

Original: Inglés

Actividades de las comisiones especializadas
COMISIÓN DE NORMAS SANITARIAS PARA LOS ANIMALES
ACUÁTICOS
Enmiendas propuestas al
Código Acuático y al Manual Acuático

(90 SG/10 SC4)

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1. PRESENTACIÓN

1.1. INTRODUCCIÓN

Desde el 89.^a Sesión General en mayo de 2022, la Comisión de Normas Sanitarias para los Animales Acuáticos (Comisión para los Animales Acuáticos) se reunió en dos ocasiones, del 14 al 21 de septiembre de 2022; el 19 de enero y del 15 al 22 de febrero de 2023. Entre sus actividades, La Comisión avanzó su trabajo de desarrollo de textos nuevos y revisados para el *Código Sanitario para los Animales Acuáticos (Código Acuático)* y el *Manual de las Pruebas de Diagnóstico para los Animales Acuáticos (Manual Acuático)*, de conformidad con su plan de trabajo. Los detalles de las actividades de la Comisión, incluyendo los textos que circularon para comentarios se publicaron en los informes de septiembre de 2022 y de febrero de 2023 de la Comisión y están disponibles en el sitio web de los Delegados y en el [sitio web de la OMSA](#).

Este documento ofrece elementos de información para cada uno de los textos nuevos y revisados del *Código Acuático* y del *Manual Acuático* que se presentarán para adopción en la 90.^a Sesión General. Los detalles del examen de los comentarios recibidos sobre los proyectos de texto que circularon para comentario figuran en los informes de [septiembre de 2022](#) y de [febrero de 2023](#) de la Comisión. La Comisión alienta a los Miembros a consultar estos informes junto con otros [informes previos de la Comisión](#) con el fin de obtener mayores precisiones sobre los textos modificados propuestos para adopción.

En los anexos de este documento se incorporan las enmiendas propuestas al *Código Acuático* y al *Manual Acuático* que se presentarán para adopción de la Asamblea Mundial de Delegados en la 90.^a Sesión General. Los números de los anexos utilizados corresponden con los números de los anexos del informe de febrero de 2023 de la Comisión para los Animales Acuáticos.

En el proceso de redacción y revisión de estas enmiendas, la Comisión para los Animales Acuáticos examinó los comentarios remitidos por los Miembros. La Comisión también trabajó en estrecha cooperación con la Comisión de Normas Sanitarias para los Animales Terrestres y con varios grupos *ad hoc*.

2. CÓDIGO ACUÁTICO – TEXTOS QUE SE PROPONDRÁN PARA ADOPCIÓN

2.1. ARTÍCULO 9.3.1. DEL CAPÍTULO 9.3. INFECCIÓN POR *HEPATOBACTER PENA EI* (HEPATOPANCREATITIS NECROTIZANTE) (ANEXO 4)

Se proponen enmiendas editoriales en el Artículo 9.3.1.

El artículo revisado ha circulado en tres oportunidades, la primer vez con el informe de febrero de 2022 de la Comisión.

El Artículo revisado 9.3.1. del capítulo 9.3. Infección por *Hepatobacter penaei* (Hepatopancreatitis necrotizante) figura en el [Anexo 4](#) y se propondrá para adopción en la 90.^a Sesión General de mayo de 2023.

2.2. ARTÍCULOS 9.4.1. Y 9.4.2. DEL CAPÍTULO 9.4. INFECCIÓN POR EL VIRUS DE LA NECROSIS HIPODÉRMICA Y HEMATOPOYÉTICA INFECCIOSA (ANEXO 5)

En el Artículo 9.4.1. se propone una actualización en la clasificación taxonómica del virus y en el Artículo 9.4.2. enmiendas editoriales menores.

Los artículos revisados han circulado tres veces, la primera vez en el informe de febrero de 2022 de la Comisión.

Los Artículos revisados 9.4.1. y 9.4.2. del Capítulo 9.4. Infección por el virus el virus de la necrosis hipodérmica y hematopoyética *infecciosa* figuran en el [Anexo 5](#) y se propondrán para adopción en la 90.^a Sesión General de mayo de 2023.

2.3. ARTÍCULO 9.5.2. DEL CAPÍTULO 9.5. INFECCIÓN POR EL VIRUS DE LA MIONECROSIS INFECCIOSA (ANEXO 6)

Se proponen enmiendas editoriales en el Artículo 9.5.2.

El artículo revisado ha circulado en dos ocasiones, la primera vez con el informe de septiembre de 2022 de la Comisión.

El Artículo revisado 9.5.2 del Capítulo 9.5. *Infección por el virus de la mionecrosis infecciosa* figura en el [Anexo 6](#) y se propondrá para adopción en la 90.^a Sesión General de mayo de 2023.

2.4. ARTÍCULO 10.9.2. DEL CAPÍTULO 10.9. INFECCIÓN POR EL VIRUS DE LA VIREMIA PRIMAVERAL DE LA CARPA (ANEXO 7)

En el Artículo 10.9.2. se propone una lista de especies susceptibles modificada.

A solicitud de la Comisión para los Animales Acuáticos, el Grupo *ad hoc* sobre la susceptibilidad de las especies de peces a las enfermedades de la lista de la OMSA evaluó la susceptibilidad de la carpa Jinsha bass (*Percocypris pingi*) a la infección por el virus de la viremia primaveral de la carpa. El grupo *ad hoc* acordó que la carpa Jinsha bass cumple los criterios para la inclusión de especies susceptibles de infección por un agente patógeno específico en la lista de especies sensibles del Artículo 10.9.2. del Capítulo 10.9. En el Anexo 10 del informe de la Comisión de [septiembre de 2022](#) se encuentran los detalles de la evaluación del grupo *ad hoc*.

El artículo revisado ha circulado en dos oportunidades, la primera vez con el informe de septiembre de 2022 de la Comisión.

El Artículo revisado 10.9.2. del capítulo 10.9. Infección por el virus de la viremia primaveral de la carpa figura en el [Anexo 7](#) y se propondrá para adopción en la 90.^a Sesión General de mayo de 2023.

2.5. NUEVO CAPÍTULO 10.X. INFECCIÓN POR EL VIRUS DE LA TILAPIA DEL LAGO (ANEXO 8)

Se propone incluir en el *Código Acuático* un nuevo capítulo específico de enfermedad para la infección por el virus de la tilapia del lago, que ha sido desarrollado tras la inclusión de esta infección en la lista de enfermedades de la OMSA en 2022.

El capítulo se ha armonizado con otros capítulos específicos de enfermedad.

El proyecto de texto ha circulado en dos oportunidades, la primera vez con el informe de septiembre de 2022 de la Comisión.

Un nuevo Capítulo 10.X. Infección por el virus de la tilapia del lago figura en el [Anexo 8](#) y se propondrá para adopción en la 90.^a Sesión General de mayo de 2023.

2.6. ARTÍCULO 11.2.2. DEL CAPÍTULO 11.2. INFECCIÓN POR *BONAMIA EXITIOSA* (ANEXO 9)

Se proponen enmiendas en la taxonomía de algunas especies susceptibles en el Artículo 11.2.2.

El artículo revisado ha circulado en dos oportunidades, la primera vez con el informe de septiembre de 2022 de la Comisión.

El Artículo revisado 11.2.2. del Capítulo 11.2. Infección por *Bonamia exitiosa* figura en el [Anexo 9](#) y se propondrá para adopción en la 90.^a Sesión General de mayo de 2023.

2.7. ARTÍCULO 11.3.2. DEL CAPÍTULO 11.3. INFECCIÓN POR *BONAMIA OSTREAE* (ANEXO 10)

Se proponen enmiendas en la taxonomía de algunas especies susceptibles en el Artículo 11.3.2.

El artículo revisado ha circulado en dos oportunidades, la primera vez con el informe de

septiembre de 2022 de la Comisión.

El Artículo revisado 11.2.2. del Capítulo 11.2. Infección por *Bonamia ostreae* figura en el [Anexo 10](#) y se propondrá para adopción en la 90.ª Sesión General de mayo de 2023.

2.8. ARTÍCULOS 11.4.1. Y 11.4.2. DEL CAPÍTULO 11.4. INFECCIÓN POR *MARTEILIA REFRINGENS* (ANEXO 11)

Se proponen enmiendas para actualizar la clasificación taxonómica en el Artículo 11.4.1. y enmiendas en la lista de especies susceptibles en el Artículo 11.4.2.

En el informe de [junio de 2022](#) del Grupo *ad hoc* sobre la susceptibilidad de las especies de moluscos a la infección por las enfermedades de la lista de la OMSA se ofrecen detalles de las evaluaciones realizadas con el fin de determinar la lista propuesta de especies susceptibles.

Los artículos revisados han circulado en dos oportunidades, la primera vez con el informe de septiembre de 2022 de la Comisión.

Los Artículos revisados 11.4.1. y 11.4.2. del Capítulo 11.4. Infección por *Marteilia refringens* figuran en el [Anexo 11](#) y se propondrán para adopción en la 90.ª Sesión General de mayo de 2023.

2.9. MODELO DE ARTÍCULOS 11.X.9. – 11.X.14. PARA LOS CAPÍTULOS ESPECÍFICOS DE LAS ENFERMEDADES DE LOS MOLUSCOS (ANEXO 12)

Se introdujeron enmiendas menores en aras de garantizar la armonización entre los capítulos específicos de las enfermedades de los moluscos en el *Código Acuático* y se presentan como modelos de Artículos 11.X.9. a 11.X.14. Estos cambios son conformes con los cambios horizontales aplicados a otros capítulos específicos de enfermedad.

Los modelos de artículo han circulado dos veces, la primera vez con el informe de septiembre de 2022 de la Comisión.

Los modelos de Artículos 11.X.9. a 11.X.14. figuran en el [Anexo 12](#) y se propondrán para adopción en la 90.ª Sesión General de mayo de 2023.

3. MANUAL ACUÁTICO – TEXTOS QUE SE PROPONDRÁN PARA ADOPCIÓN

3.1. CAPÍTULO 2.2.1. ENFERMEDAD DE LA NECROSIS HEPATOPANCREÁTICA AGUDA (ANEXO 22)

Se introdujeron amplias enmiendas para actualizar el Capítulo 2.2.1. que se reformateó utilizando el nuevo modelo de capítulo de enfermedad.

Los expertos del laboratorio de referencia de la OMSA propusieron modificaciones encaminadas a actualizar el capítulo y armonizarlo con el nuevo modelo de capítulo específico de enfermedad.

El capítulo revisado ha circulado en tres oportunidades, la primera vez con el informe de febrero de 2022 de la Comisión.

El Capítulo revisado 2.2.1. Enfermedad de la necrosis hepatopancreática aguda figura en el [Anexo 22](#) y se propondrá para adopción en la 90.ª Sesión General de mayo de 2023.

3.2. CAPÍTULO 2.2.3. INFECCIÓN POR *HEPATOBACTER PENAEI* (HEPATOPANCREATITIS NECROTIZANTE) (ANEXO 23)

Se introdujeron amplias enmiendas para actualizar el Capítulo 2.2.3. que se reformateó utilizando el nuevo modelo de capítulo de enfermedad.

Los expertos del laboratorio de referencia de la OMSA propusieron modificaciones encaminadas a actualizar el capítulo y armonizarlo con el nuevo modelo de capítulo específico de enfermedad.

El capítulo revisado ha circulado en tres oportunidades, la primera vez con el informe de febrero de 2022 de la Comisión.

El Capítulo revisado 2.2.3. Infección por *Hepatobacter penaei* (hepatopancreatitis necrotizante) figura en el [Anexo 23](#) y se propondrá para adopción en la 90.ª Sesión General de mayo de 2023.

3.3. CAPÍTULO 2.2.4. INFECCIÓN POR EL VIRUS DE LA NECROSIS HIPODÉRMICA Y HEMATOPOYÉTICA (ANEXO 24)

Se introdujeron amplias enmiendas para actualizar el Capítulo 2.2.4. que se reformateó utilizando el nuevo modelo de capítulo de enfermedad.

Los expertos del laboratorio de referencia de la OMSA propusieron modificaciones encaminadas a actualizar el capítulo y armonizarlo con el nuevo modelo de capítulo específico de enfermedad.

El capítulo revisado ha circulado en tres oportunidades, la primera vez con el informe de febrero de 2022 de la Comisión.

El Capítulo revisado 2.2.4. Infección por el virus de la necrosis hipodérmica y hematopoyética figura en el [Anexo 24](#) y se propondrá para adopción en la 90.ª Sesión General de mayo de 2023.

3.4. CAPÍTULO 2.2.5. INFECCIÓN POR EL VIRUS DE LA MIONECROSIS INFECCIOSA (ANEXO 25)

Se introdujeron amplias enmiendas para actualizar el Capítulo 2.2.5. que se reformateó utilizando el nuevo modelo de capítulo de enfermedad.

El capítulo revisado ha circulado en dos oportunidades, la primera vez con el informe de septiembre de 2022 de la Comisión.

El Capítulo revisado 2.2.5. Infección por el virus de la mionecrosis infecciosa figura en el [Anexo 25](#) y se propondrá para adopción en la 90.ª Sesión General en mayo de 2023.

3.5. CAPÍTULO 2.2.7. CAPÍTULO 2.2.7. INFECCIÓN POR EL VIRUS DEL SÍNDROME DE TAURA (ANEXO 26)

Se introdujeron amplias enmiendas para actualizar el Capítulo 2.2.7. que se reformateó utilizando el nuevo modelo de capítulo de enfermedad.

Los expertos del laboratorio de referencia de la OMSA propusieron modificaciones encaminadas a actualizar el capítulo y armonizarlo con el nuevo modelo de capítulo específico de enfermedad.

El capítulo revisado ha circulado en dos oportunidades, la primera vez con el informe de septiembre de 2022 de la Comisión.

El Capítulo revisado 2.2.7. Infección el virus del síndrome de Taura figura en el [Anexo 26](#) y se propondrá para adopción en la 90.ª Sesión General en mayo de 2023.

3.6. CAPÍTULO 2.2.8. INFECCIÓN POR EL VIRUS DEL SÍNDROME DE LAS MANCHAS BLANCAS (ANEXO 27)

Se introdujeron amplias enmiendas para actualizar el Capítulo 2.2.8. que se reformateó utilizando el nuevo modelo de capítulo de enfermedad.

Los expertos del laboratorio de referencia de la OMSA propusieron modificaciones encaminadas a actualizar el capítulo y armonizarlo con el nuevo modelo de capítulo específico de enfermedad.

El capítulo revisado ha circulado en dos oportunidades, la primera vez con el informe de septiembre de 2022 de la Comisión.

El Capítulo revisado 2.2.8. Infección por el virus del síndrome de las manchas blancas figura en el [Anexo 27](#) y se propondrá para adopción en la 90.ª Sesión General en mayo de 2023.

3.7. CAPÍTULO 2.3.1. INFECCIÓN POR *APHANOMYCES INVADANS* (SÍNDROME EPIZOÓTICO ULCERANTE) (ANEXO 28)

Se introdujeron amplias enmiendas para actualizar el Capítulo 2.3.1. que se reformateó utilizando el nuevo modelo de capítulo de enfermedad.

El capítulo revisado ha circulado en tres oportunidades, la primera vez con el informe de febrero de 2022 de la Comisión.

El Capítulo revisado 2.3.1. Infección por *Aphanomyces invadans* (síndrome epizoótico ulcerante) figura en el [Anexo 28](#) y se propondrá para adopción en la 90.ª Sesión General en mayo de 2023.

3.8. CAPÍTULO 2.3.2. INFECCIÓN POR EL VIRUS DE LA NECROSIS HEMATOPOYÉTICA EPIZOÓTICA (ANEXO 29)

Se introdujeron amplias enmiendas para actualizar el Capítulo 2.3.2. que se reformateó utilizando el nuevo modelo de capítulo de enfermedad.

Los expertos del laboratorio de referencia de la OMSA propusieron modificaciones encaminadas a actualizar el capítulo y armonizarlo con el nuevo modelo de capítulo específico de enfermedad.

El capítulo revisado ha circulado en cuatro oportunidades, la primera vez con el informe de septiembre de 2021 de la Comisión.

El Capítulo revisado 2.3.2. Infección por el virus de la necrosis hematopoyética epizoótica figura en el [Anexo 29](#) y se propondrá para adopción en la 90.ª Sesión General en mayo de 2023.

3.9. SECCIÓN 2.2.1. DEL CAPÍTULO 2.3.9. INFECCIÓN POR EL VIRUS DE LA VIREMIA PRIMAVERAL DE LA CARPA (ANEXO 30)

Se propone una lista de especies susceptibles en la Sección 2.2.1. del Capítulo 2.3.9. que refleja las enmiendas propuestas al Artículo 10.9.2 del *Código Acuático*.

La sección revisada ha circulado en dos oportunidades, la primera vez con el informe de septiembre de 2022 de la Comisión.

La Sección revisada 2.2.1. del Capítulo 2.3.9. Infección por el virus de la viremia primaveral de la carpa figura en el [Anexo 30](#) y se propondrá para adopción en la 90.ª Sesión General de mayo de 2023.

3.10. SECCIONES 2.2.1. Y 2.2.2. DEL CAPÍTULO 2.4.2. INFECCIÓN POR *BONAMIA EXITIOSA* (ANEXO 31)

Se propusieron enmiendas a la taxonomía de algunas especies susceptibles en las secciones 2.2.1. y 2.2.2. del Capítulo 2.4.2.

Las secciones revisadas han circulado en dos oportunidades, la primera vez con el informe de septiembre de 2022 de la Comisión.

Las Secciones revisadas 2.2.1. y 2.2.2. del Capítulo 2.4.2. Infección por *Bonamia exitiosa* figuran en el [Anexo 31](#) y se propondrán para adopción en la 90.ª Sesión General en mayo de 2023.

3.11. SECCIONES 2.2.1. Y 2.2.2. DEL CAPÍTULO 2.4.3. INFECCIÓN POR *BONAMIA OSTREAE* (ANEXO 32)

Se propusieron enmiendas a la taxonomía de algunas especies susceptibles en las secciones 2.2.1. y 2.2.2. del Capítulo 2.4.3.

Las secciones revisadas han circulado en dos oportunidades, la primera vez con el informe de septiembre de 2022 de la Comisión.

Las Secciones revisadas 2.2.1. and 2.2.2. del Capítulo 2.4.3. Infección por *Bonamia ostreae* figuran en el [Anexo 32](#) y se propondrán para adopción en la 90.ª Sesión General en mayo de

2023.

3.12. SECCIONES 2.2.1. Y 2.2.2. DEL CAPÍTULO 2.4.4. INFECCIÓN POR *MARTEILIA REFRINGENS* (ANEXO 33)

Se proponen enmiendas en la lista de especies susceptibles de las secciones 2.2.1. y 2.2.2. del capítulo 2.4.4

En el informe de [junio de 2022](#) del Grupo *ad hoc* sobre la susceptibilidad de las especies de moluscos a la infección por las enfermedades de la lista de la OMSA se ofrecen detalles de las evaluaciones realizadas con el fin de determinar la lista propuesta de especies susceptibles.

Las secciones revisadas circularon en dos ocasiones, la primera vez en el informe de septiembre de 2022 de la Comisión.

Las Secciones revisadas 2.2.1. y 2.2.2. del Capítulo 2.4.4. Infección por *Marteilia refringens* figuran en el [Anexo 33](#) y se propondrán para adopción en la 90.^a Sesión General en mayo de 2023.

.../...Anexos

CAPÍTULO 9.3.

INFECCIÓN POR *HEPATOBACTER PENA EI* (HEPATOPANCREATITIS NECROTIZANTE)

Artículo 9.3.1.

A efectos del Código Acuático, la infección por *Hepatobacter penaei* (hepatopancreatitis necrotizante) designa una infección causada por el agente patógeno ~~Candidatus *Hepatobacter penaei*~~ *Hepatobacter penaei*, una bacteria intracelular obligada ~~de la familia Holosporaceae~~ de la orden Rickettsiales ~~de las proteobacterias alfa~~. ~~La enfermedad se conoce comúnmente como hepatopancreatitis necrotizante.~~

La información sobre los métodos de diagnóstico figura en el Manual Acuático.

[...]

CAPÍTULO 9.4.

INFECCIÓN POR EL VIRUS DE LA NECROSIS HIPODÉRMICA Y HEMATOPOYÉTICA INFECCIOSA

Artículo 9.4.1.

A efectos del Código Acuático, la infección por el virus de la necrosis hipodérmica y hematopoyética infecciosa es la infección causada por el agente patógeno de los decápodos penstylhamaparvovirus 1 ~~del virus de la necrosis hipodérmica y hematopoyética infecciosa del género *Penstyldensevirus* *Penstylhamaparvovirus*~~ y de la familia de los Parvoviridos.

La información sobre los métodos de diagnóstico figura en el *Manual Acuático*.

Artículo 9.4.2.

Ámbito de aplicación

Las recomendaciones de este capítulo se aplican a las siguientes especies que cumplen con los criterios de inclusión en la lista de especies susceptibles de acuerdo con el Capítulo 1.5.: camarón azul (*Penaeus stylirostris*), camarón tigre gigante (*Penaeus monodon*), camarón blanco del Golfo (*Penaeus setiferus*), camarón patiamarillo (*Penaeus californiensis*), camarón tigre gigante (*Penaeus monodon*), camarón blanco nortero (*Penaeus setiferus*), camarón azul (*Penaeus stylirostris*) y camarón patiblanco (*Penaeus vannamei*) y camarón patiamarillo (*Penaeus californiensis*).

[...]

CAPÍTULO 9.5.

INFECCIÓN POR EL VIRUS DE LA MIONECROSIS INFECCIOSA

[...]

Artículo 9.5.2.

Ámbito de aplicación

Las recomendaciones de este capítulo se aplican a las siguientes especies que cumplen con los criterios de inclusión en la lista de *especies susceptibles* de acuerdo con el Capítulo 1.5.: ~~camarón tigre marrón (*Penaeus esculentus*)~~, camarón banana (*Penaeus merguensis*), langostino tigre marrón (*Penaeus esculentus*) y camarón patiblanco (*Penaeus vannamei*).

[...]

CAPÍTULO 10.9. INFECCIÓN POR EL VIRUS DE LA VIREMIA PRIMAVERAL DE LA CARPA

[...]

Artículo 10.9.2.

Ámbito de aplicación

Las recomendaciones de este capítulo se aplican a las siguientes especies que cumplen con los criterios de inclusión en la lista de *especies susceptibles* de acuerdo con el Capítulo 1.5.:

Familia	Nombre científico	Nombre común
Cyprinidae	<i>Abramis brama</i>	B <u>b</u> rema
	<i>Aristichthys nobilis</i>	C <u>c</u> arpa cabezona
	<i>Carassius auratus</i>	C <u>c</u> arpa dorada
	<i>Ctenopharyngodon idella</i>	C <u>c</u> arpa herbívora
	<i>Cyprinus carpio</i>	C <u>c</u> arpa común (todas las variedades y subespecies)
	<i>Danio rerio</i>	P <u>p</u> ez zebra
	<i>Notemigonus crysoleucas</i>	C <u>c</u> arpita dorada
	<i>Pimephales promelas</i>	P <u>p</u> iscardo
	<i>Percocypris pingi</i>	[Jinsha barbel carp] Carpa de Jinsha
	<i>Rutilus kutum</i>	P <u>p</u> escado blanco del Caspio
<i>Rutilus rutilus</i>	R <u>r</u> utilo	
Siluridae	<i>Silurus glanis</i>	S <u>s</u> irulo

[...]

CAPÍTULO 10.X.

INFECCIÓN POR EL VIRUS DE LA TILAPIA DEL LAGO

Artículo 10.X.1.

A efectos del Código Acuático, la infección por el virus de la tilapia del lago (TiLV) designa una *infección* causada por el agente patógeno *Tilapia tilapinevirus* del género *Tilapinevirus* y de la familia *Amnoonviridae*.

La información sobre los métodos de diagnóstico figura en el *Manual Acuático*.

Artículo 10.X.2.

Ámbito de aplicación

Las recomendaciones de este capítulo se aplican a las siguientes especies que cumplen con los criterios de inclusión en la lista de *especies susceptibles* de acuerdo con el Capítulo 1.5.: [tilapia azul (*Oreochromis aureus*), tilapia roja híbrida de Malasia (*Oreochromis niloticus* x *Oreochromis mossambicus*), (*Sarotherodon galilaeus*), tilapia de Mozambique (*Oreochromis mossambicus*), tilapia del Nilo (*Oreochromis niloticus*), (*Tilapia zilli*), (*Barbonymus schwanenfeldii*), (*Tristramella simonis*) y tilapia azul del Nilo, híbrido (*Oreochromis niloticus* X *Oreochromis aureus*)] (en estudio).

