~TILV~~ or disposed of in a biosecure manner in accordance with Chapters 4.4., 4.8. and 5.5.; and

3) all effluent and waste materials are treated to ensure inactivation of TiLV~~TILV~~ or disposed of in a biosecure manner in accordance with Chapters 4.4. and 4.8.

For these *aquatic animals* or *aquatic animal products* Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *aquatic animal* or *aquatic animal product* being used for any purpose other than for human consumption.

Article 10.X.12.

Importation of aquatic animals or aquatic animal products intended for uses other than human consumption, including animal feed and agricultural, industrial, research or pharmaceutical use, from a country, zone or compartment not declared free from infection with TiLV~~TILV~~

When importing *aquatic animals* of a species referred to in Article 10.X.2., or *aquatic animal products* derived thereof, intended for uses other than human consumption, including animal *feed* and agricultural, industrial, research or pharmaceutical use, from a country, *zone* or *compartment* not declared free from infection with TiLV~~THLV~~, the *Competent Authority* of the *importing country* should require that:

1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities until processed into one of the products referred to in Article 10.X.3. or other products authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport are treated to ensure inactivation of TiLV~~THLV~~ or disposed of in a biosecure manner in accordance with Chapters 4.4., 4.8. and 5.5.; and

3) all effluent and waste materials are treated to ensure inactivation of TiLV~~THLV~~ or disposed of in a biosecure manner in accordance with Chapters 4.4. and 4.8.

Article 10.X.13.

Importation of aquatic animals intended for use in laboratories or zoos from a country, zone or compartment not declared free from infection with TiLV~~THLV~~

When importing, for use in laboratories or zoos, *aquatic animals* of a species referred to in Article 10.X.2. from a country, *zone* or *compartment* not declared free from infection with TiLV~~THLV~~, the *Competent Authority* of the *importing country* should ensure:

1) the consignment is delivered directly to, and held in, *quarantine* facilities authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport are treated to ensure inactivation of TiLV~~THLV~~ or disposed of in a biosecure manner in accordance with Chapters 4.4., 4.8. and 5.5.; and

3) all effluent and waste materials from the *quarantine* facilities in the laboratories or zoos are treated to ensure inactivation of TiLV~~THLV~~ or disposed of in a biosecure manner in accordance with Chapters 4.4. and 4.8.; and

4) the carcasses are disposed of in accordance with Chapter 4.8.

Article 10.X.14.

Importation ~~(~~or transit~~)~~ of aquatic animal products for retail trade for human consumption regardless of the infection with TiLV~~THLV~~ status of the exporting country, zone or compartment

1) [*Competent Authorities* should not require any conditions related to TiLV~~THLV~~, regardless of the infection with TiLV~~THLV~~ status of the *exporting country*, *zone* or *compartment*, when authorising the importation ~~(~~or transit~~)~~ of the following ~~commodities~~ *aquatic animal products* that have been prepared and packaged for retail trade and comply with Article 5.4.2.

   a) fish fillets or steaks (chilled)] (under study).

   Certain assumptions have been made in assessing the safety of the *aquatic animal products* mentioned above. Member Countries should refer to these assumptions at Article 5.4.2. and consider whether the assumptions apply to their conditions.

   For these *aquatic animal products* Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *aquatic animal product* being used for any purpose other than for human consumption.

2) When importing *aquatic animal products*, other than those referred to in point 1 above, derived from a species referred to in Article 10.X.2. from a country, *zone* or *compartment* not declared free from infection with TiLV~~THLV~~, the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

Annex 9. Item 7.6. – Article 11.2.2. of Chapter 11.2. Infection with *Bonamia exitiosa*

CHAPTER 11.2.

INFECTION WITH *BONAMIA EXITIOSA*

[…]

Article 11.2.2.

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.: Argentinean flat oyster (*Ostrea puelchana*), Ariake cupped oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*), Australian mud oyster (*Ostrea angasi*), Chilean flat oyster (*Ostrea chilensis*), crested oyster (*Ostrea equestris*), eastern oyster (*Crassostrea virginica*), European flat oyster (*Ostrea edulis*)~~,~~ and Olympia oyster (*Ostrea lurida*) ~~and Suminoe oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*)~~.

[…]

_____

Annex 10. Item 7.6. – Article 11.3.2. of Chapter 11.3. Infection with *Bonamia ostreae*

CHAPTER 11.3.

INFECTION WITH *BONAMIA OSTREAE*

[…]

Article 11.3.2.

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.: Ariake cupped oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*)~~European flat oyster (*Ostrea edulis*)~~, Chilean flat oyster (*Ostrea chilensis*) and European flat oyster (*Ostrea edulis*)~~Suminoe oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*)~~.

[…]

_____

Annex 11. Item 7.7. – Article 11.4.1. and 11.4.2. of Chapter 11.4. Infection with *Marteilia refringens*

CHAPTER 11.4.

INFECTION WITH *MARTEILIA REFRINGENS*

Article 11.4.1.

For the purposes of the *Aquatic Code*, infection with *Marteilia refringens* means *infection* with the *pathogenic agent* *M. refringens* (including O and M types) of the Family Marteiliidae.

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

Article 11.4.2.

Scope

The recommendations in this chapter apply to: blue mussel (*Mytilus edulis*), dwarf oyster (*Ostrea stentina*), European flat oyster (*Ostrea edulis*), European razor clam (*Solen marginatus*), golden mussel (*Xenostrobus securis*), ~~Australian mud oyster *(Ostrea angasi)*,~~ Argentinean oyster ~~*(Ostrea puelchana)*,~~ Chilean flat oyster ~~*(Ostrea chilensis)*,~~ blue mussel ~~*(Mytilus edulis)*~~ ~~and~~ Mediterranean mussel *(Mytilus galloprovincialis)* and striped venus clam (*Chamelea gallina*). ~~These recommendations also apply to any other *susceptible species* referred to in the *Aquatic Manual* when traded internationally.~~

[…]

Annex 12. Item 7.8. – Model Articles 11.X.9. – 11.X.14. for mollusc disease-specific chapters

Model Articles 11.X.9. – 11.X.14. for mollusc disease-specific chapters

CHAPTER 11.X.

INFECTION WITH [PATHOGEN X]

[…]

Article 11.X.9.

Importation of aquatic animals or~~and~~ aquatic animal products from a country, zone or compartment declared free from infection with [Pathogen X]

When importing *aquatic animals* ~~and *aquatic animal products*~~ of a species referred to in Article 11.X.2., or *aquatic animal products* derived thereof, from a country, *zone* or *compartment* declared free from infection with [Pathogen X], the *Competent Authority* of the *importing country* should require that the consignment be accompanied by an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country*. The *international aquatic animal health certificate* should state that, ~~or a *certifying official* approved by the *importing country* certifying that,~~ on the basis of the procedures described in Articles 11.X.~~4~~5., 11.X.~~5~~6. or 11.X.7. (as applicable) and 11.X.~~6~~8., the place of production of the *aquatic animals* or~~and~~ *aquatic animal products* is a country, *zone* or *compartment* declared free from infection with [Pathogen X].

The *international aquatic animal health certificate* should be in accordance with the Model Certificate in Chapter 5.11.

This article does not apply to *aquatic animal products*~~*commodities*~~ ~~referred to~~listed in point 1 of Article 11.X.3.

Article 11.X.10.

Importation of aquatic animals for aquaculture from a country, zone or compartment not declared free from infection with [Pathogen X]

When importing, for *aquaculture*, *aquatic animals* of a species referred to in Article 11.X.2. from a country, *zone* or *compartment* not declared free from infection with [Pathogen X], the *Competent Authority* of the *importing country* should assess the *risk* in accordance with Chapter 2.1. and consider the *risk* mitigation measures in points 1 and 2 below.

1) If the intention is to grow out and harvest the imported *aquatic animals*, consider applying the following:

   a) the direct delivery to and lifelong holding of the imported *aquatic animals* in a *quarantine* facility; and

   b) before leaving *quarantine* (either in the original facility or following biosecure transport to another *quarantine* facility) the *aquatic animals* are killed and processed into one or more of the *aquatic animal products* referred to in point 1 of Article 11.X.3. or other products authorised by the *Competent Authority*; and

   c) the treatment of all transport water, equipment, effluent and waste materials to ~~inactive~~inactivate [Pathogen X] in accordance with Chapters 4.4., 4.8. and5.5.

OR

2) If the intention is to establish a new stock for *aquaculture*, consider applying the following:

   a) In the *exporting country*:

      i) identify potential source populations and evaluate their *aquatic animal* health records;

      ii) test source populations in accordance with Chapter 1.4. and select a founder population (F-0) of *aquatic animals* with a high health status for infection with [Pathogen X].

b) In the *importing country*:

i) import the F-0 population into a *quarantine* facility;

ii) test the F-0 population for [Pathogen X] in accordance with Chapter 1.4. to determine their suitability as broodstock;

iii) produce a first generation (F-1) population in *quarantine*;

iv) culture F-1 population in *quarantine* for a duration sufficient for, and under conditions that are conducive to, the clinical expression of infection with [Pathogen X], ~~(as described in Chapter 2.4.X. of the *Aquatic Manual*)~~and sample and test for [Pathogen X] in accordance with Chapter 1.4. of the *Aquatic Code* and Chapter 2.4.X. of the *Aquatic Manual*;

v) if [Pathogen X] is not detected in the F-1 population, it may be defined as free from infection with [Pathogen X] and may be released from *quarantine*;

vi) if [Pathogen X] is detected in the F-1 population, those animals should not be released from *quarantine* and should be killed and disposed of in a biosecure manner in accordance with Chapter 4.8.

Article 11.X.11.

Importation of aquatic animals or ~~and~~ aquatic animal products for processing for human consumption from a country, zone or compartment not declared free from infection with [Pathogen X]

When importing, for processing for human consumption, *aquatic animals* of a species referred to in Article 11.X.2., or *aquatic animal products* derived thereof, ~~of species referred to in Article 11.X.2.~~ from a country, *zone* or *compartment* not declared free from infection with [Pathogen X], the *Competent Authority* of the *importing country* should assess the *risk* and, if justified, require that:

1) the consignment is delivered directly to, and held in, *quarantine* or containment facilities until processing into one of the products referred to in point 1 of Article 11.X.3., ~~;~~ or ~~products described~~ in point 1 of Article 11.X.~~12~~14., or other products authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport ~~and all effluent and waste materials from the processing~~ are treated ~~in a manner that~~ to ensure~~s~~ inactivation of [Pathogen X] or ~~is~~ disposed of in a biosecure manner ~~that prevents contact of waste with *susceptible species*~~ in accordance with Chapters 4.4., 4.8. and 5.5.; and

3) all effluent and waste materials are treated to ensure inactivation of [Pathogen X] or disposed of in a biosecure manner in accordance with Chapters 4.4. and 4.8.

For these *aquatic animals* or *aquatic animal products* ~~*commodities*~~ Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *aquatic animal* or *aquatic animal product*~~*commodity*~~ being used for any purpose other than for human consumption.

Article 11.X.12.

Importation of aquatic animals or aquatic animal products intended for use~~s~~ other than human consumption, including ~~in~~ animal feed~~,~~ and ~~or for~~ agricultural, industrial, research or pharmaceutical use, from a country, zone or compartment not declared free from infection with [Pathogen X]

When importing *aquatic animals* of a species referred to in Article 11.X.2., or *aquatic animal products* derived thereof, intended for use~~s~~ other than human consumption, including ~~in~~ animal *feed* ~~or for~~ and agricultural, industrial, research or pharmaceutical use, ~~*aquatic animals* of species referred to in Article 11.X.2.~~ from a country, *zone* or *compartment* not declared free from infection with [Pathogen X], the *Competent Authority* of the *importing country* should require that:

1) the consignment is delivered directly to and held in *quarantine* or containment facilities ~~for slaughter and processing~~ until processed into one of the products referred to in point 1 of Article 11.X.3. or other products authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers,* and packaging material used in transport ~~and all effluent and waste materials from the processing~~ are treated ~~in a manner~~ to ~~that~~ ensure~~s~~ inactivation of [Pathogen X] or disposed of in a

biosecure manner in accordance with Chapters 4.4., 4.8. and 5.5.; and

3) all effluent and waste materials are treated to ensure inactivation of [Pathogen X] or disposed of in a biosecure manner in accordance with Chapters 4.4. and 4.8.

~~This article does not apply to *commodities* referred to in point 1 of Article 11.X.3.~~

Article 11.X.13.

[**Note**: this is a new article to align with other disease-specific chapters within the *Aquatic Code*.]

Importation of aquatic animals intended for use in laboratories or zoos from a country, zone or compartment not declared free from infection with [Pathogen X]

When importing, for use in laboratories or zoos, *aquatic animals* of a species referred to in Article ~~10.2.2.~~11.X.2. from a country, *zone* or *compartment* not declared free from infection with [Pathogen X], the *Competent Authority* of the *importing country* should ensure:

1) the consignment is delivered directly to, and held in, *quarantine* facilities authorised by the *Competent Authority*; and

2) all water (including ice), equipment, *containers* and packaging material used in transport are treated to ensure inactivation of [Pathogen X] or disposed of in a biosecure manner in accordance with Chapters 4.4., 4.8. and 5.5.; and

3) all effluent and waste materials from the *quarantine* facilities in the laboratories or zoos are treated to ensure inactivation of [Pathogen X] or disposed of in a biosecure manner in accordance with Chapters 4.4. and 4.8.; and

4) the carcasses are disposed of in accordance with Chapter 4.8.

Article 11.X.~~13~~14.

Importation ~~(~~or transit~~)~~ of ~~aquatic animals and~~ aquatic animal products for retail trade for human consumption regardless of the infection with [Pathogen X] status of the exporting country, zone or compartment~~from a country, zone or compartment not declared free from infection with [Pathogen X]~~

1) *Competent Authorities* should not require any conditions related to ~~infection with~~ [Pathogen X], regardless of the infection with [Pathogen X] status of the *exporting country*, *zone* or *compartment*, when authorising the importation or transit of the following *aquatic animal products*~~*commodities*~~ that~~which~~ have been prepared and packaged for retail trade and which comply with Article 5.4.2.

   a) […]

   Certain assumptions have been made in assessing the safety of the *aquatic animal products* mentioned above. Member Countries should refer to these assumptions at Article 5.4.2. and consider whether the assumptions apply to their conditions.

   For these *aquatic animal products* ~~*commodities*~~ Member Countries may wish to consider introducing internal measures to address the *risks* associated with the *aquatic animal products* ~~*commodity*~~ being used for any purpose other than for human consumption.

2) When importing ~~*aquatic animals* or~~ *aquatic animal products*, other than those referred to in point 1 above, derived from a ~~of~~ species referred to in Article 11.X.2. from a country, *zone* or *compartment* not declared free from infection with [Pathogen X], the *Competent Authority* of the *importing country* should assess the *risk* and apply appropriate *risk* mitigation measures.

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: fleshy prawn (*Penaeus chinensis*).

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 including 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).~~ None known.

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 of disease and mortality can start as early as 10 days post-stocking. ~~Clinical~~ Signs ~~include:~~ of disease in moribund prawns ~~sink to bottom,~~ may include pale to white hepatopancreas (HP) due to pigment loss in the connective tissue capsule (NACA, 2014). ~~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).~~ Behavioural changes such as frequent sinking to the bottom of tanks may also be noted.

2.3.3 Gross pathology

Gross pathological observations include pale-to-white 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). ~~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 WOAH WAHIS (https://wahis.woah.org/#/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.

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

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 a DNA preservative ~~DNAzol~~ for PCR testing. If material cannot be fixed it may be frozen, but repeated freezing and thawing of samples should be avoided.

Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.5.5. of Chapter 2.2.0 *General information* (diseases of crustaceans).

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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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~~ Isolation					±	±	±	NA				
Real-time PCR	++	++	++	1	++	++	++	1	++	++	++	1
Conventional PCR	++	++	++	2	++	++	++	2	~~++~~	~~++~~	~~++~~	~~2~~
Conventional PCR followed by amplicon sequencing									+++	+++	+++	~~1~~ 2
In-situ hybridisation												
Bioassay					+	+	+	NA	~~+~~	~~+~~	~~+~~	~~NA~~
LAMP		++	++	1								
Ab-ELISA												
Ag-ELISA		±	++	1		±	++	1		±	++	1
Other antigen detection methods[3]												
Other methods[3]												

LV = level of validation, refers to the stage of validation in the WOAH 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

Histological examination of AHPND infected shrimp reveals that pathological changes are limited to the hepatopancreas. 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. No B-, F- and R-cells are seen in the hepatopancreatic tubule and some nuclei of tubule epithelial cells are enlarged (karyomegaly). No significant bacterial involvement appears during this phase ~~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~~ 2014; Nunan *et al*., 2014; Soto-Rodriguez *et al*., 2015; Tran *et al*., 2013; 2014).

iii) In *Penaeus vannamei* AHPND tolerant lines, a chronic phase can be observed. 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~~ isolation

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 assays should always be run with the controls specified in Section 5.5 *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis* of Chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate.

Extraction of nucleic acids

~~Numerous~~ Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~using optical density or running a gel~~.

