

Informe de la Reunión de la Comisión de Normas Sanitarias para los Animales Acuáticos de la OMSA

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14 al 21 de septiembre de 2022
Reunión híbrida

Introducción y comentarios de los Miembros

La Comisión de Normas Sanitarias para los Animales Acuáticos (Comisión para los Animales Acuáticos) agradece a los siguientes Miembros por el envío de sus comentarios sobre los proyectos de texto para el *Código Sanitario para los Animales Acuáticos* de la OMSA (*Código Acuático*) y el *Manual de las Pruebas de Diagnóstico para los Animales Acuáticos* de la OMSA (*Manual Acuático*) que circularon en la reunión de la Comisión de febrero de 2022: Australia, Canadá, China (República Popular de), Estados Unidos de América, Japón, Noruega, Suiza, Tailandia, Taipéi Chino y los Estados miembros de la Unión Europea (UE). Igualmente, expresa su agradecimiento a numerosos expertos de la red científica de la OMSA por su valiosa participación y contribución.

La Comisión para los Animales Acuáticos examinó todos los comentarios de los Miembros que se presentaron a tiempo y que estaban acompañados por fundamentos. Dada la gran carga de trabajo, no pudo preparar una explicación detallada de las razones que la motivaron a aceptar o rechazar los comentarios recibidos y concentró sus explicaciones en los más importantes. Cuando se trata de cambios de naturaleza editorial, no se brinda ningún texto explicativo. La Comisión desea destacar que, en aras de claridad, no se aceptaron todos los textos propuestos por los Miembros; en dichos casos, consideró que el texto era claro tal y como estaba redactado. Las modificaciones se señalan del modo habitual con “subrayado doble” y “texto tachado” y figuran en los anexos del presente informe. En los anexos, los cambios propuestos en esta reunión se muestran con un fondo de color para distinguirlos de los realizados anteriormente.

Nota

La Comisión para los Animales Acuáticos comunicó a los Miembros que los informes de los grupos *ad hoc* ya no se adjuntan a su informe. En su lugar, se proporcionará un enlace para consultarlos en las páginas del sitio web de la OMSA: [Grupos ad hoc - OMSA - Organización Mundial de Sanidad Animal \(woah.org\)](#)

Observación sobre los anexos

Los textos en los Anexos 1 a 9 y 11 a 30 se presentan para comentario.

Cómo enviar los comentarios

La Comisión para los Animales Acuáticos anima encarecidamente a los Miembros y a las organizaciones que han suscrito un acuerdo de cooperación con la OMSA a participar en la elaboración de las normas internacionales de la Organización enviando sus comentarios sobre este informe.

Los comentarios deben remitirse en formato Word y no PDF, ya que este último formato es difícil de incorporar a los documentos de trabajo de la Comisión.

Los comentarios se deberán presentar en el anexo pertinente e incluir toda modificación de texto, en base a una justificación estructurada o a partir de referencias científicas publicadas. Las supresiones propuestas deberán indicarse con “texto tachado” y, las inserciones, con “subrayado doble”. Se ruega a los Miembros que no utilicen la función automática “Resaltar cambios” del procesador de texto, ya que dichos cambios se pierden en el proceso de recopilación de las propuestas de los Miembros incluidas en los documentos de trabajo de la Comisión.

Fecha límite para enviar los comentarios

Todos los comentarios sobre los textos pertinentes de este informe deberán remitirse a la sede de la OMSA hasta el 6 de enero de 2023 para que la Comisión los examine en su reunión de febrero de 2023.

Dónde enviar los comentarios

Todos los comentarios deberán remitirse por correo electrónico al Departamento de Normas: ACC.Secretariat@woah.org.

Fecha de la próxima reunión

La Comisión tomó nota de las fechas para su próxima reunión: 15 al 22 de febrero de 2023.



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1. Bienvenida

1.1. Directora general adjunta de la OMSA de Normas Internacionales y Ciencia

La Dra. Montserrat Arroyo, directora general adjunta de Normas Internacionales y Ciencia, dio la bienvenida a los integrantes de la Comisión para los Animales Acuáticos y agradeció a todos su contribución en el trabajo de la OMSA. Asimismo, felicitó a la Comisión por su ambiciosa agenda y extendió su agradecimiento a las instituciones empleadoras y a los gobiernos nacionales.

La Dra. Arroyo informó a la Comisión de la intención de celebrar la 90.^a Sesión General como una reunión presencial centrada en el reencuentro tras las anteriores sesiones generales virtuales e híbridas. Animó a los integrantes de la Comisión a presentar en los seminarios web regionales los aspectos más destacados de su informe de septiembre de 2022, por considerarlo un mecanismo excelente para reforzar el compromiso entre los Miembros. Por otro lado, indicó que el nuevo nombre “OMSA” se introducirá progresivamente en el *Código Acuático* y el *Manual Acuático*. La Dra. Arroyo presentó un resumen de las iniciativas en curso de la Organización en materia de digitalización, incluidos el desarrollo y la planificación de nuevas herramientas digitales. Evocó también la nueva Red de coordinación de investigación de la OMSA. Resumió la implementación de la *Estrategia de la OMSA para la sanidad de los animales acuáticos* (la Estrategia), al tiempo que reconoció que la Comisión recibiría presentaciones adicionales sobre las actividades de esta Estrategia durante su reunión. Por su parte, los integrantes de la Comisión agradecieron a la Dra. Arroyo el excelente respaldo de la secretaría de la OMSA.

1.2. Directora general de la OMSA

La directora general de la OMSA, la Dra. Monique Eloit, se reunió con la Comisión para los Animales Acuáticos el día 21 de septiembre y dio las gracias a cada uno de los integrantes por su compromiso a la hora de alcanzar los objetivos de la Organización. La Dra. Eloit informó a la Comisión de que la aplicación de la Estrategia había dado lugar a iniciativas positivas, pero señaló la necesidad de un equilibrio entre las actividades de apoyo al objetivo No.1 *Normas* y a los otros tres objetivos para garantizar una mejor sanidad y bienestar de los animales acuáticos en todos los ámbitos. Explicó que el apoyo y el refuerzo de las actividades regionales y el fortalecimiento de capacidades adquirirán cada vez más importancia en el futuro. Se refirió a la revisión del sistema científico de la OMSA que se está llevando a cabo y subrayó que debía ajustarse a las mejores prácticas actuales y constituir un sistema ágil y dotado de una gran capacidad de respuesta. La Dra. Eloit reafirmó el papel de la OMSA en el campo de la prevención de enfermedades, específicamente en el contexto de los animales silvestres y presentó algunos de los trabajos de la OMSA en esta área. La Comisión agradeció a la Dra. Eloit por estas actualizaciones.

2. Aprobación del orden del día

El proyecto de orden del día fue aprobado por la Comisión. El orden del día y la lista de participantes figuran en los [Anexos 1](#) y [2](#), respectivamente.

3. Cooperación con la Comisión de Normas Sanitarias para los Animales Terrestres

Las mesas (el presidente y los dos vicepresidentes) de la Comisión de Normas Sanitarias para los Animales Terrestres (Comisión del Código) y la Comisión para los Animales Acuáticos celebraron un breve encuentro el 19 de septiembre de 2022, presidido por la directora general adjunta de “Normas Internacionales y Ciencia” de la OMSA. El objetivo de la reunión fue compartir información y garantizar un enfoque armonizado para las revisiones de los capítulos horizontales, en función de las necesidades. Ambas Comisiones se comprometieron a seguir convocando reuniones de la mesa al menos una vez al año con vistas a garantizar una mayor coordinación. Las mesas debatieron temas de interés mutuo en torno al *Código Acuático* y el *Código Terrestre*, en particular:

- El enfoque adoptado por ambas Comisiones en la elaboración de sus respectivos programas de trabajo y en la definición de criterios para establecer un orden de prioridades de los ítems;
- El acercamiento a seguir para revisar el uso de las definiciones del Glosario (“autoridad competente”, “autoridad veterinaria”, “servicios veterinarios” y “servicios de sanidad de los animales acuáticos”) en ambos *Códigos*, tras la adopción de las definiciones revisadas en mayo de 2022. Las mesas se pusieron de acuerdo en coordinar la revisión del uso, con el fin de garantizar un enfoque armonizado y difundirlo para comentario en febrero de 2023 (ver ítem 6.1.);
- La propuesta de nuevos trabajos sobre la certificación electrónica. Las mesas aceptaron añadir la revisión del Capítulo 5.2. del *Código Acuático* y del *Código Terrestre* a sus respectivos programas de trabajo (ver ítem 6.3.);
- Los progresos en los trabajos respectivos de cada Comisión para revisar el Título 4. Las mesas decidieron mantenerse mutuamente informadas de los trabajos en curso;

- La revisión de los Capítulos 5.4. a 5.7. del *Código Terrestre*. La mesa de la Comisión del Código acordó compartir el mandato con la Comisión para los Animales Acuáticos y remitir los documentos de trabajo a medida que progrese su elaboración;
- La revisión del Capítulo 6.10. *Uso responsable y prudente de agentes antimicrobianos en medicina veterinaria* en el *Código Terrestre*. La mesa de la Comisión del Código acordó compartir el informe del Grupo de trabajo sobre la resistencia a los antimicrobianos, el proyecto de capítulo y los documentos de trabajo a medida que se reciban los comentarios.

4. Plan de trabajo y prioridades

Se recibieron comentarios de Australia, Canadá, Noruega y la UE.

La Comisión analizó los comentarios recibidos.

La Comisión aceptó un comentario sobre la necesidad de promover entre los Miembros mecanismos para la generación de datos sobre el tiempo/temperaturas de inactivación para las mercancías seguras. La Comisión subrayó dos mecanismos de la OMSA que ayudarán a identificar y fomentar las necesidades de investigación, el nuevo Programa de coordinación de investigación de la OMSA y la nueva Red de laboratorios de referencia que se están desarrollando a través de la Estrategia como medios para fomentar la investigación en este ámbito. Además, la Comisión modificó el modelo de informe anual para los centros de referencia, con el fin de solicitar la identificación de las lagunas de investigación. Esta información la transmitirá al Programa de Coordinación de la Investigación de OMSA. Dicha modificación permitirá recabar información y guiar estas dos nuevas iniciativas. La Comisión tomó nota del establecimiento de una consultoría dedicada a actualizar la evaluación de mercancías seguras, publicada en 2016. Las conclusiones se revisarán en la reunión de la Comisión de febrero de 2023 y servirán de base para actualizar los Artículos X.X.3. pertinentes del *Código Acuático*.

A tenor de un comentario, la Comisión aceptó que toda propuesta de modificación del Capítulo 4.2. *Zonificación y Compartimentación* esté interconectada con las modificaciones del Capítulo 4.3. *Aplicación de la compartimentación*. La Comisión recordó a los Miembros que el Capítulo 4.2. se modificaría con la intención de tratar únicamente la zonificación y que la información que actualmente figura en el capítulo sobre compartimentación se abordaría tras la adopción de las modificaciones del Capítulo 4.3. *Aplicación de la compartimentación*. La Comisión prosigue su plan de actualización del Título 4 en función de los recursos disponibles.

La Comisión agradeció a un Miembro su propuesta de ayuda en la elaboración de un nuevo capítulo sobre el comercio de material genético y señaló que este tema se debatiría a fondo en el momento que se planifique el nuevo capítulo.

La Comisión debatió las condiciones para la elaboración del Capítulo 5.X. *Comercio de animales acuáticos ornamentales*, el Capítulo 5.Y. *Comercio de materiales genéticos* y la modificación del Capítulo 4.3. *Aplicación de la compartimentación*. La Comisión añadió estos capítulos a su plan de trabajo y acordó un plan de proyecto para cada capítulo con el fin de establecer su progresión, las etapas necesarias a su finalización y el calendario de distribución para comentario de los Miembros.

La Comisión agradeció a un Miembro la presentación de nuevas pruebas científicas sobre una especie susceptible a la infección por el virus de la viremia primaveral de la carpa (ver ítem 5.5.). La Comisión observó que, para todas las enfermedades evaluadas anteriormente, a medida que se disponga de recientes pruebas científicas, sería necesario realizar evaluaciones de nuevas especies susceptibles, o una nueva evaluación de las existentes, que se añadirían a su plan de trabajo. La Comisión instó a los Miembros a transmitir, para una posterior evaluación, cualquier nueva evidencia científica sobre la susceptibilidad.

La Comisión estableció un debate con el Departamento de Estatus de la OMSA sobre el proceso de autodeclaración de ausencia de enfermedad y la modificación del procedimiento actual para la publicación de una autodeclaración de estatus zoosanitario, con el fin de garantizar una armonización con el Capítulo 1.4. *Vigilancia de las enfermedades de los animales acuáticos*, recientemente adoptado. La Comisión acordó añadir un ítem a su plan de trabajo que busca elaborar nuevas directrices en forma de un modelo de autodeclaración y así apoyar a los Miembros en la presentación de autodeclaraciones.

La Comisión revisó el avance de los temas en curso en el plan de trabajo y estableció los plazos para su finalización.

Examinó también las prioridades de los nuevos ítems de trabajo, teniendo en cuenta una serie de criterios, como la mejora esperada de las normas y su impacto, el beneficio para los Miembros, sus comentarios, la pertinencia para las actividades de la Estrategia, los comentarios de la sede de la OMSA y el progreso de los ítems del plan de trabajo en curso.

La Comisión observó que la progresión de los puntos del plan de trabajo que están sujetos a la convocatoria de los grupos *ad hoc* dependía de los progresos previstos para 2022. La lista de grupos *ad hoc* actuales y programados para 2022 se puede consultar en el sitio web de la OMSA.

El plan de trabajo actualizado figura en el [Anexo 3](#) para comentario de los Miembros.

Código Sanitario para los Animales Acuáticos de la OMSA

5. Textos para comentario de los Miembros

5.1. Capítulo 1.3. Enfermedades de la lista de la OIE – Inclusión en la lista de la infección por *Megalocytivirus*

Contexto

En su reunión de febrero de 2022, la Comisión para los Animales Acuáticos comunicó a los Miembros que otros virus del género *Megalocytivirus* también pueden causar enfermedades en los peces, pero que no entran en el ámbito de aplicación del Capítulo 2.3.7. *Infección por iridovirosis de la dorada japonesa* del *Manual Acuático*. La Comisión observó que si el virus de la necrosis infecciosa del bazo y del riñón, el iridovirus del cuerpo rojizo del rodaballo u otros megalocitivirus se incluyen en la lista, los virus tendrían que ser evaluados según los criterios de inclusión en el Capítulo 1.2. del *Código Acuático*. Si se considera que cumplen los criterios de inclusión en la lista, se podría proponer su inclusión a la Asamblea de la OMSA.

El Grupo *ad hoc* de la OMSA sobre la susceptibilidad de las especies de peces a la infección por enfermedades de la lista de la OIE se reunió en abril de 2022 para seguir trabajando en la aplicación de los criterios del Capítulo 1.5. *Criterios para la inclusión de especies susceptibles de infección por un agente patógeno específico*. En estas reuniones, el grupo *ad hoc* llevó a cabo evaluaciones preliminares de la susceptibilidad de las especies de peces a la infección por iridovirosis de la dorada japonesa y presentó un informe provisional a consideración de la Comisión.

Informes de la Comisión donde se discutió del tema

Informe de febrero de 2022 (Parte B, ítem 3.1.2.3, página 13).

Septiembre de 2022

La Comisión examinó el informe del Grupo *ad hoc* sobre la susceptibilidad de las especies de peces a la infección por enfermedades de la lista de la OIE para la infección por el iridovirosis de la dorada japonesa (RSIV). La Comisión también observó que para distinguir las especies susceptibles a la infección por RSIV se requiere un análisis de la secuencia de ácidos nucleicos y/o del árbol filogenético para determinar si el agente patógeno es el RSIV o si se trata de otro genogruppo. Por lo tanto, en ocasiones el grupo *ad hoc* carece de pruebas para identificar las especies susceptibles a nivel del genotipo. En consecuencia, el grupo *ad hoc* completó las evaluaciones preliminares de la susceptibilidad de las especies de peces a la infección por iridovirosis de la dorada japonesa (RSIV), el virus de la necrosis infecciosa del bazo y del riñón (ISKNV) y el iridovirus del cuerpo rojizo del rodaballo (TRBIV) y, en su informe provisional, recomendó a la Comisión que considerara la posibilidad de incluir el virus en la lista de *Megalocytivirus*, incluyendo el ISKNV, el RSIV y el TRBIV (salvo el virus de la enfermedad de la caída de escamas o descamación, SDDV).

La Comisión reconoció que la complejidad asociada a los diferentes genogrupos de *Megalocytivirus* justificaba una evaluación según los criterios del Artículo 1.2.2. del Capítulo 1.2. *Criterios para la inclusión de las enfermedades de los animales acuáticos en la lista de la OIE*. La Comisión acordó evaluar la especie del virus de la necrosis infecciosa del bazo y del riñón (ISKNV), incluidos sus tres genogrupos, el RSIV, el ISKNV y el TRBIV. La Comisión acordó que el genogruppo RSIV (actualmente incluido en el *Código Acuático*), así como los dos genogrupos ISKNV y TRBIV cumplen los criterios 1, 2, 3 y 4b de la lista.

La Comisión observó que los tres genogrupos (RSIV, ISKNV y TRBIV) tienen especies susceptibles que se superponen, con una epidemiología y métodos de diagnóstico similares. En consecuencia, acordó que la enfermedad de la lista propuesta debería denominarse "infección por el virus de la necrosis infecciosa del bazo y del riñón (ISKNV)". La infección por el ISKNV se definirá para incluir los tres genogrupos de la especie ISKNV (es decir, ISKNV, RSIV y TRBIV), pero excluiría la otra especie reconocida de *Megalocytivirus*, el SDDV.

La evaluación de la infección por el virus de la necrosis infecciosa del bazo y del riñón para su inclusión en el *Código Acuático* de la OMSA figura en el [Anexo 5](#) para comentario.

El Artículo revisado 1.3.1. del Capítulo 1.3. *Enfermedades de la lista de la OIE* figura en el [Anexo 4](#) para comentario.

5.2. Artículo 9.3.1. del Capítulo 9.3. Infección por *Hepatobacter penaei* (*hepatopancreatitis necrotizante*)

Se recibieron comentarios de China, Noruega, Suiza y la UE.

Contexto

En su reunión de febrero de 2022, la Comisión para los Animales Acuáticos aceptó modificar el Artículo 9.3.1. en aras de coherencia con el Capítulo 1.3. *Enfermedades de la lista de la OIE*.

Informes de la Comisión donde se discutió del tema

Informe de febrero de 2022 (Parte B: ítem 2.1.2., página 6).

Reunión de septiembre de 2022

La Comisión aceptó un comentario que proponía modificar la descripción taxonómica en el Artículo 9.3.1. y, de acuerdo con la convención utilizada en el *Código Acuático* y el *Manual Acuático*, añadió el nivel de Familia y modificó el Orden a "Rickettsiales" para reflejar las relaciones taxonómicas correctas. La Comisión también modificó la Sección 1. del Capítulo 2.2.3. del *Manual Acuático*, con fines de armonización (ver ítem 7.1.4.).

El Artículo revisado 9.3.1. del Capítulo 9.3. *Infección por Hepatobacter penaei (hepatopancreatitis necrotizante)* figura en el [Anexo 6](#) para comentario de los Miembros.

5.3. Artículos 9.4.1. y 9.4.2. del Capítulo 9.4. Infección por el virus de la necrosis hipodérmica y hematopoyética infecciosa (IHHNV)

Se recibieron comentarios de Noruega, Suiza y la UE.

Contexto

En su reunión de febrero de 2022, la Comisión para los Animales Acuáticos aceptó modificar el Artículo 9.4.1. del Capítulo 9.4. *Infección por el virus de la necrosis hipodérmica y hematopoyética infecciosa* para reflejar una actualización en la clasificación taxonómica y en aras de coherencia con otros capítulos específicos de enfermedad.

En el Artículo 9.4.2., la Comisión aceptó enmendar las especies de crustáceos susceptibles de acuerdo con la convención utilizada en el Artículo X.X.2. del *Código Acuático*, es decir, enumerar las especies susceptibles alfabéticamente según su nombre común. La Comisión también modificó la Sección 2.2.2. del Capítulo 2.2.4. de *Manual Acuático* en aras de armonización.

Informes de la Comisión donde se discutió del tema

Informe de febrero de 2022 (Parte B: ítem 2.1.3., página 6).

Reunión de septiembre de 2022

La Comisión revisó los comentarios recibidos y no propuso ninguna modificación adicional dado que los Miembros aceptaron los cambios propuestos.

Los Artículos revisados 9.4.1. y 9.4.2. del Capítulo 9.4. *Infección por el virus de la necrosis hipodérmica y hematopoyética infecciosa* figuran en el [Anexo 7](#) para comentario de los Miembros.

5.4. Artículo 9.5.2 del Capítulo 9.5. Infección por el virus de la mionecrosis infecciosa

Contexto

En un esfuerzo continuo por garantizar la concordancia entre el *Código Acuático* y el *Manual Acuático*, a medida que se aplica progresivamente el nuevo modelo de los capítulos específicos de enfermedad del *Manual Acuático*, también se actualizan, si es necesario, los artículos pertinentes de los capítulos específicos de enfermedad del *Código Acuático*.

Reunión de septiembre de 2022

En el Artículo 9.5.2., la Comisión acordó modificar la lista de especies susceptibles de acuerdo con la convención utilizada en el Artículo X.X.2. del *Código Acuático*, es decir, enumerar las especies susceptibles por orden alfabético según el nombre común. La Comisión también modificó la Sección 2.2.2. del Capítulo 2.2.5. *Infección por el virus de la mionecrosis infecciosa* del *Manual Acuático* en aras de concordancia (ver ítem 7.1.6.).

Los Artículos revisados 9.5.2. y el Capítulo 9.5. *Infección por el virus de la mionecrosis infecciosa* figura en el [Anexo 8](#) para comentario de los Miembros.

5.5. Artículo 10.9.2. del Capítulo 10.9. Infección por el virus de la viremia primaveral de la carpa

Contexto

En su reunión de febrero de 2022, la Comisión para los Animales Acuáticos acordó que, a medida que se presenten nuevas pruebas científicas sobre la susceptibilidad de las especies de animales acuáticos a las enfermedades de la lista de la OIE, sería necesario efectuar evaluaciones de nuevas especies susceptibles o reevaluar las existentes. La Comisión añadió un nuevo ítem en su plan de trabajo para tratar esta necesidad. La Comisión instó a los Miembros a que proporcionen para evaluación toda nueva prueba científica sobre la susceptibilidad.

Reunión de septiembre de 2022

En respuesta a un Miembro que aportó pruebas científicas de la susceptibilidad de una nueva especie, la Comisión solicitó al Grupo *ad hoc* sobre la susceptibilidad de las especies de peces a la infección por las enfermedades de la lista de la OIE que evaluara 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* aplicó los criterios expuestos en su informe de noviembre de 2017 para la susceptibilidad de las especies de peces a la infección por el virus de la viremia primaveral de la carpa para la evaluación de la carpa Jinsha bass (*Percocypris pingi*).

La Comisión examinó la evaluación del grupo *ad hoc* y acordó incluir la carpa Jinsha bass (*Percocypris pingi*) en la lista de especies susceptibles del Artículo 10.9.2.

El Grupo *ad hoc* de evaluación de la carpa Jinsha bass (*Percocypris pingi*) figura en el [Anexo 10](#) para información de los Miembros.

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 9](#) para comentario de los Miembros.

5.6. Nuevo Capítulo 10.X. Infección por el virus de la tilapia del lago

Contexto

Tras la adopción de la "infección por el virus de la tilapia del lago" (TiLV) en el Artículo 1.3.1. del Capítulo 1.3. *Enfermedades de la lista de la OIE*, en mayo de 2022, la Comisión para los Animales Acuáticos acordó elaborar un nuevo proyecto de capítulo para la infección por TiLV en base a la estructura de los artículos de otros capítulos específicos de enfermedad del Título 10.

Reunión de septiembre de 2022

La Comisión revisó el proyecto de Capítulo 10.X. *Infección por TiLV*, elaborado por un miembro de la Comisión.

La Comisión recuerda a los Miembros que la anotación "(en estudio)" se utiliza en el proyecto de Capítulo 10.X. *Infección por el virus de la tilapia del lago* (TiLV) y, como se explica en el apartado B.2. de la Guía del Usuario del *Código Acuático*, significa que "esta parte del texto no ha sido aprobada por la Asamblea Mundial de Delegados de la OMSA y que, por lo tanto, las disposiciones específicas no forman parte aún del *Código Acuático*".

La Comisión reconoció que las especies susceptibles del Artículo 10.X.2. se ponían "en estudio" a la espera de una evaluación con arreglo al Capítulo 1.5. *Criterios para la inclusión de especies susceptibles de infección por un agente patógeno específico*. La Comisión también acordó indicar "en estudio" en los productos de animales acuáticos enumerados en los apartados 1-2 del Artículo 10.X.3. y en el apartado 1a. del Artículo 10.X.14., a la espera de una evaluación de conformidad con el Capítulo 5.4. *Criterios para la evaluación de la inocuidad de las mercancías de animales acuáticos*. La Comisión acordó que los procesos físicos y químicos que se aplican en la producción de aceite de pescado y cuero de pescado serían suficientes para inactivar la presencia de cualquier TiLV y que, por lo

consiguiente, cumplen los criterios del apartado 2 del Artículo 5.4.1. La Comisión aceptó incluir estos productos de animales acuáticos en el Artículo 10.X.3. y no considerarlos "en estudio".

La Comisión indicó que los períodos por defecto para las condiciones elementales de bioseguridad y la vigilancia específica presentados en el Capítulo 1.4. *Vigilancia de las enfermedades de los animales acuáticos* se apliquen para la infección por TiLV hasta que se complete una evaluación de los períodos por defecto. La Comisión señaló haber solicitado el asesoramiento de expertos sobre una evaluación de estos períodos por defecto para todas las enfermedades de la lista, incluida la infección por TiLV. Una vez que la Comisión estudie el asesoramiento recibido, será posible proponer cambios en los capítulos específicos de enfermedad, cuando corresponda.

El nuevo Capítulo 10.X. *Infección por TiLV* figura en el [Anexo 11](#) para comentario de los Miembros.

5.7. Artículo 11.2.2. del Capítulo 11.2. Infección por *Bonamia exitiosa* y Artículo 11.3.2. del Capítulo 11.3. Infección por *Bonamia ostreae*

Contexto

En el informe de junio de 2022 del Grupo *ad hoc* sobre la susceptibilidad de las especies de moluscos a la infección por enfermedades de la lista de la OIE para la infección por *Bonamia exitiosa* y la infección por *Bonamia ostreae*, reconoció que *Magallana gigas* es el nombre aceptado para la ostra de copa del Pacífico por el Registro Mundial de Especies Marinas (WoRMS). El grupo *ad hoc* había mantenido el nombre de *Crassostrea gigas*, ya que las pruebas no se consideraban lo suficientemente sólidas como para apoyar un cambio taxonómico. En la reunión del grupo *ad hoc* de mayo-junio de 2022 destinada a evaluar las especies susceptibles de infección por *Marteilia refringens*, se analizaron nuevos datos y publicaciones revisadas por pares sobre el nuevo nombre de *Magallana gigas*. El grupo *ad hoc* recomendó un cambio en el nombre científico de la ostra cúpula del Pacífico para consideración de la Comisión para los Animales Acuáticos.

El informe del grupo *ad hoc* de junio de 2022 está disponible en el sitio web de la OMSA.

Reunión de septiembre de 2022

La Comisión para los Animales Acuáticos revisó el informe del Grupo *ad hoc* sobre la susceptibilidad de las especies de moluscos a la infección por enfermedades de la lista de la OIE para la infección por *Marteilia refringens* y tomó nota de que las recomendaciones del grupo *ad hoc* para la nueva nomenclatura de la ostra de copa del Pacífico acarreaban consecuencias en el Artículo 11.2.2. del Capítulo 11.2. *Infección por Bonamia exitiosa* y el Artículo 11.3.2. del Capítulo 11.3. *Infección por Bonamia ostreae*.

La Comisión aceptó modificar el nombre científico de la ostra de Suminoe por "*Magallana* (*Syn. Crassostrea*) *ariakensis*" y el de la ostra de copa del Pacífico por "*Magallana* (*Syn. Crassostrea*) *gigas*" cuando se utilicen en los capítulos específicos de enfermedad del Código Acuático y del Manual Acuático. Las enmiendas se introducirán de forma progresiva y se modificarán los Artículos 11.2.2. y 11.3.2., gracias a la evaluación realizada por el grupo *ad hoc* de las especies susceptibles. Las modificaciones de los otros Artículos 11.X.2. se modificarán a medida que el grupo *ad hoc* avance en sus evaluaciones de las otras enfermedades de los moluscos. Para los cambios asociados a las Secciones 2.2.1. y 2.2.2. del Capítulo 2.4.2. *Infección por Bonamia exitiosa* y del Capítulo 2.4.3. *Infección por Bonamia ostreae* del Manual Acuático ver ítem 7.3.1.

El Artículo enmendado 11.2.2. del Capítulo 11.2. *Infección por Bonamia exitiosa*, figura en el [Anexo 12](#) para comentario de los Miembros.

El Artículo enmendado 11.3.2. del Capítulo 11.3. *Infección por Bonamia ostreae* figura en el [Anexo 13](#) para comentario de los Miembros.

5.8. Artículos 11.4.1. y 11.4.2. del Capítulo 11.4. Infección por *Marteilia refringens*

Contexto

El Grupo *ad hoc* sobre la susceptibilidad de las especies de moluscos a la infección por enfermedades de la lista de la OMSA se reunió durante los meses de noviembre y diciembre de 2021 y de mayo y junio de 2022 para continuar su labor sobre la aplicación de los criterios del Capítulo 1.5. *Criterios para la inclusión de especies susceptibles a la infección por un patógeno específico*. En estas reuniones el grupo *ad hoc* evaluó la susceptibilidad de las especies de moluscos a la infección por *Marteilia refringens*.

Informes de la Comisión donde se discutió del tema

Informe de febrero de 2022 (Parte B: ítem 4.1., página 17).

Reunión de septiembre de 2022

La Comisión para los Animales Acuáticos examinó el informe del Grupo *ad hoc* sobre la susceptibilidad de las especies de moluscos a la infección por enfermedades de la lista de la OIE y felicitó a los integrantes por su exhaustivo trabajo.

La Comisión modificó el Artículo 11.4.1. en aras de coherencia con el enfoque adoptado en otros capítulos específicos de las enfermedades de los moluscos.

La Comisión acordó modificar la lista de especies sensibles del Artículo 11.4.2. de acuerdo con las recomendaciones del grupo *ad hoc*, es decir:

- tres especies actualmente incluidas en el Artículo 11.4.2., el mejillón azul (*Mytilus edulis*), la ostra plana europea (*Ostrea edulis*) y el mejillón mediterráneo (*Mytilus galloprovincialis*), se evaluaron y se determinó que cumplen los criterios de inclusión en la lista como susceptibles a la infección por *M. refringens* y, por lo tanto, se propone permanezcan en el Artículo 11.4.2.
- cinco nuevas especies susceptibles, la ostra enana (*Ostrea stentina*), la navaja europea (*Solen marginatus*), el mejillón dorado (*Xenostrobus securis*) y la venus rayada (*Chamelea gallina*) fueron evaluadas y se determinó que cumplen los criterios de inclusión en la lista de especies susceptibles a la infección por *M. refringens* y, por lo tanto, se propone añadirlas al Artículo 11.4.2.
- tres especies actualmente incluidas en el Artículo 11.4.2., la ostra de barro australiana (*Ostrea angasi*), la ostra argentina (*Ostrea puelchana*) y la ostra plana chilena (*Ostrea chilensis*), fueron evaluadas y se determinó que no cumplen los criterios para ser incluidas en la lista de especies susceptibles a la infección por *M. refringens*, por lo que se propone suprimirlas del Artículo 11.4.2.

La Comisión rechazó la recomendación del grupo *ad hoc* de añadir un copépodo (*Paracartia grani*) al Artículo 11.4.2. a pesar de que cumple los criterios para figurar en la lista como susceptible a la infección por *M. refringens*. La Comisión consideró que esta especie no es relevante para el comercio de moluscos o productos derivados. Sin embargo, la Comisión estimó que el copépodo (*Paracartia grani*) debía incluirse en la Sección 2.2.1. del Capítulo 2.4.4. *Infección por Marteilia refringens* en el *Manual Acuático* para asegurarse de que los Miembros sepan que se trata de una especie susceptible y que su control puede resultar de interés para los Miembros en algunas circunstancias con el fin de evitar la propagación de *Marteilia refringens*.

Las secciones pertinentes del Capítulo 2.4.4. *Infección por Marteilia refringens*, del *Manual Acuático* también se modificaron de acuerdo con las recomendaciones del grupo *ad hoc* (ver ítem 7.3.2.).

La Comisión instó a los Miembros a consultar el informe del grupo *ad hoc* de junio de 2022, disponible en el sitio web de la OMSA, y así familiarizarse con las evaluaciones realizadas por el grupo *ad hoc*.

Los Artículos revisados 11.4.1. y 11.4.2. del Capítulo 11.4. *Infección por Marteilia refringens* figuran en el [Anexo 14](#) para comentario.

5.9. Modelo de los Artículos 11.X.9. – 11.X.14. para los capítulos de enfermedades específicas de los moluscos

Contexto

En su reunión de febrero de 2018, la Comisión para los Animales Acuáticos acordó aplicar el modelo de los artículos X.X.8., X.X.9., X.X.10. y X.X.11. a todos los capítulos específicos de enfermedad de los Títulos 8, 9 y 10 del Código Acuático. La Comisión destaca que estos cambios se introducirán en el Título 11 para los capítulos específicos de las enfermedades de los moluscos cuando se modifiquen como resultado de la labor realizada por el Grupo *ad hoc* sobre la susceptibilidad de las especies de moluscos a la infección por enfermedades de la lista de la OIE.

Informes de la Comisión donde se discutió del tema

Reunión de febrero de 2018 (ítem 1.11., página 13).

Reunión de septiembre de 2022

La Comisión observó que las modificaciones aplicadas a los Títulos 8, 9 y 10 no se habían introducido sistemáticamente a los capítulos del Título 11, como acordado anteriormente.

La Comisión modificó el texto del Artículo 11.X.9. por "Artículos 11.X.5., 11.X.6. u 11.X.7. (según corresponda) y 11.X.8.", en aras de armonización con los Artículos X.X.5.-X.X.8. adoptados recientemente. Dada la modificación propuesta, la Comisión acordó que el texto se modificara en todos los capítulos específicos de enfermedad una vez adoptadas las modificaciones propuestas para los Artículos 11.X.9. a 11.X.14.

La Comisión señaló que el Artículo 11.X.13. es un nuevo artículo destinado a armonizarse con otros capítulos específicos de enfermedad en el *Código Acuático* adoptados originalmente en 2018. La Comisión también destacó que el Artículo 11.X.14. incluye información específica sobre las mercancías evaluadas que cumplen con el Artículo 5.4.2. y que están publicadas en la versión 2022 del *Código Acuático*. Las evaluaciones de estas mercancías pueden consultarse en el sitio web de la OMSA en el documento "Evaluaciones de mercancías seguras para las enfermedades de los animales acuáticos de la lista de la OIE". La Comisión informó a los Miembros de que no existe ningún cambio en esta información sobre la enfermedad y, en consecuencia, la modificación se presenta como un artículo modelo con información de la enfermedad en el apartado 1 del artículo que se muestra como [...]. La información pertinente de los actuales capítulos específicos de enfermedad se incluye en el siguiente cuadro para información de los Miembros y se incluirá en los capítulos específicos de enfermedad una vez adoptados y publicados.