Artículo 10.X.3.

Medidas para la importación o tránsito de productos de animales acuáticos cualquiera que sea el uso al que se destinan, independientemente del estatus sanitario del país, la zona o el compartimento de exportación con respecto a la infección por TiLV

Los *productos de animales acuáticos* enumerados a continuación se han evaluado y cumplen con los criterios de seguridad aplicables a este tipo de productos de acuerdo con el Artículo 5.4.1. Cuando autoricen la importación o el tránsito por su territorio de estos *productos de animales acuáticos*, las *autoridades competentes* no deberán exigir ninguna *medida sanitaria* relacionada con el TiLV, independientemente del estatus sanitario del país, la zona o el *compartimento* de exportación respecto a la infección por TiLV:

- 1) *Productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 560°C durante por lo menos ~~cinco~~ 120 minutos, o un tiempo/temperatura equivalente que inactive TiLV;
- 2) *harina* de pescado que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 560°C durante por lo menos ~~cinco~~ 120 minutos, o un tiempo/temperatura equivalente que inactive TiLV ~~(en estudio)~~;
- 3) aceite de pescado;
- 4) cueros elaborados con piel de pescado.

Artículo 10.X.4.

Requisitos para una autodeclaración de ausencia de infección por TiLV

Un País Miembro podrá hacer una autodeclaración de ausencia de infección por TiLV en todo el país, una zona o un *compartimento* con arreglo a las disposiciones de los Artículos 10.X.5. a 10.X.8., según proceda. La autodeclaración de ausencia de enfermedad también debe satisfacer otros requisitos pertinentes contemplados en el Código Acuático, entre ellos, la exigencia de que el País Miembro reúna las siguientes condiciones:

- 1) cumple las disposiciones del Capítulo 3.1.; y
- 2) utiliza métodos de *diagnóstico* apropiados, según las recomendaciones del *Manual Acuático*; y
- 3) satisface todos los requisitos del Capítulo 1.4. pertinentes para la autodeclaración.

Artículo 10.X.5.

País libre de infección por TiLV

Si el país comparte cuerpos de agua con otros países, solo podrá hacer una autodeclaración de ausencia de infección por TiLV si todos los cuerpos de agua compartidos están situados en países o zonas declarados libres de infección por TiLV.

Como se describe en el Artículo 1.4.4., un País Miembro puede hacer una autodeclaración de ausencia de infección por TiLV en la totalidad de su *territorio* si puede demostrar que:

- 1) ninguna *especie susceptible* de las mencionadas en el Artículo 10.X.2. está presente y se han reunido ininterrumpidamente las *condiciones elementales de bioseguridad* durante al menos los últimos [seis] meses;
O
- 2) no ha ocurrido ninguna infección por TiLV durante al menos los [diez] últimos años, y:
 - a) el País Miembro puede demostrar que las condiciones son propicias para la manifestación clínica de la infección por ~~TiLV~~ TiLV, de acuerdo con lo descrito en el capítulo correspondiente del *Manual Acuático*; y
 - b) se han reunido ininterrumpidamente las *condiciones elementales de bioseguridad* descritas en el Capítulo 1.4. durante al menos los [diez] últimos años;O
- 3) se ha aplicado una *vigilancia específica*, de acuerdo con lo descrito en el Capítulo 1.4., durante al menos los [dos] últimos años sin que se haya detectado la presencia de TiLV, y se han reunido e implementado ininterrumpidamente las *condiciones elementales de bioseguridad* durante al menos [un] año antes del inicio de la *vigilancia específica*;
O
- 4) había hecho previamente una autodeclaración de ausencia de infección por TiLV y perdió posteriormente su estatus libre por haberse detectado TiLV, pero se han cumplido las condiciones siguientes:
 - a) nada más haberse detectado TiLV, el área afectada ha sido declarada *zona infectada* y se ha establecido una *zona de protección*; y
 - b) las poblaciones infectadas dentro de la *zona infectada* se han sacrificado y eliminado con medios que reducen al mínimo la probabilidad de una mayor transmisión de TiLV, y se han completado los procedimientos de *desinfección* apropiados (descritos en el Capítulo 4.4.), seguidos de un periodo de *vacío sanitario* según se describe en el Capítulo 4.7.; y
 - c) las *condiciones elementales de bioseguridad* vigentes anteriormente han sido debidamente revisadas y modificadas y se han mantenido ininterrumpidamente desde la erradicación de la infección por TiLV; y
 - d) se ha aplicado una *vigilancia específica*, de acuerdo con lo descrito en el Capítulo 1.4., durante:
 - i) por lo menos los [dos] últimos años en las *especies susceptibles* silvestres y de cría sin que se haya detectado la presencia de TiLV, o
 - ii) por lo menos el [un] último año sin que se haya detectado la presencia de TiLV, si los

establecimientos de acuicultura afectados no tenían vínculos epidemiológicos con las poblaciones silvestres de especies susceptibles.

Mientras tanto, la parte del país ubicada fuera de la zona infectada y de las zonas de protección una parte o la totalidad del país, excepto las zonas infectadas y de protección, podrá ser declarada zona libre de conformidad con el Artículo 1.4.4., siempre que se hayan cumplido reúna las condiciones descritas en el las disposiciones de los apartados 4 a) a 4 c) 2 del Artículo 10.X.6.

Artículo 10.X.6.

Zona libre de infección por ~~TiLV~~ TiLV

Si una zona se extiende por el territorio de más un país, solo podrá ser declarada zona libre de infección por TiLV si todas las autoridades competentes confirman que se han reunido el conjunto de condiciones exigidas.

Como se describe en el Artículo 1.4.4., un País Miembro puede hacer una autodeclaración de ausencia de infección por ~~TiLV~~ TiLV para una zona dentro de su territorio si puede demostrar que:

- 1) ninguna especie susceptible de las mencionadas en el Artículo 10.X.2. está presente y se han reunido ininterrumpidamente las condiciones elementales de bioseguridad durante al menos los [seis] últimos meses;
O
- 2) no ha ocurrido ninguna infección por TiLV durante al menos los [diez] últimos años, y:
 - a) el País Miembro puede demostrar que las condiciones son propicias para la manifestación clínica de la infección por TiLV, de acuerdo con lo descrito en el Artículo 1.4.8. del Capítulo 1.4.; y
 - b) se han reunido ininterrumpidamente las condiciones elementales de bioseguridad descritas en el Capítulo 1.4. durante al menos los [diez] últimos años;O
- 3) se ha aplicado una *vigilancia específica* en la zona, de acuerdo con lo descrito en el Capítulo 1.4., durante al menos los [dos] últimos años sin que se haya detectado la presencia de TiLV, y se han reunido ininterrumpidamente e implementado las condiciones elementales de bioseguridad durante al menos [un] año antes del inicio de la *vigilancia específica*;
O
- 4) había hecho previamente una autodeclaración de ausencia de infección por TiLV para una zona y perdió posteriormente su estatus libre por haberse detectado TiLV en la zona, pero se han cumplido las condiciones siguientes:
 - a) nada más haberse detectado TiLV, el área afectada fue declarada *zona infectada* y se ha establecido una *zona de protección*; y
 - b) las poblaciones infectadas dentro de la *zona infectada* se han sacrificado y eliminado con medios que reducen al mínimo la probabilidad de una mayor transmisión de TiLV, y se han completado los procedimientos de *desinfección* apropiados (descritos en el Capítulo 4.4.), seguidos de un periodo de *vacío sanitario* según se describe en el Capítulo 4.7.; y
 - c) las *condiciones elementales de bioseguridad* vigentes anteriormente han sido debidamente revisadas y modificadas y se han mantenido ininterrumpidamente desde la erradicación de la infección por TiLV; y
 - d) se ha aplicado una *vigilancia específica*, de acuerdo con lo descrito en el Capítulo 1.4., durante al menos los [dos] últimos años sin que se haya detectado la presencia de TiLV.

Mientras tanto, una parte de la zona ubicada fuera de la zona infectada y de la zona de protección podrá ser declarada nueva zona libre de conformidad con el Artículo 1.4.4., siempre que se hayan cumplido las disposiciones

de los apartados 4 a) a 4 c).

Artículo 10.X.7.

Compartimento libre de infección por ~~TiLV~~ TiLV

Según se describe en el Artículo 1.4.4., un País Miembro podrá hacer una autodeclaración de ausencia de infección por TiLV en un *compartimento* dentro de su *territorio* si puede demostrar que:

- 1) se ha aplicado una *vigilancia específica* en el *compartimento*, de acuerdo con lo descrito en el Capítulo 1.4., durante al menos el [un] último año sin que se haya detectado TiLV, y se han reunido ininterrumpidamente implementado las *condiciones elementales de bioseguridad* durante al menos [un] año antes del inicio de la *vigilancia específica*;

O

- 2) había hecho previamente una autodeclaración de ausencia de infección por TiLV en un *compartimento* y perdió posteriormente su estatus libre por haberse detectado TiLV en el *compartimento*, pero se han cumplido las condiciones siguientes:
 - a) todos los *animales acuáticos* dentro del *compartimento* se han sacrificado y eliminado con medios que reducen al mínimo la probabilidad de una mayor transmisión de TiLV, se han completado los procedimientos de *desinfección* apropiados (descritos en el Capítulo 4.4.) y el *compartimento* se ha sometido a un periodo de *vacío sanitario* según se describe en el Capítulo 4.7.; y
 - b) las *condiciones elementales de bioseguridad* vigentes anteriormente, incluido el *plan de bioseguridad* en el *compartimento*, han sido debidamente revisados y modificados y se han mantenido ininterrumpidamente desde la repoblación con *animales acuáticos* procedentes de una fuente aprobada libre de patógenos conforme a los requisitos de los Artículos 10.X.9 y 10.X.10. si procede; y
 - c) se ha aplicado una *vigilancia específica*, de acuerdo con lo descrito en el Capítulo 1.4., durante al menos [un] año se ha realizado una encuesta para determinar la infección por TiLV al menos [seis meses] luego de la repoblación (tal como se describe en el Artículo 1.4.14.) sin que se haya detectado la presencia del patógeno-TiLV.

Artículo 10.X.8.

Conservación del estatus libre

Un país, *zona* o *compartimento* declarados libres de infección por TiLV, de conformidad con lo dispuesto en los Artículos 10.X.4. a 10.X.7. (según proceda), podrán conservar su estatus libre de infección por TiLV si reúnen ininterrumpidamente los requisitos descritos en el Artículo 1.4.15.

Artículo 10.X.9.

Importación de animales acuáticos o productos de animales acuáticos de un país, una zona o un compartimento declarados libres de infección por TiLV

Cuando se importen *animales acuáticos* de una de las especies mencionadas en el Artículo 10.X.2., o *productos de animales acuáticos* derivados de dichas especies, procedentes de un país, una *zona* o un *compartimento* declarados libres de infección por TiLV, la *autoridad competente* del país importador deberá exigir que la remesa este acompañada de la presentación de un *certificado sanitario internacional aplicable a los animales acuáticos* extendido por la *autoridad competente* del país exportador. El *certificado sanitario internacional aplicable a los animales acuáticos* deberá acreditar, según los procedimientos descritos en los Artículos 10.X.5., 10.X.6. o 10.X.7. (según proceda) y 10.X.8., que el lugar de producción de la remesa de *animales acuáticos* o *productos de animales acuáticos* es un país, una *zona* o un *compartimento* declarados libres de infección por TiLV.

El *certificado sanitario internacional aplicable a los animales acuáticos* deberá ser conforme al modelo de certificado que figura en el Capítulo 5.11.

Este artículo no se aplica a los *productos de animales acuáticos* enumerados en el Artículo 10.X.3.

Artículo 10.X.10.

Importación, para la acuicultura, de animales acuáticos de un país, una zona o un compartimento no declarados libres de infección por ~~TiLV~~ TiLV

Cuando se importen, para la *acuicultura*, *animales acuáticos* de una de las especies mencionadas en el Artículo 10.X.2. de un país, una *zona* o un *compartimento* no declarados libres de infección por TiLV, la *autoridad competente* del *país importador* deberá evaluar el *riesgo* de conformidad con el Capítulo 2.1. y considerar las medidas de mitigación del *riesgo* en los apartados 1 y 2 que figuran a continuación:

- 1) Si la intención es el crecimiento y la cría de *animales acuáticos* importados se considerará la aplicación de:
 - a) entrega directa de los *animales acuáticos* importados a instalaciones de *cuarentena* donde permanecerán de por vida; y
 - b) antes de salir de la *cuarentena* (ya sea en la instalación de origen o en otra instalación de *cuarentena* hasta donde han sido transportados en condiciones adecuadas de *bioseguridad*), los *animales acuáticos* se sacrifican y procesan en uno o más de los *productos de animales acuáticos* enumerados en el Artículo 10.X.3. o en otros productos autorizados por la *autoridad competente*; y
 - c) tratamiento del agua utilizada en el transporte, de los equipos, efluentes y despojos con el fin de inactivar el TiLV, ~~virus de la anemia infecciosa del salmón~~ (de conformidad con los Capítulos 4.4., 4.8. y 5.5.).

O

- 2) Si la intención es establecer nuevas poblaciones para la *acuicultura*, se tendrá en cuenta lo siguiente:
 - a) en el *país exportador*:
 - i) identificar las fuentes posibles de población y evaluar el historial sanitario de sus *animales acuáticos*;
 - ii) examinar las poblaciones de origen de acuerdo con el Capítulo 1.4. y seleccionar una población fundadora (F-0) de *animales acuáticos* con un alto estatus sanitario para la infección por TiLV;
 - b) en el *país importador*:
 - i) importar la población fundadora (F-0) a una instalación de *cuarentena*;
 - ii) examinar la población F-0 para TiLV de conformidad con el Capítulo 1.4. para determinar su idoneidad como población reproductora;
 - iii) producir una población de primera generación (F-1) en *cuarentena*;
 - iv) criar la población F-1 en *cuarentena* durante una duración suficiente, y en condiciones favorables, para la expresión clínica de la infección por TiLV, y extraer muestras y realizar pruebas para la detección de TiLV de conformidad con el Capítulo 1.4. del *Código Acuático* y el Capítulo X.X.6. del *Manual Acuático*;
 - v) si no se detecta TiLV, la población F-1 puede ser definida libre de infección por TiLV y liberada de la *cuarentena*;
 - vi) si se detecta el TiLV, la población F-1 no puede ser liberada de la *cuarentena* y deberá sacrificarse y eliminarse de manera biológicamente segura de acuerdo con el Capítulo 4.8.

Artículo 10.X.11.

Importación, para transformación para el consumo humano, de animales acuáticos o productos de animales acuáticos de un país, una zona o un compartimento no declarados libres de infección por TiLV

Cuando se importen, para transformación para el consumo humano, *animales acuáticos* de una de las especies mencionadas en el Artículo 10.X.2., o *productos de animales acuáticos* derivados de dichas especies, procedentes de un país, una zona o un *compartimento* no declarados libres de infección por TiLV, la *autoridad competente* del país importador deberá evaluar el riesgo y aplicar, si se justifican, las siguientes medidas para reducirlo:

- 1) entrega directa y mantenimiento de la remesa en instalaciones de *cuarentena* o contención hasta su procesamiento en uno de los productos enumerados en el Artículo 10.X.3. o en el apartado 1 del Artículo 10.X.14. o en otros productos autorizados por la *autoridad competente*; y
- 2) tratamiento de toda el agua (incluido el hielo), de los equipos, *contenedores* y material de embalaje utilizados para el transporte ~~de modo que~~ a fin de garantizar la inactivación de TiLV o la eliminación biosegura de acuerdo con los Capítulos 4.4., 4.8. y 5.5.; y
- 3) tratamiento de todos los efluentes y despojos de modo que garantice la inactivación de TiLV o la eliminación biosegura de acuerdo con los Capítulos 4.4. y 4.8.

En lo que se refiere a estos *animales acuáticos* o *productos de animales acuáticos*, los Países Miembros podrán considerar, si lo desean, la oportunidad de introducir medidas internas para afrontar los riesgos asociados a la utilización de cualquiera de ellos para fines que no sean el consumo humano.

Artículo 10.X.12.

Importación de animales acuáticos o productos de animales acuáticos destinados a usos distintos del consumo humano incluyendo la alimentación de los animales, la investigación y el uso agrícola, industrial o farmacéutico y procedentes de un país, una zona o un compartimento no declarados libres de infección por TiLV

Cuando se importen, para usos distintos del consumo humano incluyendo la alimentación de los animales, el uso agrícola, industrial, la investigación o farmacéutico, *animales acuáticos* de una de las especies mencionadas en el Artículo 10.X.2., o *productos de animales acuáticos* derivados de dichas especies, procedentes de un país, una zona o un *compartimento* no declarados libres de infección por TiLV, la *autoridad competente* del país importador deberá requerir:

- 1) los *animales acuáticos* o *productos de animales acuáticos* sean entregados directamente a instalaciones de *cuarentena* y mantenidos en las mismas hasta su procesamiento en uno de los productos mencionados en el Artículo 10.X.3. o en otros productos autorizados por la *autoridad competente*, y
- 2) el tratamiento de toda el agua (incluido el hielo), de los equipos, *contenedores* y material de embalaje utilizados para el transporte a fin de garantizar la inactivación del ~~TiLV~~ TiLV o la eliminación biosegura de acuerdo con los Capítulos 4.4., 4.8. y 5.5., y
- 3) el tratamiento de todos los efluentes y despojos garantice la inactivación del ~~TiLV~~ TiLV o la eliminación biosegura de acuerdo con los Capítulos 4.4. y 4.8.

Artículo 10.X.13.

Importación de animales acuáticos destinados a uso en laboratorios o zoológicos procedentes de un país, una zona o un compartimento no declarados libres de infección por TiLV

Cuando se importen, para uso en laboratorios o zoológicos, *animales acuáticos* de una de las especies mencionadas en el Artículo 10.X.2. procedentes de un país, una zona o un *compartimento* no declarados libres de infección por TiLV, la *autoridad competente* del país importador deberá garantizar:

- 1) entrega directa de la remesa a instalaciones de *cuarentena* autorizadas por la *autoridad competente* y mantenimiento en las mismas;
- 2) tratamiento de toda el agua (incluido el hielo), de los equipos, *contenedores* y material de embalaje utilizados en el transporte ~~de modo que~~ en fin de garantizar la inactivación del TiLV o la eliminación biosegura de

acuerdo con los Capítulos 4.4., 4.8. y 5.5.; y

- 3) tratamiento de todos los efluentes y despojos provenientes de las instalaciones de *cuarentena* en los laboratorios o zoológicos, de modo que garantice la inactivación del TiLV o la eliminación biosegura de acuerdo con los Capítulos 4.4. y 4.8.; y
- 4) eliminación de los animales muertos de acuerdo con el Capítulo 4.8.

Artículo 10.X.14.

Importación (o tránsito por el territorio), para venta directa al por menor para el consumo humano, de productos de animales acuáticos independientemente del estatus sanitario del país, la zona o el compartimento de exportación con respecto a la infección por TiLV

- 1) [Independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por TiLV, las *autoridades competentes* no deberán exigir ningún tipo de condición relacionada con el TiLV cuando autoricen la importación (o el tránsito por su territorio) de ~~las~~ los siguientes ~~mercancías~~ productos de animales acuáticos que han sido elaborados y envasados para la venta directa al por menor y reúnen las condiciones descritas en el Artículo 5.4.2.:

- a) filetes o rodajas de pescado (refrigerados)] (en estudio).

Se han establecido algunos supuestos a la hora de evaluar la seguridad sanitaria de los *productos de animales acuáticos* enumerados más arriba. Los Países Miembros deberán referirse a tales supuestos, que figuran en el Artículo 5.4.2., y analizar si se aplican a sus condiciones.

En lo que se refiere a estos *productos de animales acuáticos*, los Países Miembros podrán considerar, si lo desean, la oportunidad de introducir medidas internas para afrontar los *riesgos* asociados la utilización de cualquiera de ellos para fines que no sean el consumo humano.

- 2) Cuando se importen *productos de animales acuáticos*, aparte de los enumerados en el apartado 1 arriba, derivados de una de las especies mencionadas en el Artículo 10.X.2. de un país, una *zona* o un *compartimento* no declarados libres de infección por TiLV, la *autoridad competente* del *país importador* deberá evaluar el *riesgo* y aplicar medidas de mitigación del *riesgo* apropiadas.

CAPÍTULO 11.2.

INFECCIÓN POR *BONAMIA EXITIOSA*

[...]

Artículo 11.2.2.

Ámbito de aplicación

Las recomendaciones de este capítulo se aplican a las siguientes especies que cumplen con los criterios de inclusión en la lista de especies susceptibles de acuerdo con el Capítulo 1.5.: ostra plana argentina (*Ostrea puelchana*), *Magallana [syn. Crassostrea] ariakensis*, ostra legamosa australiana (*Ostrea angasi*), ostra plana chilena (*Ostrea chilensis*), *Ostrea equestris*, ostra americana (*Crassostrea virginica*), ostra plana europea (*Ostrea edulis*); y ostra Olimpia (*Ostrea lurida*) y ostra de Suminoe (*Magallana [syn. Crassostrea] ariakensis*).

[...]

CAPÍTULO 11.3.
INFECCIÓN POR *BONAMIA OSTREAE*

[...]

Artículo 11.3.2.

Ámbito de aplicación

Las recomendaciones de este capítulo se aplican a las siguientes especies que cumplen con los criterios de inclusión en la lista de especies susceptibles de acuerdo con el Capítulo 1.5.: Magallana [syn. Crassostrea] ariakensis, ostra plana europea (Ostrea edulis), ostra plana chilena (*Ostrea chilensis*) y ostra plana europea (Ostrea edulis) la ostra de Suminoe (Magallana [syn. Crassostrea] ariakensis).

[...]

CAPÍTULO 11.4.

INFECCIÓN POR *MARTEILIA REFRINGENS*

Artículo 11.4.1.

A efectos del Código Acuático, la infección por *Marteilia refringens* designa una infección causada exclusivamente por el agente patógeno *M. refringens* **(incluidos los tipos O y M)** de la familia Marteiliidae.

La información sobre los métodos de diagnóstico figura en el Manual Acuático.

Artículo 11.4.2.

Ámbito de aplicación

Las recomendaciones de este capítulo se aplican a las siguientes especies: mejillón (*Mytilus edulis*), ostra enana (*Ostrea stentina*), ostra plana europea (*Ostrea edulis*), almeja navaja (*Solen marginatus*), mejillón dorado (*Xenostrobus securis*), ostra legamosa australiana (*Ostrea angasi*), ostra argentina (*Ostrea pulchana*) y ostra plana chilena (*Ostrea chilensis*), mejillón común (*Mytilus edulis*) y mejillón mediterráneo (*Mytilus Galloprovincialis*) y chirla (*Chamelea gallina*). Estas recomendaciones se aplican también a todas las demás especies susceptibles mencionadas en el Manual Acuático que sean objeto de comercio internacional.

Modelo de Artículos 11.X.9. – 11.X.14. para los capítulos específicos de las enfermedades de los moluscos

CAPÍTULO 11.X. INFECCIÓN POR [PATÓGENO X]

[...]

Artículo 11.X.9.

Importación de animales acuáticos ~~y~~ productos de animales acuáticos de un país, una zona o un compartimento declarados libres de infección por [Patógeno X]

Cuando se importen ~~animales acuáticos y productos de animales acuáticos~~ de una de las especies mencionadas en el Artículo 11.X.2., ~~o productos de animales acuáticos derivados de dichas especies, procedentes de~~ un país, una zona o un compartimento declarados libres de infección por el [Patógeno X], la *autoridad competente* del país importador deberá exigir que la remesa esté acompañada de la presentación de un *certificado sanitario internacional aplicable a los animales acuáticos* extendido por la *autoridad competente* del país exportador. El certificado sanitario internacional aplicable a los animales acuáticos deberá acreditar, ~~o por un certificador oficial aprobado por el país importador, que acredite,~~ según los procedimientos descritos en los Artículos 11.X.45., e 11.X.56. o 11.X.7. (según proceda) y 11.1.6.8, que el lugar de producción de la remesa de *animales acuáticos* ~~o~~ *productos de animales acuáticos* es un país, una zona o un compartimento declarados libres de infección por [Patógeno X].

El *certificado sanitario internacional aplicable a los animales acuáticos* deberá ser conforme al modelo de certificado que figura en el Capítulo 5.11.

Este artículo no se aplica a ~~las mercancías~~ los productos de animales acuáticos enumerados en **el apartado 1 del** Artículo 11.X.3.

Artículo 11.X.10.