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

Pathogen/ target gene	**Primer/probe (5'–3')**	**Concentration**	**Cycling parameters**
Method 1: Han *et al.*, 2015b; GenBank Accession No.: KM067908			
pirA	**Fwd VpPirA-F: TTG-GAC-TGT-CGA-ACC-AAA-CG** **Rev VpPirA-R: GCA-CCC-CAT-TGG-TAT-TGA-ATG** **VpPirA Probe: FAM-AGA-CAG-CAA-ACA-TAC-ACC-TAT-CAT-CCC-GGA-TAMRA**	**Fwd: 0.3 µM** **Rev: 0.3 µM** **probe: 0.1 µM**	**95°C/20 sec; 45 cycles 95°C/3 sec and 60°C/30 sec**

~~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

Pathogen/ target gene	**Primer~~/probe~~ (5'–3')**	**Concentration**	**Cycling parameters**
~~Method 1 (AP1): Flegel & Lo, 2014; GenBank: KP324996; 700 bp~~			
~~*pVA1*~~	~~Fwd AP1F: 5CCT TGG GTG TGC TTA GAG GAT G~~	~~0.2 µM each~~	~~94°C/5 min; 25–30 cycles of 94°C/30 sec, 60°C/30 sec and 72°C/60 sec; final extension step at 72°C/10 min. Reaction mixture can be held at 4°C~~

	~~Rev AP1R: GCA-AAC-TAT-CGC-GCA-GAA-CAC-C~~		
~~Method 2 (AP2): Flegel & Lo, 2014; GenBank: KP324996; 700 bp~~			
~~*pVA1*~~	~~Fwd AP2F: TCA-CCC-GAA-TGC-TCG-CTT-GTG-G~~ ~~Rev AP2R: CGT-CGC-TAC-TGT-CTA-GCT-GAA-G~~	~~0.2 µM each~~	~~94°C/5 min; 25–30 cycles of 94°C/30 sec, 60°C/30 sec, 72°C/60 sec; final extension step at 72°C/10 min. Reaction mixture can be held at 4°C~~
Method 1~~3~~ (AP3): Sirikharin *et al.*, 2015; GenBank Accession No.: JALL01000066.1; amplicon size: 333 bp			
pirA^{vp}	Fwd AP3-F: ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC Rev AP3-R: GTG-GTA-ATA-GAT-TGT-ACA-GAA	0.2 µM each	94°C/5 min; 30 cycles of 94°C/30 sec, 53°C/30 sec, 72°C/40 sec; final elongation step at 72°C/7 min; Reaction mixture can be held at 4°C
Method 2~~4~~ (TUMSAT-Vp3): Tinwongger *et al.*, 2014; GenBank Accession No.: AB972427; amplicon size: 360 bp			
pVA1	Fwd TUMSAT-Vp3 F: GTG-TTG-CAT-AAT-TTT-GTG-CA Rev TUMSAT-Vp3 R: TTG-TAC-AGA-AAC-CAC-GAC-TA	0.6 µM each	95°C/2 min; 30 cycles of 95°C/30 sec, 56°C/30 sec, 72°C/30 sec
Method 3~~5~~ (VpPirA-284): Han *et al.*, 2015a; GenBank Accession No.: KM067908; amplicon size: 284 bp			
pirA^{vp}	Fwd VpPirA-284F: TGA-CTA-TTC-TCA-CGA-TTG-GAC-TG Rev VpPirA-284R: CAC-GAC-TAG-CGC-CAT-TGT-TA	0.2 µM each	94°C/3 min; 35 cycles of 94°C/30 sec, 60°C/30 sec, 72°C/30 sec; final extension 72°C/7 min
Method 4~~6~~ (VpPirB-392): Han *et al.*, 2015a; GenBank Accession No.: KM067908; amplicon size: 392 bp			
pirB^{vp}	Fwd VpPirB-392F: TGA-TGA-AGT-GAT-GGG-TGC-TC Rev VpPirB-392R: TGT-AAG-CGC-CGT-TTA-ACT-CA	0.2 µM each	94°C/3 min; 35 cycles of 94°C/30 sec, 60°C/30 sec, 72°C/30 sec; final extension 72°C/7 min
Method 5~~7~~ (AP4): Dangtip *et al.*, 2015; GenBank Accession No.: JPKS01000000; amplicon size: 1269 bp			
PirA and PirB toxin genes	Primary Fwd AP4-F1: ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC Rev AP4-R1: ACG-ATT-TCG-ACG-TTC-CCC-AA Nested Fwd AP4-F2: TTG-AGA-ATA-CGG-GAC-GTG-GG Rev AP4-R2: GTT-AGT-CAT-GTG-AGC-ACC-TTC	0.2 µM each	Primary 94°C/2 min; 30 cycles of 94°C/30 sec, 55°C/30 sec, 72°C/90 sec; final extension step at 72°C/2 min; hold at 4°C Nested 94°C/2 min; 25 cycles of 94°C/20 sec, 55°C/20 sec, 72°C/20 sec; hold at 4°C
~~Method 8 (AP4): Dangtip *et al.*, 2015; GenBank: JPKS01000000; amplicon size: 230 bp~~			
~~***PirA and PirB toxin genes***~~	~~**Fwd AP4-F2: TTG-AGA-ATA-CGG-GAC-GTG-GG**~~ ~~**Rev AP4-R2: GTT-AGT-CAT-GTG-AGC-ACC-TTC**~~	~~**0.2 µM each**~~	~~**94°C/2 min; 25 cycles of 94°C/20 sec, 55°C/20 sec, 72°C/20 sec; hold at 4°C**~~

~~**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 AP1R: GCA AAC TAT CGC GCA GAA CAC C	*pVA1*	700bp	Flegel & Lo (2014)
AP2	AP2F: TCA CCC GAA TGC TCG CTT GTG G 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 4.4.2.32.

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 AP3-R: GTG GTA ATA GAT TGT ACA GAA	*pirA*^{vp}	333bp	Sirikharin *et al.*, 2015
TUMSAT-Vp3	TUMSAT-Vp3 F: GTG TTG CAT AAT TTT GTG CA TUMSAT-Vp3 R: TTG TAC AGA AAC CAC GAC TA	*pirA*^{vp}	360bp	Tinwongger *et al.*, 2014
VpPirA-284	VpPirA-284F: TGA CTA TTC TCA CGA TTG GAC TG VpPirA-284R: CAC GAC TAG CGC CAT TGT TA	*pirA*^{vp}	284bp	Han *et al.*, 2015a
VpPirB-392	VpPirB-392F: TGA TGA AGT GAT GGG TGC TC 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~~3. ~~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 Step 1~~	~~AP4-F1: ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC AP4-R1: ACG-ATT-TCG-ACG-TTC-CCC-AA~~	~~1269~~	~~Dangtip *et al.*, 2015~~
~~AP4 Step 2~~	~~AP4-F2: TTG-AGA-ATA-CGG-GAC-GTG-GG 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. Isothermal loop-mediated amplification protocol (LAMP)

Pathogen/ target gene	**Primer/probe (5'–3')**	**Concentration**	**Cycling parameters**
Method: Koiwai *et al.*, 2017; GenBank Accession No.: AB972427.1			
Toxin PirAB-like	**F3: TGA-TAA-TGC-ATT-CTA-TCA-TCA-GC** **B3: ATT-TGA-AAG-ACC-AAA-TGA-AAC-C** **FIP-F1c: GTG-AGC-ACC-TTC-TTA-GTG-GTA-ATA** **FIP-F2: GTT-GTA-ATT-AAC-AAT-GGC-GCT-AG** **BIP-B1c: TGA-CGG-AAT-TTA-ACC-CTA-ACA-ATG-C** **BIP-B2: GCT-TTG-AAA-GCA-TAG-TTA-GGA-TC**	**F3: 5.0 pmol** **B3: 5.0 pmol** **FIP: 40 pmol** **BIP: 40 pmol**	**65°C/60 min and 80°C/5 min**

4.4.~~3~~4. 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 size of the PCR amplicon ~~is~~ should be verified, for example by agarose gel electrophoresis~~, and purified by excision from this gel~~. Both DNA strands of the PCR product must be sequenced and analysed ~~and compared~~ in comparison with ~~published~~ reference sequences.

~~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 2×10^8 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 2×10^6 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 μl^{-1} for $PirA^{vp}$ and 0.008 ng μl^{-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) and conventional PCR (Dangtip *et al.,* 2015) are ~~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 WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAH Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status [1]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. ~~Geographical~~ Hydrographical proximity to, or movement of animals or animal products or

1 For example transboundary commodities.

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 PCR

ii) A positive result by ~~or~~ conventional PCR ~~methods recommended in Table 4.1~~

iii) A positive result by LAMP

iv) Histopathology ~~or cytopathological changes~~ consistent with the ~~presence of the pathogen or the~~ disease

v) A positive result by Ag-ELISA

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 at least one of the following ~~criterion~~ criteria is met:

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

ii) Positive results by LAMP and conventional PCR followed by amplicon sequence analysis

iii) Positive results by Ag-ELISA 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~~

ii) A positive result by agent isolation

iii) A positive result by real-time PCR

iv) A positive result by conventional PCR

v) A positive result by bioassay

vi) A positive result by LAMP

vii) A positive result by Ag-ELISA

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 at least one of the following ~~criterion~~ criteria is met:

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

ii) Positive results by LAMP and conventional PCR followed by amplicon sequence analysis

iii) Positive results by Ag-ELISA 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 (no data are currently available). 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~~	~~Clinically 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 rpose	Source pulations	Tissue or sample types	Species	DSe	DSp	Reference test	Citation

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

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* *

NB: There are WOAH Reference Laboratories for acute hepatopancreatic necrosis disease
(please consult the WOAH web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratory for any further information on
acute hepatopancreatic necrosis disease

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

CHAPTER 2.2.3.

INFECTION WITH HEPATOBACTER PENAEI (NECROTISING HEPATOPANCREATITIS)

1. Scope

~~Infection with infectious salmon anaemia virus (ISAV) means infection with the pathogenic agent highly polymorphic region (HPR) deleted ISAV, or the non-pathogenic HPR0 (non-deleted HPR) ISAV of the Genus *Isavirus* and Family *Orthomyxoviridae*.~~

Infection with ~~*Candidatus* *Hepatobacter penaei*~~ means infection with the pathogenic agent *Candidatus* H. penaei*,* an obligate intracellular bacterium of the Family Holosporaceae*,* Order Rickettsiales ~~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 Family Holosporaceae ~~family~~ within the Order 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 confirms 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~~ are: 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~~ are: 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 (PL), 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* often causes ~~an~~ acute disease with very high mortalities in young juveniles, adults and broodstock. In horizontally infected young juveniles, adults and broodstock, the incubation period and severity of the disease are somewhat size or age dependent, with juveniles always being the most severely affected. 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 atrophied hepatopancreas, empty guts, soft shells and flaccid bodies; black or darkened gills; 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)~~ 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 PL that were also *H. penaei*-positive, which suggests that a transmission from broodstock to progeny can occur~~s~~ (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 WOAH WAHIS (https://wahis.woah.org/#/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: 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 and this organ should be selected preferentially (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

Hepatobacter penaei can be detected in faeces samples collected from clinically affected populations of *Penaeus vannamei* ~~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~~). However, the use of faeces samples to detect *H. penaei* ~~NHP~~ in apparently healthy shrimp has not been evaluated. ~~Faeces samples have not been validated to the same level as hepatopancreas samples.~~

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

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, but repeated freezing and thawing should be avoided.

Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.5.5. 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 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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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
Conventional PCR followed by 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 WOAH 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 screening populations for infection with ~~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*~~ in cell culture. No crustacean cell lines exist (Vincent & Lotz, 2007).

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 5.5 *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis* of chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate.

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

Extraction of nucleic acids

~~Numerous~~ Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~using optical density or running a gel~~.

~~*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).

Pathogen/ target gene	**Primer/probe (5'–3')**	**Concentration**	**Cycling parameters**
Method 1: Aranguren *et al.,* 2010; GenBank U65509			
H. penaei/16S rRNA gene	Fwd NHP1300F: CGT-TCA-CGG-GCC-TTG-TAC-AC Rev NHP1366R: GCT-CAT-CGC-CTT-AAA-GAA-AAG-ATA-A Probe: CCG-CCC-GTC-AAG-CCA-TGG-AA	300 nM 100 nM	40 cycles: 95°C/15 sec and 60°C/1 min
Method 2: Aranguren & Dhar 2018; GenBank JQAJ01000001.1			
***H. penaei*/ Flagella hook gene ~~protein~~**	**Fwd NHP FlgE3qF: AAC-ACC-CTG-TCT-CCC-CAA-TTC Rev FlgE3qR: CCA-GCC-TTG-GAC-AAA-CAC-CTT Probe: CGC-CCC-AAA-GCA-TGC-CGC**	**500 nM 100 nM**	**40 cycles: 95°C/1 sec and 60°C/20 sec**

~~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 95°C followed by 40 cycles of 1 second at 95°C and 20 seconds at 60°C. 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~~ flagella hook gene separately.

Pathogen/ target gene	**Primer~~/probe~~ (5'–3')**	**Concentration**	**Cycling parameters**
Method 1: Aranguren *et al.*, 2010; GenBank Accession No.: MH230908.1; amplicon size 379 bp			
H. penaei/16S rRNA gene	Fwd NHPF2: CGT-TGG-AGG-TTC-GTC-CTT-CAG-T Rev NHPR2: GCC-ATG-AGG-ACC-TGA-CAT-CAT-C	200 nM	35 cycles: 95°C/30 sec, 60°C/30 sec and 72°C/30 sec
Method 2: Aranguren & Dhar, 2018; GenBank Accession No.: JQAJ01000001.1; amplicon size 333 bp			
***H. penaei*/ Flagella hook gene ~~protein~~**	**Fwd FlgE 1143F: AGG-CAA-ACA-AAC-CCT-TG Rev FlgE 1475R: GCG-TTG-GGA-AAG-TT**	**~~0.2 µM~~ 200 nM**	**35 cycles,: 95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec**

~~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 μl^{-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 μl^{-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 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C 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

The size of the PCR amplicon should be verified, for example by agarose gel electrophoresis. Both DNA strands of the PCR product must be sequenced and analysed in comparison with reference sequences.

~~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~~ exist 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~~ is 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 WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAH Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status [1]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Hydrographical ~~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) A positive result by real-time PCR

ii) A 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:

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

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

iv) A positive result by conventional PCR

v) A positive result by *in-situ* hybridisation

vi) A positive result by bioassay

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:

1 For example transboundary commodities.

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 followed by amplicon sequencing

iii) ~~Histopathology consistent with *H. penaei* and positive *in-situ* hybridisation test~~ A positive result by *in-situ* hybridisation and real-time PCR

iv) A positive result by *in-situ* hybridisation and conventional PCR followed by amplicon sequencing

~~v) A positive result by bioassay followed by real-time PCR~~

~~vi) A positive result by bioassay followed by conventional PCR followed by 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

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 (no data are currently available for either). 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 a WOAH Reference Laboratory for infection with *Hepatobacter penaei*
(necrotising hepatopancreatitis)
(please consult the WOAH web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
infection with *Hepatobacter penaei* (necrotising hepatopancreatitis).

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

Annex 24. Item 10.1.3. – Chapter 2.2.4. Infection with infectious hypodermal and haematopoietic necrosis virus

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 Decapod penstylhamaparvovirus* 1, of the Genus *Penstylhamaparvovirus* and Family *Parvoviridae* ~~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 (GenBank NC_002190), 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 South-East Asia (principally the Philippines) and Type 2 is from South-East Asia. These genotypes were shown to be ~~are~~ infectious to *Penaeus vannamei* and *P. monodon* (Tang *et al.,* 2003). IHHNV genotypes in Ecuador and Peru were found to be within a separate lineage of IHHNV type 2 genotypes circulating within these countries (Aranguen Caro *et al.,* 2022). 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 (*Penaeus monodon*), northern white shrimp (*Penaeus setiferus*), blue shrimp (*Penaeus stylirostris*), and white leg shrimp (*Penaeus vannamei*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with 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*).~~

Family	Scientific name	Common name
Achiridae	*Achirus mazatlanus*	Mazatlan sole
Centropomidae	*Centropomus medius*	blackfin snook
Cichlidae	*Oreochromis* sp.	tilapias
Clupeidae	*Lile stolifera*	Pacific piquitinga
Gerreidae	*Gerres cinereus*	yellowfin mojarra
Palaemonidae	*Macrobrachium rosenbergii*	giant river prawn
Penaeidae	*Penaeus duorarum*	northern pink shrimp
	Penaeus occidentalis	western white shrimp
	Penaeus japonicus	kuruma prawn
	Penaeus semisulcatus	green tiger prawn
	Artemesia longinaris	Argentine stiletto shrimp
~~Portunoidea~~ Portunidae	*Callinectes arcuatus*	Cuata swimcrab
Varunidae	*Hemigrapsus penicillatus*	

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~~ has been detected in many crustacean and non-crustacean species however their (~~*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%. 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).

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). 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.

~~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).~~

There was no mortality or clinical signs of disease in *P. vannamei*, *P. monodon* or *P. stylirostris* when experimentally challenged with IHHNV genotypes from Ecuador and Peru ~~(Aranguen Caro *et al.,* 2022)~~. The IHHNV genotypes were found to be within a separate lineage of IHHNV type 2 genotypes circulating within these countries (Aranguen Caro *et al.,* 2022).

