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COMISIÓN DE NORMAS SANITARIAS
PARA LOS ANIMALES ACUÁTICOS

Enmiendas propuestas al *Código Acuático* y al *Manual Acuático*

[Documento técnico de trabajo]



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I. Introducción

1. Desde la 90.^a Sesión General de mayo de 2023, la Comisión de Normas Sanitarias para los Animales Acuáticos (la Comisión para los Animales Acuáticos) se reunió dos veces, del 13 al 20 de septiembre de 2023 y del 14 al 21 de febrero de 2024. Entre sus actividades, y de conformidad con su plan de trabajo, la Comisión avanzó en la elaboración de textos nuevos y revisados del *Código Sanitario para los Animales Acuáticos* de la OMSA (en adelante, el *Código Acuático*) y del *Manual de las Pruebas de Diagnóstico para los Animales Acuáticos* de la OMSA (en adelante, el *Manual Acuático*). Toda la información detallada de las reuniones de la Comisión para los Animales Acuáticos está disponible en el sitio web de los Delegados y en el [sitio web de la OMSA](#).
2. El presente documento brinda una contextualización para cada uno de los textos nuevos y revisados del *Código Acuático* y del *Manual Acuático* que se propondrán para aprobación en la 91.^a Sesión General, en mayo de 2024. En el marco de este proceso de revisión, la Comisión tuvo en cuenta los comentarios enviados por los Miembros, las recomendaciones incluidas en numerosos informes de los grupos *ad hoc*, así como las observaciones de los expertos de los laboratorios de referencia. Igualmente, la Comisión para los Animales Acuáticos trabajó en cooperación con la Comisión de Normas Sanitarias para los Animales Terrestres en todas las actividades pertinentes.
3. La respuesta detallada de la Comisión en cuanto a los comentarios recibidos sobre los proyectos de texto distribuidos para comentario se facilitó en los informes de la Comisión de [septiembre de 2023](#) y de [febrero de 2024](#). **La Comisión insta a los Miembros a consultar dichos informes para obtener más detalles sobre los textos enmendados que se propondrán para adopción.**
4. Las enmiendas al *Código Acuático* y al *Manual Acuático* presentadas en los Anexos 4 a 6, 8 a 39 y 51 a 59 se propondrán para adopción en la 91.^a Sesión General. Los números de anexo utilizados en este documento coinciden con los indicados en el informe de febrero de 2024 de la Comisión.

1. Textos del Código Acuático propuestos para adopción

1.1 Uso de las definiciones de los términos del Glosario: «servicios de sanidad de los animales acuáticos», «autoridad competente» y «autoridad veterinaria» (Anexo 4)

5. Las enmiendas propuestas tienen por objeto garantizar el uso coherente de las definiciones revisadas del Glosario utilizadas en todas las secciones pertinentes del *Código Acuático*. Las definiciones revisadas del Glosario adoptadas en 2022 son: «servicios de sanidad de los animales acuáticos», «autoridad competente» y «autoridad veterinaria».
6. Estas modificaciones se elaboraron junto con la Comisión de Normas Sanitarias para los Animales Terrestres, que propuso enmiendas para el uso de «servicios veterinarios», «autoridad competente» y «autoridad veterinaria» en todo el *Código Terrestre*.
7. El texto revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
8. El texto revisado de la definición de «servicios de sanidad para los animales acuáticos», «autoridad competente» y «autoridad veterinaria» figura en el Anexo 4 y se propondrá para adopción en la 91.ª Sesión General, en mayo de 2024.

1.2 Uso de la definición del Glosario de «productos de animales acuáticos» (Anexo 5)

9. Las enmiendas propuestas consisten en sustituir «productos derivados de animales acuáticos» por la expresión definida en el Glosario «productos de animales acuáticos» en aras de coherencia con las definiciones del Glosario en todas las secciones pertinentes del *Código Acuático*.
10. El texto revisado se difundió dos veces para comentario; la primera vez, en el informe de la Comisión de septiembre de 2023.
11. El texto revisado de la definición de «productos de animales acuáticos» figura en el Anexo 5 y se propondrá para adopción en la 91.ª Sesión General, en mayo de 2024.

1.3 Artículo 1.1.5. del Capítulo 1.1. Notificación de enfermedades y aportación de datos epidemiológicos (Anexo 6)

12. La enmienda propuesta consiste en suprimir el Artículo 1.1.5., ya que, en la actualidad, las disposiciones se abordan en el Capítulo revisado 1.4. *Vigilancia de las enfermedades de los animales acuáticos*. Igualmente, esta modificación garantiza la armonización con el Capítulo 1.1. del *Código Terrestre*.
13. El texto revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
14. El Artículo revisado 1.1.5. del Capítulo 1.1. *Notificación de enfermedades y aportación de datos epidemiológicos* figura en el Anexo 6 y se propondrá para adopción en la 91.ª Sesión General, en mayo de 2024.

1.4 Artículo 1.3.1. del Capítulo 1.3. Enfermedades de la lista de la OMSA (Anexo 8)

15. Se propuso modificar la denominación de «infección por el virus de la dorada japonesa» por «infección por las especies del virus de la necrosis infecciosa del bazo y del riñón».
16. Los virus del género *Megalocytivirus* pueden causar enfermedades importantes en los peces, y este género contiene la especie del virus de la necrosis infecciosa del bazo y los riñones (ISKNV). Un genogrupo del ISKNV denominado iridovirus de la dorada japonesa (RSIV) causa la enfermedad «infección por RSIV», que figura en la lista de la OMSA. Además del RSIV, el ISKNV también incluye el genogrupo ISKNV y el genogrupo iridovirus del cuerpo rojizo del rodaballo (TRBIV).
17. La especie del virus ISKNV, incluidos sus tres genogrupos, se evaluó con arreglo a los criterios del Artículo 1.2.2. del Capítulo 1.2. *Criterios para la inclusión de las enfermedades de los animales acuáticos*. La Comisión acordó que los tres genogrupos del virus ISKNV cumplieran los criterios de inclusión 1, 2, 3 y 4b. Se propuso cambiar el nombre de la enfermedad en la lista por «infección por las especies del virus de la necrosis infecciosa del bazo y del riñón (ISKNV)» y se definiría para incluir los tres genogrupos ISKNV, RSIV y TRBIV.
18. El texto revisado se difundió cuatro veces; la primera vez, en el informe de la Comisión de septiembre de 2022.
19. El Artículo revisado 1.3.1. del Capítulo 1.3. *Enfermedades de la lista de la OMSA* figura en el Anexo 8 y se propondrá para adopción en la 91.ª Sesión General, en mayo de 2024.

1.5 Artículo 8.1.3. del Capítulo 8.1. Infección por *Batrachochytrium dendrobatidis* (Anexo 9)

20. Las enmiendas propuestas en el Artículo 8.1.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
21. El texto revisado se difundió cuatro veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2022.
22. El Artículo revisado 8.1.3. del Capítulo 8.1. *Infección por *Batrachochytrium dendrobatidis** figura en el Anexo 9 y se propondrá para adopción en la 91.ª Sesión General, en mayo de 2024.

1.6 Artículo 8.2.3. del Capítulo 8.2. Infección por *Batrachochytrium salmandrivorans* (Anexo 10)

23. Las enmiendas propuestas en el Artículo 8.2.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
24. El texto revisado se difundió cuatro veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2022.
25. El Artículo revisado 8.2.3. del Capítulo 8.2 *Infección por *Batrachochytrium salmandrivorans** figura en el Anexo 10 y se propondrá para adopción en la 91.ª Sesión General, en mayo de 2024.

1.7 Artículo 8.3.3. del Capítulo 8.3. Infección por las especies de Ranavirus (Anexo 11)

26. Las enmiendas propuestas en el Artículo 8.3.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
27. El texto revisado se difundió cuatro veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2022.
28. El Artículo revisado 8.3.3. del Capítulo 8.3. *Infección por las especies de Ranavirus* figura en el Anexo 11 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.8 Artículo 9.3.3. del Capítulo 9.3. Infección por el virus iridiscente de los decápodos tipo 1 (Anexo 12)

29. Las enmiendas propuestas en el Artículo 9.3.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
30. El texto revisado se difundió tres veces para comentario; la primera, en el informe de la Comisión de febrero de 2023.
31. El Artículo revisado 9.3.3. del Capítulo 9.3. *Infección por el virus iridiscente de los decápodos tipo 1* figura en el Anexo 12 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.9 Artículo 9.4.3. del Capítulo 9.4. Infección por Hepatobacter penaei (hepatopancreatitis necronizante) (Anexo 13)

32. Las enmiendas propuestas en el Artículo 9.4.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
33. El texto revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
34. El Artículo revisado 9.4.3. del Capítulo 9.4. *Infección por Hepatobacter penaei (hepatopancreatitis necronizante)* figura en el Anexo 13 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.10 Artículo 9.6.3. del Capítulo 9.6. Infección por el virus de la mionecrosis infecciosa (Anexo 14)

35. Las enmiendas propuestas en el Artículo 9.6.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
36. El texto revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
37. El Artículo revisado 9.6.3. del Capítulo 9.6. *Infección por el virus de la mionecrosis infecciosa* figura en el Anexo 14 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.11 Artículo 9.7.3. del Capítulo 9.7. Infección por el nodavirus *Macrobrachium rosenbergii* (Anexo 15)

38. Las enmiendas propuestas en el Artículo 9.7.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
39. El texto revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
40. El Artículo revisado 9.7.3. del Capítulo 9.7. *Infección por el nodavirus *Macrobrachium rosenbergii** figura en el Anexo 15 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.12 Artículo 9.8.3. del Capítulo 9.8. Infección por el virus del síndrome de *Taura* (Anexo 16)

41. Las enmiendas propuestas en el Artículo 9.8.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
42. El texto revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
43. El Artículo revisado 9.8.3. del Capítulo 9.8. *Infección por el virus del síndrome de *Taura** figura en el Anexo 16 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.13 Artículo 10.1.3. del Capítulo 10.1. Infección por el virus de la necrosis hematopoyética epizoótica (Anexo 17)

44. Las enmiendas propuestas en el Artículo 10.1.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
45. El texto revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
46. El Artículo revisado 10.1.3. del Capítulo 10.1. *Infección por el virus de la necrosis hematopoyética epizoótica* figura en el Anexo 17 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.14 Artículo 10.2.3. del Capítulo 10.2. Infección por *Aphanomyces invadans* (síndrome ulcerativo epizoótico) (Anexo 18)

47. Las enmiendas propuestas en el Artículo 10.2.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
48. El texto revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.

49. El Artículo revisado 10.2.3. del Capítulo 10.2. *Infección por Aphanomyces invadans (síndrome ulcerativo epizoótico)* figura en el Anexo 18 y se propondrá para adopción en la 91.ª Sesión General, en mayo de 2024.

1.15 Artículo 10.3.3. del Capítulo 10.3. Infección por Gyrodactylus salaris (Anexo 19)

50. Las enmiendas propuestas en el Artículo 10.3.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
51. El texto revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
52. El Artículo revisado 10.3.3. del Capítulo 10.3. *Infección por Gyrodactylus salaris* figura en el Anexo 19 y se propondrá para adopción en la 91.ª Sesión General, en mayo de 2024.

1.16 Artículo 10.4.3. del Capítulo 10.4. Infección por el virus de la anemia infecciosa del salmón (Anexo 20)

53. Las enmiendas propuestas en el Artículo 10.4.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
54. El texto enmendado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
55. El Artículo revisado 10.4.3. del Capítulo 10.4. *Infección por el virus de la anemia infecciosa del salmón* figura en el Anexo 20 y se propondrá para adopción en la 91.ª Sesión General, en mayo de 2024.

1.17 Artículo 10.5.3. del Capítulo 10.5. Infección por el alfavirus de los salmónidos (Anexo 21)

56. Las enmiendas propuestas en el Artículo 10.5.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
57. El texto revisado se difundió tres veces; la primera vez, en el informe de la Comisión de febrero de 2023.
58. El Artículo revisado 10.5.3. del Capítulo 10.5. *Infección por alfavirus de los salmónidos* figura en el Anexo 21 y se propondrá para adopción en la 91.ª Sesión General, en mayo de 2024.

1.18 Artículo 10.6.3. del Capítulo 10.6. Infección por el virus de la necrosis hematopoyética infecciosa (Anexo 22)

59. Las enmiendas propuestas en el Artículo 10.6.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
60. El texto revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.

61. El Artículo revisado 10.6.3. del Capítulo 10.6. *Infección por el virus de la necrosis hematopoyética infecciosa* figura en el Anexo 22 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.19 Artículo 10.7.3. del Capítulo 10.7. Infección por el herpesvirus de la carpa koi (Anexo 23)

62. Las enmiendas propuestas en el Artículo 10.7.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
63. El texto revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
64. El Artículo revisado 10.7.3. del Capítulo 10.7. *Infección por el herpesvirus de la carpa koi* figura en el Anexo 23 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.20 Artículo 10.8.3. del Capítulo 10.8. Infección por el iridovirus de la dorada japonesa (Anexo 24)

65. Las enmiendas propuestas en el Artículo 10.8.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
66. El texto revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
67. El Artículo revisado 10.8.3. del Capítulo 10.8. *Infección por el iridovirus de la dorada japonesa* figura en el Anexo 24 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.21 Artículo 10.9.3. del Capítulo 10.9. Infección por el virus de la viremia primaveral de la carpa (Anexo 25)

68. Las enmiendas propuestas en el Artículo 10.9.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
69. El texto revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
70. El Artículo revisado 10.9.3. del Capítulo 10.9. *Infección por el virus de la viremia primaveral de la carpa* figura en el Anexo 25 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.22 Artículo 10.10.3. del Capítulo 10.10. Infección por el virus de la septicemia hemorrágica viral (Anexo 26)

71. Las enmiendas propuestas en el Artículo 10.10.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
72. El texto revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.

73. El Artículo revisado 10.10.3. del Capítulo 10.10. *Infección por el virus de la septicemia hemorrágica viral* figura en el Anexo 26 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.23 Artículo 10.11.3. del Capítulo 10.11. *Infección por el virus de la tilapia del lago* (Anexo 27)

74. Las enmiendas propuestas en el Artículo 10.11.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
75. El texto revisado se difundió dos veces para comentario; la primera vez, en el informe de la Comisión de septiembre de 2023.
76. El Artículo revisado 10.11.3. del Capítulo 10.11. *Infección por el virus de la tilapia del lago* figura en el Anexo 27 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.24 Artículo 11.1.3. del Capítulo 11.1. *Infección por el herpesvirus del abalón* (Anexo 28)

77. Las enmiendas propuestas en el Artículo 11.1.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
78. El texto revisado se difundió cuatro veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2022.
79. El Artículo revisado 11.1.3. del Capítulo 11.1. *Infección por el herpesvirus del abalón* figura en el Anexo 28 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.25 Artículo 11.2.3. del Capítulo 11.2. *Infección por Bonamia exitiosa* (Anexo 29)

80. Las enmiendas propuestas en el Artículo 11.2.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
81. El texto revisado se difundió cuatro veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2022.
82. El Artículo revisado 11.2.3. del Capítulo 11.2. *Infección por Bonamia exitiosa* figura en el Anexo 29 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.26 Artículo 11.3.3. del Capítulo 11.3. *Infección por Bonamia ostreae* (Anexo 30)

83. Las enmiendas propuestas en el Artículo 11.3.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
84. El texto revisado se difundió cuatro veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2022.
85. El Artículo revisado 11.3.3. del Capítulo 11.3. *Infección por Bonamia ostreae* figura en el Anexo 30 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.27 Artículo 11.4.3. del Capítulo 11.4. Infección por Marteilia refringens (Anexo 31)

- 86. Las enmiendas propuestas en el Artículo 11.4.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
- 87. El texto revisado se difundió cuatro veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2022.
- 88. El Artículo revisado 11.4.3. del Capítulo 11.4. *Infección por Marteilia refringens* figura en el Anexo 31 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.28 Artículo 11.5.3. del Capítulo 11.5. Infección por Perkinsus marinus (Anexo 32)

- 89. Las enmiendas propuestas en el Artículo 11.5.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
- 90. El texto enmendado se difundió cuatro veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2022.
- 91. El Artículo revisado 11.5.3. del Capítulo 11.5. *Infección por Perkinsus marinus* figura en el Anexo 32 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.29 Artículo 11.6.3. del Capítulo 11.6. Infección por Perkinsus olseni (Anexo 33)

- 92. Las enmiendas propuestas en el Artículo 11.6.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
- 93. El texto revisado se difundió cuatro veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2022.
- 94. El Artículo revisado 11.6.3. del Capítulo 11.6. *Infección por Perkinsus olseni* figura en el Anexo 33 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.30 Artículo 11.7.3. del Capítulo 11.7. Infección por Xenohalictis californiensis (Anexo 34)

- 95. Las enmiendas propuestas en el Artículo 11.7.3. se efectuaron en aras de armonización con el enfoque revisado de los tratamientos de tiempo/temperatura para las mercancías seguras.
- 96. El texto revisado se difundió cuatro veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2022.
- 97. El Artículo revisado 11.7.3. del Capítulo 11.7. *Infección por Xenohalictis californiensis* figura en el Anexo 34 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.31 Modelos de Artículos X.X.5. y X.X.6. para los capítulos específicos de enfermedad (Anexo 35)

98. Las enmiendas propuestas para el modelo de Artículos X.X.5. «país libre de infección por [agente patógeno X]» y X.X.6. «zona libre de infección por [agente patógeno X]» se aplicaron en aras de coherencia a los artículos correspondientes a los países y zonas libres en todos los capítulos específicos de enfermedad del *Código Acuático*.
99. El texto revisado se difundió dos veces para comentario; la primera vez, en el informe de la Comisión de septiembre de 2023.
100. Los modelos revisados de los Artículos X.X.5. y X.X.6. para los capítulos específicos de enfermedad figuran en el Anexo 35 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.32 Artículo 9.3.2. del Capítulo 9.3. Infección por el virus iridiscente de los decápodos tipo 1 (Anexo 36)