Importación, para la acuicultura, de animales acuáticos de un país, una zona o un compartimento no declarados libres de infección por [Patógeno X]

Cuando se importen, para la *acuicultura*, *animales acuáticos* de una de las especies mencionadas en el Artículo 11.X.2. procedentes de un país, una zona o un compartimento no declarados libres de infección por [Patógeno X]., la *autoridad competente* del país importador deberá evaluar el *riesgo* de conformidad con el Capítulo 2.1. y considerar las medidas de mitigación del *riesgo* en los apartados 1 y 2 que figuran a continuación.

- 1) Si la intención es el crecimiento y la cría de *animales acuáticos* importados se considerará la aplicación de:
 - a) entrega directa de los *animales acuáticos* importados a instalaciones de *cuarentena* donde permanecerán de por vida; **y**
 - b) antes de salir de la cuarentena (ya sea en la instalación de origen o en otra instalación de cuarentena hasta donde han sido transportados en condiciones adecuadas de bioseguridad), los animales acuáticos se sacrifican y procesan en uno o más de los productos de animales acuáticos enumerados en el apartado 1 del Artículo 11.X.3. o en otros productos autorizados por la autoridad competente; y

- c) tratamiento del agua utilizada en el transporte, de los equipos, efluentes y despojos con el fin de inactivar [Patógeno X] de conformidad con los Capítulos 4.4., 4.8. y 5.5.

O

2) Si la intención es establecer nuevas poblaciones para la *acuicultura*, se tendrá en cuenta lo siguiente:

a) en el *país exportador*:

- i) identificar las fuentes posibles de población y evaluar el historial sanitario de sus *animales acuáticos*;
- ii) examinar las poblaciones de origen de acuerdo con el Capítulo 1.4. y seleccionar una población fundadora (F-0) de *animales acuáticos* con un alto estatus sanitario para la infección por [Patógeno X];

b) en el *país importador*:

- i) importar la población fundadora (F-0) a instalaciones de *cuarentena*;
- ii) examinar la población F-0 para el [Patógeno X] de conformidad con el Capítulo 1.4. para determinar su idoneidad como población reproductora;
- iii) producir una población de primera generación (F-1) en *cuarentena*;
- iv) criar la población F-1 en *cuarentena* durante una duración suficiente, y en condiciones favorables, para la expresión clínica de la infección por [Patógeno X] (como descrito en el Capítulo 2.4.X. del Manual Acuático), y extraer muestras y realizar pruebas para la detección de [Patógeno X] de conformidad con el Capítulo 1.4. del Código Acuático y el Capítulo 2.4.X. del Manual Acuático;
- v) si no se detecta [Patógeno X], la población F-1 puede ser definida libre de infección por el [Patógeno X]. y liberada de la *cuarentena*;
- vi) si se detecta [Patógeno X], la población F-1 no puede ser liberada de la *cuarentena* y deberá sacrificarse y eliminarse de manera biológicamente segura de acuerdo con el Capítulo 4.8.

Artículo 11.X.11.

Importación, para transformación para el consumo humano, de animales acuáticos y/o productos de animales acuáticos de un país, una zona o un compartimento no declarados libres de infección por [Patógeno X]

Cuando se importen, para transformación para el consumo humano, *animales acuáticos y productos de animales acuáticos* de una de las especies mencionadas en el Artículo 11.X.2., o productos de animales acuáticos derivados de dichas especies, procedentes de un país, una zona o un compartimento no declarados libres de infección por [Patógeno X], la *autoridad competente del país importador* deberá evaluar el riesgo y aplicar, si se justifican, las siguientes medidas para reducirlo:

- 1) entrega directa y mantenimiento de los animales la remesa a en centros de *cuarentena* o contención hasta su procesamiento en uno de los productos enumerados en el apartado 1 del Artículo 11.X.3., o en productos descritos en el apartado 1 del Artículo 11.X. 2.14., o en otros productos autorizados por la *autoridad competente*; y
- 2) tratamiento de toda el agua (incluido el hielo), de los equipos, contenedores y material de embalaje utilizados para el transporte, y de todos los efluentes y despojos resultantes de la transformación de modo que a fin de garantizar la inactivación de [Patógeno X] o la eliminación biosegura de acuerdo con los Capítulos 4.4., 4.8. y 5.5.; y
- 3) tratamiento de todos los efluentes y despojos de modo que garantice la inactivación de [Patógeno X] o la eliminación biosegura de acuerdo con los Capítulos 4.4. y 4.8.

En lo que se refiere a estas mercancías estos animales acuáticos o productos de animales acuáticos, los Países Miembros podrán considerar, si lo desean, la oportunidad de introducir medidas internas para afrontar los riesgos asociados a la utilización de cualquiera de ellos para fines que no sean el consumo humano.

Artículo 11.X.12.

Importación de animales acuáticos o productos de animales acuáticos destinados a usos distintos del consumo humano incluyendo la alimentación de los animales, la investigación y el uso agrícola, industrial o farmacéutico y procedentes de un país, una zona o un compartimento no declarados libres de infección por [Patógeno X]

Cuando se importen, para usos distintos del consumo humano incluyendo la alimentación de los animales, la investigación y el uso agrícola, industrial o farmacéutico, animales acuáticos de una de las especies mencionadas en el Artículo 11.X.2., o productos de animales acuáticos derivados de dichas especies, animales acuáticos de una de las especies mencionadas en el Artículo 9.2.2. procedentes de un país, una zona o un compartimento no declarados libres de infección por [Patógeno X], la autoridad competente del país importador exigirá que:

- 1) los animales acuáticos o productos de animales acuáticos sean entregados directamente a instalaciones de cuarentena y mantenidos en las mismas hasta su procesamiento en uno de los productos mencionados en el punto 1 del Artículo 11.X.3. o en otros productos autorizados por la autoridad competente, y
- 2) el tratamiento de toda el agua (incluido el hielo), de los equipos, contenedores y material de embalaje utilizados para el transporte y el tratamiento de todos los efluentes y despojos a fin de garantizar la inactivación de [Patógeno X] o la eliminación biosegura de acuerdo con los Capítulos 4.4., y 4.8., y 5.5.; y
- 3) el tratamiento de todos los efluentes y despojos garantice la inactivación de [Patógeno X] o la eliminación biosegura de acuerdo con los Capítulos 4.4. y 4.8..

Este artículo no se aplica a las mercancías enumeradas en el apartado 1 del Artículo 11.2.3.

Artículo 11.X.13.

[Nota: este es nuevo artículo que se armoniza con otros capítulos específicos de enfermedad del Código Acuático.]

Importación de animales acuáticos destinados al uso en laboratorios y zoológicos procedentes de un país, una zona o un compartimento no declarados libres de infección por [patógeno X]

Cuando se importen, para uso en laboratorios o zoológicos, animales acuáticos de una de las especies mencionadas en el Artículo 11.X.2. procedentes de un país, una zona o un compartimento no declarados libres de [Patógeno X], la autoridad competente del país importador deberá garantizar:

- 1) entrega directa de la remesa a instalaciones de cuarentena autorizadas por la autoridad competente y mantenimiento en las mismas;
- 2) tratamiento de toda el agua (incluido el hielo), de los equipos, contenedores y material de embalaje utilizados para el transporte de modo que garantiza la inactivación de [Patógeno X] o la eliminación biosegura de acuerdo con los Capítulos 4.4., 4.8. y 5.5., y
- 3) tratamiento de todos los efluentes y despojos provenientes de las instalaciones de cuarentena en los laboratorios o zoológicos, de modo que garantice la inactivación de [Patógeno X] o la eliminación biosegura de acuerdo con los Capítulos 4.4. y 4.8., y
- 4) eliminación de los animales muertos de acuerdo con el Capítulo 4.8.

Artículo 11.X.14.

Importación ~~o tránsito por el territorio,~~ para venta directa al por menor para el consumo humano, de animales acuáticos y productos de animales acuáticos independientemente del estatus sanitario del país, la zona o el compartimento de exportación con respecto a la infección por [Patógeno X] de un país, una zona o un compartimento no declarados libres de por [Patógeno X]

- 1) Independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por [Patógeno X], las *autoridades competentes* no deberán exigir ningún tipo de condición relacionada con la infección por [Patógeno X] cuando autoricen la importación (o el tránsito por su *territorio*) de las *los* siguientes *mercancías* productos de animales acuáticos que han sido elaborados y envasados para la venta directa al por menor y reúnen las condiciones descritas en el Artículo 5.4.2.

a) [...]

Se han establecido algunos supuestos a la hora de evaluar la seguridad sanitaria de los *productos de animales acuáticos* enumerados más arriba. Los Países Miembros deberán referirse a tales supuestos, que figuran en el Artículo 5.4.2., y analizar si se aplican a sus condiciones.

En lo que se refiere a estos *productos de animales acuáticos*, los Países Miembros podrán considerar, si lo desean, la oportunidad de introducir medidas internas para afrontar los *riesgos* asociados la utilización de cualquiera de ellos para fines que no sean el consumo humano.

- 2) Cuando se importen ~~*animales acuáticos y productos de animales acuáticos*~~, aparte de los enumerados en el apartado 1 arriba, derivados de una de las especies mencionadas en el Artículo 11.X.2. de un país, una *zona* o un *compartimento* no declarados libres de infección por [Patógeno X], la *autoridad competente* del país importador deberá evaluar el *riesgo* y aplicar medidas de mitigación del riesgo apropiadas ~~para reducirlo~~.

CHAPTER 2.2.1.

ACUTE HEPATOPANCREATIC NECROSIS DISEASE

1. Scope

Acute hepatopancreatic necrosis disease (AHPND) means infection with strains of *Vibrio parahaemolyticus* (V_{AHPND}) that contain a ~70-kbp plasmid with genes that encode homologues of the *Photobacterium* insect-related (Pir) toxins, PirA and PirB. Although there are reports of the isolation of other *Vibrio* species from clinical cases of AHPND, only V_{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* (V_{AHPND}) that contain a ~70-kbp plasmid with genes that encode homologues of the *Photobacterium* 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 V_{AHPND} has been designated pVA1, and its size may vary slightly. Removal (or “curing”) of pVA1 abolishes the AHPND-causing ability of V_{AHPND} strains.

Within a population of V_{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 V_{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 V_{AHPND} . A recent report describes a naturally occurring deletion mutant of V_{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

V_{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 $28 \pm 2^\circ\text{C}$ (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 WOAHP 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 V_{PAHPND} (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.*, 2014; 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. WOAAH 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
<u>Conventional PCR followed by amplicon sequencing</u>									+++	+++	+++	± <u>2</u>
<i>In-situ</i> 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 WOAAH 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.

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

³Specify 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 V_{PAHPND} 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

V_{PAHPND} 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 V_{PAHPND} 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 V_{PAHPND} 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 V_{PAHPND} 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 V_{PAHPND} 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 decapped 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

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

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-GG	pirA	Han et al., 2015b
VpPirA-R	GCA-GCC-CAT-TGG-TAT-TGA-ATG		
VpPirA-Probe	FAM-AGA-CAG-GAA-ACA-TAG-ACC-TAT-CAT-CGC-GGA-TAMRA		

4.4.2. Conventional PCR

Pathogen/ target gene	Primer/probe (5'-3')	Concentration	Cycling parameters
<u>Method 1 (AP1): Flegel & Lo, 2014; GenBank: KP324996; 700 bp</u>			
pVA 1	Fwd AP1F: 5CCT-TGG-GTG-TGC-TTA-GAG-GAT-G Rev AP1R: GCA-AAC-TAT-CGC-GCA-GAA-CAC-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
<u>Method 2 (AP2): Flegel & Lo, 2014; GenBank: KP324996; 700 bp</u>			
pVA 1	Fwd AP2F: TCA-GCC-GAA-TGG-TGC-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
<u>Method 13 (AP3): Sirikharin et al., 2015; GenBank Accession No.: JALL01000066.1; amplicon size: 333 bp</u>			
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
<u>Method 24 (TUMSAT-Vp3): Tinwongger et al., 2014; GenBank Accession No.: AB972427; amplicon size: 360 bp</u>			
pVA 1	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
<u>Method 35 (VpPirA-284): Han et al., 2015a; GenBank Accession No.: KM067908; amplicon size: 284 bp</u>			
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
<u>Method 46 (VpPirB-392): Han et al., 2015a; GenBank Accession No.: KM067908; amplicon size: 392 bp</u>			

<i>pirB</i> <i>vr</i>	Fwd VpPirB-392F: TGA-TGA-AGT-GAT-GGG-TGC-TC Rev VpPirB-392R: TGT-AAG-CGC-CGT-TTA-ACT-CA	0.2 μ M 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 57 (AP4): Dangtip <i>et al.</i> , 2015; GenBank Accession No.: JPKS01000000; amplicon size: 1269 bp			
<i>PirA</i> and <i>PirB</i> <i>toxi</i> <i>n</i> <i>gen</i> <i>es</i>	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 μ M 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 <i>et al.</i> , 2015; GenBank: JPKS01000000; amplicon size: 230 bp			
<i>PirA</i> and <i>PirB</i> <i>toxi</i> <i>n</i> <i>gen</i> <i>es</i>	Fwd AP4-F2: TTG-AGA-ATA-CGG-GAC-GTG-GG Rev AP4-R2: GTT-AGT-CAT-GTG-AGC-ACC-TTC	0.2 μ M 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: 5GCT TGG GTG TGC TTA GAG GAT G AP1R: GCA AAC TAT CGC GCA GAA CAG C	pVA1	700bp	Flegel & Lo (2014)
AP2	AP2F: TCA CCC GAA TGC TCG CTT GTG G AP2R: CGT CGC TAG 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 μ l 10 \times PCR mix, 0.7 μ l 50 mM MgCl₂, 0.4 μ l 10 mM dNTPs, 0.5 μ l 10 μ M AP1/AP2F, 0.5 μ l 10 μ M AP1/AP2R, 0.2 μ l Taq DNA polymerase and approximately 0.01–1 ng of template DNA in a total volume of 25 μ l 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	<i>pirA</i> ^{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	<i>pirA</i> ^{VP}	360bp	Tinwongger et al., 2014
VpPirA-284	VpPirA-284 F: TGA CTA TTC TCA CGA TTG GAC TG VpPirA-284 R: CAC GAC TAG CGC CAT TGT TA	<i>pirA</i> ^{VP}	284bp	Han et al., 2015a
VpPirB-392	VpPirB-392 F: TGA TGA AGT GAT GGG TGG TG VpPirB-392 R: TGT AAG CGC CGT TTA ACT CA	<i>pirB</i> ^{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 MgCl₂, 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 MgCl₂, 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 MgCl₂, 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 10× PCR mix, 1.5 µl 50 mM MgCl₂, 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 4.4.2.73. 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-AAG-AAT-ATA-AAA-CAT-GAA-AG AP4-R1: ACG-ATT-TCG-ACG-TTC-CCC-AA	1269	Dangtip et al., 2015
AP4 Step 2	AP4-F2: TTT-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

4.4.34. 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 WOAHP 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 WOAHP 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

¹ For example transboundary commodities.

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 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* (V_{PAHPND}) 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* (V_{PAHPND}), 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	<i>Penaeus vannamei</i>	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	<i>Penaeus vannamei</i>	100	NA	Bioassay and histopathology	Han et al. 2015b

DSe = diagnostic sensitivity, DSp = diagnostic specificity, NA= Not available, PCR: = polymerase chain reaction.
¹100% sensitivity for TUMSAT-Vp3 primer set.

6.3.2. For surveillance of apparently healthy animals

Test type	Test pose	Source populations	Tissue or sample types	Species	DSe	DSp	Reference test	Citation

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

7. References

ARANGUREN CARO L.F, MAI H.N., KANRAR S., CRUZ-FLORES R. & DHAR A.K. (2020a). A mutant of *Vibrio parahaemolyticus* *pirAB*_{VP} (+) that carries binary toxin genes but does not cause acute hepatopancreatic necrosis disease. *Microorganisms*, **8**, 1549.

[Aranguren Caro L.F., Mai H.N., Noble B. & Dhar A.K. \(2020b\). Acute hepatopancreatic necrosis disease \(V_{PAHPND}\), a chronic disease in shrimp \(*Penaeus vannamei*\) population raised in latin America. J. Invertebr. Pathol., 174, 107424. doi: 10.1016/j.jip.2020.107424. Epub 2020 Jun 11.PMID: 32535000](#)

CRUZ-FLORES R., MAI H.N & DHAR A.K. (2019). Multiplex SYBR Green and duplex TaqMan real-time PCR assays for the detection of *Photobacterium* Insect-Related (Pir) toxin genes *pirA* and *pirB*. *Mol. Cell. Probes*, **43**, 20–28.

DABU I.M., LIM J.J., ARABIT P.M.T., ORENSE S.J.A.B., TABARDILLO J.A., CORRE V.L. & MANINGAS M.B.B. (2017). The first record of acute hepatopancreatic necrosis disease in the Philippines. *Aquacult. Res.*, **48**, 792–799.

DANGTIP S., SIRIKHARIN R., SANGUANRUT P., THITAMADEE S., SRITUNYALUCKSANA K., TAENGCHAIYAPHUM S., MAVICHAK R., PROESPRAWONG P. & FLEGEL T.W. (2015). AP4 method for two-tube nested PCR detection of AHPND isolates of *Vibrio parahaemolyticus*. *Aquaculture Rep.*, **2**, 158–162.

DE LA PENA L.D., CABILLON N.A.R., CATEDRAL D.D., AMAR E.C., USERO R.C., MONOTILLA W.D., CALPE A.T., FERNANDEZ D.D. & SALOMA C.P. (2015). Acute hepatopancreatic necrosis disease (AHPND) outbreaks in *Penaeus vannamei* and *P. monodon* cultured in the Philippines. *Dis. Aquat. Org.*, **116**, 251–254.

FLEGEL T.W. & LO C.F. (2014). Free release of primers for specific detection of bacterial isolates that cause acute hepatopancreatic necrosis disease (AHPND). Published by the Network of Aquaculture Centres in Asia-Pacific, Bangkok, Thailand. <https://enaca.org/enclosure/?id=88>

GOMEZ-GIL B., SOTO-RODRÍGUEZ S., LOZANO R. & BETANCOURT-LOZANO M. (2014). Draft genome sequence of *Vibrio parahaemolyticus* strain M0605, which causes severe mortalities of shrimps in Mexico. *Genome Announc.*, **2**, e00055-14.

GOMEZ-JIMENEZ S., NORIEGA-OROZCO L., SOTELO-MUNDO R.R., CANTU-ROBLES V.A., COBIAN-GUEMES A.G., COTA-VERDUGO R.G., GAMEZ-ALEJO L.A., DEL POZO-YAUNER L., GUEVARA-HERNANDEZ E., GARCIA-OROZCO K.D., LOPEZ-ZAVALA A.A. & OCHOA-LEYVA A. (2014). High-quality draft genomes of two *Vibrio parahaemolyticus* strains aid in understanding acute hepatopancreatic necrosis disease of cultured shrimps in Mexico. *Genome Announc.*, **2**, e00800-14.

HAN J.E., TANG K.F.J., TRAN L.H. & LIGHTNER D.V. (2015a). *Photorhabdus* insect related (*Pir*) toxin-like genes in a plasmid of *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. *Dis. Aquat. Org.*, **113**, 33–40.

HAN J.E., TANG K.F.J., PANTOJA C.R., WHITE B.L. & LIGHTNER D.V. (2015b). qPCR assay for detecting and quantifying a virulence plasmid in acute hepatopancreatic necrosis disease (AHPND) due to pathogenic *Vibrio parahaemolyticus*. *Aquaculture*, **442**, 12–15.

HONG X.P., XU D., ZHUO Y., LIU H.Q. & LU L.Q. (2016). Identification and pathogenicity of *Vibrio parahaemolyticus* isolates and immune responses of *Penaeus (Litopenaeus) vannamei* (Boone). *J. Fish Dis.*, **39**, 1085–1097.

JOSHI J., SRISALA J., SAKAEW W., PRACHUMWAT A., SRITUNYALUCKSANA K., FLEGEL T.W. & THITAMADEE S. (2014a). Identification of bacterial agent(s) for acute hepatopancreatic necrosis syndrome, a new emerging shrimp disease. *Suranaree J. Sci. Technol.* Available from: <http://ird.sut.ac.th/e-journal/Journal/pdf/140283.pdf>.

JOSHI J., SRISALA J., TRUONG V.H., CHEN I.T., NUANGSAENG B., SUTHIENKUL O., LO C.F., FLEGEL T.W., SRITUNYALUCKSANA K. & THITAMADEE S. (2014b). Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). *Aquaculture*, **428–429**, 297–302.

KARUNASAGAR I., KARUNASAGAR I., VENUGOPAL M.N. & NAGESHA C.N. (1987). Survival of *Vibrio parahaemolyticus* in estuarine and sea water and in association with clams. *Syst. Appl. Microbiol.*, **9**, 316–319.

KOIWAI K., TINWONGGER S., NOZAKI R., KONDO H. & HIRONO I. (2016). Detection of acute hepatopancreatic necrosis disease strain of *Vibrio parahaemolyticus* using loop-mediated isothermal amplification. *J. Fish Dis.*, **39**, 603–606.

KONDO H., TINWONGGER S., PROESPRAWONG P., MAVICHAK R., UNAJAK S., NOZAKI R. & HIRONO I. (2014). Draft genome sequences of six strains of *Vibrio parahaemolyticus* isolated from early mortality syndrome/acute hepatopancreatic necrosis disease shrimp in Thailand. *Genome Announc.*, **2**, e00221-14.

KONDO H., VAN P.T., DANG L.T. & HIRONO I. (2015). Draft genome sequences of non-*Vibrio parahaemolyticus* acute hepatopancreatic necrosis disease strain KC13.17.5, isolated from diseased shrimp in Vietnam. *Genome Announc.*, **3**, e00978-15.

- KUMAR V., BELS L.D., COUCK L., BARUAH K., BOSSIER P. & BROECK W.V.D. (2019). PirABVP Toxin Binds to Epithelial Cells of the Digestive Tract and Produce Pathognomonic AHPND Lesions in Germ-Free Brine Shrimp. *Toxins*, **11**, 717.
- LEE C.T., CHEN I.T., YANG Y.T., KO T.P., HUANG Y.T., HUANG J.Y., HUANG M.F., LIN S.J., CHEN C.Y., LIN S.S., LIGHTNER D.V., WANG A.H., WANG H.C., HOR L.I. & LO C.F. (2015). The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. *Proc. Natl Acad. Sci. USA.*, **112**, 10798–10803.
- LIGHTNER D.V. (1996). A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. World Aquaculture Society, Baton Rouge, LA, USA.
- LO C.-F., LEU J.-H., HO C.-H., CHEN C.-H., PENG S.-E., CHEN Y.-T., CHOU C.-M., YEH P.-Y., HUANG C.-J., CHOU H.-Y., WANG C.-H. & KOU G.-H. (1996). Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. *Dis. Aquat. Org.*, **25**, 133–141.
- MAI N.H., ARANGUREN L.F.C, CRUZ-FLORES R. & DHAR A.K. (2021). Development of a Recombinase Polymerase Amplification (RPA) assay for acute hepatopancreatic necrosis disease (AHPND) detection in Pacific white shrimp (*Penaeus vannamei*). *Mol. Cell. Probes*, **57**, 101710.
- MAI H.N., CRUZ-FLORES R. & DHAR A.K. (2020). Development of an indirect Enzyme Linked Immunoassay (iELISA) using monoclonal antibodies against Photorhabdus insect related toxins, PirA^{Vp} and PirB^{Vp} released from *Vibrio* spp. *J. Microbiol. Methods*, **176**, 106002.
- MUNTADA-GARRIGA J.M., RODRIGUEZ-JEREZ J.J., LOPEZ-SABATER E.I. & MORA-VENTURA M.T. (1995). Effect of chill and freezing temperatures on survival of *Vibrio parahaemolyticus* inoculated in homogenates of oyster meat. *Lett. Appl. Microbiol.*, **20**, 225–227.
- NACA (2014). Acute hepatopancreatic necrosis disease card (updated June 2014). Published by the Network of Aquaculture Centres in Asia-Pacific, Bangkok, Thailand.
- NUNAN L., LIGHTNER D., PANTOJA C. & GOMEZ-JIMENEZ S. (2014). Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. *Dis. Aquat. Org.*, **111**, 81–86.
- ~~POWERS Q.M., ARANGUREN L.F., FITZSIMMONS K.M., McLAIN J.E. & DHAR A.K. (2021). Crayfish (*Cherax quadricarinatus*) susceptibility to acute hepatopancreatic necrosis disease (AHPND). *J. Invertebr. Pathol.*, **186**, 107554.~~
- ~~SCHOFIELD P.J., NOBLE B.L., ARANGUREN CARO L.F., MAI H.N., PADILLA T.J., MILLABAS J. & DHAR A.K. (2020). Pathogenicity of Acute Hepatopancreatic Necrosis Disease (AHPND) on the freshwater prawn, *Macrobrachium rosenbergii*, and Pacific White Shrimp, *Penaeus vannamei*, at various salinities. *Aquac. Res.*, **52**, 1480–1489.~~
- SIRIKHARIN R., TAENGCHAIYAPHUM S., SANGUANRUT P., CHI T.D., MAVICHAK R., PROESPRAIWONG P., NUANGSAENG B., THITAMADEE S., FLEGEL T.W. & SRITUNYALUCKSANA K. (2015). Characterization and PCR detection of binary, Pir-like toxins from *Vibrio parahaemolyticus* isolates that cause acute hepatopancreatic necrosis disease (AHPND) in shrimp. *PLoS ONE*, **10**, e0126987. doi:10.1371/journal.pone.0126987.
- SOTO-RODRIGUEZ S.A., GOMEZ-GIL B., LOZANO-OLVERA R., BETANCOURT-LOZANO M. & MORALES-COVARRUBIAS M.S. (2015). Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in northwestern Mexico. *Appl. Environ. Microbiol.*, **81**, 1689–1699.
- THOMSON W.K. & THACKER C.L. (1973). Effect of temperature on *Vibrio parahaemolyticus* in oysters at refrigerator and deep freeze temperatures. *Can. Inst. Food Sci. Tech. J.*, **6**, 156–158.
- TINWONGGER S., PROESPRAIWONG P., THAWONSUWAN J., SRIWANAYOS P., KONGKUMNERD J., CHAWEEPCK T., MAVICHAK R., UNAJAK S., NOZAKI R., KONDO H. & HIRONO I. (2014). Development of PCR diagnosis method for shrimp acute hepatopancreatic necrosis disease (AHPND) strain of *Vibrio parahaemolyticus*. *Fish Pathol.*, **49**, 159–164.