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

Animals with this disease may show one or more of these signs, but the pathogen may still be present in the absence of any signs. Clinical 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.

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.

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).

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 WOAH WAHIS (https://wahis.woah.org/#/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

Specimens suitable for testing for infection with IHHNV include postlarvae (PL), juveniles and adults. While IHHNV may infect all life stages, virus load ~~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).

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

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~~ bioassay

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~~ molecular techniques can be found in Section B.~~2.5~~ 5.5. 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~~ 5.3 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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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~~
Conventional PCR followed by amplicon sequencing									+++	+++	+++	1
In-situ hybridisation						+	+	1		++	++	1
Bioassay					±	±	±	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 WOAH 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

PCR assays should always be run with the controls specified in Section 5.5 *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis* of Chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate.

Extraction of nucleic acids

~~Numerous~~ different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can ~~should~~ be checked using a suitable method as appropriate to the circumstances ~~optical density or running a gel~~.

There are multiple ~~geographical~~ variants of IHHNV, some of which are not detected by ~~all of the~~ some 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; Taengchaiyaphum *et al.,* 2022; 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~~ considered less susceptible to endogenisation was developed (Encinas-Garcia *et al.,* 2015). ~~More recently,~~ A TaqMan real-time assay ~~capable of~~ developed to differentiate ~~endogenous virus element~~ EVEs from infectious form of IHHNV in *P. monodon* has been reported (Cowley *et al.,* 2018); however, analysis of a *P. monodon* whole genome sequence has identified 100% primer and probe sequence matches to EVEs (Taengchaiyaphum *et al.,* 2022). The real-time PCR method ~~using TaqMan chemistry~~ described in Table 4.4.1 ~~below for IHHNV~~ generally follows the method used in Tang & Lightner (2001).

Table 4.4.1. *Primers and probes for real-time PCR detection of IHHNV*

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1* Tang & Lightner, 2001; GenBank Accession No.: ~~Acc. No~~ AF218266			
IHHNV and IHHNV-related EVEs non-structural protein	Fwd IHHNV1608F: TAC-TCC-GGA-CAC-CCA-ACC-A Rev IHHNV1688R: GGC-TCT-GGC-AGC-AAA-GGT-AA Probe: FAM-ACC-AGA-CAT-AGA-GCT-ACA-ATC-CTC-GCC-TAT-TTG-TAMRA	300 nM primers 150 nM probe	40 cycles of: 95°C/1 sec and 60°C/20 sec

***NOTE** – this method will amplify EVEs within the genome of* P. monodon*. Positive results in this species must be confirmed by a method that does not react with IHHNV EVEs.*

~~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. In addition to IHHNV, some of these methods will amplify EVEs in *Penaeus monodon*. Positive results in *P.*

monodon should be followed up with other methods that will not react with EVEs. ~~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.~~

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

Pathogen / target gene	Primer~~/probe~~ (5'–3')	Concentration	Cycling parameters
Method 1* Tang *et al.,* 2007; GenBank Accession No.: ~~Acc. No~~ AF218266; amplicon size 389 bp ~~product~~			
IHHNV and IHHNV-related EVEs Non-structural protein	Fwd 389F: CGG-AAC-ACA-ACC-CGA-CTT-TA Rev 389R: GGC-CAA-GAC-CAA-AAT-ACG-AA	200 nM	35 cycles of: 94°C/30 sec, 60°C/30 sec, and 72°C/30 sec
Method 2* Nunan *et al.,* 2000; GenBank Accession No.: ~~Acc. No~~ AF218266; amplicon size 356 bp ~~product~~			
IHHNV and IHHNV-related EVEs Between the non-structural and capsid protein-coding regions	Fwd 77012F: ~~TAC-TCC-GGA-CAC-CCA-ACC-A~~ ATC-GGT-GCA-CTA-CTC-GGA Rev 77353R: ~~GGC-TCT-GGC-AGC-AAA-GGT-AA-~~TCG-TAC-TGG-CTG-TTC-ATC	1000 nM	35 cycles of: 95°C/30 sec, 60°C/30 sec, and 72°C/30 sec
Method 3* Tang *et al.,* 2000; GenBank Accession No.: ~~Acc. No~~ AF218266; amplicon size 392 bp ~~product~~			
IHHNV and IHHNV-related EVEs Non-structural protein	Fwd 392F: GGG-CGA-ACC-AGA-ATC-ACT-TA Rev 392R: ATC-CGG-AGG-AAT-CTG-ATG-TG	300 nM	35 cycles of: 95°C/30 sec, 60°C/30 sec, and 72°C/30 sec
Method 4 Tang *et al.,* 2007; GenBank Accession No.: ~~Acc. No~~ AF218266; amplicon size 309 bp ~~product~~			
IHHNV ORF1	Fwd 309F: TCC-AAC-ACT-TAG-TCA-AAA-CCA-A Rev 309R: TGT-CTG-CTA-CGA-TGA-TTA-TCC-A	200 nM	35 cycles of: 94°C/30 sec, 55°C/30 sec, and 72°C/30 sec

***NOTE** – these methods will amplify EVEs within the genome of* P. monodon*. Positive results in this species must be confirmed by a method that does not react with IHHNV EVEs.*

~~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 µl^{-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 a real-time isothermal recombinase polymerase amplification (RPA) assay ~~are available~~ to detect ~~and confirm~~ IHHNV ~~infection~~ have been published (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

The size of the PCR amplicon should be ~~is~~ verified, for example by agarose gel electrophoresis~~, and purified by excision from this gel~~. Both DNA strands must be sequenced and analysed ~~and compared with published~~ in comparison with reference sequences.

~~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 day~~s~~.

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 molecular 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 WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAH Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status [1]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. ~~Geographical~~ Hydrographical 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 criterion ~~criteria~~ is met:

i) Positive result by real-time PCR and a positive result by conventional PCR ~~targeting non-overlapping regions of the viral genome and~~ followed by 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) Histopathology consistent with IHHNV infection

iii) Positive result by conventional PCR

~~iii~~ iv) Positive result by real-time PCR

~~iv) Histopathology consistent with IHHNV infection~~

v) Positive result by *in-situ* hybridisation

vi) Positive result by bioassay

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 a positive result by conventional PCR ~~targeting non-overlapping regions of the viral genome and~~ followed by amplicon sequencing

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

iii) ~~Histopathology consistent with IHHNV infection coupled with~~ A positive result by *in-situ* hybridisation and ~~detection of IHHNV by~~ a positive result by conventional PCR ~~and~~ followed by amplicon sequencing

1 For example transboundary commodities.

~~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 (~~none~~ no data are currently available for either). 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 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.

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 WOAH Reference Laboratories for infection with infectious hypodermal and haematopoietic necrosis virus (please consult the WOAH web site for the most up-to-date list: http://www.woah.org/en/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the WOAH 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.

CHAPTER 2.2.5.

INFECTION WITH INFECTIOUS MYONECROSIS VIRUS

1. Scope

Infection with infectious myonecrosis virus means infection with the pathogenic agent infectious myonecrosis virus (IMNV) that is tentatively assigned to the Family *Totiviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

~~Phylogenetic analysis of its RNA-dependent RNA polymerase (RdRp) gene coding sequence indicates that~~ IMNV ~~is most closely related to *Giardia lamblia virus*,~~ tentatively assigned to the family *Totiviridae* ~~a member of the family *Totiviridae*~~ (~~Fauquet *et al.,* 2005~~; Lightner, 2011; Nibert, 2007; Poulos *et al.,* 2006; Wickner *et al.,* 2011).

IMNV particles are icosahedral in shape and 40 nm in diameter, with a buoyant density of 1.366 g ml^{-1} in caesium chloride. The genome consists of a single, double-stranded (ds) RNA molecule of 8226–8230 bp (Loy *et al*., 2015; Naim *et al.,* 2015). Sequencing of the viral genome reveals two non-overlapping open reading frames (ORFs). The first ORF (ORF1, 470–5596 nt) encodes a putative RNA-binding protein and a capsid protein. The coding region of the RNA-binding protein is located in the first half of ORF1 and contains a dsRNA-binding motif in the first 60 amino acids. The second half of ORF1 encodes a capsid protein, as determined by amino acid sequencing, with a molecular mass of 106 kDa. The second ORF (ORF2, 5884–8133 nt) encodes a putative RdRp (Poulos *et al.,* 2006). The most variable region of IMNV genome is located in the first half of ORF1, coinciding with a region which probably encodes the capsid protrusions (Dantas *et al*., 2015).

The complete genomes of IMNV types originating from Brazil and Indonesia have been sequenced and found to be 99.6% identical at the nucleotide level (Poulos *et al.,* 2006; Senapin *et al.,* 2007). The 99.6% full genome sequence identity (and anecdotal information on the introduction of *Penaeus vannamei* stocks from Brazil) indicate that the disease was introduced from Brazil to Indonesia in 2006. A new genotype was analysed in infected samples in 2018 in Indonesia, including an isolate that contains a deletion of 622 amino acids (Mai *et al.,* 2019).

2.1.2. Survival and stability in processed or stored samples

No data.

2.1.3. Survival and stability outside the host

No information available.

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 IMNV according to Chapter 1.5 of *Aquatic Animal Health Code* (*Aquatic Code*) are: brown tiger prawn (*Penaeus esculentus*), banana prawn (*P. merguiensis*), and 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 IMNV according to Chapter 1.5 of the *Aquatic Code* are: giant tiger prawn (*Penaeus monodon*) and blue shrimp (*P. stylirostris*).

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: southern brown shrimp (*P. subtilis*).

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

Juveniles and subadults of *P. vannamei*, farmed in marine, brackish, and low salinity brackish water, appear to be most severely affected by infection with IMNV (Lightner, 2011; Lightner *et al.,* 2004; Nunes *et al.,* 2004; Poulos *et al.,* 2006).

2.2.4. Distribution of the pathogen in the host

The principal target tissues for IMNV include the striated muscles (skeletal and less often cardiac), connective tissues, haemocytes, and the lymphoid organ parenchymal cells (Lightner, 2011; Lightner *et al.,* 2004; Poulos *et al.,* 2006; Tang *et al.,* 2005).

2.2.5. Aquatic animal reservoirs of infection

Some members of populations of *P. vannamei* that survive IMNV infections or epizootics may carry the virus.

2.2.6. Vectors

Experimental studies have demonstrated that brine shrimp *Artemia franciscana* can act as a vector for IMNV (da Silva *et al*., 2015).

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

In early juvenile, juvenile, or adult *P. vannamei* in regions where infection with IMNV is enzootic, outbreaks of IMNV infections associated with sudden high morbidity and mortality may follow 'stress' events such as capture by cast-netting, feeding and sudden changes in water salinity or temperature (Lightner, 2011; Lightner *et al.,* 2004; Nunes *et al.,* 2004; Poulos *et al.,* 2006). Feed conversion ratios of affected populations can increase from a normal value of ~ 1.5 up to 4.0 or higher (Andrade *et al.,* 2007). Mortalities from infection with IMNV can range from 40% to 70% in cultivated *P. vannamei*.

In regions where infection with IMNV is enzootic in farmed stocks of *P. vannamei*, its prevalence may reach 100% (Andrade *et al.,* 2007; Nunes *et al.,* 2004).

2.3.2. Clinical signs, including behavioural changes

Affected shrimp present with visibly white tails. Such severely affected shrimp may have been feeding just before the onset of stress and may have a full gut. High mortality can occur suddenly and continue for several days. A sudden onset of clinical signs may ~~have a sudden onset~~ occur following stress events (e.g. capture by cast-netting, feeding, and sudden changes in temperature or salinity).

Only shrimp in the acute phase of disease present behavioural changes. Typically, severely affected shrimp become lethargic during or soon after stress events such as capture by cast-netting, feeding, sudden changes in water temperature, sudden reductions in water salinity, etc.

2.3.3 Gross pathology

Shrimp in the acute phase of disease present focal-to-extensive white necrotic areas in striated (skeletal) muscles, especially in the distal abdominal segments and tail fan, which can become necrotic and reddened in some individual shrimp.

Exposing the paired lymphoid organs (LO) by simple dissection will show that they are hypertrophied (3–4 times their normal size) (Lightner *et al.,* 2004; Poulos *et al.,* 2006).

2.3.4. Modes of transmission and life cycle

IMNV has been demonstrated to be transmitted horizontally by cannibalism (Lightner, 2011; Poulos *et al.,* 2006). Transmission via water probably occurs. Although vertical transmission is suspected from anecdotal evidence, it is not known whether this occurs via transovarial mechanism or by surface contamination of newly spawned eggs.

2.3.5. Environmental factors

Temperature and salinity effects are likely predisposing factors to disease outbreaks, but no experimental data are available (Nunes *et al.,* 2004).

2.3.6. Geographical distribution

Infection with IMNV has been reported to occur in some countries in the Americas, Asia and Africa (Aly *et al.,* 2021; Andrade *et al.,* 2007; Lightner *et al.,* 2004; Naim *et al.,* 2014; Nunes *et al.,* 2004; Poulos *et al.,* 2006; Sahul *et al*., 2017).

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

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

No effective vaccines for infection with IMNV are available.

2.4.2. Chemotherapy including blocking agents

Ctn[15-34], a cathelicidin-derived eicosapeptide was found to demonstrate antiviral activity against IMNV in primary haemocyte cultures (Vieira-Girao *et al.,* 2017).

2.4.3. Immunostimulation

No data.

2.4.4. Breeding resistant strains

There are anecdotal reports of some selected lines of *P. vannamei* having better survival and culture performance in farms where infection with IMNV is enzootic. During a 20-day controlled laboratory study in which the shrimp were challenged with IMNV, some domesticated lines of *P. vannamei* were found to survive better than other lines (White-Noble *et al.,* 2010).

Penaeus monodon and *P. stylirostris*, for which there is incomplete evidence of susceptibility (see section 2.2.2), are considered to be more resistant to infection with IMNV than *P. vannamei* (Tang *et al.,* 2005).

2.4.5. Inactivation methods

No data.

2.4.6. Disinfection of eggs and larvae

While IMNV is believed to be transmitted vertically, there are no scientific data confirming this route of transmission. Disinfection of eggs and larvae (Chen *et al.,* 1992) is a good management practice recommended to reduce the potential for transmission of a number of penaeid shrimp diseases from female spawners to their eggs or larvae, and the practice may reduce IMNV contamination of spawned eggs and larvae produced from them.

2.4.7. General husbandry

Management practices in endemic areas principally involves exclusion of IMNV from shrimp farms. Broodstock or their spawned eggs or nauplii are PCR-tested and those that test positive are discarded (Andrade *et al.,* 2007). Fallowing and restocking of affected farms or entire culture regions with IMNV-free stocks of *P. vannamei* most suited to local culture conditions has proven to be the most successful for preventing and controlling other virus diseases of shrimp, and should be applicable to control and prevent infection with IMNV (Lee & O'Bryen, 2003; Lightner, 2005; Lightner *et al.,* 2009; Moss & Moss, 2009).

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

Specimens suitable for testing for infection with IMNV using molecular methods (e.g. RT-PCR, nested RT-PCR, real-time RT-PCR, etc.) include postlarvae (PL), juveniles, subadults and adults. While IMNV may infect all life stages, infection severity, and hence virus load, may be below detection limits in spawned eggs and in larval stages, so these life stages may not be suitable for demonstrating freedom from infection with IMNV unless validated for those life stages.

3.2. Selection of organs or tissues

IMNV infects tissues of mesodermal origin. The principal target tissues in the acute phase of infection with IMNV are the striated muscles (skeletal and less commonly cardiac muscle), connective tissues, haemocytes, and the lymphoid organ tubule parenchymal cells. In chronic infections, the lymphoid organ may be the principal target tissue.

3.3. Samples or tissues not suitable for pathogen detection

IMNV replicates systemically but does not replicate in enteric tissues (e.g. the hepatopancreas, the midgut, or its caeca). Hence, enteric tissues are inappropriate samples for detection of IMNV infection.

3.4. Non-lethal sampling

Haemolymph or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary.

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

3.5. Preservation of samples for submission

Several factors can affect specimen quality during collection, handling and storage, such as exposure to light, heat, desiccation, and incomplete preservation. Hence, standard operating protocols or recommended practices should be followed at all steps of the diagnostic process.

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

3.5.1. Samples for pathogen isolation

Not applicable.

3.5.2. Preservation of samples for molecular detection

Tissue samples (pleopods, cephalothorax, muscle, haemolymph) 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.

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 2.3.0 *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. 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 fry can be pooled to obtain the minimum amount of material for 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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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					+	+	+	1				
Histopathology					++	++	++	2				
Cell culture												
Real-time RT-PCR	+	++	++	1	++	++	++	2	++	++	++	2
Conventional RT-PCR	+	++	++	1	++	++	++	1				
Conventional RT-PCR followed by amplicon sequencing									+++	+++	+++	1
In-situ hybridisation					+	+	+	1	+	++	++	1
Bioassay												
LAMP												
Ab-ELISA												
Ag-ELISA												
Other antigen detection methods												
Other methods												

LV = level of validation, refers to the stage of validation in the WOAH 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; IFAT = indirect fluorescent antibody test.
[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.
Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

Stained or unstained tissue squashes of affected skeletal muscle or of the LO may show abnormalities. Tissue squashes of skeletal muscle when examined with phase or reduced light microscopy may show loss of the normal striations. Fragmentation of muscle fibres may also be apparent. Squashes of the LO may show the presence of significant accumulations of spherical masses of cells called lymphoid organ spheroids (LOS) amongst normal LO tubules.