Mercancías que cumplen con el Artículo 5.4.2. Criterios para evaluar la seguridad de los productos de animales acuáticos importados (o en tránsito) para la venta directa al por menor para el consumo humano independientemente del estatus sanitario del país, la zona o el comportamiento de exportación con respecto a la enfermedad X

Capítulo	Agente patógeno	Mercancías
11.1.	<i>Herpesvirus de la abalón</i>	a. carne de abalón eviscerado sin concha (refrigerada o congelada).
11.2.	<i>Bonamia exitiosa</i>	a. carne de ostra refrigerada, y b. ostras refrigeradas con media concha.
11.3.	<i>Bonamia ostreae</i>	a. carne de ostra refrigerada, y b. ostras refrigeradas con media concha
11.4.	<i>Marteilia refringens</i>	a. carne de molusco (refrigerada o congelada), y b. ostras con media concha (refrigeradas o congeladas).
11.5.	<i>Perkinsus marinus</i>	a. carne de molusco (refrigerada o congelada), y b. ostras con media concha (refrigeradas o congeladas).
11.6	<i>Perkinsus olseni</i>	a. carne de molusco (refrigerada o congelada), y b. ostras con media concha (refrigeradas o congeladas).
11.7.	<i>Xenohaliotis californiensis</i>	a. abalón eviscerado sin concha (refrigerado o congelado).

Los modelos de Artículos revisados 11.X.9. a 11.X.14. figuran en el [Anexo 15](#) para comentario de los Miembros.

5.10. Cuestionario de los Miembros para la revisión del Capítulo 4.3. Aplicación de la compartimentación

Contexto

En su reunión de febrero de 2022, la Comisión identificó la revisión del Capítulo 4.3. *Aplicación de la compartimentación* como la prioridad en el marco de la revisión progresiva del Título 4 del *Código Acuático*. La Comisión acordó volver a desarrollar el Capítulo 4.3. para centrarse únicamente en la compartimentación, mejorar las orientaciones a los Miembros y en aras de armonización con otros capítulos nuevos y revisados, como el Capítulo 4.1. *Bioseguridad en los establecimientos de acuicultura*. La Comisión observó que el Capítulo 1.4. revisado y los artículos modelo asociados X.X.4. - X.X.8. en los capítulos específicos de enfermedad sobre la declaración de ausencia de enfermedad, incluyen una referencia específica a los requisitos para demostrar y mantener la ausencia de enfermedad a nivel de compartimento. La Comisión acordó que la revisión del Capítulo 4.3. *Aplicación de la compartimentación* era el siguiente paso adecuado.

Reunión de septiembre de 2022

La Comisión convino en que las experiencias de los Miembros en el uso y la implementación de las normas de compartimentación serían una información útil para la revisión del Capítulo 4.3. Por lo tanto, elaboró un breve cuestionario (cinco preguntas) e invitó a los Miembros a responder a estas preguntas. Las respuestas de los Miembros serán consideradas por la Comisión en su reunión de febrero de 2023 mientras continúa su trabajo de revisión del Capítulo 4.3. para satisfacer las necesidades de los Miembros.

El cuestionario dirigido a los Miembros figura en el [Anexo 16](#) para comentario.

6. Ítems para información de los Miembros

6.1. Definiciones del Glosario de “autoridad competente”, “autoridad veterinaria” y “servicios de sanidad de los animales acuáticos”

Contexto

En mayo de 2022, se adoptaron las definiciones revisadas del Glosario de "autoridad competente", "autoridad veterinaria" y "servicios de sanidad de los animales acuáticos". La Comisión acordó que, una vez adoptadas estas definiciones revisadas del Glosario, iniciaría una revisión exhaustiva de su uso en todo el *Código Acuático*.

Informes de la Comisión donde se discutió del tema

Informes de septiembre de 2020 (ítem 4.5.3., página 9); septiembre de 2021 (ítem 5.1.2.2., página 7) y febrero de 2022 (Parte A: ítem 4.1.2.2., página 8).

Reunión de septiembre de 2022

Durante la reunión de las mesas de la Comisión para los Animales Acuáticos y de la Comisión del Código, se acordó que ambas Comisiones coordinarían sus respectivos trabajos para revisar y modificar el uso de las definiciones revisadas en los Códigos, según corresponda.

Las dos Comisiones acordaron intercambiar sus propuestas respectivas antes de sus reuniones de febrero de 2023 y sus propuestas de modificación en sus informes de febrero de 2023.

6.2. Enfermedades emergentes

6.2.1. Infección por el virus del edema de la carpa

Se recibieron comentarios de Japón.

Contexto

En su reunión de febrero de 2020, la Comisión para los Animales Acuáticos revisó la información científica sobre la infección por el virus del edema de la carpa y acordó que esta enfermedad cumplía con la definición de la OIE de "enfermedad emergente", de acuerdo con el Artículo 1.1.4. del Capítulo 1.1. *Notificación de enfermedades y aportación de datos epidemiológicos del Código Acuático*.

En su reunión de septiembre de 2021, la Comisión revisó los comentarios de los Miembros y las últimas evidencias científicas y observó que la infección por el virus del edema de la carpa seguía afectando la producción y causando eventos de mortalidad en las poblaciones silvestres y de cría, sin tener claro por el momento la gravedad de los impactos en la producción.

En su reunión de febrero de 2022, la Comisión revisó la información científica más reciente y destacó que la mortalidad causada por la infección por el virus del edema de la carpa preocupa a científicos y productores de peces ornamentales, con más informes sobre su detección y artículos en la literatura científica publicados cada año. La Comisión también señaló que el genoma del virus del edema de la carpa publicado en 2021 ayudará a promover los estudios epidemiológicos, el análisis filogenético del virus del edema de la carpa y el desarrollo de nuevas pruebas de diagnóstico de esta infección en el futuro. Una vez más, indicó que las nuevas detecciones de la infección por el virus del edema de la carpa deberán notificarse a la OMSA como enfermedad emergente, de conformidad con el artículo 1.1.4. del *Código Acuático*.

Informes de la Comisión donde se discutió del tema

Informes de febrero de 2020 (ítem 7.3.3., página 17); septiembre de 2020 (ítem 6.3., página 17); febrero de 2021 (Parte B: ítem 2.2., página 11); septiembre de 2021 (ítem 5.2.1.1., página 27) y febrero de 2022 (Parte B: ítem 2.2.1.1., página 6).

Reunión de septiembre de 2022

La Comisión revisó las nuevas pruebas científicas de la infección por el virus del edema de la carpa y observó que, desde su reunión de febrero de 2022, se habían notificado nuevos brotes en la región de Asia-Pacífico. La Comisión reconoció que seguía existiendo cierta incertidumbre en cuanto al impacto asociado a esta infección y al alcance de su propagación a nivel mundial, en particular en Europa. Convino en que esta incertidumbre destaca la importancia de que los Miembros notifiquen las nuevas detecciones de infección por el virus del edema de la carpa como enfermedad emergente, con el fin de garantizar el proceso de recopilación de datos epidemiológicos y mantener la sensibilización en torno a la propagación de este patógeno.

Reconoció que la infección por el virus del edema de la carpa sigue cumpliendo la actual definición de "enfermedad emergente" de la OMSA. Una vez más, solicitó a los Miembros aportar toda información pertinente sobre esta infección para que la Comisión pueda decidir si aplica los criterios de inclusión en la lista (Capítulo 1.2.) o si debe dejar de considerarse como enfermedad emergente.

La Comisión recuerda a los Miembros que en el sitio web de la OMSA está disponible una ficha técnica de esta enfermedad: [Enfermedades Animales - OMSA - Organización Mundial de Sanidad Animal \(woah.org\)](#)

6.2.2. Infección por el nodavirus de la mortalidad encubierta (CMNV)

Contexto

Actualmente, como parte de su plan de trabajo, la Comisión para los Animales Acuáticos revisa la información científica disponible sobre enfermedades nuevas o emergentes con vistas a determinar si se justifica alguna acción de su parte. La revisión puede ser iniciada por la Comisión, o a pedido de la sede de la OMSA, sus centros de referencia, las representaciones regionales, los grupos *ad hoc* o los Miembros. En cada reunión de la Comisión, se lleva a cabo el análisis de enfermedades nuevas o emergentes.

Reunión de septiembre de 2022

La Comisión examinó la información disponible sobre el nodavirus de la mortalidad encubierta (CMNV) con la intención de determinar si cumple con la definición actual de "enfermedad emergente" de la OMSA. La Comisión destacó la amplia gama de especies susceptibles y de que el CMNV puede infectar y causar la enfermedad tanto en crustáceos (por ejemplo, camarón de patas blancas (*Penaeus vannamei*) y langostino gigante de río (*Macrobrachium rosenbergii*)) como en peces de aleta corvina amarilla (*Larimichthys crocea*), platija japonesa (*Paralichthys olivaceus*) y pez cebra (*Danio rerio*)).

La Comisión observó que los casos notificados de CMNV indican la presencia de este virus en Asia y que el CMNV (en crustáceos) figuraba desde 2017 en el programa de notificación trimestral de enfermedades de los animales acuáticos de Asia-Pacífico como "enfermedad viral de mortalidad encubierta" de los crustáceos.

Se notificaron casos de mortalidad e impactos en la producción asociados a la infección por CMNV. La presencia de partículas virales en las góndolas indica la posibilidad de una transmisión vertical.

La Comisión convino en que la infección por CMNV se ajusta a la definición de "enfermedad emergente" y acordó que debe notificarse a la OMSA de conformidad con el Artículo 1.1.4. del *Código Acuático*. La Comisión decidió elaborar una ficha técnica de enfermedad para la infección por CMNV, que se publicará en el sitio web de la Organización en los próximos meses.

6.3. Certificación electrónica

La secretaría comunicó a la Comisión de Animales Acuáticos que, aunque la adopción de la certificación electrónica por parte de los Miembros es aún limitada, el uso de los sistemas electrónicos está en aumento. La secretaría presentó a la Comisión las actividades llevadas a cabo por la OMSA, incluido un proyecto del Fondo para la Normalización y el Fomento del Comercio sobre la [certificación electrónica veterinaria](#). El objetivo de este proyecto fue comprender mejor las prácticas implementadas por algunos Miembros de la OMSA, tanto ya desarrolladas como en fase de desarrollo, así como otros trabajos relevantes en organizaciones internacionales sobre certificación electrónica y ventanilla única.

Dada la estrecha armonización entre el trabajo del Codex y el de la OMSA (en relación con los alimentos de origen animal) y que, en la práctica, una certificación única para la exportación puede contener información relevante para la inocuidad alimentaria y la sanidad de los animales acuáticos y que, en 2021, el Codex adoptó las [Directrices del Codex para el diseño, elaboración, expedición y uso de certificados oficiales genéricos \(CXG 38-2011\)](#), relacionadas específicamente con la migración a la certificación sin papel, la OMSA consideró el estudio del desarrollo de una orientación similar a la del Codex.

Se informó a la Comisión de que la OMSA elaboraría modelos de datos (versiones electrónicas) de los modelos de certificados de la OMSA para el comercio internacional, es decir, el Capítulo 5.11. del *Código Acuático*, similares al modelo de datos de referencia del modelo genérico de certificado oficial que figura en las directrices del Codex.

Consciente de que la introducción de la certificación electrónica facilitaría el comercio internacional, minimizando el riesgo de fraude comercial y que las perturbaciones causadas por la pandemia de COVID-19 destacaron las ventajas de este enfoque, la Comisión acordó incluir la actualización del Capítulo 5.2. en su plan de trabajo. La Comisión destacó la importancia de trabajar en estrecha colaboración con la Comisión del Código, ya que el *Código Terrestre* tiene un capítulo similar.

Manual de Pruebas de Diagnóstico para los Animales Acuáticos de la OMSA

7. Textos para comentario de los Miembros

La Comisión para los Animales Acuáticos recordó a los Miembros el inicio del proceso de modificación progresiva del formato de los capítulos específicos de enfermedad del *Manual Acuático* a partir de un nuevo modelo. Dado que los capítulos reformateados y actualizados presentan cambios sustanciales, la Comisión acordó en su reunión de septiembre de 2019 que en sus informes sólo se presentarían versiones limpias de los capítulos, sin cambios aparentes. Los cambios posteriores realizados en estas revisiones iniciales a raíz de los comentarios de los Miembros se indicarán con el estilo habitual (es decir, tachado para las supresiones y doble subrayado para las adiciones).

Se creará un documento comparativo entre la versión adoptada de un capítulo y el nuevo texto propuesto. Este documento no se incluye en el informe de la Comisión, pero estará disponible si se solicita al Departamento de Normas de la OMSA (AAC.Secretariat@WOAH.org).

Al revisar los comentarios de los Miembros y los capítulos recientemente actualizados, la Comisión observó que algunas modificaciones se aplicaban a todos los capítulos. Por lo tanto, aceptó modificar el modelo y aplicar los siguientes cambios a todos los capítulos en revisión:

1. Tabla 4.1. *Métodos de diagnóstico recomendados por la OIE y su nivel de validación para la vigilancia de animales aparentemente sanos y la investigación de animales clínicamente afectados*: a efectos de aclarar que para el diagnóstico confirmatorio, la secuenciación de amplicones siempre sigue a la reacción en cadena de la polimerasa (PCR) convencional, se añadió "PCR convencional seguida de" antes de "secuenciación de amplicones" en la fila correspondiente. Con el fin de dejar claro que la PCR convencional nunca debe utilizarse sin la secuenciación para confirmar un caso, la Sección C *Diagnóstico confirmatorio de un resultado sospechoso de vigilancia o diagnóstico presuntivo* se sombreó en gris para la fila "PCR convencional".
2. Sección 6. *Criterios de diagnóstico confirmativo* añadir el siguiente texto estándar al final del segundo párrafo:

...Se recomienda que todas las muestras que arrojen resultados positivos sospechosos en un país, zona o compartimento libre del agente patógeno se remitan inmediatamente al laboratorio de referencia de la OIE para su confirmación, independientemente de que los signos clínicos estén asociados al caso. Si un laboratorio no tiene la capacidad para realizar las pruebas de diagnóstico necesarias, debe solicitar el asesoramiento del laboratorio de referencia de la OIE correspondiente y, si es necesario, enviar las muestras a ese laboratorio para que las analice.

Al añadir este texto, se suprimirá el siguiente párrafo de las secciones 6.1.2. *Definición de caso confirmado en animales aparentemente sanos* y 6.2.2. *Definición de caso confirmado en animales clínicamente afectados*:

~~Se debe contactar con los laboratorios de referencia para la remisión de muestras cuando los laboratorios analíticos no puedan realizar ninguna de las pruebas recomendadas y se estén realizando pruebas que puedan dar lugar a una notificación a la OIE.~~

3. Sección 6.1. *Animales aparentemente sanos o de estado sanitario desconocido*, sustituir la palabra "geográfica" por la palabra "hidrográfica" en la segunda frase del primer párrafo: La proximidad geográfica hidrográfica o el desplazamiento de animales, productos o equipos de origen animal, etc., desde una población que se sabe que está infectada equivale a un vínculo epidemiológico.

La Comisión revisó el texto de la Parte 2. *Recomendaciones aplicables a enfermedades específicas, Introducción general del Manual Acuático*, adoptado en 2012 y que abarca un enfoque general de la sanidad de los animales acuáticos en materia de gestión, vigilancia y muestreo. La Comisión observó que parte del texto está desactualizado o entra en conflicto con capítulos adoptados recientemente, por ejemplo el Capítulo 1.1.1. *Gestión de los laboratorios veterinarios de diagnóstico*. La Comisión recomendó la supresión de este capítulo del *Manual Acuático* al ya no ser pertinente ni adecuado para su finalidad.

La Comisión invitó a los Miembros a comentar la supresión del capítulo "Parte 2. *Recomendaciones aplicables a enfermedades específicas, Introducción general del Manual Acuático*". No se presenta ningún anexo, ya que el capítulo se suprimiría por completo.

7.1. Sección 2.2. Enfermedades de los crustáceos

7.1.1. Capítulo 2.2.0. Información general (enfermedades de los crustáceos)

Reunión de septiembre de 2022

El Capítulo 2.2.0. *Información general (enfermedades de los crustáceos)* se actualizó tras la consulta con los expertos del laboratorio de referencia para las enfermedades de los crustáceos.

Las principales modificaciones incluyen:

- supresión de los dos párrafos de la Sección A.1. *Evaluación del estatus sanitario de la unidad epidemiológica*, ya que la información carecía de utilidad y no existía un texto similar en el Capítulo 2.3.0. *Información general (enfermedades de los peces)*;
- reorganización de las secciones de la Sección A *Muestreo* en aras de armonización con el Capítulo 2.3.0.;
- actualización en aras de claridad, del texto relativo a la selección de muestras de animales en la Sección A.1.2. *Especificaciones relativas a las poblaciones de crustáceos*;
- actualización en detalle del texto de las Secciones B.5.5. *Uso de técnicas moleculares y basadas en anticuerpos para pruebas confirmatorias y diagnóstico*, B.5.5.3. *Extracción de ácido nucleico* y B.5.5.4. *Preparación de portas para la hibridación in situ*;
- ampliación de la Sección B.6 *Información adicional que debe recogerse* para incluir un texto sobre la historia de las muestras;
- actualización de las referencias.

El Capítulo revisado 2.2.0. *Información General (enfermedades de los crustáceos)* figura en el [Anexo 17](#) para comentario.

7.1.2. Capítulo 2.2.1. Enfermedad de la necrosis hepatopancreática aguda

Se recibieron comentarios de Australia, Canadá, China (Rep. Pop. de), Estados Unidos de América, Noruega, Suiza, Taipéi Chino y la UE.

Contexto

En su reunión de febrero de 2022, la Comisión para los Animales Acuáticos revisó el Capítulo 2.2.1. *Enfermedad de la necrosis hepatopancreática aguda*, actualizado por los expertos del laboratorio de referencia de la OMSA y reformateado a partir del nuevo modelo de capítulo de enfermedad. El capítulo revisado se presentó para comentario de los Miembros en la Parte B el informe de febrero de 2022.

Informes de la Comisión donde se discutió del tema

Informe de febrero de 2022 (Parte B: ítem 3.1.1.1., página 9).

Reunión de septiembre de 2022

El capítulo actual indica que la enfermedad de la necrosis hepatopancreática aguda (AHPND) designa la infección por cepas de *Vibrio parahaemolyticus* (*Vp*_{AHPND}) que contienen un plásmido de ~70 kbp con genes

que codifican homólogos de las toxinas relacionadas con el insecto *Photobacterium Pir*, PirA y PirB. Un Miembro solicitó que se ampliara el ámbito de aplicación del capítulo para tener en cuenta los informes de la AHPND causados por otras especies *Vibrio*. La Comisión, en consulta con los dos laboratorios de referencia de la OMSA, revisará la información publicada sobre las especies no-*Vibrio parahaemolyticus* que se han asociado a la AHPND y presentará sus conclusiones en su reunión de febrero de 2023.

La Comisión acordó suprimir el texto de la Sección 2.2.5. *Reservorios de infección en animales acuáticos* y sustituirlo por "ninguno conocido", ya que el texto se refería a estudios experimentales y no a reservorios de infección confirmados (animales infectados de forma subclínica capaces de transmitir la enfermedad).

En respuesta a una necesidad de aclarar la afirmación en la Sección 2.3.1. *Mortalidad, morbilidad y prevalencia* de que "En las regiones en las que la AHPND es enzoótica en los camarones de piscifactoría, las pruebas indican una prevalencia cercana al 100 %. (Tran et al., 2014)", la Comisión confirmó que la prevalencia aquí designa a las especies de *Vibrio parahaemolyticus* que transportan los genes PirA y Pir B de la necrosis hepatopancreática aguda (AHPND).

La Comisión aceptó la propuesta de trasladar el texto sobre la patología macroscópica de la Sección 2.3.2. *Signos clínicos, incluidos los cambios de comportamiento*, a la Sección 2.3.3. *Patología macroscópica*, y eliminar el texto sobre histopatología y citopatología de la Sección 2.3.3., ya que se repite en la Sección 4.2. *Histopatología y citopatología*.

En la Sección 3.2. *Selección de órganos o tejidos*, la Comisión acordó suprimir el texto relativo a las muestras fecales, ya que se repite en la Sección 3.4. *Muestreo no letal*. La Comisión también acordó suprimir el texto de la Sección 3.5. *Conservación de las muestras para su envío*, ya que se repite en las Secciones 3.5.1. *Muestras para el aislamiento de patógenos*, 3.5.2. *Conservación de muestras para la detección molecular* y 3.5.3. *Muestras para histopatología, inmunohistoquímica o hibridación in situ*.

En la Sección 3.5.2. *Conservación de muestras para la detección molecular* se explica que si el material no puede fijarse, puede congelarse "pero deberá evitarse la congelación y descongelación repetida de las muestras".

En la Tabla 4.1. *Métodos de diagnóstico recomendados por la OIE* y su nivel de validación para la vigilancia de animales aparentemente sanos y la investigación de animales clínicamente afectados, propósito C. *Diagnóstico confirmatorio de un resultado sospechoso de vigilancia o diagnóstico presuntivo*, la Comisión acordó calificar la PCR en tiempo real como "++" para todas las etapas de la vida con el nivel de validación de 1 de tal manera que corresponda con la definición de caso de la Sección 6. *Criterios de diagnóstico confirmativo*, y elevar el nivel de validación de la PCR convencional seguida de la secuenciación de amplicones a 2, al incluirse en la tabla de la Sección 6.3.1. sobre el diagnóstico presuntivo de animales clínicamente afectados. Tras consultar a los expertos de los laboratorios de referencia, la Comisión acordó añadir el método de amplificación isotérmica mediada por bucle (LAMP) a la tabla para el propósito A. *Vigilancia de animales aparentemente sanos*, y el antígeno ELISA para los tres propósitos. También se introdujeron los cambios en la Tabla 4.1. descritos en el ítem 7 del orden del día.

La Comisión rechazó la supresión de la Sección 4.2. *Histopatología y citopatología*, ya que la información es esencial y no figura en otras secciones del capítulo.

En respuesta a la aclaración de una declaración en la Sección 4.4. *Amplificación del ácido nucleico*, sobre el número de aislados en el estudio de validación del método de PCR AP3, la Comisión acordó eliminar esa parte de la frase por causar confusión; los detalles del estudio pueden encontrarse en las referencias. Asimismo, en la Sección 4.4., un Miembro señaló que existen métodos sensibles de PCR anidada y LAMP para la detección de la AHPND y solicitó se incluyeran estos métodos en la Tabla 4.1. o que se explicaran las razones por las que no se recomiendan actualmente. Este Miembro también señaló que la Sección 4.9. *Métodos para la detección basados en anticuerpos o antígenos*, se refiere a una prueba ELISA de antígenos sensibles y específicos que tampoco se incluye en la Tabla 4.1. Tras consultar con los laboratorios de referencia, tanto el LAMP como el Ag-ELISA se añadieron a la Tabla 4.1.

En la Sección 4.4. *Amplificación del ácido nucleico*, la Comisión añadió el texto estándar sobre los controles de la PCR y la extracción de los ácidos nucleicos, junto con las tablas con los cebadores, las sondas y los parámetros de los ciclos para la PCR en tiempo real y convencional para la detección de los genes de la toxina VpAHPND, como se detalla en el ítem 7.4. del orden del día. La inclusión de las tablas estándar permitió suprimir algunos detalles de los protocolos de PCR.

En la Sección 4.4.2. *PCR convencional*, sobre el protocolo para los métodos de PCR AP1 y AP2, la Comisión añadió a la referencia el enlace actualizado correspondiente al protocolo.

En la Sección 5. *Prueba(s) recomendada(s) para la vigilancia a fin de demostrar la ausencia en poblaciones aparentemente sanas*, un Miembro señaló que tanto los métodos de PCR en tiempo real como los de PCR convencional tienen la misma calificación de uso y nivel de validación y propuso que se mencionara la PCR convencional en la Sección 5. Tras consultar con los laboratorios de referencia, se incluyó la PCR convencional en la Sección 5.

La Comisión modificó las definiciones de caso en la Sección 6. *Criterios de diagnóstico corroborativo* en aras de armonización con las modificaciones introducidas en la Tabla 4.1. En la Sección 6 también introdujo los cambios generales descritos en el ítem 7 del orden del día.

El Capítulo revisado 2.2.1. *Enfermedad de la necrosis hepatopancreática aguda* figura en el [Anexo 18](#) para comentario.

7.1.3. Capítulo 2.2.2. Infección por *Aphanomyces astaci* (plaga del cangrejo de río)

Reunión de septiembre de 2022

La Comisión para los Animales Acuáticos revisó el Capítulo 2.2.2. Infección por *Aphanomyces astaci* (plaga del cangrejo de río), actualizado por el experto del laboratorio de referencia de la OMSA y modificado a partir del nuevo modelo de capítulo de enfermedad.

Las principales enmiendas incluyen:

- información actualizada sobre el agente etiológico; debido a que el Grupo *ad hoc* sobre la susceptibilidad de las especies de crustáceos a la infección por enfermedades de la lista de la OIE no ha evaluado las especies susceptibles, el texto actual adoptado en las Secciones 2.2.1. *Especies hospedadoras susceptibles* y 2.2.2. *Especies con evidencia incompleta de susceptibilidad* sigue siendo el mismo;
- secciones actualizadas sobre la enfermedad, la bioseguridad y las estrategias de control de la enfermedad y sobre la selección y la recopilación de muestras, el transporte y la manipulación;
- actualización de la Sección 4. *Métodos de diagnóstico*, incluidos los cambios descritos en el ítem 7 del orden del día y con elementos adicionales en la Tabla 4.1 *Métodos de diagnóstico recomendados por la OIE y su nivel de validación para la vigilancia de animales aparentemente sanos y la investigación de animales clínicamente afectados*;
- supresión de la descripción detallada en la Sección 4.3. *Cultivo celular para el aislamiento*, ya que no se trata de un método de diagnóstico de rutina, sino que se utiliza para la investigación o el mantenimiento de los cultivos;
- actualización de la Sección 4.4. *Amplificación de ácido nucleico*, se añadió el texto estándar, descrito en el ítem 7 del orden del día, relativo a los controles de la prueba PCR y la extracción de ácidos nucleicos, junto con las tablas estándar que indican los cebadores, las sondas y los parámetros de ciclado; la inclusión de las tablas estándar permitió suprimir algunos detalles de los protocolos de la PCR; actualización de la Sección 4.5. *Secuenciación de amplicones* y se añadió el texto estándar (ver ítem 7); y
- definiciones revisadas de caso sospechoso y confirmado en animales aparentemente sanos y clínicamente afectados.

El Capítulo revisado 2.2.2. *Infección por Aphanomyces astaci (plaga del cangrejo de río)* figura en el [Anexo 19](#) para comentario de los Miembros.

7.1.4. Capítulo 2.2.3. Infección por *Hepatobacter penaei* (hepatopancreatitis necrotizante)

Contexto

La Comisión para los Animales Acuáticos revisó el Capítulo 2.2.3. *Infección por Hepatobacter penaei (hepatopancreatitis necrotizante)*, actualizado por los expertos del laboratorio de referencia de la OMSA y reformateado en base al nuevo modelo de capítulo de enfermedad. El capítulo revisado se presentó para comentario de los Miembros en la Parte B del informe de la Comisión de febrero de 2022.

Informe de la Comisión donde se discutió del tema

Informe de febrero de 2022 (Parte B: ítem 3.1.1.2., página 10).

Reunión de septiembre de 2022

La Comisión actualizó la taxonomía en la Sección 1. *Ámbito de aplicación*, para alinearla con el Capítulo 9.3. *Infección por Hepatobacter penaei (hepatopancreatitis necrotizante)* del *Código Acuático* e incluyó la familia Holosporaceae y el orden Rickettsiales con el fin de reflejar las relaciones taxonómicas correctas.

La Comisión acordó transferir el texto sobre la mortalidad de la Sección 2.3.3. *Patología macroscópica* a la Sección 2.3.1. *Mortalidad, morbilidad y prevalencia*. En respuesta a un comentario en el que se solicitaban detalles sobre los reproductores afectados por la mortalidad, la Comisión señaló que la información solicitada figura en las referencias por lo que decidió no incluirla en el capítulo.

La Comisión acordó suprimir el texto de las Secciones 2.3.2. *Signos clínicos, incluidos los cambios de comportamiento* y 2.3.3. *Patología macroscópica*, por no ser relevante para el tema de cada sección.

En la Sección 2.4. *Bioseguridad y estrategias de control de la enfermedad*, la Comisión acordó suprimir la última frase sobre la sensibilidad de *H. penaei* en base a una referencia publicada en 1994.

En la Sección 3.2. *Selección de órganos o tejidos*, la Comisión aclaró que el hepatopáncreas, principal órgano diana, debe seleccionarse de preferencia.

La Comisión actualizó la Sección 3.4. *Muestreo no letal* para actualizar el contenido y mejorar el texto.

En la Sección 3.5.2. *Preservación de las muestras para la detección molecular* se indica que, si el material no puede fijarse, se congela "pero deberá evitarse la congelación y descongelación repetida de las muestras".

En la Tabla 4.1. *Métodos de diagnóstico recomendados por la OIE* y su nivel de validación para la vigilancia de animales aparentemente sanos y la investigación de animales clínicamente afectados, la Comisión cambió la calificación de la PCR convencional de "+++" a "++" para el propósito A *Vigilancia de animales aparentemente sanos* por no tratarse de una prueba adecuada para la vigilancia. Igualmente, se introdujeron los cambios en la Tabla 4.1. descritos en el ítem 7 del orden del día.

En la Sección 4.4. *Amplificación del ácido nucleico*, la Comisión añadió el texto estándar sobre los controles de la PCR y la extracción de los ácidos nucleicos, junto con las tablas que indican los cebadores, las sondas y los parámetros de ciclado para la PCR en tiempo real y convencional para la detección de *H. penaei*, como se detalla en el ítem 7.4. del orden del día. La inclusión de las tablas estándar permitió suprimir algunos detalles de los protocolos de PCR. Igualmente, se insertaron los cambios en la Tabla 4.1. descritos en el ítem 7 del orden del día.

En la Sección 4.5. *Secuenciación de amplicones* se añadió el texto de las normas sobre la secuenciación de amplicones para sustituir el texto existente.

La Comisión no aceptó incluir la prueba PCR convencional junto con la PCR en tiempo real en la Sección 5. *Prueba(s) recomendada(s) para la vigilancia a fin de demostrar la ausencia de la enfermedad en poblaciones aparentemente sanas*, porque la PCR convencional no es lo ideal para la vigilancia. La Comisión corrigió la clasificación de la PCR convencional en la Tabla 4.1, como mencionado anteriormente.

La Comisión introdujo los cambios genéricos descritos en el ítem 7 del orden del día en la Sección 6 *Criterios de diagnóstico confirmativo*.

El Capítulo revisado 2.2.3. Infección por *Hepatobacter penaei* (hepatopancreatitis necrotizante) figura en el Anexo 20 para comentario.

7.1.5. Capítulo 2.2.4. Infección por el virus de la necrosis hipodérmica y hematopoyética

Se recibieron comentarios de Australia, China (Rep. Pop. de), Estados Unidos de América, Noruega, Suiza, y la UE.

Contexto

La Comisión para los Animales Acuáticos revisó el Capítulo 2.2.4. *Infección por el virus de la necrosis hipodérmica y hematopoyética*, actualizado por los expertos del laboratorio de referencia de la OMSA y

reformateado en base al nuevo modelo de capítulo de enfermedad. El capítulo revisado se presentó para comentario de los Miembros en la Parte B del informe de la Comisión de febrero de 2022.

Informe de la Comisión donde se discutió del tema

Informe de febrero de 2022 (Parte B: ítem 3.1.1.3., página 10).

Reunión de septiembre de 2022

Se actualizó la taxonomía en la Sección 1. *Ámbito de aplicación* para alinearla con el Capítulo 9.4. *Infección por el virus de la necrosis hipodérmica y hematopoyética infecciosa del Código Acuático*.

En la Sección 2.1.1. *Agente etiológico*, la Comisión añadió una referencia que verifica que dos genotipos distintos del virus de la necrosis hipodérmica y hematopoyética (Tipo 1 y Tipo 2) demostraron ser infecciosos para *Penaeus vannamei* y *P. monodon*. La Comisión también corrigió la afirmación de que el Tipo 1 ha sido identificado en Asia sudoriental y no en Asia oriental.

En la Sección 2.2.2. *Especies con evidencia incompleta de susceptibilidad*, la Comisión aceptó borrar la frase “~~Se carece de pruebas para esta especie que confirmen que la identidad del agente patógeno es el virus de la necrosis hipodérmica y hematopoyética, a través de la exposición experimental que reproduce las vías naturas de transmisión o que la presencia del agente patógeno constituye una infección~~” se había dejado de una versión anterior del capítulo y ya no se ajustaba al modelo. También en la Sección 2.2.2., la Comisión no aceptó suprimir la introducción del segundo párrafo explicando que se habían notificado resultados positivos de PCR específicos para el patógeno en los siguientes organismos, pero que no se había demostrado una infección activa. La Comisión considera que se trata de un texto explicativo importante que refleja el trabajo del Grupo *ad hoc* sobre la susceptibilidad de las especies de crustáceos a la infección por las enfermedades de la lista de la OIE y está incluida en el modelo.

La Comisión aceptó desplazar texto de la Sección 2.3.3. *Patología macroscópica* a la Sección 2.3.1. *Mortalidad, morbilidad y prevalencia*, y desplazar el tercer párrafo de la Sección 2.3.1. a la Sección 2.3.2. puesto que el texto se ajusta mejor a dichas secciones. En la Sección 2.3.1., añadió texto y una referencia a la ausencia de signos clínicos y de mortalidad en los animales infectados experimentalmente con genotipos del virus de la necrosis hipodérmica y hematopoyética infecciosa que son un linaje separado de genotipos del tipo II del virus de la necrosis hipodérmica y hematopoyética infecciosa que circulan en Ecuador y Perú. Finalmente, amplió el texto de la Sección 2.3.2. con fines de aclarar algunos signos clínicos y comportamentales causados por la infección por el virus de la necrosis hipodérmica y hematopoyética.

En la Tabla 4.1. *Métodos de diagnóstico recomendados por la OIE y su nivel de validación para la vigilancia de animales aparentemente sanos y la investigación de animales clínicamente afectados*, la Comisión suprimió las calificaciones de la histopatología para el propósito C *Diagnóstico confirmatorio de un resultado sospechoso de vigilancia o diagnóstico presuntivo* porque no es suficientemente específico para la confirmación de un caso. Se introdujeron igualmente los cambios en la Tabla 4.1. descritos en el ítem 7 del orden del día.

En la Sección 4.4. *Amplificación del ácido nucleico*, la Comisión añadió el texto estándar sobre los controles de la prueba PCR y la extracción de los ácidos nucleicos, junto con las tablas que indican los cebadores, las sondas y los parámetros de ciclado para la PCR en tiempo real y convencional para la detección del virus de la necrosis hipodérmica y hematopoyética infecciosa, como se detalla en el ítem 7.4. del orden del día. La inclusión de las tablas estándar permitió suprimir algunos detalles de los protocolos de la PCR.

En la Sección 4.5. se añadió el texto estándar sobre la secuenciación de amplicones para sustituir el texto existente (ver ítem 7.4.).

La Comisión introdujo los cambios genéricos descritos en el ítem 7 del orden del día en la Sección 6. *Criterios de diagnóstico confirmativo*.