101. Se propuso una lista modificada de especies susceptibles para el Artículo 9.3.2.
102. El informe de [marzo 2023](#) del Grupo *ad hoc* de la OMSA sobre la susceptibilidad de las especies de crustáceos a la infección por las enfermedades de la lista de la OMSA, brinda información detallada sobre las evaluaciones realizadas para determinar la lista propuesta de especies susceptibles. En su reunión de febrero de 2024, la Comisión para los Animales Acuáticos solicitó al Grupo *ad hoc* que revisara las pruebas para dos especies basándose en el comentario de un Miembro. La reevaluación de esas dos especies se incluye en el informe de [febrero de 2024](#) de la Comisión.
103. El texto revisado se difundió dos veces para comentario; la primera vez, en el informe de la Comisión de septiembre de 2023.
104. El Artículo revisado 9.3.2. del Capítulo 9.3. *Infección por el virus iridiscente de los decápodos tipo 1* figura en el Anexo 36 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.33 Artículo 10.6.2. del Capítulo 10.6. Infección por el virus de la necrosis hematopoyética infecciosa (Anexo 37)

105. Se propuso reformatear la lista de especies susceptibles del Artículo 10.6.2. para alinearla con el formato utilizado en el Artículo X.X.2. del *Código Acuático*, para enumerar las especies susceptibles en un cuadro.
106. El texto revisado se difundió dos veces para comentario; la primera vez, en el informe de la Comisión de septiembre de 2023.
107. El Artículo revisado 10.6.2. del Capítulo 10.6. *Infección por el virus de la necrosis hematopoyética infecciosa* figura en el Anexo 37 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.34 Artículo 10.11.2. del Capítulo 10.11. Infección por el virus de la tilapia del lago (Anexo 38)

108. Se propuso una lista enmendada de las especies susceptibles para el Artículo 10.11.2.

109. El informe de [abril de 2023](#) del Grupo *ad hoc* de la OMSA sobre la susceptibilidad de las especies de peces a la infección por las enfermedades de la lista de la OMSA brinda información detallada sobre las evaluaciones realizadas para determinar la lista propuesta de especies susceptibles.
110. El texto revisado se difundió dos veces para comentario; la primera vez, en el informe de la Comisión de septiembre de 2023.
111. El Artículo revisado 10.11.2. del Capítulo 10.11. *Infección por el virus de la tilapia del lago* figura en el Anexo 38 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

1.35 Artículos 11.5.1. y 11.5.2. del Capítulo 11.5. Infección por Perkinsus marinus (Anexo 39)

112. Las enmiendas propuestas para la revisión parcial del Artículo 11.5.2. tienen como finalidad garantizar la coherencia con otros capítulos específicos de las enfermedades de los moluscos. El texto «a las siguientes especies que cumplen los criterios de inclusión en la lista de especies susceptibles de acuerdo con el Capítulo 1.5.» se añadió después de «Las recomendaciones de este capítulo se aplican a» en aras de armonización con los otros capítulos específicos de las enfermedades de los moluscos.
113. Se propuso una lista modificada de las especies susceptibles para el Artículo 11.5.2.
114. El informe de [noviembre/diciembre de 2022](#) del Grupo *ad hoc* sobre la susceptibilidad de las especies de moluscos a la infección por las enfermedades de la lista de la OMSA brinda información detallada de las evaluaciones realizadas para determinar la lista propuesta de especies susceptibles.
115. El texto revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
116. Los Artículos revisados 11.5.1. y 11.5.2. del Capítulo 11.5. *Infección por Perkinsus marinus* figuran en el Anexo 39 y se propondrán para adopción en la 91.^a Sesión General, en mayo de 2024.

2. Textos del *Manual Acuático* propuestos para adopción

2.1 Capítulo 2.2.0. *Información general (enfermedades de los crustáceos) (Anexo 51)*

117. Se propuso una revisión exhaustiva del Capítulo 2.2.0. *Información general* (enfermedades de los crustáceos) para así proporcionar información actualizada.
118. El texto revisado se difundió cuatro veces para comentario; la primera vez, en el informe de la Comisión de septiembre de 2022.
119. El Capítulo revisado 2.2.0. *Información general* (enfermedades de los crustáceos) figuran en el Anexo 51 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

2.2 Capítulo 2.2.2. *Infección por Aphanomyces astaci (plaga del cangrejo de río) (Anexo 52)*

120. Una revisión exhaustiva del Capítulo 2.2.2. *Infección por Aphanomyces astaci (plaga del cangrejo de río)* incluyó la reformulación propuesta en aras de armonización con el nuevo modelo de capítulo de enfermedad.
121. El texto revisado se difundió cuatro veces para comentario; la primera vez, en el informe de la Comisión de septiembre de 2022.
122. El Capítulo revisado 2.2.2. *Infección por Aphanomyces astaci (plaga del cangrejo del río)* figura en el Anexo 52 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

2.3 Capítulo 2.2.6. *Infección por el nodavirus Macrobrachium rosenbergii (enfermedad de la cola blanca) (Anexo 53)*

123. Una revisión exhaustiva del Capítulo 2.2.6. *Infección por el nodavirus Macrobrachium rosenbergii (enfermedad de la cola blanca)* incluyó la reformulación propuesta en aras de armonización con el nuevo modelo de capítulo de enfermedad.
124. El capítulo revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
125. El Capítulo revisado 2.2.6. *Infección por el nodavirus Macrobrachium rosenbergii (enfermedad de la cola blanca)* figura en el Anexo 53 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

2.4 Capítulo 2.2.9. *Infección por el virus de la cabeza amarilla genotipo 1 (Anexo 54)*

126. Una revisión exhaustiva del Capítulo 2.2.9. *Infección por el virus de la cabeza amarilla genotipo 1* incluyó la reformulación propuesta en aras de armonización con el nuevo modelo de capítulo de enfermedad.
127. El capítulo revisado se difundió tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
128. El Capítulo revisado 2.2.9. *Infección por el virus de la cabeza amarilla genotipo 1* figura en el Anexo 54 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

2.5 Capítulo 2.2.X. Infección por el virus iridiscente de los decápodos tipo 1 (DIV1) (Anexo 55)

- 129. Se propuso un nuevo Capítulo 2.2.X. *Infección por el virus iridiscente de los decápodos 1 (DIV1)* armonizado con el nuevo modelo de capítulo de enfermedad.
- 130. El nuevo capítulo se difundió dos veces para comentario; la primera vez, en el informe de la Comisión de septiembre de 2023.
- 131. El nuevo Capítulo 2.2.X. *Infección por el virus iridiscente de los decápodos tipo 1 (DIV1)* figura en el Anexo 55 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

2.6 Capítulo 2.4.0. Información general: enfermedades de los moluscos (Anexo 56)

- 132. Se propuso una revisión exhaustiva del Capítulo 2.4.0. *Información general* sobre los moluscos con enmiendas propuestas para proporcionar información actualizada.
- 133. El capítulo revisado se difundió dos veces para comentario; la primera vez, en el informe de la Comisión de septiembre de 2023.
- 134. El Capítulo revisado 2.4.0. *Información general* (enfermedades de los moluscos) figura en el Anexo 56 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

2.7 Capítulo 2.4.1. Infección por el herpesvirus del abalón (Anexo 57)

- 135. Una revisión exhaustiva del Capítulo 2.4.1. *Infección por herpesvirus del abalón* incluyó la reformulación, en aras de armonización con el nuevo modelo de capítulo de enfermedad.
- 136. El capítulo revisado se difundió dos veces para comentario; la primera vez, en el informe de la Comisión de septiembre de 2023.
- 137. El Capítulo revisado 2.4.1. *Infección por el herpesvirus del abalón* figura en el Anexo 57 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

2.8 Capítulo 2.4.4. Infección por *Marteilia refringens* (Anexo 58)

- 138. Una revisión exhaustiva del Capítulo 2.4.4. *Infección por *Marteilia refringens** incluyó la reformulación en aras de armonización con el nuevo modelo de capítulo de enfermedad.
- 139. El capítulo revisado se difundió dos veces para comentario; la primera vez, en el informe de la Comisión de septiembre de 2023.
- 140. El Capítulo revisado 2.4.4. *Infección por *Marteilia refringens** figura en el Anexo 58 y se propondrá para adopción en la 91.^a Sesión General, en mayo de 2024.

2.9 Secciones 2.2.1. y 2.2.2. del Capítulo 2.4.5. *Infección por Perkinsus marinus*
(Anexo 59)

141. Se propusieron enmiendas a la lista de especies susceptibles de las Secciones 2.2.1. y 2.2.2.
142. El informe de [diciembre de 2022](#) del Grupo *ad hoc* sobre la susceptibilidad de las especies de moluscos a la infección por las enfermedades de la lista de la OMSA brinda información detallada sobre las evaluaciones realizadas, con el fin de determinar la lista propuesta de especies susceptibles.
143. Las secciones revisadas se difundieron tres veces para comentario; la primera vez, en el informe de la Comisión de febrero de 2023.
144. Las Secciones revisadas 2.2.1. y 2.2.2. del Capítulo 2.4.5. *Infección por Perkinsus marinus* figuran en el Anexo 59 y se propondrán para adopción en la 91.^a Sesión General, en mayo de 2024.

3. Anexos

Anexo 4. Ítem 6.1. – Definiciones del Glosario: “servicios de sanidad de los animales acuáticos”, “autoridad competente” y “autoridad veterinaria”

Artículo	Uso
Guía del usuario: C.8.	Las normas de los capítulos del Título 3 tratan del establecimiento, de la conservación y de la evaluación de los Servicios de Sanidad de <u>los Servicios de Sanidad de los Animales Acuáticos y los Animales Acuáticos</u> , incluida la comunicación. Esas normas pretenden ayudar a las autoridades competentes de los Países Miembros a cumplir sus objetivos de mejora de la sanidad de los animales acuáticos y del bienestar de los peces de cultivo, y a crear y mantener la confianza en sus certificados sanitarios internacionales aplicables a los animales acuáticos.
Guía del usuario: C.8.	Certificados sanitarios internacionales para los animales acuáticos Un certificado sanitario internacional aplicable a los animales acuáticos es un documento oficial que la autoridad competente del país exportador expide de acuerdo con lo dispuesto en los Capítulos 5.1. y 5.2. Los certificados enumeran los requisitos de sanidad de los animales acuáticos que reúne la mercancía exportada. La calidad de los Servicios Veterinarios o los Servicios de Sanidad de los Animales Acuáticos del país exportador es esencial para ofrecer garantías a los socios comerciales de la seguridad sanitaria de las mercancías de animales acuáticos exportadas. Esto incluye los principios éticos de los Servicios de Sanidad de los Animales Acuáticos <u>la autoridad competente relevante</u> en cuanto a la expedición de certificados sanitarios internacionales y sus los antecedentes de la <u>autoridad veterinaria</u> en el cumplimiento de sus obligaciones de notificación.
Glosario	NOTIFICACIÓN designa el procedimiento por el que: a. la autoridad competente <u>autoridad veterinaria</u> comunica a la Sede, b. la Sede comunica a las autoridades competentes <u>la autoridad veterinaria</u> de los Países Miembros la aparición de una <i>enfermedad</i> , según lo dispuesto en el Capítulo 1.1.
Artículo 1.1.1.	A efectos del <i>Código Acuático</i> y de conformidad con los Artículos 5, 9 y 10 de los Estatutos orgánicos de la OMSA, todos los Países Miembros reconocen a la <i>Sede</i> el derecho de comunicarse directamente con la autoridad competente <u>autoridad veterinaria</u> de su o sus territorios. Cualquier <i>notificación</i> o información enviada por la OMSA a una autoridad competente <u>autoridad veterinaria</u> se considerará enviada al Estado al que ésta pertenece y cualquier <i>notificación</i> o información enviada a la OMSA por una autoridad competente <u>autoridad veterinaria</u> se considerará enviada por el Estado al que ésta pertenece.
Artículo 1.1.3. párrafo 1	La autoridad competente <u>autoridad veterinaria</u> , bajo la responsabilidad del Delegado, deberá enviar a la <i>Sede</i> :
Artículo 1.1.4. párrafo 1	La autoridad competente <u>autoridad veterinaria</u> , bajo la responsabilidad del Delegado, deberá enviar a la <i>Sede</i> :
Artículo 1.1.5. punto 1	La autoridad competente <u>autoridad veterinaria</u> de un país en el que está ubicada una <i>zona infectada</i> o un <i>compartimento</i> infectado avisará a la <i>Sede</i> tan pronto como dicho país, <i>zona</i> o <i>compartimento</i> quede libre de la <i>enfermedad</i> .
Artículo 1.1.5. punto 3	La autoridad competente <u>autoridad veterinaria</u> de un País Miembro que establezca una o varias <i>zonas libres</i> o un o varios <i>compartimentos libres</i> deberá notificarlo a la <i>Sede</i> facilitando los datos necesarios, entre los cuales deberán figurar los criterios sobre los que se basa el establecimiento del estatus libre y las condiciones para mantenerlo e indicando con claridad la ubicación de las <i>zonas</i> o los <i>compartimentos</i> en un mapa del territorio del País Miembro.

Artículo	Uso
Artículo 3.1.2. punto 7 párrafo 3	Los Servicios de Sanidad de los Animales Acuáticos Las <u>autoridades competentes</u> deberán definir y documentar las responsabilidades y la estructura (en particular el orden jerárquico) de la organización encargada de la expedición de <i>certificados sanitarios internacionales aplicables a los animales acuáticos</i> .
Artículo 3.1.2. punto 10	<u>Información, reclamaciones y recursos</u> Los Servicios de Sanidad de los Animales Acuáticos La <u>autoridad competente relevante</u> deberán comprometerse a atender todas las peticiones de los Servicios de Sanidad de los Animales Acuáticos de la autoridad competente de los demás Países Miembros o de cualquier otra autoridad , en especial encargándose de cursar oportunamente las peticiones de información, las reclamaciones o los recursos que éstos presenten. Se llevará un registro de todas las reclamaciones y recursos presentados, así como del curso dado a los mismos por los Servicios de Sanidad de los Animales Acuáticos la <u>autoridad competente</u> .
Artículo 3.1.5. párrafo 4	El experto o los expertos facilita(n) la evaluación de los <i>Servicios de Sanidad de los Animales Acuáticos</i> del País Miembro aplicando la <i>Herramienta de la OMSA para la Evaluación de las Prestaciones de los Servicios Veterinarios y/o los Servicios de Sanidad de los Animales Acuáticos (Herramienta PVS de la OMSA: Animales acuáticos)</i> . El experto o los expertos redacta(n) un informe en colaboración con los <i>Servicios de Sanidad de los Animales Acuáticos</i> del País Miembro.
Artículo 3.2.1. párrafo 2	El reconocimiento y la incorporación de la comunicación como disciplina de los <i>Servicios de Sanidad de los Animales Acuáticos</i> resultan capitales para su funcionamiento. La combinación de la pericia en materia de sanidad de <i>animales acuáticos</i> y de comunicación es esencial para garantizar una comunicación eficaz. La comunicación entre los Servicios de Sanidad de los Animales Acuáticos y los Servicios Veterinarios (sobre todo cuando los Servicios de Sanidad de los Animales Acuáticos están separados y son independientes de los Servicios Veterinarios) es especialmente importante.
Artículo 4.2.3. punto 1	La extensión de una <i>zona</i> será determinada por el Servicio de Sanidad de los Animales Acuáticos la <u>autoridad competente</u> basándose en la definición vigente de <i>zona</i> y será publicada por vía oficial.
Artículo 4.2.3. punto 3	Los factores que definen un compartimento serán determinados por el Servicio de Sanidad de los Animales Acuáticos la <u>autoridad competente</u> basándose en criterios pertinentes, como los métodos de gestión y explotación relacionados con la <i>bioseguridad</i> , y serán publicados por vía oficial.
Artículo 4.2.3. punto 6	El <i>plan de bioseguridad</i> de un <i>compartimento</i> deberá describir la colaboración entre la industria o empresa pertinente, y el Servicio de Sanidad de los Animales Acuáticos la <u>autoridad competente</u> y los Servicios de Sanidad de los Animales Acuáticos , así como sus responsabilidades respectivas y los procedimientos para la supervisión del funcionamiento del <i>compartimento</i> por el Servicio de Sanidad de los Animales Acuáticos la <u>autoridad competente</u> .
Artículo 5.3.4. punto 2 a)	infraestructura: incluye la base legislativa (leyes sobre sanidad de los <i>animales acuáticos</i> , por ejemplo) y los sistemas administrativos (organización de los <i>Servicios Veterinarios</i> y de los Servicios de Sanidad de los Animales Acuáticos la <u>autoridad competente</u> por ejemplo);
Artículo 5.3.7. punto 1 d) i)	una evaluación de los Servicios Veterinarios <i>Servicios de Sanidad de los Animales Acuáticos</i> del país exportador;
Artículo 5.3.7. punto 2 e) i)	una evaluación de los Servicios Veterinarios <i>Servicios de Sanidad de los Animales Acuáticos</i> del país exportador;

Anexo 5. Ítem 6.2. – Definiciones del Glosario: “productos de animales acuáticos”

No se han propuesto enmiendas en español.

Anexo 6. Ítem 6.3. – Artículo 1.1.5. del Capítulo 1.1. Notificación de enfermedades y aportación de datos epidemiológicos

CAPÍTULO 1.1.

**NOTIFICACIÓN DE ENFERMEDADES Y APORTACIÓN DE DATOS
EPIDEMIOLÓGICOS**

[...]

Artículo 1.1.5.