TRAN L.H., FITZSIMMONS K. & LIGHTNER D.V. (2014). AHPND/EMS: From the academic science perspective to the production point of view. *Aquaculture Asia Pacific*, **10**, 14–18.

TRAN L., NUNAN L., REDMAN R.M., MOHNEY L.L., PANTOJA C.R., FITZSIMMONS K. & LIGHTNER D.V. (2013). Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Dis. Aquat. Org.*, **105**, 45–55.

WEISBURG W.G., BARNES S.M., PELLETIER D.A. & LANE D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.*, **173**, 697–703.

YANG Y.T., CHEN I.T., LEE C.T., CHEN C.Y., LIN S.S., HOR L.I., TSENG T.C., HUANG Y.T., SRITUNYALUCKSANA K., THITAMADEE S., WANG H.C. & LO C.F. (2014). Draft genome sequences of four strains of *Vibrio parahaemolyticus*, three of which cause early mortality syndrome/acute hepatopancreatic necrosis disease in shrimp in China and Thailand. *Genome Announc.*, **2**, e00816-14.

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* *

NB: There are WOA Reference Laboratories for acute hepatopancreatic necrosis disease
(please consult the WOA 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 WOA 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 PENA EI (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 HPRO (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. merguensis*), 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; Frelieir *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 (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; Frelieir *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 WOA H 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* 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; Frelot *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
<u>Conventional PCR followed by amplicon sequencing</u>									+++	+++	+++	1
<i>In-situ</i> 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 WOAHP 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.

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

³Specify 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 ~~Fig-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).

<u>Patho gen/ target gene</u>	<u>Primer/probe (5'–3')</u>	<u>Concentration</u>	<u>Cycling parameters</u>
<u>Method 1: Aranguren et al., 2010; GenBank U65509</u>			
<u><i>H. penaei</i></u> <u>16S</u> <u>rRNA</u> <u>gene</u>	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	<u>300 nM</u> <u>100 nM</u>	<u>40 cycles:</u> <u>95°C/15 sec</u> <u>and 60°C/1 min</u>
<u>Method 2: Aranguren & Dhar 2018; GenBank JQAJ01000001.1</u>			
<u><i>H. penaei</i></u> <u>Flagella hook</u> <u>gene</u> <u>protein</u>	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	<u>500 nM</u> <u>100 nM</u>	<u>40 cycles:</u> <u>95°C/1 sec and</u> <u>60°C/20 sec</u>

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-TAC-AC-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,N-tetramethyl-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 Fig 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 FigE3qF) and downstream (FigE3qR) primer sequences are: 5' AAG 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,N-tetramethyl 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.

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

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 ($0.1 \text{ U } \mu\text{l}^{-1}$), magnesium chloride (1.5 mM) in 10 mM Tris-HCl, pH 9.0, 50 mM KCl.

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

Protocol 2

The PCR based on flagella gene (flagella hook protein, flgE) is based on Aranguren & Dhar (2018). Primers designated as NHP FlgE 1143F (5'-AGG CAA ACA AAC CCT TG-3') and 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 ($0.1 \text{ U } \mu\text{l}^{-1}$), magnesium chloride (1.5 mM) in 10 mM Tris-HCl, pH 9.0, 50 mM KCl.
- iv) The cycling parameters are: initial denaturation at 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 WOA 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 WOA 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

¹ For example transboundary commodities.

- 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:

- 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.

7. References

- AGUIRRE-GUZMAN G., SANCHEZ-MARTINEZ J.G., PÉREZ-CASTAÑEDA R. & ORTA-RODRIGUEZ R. (2010). Detection of necrotizing hepatopancreatitis (NHP) in wild shrimp from Laguna Madre, Mexico by a multiplex polymerase chain reaction. *Thai J. Vet. Med.*, **40**, 337–341.
- ARANGUREN L.F., BRIÑEZ B., ARAGON L., PLATZ C., CARABALLO X., SUAREZ A. & SALAZAR M. (2006). Necrotizing hepatopancreatitis (NHP) infected *Penaeus vannamei* female broodstock: effect on reproductive parameters nauplii and larvae quality. *Aquaculture*, **258**, 337–343.
- ARANGUREN L.F. & DHAR ARUN K. (2018). Detection and quantification of *Hepatobacter penaei* bacteria (NHPB) by new PCR and real-time quantitative PCR assays. *Dis. Aquat. Org.*, **131**, 49–57.
- ARANGUREN L.F., TANG K.F.J. & LIGHTNER D.V. (2010). Quantification of the bacterial agent of necrotizing hepatopancreatitis (NHP-B) by real-time PCR and comparison of survival and NHP load of two shrimp populations. *Aquaculture*, **307**, 187–192.
- AVILA-VILLA L.A., GOLLAS-GALVAN T., MARTINEZ-PORCHAS M., MENDOZA-CANO F. & HERNANDEZ-LOPEZ J. (2012). Experimental infection and detection of necrotizing hepatopancreatitis bacterium in the American lobster *Homarus americanus*. *Sci. World J.*, **2012**, 979381, <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3356760/>
- BEKAVAC A., BECK A., DRAGIČEVIĆ P., DRAGUN Z., MAGUIRE I., IVANKOVIĆ D., FIKET Ž., GRAČAN R., HUDINA S. (2022). Disturbance in invasion? Idiopathic necrotizing hepatopancreatitis in the signal crayfish *Pacifastacus leniusculus* (Dana, 1852) in Croatia. *J. Fish Dis.*, **45**, 261–276.
- BRADLEY-DUNLOP D.J., PANTOJA C. & LIGHTNER D.V. (2004). Development of monoclonal antibodies for detection of necrotizing hepatopancreatitis in penaeid shrimp. *Dis. Aquat. Org.*, **60**, 233–240.
- BRINEZ B., ARANGUREN F. & SALAZAR M. (2003). Fecal samples as DNA source for the diagnosis of necrotizing hepatopancreatitis (NHP) in *Penaeus vannamei* broodstock. *Dis. Aquat. Org.*, **55**, 69–72.
- CRABTREE B.G., ERDMAN M.M., HARRIS D.L. & HARRIS I.T. (2006). Preservation of necrotizing hepatopancreatitis bacterium (NHPB) by freezing tissue collected from experimentally infected *Litopenaeus vannamei*. *Dis. Aquat. Org.*, **70**, 175–179.

- DEL RÍO-RODRIGUEZ R.E., SOTO-RODRÍGUEZ S., LARA-FLORES M., CU-ESCAMILLA A.D. & GOMEZ-SOLANO M.I. (2006). A necrotizing hepatopancreatitis (NHP) outbreak in a shrimp farm in Campeche, Mexico: A first case report. *Aquaculture*, **255**, 606–609.
- FRELIER P.F., LOY J.K. & KRUPPENBACH B. (1993). Transmission of necrotizing hepatopancreatitis in *Penaeus vannamei*. *J. Invertebr. Pathol.*, **61**, 44–48.
- FRELIER P.F., SIS R.F., BELL T.A. & LEWIS D.H. (1992). Microscopic and ultrastructural studies of necrotizing hepatopancreatitis in Pacific white shrimp (*Penaeus vannamei*) cultured in Texas. *Vet. Pathol.*, **29**, 269–277.
- GRACIA-VALENZUELA M.H., LUZ ANGELICA ÁVILA-VILLA L.A., GLORIA YEPIZ-PLASCENCIA G., HERNÁNDEZ-LÓPEZ J., MENDOZA-CANO F., GARCÍA-SANCHEZ G. & GOLLAS-GALVÁN T. (2011). Assessing the viability of necrotizing hepatopancreatitis bacterium (NHPB) stored at –20°C for use in forced-feeding infection of *Penaeus (Litopenaeus) vannamei*. *Aquaculture*, **311**, 105–109.
- IBARRA-GAMEZ J.C., GALAVÍZ-SILVA L. & MOLINA-GARZA Z.J. (2007). Distribution of necrotizing hepatopancreatitis bacterium (NHPB) in cultured white shrimp, *Litopenaeus vannamei*, from Mexico. *Cienc. Mar.*, **33**, 1–9.
- KROL R.M., HAWKINS W.E. & OVERSTREET R.M. (1991). Rickettsial and mollicute infections in hepatopancreatic cells of cultured pacific white shrimp (*Penaeus vannamei*). *J. Invertebr. Pathol.*, **57**, 362–370.
- LEYVA J.M., MARTINEZ-PORCHAS M., HERNANDEZ-LOPEZ J., VARGAS-ALBORES F. & T. GOLLAS-GALVAN (2018). Identifying the causal agent of necrotizing hepatopancreatitis in shrimp: Multilocus sequence analysis approach *Aquaculture Res.*, 1–8.
- LIGHTNER D.V. (ed.) (1996). A handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp. World Aquaculture Society, Baton Rouge, LA, USA, 304 p.
- LIGHTNER D.V. (2005). Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. *J. World Aquac. Soc.*, **36**, 229–248.
- LIGHTNER D.V. & REDMAN R.M. (1994). An epizootic of necrotizing hepatopancreatitis in cultured penaeid shrimp (Crustacea: Decapoda) in northwestern Peru. *Aquaculture*, **122**, 9–18.
- LIGHTNER D.V., REDMAN R.M., PANTOJA C.R., TANG K.F.J., NOBLE B.L., SCHOFIELD P., MOHNEY L.L., NUNAN L.M. & NAVARRO S.A. (2012). Historic emergence, impact and current status of shrimp pathogens in the Americas. *J. Invertebr. Pathol.*, **110**, 174–183.
- LOY J.K., DEWHIRST F.E., WEBER W., FRELIER P.F., GARBAR T.L., TASCIA S.I. & TEMPLETON J.W. (1996). Molecular phylogeny and *in situ* detection of the etiologic agent of necrotizing hepatopancreatitis in shrimp. *Appl. Environ. Microbiol.*, **62**, 3439–3445.
- LOY J.K. & FRELIER P.F. (1996). Specific, nonradioactive detection of the NHP bacterium in *Penaeus vannamei* by *in situ* hybridization. *J. Vet. Diagn. Invest.*, **8**, 324–331.
- LOY J.K., FRELIER P.F., VARNER P. & TEMPLETON J.W. (1996). Detection of the etiologic agent of necrotizing hepatopancreatitis in cultured *Penaeus vannamei* from Texas and Peru by polymerase chain reaction. *Dis. Aquat. Org.*, **25**, 117–122.
- MORALES-COVARRUBIAS M.S. (2010). Enfermedades del camarón. Detección mediante análisis en fresco e histopatología. Editorial Trillas, SA de CV., Av. Río Churubusco 385, Col. Pedro María Anaya, México, D.F. Segunda edición. ISBN: ISBN 978-607-17-0436-8. 1-180.
- MORALES-COVARRUBIAS M.S., OSUNA-DUARTE A.G., GARCIA-GASCA A., LIGHTNER D.V. & MOTA-URBINA J.C. (2006). Prevalence of necrotizing hepatopancreatitis in female broodstock of *Penaeus vannamei* with unilateral eyestalk ablation and hormone injection. *J. Aquat. Anim. Health*, **18**, 19–25.

MORALES-COVARRUBIAS M.S., RUIZ-LUNA A., MOURA-LEMUS A.P., SOLÍS MONTIEL V.T. & CONROY G. (2011). Prevalencia de enfermedades de camarón blanco (*Litopenaeus vannamei*) cultivado en ocho regiones de latinoamérica. *Rev. Cient. (Maracaibo)*, **XXI**, 434–446.

NUNAN L.M., PANTOJA C.R., GOMEZ-JIMENEZ S. & LIGHTNER D.V. (2013). “*Candidatus* *Hepatobacter penaei*,” an intracellular pathogenic enteric bacterium in the hepatopancreas of the marine shrimp *Penaeus vannamei* (Crustacea: Decapoda). *Appl. Environ. Microbiol.*, **79**, 1407–1409.

VINCENT A.G., BRELAND V.M. & LOTZ J.M. (2004). Experimental infection of Pacific white shrimp *Litopenaeus vannamei* with necrotizing hepatopancreatitis (NHP) bacterium by *per os* exposure. *Dis. Aquat. Org.*, **61**, 227–233

VINCENT A.G. & LOTZ J.M. (2005). Time course of necrotizing hepatopancreatitis (NHP) in experimentally infected *Litopenaeus vannamei* and quantification of NHP-bacterium using real-time PCR. *Dis. Aquat. Org.*, **67**, 163–169.

VINCENT A.G. & LOTZ J.M. (2007). Effect of salinity on transmission of necrotizing hepatopancreatitis bacterium (NHPB) to Kona stock *Litopenaeus vannamei*. *Dis. Aquat. Org.*, **75**, 265–268.

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* *

NB: There is a WOA Reference Laboratory for infection with *Hepatobacter penaei* (necrotising hepatopancreatitis)

(please consult the WOA 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 WOA Reference Laboratories for any further information on infection with *Hepatobacter penaei* (necrotising hepatopancreatitis).

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

CHAPTER 2.2.4.

INFECTION WITH INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS

1. Scope

Infection with infectious hypodermal and haematopietic 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 haematopietic 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 IHNV 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 IHNV 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 IHNV, 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
<u>Achiridae</u>	<u><i>Achirus mazatlanus</i></u>	<u>Mazatlan sole</u>
<u>Centropomidae</u>	<u><i>Centropomus medius</i></u>	<u>blackfin snook</u>
<u>Cichlidae</u>	<u><i>Oreochromis</i> sp.</u>	<u>tilapias</u>
<u>Clupeidae</u>	<u><i>Lile stolifera</i></u>	<u>Pacific piquitinga</u>
<u>Gerreidae</u>	<u><i>Gerres cinereus</i></u>	<u>yellowfin mojarra</u>
<u>Palaemonidae</u>	<u><i>Macrobrachium rosenbergii</i></u>	<u>giant river prawn</u>
<u>Penaeidae</u>	<u><i>Penaeus duorarum</i></u>	<u>northern pink shrimp</u>
	<u><i>Penaeus occidentalis</i></u>	<u>western white shrimp</u>
	<u><i>Penaeus japonicus</i></u>	<u>kuruma prawn</u>
	<u><i>Penaeus semisulcatus</i></u>	<u>green tiger prawn</u>
	<u><i>Artemesia longinaris</i></u>	<u>Argentine stiletto shrimp</u>
<u>Pertunioidea <u>Portunidae</u></u>	<u><i>Callinectes arcuatus</i></u>	<u>Cuata swimcrab</u>
<u>Varunidae</u>	<u><i>Hemigrapsus penicillatus</i></u>	

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

IHNV 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 IHNV (Motte *et al.*, 2003).

2.2.4. Distribution of the pathogen in the host

IHNV 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 IHNV 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

~~IHNV 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 IHNV without apparent signs. Although the mussel *Mytilus edulis* is an important reservoir of IHNV (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 IHNV varies among shrimp species and populations, where infections can be either acute or chronic. For example, in unselected populations of *P. stylirostris*, infection with IHNV 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 IHNV 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 IHNV 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 IHNV genotypes from Ecuador and Peru (Aranguen Caro et al., 2022). The IHNV genotypes were found to be within a separate lineage of IHNV 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 IHNV. 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 IHNV 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 IHNV has been found in various surveys to range from 0 to 100%. Some reported mean values for IHNV 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 IHNV 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 IHNV show a marked reduction in food consumption, followed by changes in behaviour and appearance. Shrimp of this species infected with IHNV 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 IHNV (~~see Section 2.3.3 Gross pathology: Infection with IHNV in *Penaeus vannamei*~~). However, this clinical sign is not always apparent in shrimp populations chronically infected with IHNV.

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

Infection with IHNV 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 IHNV in Penaeus stylirostris

Infection with IHNV 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 IHNV show a marked reduction in food consumption, followed by changes in behaviour and appearance. Shrimp of this species infected with IHNV 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 IHNV, moribund shrimp are often distinctly bluish in colour, with opaque abdominal musculature (Lightner et al., 1983).

Infection with IHNV in Penaeus vannamei

RDS, a chronic form of infection with IHNV, 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 IHNV (and thus RDS-free) usually show CVs of 10–30% (Lightner, 1996; Primavera & Quintio, 2000).

2.3.4. Modes of transmission and life cycle

Transmission of IHNV 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 IHNV 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 10² times lower viral load than shrimp held at 24°C (Montgomery-Brock et al., 2007).

2.3.6. Geographical distribution

Infection with IHNV 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 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
<u>Conventional PCR followed by amplicon sequencing</u>									+++	+++	+++	1
<i>In-situ</i> 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.

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

³Specify 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 (Saksmerprom *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 (Saksmerprom 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

<u>Pathogen/ target gene</u>	<u>Primer/probe (5'-3')</u>	<u>Concentration</u>	<u>Cycling parameters</u>
Method 1* Tang & Lightner, 2001; GenBank Accession No.: Acc. No. AF218266			
<u>IHHNV and IHHNV-related EVEs</u> <u>non-structural protein</u>	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-AGA-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 (Krabetsve et al., 2004; Nunan et 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

Primer	Product	Sequence (5'-3')	G+C%/Temp.	GenBank & References	Specificity
392R		ATC-CGG-AGG-AAT-CTG-ATG-TG	50%/71°C	(Tang <i>et al.</i> , 2000)	
309F	309 bp	TGC-AAG-ACT-TAG-TCA-AAA-CCA-A	36%/68°C	AF218266	IHHNV <u>but not</u> IHHNV-related EVEs
309R		TGT-CTG-CTA-CGA-TGA-TTA-TCC-A	40%/69°C	(Tang <i>et al.</i> , 2007)	
MG831F	831 bp	TTG-GGG-ATG-CAG-CAA-TAT-CT	45%/58°C	DQ228358	IHHNV-related EVEs <u>but not</u> IHHNV
MG831R		GTC-CAT-CCA-CTG-ATC-GGA-CT	55%/62°C	(Tang <i>et al.</i> , 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 & Lightner, 2006).

4.6. *In-situ* hybridisation

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

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

4.7. Immunohistochemistry

Not relevant.

4.8. Bioassay

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

- i) For bioassay, feed the minced shrimp tissue suspected of being infected with IHHNV to the indicator shrimp species (e.g. SPF *P. vannamei* and *P. stylirostris* at the PLs or juvenile stage) at 10% of their body weight twice daily for 1 days.
- ii) For the following, the indicator shrimp were maintained on a pelletised ration.
- iii) Examine moribund shrimp grossly or by using the methods described above. There may be no apparent mortality during the experimental period.
- iv) If at 30 days after feeding there are still no moribund shrimp and all 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 WOAHP 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 WOAHP 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

¹ For example transboundary commodities.

- iv) ~~Histopathology consistent with IHNV 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 IHNV 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 IHNV infection coupled with~~ A positive result by *in-situ* hybridisation and detection of IHNV a positive result by real-time PCR
- iii) ~~Histopathology consistent with IHNV infection coupled with~~ A positive result by *in-situ* hybridisation and detection of IHNV by a positive result by conventional PCR 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.~~

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

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with IHNV 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 IHNV, 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.

7. References

ALY S.M., MANSOUR S.M., THABET R.Y. & MABROK M. (2021). Studies on infectious myonecrosis virus (IMNV) and infectious hypodermal and hematopoietic necrosis virus (IHNV) in cultured penaeid shrimp in Egypt. *Dis. Aquat. Org.*, **143**, 57–67.

ARANGUREN CARO L.F., GOMEZ-SANCHEZ M.M., PIEDRAHITA Y., MAI H.N., CRUZ-FLORES R., ALENTON R.R.R. & DHAR A.K. (2022). Current status of infection with infectious hypodermal and hematopoietic necrosis virus (IHHNV) in the Peruvian and Ecuadorian shrimp industry. *PLoS One*, 17(8):e0272456. doi: 10.1371/journal.pone.0272456.

ARUNRUT N., PROMBUN P., SAKSMERPROME V., FLEGEL T. W. & KIATPATHOMCHAI W. (2011). Rapid and sensitive detection of infectious hypodermal and hematopoietic necrosis virus by loop-mediated isothermal amplification combined with a lateral flow dipstick. *J. Virol. Methods*, **171**, 21–25.

BELL T.A. & LIGHTNER D.V. (1984). IHHN virus: infectivity and pathogenicity studies in *Penaeus stylirostris* and *Penaeus vannamei*. *Aquaculture*, **38**, 185–194.

BELL T.A. & LIGHTNER D.V. (1987). IHHN disease of *Penaeus stylirostris*: effects of shrimp size on disease expression. *J. Fish Dis.*, **10**, 165–170.

BELL T.A. & LIGHTNER D.V. (1988). A Handbook of Normal Shrimp Histology. Special Publication No. 1, World Aquaculture Society, Baton Rouge, Louisiana, USA, 114 pp.

BONAMI J.R., TRUMPER B., MARI J., BREHELIN M. & LIGHTNER D.V. (1990). Purification and characterization of IHHN virus of penaeid shrimps. *J. Gen. Virol.*, **71**, 2657–2664.

CHAYABURAKUL K., LIGHTNER D.V., SRIURAIRATTANA S., NELSON K.T. & WITHYACHUMNARNKUL B. (2005). Different responses to infectious hypodermal and hematopoietic necrosis virus (IHHNV) in *Penaeus monodon* and *P. vannamei*. *Dis. Aquat. Org.*, **67**, 191–200.

CHAYABURAKUL K., NASH G., PRATANPIPAT P., SRIURAIRATTANA S. & WITHYACHUMNARNKUL B. (2004). Multiple pathogens found in growth-retarded black tiger shrimp *Penaeus monodon* cultivated in Thailand. *Dis. Aquat. Org.*, **60**, 89–96.

CHEN S.N., CHANG P.S. & KOU G.H. (1992). Infection route and eradication of *Penaeus monodon* baculovirus (MBV) in larval giant tiger prawns, *Penaeus monodon*. In: Diseases of Cultured Penaeid Shrimp in Asia and the United States, Fulks W. & Main K.L., eds. Oceanic Institute, Honolulu, Hawaii, USA, 177–184.

COWLEY J.A., RAO M., COMAN G.J. & COWLEY J. (2018). Real-time PCR tests to specifically detect Infectious hypodermal and haemopoietic necrosis virus (IHHNV) lineages and an IHHNV endogenous viral element (EVE) integrated in the genome of Black Tiger shrimp (*Penaeus monodon*). *Dis. Aquat. Org.*, **129**, 145–158.

DHAR A.K., ROUX M.M. & KLIMPEL K.R. (2001). Detection and quantification of Infectious hypodermal and hematopoietic necrosis virus and White spot virus in shrimp using real-time quantitative PCR and SYBR green chemistry. *J. Clin. Microbiol.*, **39**, 2835–2845.