La Comisión armonizó el texto de las Secciones 6.1.2. *Definición de caso confirmado en animales aparentemente sanos*, y 6.2.2. *Definición de caso confirmado en animales clínicamente afectados* para alinearlos con los demás capítulos y eliminó la histopatología por no ser lo suficientemente específica para la confirmación de un caso.

El Capítulo revisado 2.2.4. *Infección por el virus de la necrosis hipodérmica y hematopoyética infecciosa* figura en el [Anexo 21](#) para comentario de los Miembros.

7.1.6. Capítulo 2.2.5. Infección por el virus de la mionecrosis infecciosa

Reunión de septiembre de 2022

La Comisión revisó el Capítulo 2.2.5. *Infección por el virus de la mionecrosis infecciosa*. Sabiendo que actualmente no existe un laboratorio de referencia de la OMSA para esta enfermedad, el capítulo se reformuló utilizando el nuevo modelo de capítulo sobre enfermedades y fue revisado por la Comisión.

Las principales enmiendas incluyen:

- información actualizada sobre la taxonomía en el ámbito de aplicación del capítulo y sobre el agente etiológico;
- secciones actualizadas sobre vectores, mortalidad, morbilidad y prevalencia, distribución geográfica y quimioterapia, incluidos los agentes bloqueadores;
- actualización de la sección sobre los métodos de diagnóstico: se completó la Tabla 4.1.; se revisó la sección sobre el cultivo de células para el aislamiento, la amplificación de ácidos nucleicos y las pruebas moleculares, se añadió el texto estándar sobre los controles de la PCR, la extracción de ácidos nucleicos y la secuenciación de amplicones; se sustituyeron los protocolos de las pruebas por las nuevas tablas de secuencias de cebadores, sondas y parámetros de ciclado;
- definiciones revisadas de caso sospechoso y confirmado en animales aparentemente sanos y clínicamente afectados; y
- se completó la tabla en la sección de sensibilidad y especificidad diagnóstica para las pruebas de diagnóstico presuntivo de los animales clínicamente afectados.

El Capítulo revisado 2.2.5. *Infección por el virus de la mionecrosis infecciosa* figura en el [Anexo 22](#) para comentario de los Miembros.

7.1.7. Capítulo 2.2.7. Infección por el virus del síndrome de Taura

Reunión de septiembre de 2022

La Comisión para los Animales Acuáticos revisó el Capítulo 2.2.7. *Infección por el virus del síndrome de Taura* actualizado por el experto del laboratorio de referencia de la OMSA y reformulado utilizando el nuevo modelo de capítulo de enfermedades.

Las principales enmiendas incluyen:

- información actualizada sobre la distribución del patógeno en el hospedador, la distribución geográfica, el cultivo de cepas resistentes, el muestreo no letal;
- actualización de la sección sobre los métodos de diagnóstico: se completó la Tabla 4.1. y se revisó la sección sobre el bioensayo, la amplificación de los ácidos nucleicos y las pruebas moleculares, se añadió el texto estándar sobre los controles de la PCR, la extracción de los ácidos nucleicos y la secuenciación de los amplicones y se sustituyeron los protocolos de las pruebas por las nuevas tablas que indican las secuencias de los cebadores y las sondas y los parámetros de ciclado;
- definiciones revisadas de caso sospechoso y confirmado en animales aparentemente sanos y clínicamente afectados; y
- se actualizaron las referencias.

El Capítulo revisado 2.2.7. *Infección por el virus del síndrome de Taura* figura en el [Anexo 23](#) para comentario de los Miembros.

7.1.8. Capítulo 2.2.8. Infección por el virus del síndrome de las manchas blancas

Reunión de septiembre de 2022

La Comisión para los Animales Acuáticos revisó el Capítulo 2.2.8. *Infección por el virus del síndrome de las manchas blancas*, actualizado por los expertos del laboratorio de referencia de la OMSA y en base al nuevo modelo de capítulo sobre enfermedades.

Las principales enmiendas incluyen:

- información actualizada sobre el agente etiológico;
- secciones actualizadas sobre supervivencia y estabilidad de las muestras procesadas o almacenadas, reservorios de animales acuáticos de infección, vectores, distribución geográfica, métodos de inactivación;
- actualización de la sección sobre los métodos de diagnóstico: se completó la Tabla 4.1. y se revisó la sección sobre la amplificación de ácidos nucleicos y pruebas moleculares, se añadió el texto estándar sobre los controles de la PCR, la extracción de ácidos nucleicos y la secuenciación de amplicones y se sustituyeron los protocolos de las pruebas por las nuevas tablas que indican las secuencias de los cebadores, las sondas y los parámetros de ciclado;
- definiciones revisadas de caso sospechoso y confirmado en animales aparentemente sanos y clínicamente afectados;
- se completaron las tablas de la sección sobre sensibilidad y especificidad diagnóstica de las pruebas diagnósticas; y
- se actualizaron las referencias.

La Comisión observó que las evaluaciones completadas por el Grupo *ad hoc* sobre la susceptibilidad de las especies de crustáceos a la infección por las enfermedades de la lista de la OIE sobre la infección por el virus del síndrome de las manchas blancas en junio de 2016 no se habían aplicado antes puesto que todavía no se había adoptado el Artículo 1.5.9. del Capítulo 1.5. *Criterios para la inclusión de especies susceptibles de infección por un agente patógeno específico*. La Comisión volverá a revisar dichas evaluaciones en el marco de la aplicación del Artículo 1.5.9. La Comisión señaló que el texto actual adoptado permanecerá en la Sección 2.2.1. hasta que se complete la revisión.

La Comisión instó a los Miembros a que consulten el informe de junio de 2016, disponible en el sitio web de la OMSA, para que conozcan en detalle las evaluaciones realizadas por el grupo *ad hoc*.

El Capítulo revisado 2.2.8. *Infección por el virus del síndrome de las manchas blancas* figura en el [Anexo 24](#) para comentario de los Miembros.

7.2. Sección 2.3. Enfermedades de los peces

7.2.1. Capítulo 2.3.1. Infección por *Aphanomyces invadans* (síndrome ulcerante epizoótico)

Se recibieron comentarios de China (Rep. Pop. de), Estados Unidos de América, Tailandia y la UE.

Contexto

En su reunión de febrero de 2022, la Comisión para los Animales Acuáticos revisó el Capítulo 2.3.1. *Infección por Aphanomyces invadans (síndrome ulcerante epizoótico)*, que los expertos del laboratorio de referencia de la OMSA había actualizado y reformateado en base al nuevo modelo de capítulo sobre enfermedades. El capítulo revisado se presentó para comentario de los Miembros en la Parte B el informe de la Comisión de febrero de 2022.

Informes de la Comisión donde se discutió del tema

Informe de febrero de 2022 (Parte B: ítem 3.1.2.1., página 10).

Reunión de septiembre de 2022

La Comisión acordó suprimir la palabra "hongo" después de "oomiceto" en la Sección 1. *Ámbito de aplicación*, ya que los Oomicetos se separaron de los hongos y se incorporaron al reino de los protozoos.

La Comisión inscribió un comentario relativo a los nombres comunes que figuran en las tablas de la Sección 2.2.1. *Especies hospedadoras susceptibles*, ya que esta sección todavía está sujeta a la revisión del Grupo *ad hoc* sobre la susceptibilidad de las especies de peces a la infección por enfermedades de la lista de la OIE. Una vez que el grupo *ad hoc* finalice la lista, los nombres comunes se cotejarán con la base de datos de la FAO.

En la Sección 3.6. *Agrupación de muestras*, la Comisión modificó el texto añadiendo una frase en la que se afirma que no se ha evaluado el efecto de la agrupación en la especificidad del diagnóstico y se recomienda que los animales más grandes se procesen y analicen en forma individual; el texto se ajusta al modelo y, por tanto, es coherente con otros capítulos.

En la Tabla 4.1. *Métodos de diagnóstico recomendados por la OIE y su nivel de validación para la vigilancia de animales aparentemente sanos y la investigación de animales clínicamente afectados*, la Comisión añadió una nueva línea para los signos clínicos y los calificó para los tres fines. La Comisión también añadió calificaciones para los soportes de preparación en el propósito C. *Diagnóstico confirmatorio de un resultado sospechoso de vigilancia o diagnóstico presuntivo*. También se introdujeron los cambios en la Tabla 4.1. descritos en el ítem 7 del orden del día.

En la Sección 4.4. *Amplificación del ácido nucleico*, la Comisión añadió el texto estándar sobre los controles de la prueba PCR y la extracción de ácidos nucleicos, junto con las tablas que indican los cebadores, las sondas y los parámetros de ciclado para la PCR en tiempo real y convencional para la detección de *A. invadans* en los tejidos de los peces, como se detalla en el ítem 7.4. del orden del día. La inclusión de las tablas estándar permitió suprimir algunos detalles de los protocolos de PCR.

En la Sección 6. *Criterios de diagnóstico corroborativo*, la Comisión rechazó la inclusión del texto estándar sobre el envío de muestras sospechosas al laboratorio de referencia de la OMSA, ya que actualmente no existe tal laboratorio designado. Se volverá a incluir el texto si, en el futuro, se restablece un laboratorio de referencia de la OMSA para esta enfermedad.

La Comisión rechazó la supresión de los signos clínicos compatibles con la infección por *A. invadans* de la Sección 6.1.1. *Definición de caso sospechoso en poblaciones aparentemente sanas*, ya que la detección de signos clínicos es una prueba recomendada para la vigilancia de poblaciones sanas, como se indica en la Sección 5. *Prueba(s) recomendada(s) para la vigilancia a fin de demostrar la ausencia de la enfermedad en poblaciones aparentemente sanas*. Además, los signos clínicos observados pueden no ser patognomónicos y, por lo tanto, cumplir los criterios de un caso sospechoso. La Comisión añadió una nota de pie de página a este criterio en la que se aclara que la vigilancia de poblaciones aparentemente sanas para el síndrome ulcerante epizoótico se basa en el examen de poblaciones objetivo con miras a detectar signos clínicos de infección por *A. invadans*.

En la Sección 6.2.1 *Definición de caso sospechoso en animales clínicamente afectados*, la Comisión acordó aclarar que la observación visual es de hifas características de *A. invadans* y que el cultivo y aislamiento es de colonias tipo *A. invadans* en los criterios iv y v.

El Capítulo revisado 2.3.1. *Infección por Aphanomyces invadans (síndrome ulcerante epizoótico)* figura en el [Anexo 25](#) para comentario.

7.2.2. Capítulo 2.3.2. Infección por el virus de la necrosis hematopoyética epizoótica

Se recibieron comentarios de Estados Unidos de América, Noruega, Suiza y la UE.

Contexto

En su reunión de septiembre de 2021, la Comisión para los Animales Acuáticos revisó el Capítulo 2.3.2. *Infección por el virus de la necrosis hematopoyética epizoótica*, actualizado por los expertos del laboratorio de referencia de la OMSA y reformateado en base al nuevo modelo de capítulo de enfermedad. El capítulo revisado también se presentó para comentario de los Miembros en la Parte B del informe de la Comisión de febrero de 2022.

Informes de la Comisión donde se discutió del tema

Informes de septiembre de 2021 (ítem 6.1.3., página 31), de febrero de 2022 (Parte B: ítem 3.1.2.2., página 11).

Reunión de septiembre de 2022

En la Sección 3.6. *Agrupación de muestras*, la Comisión rechazó la supresión de dos frases sobre los procedimientos de agrupación que deben seguirse cuando no se ha evaluado en detalle el efecto de la agrupación según la sensibilidad diagnóstica. La Comisión observó que ambas frases forman parte del texto estándar de esta sección, tal y como se recoge en el nuevo modelo, y están destinadas a apoyar las recomendaciones basadas en la evidencia. Sin embargo, acordó suprimir la última frase que recomienda utilizar

un máximo de cinco peces para agrupar órganos, puesto que procede de una versión anterior del capítulo y ya no se recomienda.

En la Tabla 4.1. *Métodos de diagnóstico recomendados por la OIE y su nivel de validación para la vigilancia de animales aparentemente sanos y la investigación de animales clínicamente afectados*, la Comisión aclaró que es esencial indicar las calificaciones para el cultivo celular, la PCR en tiempo real y la PCR convencional, aunque la secuenciación de amplicones sea necesaria para el diagnóstico confirmatorio. Además, la Comisión redujo el nivel de validación de "2" a "1" para el cultivo celular para los tres fines y la PCR en tiempo real para el propósito A. *Vigilancia de animales aparentemente sanos y C. Diagnóstico confirmatorio de un resultado sospechoso de la vigilancia o el diagnóstico presuntivo*, dado que no se ha publicado la sensibilidad y especificidad del diagnóstico ni para los animales clínicamente enfermos ni para los aparentemente sanos. Se introdujeron igualmente los cambios en la Tabla 4.1. descritos en el ítem 7 del orden del día.

En la Sección 4.3.2. *Cultivo celular*, la Comisión, en consulta con el experto del laboratorio de referencia, acordó suprimir la inmunotinción, la prueba ELISA y la inmunomicroscopía electrónica de la última frase y revisó el texto para aclarar que la identidad de los virus en el cultivo celular se determina por "PCR y secuenciación de amplicones".

En la Sección 4.4. *Amplificación del ácido nucleico*, la Comisión, en consulta con el experto del laboratorio de referencia, enmendó la primera frase para indicar que, aunque se hayan descrito varios métodos convencionales de PCR o PCR cuantitativa en tiempo real para la detección de ranavirus, el virus de la necrosis hematopoyética epizoótica sólo puede detectarse cuando estos métodos se combinan con métodos que detectan específicamente el virus de la necrosis hematopoyética epizoótica. La Comisión también acordó suprimir una frase que hace referencia a un método de PCR que no se describe en el capítulo. Por último, la Comisión añadió el texto estándar sobre los controles de la PCR y la extracción de ácidos nucleicos, junto con las tablas que indican los cebadores, las sondas y los parámetros de ciclado para la PCR en tiempo real y convencional para la detección del virus de la necrosis hematopoyética epizoótica, como se detalla en el ítem 7.4. del orden del día. La inclusión de las tablas estándar permitió suprimir algunos detalles de los protocolos de PCR.

En la Sección 4.5. *Secuenciación de amplicones* se añadió el texto estándar para sustituir el texto existente (ver ítem 7.4. del orden del día).

En la Sección 5. *Prueba(s) recomendada(s) para la vigilancia a fin de demostrar la ausencia de la enfermedad en poblaciones aparentemente sanas*, la Comisión acordó aclarar que las muestras positivas a la PCR en tiempo real deben ser sometidas a la prueba PCR convencional y al análisis de la secuencia para distinguir el "virus de la necrosis hematopoyética epizoótica de otros" ranavirus.

La Comisión introdujo los cambios genéricos escritos en el ítem 7 del orden del día en la Sección 6. *Criterios de diagnóstico confirmativo*.

En la Sección 6.1.1. *Definición de caso sospechoso en animales aparentemente sanos*, la Comisión sustituyó el primer criterio "*Positive result for EHNV based on virus isolation in cell cultures*" por "*EHNV-typical CPE in cell culture*", más apropiado para alinearse con la Tabla 4.1.

El Capítulo revisado 2.3.2. *Infección por el virus de la necrosis hematopoyética epizoótica* figura en el [Anexo 26](#) para comentario de los Miembros.

7.2.3. Sección 2.2.1. del Capítulo 2.3.9. Infección por el virus de la viremia primaveral de la carpa

Reunión de septiembre de 2022

La Comisión para los Animales Acuáticos acordó incluir la carpa de Jinsha (*Percocypris pingi*) en la lista de especies susceptibles de la Sección 2.2.1. del Capítulo 2.3.9. *Infección por el virus de la viremia primaveral de la carpa* (ver ítem 5.5.).

El Grupo *ad hoc* de evaluación de la carpa de Jinsha (*Percocypris pingi*) figura en el [Anexo 10](#) para información de los Miembros.

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 27](#) para comentario de los Miembros.

7.3. Sección 2.4. Enfermedades de los moluscos

7.3.1. Secciones 2.2.1 y 2.2.2. del Capítulo 2.4.2. Infección por *Bonamia exitiosa* y Secciones 2.2.1 y 2.2.2. del Capítulo 2.4.3. Infección por *Bonamia ostreae*

Reunión de septiembre de 2022

La Comisión para los Animales Acuáticos modificó las Secciones 2.2.1 y 2.2.2 del Capítulo 2.4.2. *Infección por Bonamia exitiosa*, y el Capítulo 2.4.3. *Infección por Bonamia ostreae*, con respecto a la taxonomía de la ostra de Suminoe y la ostra de copa del Pacífico (ver ítem 5.8.).

Las Secciones enmendadas 2.2.1. y 2.2.2. del Capítulo 2.4.2. *Infección por Bonamia exitiosa* figuran en el [Anexo 28](#) para comentario de los Miembros.

Las Secciones enmendadas 2.2.1. y 2.2.2. del Capítulo 2.4.3. *Infección por Bonamia ostreae* figuran en el [Anexo 29](#) para comentario de los Miembros.

7.3.2. Secciones 2.2.1. y 2.2.2. del Capítulo 2.4.4. Infección por *Marteilia refringens*

Reunión de septiembre de 2022

La Comisión para los Animales Acuáticos modificó las Secciones 2.2.1 y 2.2.2 del Capítulo 2.4.4, *Infección por Marteilia refringens*, de acuerdo con las recomendaciones del Grupo *ad hoc* sobre la susceptibilidad de las especies de moluscos a la infección por enfermedades de la lista de la OIE (ver ítem 5.8.).

La Comisión rechazó la recomendación del Grupo *ad hoc* de añadir un copépodo (*Paracartia grani*) en el Artículo 11.4.2. del Código Acuático (ver ítem 5.8.) Sin embargo, la Comisión aceptó añadir un nuevo párrafo "Además, se comprobó que una especie de copépodo (*Paracartia grani*) cumple los criterios de inclusión en la lista como especie susceptible a la infección por *Marteilia refringens* y se considera un huésped intermedio" en la Sección 2.2.1. en aras de reflejar la situación única con el riesgo asociado a un hospedador intermedio. La Comisión consideró que la susceptibilidad de *Paracartia grani* puede ser pertinente para los Miembros en algunas circunstancias para evitar la propagación de *Marteilia refringens*.

Las Secciones enmendadas 2.2.1. y 2.2.2. del Capítulo 2.4.4. *Infección por Marteilia refringens* figuran en el [Anexo 30](#) para comentario.

8. Ítems para información de los miembros

8.1. Tabla propuesta de los parámetros de PCR para armonización de los protocolos de las pruebas PCR

Contexto

En su reunión de febrero de 2022, la Comisión para los Animales Acuáticos observó que el proceso de revisión de los capítulos actualizados y reformateados había puesto de manifiesto la amplia variación entre los capítulos en el nivel de detalle dado en la descripción de los métodos de PCR en la Sección 4.4. *Amplificación del ácido nucleico*, y su presentación. La Comisión decidió tratar esta cuestión a través de la elaboración de un modelo para la descripción de los métodos de PCR, que incluirá un texto conciso, uniforme y genérico sobre los métodos de extracción de ácido nucleico y los controles utilizados en las pruebas, al tiempo que ofrecerá toda la información necesaria sobre las secuencias de cebadores y sondas y los parámetros de ciclado presentada en una tabla.

Informes de la Comisión donde se discutió del tema

Informe de febrero de 2022 (Parte B: ítem 3.1, página 9).

Reunión de septiembre de 2022

La Comisión revisó y aprobó la tabla propuesta de secuencias de los cebadores y sondas y los parámetros de ciclado, así como el texto sobre los controles, la extracción de ácido nucleico y la secuenciación de amplicones. La tabla y el texto, que figuran a continuación, se añadirán al modelo y a todos los capítulos en revisión para que la información crítica sobre los métodos de PCR se presente de manera uniforme en todos los capítulos del *Manual Acuático*. La tabla sustituirá a los textos existentes sobre los protocolos de PCR.

Cebadores, sondas (secuencia) y parámetros de ciclado (solo ejemplos)

Patógeno / gen diana	Cebador/sonda (5'-3')	Concentración	Parámetros de ciclado
Referencia: GenBank Accession No., Product size [bp]*			
X	Fwd: TGC-GTC-CTG-CGT-ATG-GCA-CC Rev: GGC-TGG-CAT-GCC-CGA-ATA-GCA Sonda: GGC-TGG-CAT-GCC-CGA-ATA-GCA	400 nM 300 nM	50 ciclos de: 95°C/15 seg y 58°C/60 seg

*Solo para PCR convencional .

1. Sección 4.4. Amplificación del ácido nucleico

Las pruebas PCR siempre deberán realizarse según los controles especificados en la Sección X.X *Uso de técnicas moleculares y basadas en anticuerpos para pruebas confirmatorias del diagnóstico* del Capítulo 2.X.0 *Información general* (enfermedades de los crustáceos o peces, según corresponda) [Sección 2.6 *Métodos moleculares* del Capítulo 2.4.0 *Información general* (enfermedades de los moluscos)]. Cada modelo deberá analizarse por duplicado.

2. Sección 4.4. Amplificación del ácido nucleico

Extracción de ácidos nucleicos

Se pueden utilizar numerosos kits y procedimientos diferentes para la extracción de ácido nucleico. La calidad y la concentración del ácido nucleico extraído deben comprobarse mediante la densidad óptica o con gel.

3. Sección 4.5. Secuenciación de amplicones

El tamaño del amplicón de la prueba PCR se verifica mediante electroforesis en gel de agarosa y se purifica por escisión del mismo. Ambas cadenas de ADN deben ser secuenciadas, analizadas y comparadas con las secuencias publicadas.

8.2. Capítulo 2.3.7. Infección por el iridovirus de la dorada japonesa

Se recibieron comentarios de China, Estados Unidos de América, Taipéi Chino, Noruega, Suiza, y la UE.

Contexto

En su reunión de febrero de 2022, la Comisión para los Animales Acuáticos revisó el Capítulo 2.3.7. *Infección por el iridovirus de la dorada japonesa*, actualizado por los expertos del laboratorio de referencia de la OMSA y reformulado utilizando el nuevo modelo de capítulo sobre enfermedades. El capítulo revisado se presentó para comentario de los Miembros en la Parte B el informe de la Comisión de febrero de 2022.

Informes de la Comisión donde se discutió del tema

Informe de febrero de 2022 (Parte B: ítem 3.1.2.3. página 13).

Reunión de septiembre de 2022

La Comisión examinó el informe del Grupo *ad hoc* sobre la susceptibilidad de las especies de peces a la infección por enfermedades de la lista de la OIE para la infección por el iridovirus de la dorada japonesa y convino en que debía completarse una evaluación para la inclusión en la lista de la infección por el virus de la necrosis renal y del bazo, incluidos sus tres genogrupos, el iridovirus de la dorada japonesa, el virus de la necrosis renal y del bazo y el iridovirus del cuerpo rojizo del rodaballo (ver ítem 5.1.). La Comisión acordó aplazar la revisión de los comentarios recibidos sobre el Capítulo 2.3.7. hasta que se pudieran revisar los comentarios sobre la propuesta de inclusión de la infección por el virus de la necrosis renal y del bazo.

8.3. Desarrollo de un mecanismo para acelerar el proceso de presentación a los Miembros de las actualizaciones de los métodos de diagnóstico del Manual Acuático

La Comisión identificó dos situaciones relacionadas con la difusión oportuna de toda nueva información de importancia en el marco de las pruebas de diagnóstico en el *Manual Acuático*. En primer lugar, se plantea qué se ha de hacer cuando surgen problemas sobre el funcionamiento de una prueba que haya sido adoptada y que está

incluida en el *Manual Acuático*. La Comisión convino en que, en tales circunstancias, se puede añadir una nota a pie de página en la que se detalle la naturaleza del problema y se brinden instrucciones al respecto. Como la nota a pie de página no sustituye ni modifica ningún texto adoptado, se podría añadir inmediatamente al capítulo correspondiente. Esta situación ya se presentó anteriormente cuando surgieron problemas de especificidad. Además, la Comisión solicitará un asesoramiento a los laboratorios de referencia y a los puntos focales para los animales acuáticos.

En segundo lugar, se trata de la inclusión de nuevas pruebas de diagnóstico en el *Manual Acuático*. En la actualidad, las pruebas deben ser publicadas en una revista revisada por pares y, preferentemente, ser validadas al menos en el nivel 2 de la vía de validación de la OMSA. Se informó a la Comisión del trabajo de la Comisión de Normas Biológicas para elaborar un modelo de los datos de validación que se pedirían a los solicitantes que deseen incluir sus pruebas en el *Manual Terrestre* (ver ítem 5.2.2. del orden del día del informe de la reunión de la Comisión de Normas Biológicas, septiembre de 2022). Se designó a un miembro de la Comisión para los Animales Acuáticos para que revise el modelo e informe en la reunión de febrero de 2023 sobre su idoneidad y aplicabilidad al *Manual Acuático*. Se mantiene el requisito de que las pruebas se publiquen en la literatura científica, pero en una situación de urgencia, los desarrolladores de pruebas podrían presentar los datos en el modelo de validación como medida provisional, permitiendo posiblemente que la prueba se incluya en el *Manual Acuático* antes de su publicación en una revista revisada por pares.

9. Grupos *ad hoc*

9.1. Grupo *ad hoc* sobre la susceptibilidad de las especies de moluscos a la infección por las enfermedades de la lista de la OIE

El Grupo *ad hoc* sobre la susceptibilidad de las especies de moluscos a la infección por las enfermedades de la lista de la OIE se reunió en junio de 2022, con el fin de evaluar la susceptibilidad de las especies de moluscos a la infección por *Marteilia refringens* (ver ítems 5.8. y 7.3.2.).

Se informó a la Comisión de que el Grupo *ad hoc* tiene previsto volver a encontrarse en noviembre de 2022 para avanzar en su trabajo de evaluación de las especies susceptibles a la infección por *Perkinsus marinus*.

El informe del Grupo *ad hoc* sobre la susceptibilidad de las especies de moluscos a la infección por enfermedades de la lista de la OIE (junio de 2022) puede consultarse en el sitio web de la OMSA.

9.2. Grupo *ad hoc* sobre la susceptibilidad de las especies de peces a la infección por las enfermedades de la lista de la OIE

El Grupo *ad hoc* sobre la susceptibilidad de las especies de peces a la infección por las enfermedades de la lista de la OIE se reunió durante el mes de abril de 2022 con el fin de evaluar la susceptibilidad de las especies de peces a la infección por el iridovirus de la dorada japonesa.

La Comisión para los Animales Acuáticos tomó nota de que el grupo *ad hoc* no había completado sus evaluaciones del iridovirus de la dorada japonesa debido a la complejidad asociada al agente patógeno. La Comisión examinó el informe provisional del grupo *ad hoc*, en el que se describe el trabajo realizado hasta la fecha, y aportó sus comentarios. El grupo *ad hoc* tiene previsto reunirse de nuevo en noviembre de 2022 para finalizar las evaluaciones de las especies susceptibles al iridovirus de la dorada japonesa.

9.3. Grupo *ad hoc* sobre los nuevos proyectos de Capítulos 4.X. Preparación de emergencia frente a las enfermedades y 4.Y. Gestión de los brotes de enfermedad

La Comisión para los Animales Acuáticos debatió la labor del Grupo *ad hoc* sobre la preparación de emergencia frente a las enfermedades y la gestión de los brotes de enfermedades. La Comisión agradeció a los miembros por su trabajo sobre los proyectos de capítulos y consideró que el trabajo ya se había finalizado y que la Comisión podía avanzar en los dos capítulos. La Comisión continuará con la elaboración de los nuevos proyectos de capítulos 4.X. *Preparación de emergencia frente a las enfermedades* y 4.Y. *Gestión de brotes de enfermedades*, para mayor examen en su reunión de febrero de 2023.

10. Centros de referencia de la OMSA o cambio de expertos

10.1. Evaluación de las solicitudes para la designación como centro de referencia de la OIE en temas de sanidad de los animales acuáticos o cambio de expertos

La Comisión para los Animales Acuáticos revisó las solicitudes para los cambios de expertos y recomendó se aceptaran los que siguen:

Infección por septicemia hemorrágica viral

La Dra. Britt Bang Jensen reemplaza al Dr. Niels Jørgen Olesen, que se jubiló del Technical University of Denmark National Institute for Aquatic Resources, Lyngby, Dinamarca.

10.2. Llamado a candidaturas para la designación de un laboratorio de referencia de la OMSA

La Comisión para los Animales Acuáticos destacó la necesidad de designar laboratorios de referencia para las siguientes enfermedades de la lista:

Infección por *Aphanomyces invadans* (síndrome ulcerativo epizoótico)

Infección por *Batrachochytrium dendrobatis*

Infección por *Batrachochytrium salamandrivorans*

Infección por el virus de la mionecrosis

Infección por *Perkinsus marinus*

Infección por *Perkinsus olseni*

Infección por el virus de la tilapia del lago

Infección por *Xenohaliotis californiensis*

La Comisión invita a presentar solicitudes a los Miembros con la experiencia adecuada en estas enfermedades.

11. Otros asuntos

11.1. Registro de los kits de diagnóstico

La Comisión para los Animales Acuáticos revisó la situación actual del Registro de kits de diagnóstico de la OMSA con la secretaría para el registro de los kits de diagnóstico. En la actualidad, figuran 14 kits de pruebas de diagnóstico en el registro de kits de diagnóstico de la OMSA.

La Comisión debatió sobre las dos solicitudes activas de kit de diagnóstico para animales acuáticos y las dos solicitudes que requieren una renovación de cinco años del registro.

Durante el debate, la Comisión pidió que se aclarara la armonización de las pruebas registradas con los métodos de diagnóstico que figuran en el *Manual Acuático* y el *Manual Terrestre* de la OMSA. Esta solicitud se someterá a debate junto con el Departamento de resistencia antimicrobiana y productos veterinarios.

.../Anexos

Anexo 1. Ítem 2 – Orden del día aprobado

REUNIÓN DE LA COMISIÓN DE NORMAS SANITARIAS PARA LOS ANIMALES ACUÁTICOS

Reunión en formato híbrido, 14 a 21 de septiembre de 2022

Orden del día

1. BIENVENIDA DE LA DIRECTORA GENERAL ADJUNTA
2. APROBACIÓN DEL ORDEN DEL DÍA
3. REUNIÓN DE LA DIRECTORA GENERAL
4. COOPERACIÓN CON LA COMISIÓN DE NORMAS SANITARIAS PARA LOS ANIMALES TERRESTRES
5. PLAN DE TRABAJO DE LA COMISIÓN PARA LOS ANIMALES ACUÁTICOS
 - 5.1. Temas prioritarios
 - 5.1.1. Capítulo 4.3. Aplicación de la compartimentación
 - 5.1.2. Nuevo Capítulo 5.X. Animales acuáticos ornamentales
 - 5.1.3. Nuevo Capítulo 5.Y. Comercio de materiales genéticos
6. ESTRATEGIA DE LA OMSA PARA LA SANIDAD DE LOS ANIMALES ACUÁTICOS
 - 6.1. Informe sobre la implementación de la Estrategia
 - 6.1.1. Actualización de la implementación
 - 6.1.2. Observatorio de la OMSA – Resultados de la encuesta
 - 6.1.3. Redes de los centros de referencia – Departamento de Ciencia
 - 6.1.4. Actualización del plan de trabajo sobre RAM

CÓDIGO ACUÁTICO

7. Ítems para comentario de los Miembros
 - 7.1. Definiciones del Glosario (“autoridad competente”, “autoridad veterinaria”, “servicios veterinarios” y “servicios de sanidad de los animales acuáticos”) en el *Código Acuático*
 - 7.2. Mercancías seguras – Artículos X.X.3. para los capítulos específicos de enfermedad
 - 7.3. Artículos revisados 8.X.3. para los capítulos específicos de las enfermedades de los anfibios
 - 7.4. Artículos revisados 11.X.3. para los capítulos específicos de las enfermedades de los moluscos
 - 7.5. Artículo 9.3.1. del Capítulo 9.3. Infección por *Hepatobacter penaei* (hepatopancreatitis necrotizante)
 - 7.6. Artículo 9.4.1. and 9.4.2. del Capítulo 9.4. Infección por el virus de la necrosis hipodérmica y hematopoyética infecciosa (IHHNV)
 - 7.7. Artículo 10.9.2. del Capítulo 10.9. Infección por el virus de la viremia primaveral de la carpa
 - 7.8. Nuevo Capítulo 10.X. Infección por el virus de la tilapia del lago
 - 7.9. Artículos 11.4.1. y 11.4.2. del Capítulo 11.4. Infección por *Marteilia refringens*
 - 7.10. Armonización de los capítulos específicos de las enfermedades de los moluscos; Artículos 11.X.8. - 11.X.12.