- 1) ~~La autoridad competente de un país en el que está ubicada una zona infectada o un compartimento infectado avisará a la Sede tan pronto como dicho país, zona o compartimento quede libre de la enfermedad.~~
- 2) ~~Podrá considerarse que un país, una zona o un compartimento está nuevamente libre de una enfermedad determinada cuando reúna las debidas condiciones previstas en el Código Acuático.~~
- 3) ~~La autoridad competente de un País Miembro que establezca una o varias zonas libres o un o varios compartimentos libres deberá notificarlo a la Sede facilitando los datos necesarios, entre los cuales deberán figurar los criterios sobre los que se basa el establecimiento del estatus libre y las condiciones para mantenerlo e indicando con claridad la ubicación de las zonas o los compartimentos en un mapa del territorio del País Miembro.~~

Artículo 1.1.65.

- 1) Aunque los Países Miembros sólo tendrán la obligación de notificar las *enfermedades de la lista de la OMSA* y las *enfermedades emergentes*, se les alienta a brindar a la OMSA información zoonosológica significativa sobre los *animales acuáticos*.
- 2) La Sede deberá comunicar a las *autoridades competentes* por correo electrónico o a través de la interfaz de WAHIS todas las *notificaciones* recibidas en aplicación de los Artículos 1.1.2. a 1.1.54., así como cualquier otra información pertinente.

[...]

CAPÍTULO 1.3.

ENFERMEDADES DE LA LISTA DE LA OMSA

[...]

Artículo 1.3.1.

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

- Infección por *Aphanomyces invadans* (Síndrome ulcerante epizoótico)
- Infección por el alfavirus de los salmónidos
- Infección por **el todos** los genogrupos de la especie virus de la necrosis infecciosa del bazo y del riñón
- Infección por el herpesvirus de la carpa koi
- ~~Infección por el iridovirus 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 9. Ítem 6.5.1. – Artículo 8.1.3. del Capítulo 8.1. 'Infección por *Batrachochytrium dendrobatidis*'

(VERSIÓN CON SEGUIMIENTO DE CAMBIOS)

CAPÍTULO 8.1.

INFECCIÓN POR *Batrachochytrium dendrobatidis*

[...]

Artículo 8.1.3.

Medidas para la importación o tránsito por el territorio 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 *B. dendrobatidis*

1) Los siguientes 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. Independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *H. penaei*, las autoridades competentes no deberán exigir ningún tipo de condición relacionada con *H. penaei* cuando autoricen la importación o el tránsito por su territorio de estos los siguientes productos de animales acuáticos, las autoridades competentes no deberán exigir ninguna medida sanitaria relacionada con *B. dendrobatidis*, independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *B. dendrobatidis*; derivados de una de las especies mencionadas en el Artículo 9.3.2., cualquiera que sea el uso al que se destinan y siempre que reúnan las condiciones descritas en el Artículo 5.4.1.:

1) productos de animales acuáticos que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 60°C durante por lo menos cinco minutos, o un tiempo/temperatura equivalente que inactive *B. dendrobatidis*;

a) productos de anfibios termoesterilizados y sellados herméticamente (es decir, un tratamiento térmico a 121 °C durante por lo menos 3,6 minutos o una combinación equivalente de tiempo/temperatura que haya demostrado que inactiva *B. dendrobatidis*);

b) productos de anfibios cocinados que se hayan sometido a un tratamiento térmico a 100 °C durante por lo menos un minuto (o a una combinación equivalente de tiempo/temperatura que haya demostrado que inactiva);

e) productos de anfibios pasteurizados que se hayan sometido a un tratamiento térmico a 90 °C durante por lo menos diez minutos (o a una combinación equivalente de tiempo/temperatura que haya demostrado que inactiva *B. dendrobatidis*);

d)2) productos de anfibios secados por medios mecánicos que se hayan sometido a (es decir, un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos a 100 °C 60°C durante al menos 30 cinco minutos, o una combinación equivalente de tiempo/temperatura que haya demostrado que un tiempo/temperatura equivalente que inactiva *B. dendrobatidis*);

e)3) cueros elaborados con piel de anfibio.

2) Cuando autoricen la importación o el tránsito por su territorio de productos de animales acuáticos derivados de una de las especies mencionadas en el Artículo 8.1.2. que no sean unos de los enumerados en el apartado 1 del Artículo 8.1.3., las autoridades competentes deberán exigir las condiciones prescritas en los Artículos 8.1.7. a 8.1.12. que correspondan al estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *B. dendrobatidis*.

3) Cuando se considere la importación o el tránsito por su territorio de productos de animales acuáticos derivados de una especie no mencionada en el Artículo 8.1.2., pero que razonablemente se podría esperar que represente un riesgo de transmisión de *B. dendrobatidis*, la autoridad competente deberá proceder a un análisis del riesgo acorde con las

recomendaciones del Capítulo 2.1. La *autoridad competente* del país exportador deberá ser informada del resultado de este análisis.

[...]

(VERSIÓN EN LIMPIO)

CAPÍTULO 8.1.

INFECCIÓN POR *BATRACHOCHYTRIUM DENDROBATIDIS*

[...]

Artículo 8.1.3.

Medidas para la importación o tránsito por el territorio 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 *B. dendrobatidis*

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 *B. dendrobatidis*, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por *B. dendrobatidis*:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 60°C durante por lo menos cinco minutos, o un tiempo/temperatura equivalente que inactive *B. dendrobatidis*;
- 2) cueros elaborados con piel de anfibio.

[...]

Anexo 10. Ítem 6.5.1. – Artículo 8.2.3. del Capítulo 8.2. 'Infección por *Batrachochytrium salamandrivorans*'

(VERSIÓN CON SEGUIMIENTO DE CAMBIOS)

CAPÍTULO 8.2

INFECCIÓN POR *BATRACHOCHYTRIUM SALAMANDRIVORANS*

[...]

Artículo 8.2.3.

Medidas para la importación o tránsito por el territorio 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 *B. salamandrivorans*

~~1) Los siguientes 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. Independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *H. penaei*, las autoridades competentes no deberán exigir ningún tipo de condición relacionada con *H. penaei*. Cuando autoricen la importación o el tránsito por su territorio de estos los siguientes productos de animales acuáticos, las autoridades competentes no deberán exigir ninguna medida sanitaria relacionada con *B. salamandrivorans*, independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *B. salamandrivorans*: derivados de una de las especies mencionadas en el Artículo 9.3.2., cualquiera que sea el uso al que se destinan y siempre que reúnan las condiciones descritas en el Artículo 5.4.1.:~~

1) productos de animales acuáticos que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 60°C durante por lo menos cinco minutos, o a un tiempo/temperatura equivalente que inactiva *B. salamandrivorans*:

a) productos de anfibios termoesterilizados y sellados herméticamente (es decir, un tratamiento térmico a 121 °C durante por lo menos 3,6 minutos o una combinación equivalente de tiempo/temperatura que haya demostrado que inactiva *B. salamandrivorans*);

b) productos de anfibios cocinados que se hayan sometido a un tratamiento térmico a 100 °C durante por lo menos un minuto (o a una combinación equivalente de tiempo/temperatura que haya demostrado que inactiva *B. salamandrivorans*);

c) productos de anfibios pasteurizados que se hayan sometido a un tratamiento térmico a 90 °C durante por lo menos diez minutos (o a una combinación equivalente de tiempo/temperatura que haya demostrado que inactiva *B. salamandrivorans*);

~~d) productos de anfibios secados por medios mecánicos que se hayan sometido a (es decir, un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos a 100 °C 60°C durante al menos 30 cinco minutos, o una combinación equivalente de tiempo/temperatura que haya demostrado que un tiempo/temperatura equivalente que inactiva *B. salamandrivorans*);~~

e) ~~3) cueros elaborados con piel de anfibio.~~

2) ~~Cuando autoricen la importación o el tránsito por su territorio de productos de animales acuáticos derivados de una de las especies mencionadas en el Artículo 8.2.2. que no sean unos de los enumerados en el apartado 1 del Artículo 8.2.3., las autoridades competentes deberán exigir las condiciones prescritas en los Artículos 8.2.7. a 8.2.12. que correspondan al estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *B. salamandrivorans*.~~

3) ~~Cuando se considere la importación o el tránsito por su territorio de productos de animales acuáticos derivados de una especie no mencionada en el Artículo 8.2.2., pero que razonablemente se podría esperar que represente un riesgo de~~

transmisión de *B. salamandrivorans*, la *autoridad competente* deberá proceder a un *análisis del riesgo* acorde con las recomendaciones del Capítulo 2.1. La *autoridad competente* del *país exportador* deberá ser informada del resultado de este análisis.

[...]

(VERSIÓN EN LIMPIO)

CAPÍTULO 8.2.

INFECCIÓN POR BATRACHOCHYTRIUM SALAMANDRIVORANS

[...]

Artículo 8.2.3.

Medidas para la importación o tránsito por el territorio 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 *B. salamandrivorans*

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 *B. salamandrivorans*, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por *B. salamandrivorans*:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 60°C durante por lo menos cinco minutos, o un tiempo/temperatura equivalente que inactive *B. salamandrivorans*;
- 2) cueros elaborados con piel de anfibio.

[...]

Anexo 11. Ítem 6.5.1. – Artículo 8.3.3. del Capítulo 8.3. 'Infección por las especies de *Ranavirus*'

(VERSIÓN CON SEGUIMIENTO DE CAMBIOS)

CAPÍTULO 8.3.

INFECCIÓN POR LAS ESPECIES DE *RANAVIRUS*

[...]

Artículo 8.3.3.

Medidas para la importación o tránsito por el territorio 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 las especies de *Ranavirus*

1) Los siguientes 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. Independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *H. penaei*, las autoridades competentes no deberán exigir ningún tipo de condición relacionada con *H. penaei* e Cuando autoricen la importación o el tránsito por su territorio de estos los siguientes productos de animales acuáticos, las autoridades competentes no deberán exigir ninguna medida sanitaria relacionada con las especies de *Ranavirus*, independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por las especies de *Ranavirus*: derivados de una de las especies mencionadas en el Artículo 8.3.2., cualquiera que sea el uso al que se destinan y siempre que reúnan las condiciones descritas en el Artículo 5.4.1.:

1) productos de animales acuáticos que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 65/60°C durante por lo menos 30 minutos, o a un tiempo/temperatura equivalente que inactiva todas las especies de *Ranavirus*.

a) productos de anfibios termoesterilizados y sellados herméticamente (es decir, un tratamiento térmico a 121 °C durante por lo menos 3,6 minutos o una combinación equivalente de tiempo/temperatura que haya demostrado que inactiva todas las especies de *Ranavirus*);

b) productos de anfibios cocinados que se hayan sometido a un tratamiento térmico de 65 °C durante por lo menos 30 minutos (o a una combinación equivalente de tiempo/temperatura que haya demostrado que inactiva todas las especies de *Ranavirus*);

c) productos de anfibios pasteurizados que se hayan sometido a un tratamiento térmico de 90 °C durante por lo menos diez minutos (o a una combinación equivalente de tiempo/temperatura que haya demostrado que inactiva todas las especies de *Ranavirus*);

~~d) productos de anfibios secados por medios mecánicos que se hayan sometido a (es decir, un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos a 100 °C 65°C durante al menos 30 minutos, o una combinación equivalente de tiempo/temperatura que haya demostrado que un tiempo/temperatura equivalente que inactiva todas las especies de *Ranavirus*);~~

2) Cuando autoricen la importación o el tránsito por su territorio de productos de animales acuáticos derivados de una de las especies mencionadas en el Artículo 8.3.2. que no sean unos de los enumerados en el apartado 1 del Artículo 8.3.3., las autoridades competentes deberán exigir las condiciones prescritas en los Artículos 8.3.7. a 8.3.12. que correspondan al estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por las especies de *Ranavirus*.

3) Cuando se considere la importación o el tránsito por su territorio de productos de animales acuáticos derivados de una especie no mencionada en el Artículo 8.3.2., pero que razonablemente se podría esperar que represente un riesgo de transmisión de las especies de *Ranavirus*, la autoridad competente deberá proceder a un análisis del riesgo acorde con las recomendaciones del Capítulo 2.1. La autoridad competente del país exportador deberá ser informada del resultado de este análisis.

[...]

(VERSIÓN EN LIMPIO)

CAPÍTULO 8.3.

INFECCIÓN POR LAS ESPECIES DE RANAVIRUS

[...]

Artículo 8.3.3.

Medidas para la importación o tránsito por el territorio 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 las especies de *Ranavirus*

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 las especies de *Ranavirus*, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por las especies de *Ranavirus*:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 60°C durante por lo menos 30 minutos, o a un tiempo/temperatura equivalente que inactiva todas las especies de *Ranavirus*.

[...]

CAPÍTULO 9.3.

INFECCIÓN POR EL VIRUS IRIDISCENTE DE LOS DECÁPODOS TIPO 1

[...]

Artículo 9.3.3.

Medidas para la importación o tránsito por el territorio 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 DIV1

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 DIV1, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por DIV1:

- 1) ~~Los~~ *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos ~~56~~68°C durante por lo menos 30 minutos, o a una combinación equivalente de tiempo/temperatura que inactive DIV1;
- 2) *harina* de crustáceos que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos ~~56~~68°C durante por lo menos 30 minutos, o a una combinación equivalente de tiempo/temperatura que inactive DIV1;
- 3) aceite de crustáceos;
- 4) quitina extraída por medios químicos. ~~(en estudio)~~

[...]

CAPÍTULO 9.4.

INFECCIÓN POR *HEPATOBACTER*
PENAEI (HEPATOPANCREATITIS NECROTIZANTE)

[...]

Artículo 9.4.3.

Medidas para la importación o tránsito por el territorio 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 *H. penaei*

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 *H. penaei*, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por *H. penaei*:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos ~~63~~95°C durante por lo menos ~~30~~cinco minutos, o un tiempo/temperatura equivalente que inactive *H. penaei*;
- 2) *harina* de crustáceos que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos ~~63~~95°C durante por lo menos ~~30~~cinco minutos, o un tiempo/temperatura equivalente que inactive *H. penaei*;
- 3) aceite de crustáceos;
- 4) quitina extraída por medios químicos.

[...]

Anexo 14. Ítem 6.5.2. – Artículo 9.6.3. del Capítulo 9.6. ‘Infección por el virus de la mionecrosis infecciosa’

CAPÍTULO 9.6.

INFECCIÓN POR EL VIRUS
DE LA MIONECROSIS INFECCIOSA

[...]

Artículo 9.6.3.

Medidas para la importación o tránsito por el territorio 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 el virus de la mionecrosis infecciosa

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 virus de la mionecrosis infecciosa, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por el virus de la mionecrosis infecciosa:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos ~~60~~75°C durante por lo menos ~~60~~cinco minutos, o un tiempo/temperatura equivalente que inactive el virus de la mionecrosis infecciosa;
- 2) *harina* de crustáceos que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos ~~60~~75°C durante por lo menos ~~60~~cinco minutos, o un tiempo/temperatura equivalente que inactive el virus de la mionecrosis infecciosa;
- 3) aceite de crustáceos;
- 4) quitina extraída por medios químicos.

[...]

Anexo 15. Ítem 6.5.2. – Artículo 9.7.3. del Capítulo 9.7. ‘Infección por el nodavirus *Macrobrachium rosenbergii*’

CAPÍTULO 9.7.

INFECCIÓN POR EL NODAVIRUS MACROBRACHIUM
ROSENBERGII (ENFERMEDAD DE LA COLA BLANCA)

[...]

Artículo 9.7.3.

Medidas para la importación o tránsito por el territorio 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 MrNV

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 MrNV, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por MrNV:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos ~~60~~50°C durante por lo menos ~~60~~cinco minutos, o un tiempo/temperatura equivalente que inactive el MrNV;
- 2) *harina* de crustáceos que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos ~~60~~50°C durante por lo menos ~~60~~cinco minutos, o un tiempo/temperatura equivalente que inactive el MrNV;
- 3) aceite de crustáceos;
- 4) quitina extraída por medios químicos.

[...]

CAPÍTULO 9.8.

INFECCIÓN POR EL VIRUS DEL SÍNDROME DE TAURA

[...]

Artículo 9.8.3.

Medidas para la importación o tránsito por el territorio 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 el virus del síndrome de Taura

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 virus del síndrome de Taura, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por el virus del síndrome de Taura:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 70°C durante por lo menos ~~30~~108 minutos, o un tiempo/temperatura equivalente que inactive el virus del síndrome de Taura;
- 2) *harina* de crustáceos que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 70°C durante por lo menos ~~30~~108 minutos, o un tiempo/temperatura equivalente que inactive el virus del síndrome de Taura;
- 3) aceite de crustáceos;
- 4) quitina extraída por medios químicos.

[...]

Anexo 17. Ítem 6.5.3. – Artículos 10.1.3. del Capítulo 10.1. ‘infección por el virus de la necrosis hematopoyética epizoótica’

CAPÍTULO 10.1.

INFECCIÓN POR EL VIRUS DE LA NECROSIS
HEMATOPOYÉTICA EPIZOÓTICA

[...]

Artículo 10.1.3.

Medidas para la importación o tránsito por el territorio 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 el virus de la necrosis hematopoyética epizoótica

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 virus de la necrosis hematopoyética epizoótica, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por el virus de la necrosis hematopoyética epizoótica:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 60°C durante por lo menos 15 minutos, o un tiempo/temperatura equivalente que inactive el virus de la necrosis hematopoyética epizoótica;
- 2) ~~pescado eviscerado y secado por medios mecánicos que se haya sometido a una temperatura interna de al menos 60°C durante por lo menos 15 minutos, o un tiempo/temperatura equivalente que inactive el virus de la necrosis hematopoyética epizoótica;~~
- 3) *harina* de pescado que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 60°C durante por lo menos 15 minutos, o un tiempo/temperatura equivalente que inactive el virus de la necrosis hematopoyética epizoótica;
- 4) *aceite* de pescado;
- 5) *cueros elaborados* con piel de pescado.