4.2. Histopathology and cytopathology

Infection with IMNV in the acute and chronic phases can be presumptively diagnosed using histology (Bell & Lightner, 1988; Lightner, 2011; Lightner *et al.,* 2004; Poulos *et al.,* 2006). However, the lesions in striated muscles and LO are not pathognomonic for infection with IMNV. White tail disease of penaeid shrimp caused by the *P. vannamei* nodavirus (PvNV) can mimic infection with IMNV (Tang *et al.,* 2007).

Haematoxylin and eosin stained tissue sections from shrimp with acute-phase infection with IMNV present myonecrosis with characteristic coagulative necrosis of striated (skeletal) muscle fibres, often with marked oedema among affected muscle fibres. Some shrimp may present a mix of acute and older lesions. The affected muscle fibres appear to progress from presenting coagulative necrosis to liquefactive necrosis, which is accompanied by moderate infiltration and accumulation of haemocytes. In the most advanced lesions, haemocytes and inflamed muscle fibres are replaced by a loose matrix of fibrocytes and connective tissue fibres that are interspersed with haemocytes and foci of (presumed) regenerating muscle fibres (Lightner *et al.,* 2004; Poulos *et al.,* 2006).

Significant hypertrophy of the LO caused by accumulations of LOS is a highly consistent lesion in shrimp with acute or chronic-phase infection with IMNV lesions. Often, many ectopic LOS are found in other tissues not near the main body of the LO. Common locations for ectopic LOS include the haemocoelom in the gills, heart, near the antennal gland tubules, and ventral nerve cord (Lightner *et al.,* 2004; Poulos *et al.,* 2006).

4.3. Cell culture for isolation

No crustacean cell lines exist, but IMNV was observed to propagate in C6/36 subclone of *Aedes albopictus* cell line (Kumar *et al.,* 2020). Performance of the test should be confirmed before being recommended.

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 5.5 *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis* of Chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate.

Extraction of nucleic acids

~~Numerous~~ Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~using optical density or running a gel~~.

Published methods are available for the molecular detection of IMNV by *in-situ* hybridisation (ISH), nested RT-PCR and quantitative real-time RT-PCR (Andrade *et al.,* 2007; Poulos *et al.,* 2006; Tang *et al.,* 2005). A nested RT-PCR kit for detection of the virus is available commercially.

4.4.1. Real-time RT-PCR

A real-time RT-PCR method was developed to detect and quantify IMNV in shrimp tissue. The method which can detect as few as 10 IMNV RNA copies μl^{-1} total RNA (Andrade *et al.,* 2007) is summarised below.

Pathogen / target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1: Andrade *et al.,* 2007; GenBank Accession No.: AY570982			
IMNV Capsid protein gene	**Fwd IMNV412F: GGA-CCT-ATC-ATA-CAT-AGC-GTT-GCA Rev IMNV545R: AAC-CCA-TAT-CTA-TTG-TCG-CTG-GAT Probe: CCA-CCT-TTA-CTT-TCA-ATA-CTA-CAT-CAT-CCC-CGG**	**300 Nm 200 nM**	**40 cycles of: 95°C/3 sec and 60°C/30 sec**

4.4.2. Conventional PCR

The nested RT-PCR method to detect IMNV uses two PCR primer sets that produce a 328 bp one-step amplicon and139 bp two-step amplicon. The 1-step PCR can detect as little as 100 IMNV RNA copies and the 2-step PCR can detect in the order of 10 IMNV RNA copies (Poulos & Lightner, 2006).

Pathogen / target gene	Primer~~/probe~~ (5'–3')	Concentration	Cycling parameters
Method 1: Poulos & Lightner, 2006; GenBank Accession No.: KJ636783.2; amplicon size: 328/139 bp			
IMNV Capsid protein gene (nested-PCR)	**~~Outer~~ Primary** **Fwd 4587F: CGA-CGC-TGC-TAA-CCA-TAC-AA** **Rev 4914R: ACT-CGG-CTG-TTC-GAT-CAA-GT**	**200 nM**	**45 cycles of: 95°C/45 sec; 60°C/45 sec; 60°C/7 min**
	~~Inner~~ Nested **Fwd 4725 NF: GGC-ACA-TGC-TCA-GAG-ACA** **Rev 4863 NR: AGC-GCT-GAG-TCC-AGT-CTT-G**	**620 nM**	**39 cycles of: 95°C/30 sec, 65°C/30 sec, 72°C/30 sec; 72°C/2 min**

4.4.3. Other nucleic acid amplification methods

None.

4.5. Amplicon sequencing

The size of the PCR amplicon ~~is~~ should be verified, for example by agarose gel electrophoresis~~, and purified by excision from this gel~~. Both DNA strands of the PCR product must be sequenced and analysed ~~and compared~~ in comparison with ~~published~~ reference sequences.

4.6. *In-situ* hybridisation

DNA probe for ISH detection of IMNV

A cDNA library was generated from RNA extracted from purified IMNV. A IMNV-specific ISH DNA probe is prepared from clone IMNV-317 by PCR labelling with digoxigenin-11-dUTP (DIG). The PCR primers used for amplification of the 993 bp probe are IMNV993F (5'-AAC-ACA-AAA-TCT-GCC-AGC-AA-3') and IMNV993R (5'-CCC-AAC-CAC-CCA-AAT-TCA-TA-3'). Following PCR, the DIG-labelled DNA probe is precipitated with ethanol, re-suspended in water and stored at –20°C until used. The ISH procedure for detecting IMNV follows that outlined by Tang *et al.* (2005). Negative and positive controls should be sourced from PCR-confirmed uninfected and infected shrimp, respectively.

4.7. Immunohistochemistry

Monoclonal antibodies have been generated using recombinant IMNV capsid protein fragments to immunise mice (Kunanopparat et al., 2011). Immunohistochemical analysis demonstrated strong reactivity in muscle, gill, heart, LO and connective tissue derived from IMNV-infected *P. vannamei* similar to that demonstrated by *in-situ* hybidisation (Tang *et al*., 2005). There was no cross-reactivity to tissues derived from uninfected shrimp or shrimp infected with other viral pathogens such as WSSV, YHV, TSV among others.

4.8. Bioassay

Not applicable.

4.9. Antibody- or antigen-based detection methods

None are recommended, however an immunochromatographic strip test has been developed (Chaivisuthangkura *et al.*, 2013) using the monoclonal antibodies developed by Kunanopparat *et al*. (2011). While the test is simple, fast and low-cost it is approximately 300-fold less sensitive than one-step RT-PCR (Chaivisuthangkura *et al*., 2013).

4.10. Other methods

A chromatographic method for detection of PCR amplicons has been developed (Koiwai *et al.,* 2018).

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

Real-time RT-PCR is the recommended test for surveillance to demonstrate freedom of infection with IMNV in apparently healthy populations as described in Section 4.1.1.

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 WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAH Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free. There are currently no WOAH Reference Laboratories designated for IMN.

6.1. Apparently healthy animals or animals of unknown health status [1]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Hydrographical 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 IMNV shall be suspected if at least one of the following criteria is met:

~~i) Histopathology consistent with the presence of the pathogen or the disease~~

i) Positive result by real-time RT-PCR

ii) Positive result by conventional RT-PCR

6.1.2. Definition of confirmed case in apparently healthy animals

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

i) Positive result by real-time RT-PCR and positive result by conventional RT-PCR followed by amplicon sequencing

~~ii) Histopathology consistent with IMNV infection coupled with *in-situ* hybridisation and detection of IMNV in a tissue sample by real-time RT-PCR~~

~~ii) Histopathology consistent with IMNV infection coupled with *in-situ* hybridisation and detection of IMNV in a tissue sample by conventional RT-PCR followed by amplicon sequencing~~

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 IMNV shall be suspected if at least one of the following criteria is met:

1 For example transboundary commodities.

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 RT-PCR

iii) Positive result by real-time RT-PCR

iv) Histopathology consistent with the presence of the pathogen or the disease

6.2.2. Definition of confirmed case in clinically affected animals

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

i) Positive result by real-time RT-PCR and a positive result by conventional RT-PCR followed by amplicon sequencing

ii) Positive result by *in-situ* hybridisation and a positive result by real-time RT-PCR

iii) Positive result by *in-situ* hybridisation and a positive result by conventional RT-PCR followed by amplicon sequencing

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with IMNV are provided in Tables 6.3.1. and 6.3.2 (no data are currently available). This information can be used for the design of surveys for infection with IMNV, 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	Experimentally infected SPF *P. vannamei*	abdominal muscle	*P. vannamei*	100 (*n*=30)	100 (*n*=30)	Histopathology	Andrade *et al*. (2007)

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
Real-time PCR								

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

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NB: At the time of publication (2022) there was no WOAH Reference Laboratory for infection with infectious myonecrosis virus (please consult the WOAH web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 2009. MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 2.2.7.

INFECTION WITH TAURA SYNDROME VIRUS

1. Scope

Infection with Taura syndrome virus means infection with the pathogenic agent Taura syndrome virus (TSV), Genus Aparavirus, Family *Dicistroviridae*, Order Picornavirales.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

TSV was described as the cause of the disease commonly known as Taura syndrome by Hasson *et al.* (1995), Bonami *et al.* (1997) and Mari *et al.* (1998; 2002). At least four genotypes (strains) of TSV have been documented based on the gene sequence encoding VP1 the largest and presumably dominant of the three major structural proteins of the virus. Based on VP1 sequence variations, these genotypic groups are: 1) the Americas group; 2) the South-East Asian group; 3) the Belize group; and 4) the Venezuelan group (Nielsen *et al.,* 2005; Tang & Lightner, 2005; Wertheim *et al.,* 2009).

At least two distinct antigenic variants of TSV have been identified by their differential reactivity to monoclonal antibody MAb 1A1, produced using a reference isolate from the Americas (TSV USA-HI94 – GenBank AF277675) (Poulos *et al.,* 1999) as the immunogen: Type A represents those that react with MAb 1A1 (in the enzyme-linked immunosorbent assay [ELISA], Western blots and immunohistochemistry (IHC) with infected tissues) and those that do not were subdivided into Type B (TSV 98 Sinaloa, Mexico) and Type C (TSV 02 Belize), based on host species and virulence. All TSV isolates of the Americas and most, if not all, South-East Asian genotypes react with MAb 1A1. In marked contrast, none of the Belize genotype group reacts with MAb 1A1 (Robles-Sikisaka *et al.,* 2002), nor does a TSV isolate from the 2005 epizootic in Venezuelan shrimp farms.

TSV particles are 32 nm in diameter, non-enveloped icosahedrons and have a buoyant density of 1.338 g ml^{-1} in CsCl. The genome of TSV consists of a linear, positive-sense single-stranded RNA 10,205 nucleotides in length, excluding the 3' poly-A tail, and it contains two large open reading frames (ORFs). ORF 1 contains the sequence motifs for non-structural proteins, such as helicase, protease and RNA-dependent RNA polymerase. ORF 2 contains the sequences for TSV structural proteins, including the three major capsid proteins VP1, VP2 and VP3 (55, 40, and 24 kDa, respectively). The virus replicates in the cytoplasm of host cells (Bonami *et al.,* 1997; Mari *et al.,* 1998; 2002; Robles-Sikisaka *et al.,* 2001).

Other reported causes of Taura syndrome: TS in Ecuador was initially linked to fungicide contamination of shrimp farms, a contention that was supported by litigation for ~16 years after the disease was scientifically shown to have a viral aetiology (Brock *et al.,* 1995; Hasson *et al.,* 1995). Hence, several papers in the literature propose a toxic aetiology for TS (Intriago *et al.,* 1997; Jimenez, 1992; Jimenez *et al.,* 2000).

2.1.2. Survival and stability in processed or stored samples

No information available.

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 TSV according to Chapter 1.5 of *Aquatic Animal Health Code* (*Aquatic Code*) are: blue shrimp (*Penaeus stylirostris*), giant tiger prawn (*Penaeus monodon*), greasyback shrimp (*Metapenaeus ensis*), northern brown shrimp (*Penaeus aztecus*), northern white shrimp (*Penaeus setiferus*), and whiteleg shrimp (*Penaeus vannamei*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with TSV according to Chapter 1.5 of the *Aquatic Code* are: fleshy prawn (*Penaeus chinensis*), giant river prawn (*Macrobrachium rosenbergii*), the copepod *Ergasilus manicatus*, and the barnacles *Chelonibia patula* and *Octolasmis muelleri.* Evidence is lacking for these species to either confirm that the identity of the pathogenic agent is TSV, 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 species, but no active infection has been demonstrated: blue crab (*Callinectes sapidus*), the crabs *Uca vocans* and *Sesarma mederi*, gulf killifish (*Fundulus grandis*), Indo-Pacific swamp crab (*Scylla serrata*), kuruma prawn (*Penaeus japonicus*), northern pink shrimp (*Penaeus duorarum*) and southern white shrimp (*P. schmitti*).

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

Infection with TSV has been documented in all life stages (i.e. post-larvae [PL], juveniles and adults) of *P. vannamei* except eggs, zygotes and larvae (Lightner, 1996a).

2.2.4. Distribution of the pathogen in the host

Using injection and *per os* challenge experiments, Nunan *et al.* (2004) demonstrated TSV could be detected in different body parts including gills, head, whole tail, tail muscle, pleopod and tail fan (Nunan *et al.,* 2004). While there was no significant difference in the viral copy number contained in different body parts when TSV was administered via injection, there was a statistically significant difference between tail/gills, tail/head, tail/tail fan, whole tail/tail fan and pleopods/tail fan when the viral inoculum was administered *per os*. The tail samples had the lower viral copy numbers, as did the whole tail and pleopods when compared to the tail fan (Nunan *et al.,* 2004).

2.2.5. Aquatic animal reservoirs of infection

Not demonstrated unequivocally

2.2.6. Vectors

Sea birds: TSV has been demonstrated to remain infectious for up to 48 hours (after ingestion of TSV-infected shrimp carcasses) in the faeces passed by wild or captive sea gulls (*Larus atricilla*) and chickens (*Gallus gallus,* used as a laboratory surrogate for all shrimp-eating birds) thus suggesting that the virus can retain infectivity when passed through the gastro-intestinal system of any bird species. These findings implicate birds as being an important mechanical vector for the transmission of the virus within affected farms or farming regions (Garza *et al.,* 1997; Vanpatten *et al.,* 2004).

Aquatic insects: the water boatman (*Trichocorixa reticulata* [*Corixidae*], an aquatic insect that feeds on shrimp carcasses in shrimp farm ponds) have been demonstrated to transport TSV within their intestinal contents, but are not directly infected by the virus (Brock, 1997; Lightner, 1996a; 1996b; reviewed in Dhar *et al.,* 2004).

2.3. Disease pattern

Infection with TSV is best known as a disease of nursery- or grow-out-phase *P. vannamei* that occurs within ~14–40 days of stocking PLs into grow-out ponds or tanks, hence, shrimp with TSV infection are typically small (~0.05 g to <5 g) juveniles. Larger shrimp may also be affected, especially if they are not exposed to the virus until they are larger juveniles or adults (Brock, 1997; Brock *et al.,* 1995; Lightner, 1996a, 1996b; Lotz, 1997).

2.3.1. Mortality, morbidity and prevalence

At the farm level, outbreaks of infection with TSV involving stocks of *P. vannamei* (the principal host species for infection with TSV) not selected for resistance, typical cumulative mortalities range from 40 to >90% in cultured populations of PL, juvenile, and subadult life stages. TSV-resistant lines of *P. vannamei* are available which show survival rates of up to 100% in laboratory challenge with all four TSV genotypes (Lightner *et al.,* 2009).

In regions where the virus is enzootic in farmed stocks, the prevalence of infection with TSV has been found in various surveys to range from 0 to 100% (Brock, 1997; Jimenez *et al.,* 2000).

2.3.2. Clinical signs, including behavioural changes

Only acute-phase clinical infection with TSV can be presumptively diagnosed from clinical signs. See Section 4.2 for a description of gross clinical signs presented by shrimp with acute-phase clinical infection with TSV.

Only shrimp with acute-phase clinical infection with TSV present behavioural changes. Typically, severely affected shrimp apparently become hypoxic and move to the pond edges or pond surface where dissolved oxygen levels are higher. Such shrimp may attract seabirds in large numbers. In many disease outbreaks, it is the large numbers of seabirds attracted to the moribund shrimp that first indicates the presence of a serious disease outbreak (which is often either infection with TSV or white spot syndrome virus) to the farm manager.

2.3.3. Gross pathology

Infection with TSV has three distinct phases, acute, transition, and chronic, which are grossly distinguishable (Hasson *et al.,* 1999a; 1999b; Lightner, 1996a; 1996b; Lightner *et al.,* 1995). Gross signs presented by juvenile, subadult and adult shrimp in the transition phase of infection with TSV are unique and provide a suspicion of infection.