8. Ítems para debate en la Comisión para los Animales Acuáticos

- 8.1. Evaluación de los períodos por defecto en los Artículos X.X.4.-X.X.8. para los capítulos específicos de enfermedad
 - 8.2. Actualización del trabajo sobre las mercancías seguras
 - 8.3. Consideración de las enfermedades emergentes
 - 8.3.1. Infección por el virus del edema de la carpa (CEV)
 - 8.3.2. Infección por el nodavirus de la mortalidad encubierta (CMNV) en el pez cebra
 - 8.3.3. *Coxiella burnetii* en camarones (presentado por WAHIAD)
 - 8.3.4. Revisión del proyecto de POE para las enfermedades emergentes
 - 8.4. Debate sobre la certificación electrónica
 - 8.5. Estrategia sobre los animales silvestres

MANUAL ACUÁTICO

9. Ítems para comentario de los Miembros

- 9.1. Sección 2.2. Enfermedades de los crustáceos
 - 9.1.1. Capítulo 2.2.0. Información general: enfermedades de los crustáceos

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- 9.1.2. Capítulo 2.2.1. Enfermedad de la necrosis hepatopancreática aguda
 - 9.1.3. Capítulo 2.2.2. Infección por *Aphanomyces astaci* (plaga del cangrejo de río)
 - 9.1.4. Capítulo 2.2.3. Infección por *Hepatobacter penaei* (hepatopancreatitis necrotizante)
 - 9.1.5. Capítulo 2.2.4. Infección por el virus de la necrosis hipodérmica y hematopoyética
 - 9.1.6. Capítulo 2.2.5. Infección por el virus de la mionecrosis infecciosa
 - 9.1.7. Capítulo 2.2.6. Infección por *Macrobrachium rosenbergii* nodavirus (enfermedad de la cola blanca)
 - 9.1.8. Capítulo 2.2.7. Infección por el virus del síndrome de Taura
 - 9.1.9. Capítulo 2.2.8. Infección por el virus del síndrome de las manchas blancas
 - 9.1.10. Capítulo 2.2.9. Infección por el genotipo 1 del virus de la cabeza blanca
 - 9.2. Sección 2.3. Enfermedades de los peces
 - 9.2.1. Capítulo 2.3.1. Infección por *Aphanomyces invadans* (síndrome ulcerante epizoótico)
 - 9.2.2. Capítulo 2.3.2. Infección por el virus de la necrosis hematopoyética epizoótica
 - 9.2.3. Capítulo 2.3.7. Infección por el iridovirus de la dorada japonesa
 - 9.2.4. Sección 2.2.2. del Capítulo 2.3.9. Infección por el virus de la viremia primaveral de la carpa
 - 9.3. Sección 2.4. Enfermedades de los moluscos
 - 9.3.1. Sección 2.2.1. y 2.2.2. del Capítulo 2.4.4. Infección por *Marteilia refringens*
 - 9.4. Tabla propuesta de los parámetros PCR para armonizar los protocolos PCR
- 10. Ítems para debate de la Comisión para los Animales Acuáticos**
- 10.1. Desarrollar un mecanismo para acelerar el proceso de poner a disposición de los Miembros las actualizaciones de los métodos de diagnóstico del *Manual Acuático*
- 11. GRUPOS AD HOC**
- 11.1. Grupo *ad hoc* sobre la susceptibilidad de las especies de moluscos a la infección por las enfermedades de la lista de la OIE
 - 11.2. Proyecto de informe del Grupo *ad hoc* sobre la susceptibilidad de las especies de peces a la infección por las enfermedades de la lista de la OIE
 - 11.3. Proyecto de informe del Grupo *ad hoc* sobre los nuevos proyectos de capítulos sobre la preparación de emergencia frente a las enfermedades y gestión de los brotes de enfermedad
- 12. CENTROS DE REFERENCIA O CAMBIO DE EXPERTOS**
- 12.1. Evaluación de las solicitudes para la designación como centro de referencia en temas de sanidad de los animales acuáticos o cambio de expertos
- 13. OTROS TEMAS**
- 13.1. Para debate
 - 13.1.1. Registro de los kits de diagnóstico
 - 13.1.1.1. *IQ PlusTM WSSV Kit*
 - 13.1.1.2. *IQ 2000TM WSSV*
 - 13.1.1.3. *WSSV LFT*
 - 13.1.1.4. *Genic Shrimp Multipath testing package*
 - 13.1.2. Autodeclaraciones para los procedimientos de ausencia de enfermedad
 - 13.1.3. Informe de febrero – Parte A/B
 - 13.2. Para información
 - 13.2.1. Coordinación de la investigación de la OIE
 - 13.2.2. Impacto global de las enfermedades animales (GBADs)
- 14. REVISIÓN DE LA REUNIÓN**
- 15. PRÓXIMA REUNIÓN: 15–22 de febrero de 2023**

Anexo 2. Ítem 2 – Lista de participantes

REUNIÓN DE LA COMISIÓN DE NORMAS SANITARIAS PARA LOS ANIMALES ACUÁTICOS

Reunión híbrida, 14 al 21 de septiembre de 2022

MIEMBROS DE LA COMISIÓN

Dr. Ingo Ernst (Presidente) Director Aquatic Pest and Health Policy, Animal Division Department of Agriculture, Water and the Environment, Canberra, AUSTRALIA	Dra. Alicia Gallardo Lago (Vicepresidente) Senior advisor FARMAVET, University of Chile, La Pintana, CHILE	Dr. Prof. Hong Liu (miembro) Deputy Director, Animal and Plant Inspection and Quarantine Technical Centre, Shenzhen Customs District General Administration of Customs Shenzhen City, CHINA (Rep. Pop. de)
Dra. Fiona Geoghegan (Vicepresidente) Legislative Officer, European Commission, DG SANTE Brussels, BÉLGICA	Dr. Kevin William Christison (miembro) Specialist Scientist, Directorate: Aquaculture Research and Development Department of Forestry, Fisheries and the Environment, Vlaeberg, SUDÁFRICA	Dr. Espen Rimstad (miembro) Professor in Virology, Faculty of Veterinary Medicine, Department of Paraclinical Sciences (PARAFAG) Norwegian University of Life Sciences Ås, NORWAY

OTROS PARTICIPANTES

Dr. Mark Crane CSIRO Honorary Fellow, Research Group Leader AAHL Fish Diseases Laboratory Australian Centre for Disease Preparedness (ACDP) CSIRO, Geelong, AUSTRALIA

SEDE DE LA OMSA

Dra. Gillian Mylrea Jefa del Departamento de Normas	Dr. Stian Johnsen Comisionado Departamento de Normas	Sra. Sara Linnane Redacción científica – Normas Internacionales Departamento Científico
Dra. Bernita Giffin Coordinadora Científica para la Sanidad de los Animales Acuáticos Departamento de Normas		Dra. Gounalan Pavade Coordinador científico Departamento Científico

Anexo 3. Ítem 4. – Plan de trabajo y prioridades

PLAN DE TRABAJO DE LA COMISIÓN PARA LOS ANIMALES ACUÁTICOS

Trabajo en curso para adopción en 2023 o posterior

<i>Código Acuático</i>			
Capítulo/Tema	Situación		
	Septiembre de 2022	Febrero de 2023	Sesión General de mayo de 2023
Seguimiento de enfermedades emergentes y consideración de las acciones requeridas	En curso		
Definiciones del Glosario: “autoridad competente”, “autoridad veterinaria” y “servicios de sanidad de los animales acuáticos”		Revisión del uso en el Código Acuático y presentación de enmiendas para comentario	
Capítulo 1.3. Enfermedades de la lista de la OIE – Inclusión de la infección por el virus de la necrosis infecciosa del bazo y del riñón	Evaluación previa para inclusión en la lista y presentación para comentarios	Revisión de los comentarios de los Miembros	
Procedimiento operativo estándar para la autodeclaración de ausencia de enfermedad		Redacción de un modelo para orientar a los Miembros en la presentación de una autodeclaración de ausencia	
Capítulo 4.3. Aplicación de la compartimentación	Envío de cuestionario a los Miembros	Examen de las respuestas para guiar las enmiendas del Capítulo 4.3.	
Capítulo 4.X. Nuevo proyecto de capítulo sobre preparación de emergencia frente a las enfermedades		Revisión del proyecto de Capítulo 4.X.	
Capítulo 4.Y. Nuevo proyecto de capítulo sobre gestión de los brotes de enfermedad		Revisión del proyecto de Capítulo 4.Y.	
Capítulo 5.2. Procedimientos de certificación		Discusión del plan para las enmiendas	
Capítulos 5.6.–5.9.		Revisión del grupo <i>ad hoc</i> bajo el mandato de la Comisión del Código	
Capítulo 5.X. Comercio de animales acuáticos ornamentales	Desarrollo de un plan para la redacción del nuevo capítulo	Revisión del proyecto de estructura del capítulo	

Capítulo 5.Y. Comercio de materiales genéticos	Desarrollo de un plan para la redacción del nuevo capítulo	Revisión del proyecto de estructura del capítulo	
Mercancías seguras – capítulos específicos de enfermedad – Artículos X.X.3.		Revisión de las evaluaciones actualizadas sobre las mercancías seguras y los artículos modificados. Presentación de los artículos enmendados para comentario	
Evaluación de los periodos por defecto de los Artículos X.X.4.-X.X.8. para los capítulos específicos de enfermedad	Establecimiento de un enfoque para evaluar los periodos por defecto	Presentación de las evaluaciones de los periodos por defecto para comentarios	
Especies susceptibles – Enfermedades de crustáceos – Artículos 9.X.1. and 9.X.2. para: – Infección por el virus iridescente de los decápodos – Infección por <i>Aphanomyces astaci</i> (Plaga del cangrejo de río)		Convocatoria de un grupo <i>ad hoc</i>	
Artículo 9.3.1. del Capítulo 9.3. Infección por <i>Hepatobacter penaei</i> (hepatopancreatitis necrotizante)	Revisión de los comentarios (1. ^a ronda)	Revisión de los comentarios (2. ^a ronda)	Propuesta para adopción
Artículos 9.4.1. y 9.4.2. del Capítulo 9.4. Infección por el virus de la necrosis hipodérmica y hematopoyética infecciosas (IHHNV)	Revisión de los comentarios (1. ^a ronda)	Revisión de los comentarios (2. ^a ronda)	Propuesta para adopción
Artículo 9.4.2. del Capítulo 9.5. Infección por el virus de la mionecrosis infecciosa (IMNV)	Revisión del artículo enmendado y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	Propuesta para adopción
Especies susceptibles – Enfermedades de peces – Artículos 10.X.1. y 10.X.2. para – Infección por el iridovirus de la dorada japonesa – Infección por el virus de la tilapia del lago – Infección por <i>Aphanomyces invadans</i> (síndrome ulcerante epizoótico)	Revisión intermedia del informe del grupo <i>ad hoc</i> : próxima reunión del grupo <i>ad hoc</i> prevista en noviembre de 2022	Revisión de los artículos modificados para el iridovirus de la dorada japonesa	
Artículos 10.9.2. del Capítulo 10.9. Infección por el virus de la viremia primaveral de la carpa	Revisión de la evaluación del grupo <i>ad hoc</i> , artículo modificado y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	Propuesta para adopción
Especies susceptibles	En curso		

Evaluación de nuevas especies/evidencia para enfermedades anteriormente evaluadas según sea necesario			
Capítulo 10.X. Infección por el virus de la tilapia del lago	Revisión del proyecto de capítulo y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	Propuesta para adopción
Especies susceptibles – Enfermedades de los moluscos – Artículos 11.X.1. y 11.X.2. para: – Infección por <i>Marteilia refringens</i> – Infección por <i>Perkinsus marinus</i> – Infección por <i>Xenohaliotis californiensis</i> – Infección por <i>Perkinsus olseni</i>	<i>Marteilia refringens</i> : Revisión de los artículos modificados y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	Propuesta para adopción
	Próxima reunión del grupo <i>ad hoc</i> prevista en noviembre de 2022	<i>Perkinsus marinus</i> : revisión de los artículos modificados y presentación para comentarios	
Especies susceptibles – Artículos 11.2.2. del Capítulo 11.2. Infección por <i>Bonamia exitiosa</i>	Revisión del artículo modificado y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	Propuesta para adopción
Especies susceptibles – Artículos 11.3.2. del Capítulo 11.3. Infección por <i>Bonamia ostreae</i>	Revisión del artículo modificado y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	Propuesta para adopción
Modelo de Artículos 11.X.9.-11.X.12.: armonización con otros capítulos específicos de enfermedad	Revisión de los artículos modificados y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	Propuesta para adopción
Manual Acuático			
Capítulo/Tema	Situación		
	Septiembre de 2022	Febrero de 2023	Sesión General de mayo de 2023
Sección 2.2. Disposiciones generales – Crustáceos	Revisión del capítulo modificado y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	
Capítulo 2.2.1. Enfermedad de la necrosis pancreática aguda	Revisión de los comentarios (1. ^a ronda)	Revisión de los comentarios (2. ^a ronda)	Propuesta para adopción
Capítulo 2.2.2. Infección por <i>Aphanomyces astaci</i> (plaga del cangrejo de río)	Revisión del proyecto actualizado y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	
Capítulo 2.2.3. Infección por <i>Hepatobacter penaei</i> (hepatopancreatitis necrotizante)	Revisión de los comentarios (1. ^a ronda)	Revisión de los comentarios (2. ^a ronda)	Propuesta para adopción
Capítulo 2.2.4. Infección por el virus de la necrosis hipodérmica y	Revisión de los comentarios (1. ^a ronda)	Revisión de los comentarios (2. ^a ronda)	Propuesta para adopción

hematopoyética infecciosa			
Capítulo 2.2.5. Infección por el virus de la mionecrosis infecciosa	Actualización, reformateado y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	
Capítulo 2.2.6. Infección por el nodavirus <i>Macrobrachium rosenbergii</i> (enfermedad de la cola blanca)	Revisión del proyecto actualizado	Nueva revisión del proyecto actualizado y presentación para comentarios	
Capítulo 2.2.7. Infección por el virus del síndrome de Taura	Actualización, reformateado y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	
Capítulo 2.2.8. Infección por el virus del síndrome de las manchas blancas	Actualización, reformateado y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	
Capítulo 2.2.9. Infección por el virus de la cabeza amarilla genotipo 1	Actualización, reformateado y revisión	Nueva revisión del proyecto actualizado y presentación para comentarios	
Capítulo 2.2.X. Infección por el virus iridiscente de los decápodos tipo 1		Desarrollo de un proyecto de capítulo para revisión	
Capítulo 2.3.1. Infección por <i>Aphanomyces invadans</i> (síndrome ulcerante epizoótico)	Revisión de los comentarios (1. ^a ronda)	Revisión de los comentarios (2. ^a ronda)	Propuesta para adopción
Capítulo 2.3.2. Infección por el virus de la necrosis hematopoyética epizoótica	Revisión de los comentarios (2. ^a ronda)	Revisión de los comentarios (3. ^a ronda)	Propuesta para adopción
Capítulo 2.3.7. Infección por la enfermedad del iridovirus de la dorada japonesa		Revisión del informe del grupo ad hoc de acuerdo con el enfoque para la inclusión en la lista	
Sección 2.2.2. del Capítulo 2.3.9. Infección por el virus de la viremia primaveral de la carpa	Revisión de los artículos modificados y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	Propuesta para adopción
Capítulo 2.3.X. Infección por el virus de la tilapia del lago		Desarrollo de un proyecto de capítulo para revisión	
Secciones 2.2.1. y 2.2.2. del Capítulo 2.4.4. Infección por <i>Marteilia refringens</i>	Revisión de los artículos modificados y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	Propuesta para adopción
Sección 2.2.2. del Capítulo 2.4.2. Infección por <i>Bonamia exitiosa</i>	Revisión de los artículos modificados y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	Propuesta para adopción
Sección 2.2.2. del Capítulo 2.4.2. Infección por <i>Bonamia ostreae</i>	Revisión de los artículos modificados y presentación para comentarios	Revisión de los comentarios (1. ^a ronda)	Propuesta para adopción

Otros ítems prioritarios para comenzar antes de mayo de 2024

<i>Código Acuático</i>				
Capítulo/Tema	Situación	Prioridad 1	Prioridad 2	Próximas etapas
Capítulo 1.3. Enfermedades de la lista de la OIE	Revisión de toda nueva enfermedad para la inclusión o retiro de la lista según sea necesario		En curso	
Capítulo 4.2. Zonificación y compartimentación	Nuevo desarrollo del capítulo centrándose exclusivamente en la zonificación		✓	
<i>Manual Acuático</i>				
Capítulo/Tema	Situación	Prioridad 1	Prioridad 2	Próximas etapas
Título 2.4. Disposiciones generales – Moluscos	Revisión y actualización del capítulo introductorio sobre las enfermedades de los moluscos		✓	
Capítulos 2.4.X. Capítulos específicos de enfermedades de los moluscos	Actualización y reformato de los capítulos utilizando el nuevo modelo (todas las enfermedades)		✓	

**Anexo 4. Ítem 5.1. – Capítulo 1.3. Enfermedades de la lista de la OIE –
Inclusión de la infección por *Megalocytivirus***

**CAPÍTULO 1.3.
ENFERMEDADES DE LA LISTA DE LA OIE**

[...]

Artículo 1.3.1.

Están incluidas en la lista de la OIE las siguientes enfermedades de los peces:

- Infección por *Aphanomyces invadans* (Síndrome ulcerante epizoótico)
- Infección por el alfavirus de los salmónidos
- Infección por el virus de la necrosis infecciosa del bazo y del riñón
- Infección por el herpesvirosis de la carpa koi
- Infección por el iridoviresis de la dorada japonesa
- Infección por el virus de la necrosis hematopoyética epizoótica
- Infección por el virus de la necrosis hematopoyética infecciosa
- Infección por el virus de la septicemia hemorrágica viral
- Infección por el virus de la septicemia hemorrágica viral
- Infección por el virus de la tilapia del lago
- Infección por *Gyrodactylus salaris*
- Infección por las variantes con supresión en la HPR y HPRO del virus de la anemia infecciosa del salmón.

[...]

Anexo 5. Ítem 5.1. – Capítulo 1.3. Enfermedades de la lista de la OIE – Inclusión en la lista de la infección por *Megalocytivirus*

EVALUACIÓN DE LA INFECCIÓN POR EL VIRUS DE LA NECROSIS INFECCIOSA DEL BAZO Y DEL RIÑON (ISKNV) PARA INCLUSIÓN EN LA LISTA DE ENFERMEDADES DEL CÓDIGO SANITARIO PARA LOS ANIMALES ACUÁTICOS

Resumen de la evaluación

1. La Comisión de Normas Sanitarias para los Animales Acuáticos evaluó la especie del virus de la *necrosis infecciosa del bazo y del riñón*, incluidos sus tres genogrupos, el iridovirus de la dorada japonesa (RSIV), el virus de la necrosis infecciosa del bazo y del riñón (ISKNV) y el iridovirus del cuerpo rojizo del rodaballo (TRBIV), en función de los criterios de inscripción en la lista de enfermedades de los animales acuáticos del Artículo 1.2.2. del *Código Acuático*.
2. La Comisión para los Animales Acuáticos acordó que el genogrupo del iridovirus de la dorada japonesa (RSIV) (actualmente incluido en el *Código Acuático*), así como los dos genogrupos ISKNV y TRBIV cumplen los criterios de inclusión en la lista 1, 2, 3 y 4b (ver Tabla 1).
3. La Comisión para los Animales Acuáticos observó que los tres genogrupos poseen especies susceptibles que se superponen, una epidemiología y métodos de diagnóstico similares. La Comisión acordó que la enfermedad de la lista propuesta debía denominarse "infección por el virus de la necrosis infecciosa del bazo y del riñón (ISKNV)". La infección por ISKNV deberá definirse para incluir los genogrupos ISKNV, RSIV y TRBIV, pero excluir las otras especies reconocidas de *Megalocytivirus*, el virus que provoca la caída de escamas o descamación.

	Criterio de la lista						Conclusión
	1	2	3	4a	4b	4c	
Infeción por ISKNV	+	+	+	NA	+	-	La enfermedad cumple con los criterios de inclusión en la lista

NA = no se aplica.

Criterio de inclusión en la lista (Capítulo 1.2. del Código Acuático)

Los criterios para incluir una enfermedad en la lista de la OIE son los siguientes:

1. Es probable la propagación internacional del agente patógeno (a través de animales acuáticos, sus productos, vectores o fómites).

Y

2. Al menos un país puede demostrar en el país o en una zona la ausencia de enfermedad en animales acuáticos susceptibles, basándose en las disposiciones del Capítulo 1.4.

Y

3. Se dispone de una definición de caso precisa y existen métodos de detección y diagnóstico fiables.

Y

- 4a. Se ha demostrado la transmisión natural de la enfermedad al ser humano y la infección humana se asocia con consecuencias graves.

O

- 4b. Se ha demostrado que la enfermedad afecta la sanidad de los animales acuáticos de cultivo a nivel de un país o una zona lo que conlleva consecuencias significativas, por ejemplo, pérdidas de producción, morbilidad o mortalidad.

O

4c. Se ha demostrado o las pruebas científicas indican que la enfermedad puede afectar la sanidad de los animales acuáticos silvestres lo que conlleva consecuencias significativas, por ejemplo, morbilidad o mortalidad a nivel de la población, productividad reducida o impactos ecológicos.

Contexto

El *Megalocytivirus* es uno de los siete géneros de la familia *Iridoviridae* y se clasifica dentro de la subfamilia Alphairidovirinae junto con los géneros *Ranavirus* y *Lymphocystivirus* (Chinchar *et al.*, 2017; Chinchar *et al.*, 2020). Los megalocytivirus se distinguen de los ranavirus y los linfocistivirus por su capacidad de provocar una ampliación celular marcada en los tejidos infectados y por el análisis de la secuencia de genes virales clave (Chinchar *et al.*, 2017). Los megalocytivirus son los agentes etiológicos de enfermedades graves asociadas a una elevada mortalidad en una serie de especies de peces con aletas de agua dulce y marina (Kurita y Nakajima, 2012).

El ICTV reconoce dos especies de *Megalocytivirus*: El virus de la necrosis infecciosa del bazo y del riñón (ISKNV) y el virus de la enfermedad de la caída de escamas (SDDV) (Chinchar *et al.*, 2017). El SDDV es genéticamente y epidemiológicamente distinto de la especie ISKNV y no se tienen en cuenta en esta evaluación.

Dentro de la especie ISKNV, se reconocieron tres genogrupos: ISKNV, RSIV y TRBIV (Song *et al.*, 2008). Sin embargo, queda por resolver si estos genogrupos representan especies distintas o cepas de una sola especie (Chinchar *et al.*, 2017). Los megalocytivirus recibieron numerosos nombres únicos en función de las especies en las que se detectaron; sin embargo, todas las variantes de la especie ISKNV con los genomas analizados se sitúan dentro de los tres genogrupos (ISKNV, RSIV y TRBIV) (Chinchar *et al.*, 2017).

El nombre del ISKNV se utiliza para una de las dos especies reconocidas de *Megalocytivirus* y también para uno de los tres genogrupos dentro de dicha especie. En este documento, "ISKNV" designa al "genogrupo ISKNV". "La especie ISKNV" se utiliza cuando se refiere a la especie.

El iridovirus de la dorada japonesa (RSIV) fue incluido por primera vez por la OMSA en el *Código Sanitario para los Animales Acuáticos*¹ de 2003 y sigue figurando en el *Código Acuático* de 2022. La enfermedad causada por el RSIV se detectó por primera vez en besugos de acuicultura (*Pagrus major*) en Japón en 1990 (Inouye *et al.*, 1992). El iridovirus de la dorada japonesa se detectó principalmente en peces marinos. Las especies que actualmente figuran en la lista de especies susceptibles de ser infectadas por el iridovirus de la dorada japonesa en el *Código Acuático*² de la OMSA son: el besugo (*Pagrus major*), la seriola (*Seriola quinqueradiata*), la serviola (*Seriola dumerilii*), la lubina (*Lateolabrax sp.*), la lubina asiática (*Lates calcarifer*), el atún blanco (*Thunnus thynnus*), el pez loro japonés (*Oplegnathus fasciatus*), el jurel rayado (*Caranx delicatissimus*), el pez mandarín (*Siniperca chuatsi*), el corvinón rojo (*Sciaenops ocellatus*), el salmonete (*Mugil cephalus*) y los meros (*Epinephelus spp.*).

El genogrupo ISKNV no figura actualmente en el *Código Acuático*. Desde finales de los años 1980 y 1990 en especies de peces de agua dulce se notificaron viriones morfológicamente compatibles con los iridovirus y que presentan células ampliadas con cuerpos de inclusión compatibles con los megalocytivirus (por ejemplo, Armstrong y Ferguson, 1989; Anderson *et al.*, 1993). El ISKNV se detectó en muestras de peces ornamentales de archivo desde 1996 (Go *et al.*, 2006; Go *et al.*, 2016). La necrosis infecciosa del bazo y del riñón se describió en peces mandarín (*Siniperca chuatsi*; He *et al.*, 2000; He *et al.*, 2002) y en 2001 se analizó el genoma del ISKNV y se encontró que era genéticamente similar al RSIV (He *et al.*, 2001). El ISKNV se detectó en numerosas especies de peces de agua dulce, incluidas algunas asociadas al comercio de peces ornamentales (ver revisión de Johan y Zainathan, 2020). Este genotipo se observó en numerosas especies de peces ornamentales objeto de comercio internacional. El ISKNV también se notificó como causa de mortalidad masiva en especies importantes para el consumo humano (por ejemplo, Subramaniam *et al.*, 2016; Ramírez-Paredes *et al.*, 2020).

El genogrupo del iridovirus del cuerpo rojizo del rodaballo (TRBIV) no figura actualmente en el *Código Acuático*. El TRBIV fue descrito por primera vez como causante de enfermedades en el rodaballo, *Scophthalmus maximus* (Shi *et al.*, 2004). El TRBIV causa principalmente enfermedades en peces planos en China y Corea (por ejemplo, Shi *et al.*, 2004; Do *et al.*, 2005), pero también se observó en otras especies, incluso en el comercio de peces ornamentales (Go *et al.*, 2016; Koda *et al.*, 2018). El TRBIV causó la enfermedad en otras especies de peces de cultivo de importancia económica, como la perca marina asiática (*Lates calcarifer*) (Tsai *et al.*, 2020) y la cuchilla barrada (*Oplegnathus fasciatus*) (Huang *et al.*, 2011).

La Comisión para los Animales Acuáticos propuso previamente un enfoque para diferenciar las cepas de patógenos (consulte los informes de las reuniones de [febrero](#) y [octubre de 2011](#) de la Comisión). Se consideraron tres criterios

¹ El iridovirus de la dorada japonesa (RSIV) se incluyó en el *Código Acuático* antes de 2003 como "otra enfermedad significativa".

² Vale aclarar que aún no está finalizada la evaluación de las especies consideradas susceptibles a la infección por el iridovirus de la dorada japonesa (RSIV), de acuerdo con el Capítulo 1.5. del *Código Acuático*.

principales para la aplicabilidad de la diferenciación de cepas patógenas en las normas del *Código Acuático* y del *Manual Acuático*: 1) las variantes del patógeno están claramente reconocidas en la literatura científica y tienen diferentes características de la enfermedad; 2) existen métodos robustos para diferenciar las variantes de forma consistente; y 3) existe, o existe la posibilidad de que exista, una gestión diferente de las variantes en o entre países. En el caso de la especie ISKNV, el RSIV se incluyó en la lista antes de la investigación que definió los 3 genogrupos dentro de la especie ISKNV, y sus relaciones genéticas y epidemiológicas. Dado que la infección por el RSIV se incluyó en la lista, pero no ISKNV y TRBIV, esta evaluación presenta información para cada uno de estos tres genogrupos, a pesar de que los tres genogrupos se propusieron para su inclusión en la lista de forma colectiva junta como la especie ISKNV.

Evaluación con respecto a los criterios de inclusión en la lista

Criterio No. 1. Es probable la propagación internacional del agente patógeno (a través de animales acuáticos, sus productos, vectores o fómites).

Evaluación

La especie ISKNV puede transmitirse horizontalmente a través del agua y se sabe que permanece viable en los tejidos congelados del huésped. Se espera que la probabilidad de transmisión sea mayor en el comercio de peces vivos, pero también es posible en productos de animales acuáticos, especialmente si no están eviscerados.

Numerosas especies marinas y de agua dulce son susceptibles al ISKNV y son objeto de comercio internacional, ya sea como animales acuáticos vivos (para consumo humano, acuicultura o con fines ornamentales) o como productos de animales acuáticos.

Por su parte, el iridovirus de la dorada japonesa se detectó en varios países de Asia, donde se asoció a la enfermedad en especies de peces marinos de cultivo (Kurita y Nakajima, 2012). Algunas especies susceptibles se comercializan vivas para el consumo humano (por ejemplo, el besugo y los meros), otras se comercializan como productos de animales acuáticos.

El ISKNV se observó en numerosas especies comercializadas como peces ornamentales y el comercio de peces ornamentales está implicado en la propagación y los brotes de la enfermedad (por ejemplo, Jeong *et al.*, 2008; Johan & Zainathan, 2020). Los peces ornamentales infectados a veces no presentan signos clínicos (por ejemplo, Subramaniam *et al.*, 2014) y, como tales, pueden actuar como portadores del virus. El ISKNV también se detectó en importantes especies de cultivo para el consumo humano que se comercializan a escala internacional, como la tilapia (Ramírez-Paredes *et al.*, 2020). Igualmente, se detectó en peces no procesados utilizados para la alimentación de la acuicultura (Lajimin *et al.*, 2015), es decir, el pescado comercializado para la alimentación o el cebo de la acuicultura puede presentar una vía de transmisión. Se demostró la transmisión de especies de peces de agua dulce a especies de peces marinos por inoculación directa y cohabitación (Jeong *et al.*, 2008b; Go & Whittington, 2019).

El TRBIV está presente en varias especies importantes en el comercio internacional (por ejemplo, el rodaballo, la platija y la lubina asiática), incluyendo el comercio de animales vivos o como productos de animales acuáticos. El análisis filogenético indica la existencia de una reciente propagación internacional del TRBIV (Tsai *et al.*, 2020).

Se detectaron variantes de la especie ISKNV en numerosas especies marinas y de agua dulce que se comercializan a escala internacional. Cada uno de los tres genogrupos se ha detectado en productos comercializados y existen pruebas de la propagación internacional asociada con el comercio.

Conclusión

Se cumple el criterio.

Criterio No. 2. Al menos un país puede demostrar en el país o en una zona la ausencia de enfermedad en animales acuáticos susceptibles, basándose en las disposiciones del Capítulo 1.4.

Evaluación

La infección por RSIV se puede notificar a la OMSA desde 2003. Varios países siguen informando de que nunca se notificó el RSIV en su territorio (ver el Sistema Mundial de Información Zoosanitaria de la OMSA) y es probable que algunos de ellos puedan demostrar que están libres de enfermedad.

Se notificó la presencia del ISKNV en numerosas especies de peces comercializados a través del comercio de peces ornamentales y es probable que este genogrupo se propague a través de las cadenas de suministro de peces ornamentales. Sin embargo, algunos países mantienen medidas básicas de bioseguridad para el ISKNV y pueden

demostrar que están libres de enfermedad. Además, las pruebas de PCR utilizadas en la vigilancia del RSIV también detectarían ISKNV, proporcionando pruebas de la ausencia de ISKNV.

El TRBIV se ha detectado principalmente en peces planos de cultivo de China y Corea, pero también en peces ornamentales y en lubinas asiáticas de cultivo. Las pruebas PCR recomendadas en el capítulo del *Manual Acuático* de la OMSA para el RSIV pueden no incluir el TRBIV, lo que da lugar a una menor confianza en la distribución del TRBIV. Sin embargo, dado que el TRBIV demostró su patogenicidad en poblaciones de cultivo de varias especies, es probable que se detecte en esas especies. Aunque no es segura la distribución del TRBIV, es probable que al menos un país pueda declararse libre a nivel de país o de zona.

Conclusión

Se cumple el criterio.

Criterio No. 3. Se dispone de una definición de caso precisa y existen métodos de detección y diagnóstico fiables

Evaluación

Las definiciones de caso para la sospecha y la confirmación de la infección por RSIV están disponibles en el *Manual Acuático* de la OMSA. Dado que la mayoría de las pruebas PCR para RSIV (y algunos otros métodos, por ejemplo, la histopatología), incluyen el ISKNV, las definiciones de caso podían adaptarse fácilmente para incluir el ISKNV. Kawato *et al.* (2021) compararon el rendimiento analítico de cuatro métodos de PCR en tiempo real para la detección de *Megalocytivirus* (excluyendo el SDDV) y encontraron que tres de los cuatro ensayos detectaron el ISKNV, RSIV y TRBIV. Kim *et al.* (2022) informaron sobre el rendimiento de un ensayo de PCR en tiempo real con inclusión de RSIV, ISKNV y TRBIV. Existen suficientes herramientas de diagnóstico disponibles para detectar la especie ISKNV y para construir definiciones de casos que incluyan los tres genogrupos.

Conclusión

Se cumple el criterio.

Criterio No. 4a Se ha demostrado la transmisión natural de la enfermedad al ser humano y la infección humana se asocia con consecuencias graves.

Evaluación

No existe evidencias de la transmisión a los seres humanos.

Conclusión

No se aplica el criterio.

Criterio No. 4b Se ha demostrado que la enfermedad afecta la sanidad de los animales acuáticos de cultivo a nivel de un país o una zona lo que conlleva consecuencias significativas, por ejemplo, pérdidas de producción, morbilidad o mortalidad.

Evaluación

El RSIV ha causado mortalidades masivas en poblaciones de peces cultivados. La enfermedad se detectó por primera vez en el besugo en Japón y los peces afectados se volvieron letárgicos y mostraron signos de anemia grave, petequias en las branquias y agrandamiento del bazo (Inouye *et al.*, 1992; Jung *et al.*, 1997; Nakajima y Maeno, 1998). Se tomó nota de que el RSIV causa pérdidas de producción, morbilidad y mortalidad en muchas otras especies (por ejemplo, Chao *et al.*, 2004; Chen *et al.*, 2003; Girisha *et al.*, 2020; Ni *et al.*, 2021; Sumithra *et al.*, 2022).

El ISKNV se asoció a numerosos casos de enfermedad en peces ornamentales (ver la revisión de Johan & Zainathan, 2020). También se vinculó con altas mortalidades en importantes especies cultivadas para el consumo humano; por ejemplo, en la lubina asiática (Dong *et al.*, 2017; Kerddee *et al.*, 2021), la tilapia (por ejemplo, Figueiredo *et al.*, 2021; Ramírez-Paredes *et al.*, 2021) y los meros (por ejemplo, Chao *et al.*, 2004; Huang *et al.*, 2020).

El TRBIV ha producido enfermedad y una alta mortalidad en la acuicultura del rodaballo en China (por ejemplo, Shi *et al.*, 2010). Se observaron mortalidades de hasta el 90 % en granjas de lubina asiática en Taiwán (Tsai *et al.*, 2020).

Conclusión

Se cumple el criterio.

Criterio No. 4c Se ha demostrado o las pruebas científicas indican que la enfermedad puede afectar la sanidad de los animales acuáticos silvestres lo que conlleva consecuencias significativas, por ejemplo, morbilidad o mortalidad a nivel de la población, productividad reducida o impactos ecológicos.

Evaluación

Existe poca información sobre la aparición del RSIV, el ISKNV o el TRBIV en poblaciones de peces silvestres y sus consecuencias, como la morbilidad, la mortalidad o el impacto ecológico. Se ha indicado que el ISKNV fue la causa de un evento de mortalidad masiva en una población de cíclidos silvestres en la India (Swaminathan *et al.*, 2022), y también se ha detectado en muchos peces silvestres aparentemente sanos de diversas especies (Wang *et al.*, 2007).

Conclusión

El criterio no se cumple.

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Anexo 6. Ítem 5.2. – Artículo 9.3.1. del Capítulo 9.3. Infección por *Hepatobacter Penaei* (hepatopancreatitis necrotizante)

CAPÍTULO 9.3.

**INFECCIÓN POR *HEPATOBACTER PENAEI*
(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.

[...]

Anexo 7. Ítem 5.3. – Artículos 9.4.1. y 9.4.2. del Capítulo 9.4. Infección por el virus de la necrosis hipodérmica y hematopoyética infecciosa

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 Parvovíridos.

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 norteño (*Penaeus setiferus*), camarón azul (*Penaeus stylirostris*) y camarón patiblanco (*Penaeus vannamei*).

[...]

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 merguiensis*), ~~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>	Brema
	<i>Aristichthys nobilis</i>	Carpa cabezona
	<i>Carassius auratus</i>	Carpa dorada
	<i>Ctenopharyngodon idella</i>	Carpa herbívora
	<i>Cyprinus carpio</i>	Carpa común (todas las variedades y subespecies)
	<i>Danio rerio</i>	Pez zebra
	<i>Notemigonus crysoleucas</i>	Carpita dorada
	<i>Pimephales promelas</i>	Piscardo
	<i>Percocypris pingi</i>	<u>Carpa de Jinsha</u>
	<i>Rutilus kutum</i>	Pescado blanco del Caspio
Siluridae	<i>Rutilus rutilus</i>	Rutilo
	<i>Silurus glanis</i>	Sirulo

[...]

Anexo 10. Ítem 5.5. – Artículo 10.9.2. del Capítulo 10.9. Infección por el virus de la viremia primaveral de la carpa (para información)

**EVALUACIÓN DE LA CARPA DE JINSHA (*PERCOCYPRIS PINGI*) COMO ESPECIE SUSCEPTIBLE
A LA INFECCIÓN POR EL VIRUS DE LA VIREMIA PRIMAVERAL DE LA CARPA**

Contexto

En respuesta a un comentario que solicitaba una evaluación de la carpa de Jinsha (*Percocypris pingi*) como especie susceptible a la infección por el virus de la viremia primaveral de la carpa, la Comisión para los Animales Acuáticos solicitó que el Grupo *ad hoc* sobre la susceptibilidad de las especies de peces a la infección por enfermedades de la lista de la OIE (grupo *ad hoc*) revisara las pruebas científicas y presentara una recomendación a la Comisión para que tomara una decisión.

Metodología

- El grupo *ad hoc* aplicó los criterios, descritos en el Artículo 1.5.3 del Código Acuático, para evaluar la carpa de Jinsha (*Percocypris pingi*) con el fin de determinar la susceptibilidad a la infección por el virus de la viremia primaveral de la carpa (SVCV). Se aplicó a esta evaluación la misma metodología y las mismas consideraciones expuestas en el informe del grupo *ad hoc* (<https://www.woah.org/app/uploads/2021/10/a-ahg-susceptibility-of-fish-november-2017.pdf>).