ENCINAS-GARCIA T., MENDOZA-CANO F., ENRÍQUEZ-ESPINOZA T., LUKEN-VEGA L., VICHIDO-CHÁVEZ R. & SÁNCHEZ-PAZ A. (2015). An improved validated SYBR green-based real-time quantitative PCR assay for the detection of the *Penaeus stylirostris* densovirus in penaeid shrimp. *J. Virol. Methods*, **212**, 53–58.

FERNANDO M.C., ENRIQUEZ-ESPINOZA T., VALENZUELA-CASTILLO A., ENCINAS-GARCIA T. & SANCHEZ-PAZ A. (2016). High Occurrence of the Decapod Penstyldenovirus (PstDV1) Detected in Postlarvae of *Penaeus vannamei* Produced in Commercial Hatcheries of Mexico. *EcoHealth*. **13**, 591–596.

JAGADEESAN V., EZHIL PRAVEENA P., OTTA S.K. & JITHENDRAN K.P. (2019). Classical runt deformity syndrome cases in farmed *Penaeus vannamei* along the east coast of India. In: BRAQQON 2019: World Brackishwater Aquaculture Conference, Jithendran K.P., Saraswathy R., Balasubramanian C.P., Kumaraguru Vasagam K.P., Jayasankar V., Raghavan R., Alavandi S.V. & Vijayan K.K., eds. Journal of Coastal Research, Special Issue No. 86, pp. 107–111. Coconut Creek (Florida), ISSN 0749-0208.

KALAGAYAN G., GODIN D., KANNA R., HAGINO G., SWEENEY J., WYBAN J. & BROCK J. (1991). IHHN virus as an etiological factor in runt-deformity syndrome of juvenile *Penaeus vannamei* cultured in Hawaii. *J. World Aquacult. Soc.*, **22**, 235–243.

- KRABSETSVE K., CULLEN B.R. & OWENS L. (2004). Rediscovery of the Australian strain of infectious hypodermal and haematopoietic necrosis virus. *Dis. Aquat. Org.*, **61**, 153–158.
- LIGHTNER D.V. (ED.) (1996). *A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp*. World Aquaculture Society, Baton Rouge, Louisiana, USA, 304 pp.
- LIGHTNER D.V. (2005). Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. *J. World Aquaculture Soc.* **36**, 229–248.
- LIGHTNER D.V., MOHNEY L.L., WILLIAMS R.R. & REDMAN R.M. (1987). Glycerol tolerance of IHNV of penaeid shrimp. *J. World Aquac. Soc.*, **18**, 196–197.
- LIGHTNER D.V., REDMAN R.M., ARCE S. & MOSS S.M. (2009). Specific pathogen-free (SPF) shrimp stocks in shrimp farming facilities as a novel method for disease control in crustaceans. In: *Shellfish Safety and Quality*, Shumway S. & Rodrick G., eds. Woodhead Publishers, London, UK, 384–424.
- LIGHTNER D.V. & REDMAN R.M. (1998). Shrimp diseases and current diagnostic methods. *Aquaculture*, **164**, 201–220.
- LIGHTNER D.V., REDMAN R.M. & BELL T.A. (1983). Infectious hypodermal and hematopoietic necrosis a newly recognized virus disease of penaeid shrimp. *J. Invertebr. Pathol.*, **42**, 62–70.
- MARI J., BONAMI J.R. & LIGHTNER D.V. (1993). Partial cloning of the genome of infectious hypodermal and hematopoietic necrosis virus, an unusual parvovirus pathogenic for penaeid shrimps; diagnosis of the disease using a specific probe. *J. Gen. Virol.*, **74**, 2637–2643.
- MONTGOMERY-BROCK D., TACON A.G.J., POULOS B., & LIGHTNER D.V. (2007). Reduced replication of infectious hypodermal and hematopoietic necrosis virus (IHNV) in *Litopenaeus vannamei* held in warm water. *Aquaculture*, **265**, 41–48.
- MORALES-COVARRUBIAS M.S., NUNAN L.M., LIGHTNER D.V., MOTA-URBINA J.C., GARZA-AGUIRRE M.C. & CHAVEZ-SANCHEZ M.C. (1999). Prevalence of IHNV in wild broodstock of *Penaeus stylirostris* from the upper Gulf of California, Mexico. *J. Aquat. Anim. Health*, **11**, 296–301.
- MOTTE, E., YUGCHA E., LUZARDO J., CASTRO F., LECLERCQ G., RODRÍGUEZ J., MIRANDA P., BORJA O., SERRANO J., TERREROS M., MONTALVO K., NARVÁEZ A., TENORIO N., CEDEÑO V., MIALHE E. & BOULO V. (2003). Prevention of IHNV vertical transmission in the white shrimp *Litopenaeus vannamei*. *Aquaculture*, **219**, 57–70.
- NUNAN L.M., POULOS B.T. & LIGHTNER D.V. (2000). Use of polymerase chain reaction (PCR) for the detection of infectious hypodermal and hematopoietic necrosis virus (IHNV) in penaeid shrimp. *Mar. Biotechnol.*, **2**, 319–328.
- NUNAN L.M., ARCE S.M., STAHA R.J. & LIGHTNER D.V. (2001). Prevalence of Infectious hypodermal and hematopoietic necrosis virus (IHNV) and White spot syndrome virus (WSSV) in *Litopenaeus vannamei* in the Pacific Ocean off the coast of Panama. *J. World Aquacult. Soc.*, **32**, 330–334.
- PANTOJA C.R., LIGHTNER D.V. & HOLTSCHMIT K.H. (1999). Prevalence and geographic distribution of IHNV parvovirus in wild penaeid shrimp (Crustacea: Decapoda) from the Gulf of California, Mexico. *J. Aquat. Anim. Health*, **11**, 23–34.
- PENZES J.J., SODERLUND-VENERMO M., CANUTI M., EIS-HUBINGER A.M., HUGHES J., COTMORE S.F. & HARRACH B. (2020). Reorganizing the family *Parvoviridae*: a revised taxonomy independent of the canonical approach based on host association. *Arch. Virol.*, **165**, 2133–2146. <https://doi.org/10.1007/s00705-020-04632-4>
- PRIMAVERA, J.H. & QUINTIO E.T. (2000). Runt-deformity syndrome in cultured giant tiger prawn *Penaeus monodon*. *J. Crust. Biol.*, **20**, 796–802.
- SAKMERPROME V., JITRAKORN S., CHAYABURAKUL K., LAIPHROM S., BOONSUA K. & FLEGEL T.W. (2011). Additional random, single to multiple genome fragments of *Penaeus stylirostris* densovirus in the giant tiger shrimp genome have implications for viral disease diagnosis. *Virus Res.*, **160**, 180–190.

SELLARS M.J., COWLEY J.A., MUSSON D., RAO M., MENZIES M.L, COMAN G. J. & MURPHY B.S. (2019). Reduced growth performance of Black Tiger shrimp (*Penaeus monodon*) infected with infectious hypodermal and hematopoietic necrosis virus. *Aquaculture*, **499**, 160–166.

SHIKE H., DHAR A.K., BURNS J.C., SHIMIZU C., JOUSSET F.X., KLIMPEL K.R. & BERGOIN M. (2000). Infectious hypodermal and hematopoietic necrosis virus of shrimp is related to mosquito Brevideensoviruses. *Virology*, **277**, 167–177.

SUN Z.F., HU C. Q., REN C. H. & SHEN Q. (2006). Sensitive and rapid detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in shrimps by loop-mediated isothermal amplification. *J. Virol. Methods*, **131**, 41–46.

TAENGCHAIYAPHUM S., BUATHONGKAM P., SUKTHAWORN S., WONGKHALUANG P., SRITUNYALUCKSANA K. & FLEGEL T.W. (2021). Shrimp Parvovirus Circular DNA Fragments Arise From Both Endogenous Viral Elements and the Infecting Virus. *Front. Immunol.*, **12**, 729528. doi: 10.3389/fimmu.2021.729528.

TAENGCHAIYAPHUM S., WONGKHALUANG P., SITTIKANKAEW K., KAROONUTHAISIRI N., FLEGEL T.W. & SRITUNYALUCKSANA K. (2022). Shrimp genome sequence contains independent clusters of ancient and current Endogenous Viral Elements (EVE) of the parvovirus IHHNV. *BMC Genomics*, **23**, 565. doi: 10.1186/s12864-022-08802-3.

TANG K.F.J., DURAND S.V., WHITE B.L., REDMAN R.M., PANTOJA C.R. & LIGHTNER D.V. (2000). Postlarvae and juveniles of a selected line of *Penaeus stylirostris* are resistant to infectious hypodermal and hematopoietic necrosis virus infection. *Aquaculture*, **190**, 203–210.

TANG K.F.J. & LIGHTNER D.V. (2001). Detection and quantification of infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp by real-time PCR. *Dis. Aquat. Org.*, **44**, 79–85.

TANG K.F.J. & LIGHTNER D.V. (2006). Infectious hypodermal and hematopoietic necrosis virus (IHHNV) in the genome of the black tiger prawn *Penaeus monodon* from Africa and Australia. *Virus Res.*, **118**, 185–191.

TANG K.F.J., NAVARRO S.A. & LIGHTNER D.V. (2007). A PCR assay for discriminating between infectious hypodermal and hematopoietic necrosis virus (IHHNV) and the virus-related sequences in the genome of *Penaeus monodon*. *Dis. Aquat. Org.*, **74**, 165–170.

TANG K.F.J., POULOS B.T., WANG J., REDMAN R.M., SHIH H.H. & LIGHTNER D.V. (2003). Geographic variations among infectious hypodermal and hematopoietic necrosis virus (IHHNV) isolates and characteristics of their infection. *Dis. Aquat. Org.*, **53**, 91–99.

~~WEL Y.W., FAN D.D. & CHEN J. (2017). The mussel *Mytilus edulis* L. as an important reservoir of infectious hypodermal and hematopoietic necrosis virus (IHHNV). *Aquaculture Res.*, **48**, 1346–1350.~~

XIA X.X., YU Y.X., HU L.H., MANFRED W. & PAN Y.J. (2015). Rapid detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) by real-time, isothermal recombinase polymerase amplification assay. *Arch. Virol.*, **160**, 987–994.

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* *

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

Please contact the WOA 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. merguensis*), 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
<i>In-situ</i> 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.

¹For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). ²Susceptibility 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 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 <i>et al.</i> , 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
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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 and 139 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)	<u>Outer-Primary</u> 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
	<u>Inner-Nested</u> 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 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* hybridisation (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 WOA 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 WOA 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 WOA Reference Laboratories designated for IMN.

6.1. Apparently healthy animals or animals of unknown health status¹

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~~ 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~~

¹ For example transboundary commodities.

- 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:

- 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 <i>P. vannamei</i>	abdominal muscle	<i>P. vannamei</i>	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
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Real-time PCR								
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DSe = diagnostic sensitivity, DSp = diagnostic specificity, n = number of samples used in the study, PCR = polymerase chain reaction.

7. References

- ALY S.M., MANSOUR S.M., THABET R.Y. & MABROK M. (2021). Studies on infectious myonecrosis virus (IMNV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) in cultured penaeid shrimp in Egypt. *Dis. Aquat. Org.*, **143**, 57–67. doi: 10.3354/dao03556. PMID: 33570040.
- ANDRADE T.P.D., SRISUVAN T., TANG K.F.J. & LIGHTNER D.V. (2007). Real-time reverse transcription polymerase chain reaction assay using TaqMan probe for detection and quantification of infectious myonecrosis virus (IMNV). *Aquaculture*, **264**, 9–15.
- BELL T.A. & LIGHTNER D.V. (1988). *A Handbook of Normal Penaeid Shrimp Histology*. World Aquaculture Society, Baton Rouge, LA, USA, 114 p.
- CHAVISUTHANGKURA P., SENAPIN S., WANGMAN P., LONGYANT S. & SITHIGORNUL P. (2013). Simple and rapid detection of infectious myonecrosis virus using an immunochromatographic strip test. *Arch Virol.*, **158**, 1925–1930.
- CHEN S.N., CHANG P.S. & KOU G.H. (1992). Infection route and eradication of *Penaeus monodon* baculovirus (MBV) in larval giant tiger prawns, *Penaeus monodon*. In: *Diseases of Cultured Penaeid Shrimp in Asia and the United States*, Fulks W. & Main K.L., eds. Oceanic Institute, Honolulu, Hawaii, USA, 177–184.
- DANTAS M.D., CHAVANTE S.F., TEIXEIRA D.I., LIMA J.P. & LANZA D.C. (2015). Analysis of new isolates reveals new genome organization and a hypervariable region in infectious myonecrosis virus (IMNV). *Virus Res.*, **20**, 66–71. doi: 10.1016/j.virusres.2015.03.015. Epub 2015 Apr 4. PMID: 25849112.
- DA SILVA S.M.B.C., LAVANDER H.D., DE SANATANA LUNA M.M., DA SILVA A.O.M.E., GALVEZ A.O. & COIMBRA M.R.M. (2015). *Artemia franciscana* as a vector for infectious myonecrosis virus (IMNV) to *Litopenaeus vannamei* juvenile. *J. Invertebr. Pathol.*, **126**, 1–5.
- FAUQUET C.M., MAYO M.A., MANILOFF J., DESSELBERGER U. & BALL L.A., EDITORS (2005). *Totiviridae*. In: *Virus Taxonomy: Classification and Nomenclature of Viruses. Eighth Report of the International Committee on the Taxonomy of Viruses*, Elsevier, San Francisco, USA, pp. 571–580.
- KOIWAI K., KODERA T., THAWONSUWAN J., RIANI S., KAWASE M., KONDO H. & HIRONO I. (2018). Rapid diagnosis of three shrimp RNA viruses using RT-PCR-DNA chromatography. *J. Fish Dis.*, 2018 May 28. doi: 10.1111/jfd.12821. Epub ahead of print. PMID: 29806113.
- KUNANOPPARAT A., CHAVISUTHANGKURA P., SENAPIN S., LONGYANT S., RUKPRATANPORN S., FLEGEL T.W. & SITHIGORNUL P. (2011). Detection of infectious myonecrosis virus using monoclonal antibody specific to N and C fragments of the capsid protein expressed heterologously. *J. Virol. Methods*, **171**, 141–148.
- LEE C.S. & O'BRYEN P.J., EDITORS (2003). *Biosecurity in Aquaculture Production Systems: Exclusion of Pathogens and Other Undesirables*. World Aquaculture Society, Baton Rouge, LA, USA, 293 p.
- LIGHTNER D.V. (2005). Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. *J. World Aquac. Soc.*, **36**, 229–248.
- LIGHTNER D.V. (2011). Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): a review. *J. Invertebr. Pathol.*, **106**, 110–130.

- LIGHTNER D.V., PANTOJA C.R., POULOS B.T., TANG K.F.J., REDMAN R.M., PASOS DE ANDRADE T. & BONAMI J.R. (2004). Infectious myonecrosis: new disease in Pacific white shrimp. *Global Aquaculture Advocate*, **7**, 85.
- LIGHTNER D.V., REDMAN R.M., ARCE S. & MOSS S.M. (2009). Specific pathogen-free shrimp stocks in shrimp farming facilities as a novel method for disease control in crustaceans. *In: Shellfish Safety and Quality*, Shumway S. & Rodrick G., eds. Woodhead Publishers, London, UK, pp. 384–424.
- LOY D.S., LIU S., MOGLER M.A., LOY D.J., BLITVICH B.J. & BARTHOLOMAY L.C. (2015). Characterization of newly revealed sequences in the infectious myonecrosis virus genome in *Litopenaeus vannamei*. *J. Gen. Virol.*, **96** (Pt 7), 1821–1819.
- MAI H.N., HANGGONO B., CARO L.F.A., KOMARUDDIN U., NUR'AINI Y.L. & DHAR A.K. (2019). Novel infectious myonecrosis virus (IMNV) genotypes associated with disease outbreaks on *Penaeus vannamei* shrimp farms in Indonesia. *Arch. Virol.*, **164**, 3051–3057. doi: 10.1007/s00705-019-04408-5. Epub 2019 Sep 17. PMID: 31531743.
- MOSS S.M. & MOSS D.R. (2009). Chapter 17: Selective breeding of penaeid shrimp. *In: Shellfish Safety and Quality*, Shumway S. & Rodrick G., eds. Woodhead Publishers, London, UK. pp. 425–452.
- NAIM S., BROWN J.K. & NIBERT M.L. (2014). Genetic diversification of penaeid shrimp infectious myonecrosis virus between Indonesia and Brazil. *Virus Res.*, **189**, 99–105.
- NAIM S., TANG K.F.J., YANG M., LIGHTNER D.V. & NIBERT M.L. (2015). Extended genome sequences of penaeid shrimp infectious myonecrosis virus strains from Brazil and Indonesia. *Arch. Virol.*, **160**, 1579–1583.
- NIBERT M.L. (2007). '2A-like' and 'shifty heptamer' motifs in penaeid shrimp infectious myonecrosis virus, a monosegmented double-stranded RNA virus. *J. Gen. Virol.*, **88**, 1315–1318.
- NUNES A.J.P., CUNHA-MARTINS P. & VASCONSELOS-GESTEIRA T.C. (2004). Carcinicultura ameaçada. *Rev. Panoram. Aquic.*, **83**, 37–51 (in Portuguese).
- POULOS B.T. & LIGHTNER D.V. (2006). Detection of infectious myonecrosis virus (IMNV) of penaeid shrimp by reverse-transcriptase polymerase chain reaction (RT-PCR). *Dis. Aquat. Org.*, **73**, 69–72.
- POULOS B.T., TANG K.F.J., PANTOJA C.R., BONAMI J.R. & LIGHTNER D.V. (2006). Purification and characterization of infectious myonecrosis virus of penaeid shrimp. *J. Gen. Virol.*, **87**, 987–996.
- SAHUL HAMEED A.S., ABDUL MAJEED S., VIMAL S., MADAN N., RAJKUMAR T., SANTHOSH KUMAR S. & SIVAKUMAR S. (2017). Studies on the occurrence of infectious myonecrosis virus in pond-reared *Litopenaeus vannamei* (Boone, 1931) in India. *J. Fish Dis.*, **40**, 1823–1830. doi: 10.1111/jfd.12655. Epub 2017 Jun 20. PMID: 28631825
- SANTHOSH KUMAR S., SIVAKUMAR S., ABDUL MAJEED S., VIMAL S., TAJU G. & SAHUL HAMEED A.S. (2021). *In vitro* propagation of infectious myonecrosis virus in C6/36 mosquito cell line. *J. Fish Dis.*, **44**, 987–992. doi: 10.1111/jfd.13359. Epub 2021 Feb 25. PMID: 33631045.
- SENAPIN S., PHEWSAIYA K., BRIGGS M. & FLEGEL T.W. (2007). Outbreaks of infectious myonecrosis virus (IMNV) in Indonesia confirmed by genome sequencing and use of an alternative RT-PCR detection method. *Aquaculture*, **266**, 32–38.
- TANG K.F.J., PANTOJA C.R., POULOS B.T., REDMAN R.M. & LIGHTNER D.V. (2005). *In situ* hybridization demonstrates that *Litopenaeus vannamei*, *L. stylirostris* and *Penaeus monodon* are susceptible to experimental infection with infectious myonecrosis virus (IMNV). *Dis. Aquat. Org.*, **63**, 261–265.
- TANG K.F.J., PANTOJA C.R., REDMAN R.M. & LIGHTNER D.V. (2007). Development of *in situ* hybridization and RT-PCR assay for the detection of a nodavirus (PvNV) that causes muscle necrosis in *Penaeus vannamei*. *Dis. Aquat. Org.*, **75**, 183–190.

VANPATTEN K.A., NUNAN L.M. & LIGHTNER D.V. (2004). Seabirds as potential vectors of penaeid shrimp viruses and the development of a surrogate laboratory model utilizing domestic chickens. *Aquaculture*, **241**, 31–46.

VIEIRA-GIRÃO P.R.N., FALCÃO C.B., ROCHA I.R.C.B., LUCENA H.M.R., COSTA F.H.F. & RÁDIS-BAPTISTA G. (2017). Antiviral Activity of Ctn[15–34], A Cathelicidin-Derived Eicosapeptide, Against Infectious Myonecrosis Virus in *Litopenaeus vannamei* Primary Hemocyte Cultures. *Food Environ. Virol.*, **9**, 277–286. doi: 10.1007/s12560-017-9285-5. Epub 2017 Feb 16. PMID: 28210987.

WHITE-NOBLE B.L., LIGHTNER D.V., TANG K.F.J. & REDMAN R. (2010). Lab challenge for selection of IMNV-resistant white shrimp. *Global Aquaculture Advocate*, July/August, 71–73.

Wickner R.B., Ghabrial S.A., Nibert M.L., Patterson J.L. & Wang C.C. (2011). Totiviridae. In: Virus Taxonomy: Classification and Nomenclature of Viruses. Ninth Report of the International Committee on the Taxonomy of Viruses. Elsevier, San Diego, USA.

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NB: At the time of publication (2022) there was no WOA Reference Laboratory for infection with infectious myonecrosis virus (please consult the WOA 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 $\times 10$ 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 depends 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
<i>In-situ</i> 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 WOAHP 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.

¹For confirmatory diagnoses, methods need to be carried out in combination (see Section 6).

²Susceptibility 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 <i>et al.</i> , 2004); GenBank Accession No.: AF277675			
<u>TSV</u> /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 <i>et al.</i> , 1998); product amplicon size: 231 bp			

<u>TSV</u> /ORF 2	Fwd: AAG-TAG-ACA-GCC-GCG-CTT Rev:9195R: TCA-ATG-AGA-GCT-TGG-TCC	9992:	Primers/620 nM each	Reverse transcription 60°C/30 min 40 cycles: 94°C/45 sec, 60°C/45 sec
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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.
- 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 MgCl₂, 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 WOAHP 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 WOAHP 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.

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

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:

- 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

² For example transboundary commodities.

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

- AUDELO DEL VALLE J., CLEMENT-MELLADO O., MAGANA-HERNANDEZ A., FLISSER A., MONTIEL-AGUIRRE F. & BRISENO-GARCIA B. (2003). Infection of cultured human and monkey cell lines with extract of penaeid shrimp infected with Taura syndrome virus. *Emerg. Infect. Dis.*, **9**, 265–266.
- BONAMI J.R., HASSON K.W., MARI J., POULOS B.T. & LIGHTNER D.V. (1997). Taura syndrome of marine penaeid shrimp: characterization of the viral agent. *J. Gen. Virol.*, **78**, 313–319.
- BROCK J.A. (1997). Special topic review: Taura syndrome, a disease important to shrimp farms in the Americas. *World J. Microbiol Biotechnol.*, **13**, 415–418.
- BROCK J.A., GOSE R., LIGHTNER D.V. & HASSON K.W. (1995). An overview on Taura syndrome, an important disease of farmed *Penaeus vannamei*. In: *Swimming through Troubled Water, Proceedings of the Special Session on Shrimp Farming, Aquaculture '95*, Browdy C.L. & Hopkins J.S., eds. San Diego, California, 1–4 February 1995. World Aquaculture Society, Baton Rouge, Louisiana, USA, 84–94.
- CHEN S.N., CHANG P.S. & KOU G.H. (1992). Infection route and eradication of *Penaeus monodon* baculovirus (MBV) in larval giant tiger prawns, *Penaeus monodon*. In: *Diseases of Cultured Penaeid Shrimp in Asia and the United States*, Fulks W. & Main K.L., eds. Oceanic Institute, Honolulu, Hawaii, USA, 177–184.
- CLIFFORD H.C. (1998). Management of ponds stocked with blue shrimp *Litopenaeus stylirostris*. In: *Proceedings of the First Latin American Shrimp Farming Congress*, D.E. Jory, ed. Panama City, Panama, 1–11.
- DHAR A.K., COWLEY J.A., HASSON K.W. & WALKER P.J. (2004). Taura syndrome virus and yellow head virus of penaeid shrimp. *Adv. Virus Res.*, **64**, 353–421.
- DIXON H. & DORADO J. (1997). Managing Taura syndrome in Belize: a case study. *Aquaculture Magazine*, May/June, 30–42.