Acute phase: gross signs displayed by moribund *P. vannamei* with acute-phase infection with TSV include expansion of the red chromatophores giving the affected shrimp a general, overall pale reddish colouration and making the tail fan and pleopods distinctly red; hence 'red tail' disease was one of the names given by farmers when the disease first appeared in Ecuador (Lightner *et al.,* 1995). In such shrimp, close inspection of the cuticular epithelium in thin appendages (such as the edges of the uropods or pleopods) with a ×10 hand lens reveals signs of focal epithelial necrosis. Shrimp showing these gross signs of acute infection with TSV typically have soft shells, an empty gut and are often in the late D stages of the moult cycle. Acutely affected shrimp usually die during ecdysis.

Transition (*recovery*) *phase:* although only present for a few days during outbreaks of infection with TSV, the gross signs presented by shrimp in the transition phase can provide a suspicion of infection with TSV. During the transition phase (which may be occurring while many shrimp in the affected populations are still in the acute phase and daily mortalities are high), fair to moderate numbers of shrimp in affected ponds show random, multifocal, irregularly shaped melanised cuticular lesions. These melanised spots are haemocyte accumulations indicating the sites of resolving TSV lesions in the cuticular epithelium. Such shrimp may or may not have soft cuticles and red-chromatophore expansion, and may be behaving and feeding normally (Brock, 1997; Hasson *et al.,* 1999b; Lightner, 1996a).

Chronic phase: after successfully moulting, shrimp in the transition phase move into the chronic phase of infection with TSV in which persistently infected shrimp show no obvious signs of disease (Brock, 1997; Hasson *et al.,* 1999b; Lightner, 1996a; 1996b; Lightner *et al.,* 1995). However, *P. vannamei* that are chronically infected with TSV may be less resistant to normal environmental stressors (i.e. sudden salinity reductions) than uninfected shrimp.

2.3.4. Modes of transmission and life cycle

Not applicable.

2.3.5. Environmental factors

Outbreaks of infection with TSV are more frequent when salinities are below 30 ppt (Jimenez *et al.,* 2000).

2.3.6. Geographical distribution

TSV is now widely distributed in the shrimp-farming regions of the Americas, South-East Asia and the Middle East (Brock, 1997; Hasson *et al.,* 1999a; Lightner, 1996a, 1996b; Lightner *et al.,* 2012; Lotz *et al.,* 2005; Nielsen *et al.,* 2005; Tang & Lightner, 2005; Tu *et al.,* 1999; Wertheim *et al.,* 2009; Vergel *et al.,* 2019; Yu & Song, 2000).

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

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

No effective vaccines for TSV are available.

2.4.2. Chemotherapy including blocking agents

No scientifically confirmed reports of effective chemotherapy treatments.

2.4.3. Immunostimulation

No scientifically confirmed reports of effective immunostimulation treatments.

2.4.4. Breeding resistant strains

After TSV emerged in Ecuador in 1992–1994, *P. stylirostris* were found that possessed resistance to infection with TSV (genotype 1, MAb 1A1 Type A). Following on from this discovery and due to the disease occurrence in Mexico in 1994 where it caused crop failures of *P. vannamei,* selected lines of TSV-resistant *P. stylirostris* became the dominant shrimp farmed in western Mexico from 1995. However, in 1998–1999, a new 'strain' of TSV (Type B; Fegan & Clifford, 2001; Lightner, 1999; 2005; Zarain-Herzberg & Ascencio, 2001) emerged and caused massive epizootics in *P. stylirostris*. The emergence of this new 'strain' of TSV was soon followed in late 1999 by the introduction of white spot syndrome virus (WSSV) into shrimp farms in western Mexico, to which *P. stylirostris* had no resistance, effectively ending any interest in the culture of *P. stylirostris* in Mexico.

TSV-resistant domesticated stocks of *P. vannamei* and *P. stylirostris* have been developed. Some domesticated lines of TSV-resistant *P. vannamei* (that are also TSV-free) are in widespread use by the shrimp-farming industries of the Americas and South-East Asia (Clifford, 1998; White *et al.,* 2002). After the appearance of infection with TSV in Central America, improved TSV resistance was reported in wild caught *P. vannamei* PLs used to stock shrimp farms in the region. Currently all genetic lines of *P. vannamei* shrimp that are being cultured in Asia and the Americas contain varying levels of tolerance/resistance to TSV.

2.4.5. Inactivation methods

No information available.

2.4.6. Disinfection of eggs and larvae

It is possible that TSV might be transmitted vertically (transovarian transmission), despite the lack of published reports documenting this route of transmission. Disinfection of eggs and larvae (Chen *et al.,* 1992) is good management practice and it is recommended for its potential to reduce TSV contamination of spawned eggs and larvae produced from them.

2.4.7. General husbandry

Some husbandry and disease control and management practices have been used successfully to reduce the risks of infection with TSV occurring during farm grow-out. These include the application of PCR assays for pre-screening of wild or pond-reared broodstock or their spawned eggs/nauplii and discarding those that test positive for the virus (Fegan & Clifford, 2001), fallowing and restocking of entire culture regions with TSV-free stocks (Dixon & Dorado, 1997), and the development of specific pathogen-free (SPF) shrimp stocks of *P. vannamei* and *P. stylirostris* (Lightner, 1996b; 2005; Wyban 1992). The adoption of the latter technology (SPF stocks) has proven to be among the most successful husbandry practice for the prevention and control of infection with TSV.

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

Suitable specimens for testing for infection with TSV include PL, juveniles and adults. While TSV may infect all life stages, infection severity, and hence virus load, may be below detection limits in spawned eggs and in the larval stages, so these life stages may not be suitable samples for TSV detection~~ or certification of freedom from infection with TSV~~.

3.2. Selection of organs or tissues

TSV infects tissues of ectodermal and mesodermal origin. The principal target tissue in the acute phase of infection with TSV is the cuticular epithelium. In chronic infections the lymphoid organ (LO) is the principal target tissue.

Haemolymph or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary.

3.3. Samples or tissues not suitable for pathogen detection

TSV is a systemic virus, and it does not replicate in enteric tissues (e.g. the hepatopancreas, the midgut, or its caeca). Hence, enteric tissues are inappropriate samples for detection of infection with TSV.

3.4. Non-lethal sampling

Haemolymph or pleopods can be collected without killing the animals and used as non-lethal sampling of genetically valuable broodstock.

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

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~~ bioassay

The success of ~~pathogen isolation~~ bioassay depend~~s~~ strongly on the quality of samples (which is influenced 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 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 animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be preserved in ethanol it may be frozen.~~

Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.5.5. 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 Chapter 2.2.0. *General information* (*diseases of crustaceans*).

3.5.4. Samples for other tests

Haemolymph could be used for PCR-based detection of TSV.

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 animals should be processed and tested individually.

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, 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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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	+	+	+	NA				
Cell culture												
Real-time RT-PCR	+++	+++	+++	1	+++	+++	+++	1	+++	+++	+++	1
Conventional RT-PCR	++	++	++	1	++	++	++	1				
Conventional RT-PCR followed by amplicon sequencing									+++	+++	+++	1
In-situ hybridisation					+	+	+	1	+	+	+	1
Bioassay					+	+	+	1				
LAMP												
IFAT												
ELISA												
Other antigen detection methods												
Other method												

LV = level of validation, refers to the stage of validation in the WOAH Pathway (chapter 1.1.2); NA = not available; PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; IFAT = indirect fluorescent antibody test; ELISA = 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.

Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

Direct microscopy of simple unstained wet mounts from excised pieces of the gills, appendage tips, etc., examined by phase- or reduced-light microscopy may be used to demonstrate (and make a tentative diagnosis of acute-phase infection with TSV) focal lesions of acute-phase infection with TSV in cuticular epithelial cells. Preparations presenting acute-phase infection with TSV will contain numerous spherical structures (see the histopathological methods in Section 4.2.3 above), which are pyknotic and karyorrhectic nuclei and cytoplasmic remnants of necrotic cells.

4.2. Histopathology and cytopathology

Histopathology is a useful method to detect infection with TSV in the acute and chronic phases of infection (Hasson *et al.,* 1999b; Lightner, 1996a). In chronic infections with TSV, the only lesion typically presented by infected shrimp is the presence of an enlarged LO with multiple LO spheroids (LOS) (Hasson *et al.,* 1999b), which cannot be distinguished from LOS induced by chronic infections of other RNA viruses (Lightner, 1996a). When histological lesions are observed and infection with TSV is suspected, a molecular test (ISH with TSV-specific probes, or reverse-transcription [RT] PCR) must be used for confirmation of infection with TSV (see Section 6).

4.2.1. Acute phase of Taura syndrome

The acute phase of the disease is characterised by multifocal areas of necrosis in the cuticular epithelium of the general body surface, appendages, gills, hindgut, and foregut (the oesophagus, anterior and posterior chambers of the stomach). Cells of the subcuticular connective tissues and adjacent striated muscle fibres basal to affected cuticular epithelium are occasionally affected. In some severe cases of acute-phase infection with TSV, the antennal gland tubule epithelium is also destroyed. Prominent in the multifocal cuticular lesions are conspicuous foci of affected cells that display an increased eosinophilia of the cytoplasm and pyknotic or karyorrhectic nuclei. Cytoplasmic remnants of necrotic cells are often extremely abundant in these infections with TSV acute-phase lesions and these are generally presented as spherical bodies (1–20 µm in diameter) that range in staining from eosinophilic to pale basophilic. These structures, along with pyknotic and karyorrhectic nuclei, give acute-phase TS lesions a characteristic 'peppered' or 'buckshot-riddled' appearance, which is considered to be pathognomonic for the infection when there is no concurrent necrosis of the parenchymal cells of the LO tubules. The absence of necrosis of the LO in acute-phase infection with TSV distinguishes it from acute-phase infection with yellowhead virus genotype 1 in which similar patterns of necrosis to those induced by infection with TSV may occur in the cuticular epithelium and gills (Lightner, 1996a).

In TSV-infected tissues, pyknotic or karyorrhectic nuclei give a positive (for DNA) Feulgen reaction, which distinguishes them from the less basophilic to eosinophilic cytoplasmic inclusions that do not contain DNA. The absence of haemocytic infiltration or other signs of a significant host-inflammatory response distinguishes the acute phase of infection with TSV from the transitional phase of the disease (Brock, 1997; Brock *et al.,* 1995; Hasson *et al.,* 1995; 1999a; 1999b; Lightner, 1996a; Lightner *et al.,* 1995).

4.2.2. Transition (recovery) phase of infection with Taura syndrome virus

In the transitional phase of infection with TSV, typical acute-phase cuticular lesions decline in abundance and severity and are replaced by conspicuous infiltration and accumulation of haemocytes at the sites of necrosis. The masses of haemocytes may become melanised giving rise to the irregular black spots that characterise the transition phase of the disease. In H&E sections, such lesions may show erosion of the cuticle, surface colonisation and invasion of the affected cuticle and exposed surface haemocytes by *Vibrio* spp. (Hasson *et al.,* 1999b; Lightner, 1996a). Sections of the LO during the transition phase of infection with TSV may appear normal with H&E staining. However, when sections of the LO are assayed for TSV by ISH with a specific cDNA probe (or by ISH with MAb 1A1 for TSV type A, genotype 1), large quantities of TSV are shown accumulating in the more peripheral parenchymal cells of the LO tubules (Hasson *et al.,* 1999b; Srisuvan *et al.,* 2005).

4.2.3. Chronic phase of infection with Taura syndrome virus

Shrimp in the chronic phase of infection with TSV display no gross signs of infection, and histologically the only sign of infection is the presence of numerous prominent LOS, which may remain associated with the main body of the paired LO, or which may detach and become ectopic LOS bodies that lodge in constricted areas of the haemocoel (i.e. the heart, gills, in the subcuticular connective tissues, etc.). Such LOS are spherical accumulations of LO cells and haemocytes and may be distinguished from normal LO tissues by their spherical nature and the lack of the central vessel that is typical of normal LO tubules. When assayed by ISH with a cDNA probe for TSV (or with MAb 1A1 using ISH) some cells in the LOS give positive reactions to the virus, while no other target tissues react (Hasson *et al.,* 1999b; Lightner, 1996a; 1996b).

4.3. Cell culture for virus isolation

TSV has not been grown *in vitro*, as no crustacean cell lines exist (Lightner, 1996a; Pantoja *et al.,* 2004). Although one publication incorrectly reported that TSV infected human and monkey cell lines (Audelo del Valle *et al.,* 2003), two other laboratories that repeated the study both found that TSV does not infect or replicate in primate or human cell lines that are known to have susceptibility to human picornaviruses (Luo *et al.,* 2004; Pantoja *et al.,* 2004).

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 5.5 *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis* of Chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate, i.e. by testing two aliquots.

Extraction of nucleic acids

~~Numerous~~ Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~using optical density or running a gel~~.

4.4.1. Real-time reverse-transcription (RT)-PCR

Real-time RT-PCR methods have been developed for the detection of TSV. These methods have the advantage of speed, specificity and sensitivity. The sensitivity of real time RT-PCR is approximately equal to 100 copies of the target sequence from the TSV genome (Dhar *et al.,* 2002; Tang *et al.,* 2004).

The real-time RT-PCR method described below for TSV follows the method used in Tang *et al.,* 2004.

~~*Primer and probe sequences, real time RT-PCR*~~

Pathogen / target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1 (Tang *et al.,*2004); GenBank Accession No.: AFAF277675			
TSV/ORF-1 Nt 1024 to 1051	Fwd: TSV1004: TTG-GGC-ACC-AAA-CGA-CAT-T- Rev: TSV1075 GGG-AGC-TTA-AAC-TGG-ACA-CAC-TGT Probe: TSV-P1 FAM-CAG-CAC-TGA-CGC-ACA-ATA-TTC-GAG-CAT-C-TAMRA,	300 nM of each primer 100 nM of probe	Reverse transcription at 50°C/30 min 40 cycles of 95°C/3 sec and 60°C/30 sec

4.4.2. Conventional RT-PCR

Tissue samples (haemolymph, pleopods, whole small shrimp etc) may be assayed for TSV using RT-PCR. The RT-PCR method outlined below for TSV follows the method used in Nunan *et al.* (1998).

~~*Primer and probe sequences, conventional RT-PCR*~~

Pathogen / target gene	Primer ~~/probe~~ (5'–3')	Concentration	Cycling parameters
Method 1 (Nunan *et al.,* 1998); ~~product~~ amplicon size: 231 bp			
TSV/ORF 2	Fwd: 9992: AAG-TAG-ACA-GCC-GCG-CTT Rev:9195R: TCA-ATG-AGA-GCT-TGG-TCC	Primers/620 nM each	Reverse transcription 60°C/30 min 40 cycles: 94°C/45 sec, 60°C/45 sec

4.4.3. Other nucleic acid amplification methods

None currently available.

4.5. Amplicon sequencing

The size of the PCR amplicon ~~is~~ should be verified, for example by agarose gel electrophoresis~~, and purified by excision from this gel~~. Both DNA strands of the PCR product must be sequenced and analysed ~~and compared~~ in comparison with ~~published~~ reference sequences.

4.6. *In-situ* hybridisation (ISH)

4.6.1. DNA probes for ISH applications with non-radioactive cDNA probes

Non-radioactive, DIG-labelled cDNA probes for detection of TSV may be produced in the laboratory. The ISH method provides greater diagnostic sensitivity than do more traditional methods for TSV detection and diagnosis that employ classic histological methods (Hasson *et al.,* 1999a; Lightner, 1996a; 1999; Lightner & Redman 1998; Mari *et al.,* 1998). The ISH assay of routine histological sections of acute- and transition-phase lesions in the cuticular epithelium, other tissues, and of LOS in transition and chronic phase with a specific DIG-labelled cDNA probe to TSV, provides a definitive diagnosis of infection with TSV (Hasson *et al.,* 1999a; 1999b; Lightner, 1996a; 1996b). Pathognomonic TSV-positive lesions display prominent blue to blue-black areas in the cytoplasm of affected cells when reacted with the cDNA probes. Not reacting to the probe are the prominent karyorrhectic nuclear fragments and pyknotic nuclei that contribute to the pathognomonic 'buckshot riddled' appearance of TS lesions (Lightner, 1996a; Mari *et al.,* 1998). (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.)

False-negative ISH results may occur with Davidson's fixed tissues if tissues are left in fixative for more than 24–48 hours. The low pH of Davidson's fixative causes acid hydrolysis of the TSV single-stranded RNA genome, resulting in false-negative probe results. This hydrolysis can be prevented by avoiding fixation times over 24 hours (Hasson *et al.,* 1997; Lightner, 1996a; Lightner & Redman 1998).

4.7. Immunohistochemistry

Not suitable.