[...]

CAPÍTULO 10.2.

INFECCIÓN POR *APHANOMYCES INVADANS*
(SÍNDROME ULCERANTE EPIZOÓTICO)

[...]

Artículo 10.2.3.

Medidas para la importación o tránsito por el territorio 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 *A. invadans*

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 *A. invadans*, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por *A. invadans*:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos ~~60~~100°C durante por lo menos ~~cinco~~un minutos, o un tiempo/temperatura equivalente que inactive *A. invadans*;
- 2) ~~pescado eviscerado y secado por medios mecánicos que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 60°C durante por lo menos cinco minutos, o un tiempo/temperatura equivalente que inactive *A. invadans*;~~
- 3) ~~harina de pescado que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 60~~100°C durante por lo menos ~~cinco~~un minutos, o un tiempo/temperatura equivalente que inactive *A. invadans*;
- 4) aceite de pescado;
- 5) pescado eviscerado y congelado;
- 6) filetes o rodajas de pescado congelado.

[...]

CAPÍTULO 10.3.

INFECCIÓN POR *GYRODACTYLUS SALARIS*

[...]

Artículo 10.3.3.

Medidas para la importación o tránsito por el territorio 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 *G. salaris*

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 *G. salaris*, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por *G. salaris*:

- 1) ~~productos de animales acuáticos que han sido sometidos a tratamiento térmico y sellados herméticamente que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 40°C durante por lo menos un minuto, o un tiempo/temperatura equivalente que inactive *G. salaris*;~~
- 2) ~~pescado eviscerado y secado por medios mecánicos;~~
- 32) ~~pescado eviscerado y secado naturalmente (es decir, secado por el sol o el viento);~~
- 43) ~~pescado eviscerado y congelado que haya sido sometido a -18°C o a temperaturas más bajas;~~
- 54) ~~filetes o rodajas de pescado congelado que se hayan sometido a -18°C o a temperaturas más bajas;~~
- 65) ~~pescado eviscerado y refrigerado criado en agua de mar con una salinidad de al menos 25 ppt por lo menos 14 días seguidos;~~
- 76) ~~filetes o rodajas de pescado refrigerado de pescados criados en agua de mar con una salinidad de al menos 25 ppt por lo menos 14 días seguidos;~~
- 87) ~~productos de pescado refrigerado sin piel, aletas ni branquias;~~
- 98) ~~huevas de pescado no viables;~~
- 109) ~~aceite de pescado;~~
- 110) ~~harina de pescado;~~
- 1211) ~~cueros elaborados con piel de pescado.~~

[...]

CAPÍTULO 10.4.

INFECCIÓN POR EL VIRUS DE LA ANEMIA INFECCIOSA DEL SALMÓN

[...]

Artículo 10.4.3.

Medidas para la importación o tránsito por el territorio 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 el virus de la anemia infecciosa del salmón

En este artículo, todas las consideraciones se entenderán hechas a cualquier virus detectable de la anemia infecciosa del salmón, incluido el virus de la anemia infecciosa del salmón HPR0.

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 virus de la anemia infecciosa del salmón, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por el virus de la anemia infecciosa del salmón:

- 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 el virus de la anemia infecciosa del salmón;
- 2) ~~pescado eviscerado y secado por medios mecánicos que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 56°C durante al menos cinco minutos, o un tiempo/temperatura equivalente que inactive el virus de la anemia infecciosa del salmón;~~
- 3) *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 el virus de la anemia infecciosa del salmón;
- 4) aceite de pescado;
- 5) cueros elaborados con piel de pescado.

[...]

CAPÍTULO 10.5.

INFECCIÓN POR EL ALFAVIRUS DE LOS SALMÓNIDOS

[...]

Artículo 10.5.3.

Medidas para la importación o tránsito por el territorio 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 el alfavirus de los salmónidos

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 alfavirus de los salmónidos, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por el alfavirus de los salmónidos:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 60°C durante por lo menos 60 minutos, o un tiempo/temperatura equivalente que inactive el alfavirus de los salmónidos;
- 2) ~~pescado eviscerado y secado por medios mecánicos que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 60°C durante al menos 60 minutos, o un tiempo/temperatura equivalente que inactive el alfavirus de los salmónidos;~~
- 3) harina de pescado que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 60°C durante por lo menos 60 minutos, o un tiempo/temperatura equivalente que inactive el alfavirus de los salmónidos;
- 4) aceite de pescado;
- 5) cueros elaborados con piel de pescado.

[...]

Anexo 22. Ítem 6.5.3. – Artículos 10.6.3. del Capítulo 10.6. ‘infección por el virus de la necrosis hematopoyética infecciosa’

CAPÍTULO 10.6.

INFECCIÓN POR EL VIRUS DE LA NECROSIS
HEMATOPOYÉTICA INFECCIOSA

[...]

Artículo 10.6.3.

Medidas para la importación o tránsito por el territorio 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 el virus de la necrosis hematopoyética infecciosa

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 virus de la necrosis hematopoyética infecciosa, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por el virus de la necrosis hematopoyética infecciosa:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 90°C durante por lo menos 30 segundos, o un tiempo/temperatura equivalente que inactive el virus de la necrosis hematopoyética infecciosa;
- 2) ~~pescado eviscerado y secado por medios mecánicos que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 90°C durante por lo menos 30 segundos, o un tiempo/temperatura equivalente que inactive el virus de la necrosis hematopoyética infecciosa;~~
- 3) 2) *harina* de pescado que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 90°C durante por lo menos 30 segundos, o un tiempo/temperatura equivalente que inactive el virus de la necrosis hematopoyética infecciosa;
- 4) 3) aceite de pescado;
- 5) 4) cueros elaborados con piel de pescado.

[...]

CAPÍTULO 10.7.

INFECCIÓN POR EL HERPESVIRUS DE LA CARPA KOI

[...]

Artículo 10.7.3.

Medidas para la importación o tránsito por el territorio 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 el herpesvirus de la carpa koi

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 herpesvirus de la carpa koi, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por el herpesvirus de la carpa koi:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 50°C durante por lo menos ~~tres~~tres minutos, o un tiempo/temperatura equivalente que inactive el herpesvirus de la carpa koi;
- 2) ~~pescado eviscerado y secado por medios mecánicos que se haya sometido a un tratamiento térmico suficiente para alcanzar una temperatura interna de al menos 50°C durante por lo menos tres minutos, o un tiempo/temperatura equivalente que inactive el herpesvirus de la carpa koi;~~
- 3) harina de pescado que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 50°C durante por lo menos ~~tres~~tres minutos, o un tiempo/temperatura equivalente que inactive el herpesvirus de la carpa koi;
- 4) aceite de pescado.

[...]

Anexo 24. Ítem 6.5.3. – Artículos 10.8.3. del Capítulo 10.8. ‘infección por el iridovirus de la dorada japonesa’

CAPÍTULO 10.8.

INFECCIÓN POR EL IRIDOVIRUS DE LA DORADA JAPONESA

[...]

Artículo 10.8.3.

Medidas para la importación o tránsito por el territorio 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 el iridovirus de la dorada japonesa

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 iridovirus de la dorada japonesa, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por el iridovirus de la dorada japonesa:

- 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 30 minutos, o un tiempo/temperatura equivalente que inactive el iridovirus de la dorada japonesa;
- 2) ~~pescado eviscerado y secado por medios mecánicos que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 56°C durante por lo menos 30 minutos, o un tiempo/temperatura equivalente que inactive el iridovirus de la dorada japonesa;~~
- 3) *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 30 minutos, o un tiempo/temperatura equivalente que inactive el iridovirus de la dorada japonesa;
- 4) *aceite* de pescado;
- 5) *cueros elaborados* con piel de pescado.

[...]

Anexo 25. Ítem 6.5.3. – Artículos 10.9.3. del Capítulo 10.9. ‘infección por el virus de la viremia primaveral de la carpa’

CAPÍTULO 10.9.

INFECCIÓN POR EL VIRUS DE LA VIREMIA PRIMAVERAL DE LA CARPA

[...]

Artículo 10.9.3.

Medidas para la importación o tránsito por el territorio 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 el virus de la viremia primaveral de la carpa

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 virus de la viremia primaveral de la carpa, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por el virus de la viremia primaveral de la carpa:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos ~~90~~60°C durante por lo menos 60 ~~segundos~~minutos, o un tiempo/temperatura equivalente que inactive el virus de la viremia primaveral de la carpa;
- 2) ~~pescado eviscerado y secado por medios mecánicos que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 90°C durante por lo menos 60 segundos, o un tiempo/temperatura equivalente que inactive el virus de la viremia primaveral de la carpa;~~
- 3) 2) *harina* de pescado que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos ~~90~~60°C durante por lo menos 60 ~~segundos~~minutos, o un tiempo/temperatura equivalente que inactive el virus de la viremia primaveral de la carpa;
- 4) 3) *aceite* de pescado.

[...]

Anexo 26. Ítem 6.5.3. – Artículos 10.10.3. del Capítulo 10.10. ‘infección por el virus de la septicemia hemorrágica viral’

CAPÍTULO 10.10.

INFECCIÓN POR EL VIRUS DE LA SEPTICEMIA HEMORRÁGICA VIRAL

[...]

Artículo 10.10.3.

Medidas para la importación o tránsito por el territorio 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 el virus de la septicemia hemorrágica viral

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 virus de la septicemia hemorrágica viral, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por el virus de la septicemia hemorrágica viral:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos ~~90~~60°C durante por lo menos 60 ~~segundos~~minutos, o un tiempo/temperatura equivalente que inactive el virus de la septicemia hemorrágica viral;
- 2) ~~pescado eviscerado y secado por medios mecánicos que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 90°C durante por lo menos 60 segundos, o un tiempo/temperatura equivalente que inactive el virus de la septicemia hemorrágica viral;~~
- 3) *harina* de pescado que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos ~~90~~60°C durante por lo menos 60 ~~segundos~~minutos, o un tiempo/temperatura equivalente que inactive el virus de la septicemia hemorrágica viral;
- 4) pescado eviscerado y secado naturalmente (es decir, secado por el sol o el viento);
- 5) aceite de pescado;
- 6) cueros elaborados con piel de pescado.

[...]

CAPÍTULO 10.11.

INFECCIÓN POR EL VIRUS DE LA TILAPIA DEL LAGO

[...]

Artículo 10.11.3.

Medidas para la importación o tránsito por el territorio 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 de 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 5660°C durante por lo menos ~~cinco~~120 minutos, o un tiempo/temperatura equivalente que inactive TiLV;
- 2) *harina* de pescado que se haya sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 5660°C durante por lo menos ~~cinco~~120 minutos, o un tiempo/temperatura equivalente que inactive TiLV} ~~(en estudio)~~;
- 3) aceite de pescado;
- 4) cueros elaborados con piel de pescado.

[...]

Anexo 28. Ítem 6.5.4. – Artículo 11.1.3. del Capítulo 11.1. ‘Infección por el herpesvirus del abalón’

(VERSIÓN CON SEGUIMIENTO DE CAMBIOS)

CAPÍTULO 11.1.

INFECCIÓN POR EL HERPESVIRUS DEL ABALÓN

[...]

Artículo 11.1.3.

Medidas para la importación o tránsito por el territorio 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 el herpesvirus del abalón

1) Los siguientes 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. Independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *H. penaei*, las autoridades competentes no deberán exigir ningún tipo de condición relacionada con *H. penaei* e Cuando autoricen la importación o el tránsito por su territorio de estos los siguientes productos de animales acuáticos, las autoridades competentes no deberán exigir ninguna medida sanitaria relacionada con el herpesvirus del abalón, independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por el herpesvirus del abalón: derivados de una de las especies mencionadas en el Artículo 11.1.2., cualquiera que sea el uso al que se destinan y siempre que reúnan las condiciones descritas en el Artículo 5.4.1.:

- 1) productos de animales acuáticos que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 121°C durante por lo menos cinco minutos y 36 segundos, o un tiempo/temperatura equivalente que inactive el herpesvirus del abalón.
 - a) productos de abalón termoesterilizados y sellados herméticamente (es decir, un tratamiento térmico a 121 °C durante al menos 3,6 minutos o una combinación equivalente de tiempo/temperatura);
 - b2) productos de abalón secados por medios mecánicos (es decir, que se hayan sometido a un tratamiento térmico a 100 °C durante al menos 30 minutos suficiente como para alcanzar una temperatura interna de al menos 121°C durante por lo menos 3 minutos y 36 segundos, o a una combinación equivalente de un tiempo/temperatura que haya demostrado que equivalente que inactiva el herpesvirus del abalón.
- 2) Las autoridades competentes deberán exigir las condiciones prescritas en los Artículos 11.1.7. a 11.1.11. que correspondan al estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por el herpesvirus del abalón cuando autoricen la importación o el tránsito por su territorio de cualesquiera animales acuáticos y productos de animales acuáticos relacionados con las especies mencionadas en el Artículo 11.1.2. que no sean unos de los enumerados en el apartado 1 del Artículo 11.1.3.
- 3) La autoridad competente deberá proceder a un análisis del riesgo acorde con las recomendaciones del Capítulo 2.1. cuando contemple la importación o el tránsito por su territorio de animales acuáticos y productos de animales acuáticos de cualquier especie no mencionada en el Artículo 11.1.2. pero que se considere que podría plantear un riesgo de propagación de la infección por el herpesvirus del abalón. La autoridad competente del país exportador deberá ser informada del resultado de la evaluación.

[...]

(VERSIÓN EN LIMPIO)

CAPÍTULO 11.1.

INFECCIÓN POR EL HERPESVIRUS DEL ABALÓN

[...]

Artículo 11.1.3.

Medidas para la importación o tránsito por el territorio 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 el herpesvirus del abalón

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 herpesvirus del abalón, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por el herpesvirus del abalón:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 50°C durante por lo menos cinco minutos, o un tiempo/temperatura equivalente que inactive el herpesvirus del abalón.

[...]

(VERSIÓN CON SEGUIMIENTO DE CAMBIOS)

CAPÍTULO 11.2.

INFECCIÓN POR *BONAMIA EXITIOSA*

[...]

Artículo 11.2.3.

Medidas para la importación o tránsito por el territorio 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 *B. exitiosa*

Los siguientes 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. Independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *H. penaei*, las autoridades competentes no deberán exigir ningún tipo de condición relacionada con *H. penaei* e cuando autoricen la importación o el tránsito por su territorio de estos los siguientes productos de animales acuáticos, las autoridades competentes no deberán exigir ninguna medida sanitaria relacionada con *B. exitiosa*, independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *B. exitiosa*: derivados de una de las especies mencionadas en el Artículo 11.2.2., cualquiera que sea el uso al que se destinan y siempre que reúnan las condiciones descritas en el Artículo 5.4.1.:

1) productos de animales acuáticos que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 100°C durante por lo menos 15 minutos, o un tiempo/temperatura equivalente que inactive *B. exitiosa*;

a) carne de ostra congelada;

b) ostras congeladas con media concha.

2) ~~Las autoridades competentes deberán exigir las condiciones prescritas en los Artículos 11.2.7. a 11.2.11. que correspondan al estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *B. exitiosa* cuando autoricen la importación o el tránsito por su territorio de cualesquiera animales acuáticos y productos de animales acuáticos relacionados con las especies mencionadas en el Artículo 11.2.2. que no sean unos de los enumerados en el apartado 1 del Artículo 11.2.3.~~

3) ~~La autoridad competente deberá proceder a un análisis del riesgo acorde con las recomendaciones del Capítulo 2.1. cuando contemple la importación o el tránsito por su territorio de animales acuáticos y productos de animales acuáticos de cualquier especie no mencionada en el Artículo 11.2.2. pero que se considere que podría plantear un riesgo de propagación de la infección por *B. exitiosa*. La autoridad competente del país exportador deberá ser informada del resultado de la evaluación.~~

[...]

(VERSIÓN EN LIMPIO)

CAPÍTULO 11.2.

INFECCIÓN POR *BONAMIA EXITIOSA*

[...]

Artículo 11.2.3.

Medidas para la importación o tránsito por el territorio 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 *B. exitiosa*

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 *B. exitiosa*, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por *B. exitiosa*:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 100°C durante por lo menos 15 minutos, o un tiempo/temperatura equivalente que inactive *B.exitiosa*;
- 2) carne de ostra congelada;
- 3) ostras congeladas con media concha.

[...]

Anexo 30. Ítem 6.5.4. – Artículo 11.3.3. del Capítulo 11.3. 'Infección por *Bonamia ostreae*'

(VERSIÓN CON SEGUIMIENTO DE CAMBIOS)

CAPÍTULO 11.3.

INFECCIÓN POR *BONAMIA OSTREAE*

[...]

Artículo 11.3.3.

Medidas para la importación o tránsito por el territorio 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 *B. ostreae*

1) Los siguientes 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. Independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *H. penaei*, las autoridades competentes no deberán exigir ningún tipo de condición relacionada con *H. penaei* e cuando autoricen la importación o el tránsito por su territorio de estos los siguientes productos de animales acuáticos, las autoridades competentes no deberán exigir ninguna medida sanitaria relacionada con *B. ostreae*, independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *B. ostreae*: derivados de una de las especies mencionadas en el Artículo 11.3.2., cualquiera que sea el uso al que se destinan y siempre que reúnan las condiciones descritas en el Artículo 5.4.1.:

1) productos de animales acuáticos que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 100°C durante por lo menos 15 minutos, o un tiempo/temperatura equivalente que inactive *B. ostreae*;

a) 12) carne de ostra congelada;

b) 23) ostras congeladas con media concha.