Evaluaciones de la susceptibilidad de los hospedadores al virus de la viremia primaveral de la carpa

Resultados

El grupo *ad hoc* convino en que la carpa de Jinsha (*Percocypris pingi*) cumplía los criterios para figurar en la lista de especies susceptibles a la infección por el virus de la viremia primaveral del virus de la carpa, de acuerdo con el Capítulo 1.5. del Código Acuático y se propuso añadirla al Artículo 10.9.2.

Tabla 1. Evaluación de la carpa de Jinsha (*Percocypris pingi*) de susceptibilidad a la infección por el virus de la viremia primaveral de la carpa

Familia	Nombre científico	Nombre común	Etapa 1: Vía de transmisión	Etapa 2: Identificación del patógeno	Etapa 3: Pruebas de la infección				Resultado individual	Referencias
					A	B	C	D		
Puntuación 1										
Cyprinidae	<i>Percocypris pingi</i>	Carpa de Jinsha	N	Cultivo + secuenciación subsecuente	Y	Y	Y	Y	1	ZHENG <i>et al.</i> , 2018
Cyprinidae	<i>Percocypris pingi</i>	Carpa de Jinsha	E/I						ND	ZHENG <i>et al.</i> , 2018

Indicadores clave para el cuadro de evaluación

N: Infección por vías naturales.
E: Experimental (non-invasiva).
EI: Experimental (invasiva).
Sí: Demuestra que se cumple el criterio.
NO: El criterio no se cumple.
ND: No se determina.

Comentarios del grupo *ad hoc*

- El grupo *ad hoc* acordó que, a pesar de que solo existía un documento para evaluar, la evidencia proporcionada por Zheng *et al.* (2018), un único estudio sólido (brote natural con patología, aislamiento del virus e identificación del virus mediante análisis de la secuencia) con un resultado de "1", era suficiente para concluir la susceptibilidad en ausencia de evidencia conflictiva.
- El grupo *ad hoc* también consideró que la carpa de Jinsha (*Percocypris pingi*) pertenece a la familia Cyprinidae, que contiene otras especies susceptibles. El análisis de la secuencia indica que el virus pertenece al genogrupo que contiene otros aislados chinos del SVCV.
- Zheng *et al.* (2018) estudiaron un brote natural. La infección experimental formaba parte del estudio y no se incluyó en la evaluación por ser "invasiva". La evaluación del procedimiento experimental invasivo no pasó de la fase 1 (es decir, el Artículo 1.5.4.).

Referencias:

ZHENG, L.P., GENG, Y., YU, Z.H., WANG, K.Y., OU, Y.P., CHEN, D.F., HUANG, X.L., DENG, L.J., GAN, W.X., FANG, J., ZHONG, Z.J., LAI, W.M. (2018). First report of spring viremia of carp virus in *Percocypris pingi* in China. *Aquaculture*. **493**, 214-218.

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 56°C durante por lo menos cinco 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 56°C durante por lo menos cinco 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 compartidas 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, 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 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, una parte o la totalidad del país, excepto las zonas *infectadas y de protección*, podrá ser declarada zona libre, siempre que reúna las condiciones descritas en el apartado 2 del Artículo 10.X.6.

Artículo 10.X.6.

Zona libre de infección por 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 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 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.

Artículo 10.X.7.

Compartimento libre de infección por 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 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 sin que se haya detectado la presencia de 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

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 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 garantice 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 garantice la inactivación del 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 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 garantice 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 siguientes mercancías que han sido elaboradas y envasadas 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.
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Anexo 12. Ítem 5.7. – Artículo 11.2.2. del Capítulo 11.2. Infección por *Bonamia exitiosa*

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*), ostra legamosa australiana (*Ostrea angasi*), ostra plana chilena (*Ostrea chilensis*), *Ostrea equestris*, ostra americana (*Crassostrea virginica*), ostra plana europea (*Ostrea edulis*), ostra Olimpia (*Ostrea lurida*) y ostra de Suminoe (*Magallana* [syn. *Crassostrea*] *ariakensis*).

[...]

CAPÍTULO 11.3.

INFECCIÓN POR *BONAMIA OSTREAЕ*

[...]

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.: ostra plana europea (*Ostrea edulis*), ostra plana chilena (*Ostrea chilensis*) y la ostra de Suminoe (*Magallana* [syn. *Crassostrea*] *ariakensis*).

[...]

Anexo 14. Ítem 5.8. – Artículos 11.4.1. y 11.4.2. del Capítulo 11.4. Infección por *Marteilia refringens*

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 agene patógeno *M. refringens* de la familia Martelliidae.~~

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 legamesa australiana (*Ostrea angasi*), ostra argentina (*Ostrea puelchana*) y ostra plana chilena (*Ostrea chilensis*), mejillón común (*Mytilus edulis*) y mejillón mediterráneo (*Mytilus Galloprovincialis*) y chirila (*Chamelea gallina*). Estas recomendaciones se aplican también a todas las demás especies susceptibles mencionadas en el Manual Acuático que sean objeto de comercio internacional.

[...]

Anexo 15. Ítem 5.9. – Modelo de Artículos 11.X.9. – 11.X.14. para los capítulos específicos de las enfermedades de los moluscos

[...]

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

**CAPÍTULO 11.X.
INFECCIÓN POR [PATÓGENO X]**

[...]

Artículo 11.X.9.

Importación de animales acuáticos o 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 y 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 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;
 - 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

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- 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.

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- 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], y extraer muestras y realizar pruebas para la detección del virus de la septicemia hemorrágica viral 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 o 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 desritos en el apartado 1 del Artículo 11.X.12., 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 garantice 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 Artículo 11.X.3. o en otros productos autorizados por la autoridad competente, y
- 2) el agua utilizada para el transporte y todos los efluentes y despojos resultantes de la transformación sean sometidos a un tratamiento que garantizar la inactivación del [patógeno X] el tratamiento de toda el agua (incluido el hielo), de los equipos, contenedores y material de embalaje utilizados para el transporte garantice la inactivación del virus de la septicemia hemorrágica viral o la eliminación biosegura de acuerdo con los Capítulos 4.4., 4.8. y 5.5.
- 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..

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 garantice 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.1314.

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 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 [Patógeno X] cuando autoricen la importación (o el tránsito por su territorio) de las siguientes mercancías que han sido elaboradas y envasadas 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.
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**Anexo 16. – Ítem 5.10. – Cuestionario dirigido a los Miembros para la revisión del Capítulo 4.3.
Aplicación de la compartimentación**

**CUESTIONARIO PARA LOS MIEMBROS
REVISIÓN DEL CAPÍTULO 4.3. APPLICACIÓN DE LA COMPARTIMENTACIÓN**

Cuestionario desarrollado por la Comisión para los Animales Acuáticos para comentario de los Miembros.

Contexto

Desde hace algún tiempo se incluyó en el programa de trabajo de la Comisión para los Animales Acuáticos la necesidad de revisar el Capítulo 4.3. *Aplicación de la compartimentación* como parte de la revisión progresiva del Título 4 del *Código Acuático*. Durante la conferencia mundial sobre la sanidad de los animales acuáticos realizada en 2019, también se destacó esta necesidad y se incluyó como una actividad de la “Estrategia sobre la sanidad de los animales acuáticos (2021-2025)”.

En su reunión de febrero de 2022, la Comisión identificó como una prioridad en su plan de trabajo inmediato la revisión de este capítulo. El capítulo se volverá a elaborar para centrarse exclusivamente en la compartimentalización, mejorar las orientaciones brindadas a los Miembros y armonizarlo con otros capítulos nuevos y revisados tales como el Capítulo 4.1. *Bioseguridad en los establecimientos de acuicultura*. Cabe destacar la importancia del capítulo recientemente adoptado 1.4 sobre la vigilancia y los artículos modelo asociados X.X.4.-X.X.8. en los capítulos específicos de enfermedad, que tratan la declaración de ausencia de enfermedad y contienen una referencia específica a los requisitos para demostrar y mantener la ausencia de enfermedad a nivel del compartimiento. Por consiguiente, la revisión del Capítulo 4.3. *Aplicación de la compartimentación* constituye el siguiente paso lógico.

El actual Capítulo 4.2. *Zonificación y compartimentación* se adoptó en 1995 y se revisó en 2010. Por su parte, el Capítulo 4.3. *Aplicación de la compartimentación* se adoptó en 2010 y su última revisión data de 2016. En la revisión del capítulo, para la Comisión resultará útil conocer las experiencias de los Miembros a la hora de implementar esta norma y desarrollar los compartimentos. Se invita a los Miembros a comentar sus experiencias sobre las normas de compartimentación del *Código Acuático*. Para ello, le rogamos responda las siguientes preguntas.

Preguntas para los Miembros

- 1) ¿La autoridad competente o los servicios de sanidad de los animales acuáticos de su país han establecido compartimentos? De ser así, ¿con qué finalidad (cría, animales acuáticos o sus productos para consumo humano, comercio doméstico o internacional)?
- 2) Cuál ha sido la experiencia en su país al establecer compartimentos en términos de:
 - a) experiencias positivas (es decir, beneficios para la sanidad de los animales acuáticos o el comercio);
 - b) impedimentos para establecer los compartimentos;
 - c) utilidad de las normas del *Código Acuático* sobre la compartimentación (Capítulos 4.2. y 4.3.) (por ejemplo, información valiosa, brechas, énfasis u orientaciones particulares requeridas);
 - d) aceptación de los socios comerciales internacionales de los compartimentos establecidos.
- 3) ¿Usted ha desarrollado políticas o procedimientos de compartimentación a nivel nacional? De ser así, ¿podría compartir esas políticas o procedimientos con la Comisión para los Animales Acuáticos que los considerará cuando se revise el Capítulo 4.3.?
- 4) Ha consultado con la industria acerca del interés de desarrollar compartimentos? De ser así, ¿existe el interés para el establecimiento de compartimentos? ¿qué sectores de la industria han expresado interés? ¿cuál es la finalidad de los compartimentos propuestos?

SECTION 2.2.

DISEASES OF CRUSTACEANS

CHAPTER 2.2.0.

GENERAL INFORMATION

A. SAMPLING

1. Assessing the health status of the epidemiological unit

1.1. Sample material to be used for tests

Sample material and the number of samples to be collected depend on the specific disease or pathogen, the size of animals and the objective of testing (i.e. diagnosis of overt disease, detection of subclinical infection in apparently healthy animals or sampling for targeted surveillance to demonstrate freedom from infection with a specified pathogen). See the individual disease chapters in this *Aquatic Manual* for specific details of sample requirements.

1.2. Specifications according to crustacean populations

For details of animals to sample for a specific listed disease, see the relevant disease chapter in the *Aquatic Manual*. The design of a surveillance system for demonstrating disease-free status for a country, zone or compartment should be in accordance with the recommendations of the OIE *Aquatic Code* Chapter 1.4.

Animals to be sampled are selected as follows:

- i) Susceptible species should be sampled proportionately or following risk-based criteria for targeted selection of lots or populations with a history of abnormal mortality or potential exposure events (e.g. replacement with stocks of unknown disease status).
- ii) If more than one water source is used for production, animals from all water sources should be included in the sample.
- iii) For the study of presumptively diseased crustaceans select those animals that are moribund, discoloured, displaying abnormal behaviour, or otherwise abnormal.
- iv) When sampling is aimed at assessing disease occurrence (e.g. estimation of disease prevalence), the preferred selection method is probability sampling.

1.3. Specifications according to clinical status

In clinical disease episodes, carefully selected quality specimens with representative lesions should be obtained from live or moribund crustaceans. Collection of dead specimens during disease outbreaks should be avoided when possible, but recently dead samples may be suitable for some diagnostic assays. When cultured or wild crustacean stocks are presenting clinical signs of an active disease that are consistent with, or suggestive of, any one of the OIE-listed crustacean diseases, care should be taken to ensure that the samples collected are preserved appropriately for the anticipated diagnostic tests (see sample preservation section for recommended methods). In situations other than when clinical disease episodes are investigated, for the OIE-listed diseases it is highly recommended that the scheduling of sampling be planned (i.e. by farm schedule, season, etc.) so that the particular life-stage(s) are sampled at a time when the pathogen of concern is most likely to be detected. Disease-specific

recommendations are provided in Section 3 *Sample selection, sample collection, transportation and handling* of the individual chapters.

Recently dead crustaceans may be suitable (depending on their condition) for certain diagnostic assays such as nucleic acid detection techniques.

1.4. Specifications according to crustacean size

See the individual disease chapters in this *Aquatic Manual* for specific details of sample requirements.

2. General processing of samples

2.1. Macroscopic examination

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

2.2. Virological examination

2.2.1. Transportation and antibiotic treatment of samples

Culture systems for crustacean viruses are not available; antibiotic treatment of samples is not required. For transportation of samples see Section 3 of disease-specific chapters in this *Aquatic Manual*.

2.2.2. Virus isolation

For processing of tissues see Section 3 of disease-specific chapters in this *Aquatic Manual*.

2.2.3. Treatment to neutralise enzootic viruses

Not applicable.

2.3. Bacteriological examination

The strains of *Vibrio parahaemolyticus* (*Vp*_{AHPND}) that cause acute hepatopancreatic necrosis disease (AHPND) can be isolated on standard bacteriological media. *Hepatobacter penaei*, the causative agent of necrotising hepatopancreatitis (NHP) has not been cultured and, because of its very small size, bacteriological examination may be limited to Gram staining. See disease-specific chapters in this *Aquatic Manual* for identification methods.

2.4. Parasitic examination

Not applicable for currently listed diseases.

2.5. Fungal and other protists examination

See Chapter 2.2.2 *Infection with Aphanomyces astaci (Crayfish plague)*.

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

1. Crustacean viruses

1.1. Crustacean cell lines

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

1.2. Culture media

Not applicable.

1.3. Virus positive controls and antigen preparation

1.3.1. Virus nomenclature

In general, the virus nomenclature used in the disease-specific chapters follows the most recent taxonomy for viruses as given in the Report of the Committee on Taxonomy of Viruses (see: [ICTV \[ictvonline.org\]](http://ictvonline.org) for latest information). Also provided in the disease-specific chapters are the disease and virus names that are in common use by the shrimp/prawn farming industries, as well as the more common synonyms that have been used or are in current use.

1.3.2. Virus production

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

1.3.3. Virus preservation and storage

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

2. Crustacean bacteria

2.1. Culture media

See Chapter 2.2.1. Acute hepatopancreatic necrosis disease for details.

2.2. Storage of cultures

Lyophilisation or storage at -70°C is recommended for long-term storage of bacterial cultures.

3. Crustacean parasites

3.1. Culture media

Not applicable for currently listed diseases.

3.2. Storage of cultures

Not applicable for currently listed diseases.

4. Crustacean fungi and protists

4.1. Culture media

See chapter 2.2.2.

4.2. Storage of cultures

See chapter 2.2.2.

5. Techniques

The available diagnostic methods that may be selected for diagnosis of the OIE-listed crustacean diseases or detection of their aetiological agents are based on:

- i) Gross and clinical signs.
- ii) Direct bright-field, phase-contrast or dark-field microscopy with whole stained or unstained tissue wet-mounts, tissue squashes, and impression smears; and wet-mounts of faecal strands.
- iii) Histology of fixed specimens.
- iv) Bioassays of suspect or subclinical carriers using a highly susceptible host (life stage or species) as the indicator for the presence of the pathogen.
- v) Antibody-based tests for pathogen detection using specific antisera, polyclonal antibodies (PAbs) or monoclonal antibodies (MAbs).
- vi) Molecular methods (including sequencing):

DNA probes or RNA probes for *in-situ* hybridisation (ISH) assays with histological sections of fixed tissues;

Conventional and real-time PCR/RT-PCR and LAMP for direct assay with fresh tissue samples or with extracted DNA or RNA.

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 crustaceans should be processed and tested individually. However, for eggs, larvae and postlarvae pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50 to 150 postlarvae depending on their size/age) may be necessary to obtain sufficient sample material to run a diagnostic assay.

5.1. Antibody-based tests

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

5.2. Direct microscopy

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

5.3. Histological techniques

Only live or moribund specimens with clinical signs should be sampled for histology. Collect crustaceans by whatever means are available with a minimum of handling stress. Hold animals in a container appropriate for maintaining suitable water quality and supply adequate aeration to the container if the crustaceans are to be held for a short period of time before actual fixation.

5.3.1. Fixation

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

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

Davidson's AFA fixative is recommended for most histological applications. The fixative is rapid, reduces autolytic changes in decapod crustaceans (i.e. especially in crustaceans in tropical and subtropical regions), and its acidic content decalcifies the cuticle. The formulation for Davidson's AFA is (for 1 litre):

330 ml 95% ethyl alcohol

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

4. 115 ml glacial acetic acid

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

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

ii) Fixation procedures with Davidson's AFA

For larvae and postlarvae that are too small to be easily injected with fixative using a tuberculin syringe: Using a fine mesh screen or a Pasteur pipette, select and collect specimens. Immerse crustaceans selected for sampling directly in the fixative. Fix for 12–24 hours in fixative and then transfer to 70% ethyl alcohol for storage.

For juveniles that are too small to be injected: Select and collect specimens. Use a needle or fine-pointed forceps to incise the cuticle and immediately immerse crustaceans selected for sampling directly into the fixative. Fix for 12–24 hours in fixative and then transfer to 70% ethyl alcohol for storage.

For large juveniles and adults: to ensure proper fixation, kill the crustacean using a humane method, then immediately inject fixative (use 5–10% volume:weight). Fix for 12–24 hours in fixative and then transfer to 70% ethyl alcohol for storage.

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

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

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

For crustaceans larger than ~12 g: After injection of fixative, the body should then be transversely bisected, at least once, just posterior to the abdomen/cephalothorax junction, and (optional) again mid-abdominally.

*For very large crustaceans (e.g. lobsters, crabs, adult penaids, adult *Macrobrachium rosenbergii*, some species and life stages of crabs, crayfish, etc.):* The organs of interest may be excised after injection of fixative. Completion of fixation of these tissue samples is then handled as outlined previously.

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

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

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

iii) Transport and shipment of preserved samples

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

5.4. Transmission or scanning electron microscopy

Electron microscopy (EM – transmission or scanning) is a valuable research tool for the study of disease in crustaceans. However, EM methods are not routinely used for diagnosis of the diseases listed by the OIE.

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

Molecular techniques, including the use of nucleic acid probes for *in-situ* hybridisation, conventional PCR and real-time PCR, have been developed for the identification of many pathogens of aquatic animals. Real-time PCR methods, in general, have high sensitivity and specificity and, following adequate validation, can be used for direct detection of viral nucleic acids in samples prepared from crustacean tissue. The technique can be used in direct surveillance of apparently healthy populations, if they have a high level of diagnostic sensitivity, as well as in the diagnosis of clinically affected animals.

When using PCR as a diagnostic method, the design of primers and probe, the use of positive and negative controls, as well as validation of the PCR method chosen are important. Real-time PCR is a powerful technique particularly for analysing relatively high numbers of samples (e.g. for surveillance) via high-throughput testing. Several nucleic acid probe and PCR protocols are included in this version of the *Aquatic Manual* as screening, diagnostic or confirmatory methods for crustaceans and can be undertaken as the standard method. Following real-time PCR-positive results, where possible, conventional PCR with sequencing of PCR products should be used for confirmation of pathogen identity.

As with all PCR protocols, optimisation may be necessary depending on the reagents, equipment and the plasticware used. PCR is prone to false-positive and false-negative results. False-positive results (negative samples giving a positive reaction) may arise from either product carryover from positive samples or, more commonly, from cross-contamination by PCR products from previous tests. False-negative results (positive samples giving a negative result) may lead to unwanted transmission of pathogens and biosecurity failure.

Diagnostic samples should be tested in duplicate and both must produce positive results for a sample to be deemed positive. In instances where a sample produces one positive and one negative result, these are deemed indeterminate and should be retested. In addition, the following controls should be run with each assay: negative extraction control (e.g. a tissue [or equivalent sample that is under test]) sample from a known uninfected animal; positive control (preferably, one that can be distinguished from the pathogen genomic sequence [e.g. an artificial plasmid], thus allowing detection of any cross-contamination leading to a false positive result); no template control (all reagents with water replacing the template); internal positive control (internal housekeeping gene). All controls should produce their expected results in order for the diagnostic test result to be valid.

To minimise the risk of contamination, aerosol-preventing pipette tips should be used for all sample and PCR preparation steps. Additionally, all PCRs should be prepared in a clean area that is separate from the area where the amplifications and gel electrophoresis are performed. Do not share equipment (e.g. laboratory coats and consumables) between areas and, where possible, restrict access between areas. Contaminating PCR products can be carried on equipment, clothes, shoes and paper (e.g. workbooks). Also, ensure all work-tops and air-flow hoods used for the extractions and PCR set-up are regularly cleaned and decontaminated. To ensure sample integrity, always store the samples (e.g. in a freezer or refrigerator) in a location away from the molecular biology laboratory and reagents.

5.5.1. Sample preparation and types

Samples selected for nucleic acid-based or antibody-based diagnostic tests should be handled and packaged (in new plastic sample bags or bottles) with care to minimise the potential for cross-contamination among the sample set taken from different (wild or farmed) stocks, tanks, ponds, farms, etc. A water-resistant label, with the appropriate data filled out, should be placed within each package or container for each sample set.

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

- i) *Live specimens*: these may be processed in the field or shipped to the diagnostic laboratory for testing.
- ii) *Haemolymph*: this tissue is the preferred sample for certain molecular and antibody-based diagnostic tests (see disease-specific chapters). Samples may be collected by needle and syringe through cardiac

puncture, from the haemocoel (i.e. the ventral sinus in penaeids), or from a severed appendage, and immediately transferred to a tube that is half full with 90–95% ethanol or suitable nucleic acid preservative.

- iii) *Iced or chilled specimens*: these are specimens that can be transported to the laboratory for testing within 24 hours. Pack samples in sample bags surrounded by an adequate quantity of wet ice around the bagged samples in an insulated box and ship to the laboratory.
- iv) *Frozen whole specimens*: select live specimens according to the criteria listed in disease-specific chapters in this *Aquatic Manual*, quick freeze in the field using crushed dry-ice or freeze in the field laboratories using a mechanical freezer at –20°C or lower temperature. Prepare and insert the label into the container with the samples, pack samples with an adequate quantity of dry-ice in an insulated box, and ship to the laboratory.
- v) *Alcohol-preserved samples*: in regions where the storage and shipment of frozen samples is problematic, 90–95% ethanol may be used to preserve, store, and transport certain types of samples for molecular tests. Alcohol-preserved samples are generally not suitable for antibody-based tests. Whole crustaceans (any life stage provided the specimen is no larger than 2–3 g), excised tissues (i.e. pleopods) from large crustaceans, or haemolymph may be preserved in 90–95% ethanol, and then packed for shipment according to the methods described in Section 5.3.1, paragraph iii (see chapter 5.10 of the *Aquatic Code* for additional details on the international transport of such samples).

5.5.2. Preservation of RNA and DNA in tissues

For routine diagnostic testing by PCR or RT-PCR, samples must be prepared to preserve the pathogen's nucleic acid. For most purposes, preservation of samples in alcohol (80–90%) is the preferred method for subsequent molecular tests. Samples preserved in this way can be stored at 4°C for 1 month, at 25°C for 1 week or indefinitely at –20°C or below. In addition, other products (e.g. nucleic acid preservatives, various lysis buffers, etc.) are commercially available for the same purpose.

5.5.3. Nucleic acid extraction

To isolate nucleic acids from tissues preserved in ethanol or nucleic acid preservative, simply remove the tissue from the fixative or preservative and treat it as though it was just harvested. Most fresh and preserved or fixed tissues can be homogenised (e.g. with a mortar and pestle or in bead-beating tubes) directly in the lysis or extraction buffer provided with commercially available DNA and RNA extraction kits. Commercial kits should be validated or undergo equivalence testing with current validated extraction procedures prior to routine use.

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

For *in-situ* hybridisation, fixed tissues that have been transferred to 70% ethanol are embedded in paraffin according to standard histological methods. Sections are cut at a thickness of 5 µm and placed on aminoalkylsilane-coated slides, which are then baked overnight in an oven at 40°C. The sections are de-waxed by immersing in xylene for 10 minutes. This step is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each. The sections are then rehydrated by immersion in an ethanol series. The protocol may require a step of membrane permeabilisation enabling access to the target DNA. For this purpose, sections are treated with proteinase K (100 µg ml⁻¹) in TE buffer (Tris [50 mM], EDTA [10 mM]), at 37°C for 30 minutes. For *in-situ* hybridisation tests (see individual chapters for details), it is essential that both a known positive and a known negative slide be stained to eliminate false positive results due to non-specific staining/stain dropout, and false negative results due to errors in the staining protocol (Qadiri et al., 2019; Valverde et al., 2017).

For further details see disease-specific chapters in this *Aquatic Manual*.

6. Additional information to be collected

Sample information should include the collector's name, organisation, date, time, and description of the geographical location. The geographical origin of samples may be described as the name or location of the sampling site or its geographical co-ordinates. There should also be records that provide information to allow trace-backs on the sample movement from the sample site to the storage facility or laboratory and within those facilities.

A history of the specimens should also be collected and should include species, age, weight, details of clinical signs including behavioural changes, as well as observations concerning any gross pathology which has been observed.

Information on the preservation method, storage location, and date and time of storage at each storage locker or freezer along with information on the storage temperature (continuously monitored is preferable) should be collected. This information should be tracked with a unique sample code for all samples. For laboratories, the date of receipt, storage location information, date of analysis, analysis notes, and report date should be maintained for all uniquely coded samples. These data will greatly facilitate the tracking of sample problems and provide assurance that the samples were properly handled.

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

CHAPTER 2.2.1.

ACUTE HEPATOPANCREATIC NECROSIS DISEASE

1. Scope

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

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

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

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

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

2.1.2. Survival and stability in processed or stored samples

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

2.1.3. Survival and stability outside the host

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

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to AHPND according to Chapter 1.5. of the Aquatic Code are: giant tiger prawn (*Penaeus monodon*) and whiteleg shrimp (*Penaeus vannamei*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to AHPND according to Chapter 1.5. of the Aquatic Code are: fleshy prawn (*Penaeus chinensis*).

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

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

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

2.2.4. Distribution of the pathogen in the host

Gut including stomach, and hepatopancreas.

2.2.5. Aquatic animal reservoirs of infection

~~In experimental challenges, *Macrobrachium rosenbergii* and *Cherax quadricarinatus* did not show clinical signs of the disease or histopathological changes induced by AHPND but tested positive by PCR assay. However, whether these species serve as reservoirs of infection or are resistant to AHPND needs further investigation (Powers et al., 2021; Schofield et al., 2020). None known.~~

2.2.6. Vectors

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

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

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

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

2.3.2. Clinical signs, including behavioural changes

The onset of clinical signs and mortality can start as early as 10 days post-stocking. Clinical signs include: moribund prawns sink to bottom, 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).

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

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

Not available.

2.4.2. Chemotherapy including blocking agents

Not available.

2.4.3. Immunostimulation

None known to be effective.

2.4.4. Breeding resistant strains

Not available.

2.4.5. Inactivation methods

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

2.4.6. Disinfection of eggs and larvae

Not available.

2.4.7. General husbandry

As with other infectious diseases of shrimp, established good sanitary and biosecurity practices, such as improvement of hatchery sanitary conditions and PL screening are likely to be beneficial; good broodstock management, use of high-quality post-larvae and good shrimp farm management including strict feeding rate control, appropriate stocking density etc. are all well-established practices that reduce the impact of disease,

including AHPND. An AHPND-tolerant line of *P. vannamei* was recently reported, but at present (2022) no genetically improved lines are commercially available (Aranguren et al., 2020b).

3. Specimen selection, sample collection, transportation and handling

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

3.1. Selection of populations and individual specimens

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

3.2. Selection of organs or tissues

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

3.3. Samples or tissues not suitable for pathogen detection

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

3.4. Non-lethal sampling

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

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

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

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

3.5.4. Samples for other tests

Not applicable.

3.6. Pooling of samples

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

4. Diagnostic methods

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

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

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

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

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

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

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV
Wet mounts												
Histopathology		+	+	NA		+	+	NA				
Cell culture												
Real-time PCR	++	++	++	1	++	++	++	1	++	++	++	1
Conventional PCR	++	++	++	2	++	++	++	2	++	++	++	2
<u>Conventional PCR followed by amplicon sequencing</u>									+++	+++	+++	+2
<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 OIE Pathway (chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively; NA = Not available.

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

The disease has three distinct phases:

- i) The acute phase is characterised by a massive and progressive degeneration of the HP tubules from proximal to distal, with significant rounding and sloughing of HP tubule epithelial cells into the HP tubules, HP collecting ducts and posterior stomach in the absence of bacterial cells (Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013; 2014).
- ii) The terminal phase is characterised by marked intra-tubular haemocytic inflammation and development of massive secondary bacterial infections that occur in association with the necrotic and sloughed HP tubule cells (NACA, 2012; Nunan et al., 2014; Soto-Rodriguez et al., 2015; Tran et al., 2013; 2014).
- iii) The chronic phase is characterised by only a few tubules with epithelial necrosis accompanied by bacteria and inflammation. This phase resembles a septic hepatopancreatic necrosis (SHPN) (Aranguren et al., 2020b).

4.3. Cell culture for isolation

4.3.1. Enrichment of samples prior to DNA extraction

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

4.3.2. Agent purification

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

4.4. Nucleic acid amplification

PCR 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 should be checked using optical density or running a gel.

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

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

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

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

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

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

4.4.1. Real-time PCR

<u>Pathogen/ target gene</u>	<u>Primer/probe (5'-3')</u>	<u>Concentration</u>	<u>Cycling parameters</u>
<u>Method 1: Han et al., 2015b; GenBank KM067908</u>			
<u>pirA</u>	<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>95°C/20 sec; 45 cycles</u> <u>95°C/3 sec and</u> <u>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

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

4.4.2. Conventional PCR

<u>Pathogen/ target gene</u>	<u>Primer/probe (5'-3')</u>	<u>Concentration</u>	<u>Cycling parameters</u>
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Method 1 (AP1): Flegel & Lo, 2014; GenBank : KP324996; 700 bp			
pVA1	Fwd AP1F: 5CCT-TGG-GTG-TGC-TTA-GAG-GAT-G Rev AP1R: GCA-AAC-TAT-CGC-GCA-GAA-CAC-C	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
Method 2 (AP2): Flegel & Lo, 2014; GenBank : KP324996; 700 bp			
pVA1	Fwd AP2F: TCA-CCC-GAA-TGC-TCG-CTT-GTG-G Rev AP2R: CGT-CGC-TAC-TGT-CTA-GCT-GAA-G	0.2 μM each	94°C/5 min; 25–30 cycles of 94°C/30 sec, 60°C/30 sec, 72°C/60 sec; final extension step at 72°C/10 min. Reaction mixture can be held at 4°C
Method 3 (AP3): Sirikharin et al., 2015; GenBank: JALL01000066.1; 333 bp			
<u>pirA^{vp}</u>	Fwd AP3-F: ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC Rev AP3-R: GTG-GTA-ATA-GAT-TGT-ACA-GAA	0.2 μM each	94°C/5 min; 30 cycles of 94°C/30 sec, 53°C/30 sec, 72°C/40 sec; final elongation step at 72°C/7 min; Reaction mixture can be held at 4°C
Method 4 (TUMSAT-Vp3): Tinwongger et al., 2014; GenBank : AB972427; 360 bp			
pVA1	Fwd TUMSAT-Vp3 F: GTG-TTG-CAT-AAT-TTT-GTG-CA Rev TUMSAT-Vp3 R: TTG-TAC-AGA-AAC-CAC-GAC-TA	0.6 μM each	95°C/2 min; 30 cycles of 95°C/30 sec, 56°C/30 sec, 72°C/30 sec
Method 5 (VpPirA-284): Han et al., 2015a; GenBank : KM067908; 284 bp			
<u>pirA^{vp}</u>	Fwd VpPirA-284F: TGA-CTA-TTC-TCA-CGA-TTG-GAC-TG Rev VpPirA-284R: CAC-GAC-TAG-CGC-CAT-TGT-TA	0.2 μM each	94°C/3 min; 35 cycles of 94°C/30 sec, 60°C/30 sec, 72°C/30 sec; final extension 72°C/7 min
Method 6 (VpPirB-392): Han et al., 2015a; GenBank KM067908; 392 bp			
<u>pirB^{vp}</u>	Fwd VpPirB-392F: TGA-TGA-AGT-GAT-GGG-TGC-TC Rev VpPirB-392R: TGT-AAG-CGC-CGT-TTA-ACT-CA	0.2 μM each	94°C/3 min; 35 cycles of 94°C/30 sec, 60°C/30 sec, 72°C/30 sec; final extension 72°C/7 min
Method 7 (AP4): Dangtip et al., 2015; GenBank : JPKS01000000; 1269 bp			
<u>PirA and PirB toxin genes</u>	Fwd AP4-F1: ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC Rev AP4-R1: ACG-ATT-TCG-ACG-TTC-CCC-AA	0.2 μM each	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
Method 8 (AP4): Dangtip et al., 2015; GenBank : JPKS01000000; 230 bp			
<u>PirA and PirB toxin genes</u>	Fwd AP4-F2: TTG-AGA-ATA-CGG-GAC-GTG-GG Rev AP4-R2: GTT-AGT-CAT-GTG-AGC-ACC-TTC	0.2 μM each	94°C/2 min; 25 cycles of 94°C/20 sec, 55°C/20 sec, 72°C/20 sec; hold at 4°C

One-step PCR detection of pVA1 plasmid

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

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

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

Protocol for the AP1 and AP2 PCR methods

This protocol follows the method described by Flegel & Lo (2014). The PCR reaction mixture consists of 2.5 µl 10×PCR mix, 0.7 µl 50 mM MgCl₂, 0.4 µl 10 mM dNTPs, 0.5 µl 10 µM AP1/AP2F, 0.5 µl 10 µM AP1/AP2R, 0.2 µl Taq DNA polymerase and approximately 0.01–1 ng of template DNA in a total volume of 25 µl made up with distilled water. For PCR a denaturation step of 94°C for 5 minutes is followed by 25–30 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 60 seconds with a final extension step at 72°C for 10 minutes and then the reaction mixture can be held at 4°C (<https://enaca.org/publications/health/disease-cards/ahpnd-detection-method-announcement.pdf>).

One-step PCR detection of PirA/PirB toxin genes

Four one-step PCR methods (AP3, TUMSAT Vp3, VpPirA 284 and VpPirB 392) are described here for detection of Pir toxin genes in enrichment broth cultures. The primers, target gene and the size of the expected amplicons are listed in Table 4.4.2.2.

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 AG AP3 R: GTG CTA ATA GAT TGT ACA GAA	<i>pirA</i> ^{Vp}	333bp	Sirikharin et al., 2015
TUMSAT Vp3	TUMSAT Vp3 F: GTG TTG CAT AAT TTT CTG CA TUMSAT Vp3 R: TTG TAC AGA AAC CAC GAC TA	<i>pirA</i> ^{Vp}	360bp	Tinwongger et al., 2014
VpPirA-284	VpPirA-284F: TGA CTA TTC TCA CGA TTG GAC TG VpPirA-284R: CAC GAC TAG CGC CAT TGT TA	<i>pirA</i> ^{Vp}	284bp	Han et al., 2015a
VpPirB-392	VpPirB-392F: TGA TGA AGT GAT GGG TGC TC VpPirB-392R: TGT AAG CGC CGT TTA ACT GA	<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 Tinwengger 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 AAC 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: TTG AGA ATA CGG GAC GTG GG AP4 R2: GTT AGT CAT GTG AGC ACC TTC	230	

Analysis of conventional PCR products by agarose gel electrophoresis

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

4.4.3. 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 verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced, analysed and compared with published 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 recommended for demonstrating freedom from AHPND in an apparently healthy population.