4.8. Bioassay

Confirmation of infection with TSV may be accomplished by bioassay of TSV-suspect animals with SPF juvenile *P. vannamei* serving as the indicator of the virus (Garza *et al.,* 1997; Hasson *et al.,* 1999b; 1995; Lightner, 1996a; Lotz, 1997; Overstreet *et al.,* 1997). Oral or injection protocols may be used. The oral method is relatively simple to perform and is accomplished by feeding chopped carcasses of suspect shrimp to SPF juvenile *P. vannamei* in small tanks (White *et al.,* 2002). The use of a negative control tank of indicator shrimp, which receive only SPF (TSV-free) tissue and normal shrimp feed is required. When the carcass feeding (*per os*) protocol is used to bioassay for TSV, TSV-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 disease and unusual mortalities (Hasson *et al.,* 1999b; Lightner, 1996a; White *et al.,* 2002).

With the injection bioassay protocol, a variety of sample types may be tested for TSV. Whole shrimp are used if they were collected during an outbreak of infection with TSV. Heads only should be used if shrimp display gross transition-phase lesions (multifocal melanised spots on the cuticle) or no clinical signs of infection (chronic phase) as the virus, if present, will be concentrated in the LO (Hasson *et al.,* 1999b; Lightner, 1996a). For non-lethal testing of broodstock, haemolymph samples may be taken and used to expose the indicator shrimp by IM injection (Lightner, 1996a).

4.9. Antibody- or antigen-based detection methods

Not recommended.

4.10. Other methods

4.10.1. Dot-blot immunoassay method

i) For the dot-blot immunoassay method, 1 µl of test antigen (purified virus, infected shrimp haemolymph or SPF shrimp haemolymph) is dotted on to the surface of MA-HA-N45 assay plates (Millipore) [1].

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

ii) After air drying, the wells are blocked for 1 hour at room temperature with 200 µl of a buffer containing phosphate-buffered saline and 0.05% Tween 20 (PBST) mixed with 10% normal goat serum (Life Technologies) and 2% Hammersten casein (Amersham Life Sciences).

iii) The wells are washed three times with PBST and then reacted with 100 µl primary antibody (MAb or mouse polyclonal antibodies) for 30 minutes at room temperature.

iv) Alkaline-phosphatase-labelled goat anti-mouse IgG, γ chain specific, secondary antibody (Zymed) diluted 1/1000 in PBST plus 10% normal goat serum is used for detection (30 minutes at room temperature).

v) After washing three times with PBST, once with PBS and once with distilled water, the reactions are visualised by development for 15 minutes at room temperature with nitroblue tetrazolium and bromo-chloro-indoyl phosphate (Roche Diagnostics in 100 mM Tris-HCl, 100 mM NaCl buffer containing 50 mM $MgCl_2$, pH 9.5.

vi) Reactions are stopped with distilled water.

vii) The reactions are graded using a scale from 0 to +4, with the highest intensity reaction being equivalent to the reaction generated using the MAb against the reference control consisting of semi-purified TSV. A negative reaction is one in which no coloured spot is visible in the well.

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

Real-time RT-PCR is the recommended test for surveillance to demonstrate freedom of infection with TSV in apparently healthy populations as described in Section 4.1.1.

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. ~~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 WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAH Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status [2]

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

6.1.1. Definition of suspect case in apparently healthy animals

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

~~i) Histopathological changes consistent with the presence of the pathogen or the disease~~

i) A positive result by real-time RT-PCR

ii) A positive result by conventional RT-PCR

6.1.2. Definition of confirmed case in apparently healthy animals

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

2 For example transboundary commodities.

i) A positive result by real-time RT-PCR and a positive result by conventional RT-PCR followed by sequencing of the amplicon

~~ii) A positive result by *in-situ* hybridisation and a positive result by real-time RT-PCR~~

~~iii) A positive result by *in-situ* hybridisation and a positive result by conventional RT-PCR followed by amplicon sequencing~~

6.2. Clinically affected animals

No clinical signs are 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 TSV 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) Histopathological changes consistent with TSV infection

iii) Positive result by real-time RT-PCR

iv) Positive result by conventional RT-PCR

v) Positive result of a bioassay

6.2.2. Definition of confirmed case in clinically affected animals

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

i) A positive result by real-time RT-PCR and a positive result by conventional RT-PCR followed by sequencing of the amplicon

ii) A positive result by *in-situ* hybridisation and a positive result by real-time RT-PCR

iii) A positive result by *in-situ* hybridisation and a positive result by conventional RT-PCR followed by amplicon sequencing

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with TSV are provided in Tables 6.3.1 and 6.3.2. (~~none~~ no data are currently available for either). This information can be used for the design of surveys for infection with TSV, 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 surveillance 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.

7. References

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NB: There is a WOAH Reference Laboratory for infection with Taura syndrome virus
(please consult the WOAH Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact WOAH Reference Laboratories for any further information on
infection with Taura syndrome virus

NB: FIRST ADOPTED IN 2006. MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 2.2.8.

INFECTION WITH WHITE SPOT SYNDROME VIRUS

1. Scope

Infection with white spot syndrome virus means infection with the pathogenic agent white spot syndrome virus (WSSV), Genus *Whispovirus,* Family *Nimaviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

WSSV was assigned by the International Committee on Taxonomy of Viruses (ICTV) as the only member of the genus *Whispovirus* within the *Nimaviridae* family. Virions of WSSV are ovoid or ellipsoid to bacilliform in shape, have a regular symmetry, and measure 80–120 nm in diameter and 250–380 nm in length. A flagella-like extension (appendage) may be observed at one end of the virion. WSSV has been divided into three groups: isolates originating from Thailand (WSSV-TH-96-II), isolates originating from India (WSSV-IN-07-I), and another Indian isolate (WSSV-IN-06-I). Most strains of WSSV were speculated to have originated from the Indian Ocean and then spread across the world (Zeng, 2021). Today, although various geographical isolates with genotypic variability have been identified, they are all classified as a single species (WSSV) within the genus *Whispovirus* (Lo *et al.,* 2012; Wang *et al*., 2019).

2.1.2. Survival and stability in processed or stored samples

Viable WSSV was found in frozen commodity shrimp imported to Australia from Southeast Asia (McColl *et al.,* 2004). The virulence of WSSV was retained for 14 months at –80°C in a filtered tissue homogenate prepared from moribund shrimp with hepatopancreas and abdomen removed (Momoyama *et al.,* 1998). The virus originally collected from the haemolymph of moribund shrimp could maintain its virulence for at least 16 months at –80°C (Wu *et al.,* 2002). However, WSSV might be inactivated by multiple freeze-thaw cycles due to damage the viral envelopes or nucleocapsids (Durand *et al.,* 2000; Hasson *et al.,* 2006).

2.1.3. Survival and stability outside the host

The agent is viable for at least 30 days at 30°C in seawater under laboratory conditions (Momoyama *et al.*, 1998); and is viable in ponds for at least 3–4 days (Nakano *et al.*, 1998). Laboratory emulations of drainable and non-drainable ponds suggest that the virus is no longer infective after 21 days of sun-drying or after 40 days in waterlogged pond sediment (Satheesh Kumar *et al*., 2013).

WSSV with an initial viral load of 1000 virions ml^{-1} was found to be viable for a period of 12 days in seawater of 27 ppt salinity, pH of 7.5 at 29–33°C. In shrimp pond sediment (with initial viral load of 211,500 copies g^{-1}), the virus was viable and infective up to 19 days despite sun-drying. In the case of non-drainable conditions, WSSV (753,600 copies g^{-1}) remained infective for a period of 35 days (Satheesh Kumar *et al*., 2013).

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

Of all the species that have been tested to date, no decapod (order Decapoda) crustacean from marine, brackish or freshwater sources has been reported to be refractory to infection with WSSV (Flegel, 1997; Lightner, 1996; Lo & Kou, 1998; Maeda *et al.*, 2000; Stentiford *et al.,* 2009).

[Note: an assessment of species that meet the criteria for listing as susceptible to infection with WSSV in accordance with Chapter 1.5. has not yet been completed]

2.2.2. Species with incomplete evidence for susceptibility

All life stages are potentially susceptible, from eggs to broodstock (Lightner, 1996; Venegas *et al.,* 1999). WSSV genetic material has been detected in reproductive organs (Lo *et al.,* 1997), but susceptibility of the gametes to WSSV infection has not been determined definitively.

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

The best life stages of crustaceans for detection of WSSV are late postlarvae (PL) stages, juveniles and adults. Probability of detection can be increased by exposure to stressful conditions (e.g. eye-stalk ablation, spawning, moulting, changes in salinity, temperature or pH, and during plankton blooms).

2.2.4. Distribution of the pathogen in the host

The major target tissues of WSSV are of ectodermal and mesodermal embryonic origin, especially the cuticular epithelium and subcuticular connective tissues (Momoyama *et al.*, 1994; Wongteerasupaya *et al.*, 1995). Although WSSV infects the underlying connective tissue in the crustacean hepatopancreas and midgut, the tubular epithelial cells of these two organs are of endodermal origin, and they do not become infected.

2.2.5. Aquatic animal reservoirs of infection

Wild decapods known to be reservoirs of infection with WSSV include *Mysis* sp. (Huang *et al.,* 1995), *Acetes* sp., *Alpheus* sp., *Callianassa* sp., *Exopalaemon* sp., *Helice* sp., *Hemigrapsus* sp. *Macrophthalmus* sp., *Macrophthel* sp., *Metaplax* sp., *Orithyia* sp., *Palaemonoidea* sp., *Scylla* sp., *Sesarma* sp., and *Stomatopoda* sp. (Desrina *et al*., 2022; He & Zhou, 1996; Lei *et al.,* 2002). These species can express the disease under suitable environmental conditions. However, non-decapodal crustaceans, such as copepods (Huang *et al.,* 1995), rotifers (Yan *et al.,* 2004), *Balanus* sp. (Lei *et al.,* 2002), *Artemia* (Li *et al*., 2004; Zhang *et al*., 2010) and *Tachypleidue* sp. (He & Zhou, 1996) may be apparently healthy carrier animals. Other marine molluscs, polychaete worms (Vijayan *et al.,* 2005), as well as non-crustacean aquatic arthropods such as sea slaters (*Isopoda*), and Euphydradae insect larvae can mechanically carry the virus without evidence of infection (Lo & Kou, 1998).

2.2.6. Vectors

The harpacticoid copepod *Nitocra* sp. (Zhang *et al*., 2008), microalgae (Liu *et al*., 2007), and the polychaete, *Dendronereis* spp. (Peters) (Desrina *et al*., 2013; Haryadi *et al.,* 2015) are vectors for WSSV.

2.3. Disease pattern

Infection with WSSV sometimes causes clinical disease (Tsai *et al.*, 1999), depending on factors that are poorly understood but related to species tolerance and environmental triggers. With an appropriate infection dose to allow sufficient time before mortality, animals susceptible to disease show large numbers of virions circulating in the haemolymph (Lo *et al.*, 1997), but this may also occur for tolerant species that show no mortality. Thus, high viral loads *per se* do not cause disease or mortality for all susceptible species.

2.3.1. Mortality, morbidity and prevalence

All penaeid shrimp species are highly susceptible to infection with WSSV, often resulting in high mortality. Crabs, crayfish, freshwater prawns, spiny lobsters and clawed lobsters are susceptible to infection with WSSV, but morbidity and mortality as a consequence of infection are highly variable (Lo & Kou, 1998). High level infections with WSSV are known in some decapods in the absence of clinical disease.

Prevalence of infection with WSSV is highly variable, from <1% in infected wild populations to up to 100% in captive populations (Lo & Kou, 1998).

2.3.2. Clinical signs, including behavioural changes

White spots embedded within the exoskeleton are the most commonly observed clinical sign. In most shrimp, these spots range from barely visible to 3 mm in diameter, and they sometimes coalesce into larger plates. However, it should be noted that environmental stress factors, such as high alkalinity, or bacterial disease can also cause white spots on the carapace of shrimp, and that moribund shrimp with infection with WSSV may have few, if any, white spots. Therefore, the appearance of white spots is not a reliable diagnostic sign of infection with

WSSV infection. High degrees of colour variation with a predominance of reddish or pinkish discoloured shrimp are seen in diseased populations.

WSSV infections can be subclinical or manifest as clinical disease. The penaeid shrimp in aquaculture will generally show clinical signs associated with high morbidity and mortality. Some animals may die without showing any clinical signs. Non-penaeid species (e.g. crab, lobster) generally have subclinical infections under natural conditions.

The affected animals can show lethargy, decreased or absent feed consumption and abnormal swimming behaviour – slow swimming, swimming on side, swimming near water surface and gathering around edges of rearing units (Corbel *et al.,* 2001; Sahul Hameed *et al.,* 1998; 2001). A very high mortality rate in the shrimp population can be expected within a few days of the onset of behavioural signs.

2.3.3 Gross pathology

In addition to the clinical and behavioural signs in Section 2.3.2. above, the following gross pathology has been reported in clinically affected penaeid shrimp: loosened attachment of the carapace with underlying cuticular epithelium (Sanchez-Paz, 2010), so that the carapace can be easily removed (Zhan *et al.,* 1998); empty gastro-intestinal tract due to anorexia (Escobedo-Bonilla, 2008); delayed clotting of haemolymph (Heidarieh *et al.,* 2013); excessive fouling of gills (Wu *et al.,* 2013) and exoskeleton.

2.3.4. Modes of transmission and life cycle

Infection with WSSV can be transmitted horizontally by consumption of infected tissue (e.g. cannibalism, predation, fomites, etc.), by water-borne routes, and by other routes of transmission (e.g. via sea birds, anthropogenic movements, feeding, rotifer, copepods, etc) (Haryadi *et al.,* 2015; Vanpatten *et al.,* 2004; Zhang *et al.,* 2006; 2008). Transmission of WSSV can occur from apparently healthy animals in the absence of disease. Dead and moribund animals can be a source of disease transmission (Lo & Kou, 1998). Microalgae could serve as a potential horizontal transmission pathway for WSSV (Liu *et al.,* 2007).

True vertical transmission (intra-ovum) of WSSV to the progeny has not been demonstrated.

In-vitro studies with primary cell cultures and *in-vivo* studies with postlarvae show that the replication cycle is approximately 20 hours at 25°C (Chang *et al.*, 1996; Chen *et al.*, 2011; Wang *et al.*, 2000).

2.3.5. Environmental factors

Disease outbreaks may be induced by stressors, such as rapid changes in salinity. Water temperature has a profound effect on disease expression, with average water temperatures of between 18 and 30°C being conducive to WSSV outbreaks (Song *et al.*, 1996; Vidal *et al.*, 2001). Under experimental challenge condition, WSSV-induced mortality in shrimp is reduced when the temperature increases above 32°C (Vidal *et al.,* 2001).

2.3.6. Geographical distribution

Infection with WSSV has been identified from crustaceans in Asia, the Mediterranean (Stentiford & Lightner, 2011), the Middle East, Oceania (Moody *et al*., 2022) and the Americas. Zones and compartments free from infection with WSSV are known within these regions (Lo *et al.,* 2012).

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

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

No consistently effective vaccination methods have been developed for infection with WSSV.

2.4.2. Chemotherapy including blocking agents

No published or validated methods.

2.4.3. Immunostimulation

Several reports have shown that beta-glucan, vitamin C, seaweed extracts (fucoidan) and other immunostimulants may improve resistance to infection with WSSV (Chang *et al.*, 2003; Chotigeat *et al.*, 2004).

2.4.4. Breeding resistant strains

Progress in breeding *P. vannamei* for resistance to infections with WSSV has been reported (Cuellar-Anjel *et al.,* 2012; Huang *et al.,* 2012).

2.4.5. Inactivation methods

Method	Treatment	Reference
Heat	55°C/90 min 70°C/5 min	Chang *et al*., 1998
	50°C/60 min 60°C/1 min 70°C/0.2 min	Nakano *et al.*, 1998
pH	pH 3/60 min pH 12/10 min	Chang *et al*., 1998; Balasubramanian *et al.,* 2006
UV	9.30×10^5 µWs/cm^2	Chang *et al*., 1998
Ozone	0.5 µg ml^{-1}/10 min	Chang *et al*., 1998
Chlorine	100 ppm/10 min	Chang *et al*., 1998; Balasubramanian *et al.,* 2006
Iodophore	100 ppm/10 min	Chang *et al*., 1998

2.4.6. Disinfection of eggs and larvae

For transovum transmission, disinfection of egg is likely to be effective (Lo & Kou, 1998), but this has not yet been confirmed in formal scientific trials.

2.4.7. General husbandry

Management practices in endemic areas principally involve the exclusion of WSSV from production populations or avoiding risk factors for development of clinical disease. Examples include avoiding stoc king in the cold season, use of specific pathogen-free (SPF) or polymerase chain reaction (PCR)-negative seed stocks, use of biosecure water and culture systems (Withyachumnarnkul, 1999). Polyculture of shrimp and fish has been proposed to reduce WSSV transmission in infected populations (Wang *et al.*, 2021).

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 or exhibit behavioural changes (Sections 2.3) should be selected for detection of WSSV.

3.2. Selection of organs or tissues

Tissue tropism analysis from both experimentally infected shrimp and wild-captured brooders shows that tissues originating from the ectoderm and mesoderm, especially the cuticular epithelium and subcuticular connective tissues, as well as other target tissues (e.g. antennal gland, haematopoietic organ, etc.), are the main target tissues for infection with WSSV. Samples from the pleopods, gills, haemolymph, stomach or abdominal muscle are recommended for submission (Lo *et al.*, 1997).