2) ~~Las autoridades competentes deberán exigir las condiciones prescritas en los Artículos 11.2.7. a 11.2.11. que correspondan al estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *B. exitiosa* cuando autoricen la importación o el tránsito por su territorio de cualesquiera animales acuáticos y productos de animales acuáticos relacionados con las especies mencionadas en el Artículo 11.2.2. que no sean unos de los enumerados en el apartado 1 del Artículo 11.2.3.~~

3) ~~La autoridad competente deberá proceder a un análisis del riesgo acorde con las recomendaciones del Capítulo 2.1. cuando contemple la importación o el tránsito por su territorio de animales acuáticos y productos de animales acuáticos de cualquier especie no mencionada en el Artículo 11.2.2. pero que se considere que podría plantear un riesgo de propagación de la infección por *B. exitiosa*. La autoridad competente del país exportador deberá ser informada del resultado de la evaluación.~~

[...]

(VERSIÓN EN LIMPIO)

CAPÍTULO 11.3.

INFECCIÓN POR *BONAMIA OSTREAE*

[...]

Artículo 11.3.3.

Medidas para la importación o tránsito por el territorio 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 *B. ostreae*

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 *B. ostreae*, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por *B. ostreae*:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 100°C durante por lo menos 15 minutos, o un tiempo/temperatura equivalente que inactive *B. ostreae*;
- 2) carne de ostra congelada;
- 3) ostras congeladas con media concha.

[...]

(VERSIÓN CON SEGUIMIENTO DE CAMBIOS)

CAPÍTULO 11.4.

INFECCIÓN POR *MARTEILIA REFRINGENS*

[...]

Artículo 11.4.3.

Medidas para la importación o tránsito por el territorio 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 *M. refringens*

1) Los siguientes 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. Independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *H. penaei*, las autoridades competentes no deberán exigir ningún tipo de condición relacionada con *H. penaei* e cuando autoricen la importación o el tránsito por su territorio de estos los siguientes productos de animales acuáticos, las autoridades competentes no deberán exigir ninguna medida sanitaria relacionada con *M. refringens*, independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *M. refringens*: derivados de una de las especies mencionadas en el Artículo 11.4.2., cualquiera que sea el uso al que se destinan y siempre que reúnan las condiciones descritas en el Artículo 5.4.1.:

- 1) productos de animales acuáticos que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 121°C durante por lo menos tres minutos y 36 segundos, o un tiempo/temperatura equivalente que inactive *M. refringens*.
- 2) Las autoridades competentes deberán exigir las condiciones prescritas en los Artículos 11.4.7. a 11.4.11. que correspondan al estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *M. refringens* cuando autoricen la importación o el tránsito por su territorio de cualesquiera animales acuáticos y productos de animales acuáticos relacionados con las especies mencionadas en el Artículo 11.4.2. que no sean unos de los enumerados en el apartado 1 del Artículo 11.4.3.
- 3) La autoridad competente deberá proceder a un análisis del riesgo acorde con las recomendaciones del Capítulo 2.1. cuando contemple la importación o el tránsito por su territorio de animales acuáticos y productos de animales acuáticos de cualquier especie no mencionada en el Artículo 11.4.2. pero que se considere que podría plantear un riesgo de propagación de la infección por *M. refringens*. La autoridad competente del país exportador deberá ser informada del resultado de la evaluación.

[...]

(VERSIÓN EN LIMPIO)

CAPÍTULO 11.4.

INFECCIÓN POR *MARTEILIA REFRINGENS*

[...]

Artículo 11.4.3.

Medidas para la importación o tránsito por el territorio 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 *M. refringens*

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 *M. refringens*, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por *M. refringens*:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 121°C durante por lo menos tres minutos y 36 segundos, o un tiempo/temperatura equivalente que inactive *M. refringens*.

[...]

(VERSIÓN CON SEGUIMIENTO DE CAMBIOS)

CAPÍTULO 11.5.

INFECCIÓN POR *PERKINSUS MARINUS*

[...]

Artículo 11.5.3.

Medidas para la importación o tránsito por el territorio 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 *P. marinus*

1) Los siguientes 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. Independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *H. penaei*, las autoridades competentes no deberán exigir ningún tipo de condición relacionada con *H. penaei* e Cuando autoricen la importación o el tránsito por su territorio de estos los siguientes productos de animales acuáticos, las autoridades competentes no deberán exigir ninguna medida sanitaria relacionada con *P. marinus*, independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *P. marinus*: derivados de una de las especies mencionadas en el Artículo 11.5.2., cualquiera que sea el uso al que se destinan y siempre que reúnan las condiciones descritas en el Artículo 5.4.1.:

- 1) productos de animales acuáticos que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 12160°C durante por lo menos 360 minutos y 36 segundos, o un tiempo/temperatura equivalente que inactive *P. marinus*.
- 2) Las autoridades competentes deberán exigir las condiciones prescritas en los Artículos 11.5.7. a 11.5.11. que correspondan al estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *P. marinus* cuando autoricen la importación o el tránsito por su territorio de cualesquiera animales acuáticos y productos de animales acuáticos relacionados con las especies mencionadas en el Artículo 11.5.2. que no sean unos de los enumerados en el apartado 1 del Artículo 11.5.3.
- 3) La autoridad competente deberá proceder a un análisis del riesgo acorde con las recomendaciones del Capítulo 2.1. cuando contemple la importación o el tránsito por su territorio de animales acuáticos y productos de animales acuáticos de cualquier especie no mencionada en el Artículo 11.5.2. pero que se considere que podría plantear un riesgo de propagación de la infección por *P. marinus*. La autoridad competente del país exportador deberá ser informada del resultado de la evaluación.

[...]

(VERSIÓN EN LIMPIO)

CAPÍTULO 11.5.

INFECCIÓN POR *PERKINSUS MARINUS*

[...]

Artículo 11.5.3.

Medidas para la importación o tránsito por el territorio 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 *P. marinus*

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 *P. marinus*, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por *P. marinus*:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 60°C durante por lo menos 60 minutos, o un tiempo/temperatura equivalente que inactive *P. marinus*.

[...]

(VERSIÓN CON SEGUIMIENTO DE CAMBIOS)

CAPÍTULO 11.6.

INFECCIÓN POR *PERKINSUS OLSENI*

[...]

Artículo 11.6.3.

Medidas para la importación o tránsito por el territorio 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 *P. olseni*

1) Los siguientes 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. Independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *H. penaei*, las autoridades competentes no deberán exigir ningún tipo de condición relacionada con *H. penaei* e cuando autoricen la importación o el tránsito por su territorio de estos los siguientes productos de animales acuáticos, las autoridades competentes no deberán exigir ninguna medida sanitaria relacionada con *P. olseni*, independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *P. olseni*: derivados de una de las especies mencionadas en el Artículo 11.6.2., cualquiera que sea el uso al que se destinan y siempre que reúnan las condiciones descritas en el Artículo 5.4.1.:

- 1) productos de animales acuáticos que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 12160°C durante por lo menos 360 minutos y 36 segundos, o un tiempo/temperatura equivalente que inactive *P. olseni*.
- 2) Las autoridades competentes deberán exigir las condiciones prescritas en los Artículos 11.6.7. a 11.6.11. que correspondan al estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *P. olseni* cuando autoricen la importación o el tránsito por su territorio de cualesquiera animales acuáticos y productos de animales acuáticos relacionados con las especies mencionadas en el Artículo 11.6.2. que no sean unos de los enumerados en el apartado 1 del Artículo 11.6.3.
- 3) La autoridad competente deberá proceder a un análisis del riesgo acorde con las recomendaciones del Capítulo 2.1. cuando contemple la importación o el tránsito por su territorio de animales acuáticos y productos de animales acuáticos de cualquier especie no mencionada en el Artículo 11.6.2. pero que se considere que podría plantear un riesgo de propagación de la infección por *P. olseni*. La autoridad competente del país exportador deberá ser informada del resultado de la evaluación.

[...]

(VERSIÓN EN LIMPIO)

CAPÍTULO 11.6.

INFECCIÓN POR *PERKINSUS OLSENI*

[...]

Artículo 11.6.3.

Medidas para la importación o tránsito por el territorio 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 *P. olseni*

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 *P. olseni*, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por *P. olseni*:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 60°C durante por lo menos 60 minutos, o un tiempo/temperatura equivalente que inactive *P. olseni*.

[...]

(VERSIÓN CON SEGUIMIENTO DE CAMBIOS)

CAPÍTULO 11.7.

INFECCIÓN POR *XENOHALIOTIS CALIFORNIENSIS*

[...]

Artículo 11.7.3.

Medidas para la importación o tránsito por el territorio 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 *X. californiensis*

1) Los siguientes 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. Independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *H. penaei*, las autoridades competentes no deberán exigir ningún tipo de condición relacionada con *H. penaei* e Cuando autoricen la importación o el tránsito por su territorio de estos los siguientes productos de animales acuáticos, las autoridades competentes no deberán exigir ninguna medida sanitaria relacionada con *X. californiensis*, independientemente del estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *X. californiensis*: derivados de una de las especies mencionadas en el Artículo 11.7.2., cualquiera que sea el uso al que se destinan y siempre que reúnan las condiciones descritas en el Artículo 5.4.1.:

- 1) productos de animales acuáticos que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos ~~121~~95°C durante por lo menos ~~3~~cinco minutos y ~~36~~ segundos, o un tiempo/temperatura equivalente que inactive *X. californiensis*.
- 2) Las autoridades competentes deberán exigir las condiciones prescritas en los Artículos 11.7.7. a 11.7.11. que correspondan al estatus sanitario del país, la zona o el compartimento de exportación respecto de la infección por *X. californiensis* cuando autoricen la importación o el tránsito por su territorio de cualesquiera animales acuáticos y productos de animales acuáticos relacionados con las especies mencionadas en el Artículo 11.7.2. que no sean unos de los enumerados en el apartado 1 del Artículo 11.7.3.
- 3) La autoridad competente deberá proceder a un análisis del riesgo acorde con las recomendaciones del Capítulo 2.1. cuando contemple la importación o el tránsito por su territorio de animales acuáticos y productos de animales acuáticos de cualquier especie no mencionada en el Artículo 11.7.2. pero que se considere que podría plantear un riesgo de propagación de la infección por *X. californiensis*. La autoridad competente del país exportador deberá ser informada del resultado de la evaluación.

[...]

(VERSIÓN EN LIMPIO)

CAPÍTULO 11.7.

INFECCIÓN POR *XENOHALIOTIS CALIFORNIENSIS*

[...]

Artículo 11.7.3.

Medidas para la importación o tránsito por el territorio 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 *X. californiensis*

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 *X. californiensis*, independientemente del estatus sanitario del país, la *zona* o el *compartimento* de exportación respecto de la infección por *X. californiensis*:

- 1) *productos de animales acuáticos* que se hayan sometido a un tratamiento térmico suficiente como para alcanzar una temperatura interna de al menos 95°C durante por lo menos cinco minutos, o un tiempo/temperatura equivalente que inactive *X. californiensis*.

[...]

Anexo 35. Ítem 6.6. – Modelo de Artículos X.X.5. y X.X.6. para los capítulos específicos de las enfermedades

Modelo de Artículos X.X.5. y X.X.6. para los capítulos específicos de enfermedades

CAPÍTULO X.X.

INFECCIÓN POR [PATÓGENO X]

[...]

Artículo X.X.5.

País libre de infección por [patógeno X]

Si el país comparte cuerpos de agua con otros países, solo podrá hacer una autodeclaración de ausencia de infección por [patógeno X] si todos los cuerpos de agua compartidos están situados en países o *zonas* declarados libres de infección por [patógeno X] (véase el Artículo X.X.6.).

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

1) ninguna *especie susceptible* de las mencionadas en el Artículo X.X.2. está presente en el país 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 [patógeno X] 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 [patógeno X], 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 del [patógeno X], y se han reunido ininterrumpidamente e implementado las *condiciones elementales de bioseguridad* durante al menos [un] año antes del inicio de la *vigilancia específica*;

O

4) había hecho previamente una autodeclaración de ausencia de infección por [patógeno X] y perdió posteriormente su estatus libre por haberse detectado el [patógeno X], pero se han cumplido las condiciones siguientes:

a) nada más haberse detectado el [patógeno X], 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 del [patógeno X], 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 [patógeno X]; 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 del [patógeno X], o
 - ii) por lo menos el [un] último año sin que se haya detectado la presencia del [patógeno X] si los *establecimientos de acuicultura* afectados no tenían vínculos epidemiológicos con las poblaciones silvestres de *especies susceptibles*.

Mientras tanto, ~~la parte del país ubicada fuera de la zona infectada y de la zona de protección una parte o la totalidad del país, excepto las zonas infectadas y de protección,~~ podrá ser declarada *zona libre*, según se describe en el Artículo 1.4.4. siempre que reúna las condiciones descritas en el apartado 2 del Artículo X.X.6.

Artículo X.X.6.

Zona libre de infección por [patógeno X]

Si una *zona* se extiende por el *territorio* de más un país, solo podrá ser declarada *zona libre* de infección por [patógeno X] 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 [patógeno X] para una *zona* dentro de su *territorio* si puede demostrar que:

- 1) ninguna *especie susceptible* de las mencionadas en el Artículo 10.10.2. 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 [patógeno X] 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 [patógeno X], 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 del [patógeno X], y se han reunido ininterrumpidamente e implementado las *condiciones elementales de bioseguridad* durante al menos [un] año antes del inicio de la *vigilancia específica*;

O
- 4) había hecho previamente una autodeclaración de ausencia de infección por [patógeno X] para una *zona* y perdió posteriormente su estatus libre por haberse detectado [patógeno X] en la *zona*, pero se han cumplido las condiciones siguientes:
 - a) nada más haberse detectado [patógeno X], 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 del [patógeno X], 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 [patógeno X]; 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 del [patógeno X].

Mientras tanto, una parte de la zona ubicada fuera de la zona infectada y de la zona de protección podrá ser declarada una nueva zona libre, según se describe en el Artículo 1.4.4.

[...]

Anexo 36. Ítem 6.7. – Artículo 9.3.2. del Capítulo 9.3. infección por el virus iridiscente de los decápodos tipo 1

CAPÍTULO 9.3.

INFECCIÓN POR EL VIRUS IRIDISCENTE DE LOS DECÁPODOS TIPO 1

[...]

Artículo 9.3.2.

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.: langostino carnoso (*Penaeus chinensis*), jaiba gazami (*Portunus trituberculatus*), langostino de río (*Macrobrachium rosenbergii*), langostino japonés (*Penaeus japonicus*), camarón nipón (*Macrobrachium nipponense*), *Cherax quadricarinatus*, cangrejo de las marismas (*Procambarus clarkii*), camarón quilla (*Exopalaemon carinicauda*) y camarón blanco (*Penaeus vannamei*), camarón tigre gigante (*Penaeus monodon*), langosta australiana de agua dulce (*Cherax quadricarinatus*), camarón gigante de agua dulce (*Macrobrachium rosenbergii*), cangrejo rojo americano (*Procambarus clarkia*), camarón nipón (*Macrobrachium nipponense*) y camarón quilla (*Exopalaemon carinicauda*) (en estudio).

Familia	Nombre científico	Nombre común
Cambaridae	<i>Procambarus clarkii</i>	cangrejo de las marismas
Palaemonidae	<i>Macrobrachium nipponense</i>	camarón nipón
	<i>Macrobrachium rosenbergii</i>	langostino de río
	<i>Palaemon carinicauda</i>	camarón quilla
Parastacidae	<i>Cherax quadricarinatus</i>	[red claw crayfish]
Penaeidae	<i>Penaeus japonicus</i>	langostino japonés
	<i>Penaeus vannamei</i>	camarón blanco
Portunidae	<i>Portunus trituberculatus</i>	jaiba gazami

[...]

Anexo 37. Ítem 6.8. – Artículo 10.6.2. del Capítulo 10.6. infección por el virus de la necrosis hematopoyética infecciosa

CAPÍTULO 10.6.

INFECCIÓN POR EL VIRUS DE LA NECROSIS
HEMATOPOYÉTICA INFECCIOSA

[...]

Artículo 10.6.2.

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.: trucha alpina (*Salvelinus alpinus*), salmón del Atlántico (*Salmo salar*), trucha de manantial (*Salvelinus fontinalis*), trucha café (*Salmo trutta*), trucha arco iris (*Oncorhynchus mykiss*), salmón real [*Oncorhynchus tshawytscha*], rojo [*Oncorhynchus nerka*], chum [*Oncorhynchus keta*], lucio europeo (*Esox lucius*), masou [*Oncorhynchus masou*], y plateado [*Oncorhynchus kisutch*], trucha garganta cortada (*Oncorhynchus clarkii*), trucha del lago (*Salvelinus namaycush*) y trucha marmorata (*Salmo marmoratus*).

<u>Familia</u>	<u>Nombre científico</u>	<u>Nombre común</u>
<u>Esocidae</u>	<u><i>Esox lucius</i></u>	<u>lucio europeo</u>
<u>Salmonidae</u>	<u><i>Oncorhynchus clarkii</i></u>	<u>trucha garganta cortada</u>
	<u><i>Oncorhynchus keta</i></u>	<u>salmón keta</u>
	<u><i>Oncorhynchus kisutch</i></u>	<u>salmón plateado</u>
	<u><i>Oncorhynchus masou</i></u>	<u>salmón japonés</u>
	<u><i>Oncorhynchus mykiss</i></u>	<u>trucha arco iris</u>
	<u><i>Oncorhynchus nerka</i></u>	<u>salmón rojo</u>
	<u><i>Oncorhynchus tshawytscha</i></u>	<u>salmón real</u>
	<u><i>Salmo marmoratus</i></u>	<u>trucha marmorata</u>
	<u><i>Salmo salar</i></u>	<u>salmón del Atlántico</u>
	<u><i>Salmo trutta</i></u>	<u>trucha café</u>
	<u><i>Salvelinus alpinus</i></u>	<u>trucha alpina</u>
	<u><i>Salvelinus fontinalis</i></u>	<u>trucha de manantial</u>
<u><i>Salvelinus namaycush</i></u>	<u>trucha del lago</u>	

[...]