6. Corroborative diagnostic criteria

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

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

6.1. Apparently healthy animals or animals of unknown health status ³

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

³ For example transboundary commodities.

equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

6.1.1. Definition of suspect case in apparently healthy animals

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

- i) A positive result by any of the real-time PCR
- ii) A positive result by or conventional PCR methods recommended in Table 4.1
- iii) A positive result by LAMP
- iv) Histopathology or cytopathological changes consistent with the presence of the pathogen or the disease
- v) A positive result by Ag-ELISA

6.1.2. Definition of confirmed case in apparently healthy animals

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

- i) Positive results by real-time PCR and conventional PCR followed by amplicon sequence analysis
- ii) Positive results by LAMP and conventional PCR followed by amplicon sequence analysis
- iii) Positive results by Ag-ELISA and conventional PCR followed by amplicon sequence analysis

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

6.2 Clinically affected animals

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

6.2.1. Definition of suspect case in clinically affected animals

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

- i) Gross pathology or clinical signs associated with the disease as described in this chapter, with or without elevated mortality
- ii) A positive result by real-time PCR
- iii) A positive result by conventional PCR
- iv) A positive result by LAMP
- v) A positive result by Ag-ELISA

6.2.2. Definition of confirmed case in clinically affected animals

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

- i) Positive results by real-time PCR and conventional PCR followed by amplicon sequence analysis
- ii) Positive results by LAMP and conventional PCR followed by amplicon sequence analysis
- iii) Positive results by Ag-ELISA and conventional PCR followed by amplicon sequence analysis

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

6.3. Diagnostic sensitivity and specificity for diagnostic tests

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

6.3.1. For presumptive diagnosis of clinically affected animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (n)	DSp (n)	Reference test	Citation
Conventional PCR	Diagnosis	Clinically diseased and apparently healthy shrimp	AHPND causing and non-causing bacterial isolates	<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, DS_p = 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 purpose	Source populations	Tissue or sample types	Species	DSe	DSp	Reference test	Citation

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

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NB: There are OIE Reference Laboratories for acute hepatopancreatic necrosis disease
(please consult the OIE 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 OIE Reference Laboratory for any further information on
acute hepatopancreatic necrosis disease

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

CHAPTER 2.2.2.

INFECTION WITH *APHANOMYCES ASTACI* (CRAYFISH PLAGUE)

1. Scope

Infection with *Aphanomyces astaci* means infection with the pathogenic agent *A. astaci*, Phylum Oomycota. The disease is commonly known as crayfish plague.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Aphanomyces astaci is a water mould. The Oomycetida or Oomycota, are considered protists and are classified with diatoms and brown algae in a group called the Stramenopiles or Chromista.

Five groups (A–E) of *A. astaci* have been described based on random amplification of polymorphic DNA polymerase chain reaction (RAPD PCR) (Dieguez-Uribeondo et al., 1995; Huang et al., 1994; Kozubikova et al., 2011). Additional geno- or haplotypes are still being detected using molecular methods (Di Domenico et al., 2021). Group A (the so called *Astacus* strains) comprises strains isolated from several European crayfish species. These strains are thought to have been in Europe for a long period of time. Group B (*Pacifastacus* strains I) includes isolates from several European crayfish species and from the invasive *Pacifastacus leniusculus* in Europe as well as Lake Tahoe, USA. Imported to Europe, *P. leniusculus* probably introduced this genotype of *A. astaci* and infected the native European crayfish. Group C (*Pacifastacus* strains II) consists of a strain isolated from *P. leniusculus* from Pitt Lake, Canada. Another strain (Pc), isolated from *Procambarus clarkii* in Spain, sits in group D (*Procambarus* strains). This strain shows temperature/growth curves with higher optimum temperatures compared with groups A and B (Dieguez-Uribeondo et al., 1995). *Aphanomyces astaci* strains that have been present in Europe for many years (group A strains) appear to be less pathogenic than strains introduced with crayfish imports from North America since the 1960s.

2.1.2. Survival and stability in processed or stored samples

Aphanomyces astaci is poorly resistant against desiccation and does not survive long in decomposing hosts. Any treatment of the crayfish (freezing, cooking, drying) will affect the survival of the pathogen (Oidtmann et al., 2002). Isolation from processed samples is not possible, however they may be suitable for molecular methods used for pathogen detection.

2.1.3. Survival and stability outside the host

Outside the host *Aphanomyces astaci* is found as zoospores that remain motile for up to 3 days and form cysts that can survive for 2 weeks in distilled water. As *A. astaci* can go through three cycles of encystment and zoospore emergence, the maximum life span outside of a host could be several weeks. Spores remained viable in a spore suspension in clean water kept at 2°C for 2 months (Unestam, 1966). Survival time is probably shorter in natural waters.

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

All stages of crayfish species native to Europe, including the noble crayfish (*Astacus astacus*) of north-west Europe, the white clawed crayfish (*Austropotamobius pallipes*) of south-west and west Europe, the related *Austropotamobius torrentium* (mountain streams of south-west Europe) and the slender clawed or Turkish crayfish (*Pontastacus leptodactylus*) of eastern Europe and Asia Minor are highly susceptible (e.g. Holdich et al., 2009). Australian species of crayfish are also highly susceptible. North American crayfish such as the signal crayfish (*Pacifastacus leniusculus*), Louisiana swamp crayfish (*Procambarus clarkii*) and *Faxonius* spp. are infected by *A. astaci*, but under normal conditions the infection does not cause clinical disease or death. All North American crayfish species investigated to date have been shown to be susceptible to infection, demonstrated by the presence of the pathogen in host cuticle (reviewed by Svoboda et al. 2017) and it is therefore currently assumed that this is the case for any other North American species.

The only other crustacean known to be susceptible to infection by *A. astaci* is the Chinese mitten crab (*Eriocheir sinensis*).

2.2.2. Species with incomplete evidence for susceptibility

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

The host species susceptible to infection with *A. astaci* fall largely into two categories: those highly susceptible to infection with development of clinical disease and mortalities, and those that are infected without associated clinical disease or mortalities.

Highly susceptible species: clinical disease outbreaks caused by infection with *A. astaci* are generally known as 'crayfish plague' outbreaks. In such outbreaks, moribund and dead crayfish of a range of sizes (and therefore ages) can be found.

2.2.4. Distribution of the pathogen in the host

The tissue that becomes initially infected is the exoskeleton cuticle. Soft cuticle, as is found on the ventral abdomen and around joints, is preferentially affected. In the highly susceptible European crayfish species, the pathogen often manages to penetrate the basal lamina located underneath the epidermis cell layer. From there, *A. astaci* spreads throughout the body primarily by invading connective tissue and haemal sinuses; however, all tissues may be affected.

In North American crayfish species, infection is usually restricted to the cuticle. Based on PCR results, the tailfan (consisting of uropods and telson) and soft abdominal cuticle appear to be frequently infected (Oidtmann et al., 2006; Vralstad et al., 2011).

2.2.5. Aquatic animal reservoirs of infection

North American crayfish species act mostly as carriers of the infection without showing clinical signs. However, some strains, especially from group A, show lowered virulence, thus enabling normally highly susceptible European crayfish to act as carriers as well (see review by Svoboda et al., 2017).

Colonisation of habitats, initially occupied by highly susceptible species, by North American crayfish species carrying *A. astaci* is likely to result in an epizootic among the highly susceptible animals.

2.2.6. Vectors

Transportation of finfish may facilitate the spread of *A. astaci* through the presence of spores in the transport water or co-transport of infected crayfish specimens (Alderman et al., 1987; Oidtmann et al., 2002). There is also circumstantial evidence of spread by contaminated equipment (e.g. nets, boots, clothing, traps) (Alderman et al., 1987).

2.3. Disease pattern

2.3.1. Mortality, morbidity, and prevalence

When the infection first reaches a naïve population of highly susceptible crayfish species, high levels of mortality are usually observed within a short space of time, so that in areas with high crayfish densities the bottoms of lakes, rivers and streams are covered with dead and dying crayfish. A band of mortality will spread quickly from the initial outbreak site downstream, whereas upstream spread is slower. Lower water temperatures are associated with slower mortalities and a greater range of clinical signs in affected animals (Alderman et al., 1987). Observations from Finland suggest that at low water temperatures, noble crayfish (*Astacus astacus*) can be infected for several months without the development of noticeable mortalities (Viljamaa-Dirks et al., 2013).

On rare occasions, single specimens of the highly susceptible species have been found after a wave of infection with *A. astaci* has gone through a river or lake. This is most likely to be due to lack of exposure of these animals during an outbreak (animals may have been present in a tributary of a river or lake or in a part of the affected river/lake that was not reached by spores, or crayfish may have stayed in burrows during the epizootic). However, low virulent strains of *A. astaci* have been described to persist in a water way, kept alive by a weak infection in the remnant population (Viljamaa-Dirks et al., 2011). Although remnant populations of susceptible crayfish species remain in many European watersheds, the dense populations that existed 150 years ago are now heavily diminished (Souty-Grosset et al., 2006; Holdich et al., 2009). Populations of susceptible crayfish may re-establish, but once population density and geographical distribution is sufficient for susceptible animals to come into contact with spores, new outbreaks of infection with *A. astaci* and large-scale mortalities will occur.

In the highly susceptible European crayfish species, exposure to *A. astaci* spores usually leads to infection and eventually to death. Prevalence of infection within a population in the early stage of an outbreak may be low (few animals in a river population may be affected). However, the pathogen is amplified in affected animals and subsequently released into the water; usually leading to 100% mortality in a contiguous population. The rate of spread from initially affected animals depends on several factors, one being water temperature. Therefore, the time from first introduction of the pathogen into a population to noticeable crayfish mortalities can vary greatly and may range from a few weeks to months. Prevalence of infection will gradually increase over this time and usually reach 100%. Data from a noble crayfish population in Finland that experienced an acute mortality event due to infection with *A. astaci* in 2001 suggest that in sparse noble crayfish populations, spread of disease throughout the host population may take several years (Viljamaa-Dirks et al., 2011).

2.3.2. Clinical signs, including behavioural changes

Susceptible species

Gross clinical signs are variable and depend on challenge severity and water temperatures. The first sign of an epizootic may be the appearance of crayfish during daylight (crayfish are normally nocturnal), some of which may show loss of co-ordination, falling onto their backs and remaining unable to right themselves. Occasionally, the infected animals can be seen trying to scratch or pinch themselves.

Often, however, the first sign of an outbreak may be the presence of large numbers of dead crayfish in a river or lake (Alderman et al., 1987).

Infection with *A. astaci* may cause mass mortality of crayfish. However, investigation of mortality event should consider other causes such as environmental pollution (e.g. insecticides such as cypermethrin have been associated with initial misdiagnoses).

North American crayfish species

Infected North American crayfish may be subclinical carriers. Controlled exposure to a highly virulent strain has resulted in mortality in juvenile stages of *Pacifastacus leniusculus* as well as behavioural alterations in adults (Thomas et al., 2020).

2.3.3 Gross pathology

Susceptible species

Depending on a range of factors, the foci of infection in crayfish may be seen by the naked eye or may not be discernible despite careful examination. Infection foci are best viewed under a low power stereo microscope and are recognisable by localised whitening of the muscle beneath the cuticle. In some cases, a brown colouration of cuticle and muscle may occur, or hyphae may be visible in infected cuticles in the form of fine brown (melanised) tracks in the cuticle. Sites for examination include the intersternal soft ventral cuticle of the

abdomen and tail, the cuticle of the perianal region, the cuticle between the carapace and abdomen, the joints of the pereiopods (walking legs), particularly the proximal joint, eyestalks and finally the gills.

North American crayfish species

Infected North American crayfish can sometimes show melanised spots in their soft cuticle, for example, the soft abdominal cuticle and joints. These melanisations can be caused by mechanical injuries or infections with other water moulds and are non-specific. However, populations with high levels of infection can show abnormally high levels of cuticular damage in individual animals, such as missing legs and claws due to deteriorated joints.

2.3.4. Modes of transmission and life cycle

The main routes of spread of the pathogen are through 1) movement of infected crayfish, 2) movement of spores with contaminated water or equipment, as may occur during movements of finfish, or 3) through colonisation of habitats by invasive North American crayfish species.

The main route of spread of *A. astaci* in Europe between the 1960s and 2000 was through the active stocking of North American crayfish into the wild or escapes from crayfish farms. Subsequent spread occurs through expanding populations of invasive North American crayfish, accidental co-transport of specimens, and release of North American crayfish into the wild by private individuals (Holdich et al., 2009).

Transmission from crayfish to crayfish occurs through the release of zoospores from an infected animal and attachment of the zoospores to naïve crayfish. The life cycle of *A. astaci* is simple with vegetative hyphae invading and ramifying through host tissues, eventually producing extramatrical sporangia that release amoeboid primary spores. These initially encyst, but then release a biflagellate zoospore (secondary zoospore). Biflagellate zoospores swim in the water column and, on encountering a susceptible host, attach and germinate to produce invasive vegetative hyphae. The zoospores of *A. astaci* swim actively in the water column and have been demonstrated to show positive chemotaxis towards crayfish (Cerenius & Söderhäll, 1984). Zoospores are capable of repeated encystment and re-emergence, extending the period of their infectivity (Soderhall & Cerenius 1999). Growth and sporulation capacity is strain-and temperature-dependent (Dieguez-Uribeondo et al., 1995).

2.3.5. Environmental factors

Under laboratory conditions, the preferred temperature range at which the *A. astaci* mycelium grows varies slightly depending on the strain. In a study, which compared several *A. astaci* strains that had been isolated from a variety of crayfish species, mycelial growth was observed between 4 and 29.5°C, with the strain isolated from *Procambarus clarkii* growing better at higher temperatures compared to the other strains. Sporulation efficiency was similarly high for all strains tested between 4 and 20°C, but it was clearly reduced for the non-*P. clarkii* strains at 25°C and absent at 27°C. In contrast, sporulation still occurred in the *P. clarkii* strain at 27°C. The proportion of motile zoospores (out of all zoospores observed in a zoospore suspension) was almost 100% at temperatures ranging from 4–18°C, reduced to about 60% at 20°C and about 20% at 25°C in all but the *P. clarkii* strain. In the *P. clarkii* strain, 80% of the zoospores were still motile at 25°C, but no motile spores were found at 27°C (Dieguez-Uribeondo et al., 1995).

Field observations show that outbreaks of infection with *A. astaci* occur over a wide temperature range, and at least in the temperature range 4–20°C. The rate of spread within a population depends on several factors, including water temperature. In a temperature range between 4 and 16°C, the speed of an epizootic is enhanced by higher water temperatures.

In buffered, redistilled water, sporulation occurs between pH 5 and 8, with the optimal range being pH 5–7. The optimal pH range for swimming of zoospores appears to be pH 6.0–7.5, with a maximum range between pH 4.5 and 9.0 (Unestam, 1966).

Zoospore emergence is influenced by the presence of certain salts in the water. CaCl_2 stimulates zoospore emergence from primary cysts, whereas MgCl_2 has an inhibitory effect. In general, zoospore emergence is triggered by transferring the vegetative mycelium into a medium where nutrients are absent or low in concentration (Cerenius et al., 1988).

2.3.6. Geographical distribution

In Europe the reports of large mortalities of crayfish go back to 1860. The reservoir of the original infections in the 19th century was never established. *Faxonius* (*Orconectes*) spp. were not known to have been introduced into Europe until the 1890s, but the post-1960s extensions are largely linked to more recent introductions of North American crayfish for farming (Alderman, 1996; Holdich et al. 2009). *Pacifastacus leniusculus* and *Procambarus clarkii* are now widely naturalised in many parts of Europe.

In recent years, crayfish plague has been reported in Asia and also in North- and South America (see e.g. references in Di Domenico et al. 2021). The distribution of *A. astaci* in North America is likely to be much wider than reported.

Any geographical area where North American crayfish species were introduced must be considered as potentially infected if not proven otherwise. Lack of clinical disease in these carrier species may hamper the reliability in reporting the infection. For the highly susceptible species, see OIE WAHIS (<https://wahis.woah.org/#/home>) for recent information on distribution at the country level. However, even high mortalities can go unnoticed in wild populations.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

No vaccines are available.

2.4.2. Chemotherapy including blocking agents

No treatments are currently known that can successfully treat the highly susceptible crayfish species, once infected.

2.4.3. Immunostimulation

No immunostimulants are currently known that can successfully protect the highly susceptible crayfish species against infection and consequent disease due to *A. astaci* infection.

2.4.4. Breeding resistant strains

A few studies suggest that there might be differences in resistance between populations of highly susceptible species (reviewed by Martin-Torrijos et al., 2017; Svoboda et al., 2017). The fact that North American crayfish generally do not develop clinical disease suggests that selection for resistance may be possible and laboratory studies using attenuated strains of *A. astaci* might be successful. However, there are currently no published data from such studies.

2.4.5. Inactivation methods

Aphanomyces astaci, both in culture and in infected crayfish, is inactivated by a short exposure to temperatures of 60°C or to temperatures of -20°C (or below) for 48 hours (or more) (Oidtmann et al., 2002). Sodium hypochlorite at 100 ppm, free chlorine and iodophors at 100 ppm available iodine, are effective for disinfection of contaminated equipment. Equipment must be cleaned prior to disinfection since organic matter decreases the effectiveness of disinfectants (Alderman & Polglase, 1985). Thorough drying of equipment (>24 hours) is also effective as *A. astaci* is not resistant to desiccation (Rennerfelt, 1936).

2.4.6. Disinfection of eggs and larvae

No information available.

2.4.7. General husbandry

If a crayfish farm for highly susceptible species is being planned, it should be carefully investigated whether North American crayfish species are in the vicinity of the planned site or present upstream. If North American crayfish are present, there is a high likelihood that susceptible farmed crayfish will eventually become infected.

In an endemic area where the highly susceptible species are being farmed, the following biosecurity recommendations should be followed to avoid an introduction of *A. astaci* onto the site:

1. General biosecurity should be in place (e.g. controlled access to premises; disinfection of boots when entering the site; investigation of mortalities if they occur; introduction of live animals (crayfish, finfish) only from sources known to be free from infection with *A. astaci*).
2. Movements of potentially infected live or dead crayfish, potentially contaminated water, equipment or any other item that might carry the pathogen from an infected to an uninfected site holding susceptible species should be prevented.
3. If transfers of finfish or crayfish are being planned, these should not come from streams or other waters that harbour potentially infected crayfish (either susceptible crayfish populations that are going through a current outbreak of infection with *A. astaci* or North American carrier crayfish).
4. North American crayfish should not be brought onto the site.
5. Finfish obtained from unknown freshwater sources or from sources, where North American crayfish may be present or a current outbreak of infection with *A. astaci* may be taking place, must not be used as bait or feed for crayfish, unless they have been subject to a temperature treatment to kill *A. astaci* (see Section 2.4.5. *Inactivation methods*).
6. Any equipment that is brought onto site should be disinfected.

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

For a suspected outbreak of infection with *A. astaci* in a population of highly susceptible crayfish species, sample crayfish should ideally consist of: a) live crayfish showing signs of disease, and b) live, apparently healthy crayfish. Freshly dead crayfish may also be suitable, although this will depend on their condition.

Live crayfish should be transported using insulated containers equipped with small holes to allow aeration. The temperature in the container should not exceed 16°C.

Crayfish should be transported in a moist atmosphere, for example using moistened wood shavings/wood wool, newspaper, or grass/hay. Unless transport water is sufficiently oxygenated, live crayfish should not be transported in water, as they may suffocate.

The time between sampling of live animals and delivery to the investigating laboratory should not exceed 24 hours.

Should only dead animals be found at the site of a suspected outbreak, freshly dead animals should be selected for diagnosis. Dead animals can either be: a) transported chilled (if they appear to have died only very recently), or, b) placed in non-methylated ethanol (minimum concentration 70%; see 3.5. *Preservation of samples for submission*), or c) placed in freezer at -20°C to avoid further decay and transported frozen.

When testing any population outside an acute mortality event for the presence of crayfish plague, as many individuals as possible should be inspected visually for signs of cuticular damage. Crayfish that have melanized spots or missing limbs should be selected in the first place for further analysis.

3.2. Selection of organs or tissues

In highly susceptible species, the tissue recommended for sampling is the soft abdominal cuticle, which can be found on the ventral side of the abdomen. Any other soft part of the exoskeleton can be included as well. If any melanized spots or whitened areas are detected, these should be included in the sampling. From diseased animals, samples should be aseptically collected from the soft abdominal cuticle. For identification of carriers, samples should be aseptically collected from soft abdominal cuticle, and telson and uropods, separately.

In the North American crayfish species, sampling of soft abdominal cuticle, uropods and telson are recommended. Any other soft part of the exoskeleton can be included as well. If any melanized spots are detected, these should be included in the sampling.

3.3. Samples or tissues not suitable for pathogen detection

Autolysed material is not suitable for analysis.

3.4. Non-lethal sampling

A non-destructive sampling method that detects *A. astaci* DNA in the microbial biofilm associated with the cuticle of individual crayfish through vigorous scrubbing has been described (Pavic et al., 2020), and could be considered in case of testing vulnerable populations.

3.5. Preservation of samples for submission

The use of non-preserved crayfish is preferred, as described above. If transport of recently dead or moribund crayfish cannot be arranged, crayfish may be frozen or fixed in ethanol (minimum 70%). However, fixation may reduce test sensitivity. The crayfish:ethanol ratio should ideally be 1:10 (1 part crayfish, 10 parts ethanol).

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

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). Isolation is best attempted from crayfish with clinical signs delivered alive (see Section 3.1.). Fresh specimens should be kept chilled and preferably sent to the laboratory within 24 hours of collection.

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.

3.5.3. Samples for histopathology

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

3.5.4. Samples for other tests

Sensitive molecular methods can be used to detect *A. astaci* DNA directly from water samples (Strand et al. 2011, 2012) These methods require validation for diagnostic use.

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 animals should be processed and tested individually. Small life stages such as PL, 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.

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

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

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV
Wet mounts						+	+	NA				
Histopathology						+	+	NA				
Cell-Culture						+	+	NA				
Real-time PCR	++	++	++	1	++	++	++	1	++	++	++	1
Conventional PCR	+	+	+	1	++	++	++	1				
Conventional PCR followed by amplicon sequencing									+++	+++	+++	1
<i>In-situ</i> hybridisation												
Bioassay												
LAMP												
Ab-ELISA												
Ag-ELISA												
Other antigen detection methods ³												
Other methods ³												

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

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

4.1. Wet mounts

Small pieces of soft cuticle excised from the regions mentioned above (Section 2.3.3 *Gross pathology*) and examined under a compound microscope using low-to-medium power will confirm the presence of aseptate fungal-like hyphae 7–9 µm wide. The hyphae can usually be found pervading the whole thickness of the cuticle, forming a three-dimensional network of hyphae in heavily affected areas of the cuticle. The presence of host haemocytes and possibly some melanisation closely associated with and encapsulating the hyphae give good presumptive evidence that the hyphae represent a pathogen rather than a secondary opportunist invader. In some cases, examination of the surface of such mounted cuticles will demonstrate the presence of characteristic *A. astaci* sporangia with clusters of encysted primary spores (see Section 4.3 *Culture for isolation*).

4.2. Histopathology

Unless the selection of tissue for fixation has been well chosen, *A. astaci* hyphae can be difficult to find in stained preparations. A histological staining technique, such as the Grocott silver stain counterstained with conventional haematoxylin and eosin, can be used. However, such material does not prove that any hyphae observed are those of *A. astaci*, especially when the material comes from animals already dead by sampling.

See also Section 4.1 *Wet mounts*.

4.3. Culture for isolation

Isolation is not recommended as a routine diagnostic method (Alderman & Polglase, 1986; Cerenius et al., 1987; Viljamaa-Dirks, 2006). Test sensitivity and specificity of the cultivation method can be very variable depending on the experience of the examiner, but in general will be lower than the PCR. Isolation of *A. astaci* by culture from apparently healthy crayfish is challenging and molecular methods are recommended. A detailed description of this test is available from the Reference Laboratory⁴.

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. Shrimp tissues may be used as negative controls.

Live crayfish can be killed using chloroform, electric current or by mechanical destroying the nerve cords. If live or moribund animals are not available, only recently dead animals should be used for DNA extraction. The soft abdominal cuticle is the preferred sample tissue for DNA extraction. Any superficial contamination should first be removed by thoroughly wiping the soft abdominal cuticle with wet (using autoclaved H₂O) clean disposable swabs. The soft abdominal cuticle is then excised and 30–50 mg ground using a pestle and mortar.

Several PCR assays have been developed with varying levels of sensitivity and specificity. Two assays are described here. Both assays target the ITS (internal transcribed spacer) region of the nuclear ribosomal gene cluster within the *A. astaci* genome.

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 should be checked using optical density or running a gel.

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.

⁴

<https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>

Pathogen/ target gene	Primer/probe (5'-3')	Concentration	Cycling conditions
Method 1*: Vralstad et al., 2009, Strand, 2013; GenBank Accession No. AM947024			
Aphanomyces astacus & <i>A. fennicus</i> ITS	Fwd: AAG-GCT-TGT-GCT-GGG-ATG-TT Rev: CTT-CTT-GCG-AAA-CCT-TCT-GCT-A Probe: 6-FAM-TTC-GGG-ACG-ACC-C-MGBNFQ	500 nM 200 nM	50 cycles of: 95°C/15 sec and 60°C/30 sec
Alternative method 2: Strand et al. to be published; GenBank Accession No. AM947024			
Aphanomyces astacus ITS	Fwd: TAT-CCA-CGT-GAA-TGT-ATT-CTT-TAT Rev: GCT-AAG-TTT-ATC-AGT-ATG-TTA-TTT-A Probe: FAM-AAG-AAC-ATC-CCA-GCA-C-MGBNFQ	500 nM 200 nM	50 cycles of: 95°C/15 sec and 60°C/30 sec

*These ITS-based methods have been found to give positive results for the species *Aphanomyces fennicus* (Viljamaa-Dirks & Heinikainen 2019).

The absolute limit of detection of method 1 was reported as approximately 5 PCR forming units (= target template copies), which is equivalent to less than one *A. astaci* genome (Vralstad et al., 2009). Another study reported consistent detection down to 50 fg DNA using this assay (Tuffs & Oidtmann, 2011).

Analytical test specificity has been investigated (Tuffs & Oidtmann, 2011; Vralstad et al., 2009) and no cross-reaction was observed in these studies. However, a novel species, *Aphanomyces fennicus*, isolated from noble crayfish was reported in 2019 (Viljamaa-Dirks & Heinikainen, 2019) that gave a positive reaction in this test at the same level as *A. astaci*. Due to this problem in specificity, the assay has been modified according to the alternative method 2 (Strand et al., manuscript in preparation):

Owing to the repeated discovery of new *Aphanomyces* strains, sequencing is required to determine the species of *Aphanomyces*. In the case of the real-time PCR assay, this requires separate amplification of a PCR product using primers ITS-1 and ITS-4 (see Section 4.5 Amplicon sequencing).

4.4.2. Conventional PCR

Pathogen/ target gene	Primer/probe (5'-3')	Concentration	Cycling conditions
Method 1*: Oidtmann et al., 2006; GenBank Accession No. AY310499 Product size: 569 bp			
Aphanomyces astacus & <i>A. fennicus</i> ITS	Fwd: GCT-TGT-GCT-GAG-GAT-GTT-CT Rev: CTA-TCC-GAC-TCC-GCA-TTC-TG-	500 nM	40 cycles of: 1 min/96°C, 1 min/59°C and 1 min/72°C

*This ITS-based method has been found to give positive results for the species *Aphanomyces fennicus* (Viljamaa-Dirks & Heinikainen 2019).

Confirmation of the identity of the PCR product by sequencing is required as a novel species, *A. fennicus*, isolated from noble crayfish was reported in 2019 (Viljamaa-Dirks & Heinikainen, 2019) that gave a positive reaction in this assay.

The assay consistently detects down to 500 fg of genomic target DNA or the equivalent amount of ten zoospores submitted to the PCR reaction (Tuffs & Oidtmann, 2011).

4.4.3. Other nucleic acid amplification methods

Several genotype-specific molecular methods have been developed that, instead of requiring a pure growth as sample material like the RAPD-PCR assay, can be used to analyse crayfish tissue directly (Di Domenico et al.,

2021; Grandjean et al., 2014; Makkonen et al., 2018; Minardi et al., 2018; 2019). Detection of a known genotype group combined with a positive result by a recommended conventional or real-time PCR can be used as a confirmative test in geographical areas where crayfish plague is known to be present. However, the current knowledge of the genotype variation is mostly limited to a few original host species and new genotypes or subtypes are expected to be found. Thus, the suitability of these methods is limited for initial excluding diagnosis or as confirmatory tests in geographical areas not known to be infected.

PCR targeting mitochondrial DNA with *A. astaci* genotype specific primers have been shown to detect the known genotypes of *A. astaci*, but these assays may also provide positive results for some other oomycete genera (Cassabella-Herrero et al., 2021).

4.5. Amplicon sequencing

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

4.6. *In-situ* hybridisation

Not available.

4.7. Immunohistochemistry

Not available

4.8. Bioassay

No longer used for diagnostic purposes (see Cerenius et al., 1988).

4.9. Antibody- or antigen-based detection methods

Not available.

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

The recommended method for surveillance is real-time PCR, the modified assay by Strand et al. (manuscript in preparation).

6. Corroborative diagnostic criteria

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

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

6.1. Apparently healthy animals or animals of unknown health status⁵

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

⁵ For example transboundary commodities.

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 *Aphanomyces astaci* shall be suspected if at least the following criterion is met:

- i. Positive result by real-time PCR
- ii. Positive result by conventional PCR

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with *Aphanomyces astaci* is considered to be confirmed if the following criterion is met:

- i) Positive result by real-time PCR and positive result 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 *Aphanomyces astaci* 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) Visual observation of hyphae indicative of *A. astaci*
- iii) Observation of hyphae indicative of *A. astaci* in stained histological sections
- iv) Culture and isolation of the pathogen
- v) Positive result by real-time PCR
- vi) Positive result by conventional PCR

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with *Aphanomyces astaci* is confirmed if the following criterion is met:

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

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *Aphanomyces astaci* are provided in Tables 6.3.1. and 6.3.2 (none available). This information can be used for the design of surveys for infection with *Aphanomyces astaci*, 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, DS_p = diagnostic specificity, n = number of samples used in the study.

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, DS_p = diagnostic specificity, n = number of samples used in the study.

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*
* *

NB: There is an OIE Reference Laboratory for infection with *Aphanomyces astaci* (crayfish plague)
(please consult the OIE 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 OIE Reference Laboratories for any further information on
infection with *Aphanomyces astaci* (crayfish plague)

NB: FIRST ADOPTED IN 1995; MOST RECENT UPDATES ADOPTED IN 2017.

CHAPTER 2.2.3.

INFECTION WITH HEPATOBACTER PENAEI (NECROTISING HEPATOPANCREATITIS)

1. Scope

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

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

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

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

2.1.2. Survival and stability in processed or stored samples

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

2.1.3. Survival and stability outside the host

No information available.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with *H. penaei* according to Chapter 1.5. of the Aquatic Animal Health Code (Aquatic Code) include—are: whiteleg shrimp (*P. vannamei*)

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with *H. penaei* according to Chapter 1.5. of the Aquatic Code include—are: aloha prawn (*P. marginatus*), banana prawn

(*P. merguiensis*), blue shrimp (*P. stylirostris*), giant tiger prawn (*P. monodon*), northern brown shrimp (*P. aztecus*), northern pink shrimp (*P. duorarum*) and northern white shrimp (*P. setiferus*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but an active infection has not been demonstrated: American lobster (*Homarus americanus*) (Avila-Villa et al., 2012; Bekavac et al., 2022).

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

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

2.2.4. Distribution of the pathogen in the host

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

2.2.5. Aquatic animal reservoirs of infection

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

2.2.6. Vectors

No vectors are known in natural infections.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Infection with *H. penaei* often causes an acute disease with very high mortalities in young juveniles, adults and broodstock. In horizontally infected young juveniles, adults and broodstock, the incubation period and severity of the disease are somewhat size or age dependent. 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 epibionts; 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 discolouration of the hepatopancreas with further size reduction.

2.3.4. Modes of transmission and life cycle

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

2.3.5. Environmental factors

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

2.3.6. Geographical distribution

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

See OIE WAHIS (<https://wahis.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 ($\text{Ca}(\text{OH})_2$) to treat the bottom of ponds during pond preparation before stocking can help reduce infection with *H. penaei*.

2.4.6. Disinfection of eggs and larvae

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

2.4.7. General husbandry

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

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

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

3.2. Selection of organs or tissues

Hepatobacter penaei infects most enteric tissue. The principal target tissue for *H. penaei* is the hepatopancreas and this organ should be selected preferentially (Lightner, 2012).

3.3. Samples or tissues not suitable for pathogen detection

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

3.4. Non-lethal sampling

Hepatobacter penaei can be detected in faeces samples collected from clinically affected populations of *Penaeus vannamei* may be collected and used for testing (usually by PCR), when non-lethal testing of valuable broodstock is necessary (Brinez et al., 2003; Frelier et al., 1993; Lightner, 1996). However, the use of faeces samples to detect NHP in apparently healthy shrimp has not been evaluated. Faeces samples have not been validated to the same level as hepatopancreas samples.

3.5. Preservation of samples for submission

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

3.5.1. Samples for pathogen isolation

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

3.5.2. Preservation of samples for molecular detection

Tissue samples of hepatopancreas or faeces for PCR testing should be preserved in 70–95% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1. The use of

lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen, but repeated freezing and thawing should be avoided.

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

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

3.5.4. Samples for other tests

No scientifically confirmed reports.

3.6. Pooling of samples

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

4. Diagnostic methods

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

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

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

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

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

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

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV
Wet mounts						+	+	NA				
Histopathology						++	++	NA				
Cell culture												
Real-time PCR	++	+++	+++	1	++	+++	+++	1	++	++	++	1
Conventional PCR	++	+++	+++	1	++	+++	+++	1	++	+++	+++	+
<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 OIE Pathway (chapter 1.1.2); NA = not available; PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.

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 initial *H. penaei* detection (e.g. by PCR or application of *H. penaei*-specific DNA probes or *in-situ* hybridisation [*ISH*] of histological sections).

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

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

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

4.3. Cell culture for isolation

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

4.4. Nucleic acid amplification

PCR 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 Flg E genes (Aranguren & Dhar, 2018; Aranguren et al., 2010; Loy et al., 1996).