3.3. Samples or tissues not suitable for pathogen detection

Although WSSV infects the underlying connective tissue in the shrimp hepatopancreas and midgut, the columnar epithelial cells of these two organs are of endodermal embryonic origin (Lo *et al.*, 1997) and are not appropriate tissues for detection. The compound eye may contain a PCR inhibitor (Lo *et al.*, 1997) and is therefore not suitable for PCR-based diagnosis.

3.4. Non-lethal sampling

Gill, haemolymph or pleopod are suitable tissues for non-lethal sampling and screening by PCR.

If non-lethal tissue sample types differ from recommended tissues (see Section 3.2.), or from the tissue samples used in validation studies, the effect on diagnostic performance should be considered.

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 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 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 histological techniques can be found in Section B.5.5. 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 Chapter 2.2.0 *General information* (diseases of crustaceans).

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 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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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				
Cell culture												
Real-time PCR	+++	+++	+++	4	+++	+++	+++	4	+++	+++	+++	4
Conventional PCR	++	++	++	2	++	++	++	2				
Conventional PCR followed by amplicon sequencing									+++	+++	+++	2
In-situ hybridisation					+	+	+	1	+	+	+	1
Bioassay					+	+	+	1				
LAMP	++	++	++	1	++	++	++	1	+	+	+	1
Ab-ELISA					+	+	+	1				
Ag-ELISA					+	+	+	1				
Other antigen detection methods					+	+	+	1				
Other methods												

LV = level of validation, refers to the stage of validation in the WOAH 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; IFAT = indirect fluorescent antibody test.
[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.
Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

Demonstration of hypertrophied nuclei in squash preparations of the gills and/or cuticular epithelium, which may be stained or unstained.

T-E staining

A T-E staining solution may be prepared from Trypan blue 0.6%, Eosin Y 0.2%, NaCl 0.5%, phenol 0.5%, and glycerol 20% (Huang & Yu, 1995) and used as follows:

i) Place a piece of diseased tissue (e.g. a piece of gill or stomach epithelium without the cuticle) on a slide and mince with a scalpel.

ii) Add 1–2 drops of the T-E staining solution to the minced tissue, mix and allow to stain for 3–5 minutes.

iii) Lay a cover glass over the stained tissue and cover with several pieces of absorbent paper. Use a thumb to squash the mince into a single layer of cells.

If the sample was taken from a heavily infected shrimp, hypertrophied nuclei and intranuclear eosinophilic or vacuolation-like inclusion bodies can be observed using light microscopy (400–1000× magnification).

4.2. Histopathology and cytopathology

Smears

Demonstration of aggregates of WSSV virions in unstained smear preparations of haemolymph by dark-field microscopy.

NOTE: This is the simplest of the microscopic techniques and is recommended for people with limited expertise in diagnosing infection with WSSV. The aggregates appear as small reflective spots of 0.5 µm in diameter (Momoyama *et al.*, 1995).

Fixed sections

Histological changes commonly reported in susceptible species include: Hypertrophied nuclei with marginated chromatin material in virus-infected cells; eosinophilic to pale basophilic (with haematoxylin & eosin stain) stained intranuclear viral inclusions within hypertrophied nuclei and multifocal necrosis associated with pyknotic and karyorrhectic nuclei in affected tissues of ectodermal and mesodermal origin. The infection with infectious hypodermal and hematopoietic necrosis virus, another DNA virus, produces similar inclusions that need to be differentiated from those of WSSV.

4.3. Cell culture for isolation

WSSV can be isolated from primary cultures of lymphoid or ovary cells. However, it is NOT recommended to use cell culture as a routine isolation method because of: 1) the high risk of contamination, and, 2) the composition of the medium varies depending on the tissue type, host species and experimental purpose; that is, to date there is no standard or recognised medium that can be recommended. As primary cell culture is so difficult to initiate and maintain for virus isolation purposes, bioassay should be the primary means for virus propagation.

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 5.5 *Use of molecular and antibody-based techniques for confirmatory testing and diagnosis* of Chapter 2.2.0 *General information* (diseases of crustaceans). Each sample should be tested in duplicate.

Extraction of nucleic acids

~~Numerous~~ Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~using optical density or running a gel~~.

4.4.1. Real-time PCR

The real-time PCR methods described by Durand & Lightner (2002) and Sritunyalucksana *et al.* (2006) are described here as modified and validated by Moody *et al.,* (2022).

Pathogen/ Target	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1: Durand & Lightner, 2002[1]; GenBank Accession No.: NC_003225			
WSSV/ ~~ORF X~~Capsid protein	Fwd WSS1011F: TGG-TCC-CGT-CCT-CAT-CTC-AG Rev WSS1079R: GCT-GCC-TTG-CCG-GAA-ATT-A Probe: 6FAM-AGC-CAT-GAA-GAA-TGC-CGT-CTA-TCA-CAC-A-TAMRA	900 nM 900 nM 250 nM	~~45 cycles of: 95°C/15 sec and 60°C/1 min~~ 50°C/2 min, 95°C/10 min, then 45 cycles of: 94°C/15 sec and 60°C/1 min
Method 2: Sritunyalucksana, 2006[1]; GenBank Accession No.: AF440570			
WSSV/ ~~ORF X~~Capsid protein	Fwd CSIRO WSSV-F: CCG-ACG-CCA-AGG-GAA-CT Rev CSIRO WSSV-R: TTC-AGA-TTC-GTT-ACC-GTT-TCC-A Probe: 6FAM-CGC-TTC-AGC-CAT-GCC-AGC-CG-TAMRA	900 nM 900 nM 250 nM	~~45 cycles of: 95°C/15 sec and 60°C/1 min~~ 50°C/2 min, 95°C/10 min, then 45 cycles of: 94°C/15 sec and 60°C/1 min

[1]Method described here as modified and validated by Moody *et al.,* 2022

4.4.2. Conventional PCR

Pathogen/ Target	Primer~~/probe~~ (5'–3')	Concentration	Cycling parameters
Method 1: Lo *et al.*, 1996a; GenBank Accession No.: AF440570; amplicon size: 1447/941 bp			
WSSV ~~(nested PCR)~~	~~Outer~~ Primary Fwd 146F1: ACT-ACT-AAC-TTC-AGC-CTA-TCTAG Rev 146R1: TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A	100 pmol 100 pmol	39 cycles of 94°C/1 min, 55°C/1 min, 72°C/2 min
	~~Inner~~ Nested Fwd 146F2: GTA-ACT-GCC-CCT-TCC-ATC-TCC-A Rev 146R2: TAC-GGC-AGC-TGC-TGC-ACC-TTG	100 pmol 100 pmol	39 cycles of 94°C/1 min, 55°C/1 min, 72°C/2 min

Commercial PCR kits are available. Please consult the WOAH Register for kits that have been certified by WOAH (https://www.woah.org/en/what-we-offer/veterinary-products/#ui-id-5).

4.4.3. Loop-mediated isothermal amplification (LAMP) method

The protocol described here is from Kono *et al.* (2004). The LAMP method is sensitive and rapid, and it amplifies the target nucleic acids under isothermal conditions, therefore needing no sophisticated machine for thermal cycling.

DNA extraction

DNA extraction could be performed according to the protocol described in Section 4.4.2 *Conventional PCR* or by other suitable methods or by commercial kits.

LAMP reaction

i) Add DNA to a tube to set up a 25 µl reaction mixture (20 mM Tris/HCl, pH 8.8, 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8M Betaine, 1.4 mM of each dNTP, 40 pmol of WSSV-FIP and -BIP primers, 5 pmol of WSSV-F3 and -B3 primers).

ii) The primer sequences are WSSV-FIP: 5'-GGG-TCG-TCG-AAT-GTT-GCC-CAT-TTT-GCC-TAC- GCA-CCA-ATC-TGT-G-3', WSSV-BIP: 5'-AAA-GGA-CAA-TCC-CTC-TCC-TGC-GTT-TTA-GAA-CGG-AAG-AAA-CTG-CC-TT-3', WSSV-F3: ACG-GAC-GGA-GGA-CCC-AAA-TCG-A-3', WSSV-B3: 5'-GCC-TCT-GCA-ACA-TCC-TTT-CC-3'.

iii) Heat the mixture at 50°C for 5 minutes and at 95°C for 5 minutes, then chill on ice, and add 1 µl (8 U) of *Bst* DNA polymerase.

iv) Incubate the mixture at 65°C for 60 minutes, and then terminate the reaction at 80°C for 10 minutes.

v) To visualise, electrophorese 2 µl LAMP reaction products on 2% agarose gels containing ethidium bromide at a concentration of 0.5 µg ml^{-1}. This reaction produces WSSV-specific LAMP products with multiple bands of various sizes from approximately 200 bp to the loading well.

Reliable LAMP commercial kits may be an alternative for WSSV diagnosis.

4.5. Amplicon sequencing

The size of the PCR amplicon ~~is~~ should be verified, for example by agarose gel electrophoresis~~, and purified by excision from this gel~~. Both DNA strands of the PCR product must be sequenced and analysed ~~and compared~~ in comparison with ~~published~~ reference sequences.

4.6. *In-situ* hybridisation

Use of WSSV-specific DNA probes with histological sections is useful to demonstrate the presence of WSSV nucleic acid in infected cells (Nunan & Lightner, 1997). See Chapter 2.2.0 Section 5.5.4 for general comments on *in-situ* hybridisation.

4.7. Immunohistochemistry

See Section 4.9.

4.8. Bioassay

If SPF shrimp are available, the bioassay method based on Nunan *et al.* (1998) and Durand *et al*. (2000), is suitable for WSSV diagnosis.

4.9. Antigen detection methods

Both polyclonal and monoclonal antibodies raised against either the virus or a recombinant viral structural protein have been used in various immunological assays including western blot analysis, immunodot assay, indirect fluorescent antibody test (IFAT), immunohistochemistry (IHC) or enzyme-linked immunosorbent assay (ELISA) to detect WSSV (Huang *et al*., 1995; Poulos *et al.*, 2001; Sithigorngul *et al.*, 2006; Yoganandhan *et al.*, 2004).

4.10. Other methods

Lateral flow tests are commercially available but their performance needs to be evaluated before they can be recommended.

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

Real-time PCR is the recommended test for surveillance to demonstrate freedom of infection with WSSV in apparently healthy populations as described in Section 4.4.1.

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 WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAH Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status [1]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Hydrographical 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 WSSV 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

iii) Positive result by LAMP method

6.1.2. Definition of confirmed case in apparently healthy animals

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

i) Positive results by real-time PCR and conventional PCR followed by amplicon sequencing

ii) Positive results by LAMP and conventional PCR method followed by amplicon sequencing

~~iii) Positive results by *in-situ* hybridisation and detection of WSSV by real-time PCR~~

~~iv) Positive results by *in-situ* hybridisation and detection of WSSV by conventional PCR followed by amplicon sequencing~~

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 WSSV shall be suspected if at least one of the following criteria is met:

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

ii) Histopathology consistent with WSSV infection

iii) Positive result by conventional PCR

iv) Positive result by real-time PCR

v) Positive result by LAMP method

vi) Positive result by *in-situ* hybridisation

6.2.2. Definition of confirmed case in clinically affected animals

1 For example transboundary commodities.

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

i) Positive results by real-time PCR and conventional PCR followed by amplicon sequencing

ii) Positive results by LAMP and conventional PCR method followed by amplicon sequencing

iii) Positive results by *in-situ* hybridisation and detection of WSSV by real-time PCR

iv) Positive results by *in-situ* hybridisation and detection of WSSV by conventional PCR followed by amplicon sequencing

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with WSSV are provided in Tables 6.3.1. and 6.3.2. This information can be used for the design of surveys for infection with WSSV, 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 (Durand & Lightner, 2002)	Diagnosis	Clinically diseased shrimp from farms	Gill, pleopod	*Penaeus monodon*	100% (*n*=71)	100% (*n*=71)	Real-time PCR	Moody *et al.,* 2022
Real-time PCR (Sritunyalucksana *et al.,* 2006)	Diagnosis	Clinically diseased shrimp from farms	Gill, pleopod	*Penaeus monodon*	100% (*n*=71)	100% (*n*=71)	Real-time PCR	Moody *et al.,* 2022

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study,
PCR: = polymerase chain reaction.
*The nested PCR (Lo *et al.,* 1996a) is linked to false positives for WSSV when they are used to test species of *Cherax quadricarinatus* (Claydon *et al.,* 2004).

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**
Real-time PCR (Durand & Lightner, 2002)	Surveillance in apparently healthy animals	Wild populations of crustaceans	Gill, pleopod	*Penaeus merguiensis, P. esculentus, P. plebejus, Metapenaeus endeavouri, M. bennettae*	76.8% (*n*=1591)	99.7% (*n*=1591)	Bayesian latent class analysis	Moody *et al.,* 2022
Real-time PCR (Sritunyalucksana et al., 2006)	Surveillance in apparently healthy animals	Wild populations of crustaceans	Gill, pleopod	*Penaeus merguiensis, P. esculentus, P. plebejus, Metapenaeus endeavouri, M. bennettae*	82.9% (*n*=1591)	99.7% (*n*=1591)	Bayesian latent class analysis	Moody *et al.,* 2022
Two real-time PCR methods in parallel (Sritunyalucksana *et al.,* 2006 and Durand & Lightner, 2002)	Surveillance in apparently healthy animals	Wild populations of crustaceans	Gill, pleopod	*Penaeus merguiensis, P. esculentus, P. plebejus, Metapenaeus endeavouri, M. bennettae*	98.3% (*n*=1591)	99.4% (*n*=1591)	Bayesian latent class analysis	Moody *et al.,* 2022

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 WOAH Reference Laboratories for infection with white spot syndrome virus
(please consult the WOAH web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
infection with white spot syndrome virus

NB: FIRST ADOPTED IN 1997 AS WHITE SPOT DISEASE. MOST RECENT UPDATES ADOPTED IN 2018.

Annex 28. Item 10.2.1. – Chapter 2.3.1. Infection with *Aphanomyces invadans* (epizootic ulcerative syndrome)

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 *A. 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

[Note: an assessment of species that meet the criteria for listing as susceptible to infection with *A. invadans* in accordance with Chapter 1.5. has not yet been completed]

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
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

Family	Scientific name	Common name
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*	wels 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
	Therapon sp.	therapon
Toxotidae	*Toxotes chatareus*	common archerfish
	Toxotes lorentzi	primitive acherfish

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 (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*), revealed resistance to *A. invadans* (Pradhan *et al.,* 2007), even though they are naturally susceptible species. Experimental infections demonstrated that goldfish (*Carassius auratus*) are susceptible (Hatai *et al.,* 1977; 1994), but common carp (*Cyprinus carpio*) (Wada *et al.,* 1996), Nile tilapia (*Oreochromis niloticus*) (Khan *et al.,* 1998) and European eel (*Anguilla anguilla*), (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 *A.~~phanomyces~~ 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.

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

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~~ There are no published protocols for *A. invadans* 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.

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).

3.3. 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~~ *A. invadans* 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 have been evaluated and found to be suitable. The effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, therefore, larger animals should be processed and tested individually ~~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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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~~ Clinical signs	±	±	±	NA	+	+	+	NA				
Squash mounts					±	±	±	1	±	±	±	1
Histopathology					++	++	++	1	++	++	++	1
Cytopathology												
Cell or artificial media culture					++	++	++	1	+	+	+	1
Real-time PCR												
Conventional PCR					++	++	++	1				
Conventional PCR followed by amplicon sequencing									+++	+++	+++	1
In-situ hybridisation									++	++	++	1
Bioassay												
LAMP												
Ab ELISA												
Ag ELISA												
Other antigen detection methods												
Other method												

LV = level of validation, refers to the stage of validation in the WOAH 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.

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~~ Observation for clinical signs

Using observational data of clinical signs (see Section 2.3.2 *Clinical signs, including behavioural changes*) for targeted surveillance, a 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*. 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.

4.2. 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~~3. 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~~3.1. 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~~3.2 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~~4. Cell culture for isolation

4.~~3~~4.1. 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 glucose/yeast extract (GY) agar (see Table 4.1) and transferred to a fresh GY agar tube once every 1–2 weeks (Hatai & Egusa, 1979).

4.~~3~~4.2. 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~~4.3. 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 glucose/peptone/yeast (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 4.4.3.1), 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 4.4.3.1. *Media for isolation, growth and sporulation of* Aphanomyces invadans *cultures*

GP (glucose/peptone) medium	GPY (glucose/peptone/ yeast) broth	GPY agar	GY agar (glucose/ yeast)	Autoclaved pond water
3 g $litre^{-1}$ glucose 1 g $litre^{-1}$ peptone 0.128 g $litre^{-1}$ $MgSO_4.7H_2O$ 0.014 g $litre^{-1}$ KH_2PO_4 0.029 g $litre^{-1}$ $CaCl_2.2H_2O$ 2.4 mg $litre^{-1}$ $FeCl_3.6H_2O$ 1.8 mg $litre^{-1}$ $MnCl_2.4H_2O$ 3.9 mg $litre^{-1}$ $CuSO_4.5H_2O$ 0.4 mg $litre^{-1}$ $ZnSO_4.7H_2O$	GP broth + 0.5 g $litre^{-1}$ yeast extract	GPY broth + 12 g $litre^{-1}$ technical agar	1% glucose, 0.25% yeast extract, 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

PCR assays should always be run with the controls specified in Section 2.5 *Use of molecular techniques for surveillance testing, confirmatory testing and diagnosis* of Chapter 2.3.0 *General information* (diseases of fish). Each sample should be tested in duplicate.