CAPÍTULO 10.11.

INFECCIÓN POR EL VIRUS DE LA TILAPIA DEL LAGO

[...]

Artículo 10.11.2.

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 azul del Nilo, híbrido (*Oreochromis aureus* X *Oreochromis niloticus*), *Sarotherodon galilaeus*, tilapia de Mozambique (*Oreochromis mossambicus*), tilapia del Nilo (*Oreochromis niloticus*) y 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).

Familia	Nombre científico	Nombre común
Cichlidae	<i>Oreochromis aureus</i> x <i>O. niloticus</i>	tilapia azul-del Nilo híbrido
	<i>Oreochromis mossambicus</i>	tilapia de Mozambique
	<i>Oreochromis niloticus</i>	tilapia del Nilo
	<i>Oreochromis niloticus</i> x <i>O. mossambicus</i>	híbrido de tilapia roja
	<i>Sarotherodon galilaeus</i>	[mango tilapia]

[...]

CAPÍTULO 11.5.
INFECCIÓN POR *PERKINSUS MARINUS*

Artículo 11.5.1.

A efectos del *Código Acuático*, la infección por *Perkinsus marinus* designa una *infección* causada exclusivamente por el agente patógeno *P. marinus* de la familia *Perkinsidae*.

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

Artículo 11.5.2.

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: la ostra americana (*Crassostrea virginica*), la ostra del Pacífico (*Crassostrea gigas*) y la ostra de Suminoe (*Crassostrea ariakensis* (*Magallana* [*Syn. Crassostrea*] *ariakensis*), la ostra de Cortés (*Crassostrea corteziensis*) y la otra palmeada (*Saccostrea palmula*), la almeja de río (*Mya arenaria*), *Macoma balthica* y la almeja americana (*Mercenaria mercenaria*). Estas recomendaciones se aplican también a todas las demás especies susceptibles mencionadas en el *Manual Acuático* que sean objeto de *comercio internacional*.

<u>Familia</u>	<u>Nombre científico</u>	<u>Nombre común</u>
<u>Ostreidae</u>	<u><i>Crassostrea corteziensis</i></u>	<u>ostra de Cortés</u>
	<u><i>Crassostrea virginica</i></u>	<u>ostra americana</u>
	<u><i>Magallana</i> [<i>syn. Crassostrea</i>] <i>ariakensis</i></u>	<u>[Ariake cupped oyster]</u>
	<u><i>Saccostrea palmula</i></u>	<u>otra palmeada</u>

[...]

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, the diagnostic method to be used and the objective of testing (i.e. surveillance of apparently healthy animals, presumptive diagnosis of clinically affected animals or confirmatory diagnosis of a suspect result from surveillance or presumptive 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 WOAHA *Aquatic Code* Chapter 1.4. *Aquatic animal disease surveillance*.

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, epidemiological units 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 collection.
- iii) ~~For the study of presumptively diseased crustaceans select those animals that are moribund, discoloured, displaying abnormal behaviour, or otherwise abnormal. If weak, abnormally behaving, discoloured or freshly dead (not decomposed) animals are present, such animals should be selected. If such animals are not present, animals should be selected in such a way that all epidemiological units of the farm or waterbody are proportionately represented in the sample.~~
- 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 (including 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 provided they are not decomposed. 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 WOAHA-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 WOA-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

Virological examination by virus isolation in cell culture of crustaceans is not routinely used for listed diseases of crustaceans. ~~*Macrobrachium rosenbergii* has been isolated in insect cell lines, but it is not a recommended method.~~

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*. Not applicable.~~

2.2.2. Virus isolation

~~For processing of tissues see Section 3 of disease-specific chapters in this *Aquatic Manual*. Not applicable.~~

2.2.3. Treatment to neutralise enzootic viruses

Not applicable.

2.3. Bacteriological examination

Bacteriological examination of crustaceans is not routinely used for listed diseases, but it may be used for the strains of *Vibrio parahaemolyticus* (*Vp*_{AHPND}) that cause acute hepatopancreatic necrosis disease (AHPND). ~~and for 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 for experimental purposes

As no cell lines (crustacean, arthropod, or vertebrate) are known that can be used to produce crustacean viruses, stocks *in vitro*, infection of known susceptible host species (which are free ~~of~~ from infection ~~by~~ with the pathogenic agent in question) is the preferred method for virus production for experimental purposes or for the development of positive control material.

1.3.3. Virus preservation and storage

Infectivity of all of the WOAHL-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. *Infection with Aphanomyces astaci (crayfish plague)*

4.2. Storage of cultures

See Chapter 2.2.2.

5. Techniques

The available diagnostic methods that may be selected for diagnosis of the WOA-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~~ is only 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 individuals (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 **(including 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)
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 penaeids, 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 WOAHA.

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~~ extracted from crustacean tissue. ~~The Molecular~~ techniques can be used in direct surveillance of crustacean diseases in 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.

Each diagnostic samples should be tested in duplicate, i.e. by testing two aliquots. ~~and Both aliquots~~ 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 barrier~~ pipette tips should be used for all sample preparation and PCR ~~preparation~~ steps. Additionally, all PCRs should be prepared in a clean area that is separate from the area where ~~the nucleic acid extraction~~, amplifications and gel electrophoresis are performed. Do not share equipment (e.g. pipettes, laboratory coats and consumables) between areas and, where possible, restrict access between areas. Contaminating PCR products can be carried on equipment, clothes, shoes, pens/marker pens and paper (e.g. workbooks). Also, ensure all work-tops and air-flow cabinets/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~~ separate from the molecular biology laboratory and reagents.

Nested PCR involves two rounds of PCR and may be used to achieve increased sensitivity and specificity; however, it increases the risk of contamination. Contaminants from previous reactions can carry over and lead to false-positive results. Strict laboratory practices such as separate workspaces, dedicated equipment, and meticulous pipetting techniques are essential to mitigate this risk. In conclusion, nested PCR is not recommended for surveillance but may sometimes be used for confirmative studies.

5.5.1. Sample preparation and types

Samples should be prepared to preserve the nucleic acid of the pathogen and should be handled and packaged with the greatest care to minimise the potential for cross-contamination among the samples or target degradation before the assay can be performed. ~~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%~~ 80% analytical grade 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 or freezer bricks 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*. In situations where it is not possible to get the specimens to the laboratory alive, they may be quick freeze-frozen in the field using crushed dry-ice or freeze-frozen 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%~~ 80% analytical grade 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%~~ 80% analytical grade 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).
- vi) *Fixed tissues for in-situ hybridisation*: For this purpose, classic methods for preservation of the tissues are adequate. Neutral-buffered formalin-Davidson’s fixative is usually a good choice. Samples should be fixed for 24–48 hours; fixation for ~~over 24~~ more than 48 hours in Davidson’s fixative should be avoided. Samples should be transferred to 80% analytical grade ethanol following Davidson’s fixation treatment.

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 analytical grade ethanol ~~alcohol~~ (80–90%) is the preferred method for subsequent molecular tests. Samples preserved in this way can be stored for up to 1 week at 4°C ~~for 1 month, at~~ or 25°C for 1 week or ~~indefinitely for extended periods~~ at –20°C or below. In addition, other products (e.g. nucleic acid preservatives, various lysis buffers, etc.) are acceptable and 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%~~ 80% analytical grade 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 of the place of origin. The geographical location of the place of origin of samples may be described as the name or location of the sampling site from which the sample has originated, 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 of origin 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.

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

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

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. North American host species spiny-cheek crayfish (*Orconectes limosus*) has been shown to be a carrier of Group E (Kozubiková *et al.*, 2011).

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

The recommendations in this chapter apply to all species of crayfish in all three crayfish families (Cambaridae, Astacidae and Parastacidae).

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

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

[Under study]

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 that development of clinical disease and experience mortality mortalities, and those that are infected without associated but do not display any significant clinical disease or experience mortality mortalities. All life stages of susceptible species are considered susceptible to infection with *A. astaci*.

Species that develop clinical disease and experience mortality mortalities include 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 Danube crayfish (*Pontastacus leptodactylus*) of eastern Europe and Asia Minor (e.g. Holdich *et al.*, 2009). Australian species of freshwater crayfish are also considered vulnerable to clinical disease and mortality mortalities.

Species that can be infected but do not normally develop clinical disease include North American crayfish species such as the signal crayfish (*Pacifastacus leniusculus*), Louisiana swamp crayfish (*Procambarus clarkii*) and *Faxonius* spp. All North American crayfish species that have been investigated have been shown to be susceptible to infection, demonstrated by the presence of the pathogen in host cuticle (reviewed by Svoboda *et al.* 2017).

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.

The only non-crayfish crustacean species known to be susceptible to infection by *A. astaci* is the Chinese mitten crab (*Eriocheir sinensis*) (Schrimpf *et al.*, 2014).

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, which are prone to development of clinical disease, 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 (Oldtmann *et al.*, 2006; Vralstad *et al.*, 2011).

2.2.5. Aquatic animal reservoirs of infection

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

~~Colonisation of habitats, initially by North American crayfish species carrying A. astaci occupied by highly susceptible is likely to result in an epizootic if crayfish species that are prone to expression of clinical disease are present 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; Oldtmann *et al.*, 2002). There is also circumstantial evidence of spread by contaminated equipment (e.g. nets, boots, clothing, traps) (Alderman *et al.*, 1987). None known.

2.3. Disease pattern

2.3.1. Mortality, morbidity, and prevalence

When the infection first reaches a naïve population of highly susceptible crayfish species that are prone to clinical disease, high levels of mortality are usually observed within a short space of time, ~~so that in and~~ In areas with high crayfish densities the bottoms of lakes, rivers and streams are become 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 a lower rate of~~ 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~~ any noticeable mortalities (Viljamaa-Dirks *et al.*, 2013).

On rare occasions, single specimens of ~~the highly susceptible~~ species that are prone to clinical disease 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 waterway, 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 may occur.

~~In the highly susceptible~~ European crayfish species, which are prone to clinical disease, 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~~ amplifies in affected animals and is 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 prone to clinical disease

Gross clinical signs are variable and depend on challenge severity and water temperatures. The first sign of an epizootic outbreak 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 **Susceptible** species that do not normally develop clinical disease

Infected North American crayfish may be subclinical **carriers-reservoirs**. 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 prone to clinical disease

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 intestinal 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 pereopods (walking legs), particularly the proximal joint, eyestalks and finally the gills.

North American crayfish **Susceptible** Species that do not normally develop clinical disease

Infected North American crayfish ~~do not usually show signs of disease 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, upon 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-Urbeondo *et al.*, 1995).

The main routes of spread of the pathogen are through 1) movement of infected crayfish, 2) movement of spores with contaminated water or equipment, or 3) through colonisation of non-native 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 occurred 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).

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).

2.3.5. 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. CaCl₂ stimulates zoospore emergence from primary cysts, whereas MgCl₂ 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 (Martin-Torrijos *et al.*, 2021).

~~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 WOAHA 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 crayfish species that are prone to clinical disease (reviewed by Martín-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 crayfish species that are prone to clinical disease 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 prone to expression of clinical disease 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 susceptible species or vectors animals (crayfish, finfish) only from sources known to be free from infection with *A. astaci*).
2. Prevent movements of potentially infected live or dead crayfish, potentially contaminated water, equipment or any other items that might carry the pathogen from an infected to an uninfected site holding susceptible species should be prevented.
3. If Do not transfers of finfish or crayfish are being planned, these should not come susceptible species or vectors 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 species).
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 that are prone to clinical disease, sampled 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 environment-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.

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 that are prone to clinical disease, 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 and eyestalks can be included as well.

If any melanised 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-reservoirs, 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.

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

3.5. Preservation of samples for submission

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 *General information (diseases of crustaceans)*

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.

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

3.5.3. Samples for histopathology

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~~ is only 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.

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

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

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV
Wet mounts						+	+	NA				
Histopathology						+	+	NA				
Cell-Culture						+	+	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 Immunohistochemistry												
Ab-ELISA												
Ag-ELISA												
Other antigen detection methods ³												
Other methods ³												

LV = level of validation, refers to the stage of validation in the WOAHP Pathway (chapter 1.1.2); NA = not available. PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification;

Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively; 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 **to visualise *A. astaci* in tissues of crayfish species prone to clinical disease**. 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 Laboratory.¹

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 **of tissue is homogenised using standard physical methods 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 is important and should can be checked using a suitable method as appropriate to the circumstances using optical density or running a gel.

4.4.1. Real-time PCR

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

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling conditions ^(a)
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¹ <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>

Method 1*: Vralstad <i>et al.</i> , 2009, Strand, 2013; GenBank Accession No.: AM947024			
<i>Aphanomyces astacus-astaci</i> & <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 <u>500 nM</u> 200 nM	50 cycles of: 95°C/15 sec and <u>60.58°C/30-60</u> sec
Method 2: Strand <i>et al.</i> , 2023; GenBank Accession No.: AM947024			
<u><i>Aphanomyces astacus astaci</i>/ITS</u>	<u>Fwd: TAT-CCA-CGT-GAA-TGT-ATT-CTT-TAT</u> <u>Rev: GCT-AAG-TTT-ATC-AGT-ATG-TTA-TTT-A</u> <u>Probe: FAM-AAG-AAC-ATC-CCA-GCA-C-MGBNFQ</u>	<u>500 nM</u> <u>500 nM</u> <u>100 nM</u>	<u>50 cycles of:</u> <u>95°C/15 sec and 60°C/30 sec</u>

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

^(a)A denaturation step prior to cycling has not been included.

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, A modified alternative method for the assay will be included once it has been published has been modified according to the alternative method 2 (Strand *et al.*, 2023 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 non-negative-an amplification product in the real-time PCR assay result. 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 ^(a)
Method 1*: Oidtmann <i>et al.</i> , 2006; GenBank Accession No.: AY310499; <u>Product amplicon</u> size: 569 bp			
<i>Aphanomyces astacus-astaci</i> & <i>A. fennicus</i> / ITS	Fwd: GCT-TGT-GCT-GAG-GAT-GTT-CT Rev: CTA-TCC-GAC-TCC-GCA-TTC-TG-	500 nM <u>500 nM</u>	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).

^(a)A denaturation step prior to cycling has not been included.

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 confirmative 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 (Casabella-Herrero *et al.*, 2021).

4.5. Amplicon sequencing

~~, and purified by excision from this gel. Both DNA strands of the PCR product must be sequenced and analysed and compared in comparison with published reference sequences.~~

4.6. *In-situ* hybridisation

Not 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 utilising the modified assay by Strand *et al.* ([2023 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 suspect and confirmed cases have been developed to support decision-making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. ~~It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the WOAHP Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.~~ If a laboratory Competent Authority does not have the capacity capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAHP Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free.

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

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

6.1.1. Definition of suspect case in apparently healthy animals

² For example transboundary commodities.

The presence of infection with *Aphanomyces astaci* shall be suspected if at least one of the following ~~criteria~~ criteria is met:

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

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with *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* in wet mounts
- 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 no data are currently available for either~~). ~~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
<u>Real-time PCR</u>	<u>Distinguish between <i>A. astaci</i> and <i>A. finnicus</i></u>		<u>Mycelium, tissue samples</u>	<u><i>Astacus astacus</i></u>		<u>Only detected <i>A. astacus</i></u>		<u>Strand et al., 2023</u>

DSe = diagnostic sensitivity, DSp = diagnostic specificity, n = samples number of animals used in the validation 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
Real-time PCR	Distinguish between <i>A. astaci</i> and <i>A. finnicus</i>		Tissue samples, environmental DNA	<i>Astacus astacus</i>		Only detected <i>A. astacus</i>		Strand et al., 2023

DSe = diagnostic sensitivity, DSp = diagnostic specificity, n = samples number of animals used in the validation study.

7. References

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NB: There is a WOAHP Reference Laboratory for infection with *Aphanomyces astaci* (crayfish plague)
(please consult the WOAHP 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 WOAHP 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 2.2.6.

INFECTION WITH
MACROBRACHIUM ROSENBERGII NODAVIRUS (WHITE
TAIL DISEASE)

1. Scope

Infection with *Macrobrachium rosenbergii* nodavirus means infection with the pathogenic agent *Macrobrachium rosenbergii* nodavirus (MrNV) in the Family *Nodaviridae*. The disease is commonly known as white tail disease (WTD).

Extra small virus (XSV) is associated with disease but its role has not been determined.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Two viruses are associated with WTD, namely MrNV (primary) and extra small virus (XSV) (associate) (Qian *et al.*, 2003; Romestand & Bonami, 2003). MrNV is a necessary cause of WTD in prawns, however, the role of XSV in pathogenicity remains unclear.

MrNV belongs in the family *Nodaviridae* (Bonami *et al.*, 2005). While the physico-chemical properties of MrNV are consistent with those of other members of the *Nodaviridae*, it differs structurally and genetically from other nodaviruses within the two recognised genera, *Alphanodavirus* and *Betanodavirus* (Ho *et al.*, 2017, 2018; Naveenkumar *et al.*, 2013). Consequently, a third genus, *Gammanodavirus*, has been proposed for nodaviruses that infect crustaceans, including MrNV and *Penaeus vannamei* nodavirus (PvNV) (Naveenkumar *et al.*, 2013).

XSV is the first sequenced satellite virus in aquatic animals and it is also the first record of a satellite-nodavirus association (Bonami *et al.*, 2005). XSV has been classified by the ICTV as *Macrobrachium satellite virus 1* of the family *Sarothroviridae*.

2.1.2. Survival and stability in processed or stored samples

Both viral pathogens (MrNV and XSV) are stable in processed or stored samples stored at –20 or –80°C. Storing the samples at –80°C is recommended for long-time storage and maintenance of pathogen virulence (Sahul Hameed & Bonami, 2012). The infected samples should be processed at low temperature to maintain the stability of the viruses. Viral inoculum prepared from infected prawn stored at –20°C caused 100% mortality in postlarvae (PL) of *M. rosenbergii* by immersion challenge (Qian *et al.*, 2003; Sahul Hameed *et al.*, 2004a). Ravi & Sahul Hameed (2016) found that MrNV in tissue suspensions was inactivated after exposure to 50°C for at least 5 min.