- FEAGAN D.F. & CLIFFORD H.C. III. (2001). Health management for viral diseases in shrimp farms. *In: The New Wave, Proceedings of the Special Session on Sustainable Shrimp Culture. Aquaculture 2001*, Browdy C.L. & Jory D.E., eds. The World Aquaculture Society, Baton Rouge, Louisiana, USA, 168–198.
- GARZA J.R., HASSON K.W., POULOS B.T., REDMAN R.M., WHITE B.L. & LIGHTNER D.V. (1997). Demonstration of infectious taura syndrome virus in the feces of sea gulls collected during an epizootic in Texas. *J. Aquat. Anim. Health*, **9**, 156–159.
- HASSON K.W., HASSON J., AUBERT H., REDMAN R.M. & LIGHTNER D.V. (1997). A new RNA-friendly fixative for the preservation of penaeid shrimp samples for virological detection using cDNA genomic probes. *J. Virol. Methods*, **66**, 227–236.
- HASSON K.W., LIGHTNER D.V., MARI J. & BONAMI J.R., POULOS B.T., MOHNEY L.L., REDMAN R.M. & BROCK J.R. (1999a). The geographic distribution of Taura Syndrome Virus (TSV) in the Americas: determination by histology and *in situ* hybridization using TSV-specific cDNA probes. *Aquaculture*, **171**, 13–26.
- HASSON K.W., LIGHTNER D.V., MOHNEY L.L., REDMAN R.M., POULOS B.T. & WHITE B.M. (1999b). Taura syndrome virus (TSV) lesion development and the disease cycle in the Pacific white shrimp *Penaeus vannamei*. *Dis. Aquat. Org.*, **36**, 81–93.
- HASSON K.W., LIGHTNER D.V., POULOS B.T., REDMAN R.M., WHITE B.L., BROCK J.A. & BONAMI J.R. (1995). Taura syndrome in *Penaeus vannamei*: demonstration of a viral etiology. *Dis. Aquat. Org.*, **23**, 115–126.
- INTRIAGO P., JIMENEZ R., MACHUCA M., BARNIOL R., KRAUSS E. & SALVADOR X. (1997). Experiments on toxicosis as the cause of Taura Syndrome in *Penaeus vannamei* (Crustacea: Decapoda) in Ecuador. *In: Diseases in Asian Aquaculture III*, Flegel T.W. & MacRae I.H., eds. Fish Health Section, Asian Fisheries Society, Manila, the Philippines, 365–379.
- JIMENEZ R. (1992). Síndrome de Taura (Resumen). *In: Acuicultura del Ecuador. Cámara Nacional de Acuicultura*, Guayaquil, Ecuador, 1–16.
- JIMENEZ R., BARNIOL R., DE BARNIOL L. & MACHUCA M. (2000). Periodic occurrence of epithelial viral necrosis outbreaks in *Penaeus vannamei* in Ecuador. *Dis. Aquat. Org.*, **42**, 91–99.
- LIGHTNER D.V. (ED.) (1996a). A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp. World Aquaculture Society, Baton Rouge, Louisiana, USA, 304 pp.
- LIGHTNER D.V. (1996b). Epizootiology, distribution and the impact on international trade of two penaeid shrimp viruses in the Americas. *Rev. sci. tech. Office int. Epiz.*, **15**, 579–601.
- LIGHTNER D.V. (1999). The penaeid shrimp viruses TSV, IHHNV, WSSV, and YHV: current status in the Americas, available diagnostic methods and management strategies. *J. Appl. Aquac.*, **9**, 27–52.
- LIGHTNER D.V. (2005). Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. *J. World Aquac. Soc.*, **36**, 229–248.
- LIGHTNER D.V. & REDMAN R.M. (1998). Shrimp diseases and current diagnostic methods. *Aquaculture*, **164**, 201–220.
- LIGHTNER D.V., REDMAN R.M., ARCE S. & MOSS S.M. (2009). Specific pathogen-free shrimp stocks in shrimp farming facilities as a novel method for disease control in crustaceans. *In: Shellfish Safety and Quality*, Shumway S. & Rodrick G., eds. Woodhead Publishers, London, UK. pp. 384–424.
- LIGHTNER D.V., REDMAN R.M., HASSON K.W. & PANTOJA C.R. (1995). Taura syndrome in *Penaeus vannamei* (Crustacea: Decapoda): gross signs, histopathology and ultrastructure. *Dis. Aquat. Org.*, **21**, 53–59.
- LIGHTNER D.V., REDMAN R.M., PANTOJA C.R., TANG K.F.J., NOBLE B.L., SCHOFIELD P., MOHNEY L.L., NUNAN L.M. & NAVARRO S.A. (2012). Historic emergence, impact and current status of shrimp pathogens in the Americas. *J. Invertebr. Pathol.*, **110**, 174–183.

- LOTZ J.M. (1997). Effect of host size on virulence of Taura virus to the marine shrimp *Penaeus vannamei* (Crustacea: Penaeidae). *Dis. Aquat. Org.*, **30**, 45–51.
- LOTZ J.M., ANTON, L.S. & SOTO M.A. (2005). Effect of chronic Taura syndrome virus infection on salinity tolerance of *Litopenaeus vannamei*. *Dis. Aquat. Org.*, **65**, 75–78.
- LUO P., HU C.Q., REN C.H. & SUN Z.F. (2004). Taura syndrome virus and mammalian cell lines. *Emerg. Infect. Dis.*, **10**, 2260–2261.
- MARI J., BONAMI J.R. & LIGHTNER D.V. (1998). Taura syndrome of penaeid shrimp: cloning of viral genome fragments and development of specific gene probes. *Dis. Aquat. Org.*, **33**, 11–17.
- NAVARRO S.A., TANG K.F.J & LIGHTNER D.V. (2009). An improved Taura syndrome virus (TSV) RT-PCR using newly designed primers. *Aquaculture*, **293**, 290–292.
- NIELSEN L., SANG-OUW W., CHEEVADHANARAK S. & FLEGEL T.W. (2005). Taura syndrome virus (TSV) in Thailand and its relationship to TSV in China and the Americas. *Dis Aquat. Org*, **63**, 101–106.
- NUNAN L.M., POULOS B.T. & LIGHTNER D.V. (1998). Reverse transcription polymerase chain reaction (RT-PCR) used for the detection of Taura Syndrome Virus (TSV) in experimentally infected shrimp. *Dis. Aquat. Org.*, **34**, 87–91.
- NUNAN L.M., TANG-NELSON K. & LIGHTNER D.V. (2004). Real-time RT-PCR determination of viral copy number in *Penaeus vannamei* experimentally infected with Taura syndrome virus. *Aquaculture*, **229**, 1–10.
- OVERSTREET R.M., LIGHTNER D.V., HASSON K.W., MCLWAIN S. & LOTZ J. (1997). Susceptibility to TSV of some penaeid shrimp native to the Gulf of Mexico and southeast Atlantic Ocean. *J. Invertebr. Pathol.*, **69**, 165–176.
- PANTOJA C.R., NAVARRO S.A., NARANJO J., LIGHTNER D.V. & GERBA C.P. (2004). Nonsusceptibility of primate cells to Taura syndrome virus. *Emerg. Infect. Dis.*, **10**, 2106–2112.
- POULOS B.T., KIBLER R., BRADLEY-DUNLOP D., MOHNEY L.L. & LIGHTNER D.V. (1999). Production and use of antibodies for the detection of Taura syndrome virus in penaeid shrimp. *Dis. Aquat. Org.*, **37**, 99–106.
- ROBLES-SIKISAKA R., HASSON K.W., GARCIA D.K., BROVONT K., CLEVELAND K., KLIMPEL K.R. & DHAR A.K. (2002). Genetic variation and immunohistochemical differences among geographical isolates of Taura syndrome virus of penaeid shrimp. *J. Gen. Virol.*, **83**, 3123–3130.
- ROBLES-SIKISAKA R., GARCIA D.K., KLIMPEL K.R. & DHAR A.K. (2001). Nucleotide sequence of 3'-end of the genome of Taura syndrome virus of shrimp suggests that it is related to insect picornaviruses. *Arch. Virol.*, **146**, 941–952.
- SRISUVAN T., TANG K.F.J. & LIGHTNER D.V. (2005). Experimental infection of *Penaeus monodon* with Taura syndrome virus (TSV). *Dis. Aquat. Org.*, **67**, 1–8.
- TANG K.F.J. & LIGHTNER D.V. (2005). Phylogenetic analysis of Taura syndrome virus isolates collected between 1993 and 2004 and virulence comparison between two isolates representing different genetic variants. *Virus Res.*, **112**, 69–76.
- TANG K.F.J., WANG J. & LIGHTNER D.V. (2004). Quantitation of Taura syndrome virus by real-time RT-PCR with a TaqMan assay. *J. Virol. Methods*, **115**, 109–114.
- TU C., HUANG H.T., CHUANG S.H., HSU J.P., KUO S.T., LI N.J., HUS T.L., LI M.C. & LIN S.Y. (1999). Taura syndrome in Pacific white shrimp *Penaeus vannamei* cultured in Taiwan. *Dis. Aquat. Org.*, **38**, 159–161.
- VANPATTEN K.A., NUNAN L.M. & LIGHTNER D.V. (2004). Seabirds as potential vectors of penaeid shrimp viruses and the development of a surrogate laboratory model utilizing domestic chickens. *Aquaculture*, **241**, 31–46.

VERGEL J.C.V., CABAWATAN L.D.P., MADRONA V.A.C., ROSARIO A.F.T., STA. ANA J.B.M., TARE M.V.R. & MANINGAS M.B.B. (2019). Detection of Taura Syndrome Virus (TSV) in *Litopenaeus vannamei* in the Philippines. *Philipp. J. Fish.*, **26**, 8–14.

WERTHEIM J.O., TANG K.F.J., NAVARRO S.A. & LIGHTNER D.V. (2009). A quick fuse and the emergence of Taura syndrome virus. *Virology*, **390**, 324–329.

WHITE B.L., SCHOFIELD P.J., POULOS B.T. & LIGHTNER D.V. (2002). A laboratory challenge method for estimating Taura Syndrome virus resistance in selected lines of Pacific White Shrimp *Penaeus vannamei*. *J. World Aquac. Soc.*, **33**, 341–348.

WYBAN J.A. (1992). Selective breeding of specific pathogen-free (SPF) shrimp for high health and increased growth. *In: Diseases of Cultured Penaeid Shrimp in Asia and the United States*, Fulks W. & Main K.L., eds. The Oceanic Institute, Honolulu, Hawaii, USA, 257–268.

YU C.I. & SONG Y.L. (2000). Outbreaks of Taura syndrome in Pacific white shrimp *Penaeus vannamei* cultured in Taiwan. *Fish Pathol.*, **5**, 21–24.

ZARAIN-HERZBERG M. & ASCENCIO F. (2001). Taura syndrome in Mexico: follow-up study in shrimp farms of Sinaloa. *Aquaculture*, **193**, 1–9.

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NB: There is a WOA Reference Laboratory for infection with Taura syndrome virus
(please consult the WOA 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 WOA 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 $1000\text{ virions ml}^{-1}$ was found to be viable for a period of 12 days in seawater of 27 ppt salinity, pH of 7.5 at $29\text{--}33^{\circ}\text{C}$. In shrimp pond sediment (with initial viral load of $211,500\text{ copies g}^{-1}$), the virus was viable and infective up to 19 days despite sun-drying. In the case of non-drainable conditions, WSSV ($753,600\text{ copies g}^{-1}$) 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., *Callinassa* sp., *Exopalaemon* sp., *Helice* sp., *Hemigrapsus* sp. *Macrophthalmus* sp., *Macrophthel* sp., *Metaplex* 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 <i>et al.</i> , 1998
	50°C/60 min 60°C/1 min 70°C/0.2 min	Nakano <i>et al.</i> , 1998
pH	pH 3/60 min pH 12/10 min	Chang <i>et al.</i> , 1998; Balasubramanian <i>et al.</i> , 2006
UV	$9.30 \times 10^5 \mu\text{Ws}/\text{cm}^2$	Chang <i>et al.</i> , 1998
Ozone	$0.5 \mu\text{g ml}^{-1}/10 \text{ min}$	Chang <i>et al.</i> , 1998
Chlorine	100 ppm/10 min	Chang <i>et al.</i> , 1998; Balasubramanian <i>et al.</i> , 2006
Iodophore	100 ppm/10 min	Chang <i>et al.</i> , 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 stocking 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. WOAAH 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
<i>In-situ</i> hybridisation					+	+	+	1	+	+	+	1
Bioassay					+	+	+	1				
LAMP	++	++	++	1	++	++	++	1	+	+	+	1
Ab-ELISA					+	+	+	1				
Ag-ELISA					+	+	+	1				
Other antigen detection methods					+	+	+					
Other methods												

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

¹For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). ²Susceptibility 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: <u>6FAM-AGC-CAT-GAA-GAA-TGC-CGT-CTA-TCA-CAC-A-TAMRA</u>	900 nM 900 nM 250 nM	45 cycles of: <u>95°C/15 sec and</u> <u>60°C/1 min,</u> <u>50°C/2 min,</u> <u>95°C/10 min,</u> <u>then 45 cycles</u> <u>of:</u> <u>94°C/15 sec and</u> <u>60°C/1 min</u>
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: <u>95°C/15 sec and</u> <u>60°C/1 min</u> <u>50°C/2 min,</u> <u>95°C/10 min,</u> <u>then 45 cycles</u> <u>of:</u> <u>94°C/15 sec and</u> <u>60°C/1 min</u>

¹Method 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)	<u>Outer Primary</u> Fwd <u>146F1</u> : 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
	<u>Inner Nested</u> 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 WOAHP Register for kits that have been certified by WOAHP (<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 MgSO₄, 10 mM (NH₄)₂SO₄, 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 WOAHP 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 WOAHP 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 status¹

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

¹ For example transboundary commodities.

- 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

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	<i>Penaeus monodon</i>	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	<i>Penaeus monodon</i>	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	<i>Penaeus merguensis</i> , <i>P. esculentus</i> , <i>P. plebejus</i> , <i>Metapenaeus endeavouri</i> , <i>M. bennettiae</i>	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	<i>Penaeus merguensis</i> , <i>P. esculentus</i> , <i>P. plebejus</i> , <i>Metapenaeus endeavouri</i> , <i>M. bennettiae</i>	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	<i>Penaeus merguensis</i> , <i>P. esculentus</i> , <i>P. plebejus</i> , <i>Metapenaeus endeavouri</i> , <i>M. bennettiae</i>	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.

7. References

- BALASUBRAMANIAN G., SUDHAKARAN R., SYED MUSTHAQ S., SARATHI M. & SAHUL HAMEED A.S. (2006). Studies on the inactivation of white spot syndrome virus of shrimp by physical and chemical treatments, and seaweed extracts tested in marine and freshwater animal models. *J. Fish Dis.*, **29**, 569–572.
- CHANG C.-F., SU M.-S., CHEN H.-Y. & LIAO I.C. (2003). Dietary β -1,3-glucan effectively improves immunity and survival of *Penaeus monodon* challenged with white spot syndrome virus. *Fish Shellfish Immunol.*, **15**, 297–310.
- CHANG P.S., CHEN H.C. & WANG Y.C. (1998). Detection of white spot syndrome associated baculovirus in experimentally infected wild shrimp, crab and lobsters by *in situ* hybridization. *Aquaculture*, **164**, 233–242.
- CHANG P.S., LO C.F., WANG Y.C. & KOU G.H. (1996). Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by *in situ* hybridization. *Dis. Aquat. Org.*, **27**, 131–139.
- CHANG Y., CHEN T., LIU W., HWANG J. & LO C. (2011). Assessment of the roles of copepod *Apocyclops royi* and bivalve mollusk *Meretrix lusoria* in white spot syndrome virus transmission. *Mar. Biotechnol.*, **13**, 909–917.
- CHEN I.T., AOKI T., HUANG Y.T., HIRONO I., CHEN T.C., HUANG J.Y., CHANG G.D., LO C.F., WANG H.C. (2011). White spot syndrome virus induces metabolic changes resembling the Warburg effect in shrimp hemocytes in the early stage of infection. *J. Virol.*, **85**, 12919–12928.
- CHEN W.Y., ZHANG H., GU L., LI F. & YANG F. (2012). Effects of high salinity, high temperature and pH on capsid structure of white spot syndrome virus. *Dis. Aquat. Org.*, **101**, 167–171.
- CHOTIGEAT W., TONGSUPA S., SUPAMATAYA K. & PHONGDARA A. (2004). Effect of fucoidan on disease resistance of black tiger shrimp. *Aquaculture*, **233**, 23–30.
- CLAYDON K., CULLEN B. & OWENS L. (2004). OIE white spot syndrome virus PCR gives false-positive results in *Cherax quadricarinatus*. *Dis. Aquat. Org.*, **62**, 265–268.
- CORBEL V., ZUPRIZAL Z., SHI C., HUANG, SUMARTONO, ARCIER J.-M. & BONAMI J.-R. (2001). Experimental infection of European crustaceans with white spot syndrome virus (WSSV). *J. Fish Dis.*, **24**, 377–382.
- CUELLAR-ANJEL J., WHITE-NOBLE B., SCHOFIELD P., CHAMORRO R. & LIGHTNER D.V. (2012). Report of significant WSSV-resistance in the Pacific white shrimp, *Litopenaeus vannamei*, from a Panamanian breeding program. *Aquaculture*, **368–369**, 36–39.
- DESRINA, PRAYITNO S.B, VERDEGEM M.C.J, VERRETH J.A.J. & VLAK J.M. (2022). White spot syndrome virus host range and impact on transmission. *Rev. Aquacult.*, 1–18.

- DESRINA, VERRETH J.A.J., PRAYITNO S.B., ROMBOUT J.H.W.M., VLAK J.M. & VERDEGEM M.C.J. (2013). Replication of white spot syndrome virus (WSSV) in the polychaete *Dendronereis* spp. *J. Invertebr. Pathol.*, **114**, 7–10.
- DURAND S.V. & LIGHTNER D.V. (2002). Quantitative real time PCR for the measurement of white spot syndrome virus in shrimp. *J. Fish Dis.*, **25**, 381–389.
- DURAND S.V., TANG K.F.J. & LIGHTNER D.V. (2000). Frozen commodity shrimp: potential avenue for introduction of white spot syndrome virus and yellow head virus. *J. Aquat. Anim. Health*, **12**, 128–135.
- EAST I.J. (2008). Addressing the problems of using the polymerase chain reaction technique as a stand-alone test for detecting pathogens in aquatic animals. *Sci. Tech. Rev.*, **27**, 829–837.
- ESCOBEDO-BONILLA C. M., ALDAY-SANZ V., WILLE M., SORGELOOS P., PENZAERT M.B. & NAUWYNCK H.J. (2008). A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. *J. Fish Dis.*, **31**, 1–18.
- FLEGEL T.W. (1997). Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. *World J. Microbiol. Biotechnol.*, **13**, 433–442.
- HASSON K.W., FAN Y., REISINGER T., VENUTI J. & VARNER P.W. (2006). White-spot syndrome virus (WSSV) introduction into the Gulf of Mexico and Texas fresh water systems through imported, frozen bait-shrimp. *Dis. Aquat. Org.*, **71**, 91–100.
- HARYADI D., VERRETH J.A.J., VERDEGEM M.C.J. & VLAK J.M. (2015). Transmission of white spot syndrome virus (WSSV) from *Dendronereis* spp. (Peters) (Nereididae) to penaeid shrimp. *J. Fish Dis.*, **38**, 419–428.
- HE J. & ZHOU H. (1996). Infection route and host species of white spot syndrome baculovirus. *Acta Sci. Natur. Univ. Sunyatseni*, **38**, 65–69.
- HEIDARIEH M., SOLTANI M., MOTAMEDI SEDEH F. & SHEIKHZADEH N. (2013). Low water temperature retards white spot syndrome virus replication in *Astacus leptodactylus* Crayfish. *Acta Sci. Vet.*, **41**, 1–6.
- HUANG J. & YU J. (1995). A new staining method for on-site observation of viral inclusion bodies of penaeid shrimp. (*Chinese J.*) *Mar. Fish. Res.*, **16**, 31–39.
- HUANG J., YU J., WANG X.-H., SONG X.-L., MA C.-S., ZHAO F.-Z. & YANG C.-H. (1995). Survey on the pathogen and route of transmission of baculoviral hypodermal and hematopoietic necrosis in shrimp by ELISA of monoclonal antibody. (*Chinese J.*) *Mar. Fish. Res.*, **16**, 40–50.
- HUANG Y., YIN Z., WENG S., HE J. & LI S. (2012). Selective breeding and preliminary commercial performance of *Penaeus vannamei* for resistance to white spot syndrome virus (WSSV). *Aquaculture*, **364–365**, 111–117.
- KONO T., SAVAN R., SAKAI M., & ITAMI T. (2004). Detection of white spot syndrome virus in shrimp by loop-mediated isothermal amplification. *J. Virol. Methods*, **115**, 59–65.
- LEI Z.-W., HUANG J., SHI C.-Y., ZHANG L.-J. & YU K.-K. (2002). Investigation into the hosts of white spot syndrome virus (WSSV). *Oceanol. Limnol. Sin.*, **33**, 250–258.
- LI Q., ZHANG J.H., CHEN Y.J. & YANG F. (2004). White spot syndrome virus (WSSV) infectivity for *Artemia* at different developmental stages. *Dis. Aquat. Org.*, **57**, 261–264.
- LIGHTNER D.V. (1996). A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. Baton Rouge, Louisiana, USA: World Aquaculture Society, 1996.
- LIU B., YU Z.M., SONG X.X. & GUAN Y.Q. (2007). Studies on the transmission of WSSV (white spot syndrome virus) in juvenile *Marsupenaeus japonicus* via marine microalgae. *J. Invertebr. Pathol.*, **95**, 87–92.
- LO C.F., AOKI T., BONAMI J.R., FLEGEL T.W., LEU J.H., LIGHTNER D.V., STENTIFORD G., SÖDERHÄLL K., WALKER P.W. WANG H.C., XUN X., YANG F. & VLAK J.M. (2012). *Nimaviridae*. In: *Virus Taxonomy: Classification and Nomenclature of*

Viruses: Ninth Report of the International Committee on Taxonomy of Viruses, King A.M.Q., Adams M.J., Carstens E.B., & Lefkowitz E.J., eds. Elsevier Academic Press, San Diego, CA, USA, pp 229–234.

LO C.F., HO C.H., CHEN C.H., LIU K.F., CHIU Y.L., YEH P.Y., PENG S.E., HSU H.C., LIU H.C., CHANG C.F., SU M.S., WANG C.H. & KOU G.H. (1997). Detection and tissue tropism of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. *Dis. Aquat. Org.*, **30**, 53–72.

LO C.F., HO C.H., PENG S.E., CHEN C.H., HSU H.C., CHIU Y.L., CHANG C.F., LIU K.F., SU M.S., WANG C.H. & KOU G.H. (1996b). White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. *Dis. Aquat. Org.*, **27**, 215–225.

LO C.F. & KOU G.H. (1998). Virus-associated white spot syndrome of shrimp in Taiwan: a review. *Fish Pathol.*, **33**, 365–371.

LO C.F., LEU J.H., HO C.H., CHEN C.H., PENG S.E., CHEN Y.T., CHOU C.M., YEH P.Y., HUANG C.J., CHOU H.Y., WANG C.H. & KOU G.H. (1996a). Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. *Dis. Aquat. Org.*, **25**, 133–141.

MAEDA M., ITAMI T., MIZUKI E., TANAKA R., YOSHIZU Y., DOI K., YASUNAGA-AOKI C., TAKAHASHI Y. & KAWARABATA T. (2000). Red swamp crawfish (*Procambarus clarkii*): an alternative experimental host in the study of white spot syndrome virus. *Acta Virol.*, **44**, 371–374.

MCCOLL K.A., SLATER J., JEYASEKARAN G., HYATT A.D. & CRANE M.ST.J. (2004). Detection of White Spot Syndrome virus and Yellow head virus in prawns imported into Australia. *Australian Vet. J.*, **82**, 69–74.

MOMOYAMA K., HIRAOKA M., INOUE K., KIMURA T. & NAKANO H. (1995). Diagnostic techniques of the rod-shaped nuclear virus infection in the kuruma shrimp, *Penaeus japonicus*. *Fish Pathol.*, **30**, 263–269.

MOMOYAMA K., HIRAOKA M., NAKANO H., KOUBE H., INOUE K. & OSEKO N. (1994). Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: Histopathological study. *Fish Pathol.*, **29**, 141–148.

MOMOYAMA K., HIRAOKA M., NAKANO H. & SAMESHIMA M. (1998). Cryopreservation of penaeid rod-shaped DNA virus (PRDV) and its survival in sea water at different temperatures. *Fish Pathol.*, **33**, 95–96.

MOODY N.J.G., MOHR P.G., WILLIAMS L.M., CUMMINS D.M., HOAD J., SLATER J., VALDETER S.T., COLLING A., SINGANALLUR N.B., GARDNER I.A., GUDKOV N. & CRANE M.ST.J. (2022). Performance characteristics of two real-time, TaqMan polymerase chain reaction assays for the detection of WSSV in clinically diseased and apparently health prawns. *Dis. Aquat. Org.*, <https://www.int-res.com/prepress/d03687.html>.