Extraction of nucleic acids

Numerous different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid should be checked 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>Pathogen/ 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>16S rRNA gene</u>	<u>Fwd NHP1300F: CGT-TCA-CGG-GCC-TTG-TAC-AC</u> <u>Rev NHP1366R: GCT-CAT-CGC-CTT-AAA-GAA-AAG-ATA-A</u> <u>Probe: CCG-CCC-GTC-AAG-CCA-TGG-AA</u>	<u>300 nM</u> <u>100 nM</u>	<u>40 cycles:</u> <u>95°C/15 sec and</u> <u>60°C/1 min</u>
<u>Method 2: Aranguren & Dhar 2018; GenBank JQAJ01000001.1</u>			
<u>Flagella hook protein</u>	<u>Fwd NHP FlgE3qF: AAC-ACC-CTG-TCT-CCC-CAA-TTC</u> <u>Rev FlgE3qR: CCA-GCC-TTG-GAC-AAA-CAC-CTT</u> <u>Probe: CGC-CCC-AAA-GCA-TGC-CGC</u>	<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>

Protocol 1

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

- i) The PCR primers and TaqMan probe are selected from the 16S rRNA gene of *H. penaei* (GenBank U65509) (Ley & Frelier, 1996). The primers and TaqMan probe were designed by the Primer Express software version 2.0 (Applied Biosystems). The upstream (NHP1300F) and downstream (NHP1366R) primer sequences are: 5'-CGT-TCA-CGG-GCC-TTG-TACAC-3' and 5'-GCT-CAT-CGC-CTT-AAA-GAA-AAG-ATA-A-3', respectively. The TaqMan probe NHP: 5'-CCG-CCC-GTC-AAG-CCA-TGG-AA-3', which corresponds to the region from nucleotides 1321–1340, is synthesised and labelled with fluorescent dyes 6-carboxyfluorescein (FAM) on the 5' and N,N,N,Ntetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end.
- ii) The real time PCR reaction mixture contains: TaqMan One-step real time PCR SuperMix (Quanta Biosciences), 0.3 µM of each primer, 0.1 µM of TaqMan probe, 5–50 ng DNA, and water in a reaction volume of 25 µl. For optimal results, the reaction mixture should be vortexed and mixed well.
- iii) Amplification is performed with the master cycler Realplex 2.0 (Eppendorf). The cycling consists of initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. After each cycle, the levels of fluorescence are measured.
- iv) It is necessary to include a 'no template control' in each reaction run. This is to rule out the presence of fluorescence contaminants in the reaction mixture and also to rule out reagent contamination with the specific target of the assay. A positive control should also be included, and this can be plasmid DNA containing the target sequence, purified bacteria, or DNA extracted from *H. penaei* infected hepatopancreas.

Protocol 2

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

- i) The PCR primers and TaqMan probe were selected from the Flg E gene of *H. penaei* (GenBank JQAJ01000001.1) (Aranguren & Dhar, 2018). The primers and TaqMan probe were designed by the Primer Express software version 3.0 (Applied Biosystems). The upstream (NHP FlgE3qF) and downstream (FlgE3qR)

~~primer sequences are: 5' AAC ACC CTC TCT CCC CAA TTC 3'; and 5' CCA GCC TTG GAC AAA CAC CTT 3', respectively. The TaqMan probe NHP: 5' CGC CCC AAA GCA TGC CGC 3', is synthesised and labelled with fluorescent dyes 6-carboxyfluorescein (FAM) on the 5' and N,N,N,Ntetramethyl 6-carboxyrhodamine (TAMRA) on the 3' end.~~

- ii) ~~The real-time PCR reaction mixture contains: The amplification reactions were conducted as follows: 0.5 µM of each primer, 0.1 µM TaqMan probe, 1× TaqMan Fast Virus 1 Step Master Mix (Life Technologies), 5–50 ng DNA template and HPLC water in a reaction volume of 10 µl. For optimal results, the reaction mixture should be vortexed and mixed well.~~
- iii) ~~The real-time PCR profile consists of 20 seconds at 95°C followed by 40 cycles of 1 second at 95°C and 20 seconds at 60°C. Amplification detection and data analysis for real-time PCR assays are carried out with the StepOnePlus real-time PCR system (Life Technologies).~~
- iv) ~~It is necessary to include a 'no template control' in each reaction run. This is to rule out the presence of fluorescence contaminants in the reaction mixture and also to rule out reagent contamination with the specific target of the assay. A positive control should also be included, and this can be plasmid DNA containing the target sequence, or DNA extracted from *H. penaei*-infected hepatopancreas.~~

4.4.2. Conventional PCR

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

<u>Pathogen/ target gene</u>	<u>Primer/probe (5'-3')</u>	<u>Concentration</u>	<u>Cycling parameters</u>
<u>Method 1: Aranguren et al., 2010; GenBank: MH230908.1; 379 bp</u>			
<u>16S rRNA gene</u>	<u>Fwd NHPF2: CGT-TGG-AGG-TTC-GTC-CTT-CAG-T Rev NHPR2: GCC-ATG-AGG-ACC-TGA-CAT-CAT-C</u>	<u>200 nM</u>	<u>35 cycles: 95°C/30 sec, 60°C/30 sec and 72°C/30 sec</u>
<u>Method 2: Aranguren & Dhar, 2018; JQAJ01000001.1; 333 bp</u>			
<u>Flagella hook protein</u>	<u>Fwd FlgE 1143F: AGG-CAA-ACA-AAC-CCT-TG Rev FlgE 1475R: GCG-TTG-GGA-AAG-TT</u>	<u>0.2 µM</u>	<u>35 cycles: 95°C for 30 sec, 62°C for 30 sec, and 72°C 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 U µl⁻¹), magnesium chloride (1.5 mM) in 10 mM Tris-HCl, pH 9.0, 50 mM KCl.~~

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

Protocol 2

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

- i) The following controls should be included when performing the PCR assay a) known *H. penaei* negative tissue sample; b) a known *H. penaei* positive sample (hepatopancreas); and c) a 'no template' control.
- ii) The PuReTaq™ Ready To Go PCR Bead (RTG beads, GE Healthcare) is used for all amplification reactions described here.
- iii) The optimised PCR conditions (5–50 ng DNA) (final concentrations in 25 µl total volume) for detection of *H. penaei* in shrimp hepatopancreas samples are: primers (0.2 µM each), dNTPs (200 µM each), Taq polymerase (0.1 U µl⁻¹), magnesium chloride (1.5 mM) in 10 mM Tris HCl, pH 9.0, 50 mM KCl.
- iv) The cycling parameters are: initial denaturation at 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 5 minutes followed by 4°C infinite hold.

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

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

4.4.3. Other nucleic acid amplification methods

None.

4.5. Amplicon sequencing

The size of the PCR amplicon is verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced, analysed and compared with published 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 & Letz, 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 OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory, and if necessary, refer samples to that laboratory for testing.

6.1. Apparently healthy animals or animals of unknown health status⁶

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

6.1.1. Definition of suspect case in apparently healthy animals

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

- i) A positive result by real-time PCR
- ii) A positive result by conventional PCR

⁶ For example transboundary commodities.

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 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. 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, DS_p = 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, DS_p = diagnostic specificity, n = number of samples used in the study,
PCR: = polymerase chain reaction.

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*
* *

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

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

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

CHAPTER 2.2.4.

INFECTION WITH INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS VIRUS

1. Scope

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

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

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

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

2.1.2. Survival and stability in processed or stored samples

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

2.1.3. Survival and stability outside the host

No data.

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with IHHNV according to Chapter 1.5 of Aquatic Animal Health Code (Aquatic Code) are: yellowleg shrimp (*Penaeus californiensis*), giant tiger prawn

(*Penaeus monodon*), northern white shrimp (*Penaeus setiferus*), blue shrimp (*Penaeus stylirostris*), and white leg shrimp (*Penaeus vannamei*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with IHHNV according to Chapter 1.5 of the Aquatic Code are: northern brown shrimp (*Penaeus aztecus*). Evidence is lacking for this species to either confirm that the identity of the pathogenic agent is IHHNV, transmission mimics natural pathways of infection, or presence of the pathogenic agent constitutes an infection.

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following organisms, but an active infection has not been demonstrated: giant river prawn (*Macrobrachium rosenbergii*), northern pink shrimp (*Penaeus duorarum*), western white shrimp (*P. occidentalis*), kuruma prawn (*P. japonicus*), green tiger prawn (*P. semisulcatus*), *Hemigrapsus penicillatus*, Argentine stiletto shrimp (*Artemesia longinaris*), Cuata swimercrab (*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><i>Penaeus duorarum</i></u>	<u>northern pink shrimp</u>
	<u><i>Penaeus occidentalis</i></u>	<u>western white shrimp</u>
<u>Penaeidae</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>Portunoidea</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

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

2.2.4. Distribution of the pathogen in the host

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

2.2.5. Aquatic animal reservoirs of infection

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

2.2.6. Vectors

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

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

The effects of infection with IHHNV varies among shrimp species and populations, where infections can be either acute or chronic. For example, in unselected populations of *P. stylirostris*, infection with IHHNV results in acute, usually catastrophic, disease with mortalities approaching 100%. Vertically infected larvae and early postlarvae do not become diseased, but in approximately 35-day-old or older juveniles, gross signs of the disease may be observed, followed by mass mortalities. In horizontally infected juveniles, the incubation period and severity of the disease is somewhat size- or age-dependent, with young juveniles always being the most severely affected. Infected adults seldom show signs of the disease or mortalities (Bell & Lightner, 1984; 1987; Lightner, 1996; Lightner et al., 1983).

In contrast, in populations of *P. vannamei*, some selected lines of *P. stylirostris*, and some populations of *P. monodon*, infection with IHHNV results in a more subtle, chronic disease, runt-deformity syndrome (RDS), in which high mortalities are unusual, but where growth suppression and cuticular deformities are common (Kalagayan et al., 1991; Sellars et al., 2019). The severity and prevalence of RDS in infected populations of juvenile or older *P. vannamei* may be related to infection during the larval or early postlarval stages.

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

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

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

In regions where the virus is enzootic in wild stocks, the prevalence of IHHNV has been found in various surveys to range from 0 to 100%. Some reported mean values for IHHNV prevalence in wild stocks are: 26% and 46% in *P. stylirostris* in the lower and upper Gulf of California, respectively (Pantoja et al., 1999); 100% and 57%, respectively, in adult female and adult male *P. stylirostris* from the mid-region of the Gulf of California (Morales-Covarrubias et al., 1999); 28% in wild *P. vannamei* collected from the Pacific coast of Panama (Nunan et al., 2001); from 51 to 63% in *P. vannamei* collected from the Pacific coasts of Ecuador, Colombia and Panama (Motte et al., 2003), and from 6 to 63% in *P. vannamei* broodstock and 49.5% in post-larvae from Mexico (Fernando et al., 2016). In farms where IHHNV is present, its prevalence can range from very low to 100%, but high prevalence is typical (Aly et al., 2021; Chayaburakul et al., 2004; Lightner, 1996; Lightner et al., 1983).

2.3.2. Clinical signs, including behavioural changes

Animals with this disease may show one or more of these signs, but the pathogen may still be present in the absence of any signs. Clinical signs are non-specific, but juvenile *P. stylirostris* with acute infection with IHHNV show a marked reduction in food consumption, followed by changes in behaviour and appearance. Shrimp of this species infected with IHHNV have been observed to rise slowly in culture tanks to the water surface, where they become motionless and then roll-over and slowly sink (ventral side up) to the tank bottom. Shrimp exhibiting this behaviour may repeat the process for several hours until they become too weak to continue, or until they are attacked and cannibalised by their healthier siblings.

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

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

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

2.3.3. Gross pathology

*Infection with IHHNV in *Penaeus stylirostris**

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

*Infection with IHHNV in *Penaeus vannamei**

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

2.3.4. Modes of transmission and life cycle

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

2.3.5. Environmental factors

The replication rate of IHHNV at high water temperatures was significantly reduced in a study in which viral replication was compared in *P. vannamei* experimentally infected and held at 24°C and 32°C. After a suitable incubation period, shrimp held at 32°C had approximately 10² times lower viral load than shrimp held at 24°C (Montgomery-Brock et al., 2007).

2.3.6. Geographical distribution

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

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

See OIE WAHIS (<https://wahis.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

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

3.2. Selection of organs or tissues

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

3.3. Samples or tissues not suitable for pathogen detection

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

3.4. Non-lethal sampling

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

3.5. Preservation of samples for submission

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

3.5.1. Samples for pathogen isolation

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

3.5.2. Preservation of samples for molecular detection

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

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

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

3.5.4. Samples for other tests

Not relevant.

3.6. Pooling of samples

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

4. Diagnostic methods

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

Ratings for purposes of use. For each recommended assay a qualitative rating for the purpose of use is provided. The ratings are determined based on multiple performance and operational factors relevant to application of an assay for a

defined purpose. These factors include appropriate diagnostic performance characteristics, level of assay validation, availability cost, timeliness, and sample throughput and operability. For a specific purpose of use, assays are rated as:

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

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

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

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

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

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); NA = not available; PCR = polymerase chain reaction;

LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.

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-margined, 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 should be checked using optical density or running a gel.

There are multiple geographical variants of IHHNV, some of which are not detected by all of the some available methods. Two primer sets, 392F/R and 389F/R, are the most suitable for detecting all the known genetic variants of IHHNV (Tang et al., 2000; 2007). However, these tests also detect non-infectious endogenous viral elements (EVE) within the *P. monodon* genome (previously known as types 3A and 3B), which are inserted into the genome of certain stocks of *P. monodon* from the western Indo-Pacific, East Africa, Australia and India (Saksmerprome et al., 2011; Taengchaiyaphum et al., 2022; Tang & Lightner, 2006; Tang et al., 2007). As these PCR methods may result in positive test results in uninfected *P. monodon*, positive results should be confirmed by a method that detects IHHNV but not the IHHNV-related EVEs.

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

4.4.1. Real-time PCR

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

Real-time PCR methods have been developed for the detection of IHHNV (Dhar et al., 2001; Tang & Lightner, 2001). A highly sensitive SYBR Green real-time PCR targeting a segment of the IHHNV genome that is considered less susceptible to endogenisation was developed (Encinas-Garcia et al., 2015). More recently, a TaqMan real-time assay capable of differentiating 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 Acc. No AF218266			
IHHNV and IHHNV-related EVEs non-structural protein	Fwd IHHNV1608F: TAC-TCC-GGA-CAC-CCA-ACC-A Rev IHHNV1688R: GGC-TCT-GGC-AGC-AAA-GGT-AA Probe: FAM-ACC-AGA-CAT-AGA-GCT-ACA-ATC-CTC-GCC-TAT-TTG-TAMRA	300 nM primers 150 nM probe	40 cycles of: 95°C/1 sec and 60°C/20 sec

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

- i) The PCR primers and TaqMan probe are selected from a region of the IHHNV genomic sequence (GenBank AF218266) that encodes for a non-structural protein. The upstream (IHHNV1608F) and downstream (IHHNV1688R) primer sequences are: 5' TAC TCC GGA CAC CCA ACC A 3' and 5' GGC TCT GGC AGC AAA GGT AA 3', respectively. The TaqMan probe 5' ACC AGA CAT AGA GCT ACA ATC CTC GCC TAT TTG 3', is synthesised and labelled with FAM on the 5' end and TAMRA on the 3' end.
- ii) Preparation of DNA template: DNA extracted from tissues or haemolymph that was preserved in 95% ethanol and then dried. A control consisting of tissues or haemolymph from known negative animals should be included during the DNA extraction step. The DNA can be extracted by a variety of methods. Commercial DNA extraction kits include QIAamp DNA Mini Kit (Qiagen), MagMax™ Nucleic Acid kits (Life Technologies), or Maxwell® 16 Cell LEV DNA Purification Kit (Promega), or DNazol (Life Technologies). Spectrophotometric readings of the final DNA will indicate the purity of the DNA and the amount of total DNA extracted from the sample.
- iii) The real-time PCR reaction mixture contains: TaqMan Fast virus 1 step Master Mix (Life Technologies, or commercially available equivalent reagents), 0.3 µM of each primers, 0.15 µM of TaqMan probe, 5–50 ng DNA, and water in a reaction volume of 20 µl. For optimal results, the reaction mixture should be vortexed and mixed well.
- iv) The cycling profile is: initial denaturation of 20 seconds at 95°C, followed by 40 cycles of denaturation at 95°C for 1 second and annealing/extension at 60°C for 20 seconds.

4.4.2. Conventional PCR

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

Several one-step PCR methods (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

<u>Pathogen / target gene</u>	<u>Primer/probe (5'-3')</u>	<u>Concentration</u>	<u>Cycling parameters</u>
Method 1* Tang et al., 2007; GenBank Acc. No. AF218266; 389bp 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	<u>35 cycles of:</u> <u>94°C/30 sec, 60°C/30 sec,</u> <u>and 72°C/30 sec</u>
Method 2* Nunan et al., 2000; GenBank Acc. No AF218266; 356bp product			
IHHNV and IHHNV-related EVEs Between the non-structural and capsid protein-coding regions	Fwd 77012F: TAC-TCC-GGA-CAC-CCA-ACC-A Rev 77353R: GGC-TCT-GGC-AGC-AAA-GGT-AA	1000 nM	<u>35 cycles of:</u> <u>95°C/30 sec, 60°C/30 sec,</u> <u>and 72°C/30 sec</u>
Method 3* Tang et al., 2000; GenBank Acc. No AF218266; 392bp 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	<u>35 cycles of:</u> <u>95°C/30 sec, 60°C/30 sec,</u> <u>and 72°C/30 sec</u>
Method 4 Tang et al., 2007; GenBank Acc. No AF218266; 309bp 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	<u>35 cycles of:</u> <u>94°C/30 sec, 55°C/30 sec,</u> <u>and 72°C/30 sec</u>

***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 et al., 2000)	
309F	309 bp	TCC-AAC-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 et al., 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 et al., 2007)	

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

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

- i) Use as a template, the extraction of DNA templates is the same as that described above. Use 1–5 µl of extracted DNA as a template per 25 µl reaction volume.
- ii) The following controls should be included in every PCR assay for IHHNV: (a) DNA from a known negative tissue sample; (b) DNA from a known positive sample (either from tissue or haemolymph or from a plasmid clone that contains the fragment that the specific set of primers amplifies); and (c) a ‘no template’ control.
- iii) Use as primers, primers 389F and 389R, which elicit a band 389 bp in size from IHHNV-infected material, or primers 77012F and 77353R, which elicit a band 356 bp in size from IHHNV infected material. Prepare primers at 10 µM in distilled water.
- iv) If PuReTaq™ Ready To Go PCR Beads (GE Healthcare) are used, the PCR profile involves a 3–5 minutes at 95°C to denature DNA followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and final extension at 72°C for 5 minutes.
- v) Prepare a ‘Master Mix’ consisting of water and primers.
- vi) For a 25 µl reaction mix, add 24 µl Master Mix to each tube and then add 1 µl of the DNA template to be tested.
- vii) Vortex each tube, spin quickly to bring down all liquid. Insert tubes into the thermal cycler and start the PCR program.
- viii) After PCR, run 6–10 µl of the sample in a 1.5% agarose gel (containing SYBR™ Safe (Thermo Fisher Scientific) or equivalent to stain the DNA). Look for the 389 bp band (if using primers 389F and 389R) or for the 356 bp band (if using primers 77012F and 77353R). Bands are not always seen, as it is necessary to have at least 10 ng DNA µl⁻¹ to see DNA in a gel. A direct sequencing of amplified products can be performed through gel extraction of a PCR band with correct size and the sequencing primer(s) used for amplification to confirm the presence of IHHNV.

4.4.3. Other nucleic acid amplification methods

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

4.5. Amplicon sequencing

The size of the PCR amplicon is verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced, analysed and compared with published 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 test results are negative, then it is safe to conclude that the bioassay results are negative.

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

4.9. Antibody- or antigen-based detection methods

None has been successfully developed.

4.10. Other methods

Not available.

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

populations

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

6. Corroborative diagnostic criteria

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

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

6.1. Apparently healthy animals or animals of unknown health status⁷

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 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) ~~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

⁷ For example transboundary commodities.

- iii) Positive result by conventional PCR
- iii iv) Positive result by real-time PCR
- iv) ~~Histopathology consistent with IHHNV infection~~
- v) Positive result by *in-situ* hybridisation

6.2.2. Definition of confirmed case in clinically affected animals

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

- i) Positive result by real-time PCR and a positive result by conventional PCR targeting non-overlapping regions of the viral genome and followed by amplicon sequencing
- ii) ~~Histopathology consistent with IHHNV infection coupled with A positive result by~~ *in-situ* hybridisation and detection of IHHNV a positive result by real-time PCR
- iii) ~~Histopathology consistent with IHHNV infection coupled with A positive result by~~ *in-situ* hybridisation and detection of IHHNV by a positive result by conventional PCR and followed by amplicon sequencing

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

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

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with IHHNV is provided in Table 6.3.1 (none available). This information can be used for the design of surveys for infection with IHHNV, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data are only presented where tests are validated to at least level 2 of the validation pathway described in Chapter 1.1.2 and the information is available within published diagnostic accuracy studies.

6.3.1. For presumptive diagnosis of clinically affected animals

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

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

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* *

NB: There are OIE Reference Laboratories for infection with infectious hypodermal and haematopoietic necrosis virus
(please consult the OIE web site for the most up-to-date list:

<http://www.woah.org/en/scientific-expertise/reference-laboratories/list-of-laboratories/>).

Please contact the OIE Reference Laboratories for any further information on
infection with infectious hypodermal and haematopoietic necrosis virus

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

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, a member of the family *Totiviridae* (Fauquet et al., 2005; Lightner, 2011; Nibert, 2007; Poulos et al., 2006).

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

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

2.1.2. Survival and stability in processed or stored samples

No data.

2.1.3. Survival and stability outside the host

No information available.

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with IMNV according to Chapter 1.5 of *Aquatic Animal Health Code* (*Aquatic Code*) are: brown tiger prawn (*Penaeus esculentus*), banana prawn (*P. merguiensis*), and whiteleg shrimp (*P. vannamei*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with IMNV according to Chapter 1.5 of the *Aquatic Code* are: giant tiger prawn (*Penaeus monodon*) and blue shrimp (*P. stylirostris*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following organisms, but an active infection has not been demonstrated: southern brown shrimp (*P. subtilis*).

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

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

2.2.4. Distribution of the pathogen in the host

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

2.2.5. Aquatic animal reservoirs of infection

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

2.2.6. Vectors

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

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

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

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

2.3.2. Clinical signs, including behavioural changes

Affected shrimp present with visibly white tails. Such severely affected shrimp may have been feeding just before the onset of stress and may have a full gut. High mortality can occur suddenly and continue for several days. Clinical signs may have a sudden onset 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.

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.

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

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

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV
Wet mounts					+	+	+	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 OIE Pathway (chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification;

Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively; IFAT = indirect fluorescent antibody test. [give definitions of abbreviations as appropriate; nPCR = nested PCR, etc.

NB "RT-PCR" is reserved for reverse-transcription polymerase chain reaction methods. "real-time PCR" should always be stated in full and refers to probe-based and SYBR green assays]

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 should be checked using optical density or running a gel.

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

4.4.1. Real-time RT-PCR

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

Pathogen / target gene	Primer/probe (5'-3')	Concentration	Cycling parameters
Method 1: Andrade et al., 2007; GenBank Accession No. AY570982			

IMNV Capsid protein gene	Fwd IMNV412F: GGA-CCT-ATC-ATA-CAT-AGC-GTT-GCA Rev IMNV545R: AAC-CCA-TAT-CTA-TTG-TCG-CTG-GAT Probe: CCA-CCT-TTA-CTT-TCA-ATA-CTA-CAT-CAT-CCC-CGG	300 nM 200 nM	40 cycles of: 95°C/3 sec and 60°C/30 sec
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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 : KJ636783.2; 328/139 bp			
IMNV Capsid protein gene (nested-PCR)	Outer Fwd 4587F: CGA-CGC-TGC-TAA-CCA-TAC-AA Rev 4914R: ACT-CGG-CTG-TTC-GAT-CAA-GT Inner Fwd 4725 NF: GGC-ACA-TGC-TCA-GAG-ACA Rev 4863 NR: AGC-GCT-GAG-TCC-AGT-CTT-G	200 nM 620 nM	45 cycles of: 95°C/45 sec; 60°C/45 sec; 60°C/7 min 39 cycles of: 95°C/30 sec, 65°C/30 sec, 72°C/30 sec; 72°C/2 min

4.4.3. Other nucleic acid amplification methods

None.

4.5. Amplicon sequencing

The size of the PCR amplicon is verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced and analysed and compared with published 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 (Kunannopparat 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 a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory, and if necessary, refer samples to that laboratory for testing.

6.1. Apparently healthy animals or animals of unknown health status⁸

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
- ii) Positive result by real-time RT-PCR
- iii) 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
- iii) 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

⁸ For example transboundary commodities.

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
- iii) 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. 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 (30)	100 (30)	Histopathology	Andrade et al. (2007)

DSe = diagnostic sensitivity, DS_p = diagnostic specificity, n = number of samples used in the study,

PCR = polymerase chain reaction.

6.3.2. For surveillance of apparently healthy animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (n)	DSp (n)	Reference test	Citation
Real-time PCR								

DSe = diagnostic sensitivity, DS_p = diagnostic specificity, n = number of samples used in the study,

PCR = polymerase chain reaction.

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NB: At the time of publication (2022) there was no OIE Reference Laboratory for infection with infectious myonecrosis virus (please consult the OIE 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 sacrificing the animals and used as non-lethal sampling of genetically valuable broodstock.

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 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.

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.

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

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

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV
Wet mounts						+	+	NA				
Histopathology		+	+	NA	+	+	+	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 OIE 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 MAAb 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.

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 should be checked using optical density or running a gel.

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

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

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

Primer and probe sequences, real time RT-PCR

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

4.4.2. Conventional RT-PCR

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

Primer and probe sequences, conventional RT-PCR

Pathogen / target gene	Primer/probe (5'-3')	Concentration	Cycling parameters
Method 1 (Nunan et al., 1998); product size 231 bp			
ORF 2	Fwd: 9992: AAG-TAG-ACA-GCC-GCG-CTT	Primers/620 nM each	Reverse transcription 60°C/30 min 40 cycles:

	Rev:9195R: TCA-ATG-AGA-GCT-TGG-TCC		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 verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced and analysed and compared with published 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)⁹.
- 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 bromochloro-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 a suspect and confirmed case have been developed to support decision-making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory, and if necessary, refer samples to that laboratory for testing.

6.1. Apparently healthy animals or animals of unknown health status¹⁰

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.

⁹ Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this *Aquatic Manual*.

¹⁰ For example transboundary commodities.

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
- ii) A positive result by real-time RT-PCR
- iii) 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 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 one of the following criteria is met:

- i) A positive result by real-time RT-PCR and a positive result by conventional RT-PCR followed by sequencing of the amplicon
- ii) A positive result by *in-situ* hybridisation and a positive result by real-time RT-PCR
- iii) A positive result by *in-situ* hybridisation and a positive result by conventional RT-PCR followed by amplicon sequencing

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with TSV are provided in Table 6.3.1 (none currently available). 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, DS_p = 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, DS_p = diagnostic specificity.

7. References

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NB: There is an OIE Reference Laboratory for infection with Taura syndrome virus

(please consult the OIE 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 OIE Reference Laboratories for any further information on
infection with Taura syndrome virus

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

CHAPTER 2.2.8.

INFECTION WITH WHITE SPOT SYNDROME VIRUS

1. Scope

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

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

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

2.1.2. Survival and stability in processed or stored samples

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

2.1.3. Survival and stability outside the host

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

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

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

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

2.2.2. Species with incomplete evidence for susceptibility

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

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

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

2.2.4. Distribution of the pathogen in the host

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

2.2.5. Aquatic animal reservoirs of infection

Wild decapods known to be reservoirs of infection with WSSV include *Mysis* sp. (Huang et al., 1995), *Acetes* sp., *Alpheus* sp., *Callianassa* sp., *Expalaemon* sp., *Helice* sp., *Hemigrapsus* sp., *Macrophthalmus* sp., *Macrobrachium* sp., *Metaplexus* sp., *Orithya* 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 *Tachypleid* 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 Euphydriidae 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 gastrointestinal tract due to anorexia (Escobedo-Bonilla, 2008); delayed clotting of haemolymph (Heidarieh et al., 2013); excessive fouling of gills (Wu et al., 2013) and exoskeleton.

2.3.4. Modes of transmission and life cycle

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

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

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

2.3.5. Environmental factors

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

2.3.6. Geographical distribution

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

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

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

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

2.4.2. Chemotherapy including blocking agents

No published or validated methods.

2.4.3. Immunostimulation

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

2.4.4. Breeding resistant strains

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

2.4.5. Inactivation methods

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

2.4.6. Disinfection of eggs and larvae

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

2.4.7. General husbandry

Management practices in endemic areas principally involve the exclusion of WSSV from production populations or avoiding risk factors for development of clinical disease. Examples include avoiding 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.

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 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.

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

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

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV
Wet mounts												
Histopathology					+	+	+	1				
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					+	+	+	1				
Other methods												

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification;

Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively; IFAT = indirect fluorescent antibody test. [give definitions of abbreviations as appropriate; nPCR = nested PCR, etc.

NB "RT-PCR" is reserved for reverse-transcription polymerase chain reaction methods. "real-time PCR" should always be stated in full and refers to probe-based and SYBR green assays]

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 \times 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 should be checked 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. [to be included])			
WSSV ORF X	Fwd WSS1011F: TGG-TCC-CGT-CCT-CAT-CTC-AG Rev WSS1079R: GCT-GCC-TTG-CCG-GAA-ATT-A Probe: AGC-CAT-GAA-GAA-TGC-CGT-CTA-TCA-CAC-A	900 nM 900 nM 250 nM	45 cycles of: 95°C/15 sec and 60°C/1 min
Method 2 (Sritunyalucksana, 2006 ¹ ; GenBank Accession No. [to be included])			
WSSV ORF X	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 AGCCG-TAMRA	900 nM 900 nM 250 nM	45 cycles of: 95°C/15 sec and 60°C/1 min

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. , 1447/941 bp)			
WSSV (nested PCR)	Outer Fwd: ACT-ACT-AAC-TTC-AGC-CTA-TCTAG Rev: 146R1, 5'-TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A Inner Fwd 146F2: GTA-ACT-GCC-CCT-TCC-ATC-TCC-A Rev 146R2 TAC-GGC-AGC-TGC-TGC-ACC-TTG	100 pmol 100 pmol 100 pmol 100 pmol	39 cycles of 94°C/1 min, 55°C/1 min, 72°C/2 min 39 cycles of 94°C/1 min, 55°C/1 min, 72°C/2 min

Commercial PCR kits are available. Please consult the OIE Register for kits that have been certified by the OIE (<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

- 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).

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- ii) The primer sequences are WSSV-FIP: 5'-GGG-TCG-TCG-AAT-GTT-GCC-CAT-TTC-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-TTC-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, electrophoresis 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 verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced and analysed and compared with published 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 a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory, and if necessary, refer samples to that laboratory for testing.

6.1. Apparently healthy animals or animals of unknown health status¹¹

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

6.1.1. Definition of suspect case in apparently healthy animals

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

- i) Positive result by conventional PCR
- ii) Positive result by real-time PCR
- iii) Positive result by LAMP method

6.1.2. Definition of confirmed case in apparently healthy animals

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

- i) Positive results by real-time PCR and conventional PCR followed by amplicon sequencing
- ii) Positive results by LAMP and conventional PCR method followed by amplicon sequencing
- iii) Positive results by *in-situ* hybridisation and detection of WSSV by real-time PCR
- iv) Positive results by *in-situ* hybridisation and detection of WSSV by conventional PCR followed by amplicon sequencing

6.2 Clinically affected animals

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

6.2.1. Definition of suspect case in clinically affected animals

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

- i) Gross pathology or clinical signs consistent with the disease as described in this chapter, with or without elevated mortality
- ii) Histopathology consistent with WSSV infection
- iii) Positive result by conventional PCR
- iv) Positive result by real-time PCR
- v) Positive result by LAMP method
- vi) Positive result by *in-situ* hybridisation

6.2.2. Definition of confirmed case in clinically affected animals

¹¹ For example transboundary commodities.

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

6.3. 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%	100%	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%	100%	Real-time PCR	Moody et al., 2022

DSe = diagnostic sensitivity, DS_p = 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 merguiensis</i> , <i>P. esculentus</i> , <i>P. plebejus</i> , <i>Metapenaeus endeavouri</i> , <i>M. bennetiae</i>	76.8%	99.7%	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 merguiensis</i> , <i>P. esculentus</i> , <i>P. plebejus</i> , <i>Metapenaeus endeavouri</i> , <i>M. bennetiae</i>	82.9%	99.7%	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 merguiensis</i> , <i>P. esculentus</i> , <i>P. plebejus</i> , <i>Metapenaeus endeavouri</i> , <i>M. bennettae</i>	98.3%	99.4%	Bayesian latent class analysis	Moody et al., 2022
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DSe = diagnostic sensitivity, DS_p = diagnostic specificity, n = number of samples used in the study,
PCR = polymerase chain reaction.

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NB: There are OIE Reference Laboratories for infection with white spot syndrome virus
(please consult the OIE web site:

<https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/>.

Please contact the OIE 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

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
Belontiidae	<i>Strongylura kreffti</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 macrourus</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
	<i>Tilapia sparrmanii</i>	banded tilapia
Clariidae	<i>Clarias gariepinus</i>	sharptooth African catfish
	<i>Clarias ngamensis</i>	blunt-toothed African catfish
	<i>Clarius batrachus</i>	walking catfish
Clupeidae	<i>Alosa sapidissima</i>	American shad
	<i>Brevoortia tyrannus</i>	Atlantic menhaden
	<i>Nematalosa erebi</i>	bony bream
Cyprinidae	<i>Barbus paludinosus</i>	straightfin barb
	<i>Barbus poecilii</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
Eleotridae	<i>Rohtee sp.</i>	keti-Bangladeshi
	<i>Oxyeleotris lineolatus</i>	sleepy cod
	<i>Oxyeleotris marmoratus</i>	marble goby

Family	Scientific name	Common name
Gobiidae	<i>Glossogobius giuris</i>	bar-eyed goby
	<i>Glossogobius</i> sp.	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>	mullets
	<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</i> sp.	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
Sillaginæ	<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</i> sp.	therapon
Toxotidae	<i>Toxotes chatareus</i>	common archerfish

Family	Scientific name	Common name
	<i>Toxotes lorenzii</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. phanomyces invadans*, hyphae have also been observed in kidney, liver, spleen, pancreatic tissue, gut, parietal peritoneum, swim bladder, gonads, spinal cord, meninges, vertebrae, inter-muscular bones, the mouth region, and the orbits (Chinabut & Roberts, 1999; Vishwanath et al., 1998; Wada et al., 1996).

2.2.5. Aquatic animal reservoirs of infection

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

2.2.6. Vectors

No data available.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

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

2.3.2. Clinical signs, including behavioural changes

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

2.3.3 Gross pathology

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

2.3.4. Modes of transmission and life cycle

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

2.3.5. Environmental factors

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

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

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

2.3.6. Geographical distribution

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

in wild brown bullhead fish in North America. Infection with *A. invadans* has been reported from more than 20 countries in four continents: North America, Southern Africa, Asia and Australia.

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

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

There is no protective vaccine available.

2.4.2. Chemotherapy including blocking agents

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

2.4.3. Immunostimulation

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

2.4.4. Breeding resistant strains

No data available.

2.4.5. Inactivation methods

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

2.4.6. Disinfection of eggs and larvae

Routine 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 (Ibrahim et al., 2018). Samples should not be taken from the middle of large lesions as these are likely to be devoid of visible and viable hyphae. Instead, samples should be taken from the leading edge of the infected area or lesion and where possible, multiple samples should be taken from an infected individual to obtain viable hyphae. Fungal hyphae can be seen in tissue squash mounts and histological sections at the leading edge of the infected area. Attempting to culture *A. invadans* from severe ulcers is often constrained because of contaminating bacteria, but still should be attempted. PCR on tissue taken from the leading edge of the ulcer also should be attempted.

3.3. Samples or tissues not suitable for pathogen detection

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

3.4. Non-lethal sampling

None available.