2.1.3. Survival and stability outside the host

Survival outside the host is not known.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with MrNV according to Chapter 1.5. of the *Aquatic Animal Health Code (Aquatic Code)* are: giant river prawn (*Macrobrachium rosenbergii*).

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 MrNV according to Chapter 1.5. of the *Aquatic Code* are: white leg shrimp (*Penaeus vannamei*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results (but not active infection) have been reported in the following species:

Family	Scientific name	Common name
<i>Aeshnidae</i>	<i>Aeshna</i> sp.	dragonfly
<i>Artemiidae</i>	<i>Artemia</i> sp.	brine shrimps
<i>Belostomatidae</i>	<i>Belostoma</i> sp.	giant water bug
<i>Dytiscidae</i>	<i>Cybister</i> sp.	beetle
<i>Notonectidae</i>	<i>Notonecta</i> sp.	backswimmer
<i>Palaemonidae</i>	<i>Macrobrachium rude</i>	hairy river prawn
	<i>Macrobrachium malcolmsonii</i>	monsoon river prawn
<i>Parastacidae</i>	<i>Cherax quadricarinatus</i>	red claw crayfish
<i>Penaeidae</i>	<i>Penaeus japonicus</i>	kuruma prawn
	<i>Penaeus indicus</i>	Indian white prawn
	<i>Penaeus monodon</i>	giant tiger prawn

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

Experimental pathogenicity studies revealed that larvae, PL and early juveniles of *M. rosenbergii* are susceptible to MrNV/XSV, whereas adults are resistant ([Gangnonngiwa et al., 2020](#); Qian et al., 2003; Sahul Hameed et al., 2004a).

No mortality was observed either in naturally or experimentally (MrNV/XSV) infected subadult and adult prawns. Experimental studies confirmed vertical transmission from infected broodstock to PL (Sudhakaran et al., 2007a).

2.2.4. Distribution of the pathogen in the host

MrNV and XSV have been demonstrated in gill tissue, head muscle, heart, abdominal muscle, ovaries, pleopods and tail muscle, but not the hepatopancreas or eyestalk (Sahul Hameed et al., 2004a; Sri Widada et al., 2003).

2.2.5. Aquatic animal reservoirs of infection

~~One study has~~ Studies have indicated the possibility that marine shrimp may act as a reservoirs for MrNV and XSV and that these viruses maintain virulence in the shrimp tissue system ([Senapin et al., 2012](#); Sudhakaran et al., 2006).

2.2.6. Vectors

Aquatic insects such as dragonfly (*Aeshna* sp.), giant water bug (*Belostoma* sp.), beetle (*Cybister* sp.) and backswimmer (*Notonecta* sp.) may act as mechanical carriers for MrNV/XSV and are a potential transmission risk to cultivated *Macrobrachium rosenbergii* (Sudhakaran et al., 2008). It is recommended to remove these insects from freshwater prawn culture systems, especially at larval-rearing centres. Sudhakaran et al. (2008) demonstrated RT-PCR positives from insects, and infected C6/36 insect cell line with tissue homogenates from the insects. Viral replication was confirmed through EM and RT-PCR, but transmission from insects to naïve shrimp was not demonstrated.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Larvae, PL and juveniles of *M. rosenbergii* are susceptible to infection with MrNV, which often causes high mortalities in these life stages. Mortality may reach a maximum in about 5 or 6 days after the appearance of the first clinical signs. Very few PL with infection with MrNV survive beyond 15 days in an outbreak, but PL that survive may grow to market size. Adults are resistant to infection with MrNV, but act as carriers (Qian et al., 2003; Sahul Hameed et al., 2004a). Prevalence is variable from 10% to 100% in hatchery, nursery and grow-out systems (Arcier et al., 1999; Qian et al., 2003; Sahul Hameed et al., 2004a; 2004b).

2.3.2. Clinical signs, including behavioural changes

Infected PL become opaque and develop a whitish appearance, particularly in the abdominal region. The whitish discoloration appears first in the second or third abdominal segment and gradually diffuses both anteriorly and posteriorly. In severe cases, degeneration of telson and uropods may occur. Floating exuviae (moult) in the tanks appear abnormal and resemble 'mica flakes' (Arcier *et al.*, 1999). The infected PL show progressive weakening of their feeding and swimming ability (Sahul Hameed *et al.*, 2004a).

2.3.3. Gross pathology

Infection with MrNV is indicated by the whitish coloration of abdominal muscle.

2.3.4. Modes of transmission and life cycle

Transmission is vertical by trans-ovum and horizontal by the waterborne route (Qian *et al.*, 2003; Sahul Hameed *et al.*, 2004a; Sudhakaran *et al.*, 2007a).

2.3.5. Environmental factors

Not available.

2.3.6. Geographical distribution

The disease was first reported in the ~~French West Indies-Caribbean~~ (Arcier *et al.*, 1999), and later in Asia-Pacific (Murwantoko *et al.*, 2016; Owens *et al.*, 2009; Qian *et al.*, 2003; Saedi *et al.*, 2012; Sahul Hameed *et al.*, 2004b; Wang *et al.*, 2008; Yoganandhan *et al.*, 2006).

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

2.4. Biosecurity and disease control strategies

Preventive measures, such as screening of broodstock and PL, and good management practices may help to prevent infection with MrNV in culture systems. As the life cycle of *M. rosenbergii* is completed under controlled conditions, specific pathogen-free (SPF) broodstock and PL can be produced (Romestand & Bonami, 2003; Sri Widada *et al.*, 2003; Yoganandhan *et al.*, 2005).

2.4.1. Vaccination

Not available

2.4.2. Chemotherapy including blocking agents

No known chemotherapeutic agents are reported to treat MrNV-infected prawn.

2.4.3. Immunostimulation

The immunomodulatory effect of recombinant capsid protein and recombinant RNA-dependent RNA polymerase (RdRp) protein of MrNV has been studied and the protection of viral challenged post-larvae from MrNV infection has been demonstrated (Farook *et al.*, 2014; NaveenKumar *et al.*, 2021).

2.4.4. Breeding resistant strains

None reported.

2.4.5. Inactivation methods

A viral suspension treated with heat at 65°C for 2 hours destroyed infectivity of MrNV and XSV in challenge experiments (Qian *et al.*, 2003). The viral inoculum exposed to UV irradiation for a period of 5 minutes and more was totally inactivated and failed to cause mortality in prawn PL of prawn (Ravi & Sahul Hameed, 2016).

2.4.6. Disinfection of eggs and larvae

Routine disinfection procedures followed for crustacean viral disease control are suggested.

2.4.7. General husbandry

MrNV is transmitted both horizontally and vertically in culture systems (Qian *et al.*, 2003; Sahul Hameed *et al.*, 2004a; Sudhakaran *et al.*, 2007a). Good husbandry practices, such as proper disinfection of tanks and water may help to prevent infection. It is recommended to remove insects from freshwater prawn culture systems, especially at larval-rearing centres. Specific pathogen-free (SPF) broodstock and PL can be obtained from disease free populations or by RT-PCR screening and selection of negative broodstock (Romestand & Bonami, 2003; Sri Widada *et al.*, 2003; Yoganandhan *et al.*, 2005).

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

PLs are most suitable for detection of MrNV. PL showing clinical signs of disease can be sampled preferentially. Adults and juveniles can be sampled for MrNV however prevalence in these lifestages may be lower (see Section 2.3.1).

3.2. Selection of organs or tissues

The tissues most affected in moribund PLs/early juveniles are striated muscles of the abdomen, cephalothorax and tail. The whole PL body is preferred for detection of MrNV (Sahul Hameed *et al.*, 2004b; Sri Widada *et al.*, 2003; Yoganandhan *et al.*, 2005). All organs of adult *M. rosenbergii* except eyestalks and the hepatopancreas, are best for screening the viruses by RT-PCR.

3.3. Samples or tissues not suitable for pathogen detection

Eyestalks and the hepatopancreas of adult prawns are not suitable (Sri Widada *et al.*, 2003; Sahul Hameed *et al.*, 2004a).

3.4. Non-lethal sampling

Pleopods (swimming legs) are a convenient source of RNA for non-destructive screening of MrNV in adult prawn (Sahul Hameed *et al.*, 2004a).

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

3.5. Preservation of samples for submission

Infected larvae or PL with prominent signs of whitish muscle in the abdominal region are collected from disease outbreak areas. Samples are washed in sterile saline, transferred to sterile tubes, and transported to the laboratory. For general 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

Moribund or frozen PL samples can be used for isolation of viral pathogens using cell lines (C6/36 mosquito cell line (Sudhakaran *et al.*, 2007b).

3.5.2. Preservation of samples for molecular detection

Infected samples stored at -80°C or samples preserved in 80% (v/v) analytical/reagent-grade (undenatured) ethanol should be used for RT-PCR for detection of MrNV (Sri Widada *et al.*, 2003; Sahul Hameed *et al.*, 2004b; Yoganandhan *et al.*, 2005).

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

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

Tissue samples for histopathology, immunohistochemistry or *in-situ* hybridisation should be fixed immediately after collection in neutral-buffered formalin or modified Davidson's fixative (Sri Widada *et al.*, 2003). 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 5.3. of 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~~ is only 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. 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 identifying infection 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 life stage.

Ratings against purposes of use. For each recommended assay a qualitative rating against 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, cost, timeliness, and sample throughput. For a specific purpose of use, assays are rated as:

+++ =	Most suitable methods – desirable performance and operational characteristics;
++ =	Suitable method(s) acceptable performance and operational characteristics under most circumstances;
+ =	Less suitable methods – performance or operational characteristics may significantly limit application;
Shaded boxes =	Not appropriate for this purpose.

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

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

Table 4.1. WOAH recommended diagnostic methods for MrNV 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				
Cell culture												
Real-time RT-PCR	+++	+++	+++	1	+++	+++	+++	1	+++	+++	+++	1
Conventional RT-PCR	++	++	++	1	+++	+++	+++	2				
Conventional RT-PCR followed by amplicon sequencing									+++	+++	+++	2
<i>In-situ</i> hybridisation						++	++	1		++	++	1
Bioassay												
LAMP	++	++	++	1	++	++	++	1				
Ab-ELISA												
Ag-ELISA					++	++	++	1				
Lateral flow assay					++	++	++	2				
Other methods ³												

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

None to date

4.2. Histopathology and cytopathology

The most affected tissue in infected PL is striated muscle of the cephalothorax, abdomen and tail. Histological features include the presence of acute Zenker's necrosis of striated muscles, characterised by severe hyaline degeneration, necrosis and muscular lysis. Moderate oedema and abnormal open spaces among the affected muscle cells are also observed, as is the presence of large oval or irregular basophilic cytoplasmic inclusion bodies in infected muscles (Arcier *et al.*, 1999; Hsieh *et al.*, 2006).

4.3. Cell culture for isolation

MrNV has been isolated in insect cell lines, but this is not a recommended method (Hernandez-Herrera *et al.*, 2007; Sudhakaran *et al.*, 2007b).

4.4. Nucleic acid amplification

PCR methods for MrNV and XSV are included in this section for completeness. However, the case definitions in Section 6 are based on detection methods for MrNV only.

PCR assays should always be run with the controls specified in Section 5.5. *Use of molecular 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

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

4.4.1. Real-time RT-PCR

Real-time RT-PCR assay can be performed using the SYBR Green dye based on the method described by Hernandez-Herrera *et al.* (2007) or the TaqMan assay described by Zhang *et al.* (2006).

Pathogen / target gene	Primer/probe (5'–3')	Concentration	Cycling parameters ^(a)
Method 1: Hernandez-Herrera <i>et al.</i> (2007); GenBank Accession No.: AY222839)			
MrNV/RNA1	Fwd: AGG-ATC-CAC-TAA-GAA-CGT-GG Rev: CAC-GGT-CAC-AAT-CCT-TGC-G	500 nM 500 nM	40 cycles of: 95°C/15 sec, 60°C/5 sec and 72°C/10 sec
Method 2: Zhang <i>et al.</i> (2006); GenBank Accession No.: AY231436)			
MrNV/RNA1	Fwd: CAA-CTC-GGT-ATG-GAA-CTC-AAG-GT Rev: AGG-AAA-TAC-ACG-AGC-AAG-AAA-AGT-C Probe: FAM-ACC-CTT-CGA-CCC-CAG-CAA-TGG-TG-TAMARA	1000 nM 1000 nM 400 nM	50 cycles of: 94°C/30 sec and 58°C/30 sec
Method 3: Zhang <i>et al.</i> (2006); GenBank Accession No.: DQ174318)			
XSV	Fwd: AGC-CAC-ACT-CTC-GCA-TCT-GA Rev: CTC-CAG-CAA-AGT-GCG-ATA-CG Probe: FAM-CAT-GCC-CCA-TGA-TCC-TCG-CA-TAMARA	1000 nM 1000 nM 400 nM	50 cycles of: 94°C/30 sec and 58°C/30 sec

^(a)A denaturation step prior to cycling has not been included.

4.4.2. Conventional RT-PCR

The protocol for the conventional RT-PCR for detection of MrNV/XSV developed by Sri Widada *et al.* (2003), Sahul Hameed *et al.* (2004a; 2004b) and Sudhakaran *et al.* (2007a) is recommended. MrNV and XSV can be detected by conventional RT-PCR separately using a specific set of primers or these two viruses can be detected simultaneously using a single-tube one-step multiplex RT-PCR (Yoganandhan *et al.*, 2005). ~~Conventional real time RT-PCR is recommended in situations where high sensitivity is required.~~

Pathogen / target gene	Primer (5'–3')	Concentration	Cycling parameters ^(a)
Method 1: One step RT-PCR (Sri Widada <i>et al.</i> , 2003; Sahul Hameed <i>et al.</i> , 2004a, b; Sudhakaran <i>et al.</i> , 2007a) GenBank Accession No.: AY222840 (MrNV) and AY247793 (XSV); <u>amplicon size: 425 bp (MrNV) and 546 bp (XSV)</u>			
MrNV	Fwd: GCG-TTA-TAG-ATG-GCA-CAA-GG Rev: AGC-TGT-GAA-ACT-TCC-ACT-GG	0.02 nM <u>400 nM</u> 0.02 nM <u>400 nM</u>	30 cycles of: 94°C/40 sec, 55°C/40sec and 68°C/60 sec
XSV	Fwd: CGC-GGA-TCC-GAT-GAA-TAA-GCG-CAT-TAA-TAA Rev: CCG-GAA-TTC-CGT-TAC-TGT-TCG-GAG-TCC-CAA	0.02 nM <u>400 nM</u> 0.02 nM <u>400 nM</u>	30 cycles of: 94°C/40 sec, 55°C/40 sec and 68°C/60 sec
Method 2: nested RT-PCR using above-mentioned primers as external primers (Sudhakaran <i>et al.</i> , 2007a); <u>amplicon size: 205 bp (MrNV) and 236 bp (XSV)</u>			
MrNV	<u>External primers: as for Method 1.</u> Internal primers: Fwd: GAT-GAC-CCC-AAC-GTT-ATC-CT Rev: GTG-TAG-TCA-CTT-GCA-AGA-GG	0.02 nM <u>1000 nM</u> 0.02 nM <u>1000 nM</u>	30 cycles of: 94°C/60 sec, 55°C/60 sec and 72°C/60 sec
XSV	<u>External primers: as for Method 1.</u> Internal primers: Fwd: ACA-TTG-GCG-GTT-GGG-TCA-TA Rev: GTG-CCT-GTT-GCT-GAA-ATA-CC-3	0.02 nM <u>1000 nM</u> 0.02 nM <u>1000 nM</u>	30 cycles of: 94°C/60 sec, 55°C/60 sec and 72°C/60 sec
Method 3: Multiplex RT-PCR (Yoganandhan <i>et al.</i> , 2005); GenBank Accession No.: AY222840 (MrNV) and AY247793 (XSV); <u>amplicon size: 681 bp (MrNV) and 500 bp (XSV)</u>			
MrNV	Fwd: GAT-ACA-GAT-CCA-CTA-GAT-GAC-C Rev: GAC-GAT-AGC-TCT-GAT-AAT-CC	0.02 nM <u>400 nM</u> 0.02 nM <u>400 nM</u>	30 cycles of: 94°C/40 sec, 55°C/40 sec and 68°C/60 sec
XSV	Fwd: GGA-GAA-CCA-TGA-GAT-CAC-G Rev: CTG-CTC-ATT-ACT-GTT-CGG-AGT-C	0.02 nM <u>400 nM</u> 0.02 nM <u>400 nM</u>	30 cycles of: 94°C/40 sec, 55°C/40 sec and 68°C/60 sec

^(a)A denaturation step prior to cycling has not been included.

4.4.3. Other nucleic acid amplification methods: Loop-mediated isothermal amplification (LAMP)

Haridas *et al.* (2010) have applied loop-mediated isothermal amplification (LAMP) for rapid detection of MrNV and XSV in the freshwater prawn. A set of four primers, two outer primers and two inner primers, have been designed separately for detection of MrNV and XSV.

4.5. Amplicon sequencing

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

4.6. *In-situ* hybridisation

The presence of MrNV in infected cells can be demonstrated in histological sections using a DIG-labelled DNA *in-situ* hybridisation probe specific for MrNV (Sri Widada *et al.*, 2003).

4.7. Immunohistochemistry

None developed.

4.8. Bioassay

Not used for diagnostic purposes.

4.9. Antibody- or antigen-based detection methods

4.9.1. ELISA

Antibody-based diagnostic methods for MrNV include the ELISA described by Romestand & Bonami (2003) or the triple-antibody sandwich (TAS) ELISA based on a monoclonal antibody (Qian *et al.*, 2006).