Extraction of nucleic acids

~~Numerous~~ Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~using optical density or running a gel~~.

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*. Oidtmann *et al.* (2008) demonstrated cross reactivity of the Phadee *et al*. (2004b) assay with *A. frigidophilus* when more than 10 ng of template DNA of *A. frigidophilus* was used in the PCR.

Pathogen/ target gene	Primer~~/probe~~ (5'–3')	Concentration	Cycling parameters
Method 1: Vandersea *et al.*, 2006); GenBank Accession No.: AF396684; ~~Product~~ amplicon size: 234bp			
Aphanomyces invadans (ITS1)	Fwd Ainvad-2F: TCA-TTG-TGA-GTG-AAA-CGG-TG Rev Ainvad-ITSR1: GCT-AAG-GTT-TCA-GTA-TGT-AG	0.025 nM 0.025 nM	35 cycles: 95°C/30 sec, 56°C/45 sec, 95°C/30 sec, 72°C/2.5 min, 95°C/30 sec
Method 2: Phadee *et al.*, 2004b; GenBank Accession No.: ~~AF396683~~ AF396684; ~~Product~~ amplicon size: 550bp			
Aphanomyces invadans (ITS1-ITS2)	Fwd ITS11: GCC-GAA-GTT-TCG-CAA-GAA-AC Rev ITS23: CGT-ATA-GAC-ACA-AGC-ACA-CCA	500 nM 500 nM	35 cycles: 94°C/30 sec, 65°C/45 sec, 72°C/1 min
Method 3: Oidtmann *et al.*, 2008; GenBank Accession No.: EU422990; ~~Product~~ amplicon size: 564			
Aphanomyces invadans (ITS1-ITS2)	Fwd BO73: CTT-GTG-CTG-AGC-TCA-CAC-TC Rev BO639: ACA-CCA-GAT-TAC-ACT-ATC-TC	600 nM 600 nM	35 cycles: 96°C/1 min, 58°C/1 min, 72°C/1 min

~~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).

The size of the PCR amplicon ~~is~~ should be verified, for example by agarose gel electrophoresis~~, and purified by excision from this gel~~. Both DNA strands of the PCR product must be sequenced and analysed ~~and compared~~ in comparison with ~~published~~ reference sequences.

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~~ were 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* (as described in Section 4.1 *Observation for clinical signs*). ~~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. If a Competent Authority does not have the capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAH Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free. There are currently no WOAH Reference Laboratories designated for EUS.

6.1. Apparently healthy animals or animals of unknown health status [1]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. ~~Geographical~~ Hydrographical 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*[2]

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 of hyphae characteristic (direct or by microscopy) of *A. invadans*

v) Culture and isolation of *A. invadans*-type colonies

6.2.2. Definition of confirmed case in clinically affected animals

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

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1 For example transboundary commodities.

2 Note that surveillance of apparently healthy populations for EUS is based on examination of target populations for clinical signs of infection with *A. invadans* (see Section 5 *Test[s] recommended for surveillance to demonstrate freedom in apparently healthy populations*).

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. and 6.3.2. (no data are currently available for either). 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 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 currently (2022) no WOAH Reference Laboratories for infection with *Aphanomyces invadans*
(please consult the WOAH web site for the most up-to-date list:
https://www.woah.org/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.

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

None known

~~*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

None demonstrated. ~~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). European perch from distinct geographical areas with and without a history of EHNV have been tested under experimental conditions and have demonstrated susceptibility to EHN (Becker *et al.,* 2016). 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 (Becker *et al.,* 2019; 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 and sporadic outbreaks involving small numbers of European perch (Becker *et al,* 2019; Whittington *et al*., 2010).

See WOAH WAHIS (https://wahis.woah.org/#/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 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 i.e. European perch where these are available, otherwise rainbow trout or the other susceptible species listed in Section 2.2.1 should be sampled proportionally.

ii) Risk-based criteria should be employed to preferentially sample epidemiological units ~~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~~ apparently healthy fish collected in such a way that all parts of the farm or affected waterbody 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

~~Non-lethal samples (blood, fin, gill, integument or mucous) are unsuitable for testing EHNV~~ Not applicable.

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.

WOAH 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 WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. *WOAH 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~~ 1	++ ~~+~~	++ ~~+~~	+++	~~2~~ 1	± ~~+~~	± ~~+~~	++	~~2~~ 1
Immunohistochemistry					+	+	+	1				
Real-time PCR	+++	+++	+++	~~2~~ 1	+++	+++	+++	2	++	++	++	~~2~~ 1
Conventional PCR	+	+	+	1	++	++	++	1	~~++~~	~~++~~	~~++~~	~~1~~
Conventional PCR followed by 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 WOAH 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.

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~~ lines for virus isolation ~~/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; and BF-2, EPC, or CHSE 214 incubated at 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 WOAH 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 and amplicon sequencing.~~

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.

The identity of viruses in cell culture is determined by PCR and amplicon sequencing.

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 for the detection of ranaviruses (Jaramillo *et al.,* 2012; Pallister *et al,* 2007; Stilwell *et al.,* 2018), EHNV can only be detected when these methods are combined with methods that specifically detect EHNV. ~~none has been adequately 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.~~

PCR assays should always be run with the controls specified in Section 2.5 *Use of molecular techniques for surveillance testing, confirmatory testing and diagnosis* of Chapter 2.3.0 *General information* (diseases of fish). Each diagnostic sample should be tested in duplicate, i.e. by testing two aliquots.

Extraction of nucleic acids

~~Numerous~~ Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and ~~should~~ can be checked using a suitable method as appropriate to the circumstances ~~using optical density or running a gel~~.

4.4.1. Real-time PCR

The ranavirus real-time screening protocol in use at the WOAH Reference Laboratory is based on Pallister *et al.,* 2007. 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) detects multiple ranavirus species using hydrolysis probe detection chemistry.

~~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**~~		~~Jaramillo *et al.*, 2012~~
~~C1096~~	~~GAC TGA CCA ACG CCA GCC TTA ACG~~	
~~C1097~~	~~GCG GTG GTG TAC CCA GAG TTG TCG~~	
~~**Primer**~~		~~Stilwell *et al.*, 2018~~
~~RanaF1~~	~~CCA GCC TGG TGT ACG AAA ACA~~	
~~RanaR1~~	~~ACT GGG ATG GAG GTG GCA TA~~	
~~**Probe**~~		
~~RanaP1~~	~~6FAM TGG GAG TCG AGT ACT AC MGB~~	

~~*Primer and probe sequences*~~

Pathogen / target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1 (Pallister *et al.*, 2007); GenBank Accession No.: DQ457105			
Ranavirus/MCP	Fwd: RANA CON: CTC-ATC-GTT-CTG-GCC-ATC-A Rev: RANA CON: TCC-CAT-CGA-GCC-GTT-CA Probe: RANA CON Pr FAM-CAC-AAC-ATT-ATC-CGC-ATC-MGB	900 nM for each primer, 250 nM for probe	45 cycles of 95°C/15 sec; 60°C/60 sec
Method 2 (Jaramillo *et al.*, 2012); GenBank Accession No.:			
Ranavirus/MCP	C1096 GAC-TGA-CCA-ACG-CCA-GCC-TTA-ACG C1097 GCG-GTG-GTG-TAC-CCA-GAG-TTG-TCG	12.5 pM for each primer	40 cycles of 95°C/30 sec; 58°C/30 sec
Method 3 (Stilwell *et al.*, 2018); GenBank Accession No.:			

Ranavirus/MCP	Fwd: RanaF1: CCA-GCC-TGG-TGT-ACG-AAA-ACA Rev: RanaR1 ACT-GGG-ATG-GAG-GTG-GCA-TA Probe: RanaP1 FAM-TGG-GAG-TCG-AGT-ACT-AC-MGB	900 nM for each primer, 250 nM for probe	40 cycles of 95°C/30 sec; 60°C/45 sec

~~The ranavirus real-time screening protocol in use at the OIE Reference Laboratory, based on Pallister *et al.*, 2007. 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) detects multiple ranavirus species using hydrolysis probe detection chemistry.~~

~~Details of the controls to be run with each assay are set out in Section 5.5. of Chapter 2.2.1. of Section 2.2.~~

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. Both MCP1 and MCP2 target a region within the capsid protein gene (Marsh *et al.*, 2002).

~~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 μl^{-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}~~

Ingredients	Amount	Final concentration in 50 µl PCR mix
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. 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.

Primer and probe sequences

Pathogen / target gene	Primer/probe (5'–3')	Concentration	Cycling parameters
Method 1 (Marsh *et al.*, 2002): Product amplicon size MCP-1 is 321 bp and product amplicon size MCP-2 is 625 bp			
MCP-1 Gene location: 266-586 MCP-2 Gene location: 842-1466	M151: AAC-CCG-GCT-TTC-GGG-CAG-CA M152: CGG-GGC-GGG-GTT-GAT-GAG-AT M153: ATG-ACC-GTC-GCC-CTC-ATC-AC MI54: CCA-TCG-AGC-CGT-TCA-TGA-TG	250 ng of each primer	35 cycles of 50°C for 30 sec NOTE: the annealing temperature may be increased to 60 or 62°C to reduce non-specific amplification when the assay is used to test fish tissues.

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.

The size of the PCR amplicon is should be verified, for example by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands of the PCR product must be sequenced and analysed and compared in comparison with published reference sequences.

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 [1]. The primary affinity purified ~~rabbit~~ anti-EHNV antibody (Lot No. M708) is supplied freeze-dried by the WOAH 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 antibody 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 WOAH Reference Laboratory.

4.8. Bioassay

1 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 http://www.dakosytomahon.com for links to other countries.

Not applicable.

4.9. Antibody- or antigen-based detection methods

An antigen ELISA for detection of EHNV and an EHNV antibody detection ELISA have been ~~described~~ reported (Whittington & Steiner, 1993). 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 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 EHNV from other 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 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 WOAH Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a ~~laboratory~~ Competent Authority does not have the ~~capacity~~ capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAH Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status [2]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link to an infected population. ~~Geographic~~ Hydrographical 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) EHNV-typical CPE in cell culture ~~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

[2] For example transboundary commodities.

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;

ii) A positive result in tissue samples by real-time PCR and identification of EHNV by conventional PCR followed by 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 followed by 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. (no data are currently available). 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	94.3%* (*n* = 105)	100% (*n* = 441)	Virus isolation in BF-2 cell culture	Jaramillo *et al.,* (2012)

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
				perch (*Macquaria australasica*) rainbow trout (*Oncorhynchus mykiss*)				
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*)	95%* (*n* = 106)	100% (*n* = 80)	Virus isolation in BF-2 cell culture	Stilwell *et al.,* 2018

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 a WOAH Reference Laboratory for infection with epizootic haematopoietic necrosis virus (EHNV)
(please consult the WOAH web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on infection with EHNV.
The WOAH 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.

CHAPTER 2.3.9.

INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

[…]

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with SVCV according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are:

Family	Scientific name	Common name
Cyprinidae	*Abramis brama*	~~B~~bream
	Aristichthys nobilis	~~B~~bighead carp
	Carassius auratus	~~G~~goldfish
	Ctenopharyngodon idella	~~G~~grass carp
	Cyprinus carpio	~~C~~common carp (all varieties and subspecies)
	Danio rerio	~~Z~~zebrafish
	Notemigonus crysoleucas	~~G~~golden shiner
	Pimephales promelas	~~F~~fathead minnow
	Percocypris pingi	Jinsha ~~bass~~barbel carp
	Rutilus kutum	Caspian white fish
	Rutilus rutilus	~~R~~roach
Siluridae	*Silurus glanis*	Wels catfish

[…]

Annex 31. Item 10.3.1. – Section 2.2.1. and 2.2.2. of Chapter 2.4.2. Infection with *B. exitiosa*

CHAPTER 2.4.2.

INFECTION WITH *BONAMIA EXITIOSA*

[…]

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with *Bonamia exitiosa* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are: Argentinean flat oyster (*Ostrea puelchana*), Ariake cupped oyster (*Magallana* (syn. *Crassostrea*) *ariakensis*), Australian mud oyster (*Ostrea angasi*), Chilean flat oyster (*Ostrea chilensis*), crested oyster (*Ostrea equestris*), eastern oyster (*Crassostrea virginica*), European flat oyster (*Ostrea edulis*)~~,~~ and Olympia oyster (*Ostrea lurida*) ~~and Suminoe oyster (*Magallana* (syn. *Crassostrea*) *ariakensis*)~~.

2.2.~~1~~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 *B. exitiosa* according to Chapter 1.5 of the *Aquatic Code* are: dwarf oyster (*Ostrea stentina*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: Pacific cupped oyster (*Magallana* [syn. *Crassostrea*] *gigas*) and Sydney rock oyster (*Saccostrea glomerata*).

[…]

Annex 32. Item 10.3.1. – Section 2.2.1. and 2.2.2. of Chapter 2.4.3. Infection with *B. ostreae*

CHAPTER 2.4.3.

INFECTION WITH *BONAMIA OSTREAE*

[. . .]

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with *Bonamia ostrea* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are: Ariake cupped oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*), European flat oyster (*Ostrea edulis*)~~,~~ and Chilean flat oyster (*Ostrea chilensis*)~~, and Suminoe oyster (*Magallana* [syn. *Crassostrea*] *ariakensis*)~~.

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 *B. ostreae* according to Chapter 1.5 of the *Aquatic Code* are: Argentinean flat oyster (*Ostrea puelchana*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: beadlet anemone (*Actina equina*), brittle star (*Ophiothrix fragilis*), European sea squirt (*Ascidiella aspersa*), grouped zooplankton and Pacific cupped oyster (*Magallana* [syn. *Crassostrea*] *gigas*).

[…]

Annex 33. Item 10.3.2. – Section 2.2.1. and 2.2.2. of Chapter 2.4.4. Infection with *M. refringens*

CHAPTER 2.4.4.

INFECTION WITH *MARTEILIA REFRINGENS*

[…]

2.2. Host factors

2.2.1. Susceptible host species

~~*Oyster* species: *Ostrea edulis* (Grizel *et al*., 1974); and mussel species: *Mytilus* species including *M. edulis* (Le Roux *et al*., 2001) and *M. galloprovincialis* (López-Flores *et al*., 2004; Novoa *et al*., 2005; Robledo *et al*., 1995a; Villalba *et al*., 1993b).~~

~~Infection with *M. refringens* was demonstrated in the oyster *Ostrea stentina*, the clam species *Solen marginatus* (López-Flores *et al*., 2008a) and *Chamelea gallina* (López-Flores *et al*., 2008b) and the mussel *Xenostrobus securis* (Pascual *et al*., 2010).~~

~~Other *Ostrea* species including *O. chilensis, O. puelchana, O. angasi*, and *O. denselamellosa* were found to be infected with *Marteilia* sp. when deployed in an infected area (Berthe *et al*., 2004; Martin, 1993). However, in these cases, the parasite identification was not done at the molecular level.~~

~~In addition, different stages, including mature stages, of parasites looking like *M. refringens,* were observed by histology in cockles (*Cerastoderma edule*), clam species (*Ruditapes decussatus R. philippinarum, Tapes rhomboides, T. pullastra, Ensis minor, E. siliqua*), and oysters (*Crassostrea virginica*) among other bivalve species (Berthe *et al*., 2004; López-Flores *et al*., 2008b). In all these cases, parasite identification is uncertain.~~

~~Lastly, the copepod *Paracartia grani* was shown to be susceptible to *M. refringens* and this species could participate in the transmission of the parasites between bivalves (see 2.3.1)~~

Species that fulfil the criteria for listing as susceptible to infection with *Marteilia refringens* according to Chapter 1.5. of the *Aquatic Animal Health Code* (*Aquatic Code*) are: blue mussel (*Mytilus edulis*), dwarf oyster (*Ostrea stentina*), European flat oyster *(Ostrea edulis)*, European razor clam (*Solen marginatus*), golden mussel (*Xenostrobus securis*), Mediterranean mussel (*Mytilus galloprovincialis*) and striped venus clam (*Chamelea gallina*).

Additionally, a copepod species (*Paracartia grani*) has been found to meet the criteria for listing as susceptible to infection with *Marteilia refringens* and is considered an intermediate host.

2.2.2. ~~Susceptible stages of the host~~ Species with incomplete evidence for susceptibility

~~Juveniles and older life stages are known to be susceptible (Grizel, 1985).~~

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with *M.refringens* according to Chapter 1.5. of the *Aquatic Code* are: Chilean flat oyster (*Ostrea chilensis*), a copepod (*Paracartia latisetosa*) and Japanese flat oyster (*Ostrea denselamellosa*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: Cortez oyster (*Crassostrea corteziensis*), grooved carpet shell (*Ruditapes decussatus*), Pacific cupped oyster (*Magallana* [syn. *Crassostrea] gigas*) and zooplankton (*Acartia discaudata, Centropages typicus, Euterpina acutifrons*, unidentified *Oithona sp., Penilia avirostris*).

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