3.5. Preservation of samples for submission

Fish specimens should be transported to the laboratory live or in ice-cooled boxes for further diagnosis. Samples must not be frozen since the fungus—*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.

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

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

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

LV = level of validation, refers to the stage of validation in the OIE Pathway (Chapter 1.1.2); NA = not available; PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification;

Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.

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

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

- i) Remove ulcer surface using a sharp scalpel blade.
- ii) Cut the muscular tissue at the edge of the ulcer.
- iii) Place the pieces of tissue on a cutting board then make thin slices using a sharp scalpel blade.
- iv) Place the thinly sliced tissue between two glass slides and squeeze gently with fingers.
- v) Remove one of the glass slides and cover the tissue with a cover-slip. View under a light microscope to find the nonseptate hyphae structure of *A. invadans* (12–25 µm in diameter).

4.2. Histopathology and cytopathology

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

- i) Sample only live or moribund specimens of fish with clinical lesions.
- ii) Take samples of skin/muscle (<1 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.

4.2.1. Histological procedure

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

4.2.2 Histopathological changes

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

4.3. Cell culture for isolation

4.3.1. Isolation of *Aphanomyces invadans* from internal tissues

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

Method 1: Moderate, pale, raised, dermal lesions are most suitable for oomycete isolation attempts. Remove the scales around the periphery of the lesion and sear the underlying skin with a red-hot spatula so as to sterilise the surface. Using a sterile scalpel blade and sterile fine-pointed forceps, cut through the stratum compactum underlying the seared area and, by cutting horizontally and reflecting superficial tissues, expose the underlying muscle. Ensure the instruments do not make contact with the contaminated external surface and thereby contaminate the underlying muscle. Using aseptic techniques, carefully excise pieces of affected muscle, approximately 2 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).

4.3.2. Identification of *Aphanomyces invadans*

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

4.3.3. Inducing sporulation in *Aphanomyces invadans* cultures

The induction of asexual reproductive structures is necessary for identifying oomycete cultures as members of the genus *Aphanomyces*. To induce sporulation, place an agar plug (3–4 mm in diameter) of actively growing mycelium in a Petri dish containing glucose/peptone/yeast (GY) broth and incubate for 4 days at approximately 20°C. Wash the nutrient agar out of the resulting mat by sequential transfer through five Petri dishes containing autoclaved pond water (Table 4.1.2), and leave overnight at 20°C in autoclaved pond water. After about 12 hours, the formation of achlyoid clusters of primary cysts and the release of motile secondary zoospores should be apparent under the microscope.

Table 4.1.2. Media for isolation, growth and sporulation of *Aphanomyces invadans* cultures

GP (glucose/peptone) medium	GPy (glucose/peptone/ yeast) broth	GY agar	GY agar (glucose/ yeast)	Autoclaved pond water
3 g litre ⁻¹ glucose	GP broth +	GY broth +	1% glucose,	Sample pond/lake water
1 g litre ⁻¹ peptone	0.5 g litre ⁻¹ yeast	12 g litre ⁻¹	0.25% yeast	known to support
0.128 g litre ⁻¹ MgSO ₄ .7H ₂ O	extract	technical agar	extract,	oomycete growth.
0.014 g litre ⁻¹ KH ₂ PO ₄			1.5% agar	Filter through Whatman
0.029 g litre ⁻¹ CaCl ₂ .2H ₂ O				541 filter paper. Combine
2.4 mg litre ⁻¹ FeCl ₃ .6H ₂ O				one part pond water with
1.8 mg litre ⁻¹ MnCl ₂ .4H ₂ O				two parts distilled water
3.9 mg litre ⁻¹ CuSO ₄ .5H ₂ O				and autoclave. pH to 6–7.
0.4 mg litre ⁻¹ ZnSO ₄ .7H ₂ O				

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 should be checked 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*.

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

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

Method 2

The species specific primer sites are located in the ITS1 and ITS2 regions. The forward primer is ITS11 (5'-GCC GAA GTT TCG CAA GAA AC 3') and the reverse is ITS23 (5'-CGT ATA GAC ACA AGC ACA CCA 3'). The PCR mixture contains 0.5 µM of each primer, 0.2 mM each deoxynucleoside triphosphate, 1.5 mM 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 CTG CTC ACC TCA CAC TC 3') and the reverse is BO639 (5' ACA CCA GAT TAC ACT ATC TC 3'). The PCR mixture contains 0.6 μ M 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 μ l of DNA template extracted from 25 mg of infected tissue and suspended in 100 μ l buffer) in a 50 μ l 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 verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced, analysed and compared with published 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 \times SET buffer [3.75 M NaCl, 25 mM EDTA (ethylene diamine tetra-acetic acid), 0.5 M Tris/HCl, pH 7.8]) containing 3% polyoxyethyl-enesorbitan monolaurate (Tween 20) is added to enhance tissue permeabilisation. The microtitre plate is gently agitated at room temperature on an orbital shaker (30 rpm) for 1.5 hours. The fixed tissues are rinsed (twice for 15 minutes each time) with 0.5 ml of hybridisation buffer (5 \times SET, 0.1% [v/v] Igepal-CA630 and 25 μ g 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 \times SET buffer containing 3% Tween 20 to remove residual probe. The tissue specimens are mounted onto poly-L-lysine-coated microscope slides. One drop of the light anti-fade solution is placed on the specimens, which are then overlaid with a cover-slip. Analyses are performed by light and epifluorescence microscopy. The camera and microscope settings for epifluorescent analyses are held constant so that comparative analyses of relative fluorescence intensity can be made between probed and non-probed specimens. The fluorescent oomycete hyphae appear as green fluorescence against the dark tissue background. The above detailed protocols are published by Vandersea et al. (2006). Using the FISH technique, *A. invadans* can be visualised very well in thinly sliced tissue compared with freshly squashed tissue.

4.7. Immunohistochemistry

None.

4.8. Bioassay

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

4.9. Antibody or antigen detection methods

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

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

4.10. Other methods

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

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

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

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

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

6. Corroborative diagnostic criteria

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

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

6.1. Apparently healthy animals or animals of unknown health status ¹²

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

¹² For example transboundary commodities.

equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

6.1.1. Definition of suspect case in apparently healthy 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*¹³
- ii) A positive result obtained by any of the diagnostic techniques described in Section 4.

6.1.2. Definition of confirmed case in apparently healthy populations

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

- i) Histopathology consistent with infection with *A. invadans* and positive result by PCR and amplicon sequencing
- ii) Histopathological changes consistent with infection with *A. invadans* and positive result for *in-situ* hybridisation
- iii) Artificial media culture and positive result by PCR and sequencing of the amplicon

6.2 Clinically affected animals

6.2.1. Definition of suspect case in clinically affected animals

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

- i) Gross pathology or clinical signs associated with infection with *A. invadans* as described in this chapter, with or without elevated mortality
- ii) Positive result by a recommended molecular detection test
- iii) Histological changes consistent with infection with *A. invadans*
- iv) Visual observation of hyphae characteristic (direct or by microscopy) of *A. invadans*
- v) Culture and isolation of *A. invadans*-type colonies

6.2.2. Definition of confirmed case in clinically affected animals

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

- i) Visualisation of hyphae under squash mounts and a positive result by PCR and sequencing of the amplicon
- ii) Histopathological changes consistent with infection with *A. invadans* and a positive result by PCR and sequencing of the amplicon
- iii) Histopathological changes consistent with infection with *A. invadans* and positive result for *in-situ* hybridisation
- iv) Artificial media culture and a positive result by PCR and sequencing of the amplicon
- v) Positive result for *in-situ* hybridisation and a positive result by PCR and sequencing of the amplicon

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

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *A. invadans* is provided in Table 6.3.1. (note: no data are currently available). This information can be used for the design of surveys

¹³ 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).

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, DS_p = 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, DS_p = diagnostic specificity.

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* *

NB: There is currently (2022) no OIE Reference Laboratories for infection with *Aphanomyces invadans*
(please consult the OIE 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.

Anexo 26. Ítem 7.2.2. – Capítulo 2.3.2. Infección por el virus de la necrosis hematopoyética epizoótica

CHAPTER 2.3.2.

INFECTION WITH EPIZOOTIC HAEMATOPOIETIC NECROSIS VIRUS

1. Scope

Infection with epizootic haematopoietic necrosis virus means infection with the pathogenic agent epizootic haematopoietic necrosis virus (EHNV) of the Genus *Ranavirus* of the Family *Iridoviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

EHNV is a species of the genus *Ranavirus* in the Family *Iridoviridae* (Chinchar et al., 2005). In addition to fish, ranaviruses have been isolated from healthy or diseased frogs, salamanders and reptiles in America, Europe and Australia (Chinchar, 2002; Drury et al., 2002; Fijan et al., 1991; Hyatt et al., 2002; Speare & Smith, 1992; Whittington et al., 2010; Wolf et al., 1968; Zupanovic et al., 1998). Ranaviruses have large (150–180 nm), icosahedral virions, a double-stranded DNA genome 150–170 kb, and replicate in both the nucleus and cytoplasm with cytoplasmic assembly (Chinchar et al., 2005).

Since the recognition of disease due to EHNV in Australia in 1986, similar systemic necrotising iridovirus syndromes have been reported in farmed fish. These include catfish (*Ictalurus melas*) in France (European catfish virus, ECV) (Pozet et al., 1992), sheatfish (*Silurus glanis*) in Germany (European sheatfish virus, ESV) (Ahne et al., 1989; 1990), turbot (*Scophthalmus maximus*) in Denmark (Bloch & Larsen, 1993), and cod (*Gadus morhua*) in Denmark (Cod iridovirus, CodV) (Ariel et al., 2010). EHNV, ECV, ESV, and CodV share >98% nucleotide identity across concatenated sequences across the RNR- α , DNAPol, RNR- β , RNase II and MCP gene regions (Ariel et al., 2010).

EHNV and ECV can be differentiated using genomic analysis (Ahne et al., 1998; Holopainen et al., 2009; Hyatt et al., 2000; Mao et al., 1996; 1997; Marsh et al., 2002). This enables epidemiological separation of disease events in finfish in Australia (EHNV) and Europe (ECV), and differentiation of these from ranavirus occurrences in amphibians.

2.1.2. Survival and stability in processed or stored samples

EHNV can persist in frozen fish tissues for more than 2 years (Langdon, 1989) and frozen fish carcases for at least a year (Whittington et al., 1996).

2.1.3. Survival and stability outside the host

EHNV is resistant to drying and remained infective for 97 days at 15°C and 300 days at 4°C in water (Langdon, 1989). For these reasons, it is presumed that EHNV would persist for months to years on a fish farm in water and sediment, as well as on plants and equipment.

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

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

Family	Scientific name	Common name
Esocidae	<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

Rainbow trout: The high case fatality rate and low prevalence of infection with EHNV in natural infections in rainbow trout means that the recruitment rate of carriers is likely to be very low (<2%) (Whittington et al., 1994). EHNV has been detected in growout fish but histopathological lesions consistent with infection with EHNV indicated an active infection rather than a carrier state (Whittington et al., 1999). Anti-EHNV serum antibodies were not detected in fingerlings during or after an outbreak but were detected in a low proportion of growout fish, hence, it is uncertain whether these were survivors of the outbreak (Whittington et al., 1994; 1999). There are data for European stocks of rainbow trout in experimental infections where potential carriers were identified (Ariel & Bang Jensen, 2009).

European perch: EHNV was isolated from 2 of 40 apparently healthy adult European perch during epizootics in juveniles in Victoria, Australia (Langdon & Humphrey, 1987), but as the incubation period extends for up to 28 days (Whittington & Reddacliff, 1995), these fish may have been in the preclinical phase.

2.2.6. Vectors

~~None demonstrated. Birds are potential vectors for EHNV, it being carried in the gut, on feathers, feet and the bill (Whittington et al., 1996).~~

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Rainbow trout: It appears that under natural farm conditions EHNV is poorly infective but once infected, ~~most fish succumb to the disease~~ has a high case fatality rate. Infection with EHNV may be present on a farm without causing suspicion because the mortality rate may not rise above the usual background rate. Infection with EHNV has most often been reported in young fingerlings <125 mm fork length with daily mortality of less than 0.2% and total mortality of up to 4%. However, rainbow trout of all ages may be susceptible, although infection has not yet been seen in broodstock (Whittington et al., 1994; 1999). There is a low direct economic impact because of the low mortality rate. Differences in susceptibility between European and Australian stocks of rainbow trout may exist (Ariel & Bang Jensen, 2009).

European perch: There is a very high rate of infection and mortality in natural outbreaks that, over time, leads to loss in wild fish populations (Langdon & Humphrey, 1987; Langdon et al., 1986; Whittington et al., 1996). Experimental bath inoculation with as few as 0.08 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). Differences in susceptibility between European and Australian stocks of European perch may exist (Ariel & Bang Jensen, 2009).

2.3.2. Clinical signs, including behavioural changes

Moribund fish may have loss of equilibrium, flared opercula and may be dark in colour (Reddacliff & Whittington, 1996). Clinical signs are usually more obvious in fingerlings and juvenile fish than adults of both rainbow trout and European perch. There may be clinical evidence of poor husbandry practices, such as overcrowding and suboptimal water quality, manifesting as skin, fin and gill lesions (Reddacliff & Whittington, 1996).

2.3.3 Gross pathology

There may be no gross lesions in affected fish. A small proportion of fish may have enlargement of kidney, liver or spleen. There may be focal white to yellow lesions in the liver corresponding to areas of necrosis (Reddacliff & Whittington, 1996).

2.3.4. Modes of transmission and life cycle

Rainbow trout: EHNV has spread between rainbow trout farms by transfer of infected fingerlings and probably transport water (Langdon et al., 1988; Whittington et al., 1994; 1999). The low prevalence of infection in rainbow trout means that active infection can easily go unrecognised in a population and be spread by trading fish. There are no data on possible vertical transmission of EHNV on or within ova, and disinfection protocols for ova have not been evaluated. EHNV has not yet been isolated from ovarian tissues or from broodstock. Annual recurrence in farmed rainbow trout may be due to reinfection of successive batches of fish or from wild European perch present in the same catchment.

European perch: The occurrence of infection with EHNV in European perch in widely separated river systems and impoundments suggested that EHNV was spread by translocation of live fish or bait by recreational fishers (Whittington et al., 2010).

The route of infection is unknown. European perch and rainbow trout are susceptible to immersion exposure. The virus infects a range of cell types including hepatocytes, haematopoietic cells and endothelial cells in many organs (Reddacliff & Whittington, 1996). Virus is shed into water from infected tissues and carcasses as they disintegrate.

2.3.5. Environmental factors

Rainbow trout: Outbreaks appear to be related to poor husbandry, particularly overcrowding, inadequate water flow and fouling of tanks with feed. Damage to skin may provide a route of entry for EHNV. Outbreaks have been seen on farms at water temperatures ranging from 11 to 20°C (Whittington et al., 1994; 1999). The incubation

period after intraperitoneal inoculation was 3–10 days at 19–21°C compared with 14–32 days at 8–10°C (Whittington & Reddacliff, 1995).

European perch: Natural epizootics of infection with EHNV affecting juvenile and adult European perch occur mostly in summer (Langdon & Humphrey, 1987; Langdon et al., 1986; Whittington et al., 1994). It has been assumed that the disease in juvenile fish is related to the annual appearance of large numbers of non-immune young fish and their subsequent exposure to the virus while schooling in shallow waters; adults are uncommonly involved in these outbreaks. It is possible that environmental temperature is the trigger for outbreaks as juvenile fish feed in warm shallow waters on planktonic fauna, whereas adults feed on benthic invertebrates and larger prey in deeper cooler water (Whittington & Reddacliff, 1995). Experimentally, the incubation period ranged from 10 to 28 days at 12–18°C compared with 10–11 days at 19–21°C, and adult perch were refractory to infection at temperatures below 12°C (Whittington & Reddacliff, 1995). European stocks of European perch also displayed temperature-dependent susceptibility (Ariel & Bang Jensen, 2009).

2.3.6. Geographical distribution

Infection with EHNV has been reported from rainbow trout farms within two river catchments in New South Wales, Australia (Whittington et al., 2010). Infection with EHNV is endemic in south-eastern Australia, with a discontinuous distribution (Whittington et al., 2010).

See WAHIS (<https://wahis.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 a bath exposure have shown that European perch from water bodies in New South Wales, Australia with previous EHNV infections showed lower mortality compared with European perch from neighbouring and distant water bodies in Australia that have no previous history of EHNV (Becker et al., 2016).

2.4.5. Inactivation methods

EHNV is susceptible to 70% ethanol, 200 mg litre⁻¹ sodium hypochlorite or heating to 60°C for 15 minutes (Langdon, 1989). Data for the inactivation of amphibian ranavirus may also be relevant: 150 mg/litre chlorhexidine and 200 mg/litre potassium peroxymonosulphate were effective after 1 minute contact time (Bryan et al., 2009). If it is first dried, EHNV in cell culture supernatant is resistant to heating to 60°C for 15 minutes (Whittington et al., 2010).

2.4.6. Disinfection of eggs and larvae

Not tested.

2.4.7. General husbandry

Disease control in rainbow trout at the farm level relies on reducing the impact of infection by maintaining low stocking rates and adequate water quality. Investigations on one rainbow trout farm indicated that ponds with

high stocking rates and low water flow, and thus poorer water quality, may result in higher levels of clinical disease compared with ponds on the same farm with lower stocking rates and higher water flow (Whittington et al., 1994). The mechanism of protection may be through maintenance of healthy integument (Whittington et al., 1994).

3. Specimen selection, sample collection, transportation and handling

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

3.1. Selection of populations and individual specimens

Clinical inspections should be carried out during a period when water temperature is conducive to development of clinical disease (see Section 2.3.5). All production units (ponds, tanks, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. For the purposes of disease surveillance, fish to be sampled are selected as follows:

- i) The most susceptible species (e.g. rainbow trout and European perch) should be sampled preferentially. Other susceptible species listed in Section 2.2.1 should be sampled proportionally.
- ii) Risk-based criteria should be employed to preferentially sample lots or populations with a history of abnormal mortality, potential exposure events or where there is evidence of poor water quality or husbandry. If more than one water source is used for fish production, fish from all water sources should be included in the sample.
- iii) If weak, abnormally behaving or freshly dead fish are present, such fish should be selected. If such fish are not present, the fish selected should include normal appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample.

For disease outbreak investigations, moribund fish or fish exhibiting clinical signs of infection with EHNV should be collected. Ideally fish should be collected while alive, however recently dead fish can also be selected for diagnostic testing. It should be noted however, that there will be a significant risk of contamination with environmental bacteria if the animals have been dead for some time.

3.2. Selection of organs or tissues

Liver, anterior kidney and spleen from individual fish are pooled (Jaramillo et al., 2012).

3.3. Samples or tissues not suitable for pathogen detection

Inappropriate tissues include gonads, gonadal fluids, milt and ova, ~~since because~~ there is no evidence of reproductive tract infection.

3.4. Non-lethal sampling

~~No~~-Non-lethal samples (blood, fin, gill, integument or mucous) are unsuitable for testing EHNV.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0.

3.5.1. Samples for pathogen isolation

For recommendations on transporting samples for virus isolation to the laboratory, see Section B.2.4 of Chapter 2.3.0 *General information (diseases of fish)*.

3.5.2. Preservation of samples for molecular detection

~~Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent grade (undiluted) 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 — <u>are most suitable with</u> desirable performance and operational characteristics.
++ =	Suitable Method(s) <u>are suitable with</u> acceptable performance and operational characteristics under most circumstances.
+ =	Less suitable Methods — <u>are suitable, but</u> performance or operational characteristics may significantly limit application <u>under some circumstances</u> .
Shaded boxes =	Not appropriate for this purpose.

~~The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities, repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.~~

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

~~OIE Reference Laboratories welcome feedback on diagnostic performance of recommended assays, in particular PCR methods. Of particular interest are any factors affecting expected assay sensitivity (e.g. tissue components inhibiting amplification) or expected specificity (e.g. failure to detect particular genotypes, detection of homologous sequences~~

within the host genome). These issues should be communicated to the OIE Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

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

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV
Wet mounts												
Histopathology					++	++	++	1				
Cytopathology												
Cell culture	+++	+++	+++	21	+++	+++	+++	21	±	±	±	21
Immunohistochemistry					+	+	+	1				
Real-time PCR	+++	+++	+++	21	+++	+++	+++	2	±	±	±	21
Conventional PCR	+	+	+	1	++	++	++	1	++	++	++	+
<u>Conventional PCR followed by amplicon sequencing</u>									+++	+++	+++	3-1
<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 OIE Pathway (chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.

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

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 ($175\text{ nm} \pm 6\text{ nm}$) nonenveloped icosahedral viruses are apparent; single viruses are also present. Complete viruses (containing electron-dense cores) bud/egress from the infected cells through the plasma membrane. The nuclei of infected cells are frequently located peripherally and are distorted in shape.

4.3. Cell culture for isolation

4.3.1. Preparation of fish tissues for virus isolation

A simple method for preparation of fish tissues for cell culture and ELISA has been validated (Whittington & Steiner, 1993) (see sampling Section 3).

- i) Freeze tubes containing tissues at –80°C until needed.
- ii) Add 0.5 ml of homogenising medium (minimal essential medium Eagle, with Earle's salts with glutamine) [MEM] with 200 International Units [IU] ml⁻¹ penicillin, 200 µg ml⁻¹ streptomycin and 4 µg ml⁻¹ amphotericin B) to each tube. Grind tissue to a fine mulch with a sterile fitted pestle.
- iii) Add another 0.5 ml of homogenising medium to each tube and mix with a pestle.
- iv) Add three sterile glass beads to each tube (3 mm diameter) and close the lid of the tube.
- v) Vortex the suspension vigorously for 20–30 seconds and place at 4°C for 2 hours.
- vi) Vortex the suspension again as above and centrifuge for 10 minutes at 2500 *g* in a benchtop microcentrifuge.
- vii) Transfer the supernatant, now called clarified tissue homogenate, to a fresh sterile tube. Homogenates may be frozen at –80°C until required for virus isolation and ELISA.

4.3.2. Cell culture/~~artificial media~~

EHNV ~~grows replicates~~ well in many fish cell lines including BF-2 (bluegill fry ATCC CCL 91), FHM (fathead minnow; ATCC CCL 42), EPC (*epithelioma papulosum cyprini* [Cinkova et al., 2010]), and CHSE-214 (Chinook salmon embryo cell line; ATCC CRL 1681) at temperatures ranging from 15 to 22°C (Crane et al., 2005). Incubation temperatures of 20°C or 24°C result in higher titres than 15°C; ~~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 OIE Reference Laboratory with an incubation temperature of 22°C. The procedure for BF-2 cells is provided below. A procedure for CHSE-214 cells is provided under immunoperoxidase staining below (Section 4.7). The identity of viruses in cell culture is determined by immunostaining, ELISA, immuno-electron microscopy, PCR 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 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 EHNV. P2 is incubated as above, and a third pass is conducted if necessary.

4.3.4. Interpretation of results

CPE is well developed and consists of focal lysis surrounded by rounded granular cells. This change extends rapidly to involve the entire monolayer, which detaches and disintegrates. Cell cultures can be tested for EHNV DNA using real-time PCR and conventional PCR with sequence analysis as described in Section 4.4. Antigen can be detected using immunocytochemistry in cell cultures with polyclonal antibodies and protocol available from the reference laboratory.

Cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

4.4. Nucleic acid amplification

Although several conventional PCR or quantitative real-time PCR methods have been described for the detection of ranaviruses (Jaramillo et al., 2012; Pallister et al., 2007; Stilwell et al., 2018), EHNV can only be detected when these methods are combined with methods that specifically detect EHNV, none has been adequately validated according to OIE guidelines for primary detection of EHNV. However, identification of ranavirus at genus and species level is possible using several published PCR strategies.

Samples can be screened by real-time PCR, but as the assays described are not specific for EHNV, identification of EHNV by conventional PCR and amplicon sequencing must be undertaken on any samples screening positive by real-time PCR. For testing by conventional PCR, two PCR assays using MCP primers are used with amplicon sequencing required to differentiate EHNV from ECV, FV3 and BIV (Marsh et al., 2002). Alternatively, PCR of the DNA polymerase gene and neurofilament triplet H1-like protein genes can be used (Holopainen et al., 2011) (this method is not described in this chapter).

Samples: virus from cell culture or direct analysis of tissue homogenate.

PCR assays should always be run with the controls specified in Section 2.5 Use of molecular techniques for surveillance testing, confirmatory testing and diagnosis of Chapter 2.3.0 General information (diseases of fish). Each 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 should be checked using optical density or by running a gel.

4.4.1. Real-time PCR

The ranavirus real-time screening protocol in use at the OIE 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 EHV-1 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 EHV-1 and other ranaviruses. The assay described by Jaramillo et al. (2012) uses SYBR Green detection chemistry and the assay described by Stilwell et al. (2018) was designed to detect multiple ranavirus species using hydrolysis probe detection chemistry.

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

Table 4.4.1.1. Ranavirus primer and probe sequences

Primer	Sequence (5'–3')	Reference
RANA-CON-F	5'-CTC ATC GTT CTG GCC ATC A-3'	
RANA-CON-R	5'-TCC CAT CGA GCC GTT CA-3'	Pallister et al., 2007
Probe		
RANA-CON-Pr	5'-6FAM-GAC AAC ATT ATC CGC ATC MGB-3'	
Primer		
C1096	GAC TGA CCA ACG CCA GCC TTA ACG	Jaramillo et al., 2012
C1097	GCG GTG GTG TAC CCA GAG TTG TCG	
Primer		
RanaF1	CCA GCC TGG TGT ACG AAA ACA	
RanaR1	ACT GGG ATG GAG GTG GCA TA	Stilwell et al., 2018
Probe		
RanaP1	6FAM-TGG GAG TCG AGT ACT AC-MGB	

Primer and probe sequences

Pathogen / target gene	Primer/probe (5'-3')	Concentration	Cycling parameters
Method 1 (Pallister et al., 2007)			

Ranavirus	Fwd: RANA CON: <u>CTC-ATC-GTT-CTG-GCC-ATC-A</u> Rev: RANA CON: <u>TCC-CAT-CGA-GCC-GTT-CA</u> Probe: RANA CON Pr <u>FAM-CAC-AAC-ATT-ATC-CGC-ATC-MGB</u>	<u>900 nM for each primer, 250 nM for probe</u>	<u>45 cycles of 95°C/15 sec; 60°C/60 sec</u>
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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.

Preparation of reagents

EHNV purified DNA and BIV purified DNA PCR control reagents are supplied by the reference laboratory in freeze-dried form. Reconstitute using 0.5 ml of Tris-EDTA (TE) buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and allow the vial to stand at RT for 2 minutes. Mix the vial very gently. For routine use, as a PCR control, it is recommended that working stocks be prepared as a 1/10 dilution in TE buffer (pH 8.0). Aliquots of 250 µl should be stored at -20°C. Each aliquot is sufficient for at least 50 reactions (1 to 5 µl added to cocktail) and has a minimum shelf life of 6 months from date of diluting.

Primers M151 and M152 (MCP-1, 321 bp), M153 and M154 (MCP-2, 625 bp) are supplied in working strength (100 ng µl⁻¹) and should be stored at -20°C. Primers can also be ordered from commercial suppliers. For primer sequences, refer to Table 4.4.2.1.

Table 4.4.2.1. MCP-1 and MCP-2 primer sequences

PCR assay	Primer	Sequence (5'–3')	Product size	Gene location
MCP-1	M151	AAC CCG GCT TTC GGG CAG CA	321 bp	266–586
	M152	CGG GGC GGG GTT GAT GAG AT		
MCP-2	M153	ATG ACC GTC GCC CTC ATC 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

Ingredients	Amount	Final concentration in 50 µl PCR mix
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. EHV-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>
Method 1: Product size MCP-1 is 321 bp and product size MCP-2 is 625 bp			
MCP-1 Gene location: <u>266-586</u>	M151: AAC-CCG-GCT-TTC-GGG-CAG-CA M152: CGG-GGC-GGG-GTT-GAT-GAG-AT	250 ng of each primer	35 cycles of 50°C for 30 sec NOTE: the annealing temperature may be increased to 60 or 62°C to reduce nonspecific amplification when the assay is used to test fish tissues.
MCP-2 Gene location: <u>842-1466</u>	M153: ATG-ACC-GTC-GCC-CTC-ATC-AC M154: CCA-TCG-AGC-CGT-TCA-TGA-TG		

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 verified by agarose gel electrophoresis, and purified by excision from this gel. Both DNA strands must be sequenced and analysed and compared with published 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 DAKO¹⁴. The primary affinity purified rabbit anti-EHNV antibody (Lot No. M708) is supplied freeze-dried by the OIE Reference Laboratory.

- i) Cut 5 µm sections and mount on SuperFrost® Plus G/Edge slides (Menzel-Glaser, HD Scientific Cat. No. HD 041300 72P3). Mark around the section with a diamond pencil to limit the spread of reagents.
- ii) Deparaffinise the section:
 - Preheat slides in a 60°C incubator for 30 minutes.
 - Place slides in a xylene bath and incubate for 5 minutes. Repeat once. Note that xylene replacements can be used without deleterious effects.
 - Tap off excess liquid and place slides in absolute ethanol for 3 minutes. Repeat once.
 - Tap off excess liquid and place slides in 95% ethanol for 3 minutes. Repeat once.
 - Tap off excess liquid and place slides in distilled or deionised water for 30 seconds.
- iii) Expose antigens using a protease treatment. Flood slide with proteinase K (5–7 µg ml⁻¹) and incubate for 20 minutes (ready-to-use solution, DakoCytomation Cat. No. S3020). Rinse slide by immersing three times in water. Place in a PBST bath for 5 minutes (PBS pH 7.2, 0.05% [v/v] Tween 20). Tap off the excess wash solution and carefully wipe around the section.
- iv) Perform the immunostaining reaction using the Universal DAKO LSAB[®] Kit, Peroxidase (DakoCytomation Cat. No. K0679). Ensuring the tissue section is completely covered, add the following reagents to the slide. Avoid drying out.
- v) 3% hydrogen peroxide: cover the section and incubate for 5 minutes. Rinse gently with PBST and place in a fresh wash bath.
- vi) Primary antibody (affinity purified rabbit anti-EHNV 1:1500 Lot No. M708) and negative control reagent (non-immune rabbit serum at a dilution of 1/1500) on a second slide. Cover the section and incubate for 15 minutes. Rinse slides.
- vii) Biotin-labelled secondary link antibody: Link: cover the section and incubate for 15 minutes. Rinse slides.
- viii) Streptavidin peroxidase: cover the section and incubate for 15 minutes. Rinse slides.
- ix) Substrate-chromogen solution: cover the section and incubate for 5 minutes. Rinse slides gently with distilled water.
- x) Counterstain by placing slides in a bath of DAKO® Mayer's Haematoxylin for 1 minute (Lillie's Modification, Cat. No. S3309). Rinse gently with distilled water. Immerse 10 times into a water bath. Place in distilled or deionised water for 2 minutes.
- xi) Mount and cover-slip samples with an aqueous-based mounting medium (DAKO® Faramount Aqueous Mounting Medium Cat. No. S3025).

Interpretation of results

¹⁴ 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.dakosytmahon.com> for links to other countries.

EHNV antigen appears as a brown stain in the areas surrounding degenerate and necrotic areas in parenchymal areas. There should be no staining with negative control rabbit serum on the same section.

Availability of test and reagents: antibody reagents and test protocols are available from the OIE Reference Laboratory.

4.8. Bioassay

Not applicable.

4.9. Antibody- or antigen-based detection methods

An antigen ELISA for detection of EHNV and an EHNV antibody detection ELISA have been described (Whittington & Steiner, 1993). The same antibodies are suitable for immunohistochemistry on fixed tissues and for detection of ranavirus antigen in cell culture. Reagents and protocols are available from the reference laboratory. It should be noted that polyclonal antibodies used in all related methods (immunoperoxidase, antigen-capture ELISA and immunoelectron microscopy) cross-react with all known ranaviruses except Santee Cooper ranaviruses (Ahne et al., 1998; Cinkova et al., 2010; Hedrick et al., 1992; Hyatt et al., 2000).

4.10. Other methods

Neutralising antibodies have not been detected in fish or mammals exposed to EHNV. Indirect ELISA for detection of antibodies induced following exposure to EHNV has been described for rainbow trout and European perch (Whittington et al., 1994; 1999; Whittington & Reddacliff, 1995). The sensitivity and specificity of these assays in relation to a standard test are not known and interpretation of results is difficult. Protocols and specific anti-immunoglobulin reagents required to conduct these tests are available from the reference laboratory.

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

Real-time PCR is the most appropriate method of screening healthy fish populations for EHNV; however, the available methods are not specific for EHNV. Any real-time PCR positive samples should be tested by conventional PCR and sequence analysis to distinguish EHNV from other ranaviruses.

6. Corroborative diagnostic criteria

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

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

6.1. Apparently healthy animals or animals of unknown health status¹⁵

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.

¹⁵ For example transboundary commodities.

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 and sequence analysis of the amplicon.

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

6.2 Clinically affected animals

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

6.2.1. Definition of suspect case in clinically affected animals

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

- i) Histopathology consistent with EHNV;
- ii) EHNV-typical CPE in cell cultures;
- iii) Positive real-time or conventional PCR result.

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with EHNV is considered to be confirmed if, in addition to the criteria in Section 6.2.1, at least one of the following criteria is met:

- i) EHNV-typical CPE in cell culture followed by identification of EHNV by conventional PCR and sequence analysis of the amplicon;
- ii) A positive result in tissue samples by real-time PCR and identification of EHNV by conventional PCR and sequence analysis of the amplicon.

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

6.3. Diagnostic sensitivity and specificity for diagnostic tests

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

6.3.1. For presumptive diagnosis of clinically affected animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (n)	DSp (n)	Reference test	Citation
Real-time PCR	Diagnosis	Clinically diseased fish (multiple species) from disease outbreaks and experimental infections	Pool of kidney, liver and spleen from individual fish	European perch (<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= 441)	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, DS_p = 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, DS_p = diagnostic specificity, qPCR: = real-time polymerase chain reaction.

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*
* *

NB: There is an OIE Reference Laboratory for infection with epizootic haematopoietic necrosis virus (EHNV)
(please consult the OIE 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 OIE Reference Laboratories for any further information on infection with EHNV.

The OIE Reference Laboratory can supply purified EHNV DNA, heat killed EHNV antigen
and polyclonal antibodies against EHNV together with technical methods.

A fee is charged for the reagents to cover the costs of operating the laboratory.

NB: FIRST ADOPTED IN 1995 AS EPIZOOTIC HAEMATOPOIETIC NECROSIS; MOST RECENT UPDATES ADOPTED IN 2018.

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>	<u>Jinsha bass carp</u>
	<i>Rutilus kutum</i>	Caspian white fish
Siluridae	<i>Rutilus rutilus</i>	Roach
	<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*), Australian mud oyster (*Ostrea angasi*), Chilean flat oyster (*Ostrea chilensis*), crested oyster (*Ostrea equestris*), eastern oyster (*Crassostrea virginica*), European flat oyster (*Ostrea edulis*), Olympia oyster (*Ostrea lurida*) and Suminoe oyster (*Magallana* [*syn. Crassostrea*] *ariakensis*).

2.2.1. 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 OSTREAЕ*

[...]

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with *Bonamia ostrea* according to Chapter 1.5. of the Aquatic Animal Health Code (Aquatic Code) are: European flat oyster (*Ostrea edulis*), 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 (*Ascidia 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; Nevea 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 (*Chamelea gallina*).

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

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

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

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

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

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