NAKANO H., HIRAOKA M., SAMESHIMA M., KIMURA T. & MOMOYAMA K. (1998). Inactivation of penaeid rod-shaped DNA virus (PRDV), the causative agent of penaeid acute viraemia (PAV), by chemical and physical treatments. *Fish Pathol.*, **33**, 65–71.

NUNAN L.M. & LIGHTNER D.V. (1997). Development of a non-radioactive gene probe by PCR for detection of white spot syndrome virus (WSSV). *J. Virol. Methods*, **63**, 193–201.

NUNAN L.M. & LIGHTNER D.V. (2011). Optimized PCR assay for detection of white spot syndrome virus (WSSV). *J. Virol. Methods*, **171**, 318–321.

NUNAN L.M., POULOS B.T. & LIGHTNER D.V. (1998). The detection of white spot syndrome virus (WSSV) and yellow head virus (YHV) in imported commodity shrimp. *Aquaculture*, **160**, 19–30.

POULOS B.T., PANTOJA C.R., BRADLEY-DUNLOP D., AGUILAR J. & LIGHTNER D.V. (2001). Development and application of monoclonal antibodies for the detection of white spot syndrome virus of penaeid shrimp. *Dis. Aquat. Org.*, **47**, 13–23.

SAHUL HAMEED A.S., ANILKUMAR M., STEPHEN RAJ M.L. & JAYARAMAN K. (1998). Studies on the pathogenicity of systemic ectodermal and mesodermal baculovirus and its detection in shrimp by immunological methods. *Aquaculture*, **160**, 31–45.

- SAHUL HAMEED A.S., YOGANANDHAN K., SATHISH S., RASHEED M., MURUGAN V. & JAYARAMAN K. (2001). White spot syndrome virus (WSSV) in two species of freshwater crabs (*Paratelphusa hydrodomous* and *P. pulvinata*). *Aquaculture*, **201**, 179–186.
- SANCHEZ-PAZ A. (2010). White spot syndrome virus: an overview on an emergent concern. *Vet. Res.*, **41**, 43.
- SATHEESH KUMAR S., ANANDA BHARATHI R., RAJAN J.J.S., ALAVANDI S.V., POORNIMA M., BALASUBRAMANIAN C.P. & PONNIAH A.G. (2013). Viability of white spot syndrome virus (WSSV) in sediment during sun-drying (drainable pond) and under non-drainable pond conditions indicated by infectivity to shrimp. *Aquaculture*, **402–403**, 119–126.
- SITHIGORNGUL W., RUKPRATANPORN S., PECHARABURANIN N., LONGYANT S., CHAVISUTHANGKURA P. & SITHIGORNGUL P. (2006). A simple and rapid immunochromatographic test strip for detection of white spot syndrome virus (WSSV) of shrimp. *Dis. Aquat. Org.*, **72**, 101–106.
- SONG X., HUANG J., WANG C., YU J., CHEN B. & YANG C. (1996). Artificial infection of brood shrimp of *Penaeus chinensis* with hypodermal and hematopoietic necrosis baculovirus. *J. Fish. China*, **20**, 374–378.
- SRITUNYALUCKSANA K., SRISALA J., MCCOLL K., NIELSEN L. & FLEGEL T.W. (2006). Comparison of PCR methods for white spot syndrome virus (WSSV) infections in penaeid shrimp. *Aquaculture*, **255**, 95–104.
- STENTIFORD G.D., BONAMI J.R. & ALDAY-SANZ V. (2009). A critical review of susceptibility of crustaceans to Taura Syndrome, yellowhead disease and white spot disease and implications of inclusion of these diseases in European legislation. *Aquaculture*, **291**, 1–17.
- STENTIFORD G.D. & LIGHTNER D.V. (2011). Cases of white spot disease (WSD) in European shrimp farms. *Aquaculture*, **319**, 302–306.
- TSAI M.F., KOU G.H., LIU H.C., LIU K.F., CHANG C.F., PENG S.E., HSU H.C., WANG C.H. & LO C.F. (1999). Long-term presence of white spot syndrome virus (WSSV) in a cultivated shrimp population without disease outbreaks. *Dis. Aquat. Org.*, **38**, 107–114.
- VANPATTEN K.A., NUNAN L.M. & LIGHTNER D.V. (2004). Sea birds as potential vectors of penaeid shrimp viruses and the development of a surrogate laboratory model utilizing domestic chickens. *Aquaculture*, **241**, 31–46.
- VENEGAS C.A., NONAKA L., MUSHIAKE K., SHIMIZU K., NISHIZAWA T. & MUROGA K. (1999). Pathogenicity of penaeid rod-shaped DNA virus (PRDV) to kuruma prawn in different developmental stages. *Fish Pathol.*, **34**, 19–23.
- VIDAL O.M., GRANJA C.B., ARANGUREN F., BROCK J.A. & SALAZAR M. (2001). A profound effect of hyperthermia on survival of *Litopenaeus vannamei* juveniles infected with white spot syndrome virus. *J. World Aquac. Soc.*, **32**, 364–372.
- VIJAYAN K.K., STALIN RAJ V., BALASUBRAMANIAN C.P., ALAVANDI S.V., THILLAI SEK HAR V. & SANTIAGO T.C. (2005). Polychaete worms – a vector for white spot syndrome virus (WSSV). *Dis. Aquat. Org.*, **63**, 107–111.
- WANG C.H., YANG H.N., TANG C.Y., LU C.H., KOU G.H. & LO C.F. (2000). Ultrastructure of white spot syndrome virus development in primary lymphoid organ cell cultures. *Dis. Aquat. Org.*, **41**, 91–104.
- WANG H.C., HIRONO I, MANINGAS M.B.B., SOMBOONWIWA K., STENTIFORD G. & ICTV REPORT CONSORTIUM. (2019). ICTV Virus Taxonomy Profile: *Nimaviridae*. In: *Virus Taxonomy: The ICTV 10th Report on Virus Classification and Taxon Nomenclature*. The ICTV website (www.ictv.global/report/nimaviridae).
- WANG M., CHEN Y., ZHAO Z., WENG S., YANG J., LIU S., LIU C., YUAN F., AI B., ZHANG H., ZHANG M., LU L., YUAN K., YU Z., MO B., LIU X., GAI C., LI Y., LU R., ZHONG Z., ZHENG L., FENG G., LI S.C. & HE J. (2021). A convenient polyculture system that controls a shrimp viral disease with a high transmission rate. *Commun Biol.*, **4**, 1276.
- WITHYACHUMNARNKUL B. (1999). Results from black tiger shrimp *Penaeus monodon* culture ponds stocked with postlarvae PCR-positive or -negative for white-spot syndrome virus (WSSV). *Dis. Aquat. Org.*, **39**, 21–27.

WONGTEERASUPAYA C., VICKERS J.E., SRIURAIRATANA S., NASH G.L., AKARAJAMORN A., BOONSAENG V., PANYIM S., TASSANAKAJON A., WITHYACHUMNARNKUL B. & FLEGEL T.W. (1995). A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. *Dis. Aquat. Org.*, **21**, 69–77.

WU J.L., SUZUKI K., ARIMOTO M., NISHIZAWA T. & MUROGA K. (2002). Preparation of an Inoculum of White Spot Syndrome Virus for Challenge Tests in *Penaeus japonicus*. *Fish Pathol.*, **37**, 65–69.

WU W., WU B., YE T., HUANG H., DAI C., YUAN J. & WANG W. (2013). TCTP is a critical factor in shrimp immune response to virus infection. *PLoS One*, **8**, e74460.

YAN D.C., DONG S.L., HUANG J., YU X.M., FENG M.Y. & LIU X.Y. (2004). White spot syndrome virus (WSSV) detected by PCR in rotifers and rotifer resting eggs from shrimp pond sediments. *Dis. Aquat. Org.*, **59**, 69–73.

YAN D.C., DONG S.L., HUANG J. & ZHANG J.S. (2007). White spot syndrome virus (WSSV) transmission from rotifer inoculum to crayfish. *J. Invertebr. Pathol.*, **94**, 144–148.

YOGANANDHAN K., SYED MUSTHAQ S., NARAYANAN R.B. & SAHUL HAMEED A.S. (2004). Production of polyclonal antiserum against recombinant VP28 protein and its application for the detection of white spot syndrome virus in crustaceans. *J. Fish Dis.*, **27**, 517–522.

ZENG Y. (2021). Molecular epidemiology of white spot syndrome virus in the world. *Aquaculture*, **537**, 736509. <https://doi.org/10.1016/j.aquaculture.2021.736509>.

ZHAN W.B., WANG Y.H., FRYER J.L., YU K.K., FUKUDA H. & MENG Q.X. (1998). White Spot Syndrome Virus Infection of Cultured Shrimp in China. *J. Aquat. Anim. Health*, **10**, 405–410.

ZHANG J.S., DONG S.L., DONG Y.W., TIAN X.L., CAO Y.C. & LI Z.J., YAN D.C. (2010). Assessment of the role of brine shrimp *Artemia* in white spot syndrome virus (WSSV) transmission. *Vet. Res. Commun.*, **34**, 25–32.

ZHANG J.S., DONG S.L., DONG Y.W., TIAN X.L. & HOU C.Q. (2008). Bioassay evidence for the transmission of WSSV by the harpacticoid copepod *Nitocra* sp. *J. Invertebrate Pathol.*, **97**, 33–39.

ZHANG J.S., DONG S.L., TIAN X.L., DONG Y.W., LIU X.Y. & YAN D.C. (2006). Studies on the rotifer (*Brachionus urceus* Linnaeus, 1758) as a vector in white spot syndrome virus (WSSV) transmission. *Aquaculture*, **261**, 1181–1185.

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* *

NB: There are WOA Reference Laboratories for infection with white spot syndrome virus
(please consult the WOA web site:
<https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).
Please contact the WOA 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.

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

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

Family	Scientific name	Common name
Toxotidae	<i>Toxotes chatareus</i>	common archerfish
	<i>Toxotes lorentzi</i>	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. phaneroomyces 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; Ibrahimi *et al.*, 2018). In advanced stages of the disease, the severity of the disease results in death of the fish (Hawke *et al.*, 2003; Ibrahimi *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; Ibrahimi *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 prevent 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 anaesthetised 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 <u>Clinical signs</u>	±	±	±	NA	+	+	+	NA				
<u>Squash mounts</u>					±	±	±	1	±	±	±	1
Histopathology					++	++	++	1	++	++	++	1
Cytopathology												
Cell or artificial media culture					++	++	++	1	+	+	+	1
Real-time PCR												
Conventional PCR					++	++	++	1				
<u>Conventional PCR followed by amplicon sequencing</u>									+++	+++	+++	1
<i>In-situ</i> 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.

¹For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). ²Susceptibility 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).

4.23. 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 cm³), 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.

4.23.1. 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.

4.23.2 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.

4.34. Cell culture for isolation

4.34.1. 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 mm³, 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 mm³) 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).

4.34.2. 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*.

4.34.3. 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/p eptone/ yeast) broth	GPY agar	GY agar <u>(gluco se/ yeast)</u>	Autoclaved pond water
3 g litre ⁻¹ glucose 1 g litre ⁻¹ peptone 0.128 g litre ⁻¹ MgSO ₄ .7H ₂ O 0.014 g litre ⁻¹ KH ₂ PO ₄ 0.029 g litre ⁻¹ CaCl ₂ .2H ₂ O 2.4 mg litre ⁻¹ FeCl ₃ .6H ₂ O 1.8 mg litre ⁻¹ MnCl ₂ .4H ₂ O 3.9 mg litre ⁻¹ CuSO ₄ .5H ₂ O	GP broth + 0.5 g litre ⁻¹ yeast extract	GPY broth + 12 g litre ⁻¹ technic al agar	1% glucos e, 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

0.4 mg litre⁻¹
ZnSO₄·7H₂O

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.

<u>Pathogen/ target gene</u>	<u>Primer/probe (5'–3')</u>	<u>Concentration</u>	<u>Cycling parameters</u>
Method 1: Vandersea <i>et al.</i> , 2006; GenBank Accession No.: AF396684; Product amplicon size: 234bp			

<u><i>Aphanomyces invadans</i> (ITS1)</u>	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			
<u><i>Aphanomyces invadans</i> (ITS1-ITS2)</u>	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			
<u><i>Aphanomyces invadans</i> (ITS1-ITS2)</u>	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 µM 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 MgCl₂, 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 MgCl₂, 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 H₂O, and 6 ml of 25 × SET buffer [3.75 M NaCl, 25 mM EDTA (ethylene diamine tetraacetic acid), 0.5 M Tris/HCl, pH 7.8]) containing 3% polyoxyethyl-onesorbitan 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/compartments/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 WOA 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 WOA 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.

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*).

6.1.2. 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 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

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.

7. References

- ADIL B., SHANKAR K.M., NAVEEN KUMAR B.T., PATIL R., BALLYAYA A., RAMESH K.S., POOJARY S.R., BYADGI O.V. & SIRIYAPPAGOUDE P. (2013). Development and standardization of a monoclonal antibody-based rapid flow-through immunoassay for the detection of *Aphanomyces invadans* in the field. *J. Vet. Sci.*, **14**, 413–419.
- AFZALI S.F., HASSAN M.D., ABDUL-RAHIM A.M., SHARIFPOUR I. & SABRI J. (2013). Isolation and identification of *Aphanomyces* species from natural water bodies and fish farms in Selangor, Malaysia. *Malaysian Appl. Biol.*, **42**, 21–31.
- ANDREW T., HUCHZERMAYER K., MBEHA B. & NENGU S. (2008). Epizootic ulcerative syndrome affecting fish in the Zambezi river system in Southern Africa. *Vet. Rec.*, **163**, 629–632.
- BALDOCK F.C., BLAZER V., CALLINAN R., HATAI K., KARUNASAGAR I., MOHAN C.V. & BONDAD-REANTASO M.G. (2005). Outcomes of a short expert consultation on epizootic ulcerative syndrome (EUS): Re-examination of causal factors, case definition and nomenclature. *In: Diseases in Asian Aquaculture V*, Walker P., Lester R. & Bondad-Reantaso M.G., eds. Fish Health Section, Asian Fisheries Society, Manila, Philippines, 555–585.
- BALASURIYA L.K.S.W. (1994). Epizootic ulcerative syndrome in fish in Sri Lanka, country status report. *In: Proceeding of the ODA Regional Seminar on Epizootic Ulcerative*, Robert R.J., Campbell B. & MacRae I.H., eds. Aquatic Animal Health Research Institute, Bangkok, Thailand, pp 39–47.
- BLAZER V.S., VOGELBEIN W.K., DENSMORE C.L., MAY E.B., LILLEY J.H. & ZWERNER D.E. (1999). *Aphanomyces* as a cause of ulcerative skin lesions of menhaden from Chesapeake Bay tributaries. *J. Aquat. Anim. Health*, **11**, 340–349.
- BONDAD-REANTASO M.G., LUMANLAN S.C., NATIVIDAD J.M. & PHILLIPS M.J. (1992). Environmental monitoring of the epizootic ulcerative syndrome (EUS) in fish from Munoz, Nueva Ecija in the Philippines. *In: Diseases in Asian Aquaculture 1*, Shariff M., Subasinghe R.P. & Arthur J.R., eds. Fish Health Section, Asian Fisheries Society, Manila, The Philippines, 475–490.
- CATAP E.S. & MUNDAY B.L. (1998). Effects of variations of water temperature and dietary lipids on the expression of experimental epizootic ulcerative syndrome (EUS) in sand whiting, *Sillago ciliata*. *Fish Pathol.*, **33**, 327–335.
- CAVALIER-SMITH T. & CHAO E.E.Y. (2006). Phylogeny and Megasystematics of Phagotrophic Heterokonts (Kingdom Chromista). *J. Mol. Evol.*, **62**, 388–420.
- CHINABUT S. & ROBERTS R.J. (1999). Pathology and Histopathology of Epizootic Ulcerative Syndrome (EUS). Aquatic Animal Health Research Institute, Department of Fisheries, Royal Thai Government, Bangkok, Thailand, 33 pp. ISBN 974-7604-55-8.
- CHINABUT S., ROBERTS R.J., WILLOUGHBY G.R. & PEARSON M.D. (1995) Histopathology of snakehead, *Channa striatus* (Bloch), experimentally infected with the specific *Aphanomyces* fungus associated with epizootic ulcerative syndrome (EUS) at different temperatures. *J. Fish Dis.*, **18**, 41–47.
- CRUZ-LACIERDA E.R. & SHARIFF M. (1995). Experimental transmission of epizootic ulcerative syndrome (EUS) in snakehead, *Ophicephalus striatus*. *Dis. Asian Aquac.*, **11**, 327–336. DIEGUEZ-URIBEONDO J., GARCIA M.A.,

- CERENIUS L., KOZUBÍKOVÁ E., BALLESTEROS I., WINDELS C., WEILAND J., KATOR H., SÖDERHÄLL K. & MARTÍN M.P. (2009). Phylogenetic relationships among plant and animal parasites, and saprotrophs in *Aphanomyces* (Oomycetes). *Fungal Genetics and Biology*, **46**, 365–376.
- EGUSA S. & MASUDA N. (1971). A new fungal disease of *Plecoglossus altivelis*. *Fish Pathol.*, **6**, 41–46.
- EUROPEAN FOOD SAFETY AUTHORITY EFSA (2011a). Scientific Opinion on Epizootic Ulcerative Syndrome. *EFSA J.*, **9**, 2387.
- EUROPEAN FOOD SAFETY AUTHORITY (EFSA) (2011b). Report of the technical hearing meeting on Epizootic Ulcerative Syndrome (EUS). *EFSA Support. Publ.*, **8**, 1–16.
- FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO) (1986). Report of the expert consultation on ulcerative fish diseases in the Asia-Pacific region (TCP/RAS/4508). Bangkok, August 1986. FAO, Regional Office for Asia and the Pacific, Bangkok, Thailand.
- FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO) (2009). Report of the international emergency disease investigation task force on a serious fish disease in Southern Africa, 18–26 May 2007, FAO, Rome, Italy, 70 pp.
- FRASER G.C., CALLINAN R.B. & CALDER L.M. (1992). *Aphanomyces* species associated with red spot disease: an ulcerative disease of estuarine fish from eastern Australia. *J. Fish Dis.*, **15**, 173–181.
- GOMO C., HANYIRE T., MAKAYA P. & SIBANDA S. (2016). Outbreak of epizootic ulcerative syndrome (EUS) in *Seranochromis robustus* fish species in Darwendale dam, Zimbabwe. *African J. Fish. Sci.*, **4**, 204–205.
- HANJAVANIT C. (1997). Mycotic granulomatosis found in two species of ornamental fishes imported from Singapore. *Mycoscience*, **38**, 433–436.
- HATAI K. & EGUSA S. (1979). Studies on pathogenic fungus of mycotic granulomatosis III. Development of the medium for MG-fungus. *Fish Pathol.*, **13**, 147–152.
- HATAI K., EGUSA S., TAKAHASHI S. & OOE K. (1977). Study on the pathogenic fungus of mycotic granulomatosis – I. Isolation and pathogenicity of the fungus from cultured-ayu infected with the disease. *Fish Pathol.*, **12**, 129–133.
- HATAI K., NAKAMURA K., AN RHA S., YUASA K. & WADA S. (1994). *Aphanomyces* infection in dwarf gourami (*Colisa lalia*). *Fish Pathol.*, **29**, 95–99.
- HAWKE J.P., GROOTERS A.M. & CAMUS A.C. (2003). Ulcerative Mycosis Caused by *Aphanomyces invadans* in Channel Catfish, Black Bullhead, and Bluegill from Southeastern Louisiana. *J. Aquat. Anim. Health.*, **15**, 120–127.
- HERBERT B., JONES J.B.B., MOHAN C.V. V. & PERERA R.P.P. (2019). Impacts of epizootic ulcerative syndrome on subsistence fisheries and wildlife. *Rev. Sci. Tech.*, **38**, 459–475.
- HUCHZERMAYER C.F., HUCHZERMAYER K.D.A., CHRISTISON K.W., MACEY B.M., COLLY P.A., HANG’OMBE B.M. & SONGE M.M. (2018). First record of epizootic ulcerative syndrome from the Upper Congo catchment: An outbreak in the Bangweulu swamps, Zambia. *J. Fish Dis.*, **41**, 87–94.
- HUCHZERMAYER K.D.A. & VAN DER WAAL B.C.W. (2012). Epizootic ulcerative syndrome: Exotic fish disease threatens Africa’s aquatic ecosystems. *J. S. Afr. Vet. Assoc.*, **83**, 1–6.
- IBERAHIM N.A., TRUSCH F. & VAN WEST P. (2018). *Aphanomyces invadans*, the causal agent of Epizootic Ulcerative Syndrome, is a global threat to wild and farmed fish. *Fungal Biol. Rev.*, **44**, 1–13.
- KHAN M.H., MARSHALL L., THOMPSON K.D., CAMPBELL R.E. & LILLEY J.H. (1998). Susceptibility of five fish species (Nile tilapia, rosy barb, rainbow trout, stickleback and roach) to intramuscular injection with the Oomycete fish pathogen, *Aphanomyces invadans*. *Bull. Eur. Assoc. Fish Pathol.*, **18**, 192–197.

- KIRYU Y., SHIELDS J.D., VOGELBEIN W.K., KATOR H. & BLAZER V.S. (2003). Infectivity and pathogenicity of the oomycete *Aphanomyces invadans* in Atlantic menhaden *Brevoortia tyrannus*. *Dis. Aquat. Org.*, **54**, 135–146.
- KUMAR P., SARKAR P., STEFI RAJU V., MANIKANDAN V., GURU A., ARSHAD A., ELUMALAI P. & AROCKIARAJ J. (2020). Pathogenicity and Pathobiology of Epizootic Ulcerative Syndrome (EUS) Causing Fungus *Aphanomyces invadans* and Its Immunological Response in Fish. *Rev. Fish. Sci. Aquac.*, **28**, 358–375.
- LILLEY J.H., CALLINAN R.B., CHINABUT S., KANCHANAKHAN S., MACRAE I.H. & PHILLIPS M.J., FALLIS A., LILLEY J.H., CALLINAN R.B., CHINABUT S., KANCHANAKHAN S., MACRAE I.H. & PHILLIPS M.J. (1998). Epizootic ulcerative syndrome (EUS) technical handbook. Bangkok: The Aquatic Animal Health Research Institute.
- LILLEY J.H., HART D., PANYAWACHIRA V., KANCHANAKHAN S., CHINABUT S., SÖDERHÄLL K. & CERENIUS L. (2003). Molecular characterization of the fish-pathogenic fungus *Aphanomyces invadans*. *J. Fish Dis.*, **26**, 263–275.
- LILLEY J.H., HART D., RICHARDS R.H., ROBERTS R.J., CERENIUS L. & SODERHALL K. (1997a). Pan-Asian spread of single fungal clone results in large scale fish kills. *Vet. Rec.*, **140**, 653–654.
- LILLEY J.H., PETCHINDA T. & PANYAWACHIRA V. (2001). *Aphanomyces invadans* zoospore physiology: 4. *In vitro* viability of cysts. *The AAHRI Newsletter*, **10**, 1–4.
- LILLEY J.H. & ROBERTS R.J. (1997). Pathogenicity and culture studies comparing the *Aphanomyces* involved in epizootic ulcerative syndrome (EUS) with other similar fungi. *J. Fish Dis.*, **20**, 135–144.
- LILLEY J.H., THOMPSON K.D. & ADAMS A. (1997b). Characterization of *Aphanomyces invadans* by electrophoretic and Western blot analysis. *Dis. Aquat. Org.*, **30**, 187–197.
- LUMANLAN-MAYO S.C., CALLINAN R.B., PACLIBARE J.O., CATAP E.S. & FRASER, G.C. (1997). Epizootic ulcerative syndrome (EUS) in rice-fish culture systems: an overview of field experiments 1993-1995. In: Diseases in Asian Aquaculture III, Flegel T.W. & MacRae I.H., eds. Fish Health Section, Asian Fisheries Society, Manila, The Philippines, 129–138.
- MCHUGH K.J., CHRISTISON K.W., WEYL O.L.F. & SMIT N.J. (2014). Histological Confirmation of Epizootic Ulcerative Syndrome in Two Cyprinid Species from Lake Liambezi, Zambezi Region, Namibia. *African Zool.*, **49**, 311–316.
- MCKENZIE R.A. & HALL W.T.K. (1976). Dermal ulceration of mullet (*Mugil cephalus*). *Aust. Vet. J.*, **52**, 230–231.
- MILES D.J.V., POLCHANA J., LILLEY J.H., KANCHANAKHAN S., THOMPSON K.D. & ADAMS A. (2001). Immunostimulation of striped snakehead *Channa striata* against epizootic ulcerative syndrome. *Aquaculture*, **195**, 1–15.
- MILES D.J.C., THOMPSON K.D., LILLEY J.H. & ADAMS A. (2003). Immunofluorescence of the epizootic ulcerative syndrome pathogen, *Aphanomyces invadans*, using a monoclonal antibody. *Dis. Aquat. Org.*, **55**, 77–84.
- NOGA E.J. & DYKSTRA M.J. (1986). Oomycete fungi associated with ulcerative mycosis in menhaden, *Brevoortia tyrannus* (Latrobe). *J. Fish Dis.*, **9**, 47–53.
- OIDTMANN B. (2012). Review of biological factors relevant to import risk assessments for epizootic ulcerative syndrome (*Aphanomyces invadans*). *Transbound. Emerg. Dis.*, **59**, 26–39.
- OIDTMANN B., STEINBAUER GEIGER S. & HOFFMANN R.W. (2008). Experimental infection and detection of *Aphanomyces invadans* in European catfish, rainbow trout and European eel. *Dis. Aquat. Org.*, **82**, 185–207.
- PAGRUT N.K., GANGULY S., JAISWAL V. & SINGH C. (2017). An overview on epizootic ulcerative syndrome of fishes in India: A comprehensive report. *J. Entomol. Zool. Stud.*, **5**, 1941–1943.
- PHADEE P., KURATA O. & HATAI K. (2004a). A PCR method for the detection of *Aphanomyces piscicida*. *Fish Pathol.*, **39**, 25–31.

PHADEE, P., KURATA, O., HATAI K., HIRONO I. & AOKI T. (2004b). Detection and identification of fish-pathogenic *Aphanomyces piscicida* using polymerase chain reaction (PCR) with species-specific primers. *J. Aquat. Anim. Health*, **16**, 220–230.

PRADHAN P.K., MOHAN C.V., SHANKAR K.M., KUMAR B.M. & DEVARAJA G. (2007). Yearlings of Indian major carps resist infection against the epizootic ulcerative syndrome pathogen, *Aphanomyces invadans*. *Current Science*, **92**, 1430–1434.

TONGUTHAI K. (1985). A preliminary account of ulcerative fish diseases in the Indo-Pacific region (a comprehensive study based on Thai experiences). National Inland Fisheries Institute, Bangkok, Thailand, 39 pp.

TSUI C.K.M., MARSHALL W., YOKOYAMA R., HONDA D., LIPPMEIER J.C., CRAVEN K.D., PETERSON P.D. & BERBEE M.L. (2009). Labyrinthulomycetes phylogeny and its implications for the evolutionary loss of chloroplasts and gain of ectoplasmic gliding. *Mol. Phylogenet. Evol.*, **50**, 129–140.

VANDERSEA M.W., LITAKER R.W., YONNISH B., SOSA E., LANDSBERG J.H., PULLINGER C., MOON-BUTZIN P., GREEN J., MORRIS J.A., KATOR H., NOGA E.J. & TESTER P.A. (2006). Molecular assays for detecting *Aphanomyces invadans* in ulcerative mycotic fish lesions. *Appl. Environ. Microbiol.*, **72**, 1551–1557.

VISHWANATH T., MOHAN C. & SHANKAR K. (1998). Epizootic Ulcerative Syndrome (EUS), associated with a fungal pathogen, in Indian fishes: histopathology – ‘a cause for invasiveness’. *Aquaculture*, **165**, 1–9.

WADA S., AN RHA S., KONDOH T., SUDA H., HATAI K. & ISHII H. (1996). Histopathological comparison between ayu and carp artificially infected with *Aphanomyces piscicida*. *Fish Pathol.*, **31**, 71–80.

WILLOUGHBY L.G. & ROBERTS R.J. (1994). Improved methodology for isolation of the *Aphanomyces* fungal pathogen of epizootic ulcerative syndrome (EUS) in Asian fish. *J. Fish Dis.*, **17**, 541–543.

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* *

NB: There is currently (2022) no WOA Reference Laboratories for infection with *Aphanomyces invadans* (please consult the WOA 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 haematopietic necrosis virus means infection with the pathogenic agent *epizootic haematopietic 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 carcasses 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	<i>Esox lucius</i>	Northern pike
Galaxiidae	<i>Galaxias olidus</i>	Mountain galaxias
Ictaluridae	<i>Ameiurus melas</i>	Black bullhead
Melanotaeniidae	<i>Melanotaenia fluviatilis</i>	Crimson spotted rainbow fish
Percidae	<i>Perca fluviatilis</i>	European perch
	<i>Sander lucioperca</i>	Pike-perch
Percichthyidae	<i>Macquaria australasica</i>	Macquarie perch
Poeciliidae	<i>Gambusia holbrooki</i>	Eastern mosquito fish
	<i>Gambusia affinis</i>	Mosquito fish
Salmonidae	<i>Oncorhynchus mykiss</i>	Rainbow trout
Terapontidae	<i>Bidyanus bidyanus</i>	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 EHN, 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 EHN is poorly infective but once infected, most fish succumb to the disease has a high case fatality rate. Infection with EHN may be present on a farm without causing suspicion because the mortality rate may not rise above the usual background rate. Infection with EHN 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 TCID₅₀ 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 EHN 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: EHN 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 EHN on or within ova, and disinfection protocols for ova have not been evaluated. EHN 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 EHN in European perch in widely separated river systems and impoundments suggested that EHN 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 WOAHS 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 WOA 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	+ +++	+ +++	++ +	<u>2</u> 1	++ +	++ +	+++	<u>2</u> 1	<u>+</u> +	<u>+</u> +	<u>++</u>	<u>2</u> 1
Immunohistochemistry					+	+	+	1				
Real-time PCR	+++	+++	+++	<u>2</u> 1	+++	+++	+++	2	<u>++</u>	<u>++</u>	<u>++</u>	<u>2</u> 1
Conventional PCR	+	+	+	1	++	++	++	1	<u>++</u>	<u>++</u>	<u>++</u>	1
<u>Conventional PCR followed by amplicon sequencing</u>									+++	+++	+++	<u>3</u> 1
<i>In-situ</i> hybridisation												
Bioassay												
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 WOH 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 WOAHP 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 [FCBS]) 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% FCBS 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 EHN. 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 EHN 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), EHN can only be detected when these methods are combined with methods that specifically detect EHN. none has been adequately validated according to OIE guidelines for primary detection of EHN. 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 EHN, identification of EHN 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 EHN 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 RANA CON-R Probe RANA CON-Pr	5'-CTC-ATC-GTT-CTG-GCC-ATC-A-3' 5'-TCC-CAT-CGA-GCC-GTT-CA-3' 5'-6FAM-CAC-AAC-ATT-ATC-CGC-ATC-MGB-3'	Pallister et al., 2007
Primer G1096 G1097	GAG-TGA-CCA-ACG-GCA-GCC-TTA-ACG GCG-GTG-GTG-TAG-CCA-GAG-TTG-TCG	Jaramillo et al., 2012
Primer RanaF1 RanaR1 Probe RanaP1	CCA-GCC-TGG-TGT-ACG-AAA-ACA ACT-GGG-ATG-GAG-GTG-GCA-TA 6FAM-TGG-GAG-TCG-AGT-ACT-AC-MGB	Stilwell et al., 2018

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-GA	321 bp	266–586
	M152	CGG-GGC-GGG-GTT-GAT-GAG-AT		
MCP-2	M153	ATG-ACC-GTC-GCC-CTC-ATC-AG	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 (NH₄)₂SO₄, 2.5 mM MgCl₂, 1.65 mg ml⁻¹ BSA, 10 mM beta-mercaptoethanol) and 2 U Taq polymerase. Instructions on preparation of 10 × PCR buffer are included in Table 4.4.2.2.

Table 4.4.2.2. 10 × PCR buffer preparation

Ingredients	Amount	Final concentration in 50 µl PCR mix
Tris	4.050 g	66.6 mM
Ammonium sulphate	1.100 g	16.6 mM
BSA (albumin bovine fraction V fatty acid free)	0.825 g	1.65 mg ml ⁻¹
Magnesium chloride	1.25 ml	2.5 mM
TE buffer (sterile)	50 ml	

NOTE: alternative commercial buffers may also be used.

Two negative controls are included, one comprising PCR cocktail only and the second containing 5 µl TE buffer.

The MCP-1 and MCP-2 reactions have the following profile: 1 cycle of denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 1 minute; a final extension of 72°C for 5 minutes, and cooling to 4°C.

NOTE: the annealing temperature may be increased to 60 or 62°C to reduce nonspecific amplification when the assay is used to test fish tissues.

PCR results are assessed by electrophoresis in 2% agarose gels stained with ethidium bromide. 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

<u>Pathogen / target gene</u>	<u>Primer/probe (5'–3')</u>	<u>Concentration</u>	<u>Cycling parameters</u>
<u>Method 1 (Marsh et al., 2002): Product amplicon size MCP-1 is 321 bp and product amplicon size MCP-2 is 625 bp</u>			
<u>MCP-1</u> <u>Gene location:</u> <u>266-586</u>	<u>M151: AAC-CCG-GCT-TTC-GGG-CAG-CA</u> <u>M152: CGG-GGC-GGG-GTT-GAT-GAG-AT</u>	<u>250 ng of each</u> <u>primer</u>	<u>35 cycles of 50°C for 30 sec</u> <u>NOTE: the annealing</u> <u>temperature may be</u> <u>increased to 60 or 62°C to</u> <u>reduce non-specific</u> <u>amplification when the</u> <u>assay is used to test fish</u> <u>tissues.</u>
<u>MCP-2</u> <u>Gene location:</u> <u>842-1466</u>	<u>M153: ATG-ACC-GTC-GCC-CTC-ATC-AC</u> <u>M154: CCA-TCG-AGC-CGT-TCA-TGA-TG</u>		

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 DAKO¹. The primary affinity purified rabbit-anti-EHNV antibody (Lot No. M708) is supplied freeze-dried by the WOAHP 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.

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.

- 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

EHNH 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 WOA Reference Laboratory.

4.8. Bioassay

Not applicable.

4.9. Antibody- or antigen-based detection methods

An antigen ELISA for detection of EHNH and an EHNH antibody detection ELISA have been ~~described~~ reported (Whittington & Steiner, 1993). Indirect ELISA for detection of antibodies induced following exposure to EHNH 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 EHNH. Indirect ELISA for detection of antibodies induced following exposure to EHNH 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 EHNH; however, the available methods are not specific for EHNH. Any real-time PCR positive samples should be tested by conventional PCR and sequence analysis to distinguish EHNH 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 WOA 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 WOA 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

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

² For example transboundary commodities.

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 (<i>Perca fluviatilis</i>), river blackfish (<i>Gadopsis marmoratus</i>), golden perch (<i>Macquaria ambigua</i>), trout cod (<i>Maccullochella macquariensis</i>), freshwater catfish (<i>Tandanus tandanus</i>), Macquarie perch (<i>Macquaria australasica</i>) rainbow trout (<i>Oncorhynchus mykiss</i>)	94.3%* (n = 105)	100% (n = 44)	Virus isolation in BF-2 cell culture	Jaramillo et al., (2012)
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 (<i>Perca fluviatilis</i>), river blackfish (<i>Gadopsis marmoratus</i>), golden perch (<i>Macquaria ambigua</i>), trout cod (<i>Maccullochella macquariensis</i>), freshwater catfish (<i>Tandanus tandanus</i>), Macquarie perch (<i>Macquaria australasica</i>) rainbow trout (<i>Oncorhynchus mykiss</i>)	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.

7. References

AHNE W., BEARZOTTI M., BREMONT M. & ESSBAUER S. (1998). Comparison of European systemic piscine and amphibian iridoviruses with epizootic haematopoietic necrosis virus and frog virus 3. *J. Vet. Med. [B]*, **45**, 373–383.

AHNE W., OGAWA M. & SCHLOTFELDT H.J. (1990). Fish viruses: transmission and pathogenicity of an icosahedral cytoplasmic deoxyribovirus isolated from sheatfish *Silurus glanis*. *J. Vet. Med. [B]*, **37**, 187–190.

AHNE W., SCHLOTFELDT H.J. & THOMSEN I. (1989). Fish viruses: isolation of an icosahedral cytoplasmic deoxyribovirus from sheatfish (*Silurus glanis*). *J. Vet. Med. [B]*, **36**, 333–336.

ARIEL E. & BANG JENSEN B. (2009). Challenge studies of European stocks of redbfin perch, *Perca fluviatilis* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum), with epizootic haematopoietic necrosis virus. *J. Fish Dis.*, **32**, 1017–1025.

Ariel E, Holopainen R, Olenen NJ & Tapiovaara H (2010). Comparative study of ranavirus isolates from cod (*Gadua morhua*) and turbot (*Psetta maxima*) with reference to other ranaviruses. *Archives of Virology* **155**, 1261-1271

ARIEL E., NICOLAISEN N., CHRISTOPHERSEN M.-B., HOLOPAINEN R., TAPIOVAARA H. & BANG JENSEN B. (2009). Propagation and isolation of ranaviruses in cell culture. *Aquaculture*, **294**, 159–164.

BECKER J.A., GILLIGAN D., ASMUS M., TWEEDIE A. & WHITTINGTON R.J. (2019). Geographic distribution of Epizootic haematopoietic necrosis virus (EHNV) in freshwater fish in south eastern Australia: lost opportunity for a notifiable pathogen to expand its geographic range. *Viruses*, **11**, 315 doi:10.3390/v11040315

BECKER J.A., TWEEDIE A., GILLIGAN D., ASMUS M. & WHITTINGTON R. J. (2016). Susceptibility of Australian Redfin Perch *Perca fluviatilis* Experimentally Challenged with Epizootic Hematopoietic Necrosis Virus (EHNV). *J. Aquat. Anim. Health*, **28**, 122–130.

BLOCH B. & LARSEN J.L. (1993). An iridovirus-like agent associated with systemic infection in cultured turbot *Scophthalmus maximus* fry in Denmark. *Dis. Aquat. Org.*, **15**, 235–240.

BRYAN L.K., BALDWIN C.A., GRAY M.J. & MILLER D.L. (2009). Efficacy of select disinfectants at inactivating Ranavirus. *Dis. Aquat. Org.*, **84**, 89–94.

CHINCHAR V.G. (2002). Ranaviruses (family Iridoviridae): emerging cold-blooded killers – brief review. *Arch. Virol.*, **147**, 447–470.

CHINCHAR G., ESSBAUER S., HE J.G., HYATT A., MIYAZAKI T., SELIGY V. & WILLIAMS T. (2005). Family Iridoviridae. In: *Virus Taxonomy. Classification and Nomenclature of Viruses. Eight Report of the International Committee on the Taxonomy of Viruses*, Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U. & Ball L.A., eds. Academic Press, San Diego, California, USA, 145–161.

CINKOVA K., RESCHOVA S., KULICH P. & VESELY T. (2010). Evaluation of a polyclonal antibody for the detection and identification of ranaviruses from freshwater fish and amphibians. *Dis. Aquat. Org.*, **89**, 191–198.

CRANE M.S.J., YOUNG J. & WILLIAMS L. (2005). Epizootic haematopoietic necrosis virus (EHNV): growth in fish cell lines at different temperatures. *Bull. Eur. Assoc. Fish Pathol.*, **25**, 228–231.

- DRURY S.E.N., GOUGH R.E. & CALVERT I. (2002). Detection and isolation of an iridovirus from chameleons (*Chamaeleo quadricornis* and *Chamaeleo hoehnelli*) in the United Kingdom. *Vet. Rec.*, **150**, 451–452.
- FJIAN N., MATASIN Z., PETRINEC Z., VALPOTIC I. & ZWILLENBERG L.O. (1991). Isolation of an iridovirus-like agent from the green frog (*Rana esculenta* L.). *Veterinarski Arhiv*, **61**, 151–158.
- HEDRICK R.P., MCDOWELL T.S., AHNE W., TORHY C. & DE KINKELIN P. (1992). Properties of three iridovirus-like agents associated with systemic infections of fish. *Dis. Aquat. Org.*, **13**, 203–209.
- ~~HOLOPAINEN R., HONKANEN J., JENSEN B.B., ARIEL E. & TAPIOVAARA H. (2011). Quantitation of ranaviruses in cell culture and tissue samples. *J. Virol. Methods*, **171**, 225–233.~~
- HOLOPAINEN R., OHLEMEYER S., SCHÜTZE H., BERGMANN S.M. & TAPIOVAARA H. (2009). Ranavirus phylogeny and differentiation based on major capsid protein, DNA polymerase and neurofilament triplet H1-like protein genes. *Dis. Aquat. Org.*, **85**, 81–91.
- HYATT A.D., GOULD A.R., ZUPANOVIC Z., CUNNINGHAM A.A., HENGSTBERGER S., WHITTINGTON R.J., KATTENBELT J. & COUPAR B.E.H. (2000). Comparative studies of piscine and amphibian iridoviruses. *Arch. Virol.*, **145**, 301–331.
- HYATT A.D., WILLIAMSON M., COUPAR B.E.H., MIDDLETON D., HENGSTBERGER S.G., GOULD A.R., SELLECK P., WISE T.G., KATTENBELT J., CUNNINGHAM A.A. & LEE J. (2002). First identification of a ranavirus from green pythons (*Chondropython viridis*). *J. Wildl. Dis.*, **38**, 239–252.
- JARAMILLO D., TWEEDIE A., BECKER J.A., HYATT A., CRAMERI S. & WHITTINGTON R.J. (2012). A validated quantitative polymerase chain reaction assay for the detection of ranaviruses (Family Iridoviridae) in fish tissue and cell cultures, using EHNV as a model. *Aquaculture*, **356–357**, 186–192.
- LANGDON J.S. (1989). Experimental transmission and pathogenicity of epizootic haematopoietic necrosis virus (EHNV) in redfin perch, *Perca fluviatilis* L., and 11 other teleosts. *J. Fish Dis.*, **12**, 295–310.
- LANGDON J.S. & HUMPHREY J.D. (1987). Epizootic Hematopoietic Necrosis a New Viral Disease in Redfin Perch *Perca fluviatilis* L. in Australia. *J. Fish Dis.*, **10**, 289–298.
- LANGDON J.S., HUMPHREY J.D. & WILLIAMS L.M. (1988). Outbreaks of an EHNV-like iridovirus in cultured rainbow trout, *Salmo gairdneri* Richardson, in Australia. *J. Fish Dis.*, **11**, 93–96.
- LANGDON J.S., HUMPHREY J.D., WILLIAMS L.M., HYATT A.D. & WESTBURY H.A. (1986). First virus isolation from Australian fish: an iridovirus-like pathogen from redfin perch, *Perca fluviatilis* L. *J. Fish Dis.*, **9**, 263–268.
- MAO J., THAM T.N., GENTRY G.A., AUBERTIN A. & CHINCHAR V.G. (1996). Cloning, sequence analysis, and expression of the major capsid protein of the iridovirus frog virus 3. *Virology*, **216**, 431–436.
- MAO J.H., HEDRICK R.P. & CHINCHAR V.G. (1997). Molecular characterisation, sequence analysis and taxonomic position of newly isolated fish iridoviruses. *Virology*, **229**, 212–220.
- MARSH I.B., WHITTINGTON R.J., O'ROURKE B., HYATT A.D. & CHISHOLM O. (2002). Rapid differentiation of Australian, European and American ranaviruses based on variation in major capsid protein gene sequence. *Molec. Cell. Probes*, **16**, 137–151.
- PALLISTER J., GOULD A., HARRISON D., HYATT A., JANCOVICH J. & HEINE H. (2007). Development of real-time PCR assays for the detection and differentiation of Australian and European ranaviruses. *J. Fish Dis.*, **30**, 427–438.
- POZET F., MORAND M., MOUSSA A., TORHY C. & DE KINKELIN P. (1992). Isolation and preliminary characterization of a pathogenic icosahedral deoxyribovirus from the catfish (*Ictalurus melas*). *Dis. Aquat. Org.*, **14**, 35–42.
- REDDACLIFF L.A. & WHITTINGTON R.J. (1996). Pathology of epizootic haematopoeitic necrosis virus (EHNV) infection in rainbow trout (*Oncorhynchus mykiss* Walbaum) and redfin perch (*Perca fluviatilis* L.). *J. Comp. Pathol.*, **115**, 103–115.

RIMMER A.E., BECKER J.A., TWEEDIE A. & WHITTINGTON R.J. (2012). Validation of high throughput methods for tissue disruption and nucleic acid extraction for ranaviruses (family Iridoviridae). *Aquaculture*, **338–341**, 23–28.

SPEARE R. & SMITH J.R. (1992). An iridovirus-like agent isolated from the ornate burrowing frog *Limnodynastes ornatus* in northern Australia. *Dis. Aquat. Org.*, **14**, 51–57.

STILWELL N.K., WHITTINGTON R.J., HICK P.M., BECKER J.A., ARIEL E., VAN BEURDEN S., VENDRAMIN N., OLESEN N.J. & WALTZEK T.B. (2018). Partial validation of a TaqMan real-time quantitative PCR for the detection of ranaviruses. *Dis. Aquat. Org.*, **128**, 105–116.

WHITTINGTON R.J., BECKER J.A. & DENNIS M.M. (2010). Iridovirus infections in finfish – critical review with emphasis on ranaviruses. *J. Fish Dis.*, **33**, 95–122.

WHITTINGTON R.J., KEARNS C., HYATT A.D., HENGSTBERGER S. & RUTZOU T. (1996). Spread of epizootic haematopoietic necrosis virus (EHNV) in redfin perch (*Perca fluviatilis*) in southern Australia. *Aust. Vet. J.*, **73**, 112–114.

WHITTINGTON R.J., PHILBEY A., REDDAKLIFF G.L. & MACGOWN A.R. (1994). Epidemiology of epizootic haematopoietic necrosis virus (EHNV) infection in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum): findings based on virus isolation, antigen capture ELISA and serology. *J. Fish Dis.*, **17**, 205–218.

WHITTINGTON R.J. & REDDAKLIFF G.L. (1995). Influence of environmental temperature on experimental infection of redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) with epizootic haematopoietic necrosis virus, an Australian iridovirus. *Aust. Vet. J.*, **72**, 421–424.

WHITTINGTON R.J., REDDAKLIFF L.A., MARSH I., KEARNS C., ZUPANOVIC Z. & CALLINAN R.B. (1999). Further observations on the epidemiology and spread of epizootic haematopoietic necrosis virus (EHNV) in farmed rainbow trout *Oncorhynchus mykiss* in southeastern Australia and a recommended sampling strategy for surveillance. *Dis. Aquat. Org.*, **35**, 125–130.

WHITTINGTON R.J. & STEINER K.A. (1993). Epizootic haematopoietic necrosis virus (EHNV): improved ELISA for detection in fish tissues and cell cultures and an efficient method for release of antigen from tissues. *J. Virol. Methods*, **43**, 205–220.

WOLF K., BULLOCK G.L., DUNBAR C.E. & QUIMBY M.C. (1968). Tadpole edema virus: a viscerotropic pathogen for anuran amphibians. *J. Infect. Dis.*, **118**, 253–262.

ZUPANOVIC Z., MUSSO C., LOPEZ G., LOURIERO C.L., HYATT A.D., HENGSTBERGER S. & ROBINSON A.J. (1998). Isolation and characterisation of iridoviruses from the giant toad *Bufo marinus* in Venezuela. *Dis. Aquat. Org.*, **33**, 1–9.

*
* *

NB: There is a WOA Reference Laboratory for infection with epizootic haematopoietic necrosis virus (EHNV)

(please consult the WOA 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 WOA Reference Laboratories for any further information on infection with EHNV.

The WOA 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	<i>Abramis brama</i>	Bream
	<i>Aristichthys nobilis</i>	Bighead carp
	<i>Carassius auratus</i>	Goldfish
	<i>Ctenopharyngodon idella</i>	Grass carp
	<i>Cyprinus carpio</i>	Common carp (all varieties and subspecies)
	<i>Danio rerio</i>	Zebrafish
	<i>Notemigonus crysoleucas</i>	Golden shiner
	<i>Pimephales promelas</i>	Fathead minnow
	<i>Percocypris pingi</i>	Jinsha bassbarbel carp
	<i>Rutilus kutum</i>	Caspian white fish
	<i>Rutilus rutilus</i>	Roach
Siluridae	<i>Silurus glanis</i>	Wels catfish

[...]

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*).

2.2.2. 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*).

[...]

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 ostreae* 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 (*Ascidella aspersa*), grouped zooplankton and Pacific cupped oyster (*Magallana [syn. Crassostrea] gigas*).

[...]

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 discadata*, *Centropages typicus*, *Euterpina acutifrons*, unidentified *Oithona* sp., *Penilia avirostris*).

[...]