4.9.2. Lateral flow assay (LFA)

An antibody-based lateral flow assay (LFA) has been developed for the early detection of MrNV in the PL stage (Jamalpure *et al.*, 2021).

4.10. Other methods

None

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

Real-time RT-PCR is recommended for targeted surveillance to declare freedom from infection with MrNV.

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. If a Competent Authority does not have the capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAHP Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status³

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

- i) Positive result by real-time RT-PCR
- ii) Positive result by conventional RT-PCR
- iii) Positive result by LAMP

³ For example transboundary commodities.

6.1.2. Definition of confirmed case in apparently healthy animals

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

- i) Positive result by real-time RT-PCR result and positive result by conventional RT-PCR and sequence analysis

6.2 Clinically affected animals

6.2.1. Definition of suspect case in clinically affected animals

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

- i) Clinical signs consistent with infection by MrNV
- ii) Histopathology consistent with infection by MrNV
- iii) Positive result by real-time RT-PCR
- iv) Positive result by conventional RT-PCR
- v) Positive result by *in situ* hybridisation
- vi) Positive result by LAMP
- vii) Positive result by Ag ELISA
- viii) Positive result by lateral flow assay

6.2.2. Definition of confirmed case in clinically affected animals

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

- i) Positive result by real time RT-PCR and positive result by conventional RT-PCR with sequence analysis
- ii) Positive result by ISH followed by positive result by conventional RT-PCR with sequence analysis
- iii) Positive result by ISH followed by positive result by real-time RT-PCR

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with MrNV are provided in Tables 6.3.1. and 6.3.2 (no data are currently available). This information can be used for the design of surveys for infection with MrNV, 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
RT-PCR	Diagnosis	Clinically affected PL from hatchery and nursery	Whole post-larvae	<i>Macrobrachium rosenbergii</i>	100 (n=20)	100 (n=20)	Western blot or ELISA	Sri Widada <i>et al.</i> (2003); Sahul Hameed <i>et al.</i> (2011)
Lateral flow immune-assay	Surveillance	PL from prawn hatcheries	Whole post-larvae	<i>Macrobrachium rosenbergii</i>	100 (n=80)	90 (n=80)	RT-PCR	Jamalpure <i>et al.</i> (2021)

DSe = diagnostic sensitivity, DSp = diagnostic specificity, n = samples-number of animals used in the validation study, RT-PCR: = reverse transcription polymerase chain reaction.

6.3.2. For surveillance of apparently healthy animals

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

DSe = diagnostic sensitivity, DSp = diagnostic specificity, $n = n =$ **samples number of animals** used in the **validation** study, RT-PCR: = reverse transcription polymerase chain reaction.

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* *

NB: There is a WOA Reference Laboratory for infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)
(please consult the WOA web site:

<https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>) any further information on
infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)

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

CHAPTER 2.2.9
INFECTION WITH
YELLOW HEAD VIRUS GENOTYPE 1

1. Scope

Infection with yellow head virus genotype 1 means infection with the pathogenic agent yellow head virus genotype 1 (YHV1) of the Genus *Okavirus* and Family *Roniviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Yellow head virus genotype 1 (YHV1; species *Yellow head virus*) is one of eight known genotypes in the yellow head complex of viruses and is the only known genotype that causes yellow head disease. YHV1 forms enveloped, rod-shaped particles 40–50 nm × 150–180 nm (Chantanachookin *et al.*, 1993; Wongteerasupaya *et al.*, 1995). Envelopes are studded with prominent peplomers projecting approximately 11 nm from the surface. Nucleocapsids appear as rods (diameter 20–30 nm) and possess a helical symmetry with a periodicity of 5–7 nm. Virions comprise three structural proteins (nucleoprotein p20 and envelope glycoproteins gp64 and gp116) and a ~26 kb positive-sense single-stranded RNA genome. The nucleotide sequence of the ORF1b region of the viral genome has been used to determine the phylogenetic relationships of YHV1 and other yellow head virus genotypes (Dong *et al.*, 2017; Mohr *et al.*, 2015; Wijegoonawardane *et al.*, 2008a).

YHV1, yellow head virus genotype 2 (YHV2; species *Gill-associated virus*) and yellow head virus genotype 8 (YHV8; species *Okavirus 1*) have been formally classified by the International Committee on Taxonomy of Viruses (Walker *et al.*, 2021). Four other genotypes in the complex (YHV3–YHV6) occur commonly in healthy *Penaeus monodon* in East Africa, Asia and Australia and are rarely or never associated with disease (Walker *et al.*, 2001; Wijegoonawardane *et al.*, 2008a). Of the remaining two yellow head virus genotypes, YHV7 was detected in diseased *P. monodon* in Australia (Mohr *et al.*, 2015) and YHV8 was detected in *P. chinensis* suspected of suffering from acute hepatopancreatic necrosis disease (Liu *et al.*, 2014). There is evidence of genetic recombination between genotypes (Wijegoonawardane *et al.*, 2009).

2.1.2. Survival and stability in processed or stored samples

YHV1, identified by either transmission electron microscopy (TEM) (Nunan *et al.*, 1998), or molecular methods (Durand *et al.*, 2000; McColl *et al.*, 2004), has been detected in frozen commodity prawns with infectivity demonstrated by bioassay.

2.1.3. Survival and stability outside the host

YHV1 remains viable in aerated seawater for up to 72 hours (Flegel *et al.*, 1995b).

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 YHV1 according to Chapter 1.5 of the *Aquatic Animal Health Code (Aquatic Code)* are: blue shrimp (*Penaeus stylirostris*), dagger blade grass shrimp (*Palaemonetes pugio*), giant tiger prawn (*Penaeus monodon*), jinga shrimp (*Metapenaeus affinis*) and whiteleg shrimp (*Penaeus vannamei*).

Family	Scientific name	Common name
Palaemonidae	<i>Palaemonetes pugio</i>	dagger blade grass shrimp
Penaeidae	<i>Metapenaeus affinis</i>	jinga shrimp
	<i>Penaeus monodon</i>	giant tiger prawn
	<i>Penaeus stylirostris</i>	blue shrimp
	<i>Penaeus vannamei</i>	whiteleg shrimp

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 YHV1 according to Chapter 1.5 of the *Aquatic Code* are:

Family	Scientific name	Common name
Palaemonidae	<i>Palaemon serrifer</i>	carpenter prawn
	<i>Palaemon styliferus</i>	Pacific blue prawn
	<i>Macrobrachium sintangense</i>	Sunda river prawn
Parastacidae	<i>Cherax quadricarinatus</i>	red claw crayfish
Penaeidae	<i>Metapenaeus brevicornis</i>	yellow shrimp
	<i>Penaeus aztecus</i>	northern brown shrimp
	<i>Penaeus duorarum</i>	northern pink shrimp
	<i>Penaeus japonicus</i>	kuruma prawn
	<i>Penaeus merguensis</i>	banana prawn
	<i>Penaeus setiferus</i>	northern white shrimp

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: acorn barnacle (*Chelonibia patula*), blue crab (*Callinectes sapidus*), cyclopoid copepod (*Ergasilus manicatus*), gooseneck barnacle (*Octolasmis muelleri*), Gulf killifish (*Fundulus grandis*) and paste shrimp (*Acetes sp.*).

Family	Scientific name	Common name
Chelonibiidae	<i>Chelonibia patula</i>	acorn barnacle
Ergasilidae	<i>Ergasilus manicatus</i>	cyclopoid copepod
Fundulidae	<i>Fundulus grandis</i>	gulf killifish
Poecilasmatidae	<i>Octolasmis muelleri</i>	gooseneck barnacle
Portunidae	<i>Callinectes sapidus</i>	blue crab
Sergestidae	<i>Acetes sp.</i>	paste shrimp

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

Penaeus monodon are susceptible to YHV1 infection beyond PL15 (Khongpradit *et al.*, 1995). Lightner *et al.* (1998) YHV1 challenge caused disease in juveniles of *Penaeus aztecus*, *P. duorarum*, *P. setiferus*, and *P. vannamei* but postlarvae appeared resistant (Lightner *et al.* 1998). YHV1 infections have been detected in healthy wild populations of *P. stylirostris* (Castro-Longoria *et al.*, 2008). Natural YHV1 infections have been detected in *P. japonicus*, *P. merguensis*, *P. setiferus*, *M. ensis*, and *P. styliferus* (Cowley *et al.*, 2002; Flegel *et al.*, 1995a; 1995b).

2.2.4. Distribution of the pathogen in the host

YHV1 targets tissues of ectodermal and mesodermal origin including lymphoid organ, haemocytes, haematopoietic tissue, gill lamellae and spongy connective tissue of the subcutis, gut, antennal gland, gonads, nerve tracts and ganglia (Chantanachookin *et al.*, 1993; Lightner, 1996).

2.2.5. Aquatic animal reservoirs of infection

YHV1 was detected by reverse-transcription polymerase chain reaction (RT-PCR) in clinically normal wild *P. stylirostris* collected for surveillance purposes in the Gulf of California in 2003 (Castro-Longoria *et al.*, 2008). The infectious nature of the YHV1 detected was confirmed by experimental infections. There is also evidence that YHV1 can persist in survivors of experimental infection (Longyant *et al.*, 2005; 2006).

2.2.6. Vectors

There are no known vectors of YHV1.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

In farmed *P. monodon*, YHV disease can cause up to 100% mortality within 3–5 days of the first appearance of clinical signs (Chantanachookin *et al.*, 1993). Mortalities can be induced by experimental exposure of *P. monodon* to YHV1 (Oanh *et al.*, 2011).

2.3.2. Clinical signs, including behavioural changes

Shrimp from late postlarvae (PL) stages onwards can be infected experimentally with YHV1. In cultured shrimp, infection can result in mass mortality occurring, usually in early to late juvenile stages. Moribund shrimp may exhibit a bleached overall appearance and a yellowish discoloration of the cephalothorax. However, these disease features are not particularly distinctive, and gross signs are not reliable even for preliminary diagnosis of YHV1.

Exceptionally high feeding activity followed by an abrupt cessation of feeding may occur within 2–4 days of the appearance of gross clinical signs of disease and mortality. Moribund shrimp may congregate at pond edges near the surface (Chantanachookin *et al.*, 1993).

2.3.3 Gross pathology

The yellow hepatopancreas of diseased shrimp may be exceptionally soft when compared with the brown hepatopancreas of a healthy shrimp (Chantanachookin *et al.*, 1993).

2.3.4. Modes of transmission and life cycle

YHV1 can be transmitted horizontally by ingestion of infected tissue, immersion in membrane-filtered tissue extracts, or by cohabitation with infected shrimp (Walker & Sittidilokratna, 2008). YHV1 replicates in the cytoplasm of infected cells in which long filamentous pre-nucleocapsids are abundant and virions bud into cytoplasmic vesicles in densely packed paracrystalline arrays for egress at the cytoplasmic membrane (Chantanachookin *et al.*, 1993).

2.3.5. Environmental factors

Elevated virus infection levels accompanied by disease can be precipitated by physiological stress induced by sudden changes in pH or dissolved oxygen levels, or other environmental factors (Flegel *et al.*, 1997).

2.3.6. Geographical distribution

YHV1 has been reported in South-East Asia (Walker *et al.*, 2001). YHV1 has also been detected in *P. stylirostris* and *P. vannamei* in the Americas (Castro-Longoria *et al.*, 2008; Sanchez-Barajas *et al.*, 2009).

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

None available.

2.4.2. Chemotherapy including blocking agents

No effective commercial anti-viral product is yet available.

2.4.3. Immunostimulation

A multi-target dsRNA for simultaneous inhibition of YHV1 and white spot syndrome virus demonstrated inhibition of the two viruses when administered to shrimp by injection (Chaimongkon *et al.*, 2020)

2.4.4. Breeding resistant strains

Not reported.

2.4.5. Inactivation methods

YHV1 can be inactivated by heating at 60°C for 15 minutes (Flegel *et al.*, 1995b). Little information is available on other inactivation methods but the virus appears to be susceptible to treatment with chlorine at 30 parts per million (0.03 mg ml⁻¹) (Flegel *et al.*, 1997).

2.4.6. Disinfection of eggs and larvae

Not reported.

2.4.7. General husbandry

The focus is to exclude YHV1 from entering production systems; for example, by using specific pathogen-free (SPF) stock, batch testing stock and biosecurity measures to reduce entry into culture systems.

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

For diagnosis during a disease outbreak, moribund shrimp collected from pond edges are the preferred source of material for examination. Apparently healthy shrimp should also be collected from the same ponds. For surveillance in populations of apparently healthy shrimp, life stages from PL stage 15 onwards can provide tissue sources useful for testing.

3.2. Selection of organs or tissues

In moribund shrimp suspected to be infected with YHV1, pleopods, gill and lymphoid organ are the most suitable sample tissues. For screening or surveillance of juvenile or adult shrimp that appear grossly normal, pleopods or gills are preferred.

3.3. Samples or tissues not suitable for pathogen detection

Not determined.

3.4. Non-lethal sampling

Haemolymph can be used for non-lethal sampling.

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

3.5. Preservation of samples for submission

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

3.5.1. Samples for bioassay

The success of bioassay 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.

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 (absolute) 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 can be frozen at –20°C or below for 1 month or less; for long-term storage, –80°C is recommended.

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

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

Standard sample collection, preservation and processing methods for histological techniques can be found in Section B.2.2 of 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 is only 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 specimens should be processed and tested individually. Small life stages can be pooled to obtain the minimum amount of material for molecular detection.

4. Diagnostic methods

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

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

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

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

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

Table 4.1. WOH 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 RT-PCR	++	+++	+++	1	++	+++	+++	4	++	+++	+++	4
Conventional RT-PCR	++	++	++	1	++	++	++	1				
Conventional PCR followed by amplicon sequencing									++	+++	+++	1
<i>In-situ</i> hybridisation						++	++	1				
Bioassay					+	+	+	1				
LAMP Immunohistochemistry												
Ab-ELISA												
Ag-ELISA												
Other antigen detection methods ³												
Other methods ³												

LV = level of validation, refers to the stage of validation in the WOH Pathway (chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop mediated isothermal amplification;

Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively; 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

Not applicable.

4.2. Histopathology and cytopathology

Fix the cephalothorax tissues of moribund shrimp suspected to be affected by YHV1 in Davidson's fixative, prepare tissue sections and stain with Meyer's haematoxylin and eosin (H&E) using standard histological procedures (Lightner, 1996). Examine tissues of ectodermal and mesodermal origin by light microscopy for the presence of moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusions approximately 2 µm in diameter or smaller (Chantanachookin *et al.*, 1993). Tissues of the lymphoid organ, stomach subcuticulum and gills are particularly informative.

Lymphoid organ spheroids are commonly observed in healthy *P. monodon* chronically infected with YHV1 or GAV and lymphoid organ necrosis often accompanies disease (Spann *et al.*, 1997). However, spheroid formation and structural degeneration of lymphoid organ tissue also result from infection by other shrimp viruses (Lightner, 1996).

4.3. Cell culture for isolation

Although primary shrimp cell culture methods are available, they are not recommended to isolate and identify YHV1 as a routine diagnostic method. No continuous cell lines suitable for YHV1 culture are available.

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

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

4.4.1. Real-time PCR

Not available.

The protocol for the real-time RT-PCR for detection of YHV1 has been developed by the WOAHP Reference Laboratory for YHV1. This assay is specific for genotype 1. Validation data are provided in the submitted validation report for diagnostic tests and confirm suitability to be recommended for inclusion in the *Aquatic Manual* ([ADD LINK OR AGREED REFERENCE](#)).

<u>Pathogen/ target gene</u>	<u>Primer/probe (5'–3')</u>	<u>Concentration</u>	<u>Cycling conditions^(a)</u>
<u>YHV1 /ORF1</u>	<u>YHV1-12-qF: AGT-CTA-CAG-TGC-TCT-GAT-CT</u> <u>YHV1-12-qR: GAT-TCT-TGA-AGC-GCA-TGA-GT</u> <u>YHV1-12-qPr: FAM-TCT-CAT-GTG/ZEN/TCA-TGA-</u> <u>TAT-TCT-CAA-GCG-AGT-IABkFQ</u>	<u>900 nM of each primer</u> <u>250 nM of probe</u>	<u>Reverse transcription at</u> <u>48°C/30 min</u> <u>1 cycle 95°C/10 min</u> <u>45 cycles of 95°C/15 sec and</u> <u>60°C/60 sec</u>

^(a)A denaturation step prior to cycling has not been included.

4.4.2. Conventional RT-PCR

Three RT-PCR protocols are described. For all conventional RT-PCR protocols, assignment to YHV genotype 1 can be achieved by nucleotide sequence analysis of the RT-PCR amplicon. Reference sequences for YHV1 include:

Protocol 1 is a 1-step RT-PCR that can be used to detect YHV1 in affected shrimp. The protocol is as described by Mohr *et al.* (2015) and adapted from Wongteerasupaya *et al.* (1997). This protocol will detect YHV1 but not GAV or any of the other genotypes currently recognised.

Protocol 2 is a more sensitive multiplex nested RT-PCR that can be used to differentiate YHV1 from GAV. The protocol is as described by Mohr *et al.* (2015) and adapted from Cowley *et al.* (2004). The first stage of the multiplex nested RT-PCR (primary RT-PCR) was designed to detect YHV1 and GAV but has been reported to also detect YHV7 (Mohr *et al.*, 2015). Both the primary RT-PCR and the nested PCR detected the novel YHV genotype from China (People's Rep. of) (Liu *et al.*, 2014). In the second PCR step, a 277 bp product indicates detection of YHV and a 406 bp product indicates detection of GAV. The presence of both 406 bp and 277 bp products indicates a dual infection with GAV and YHV1. The nested PCR can be run as two separate assays specific for YHV1 or GAV by omitting either the G6 or Y3 primer, respectively. **NOTE:** Due to reported problems with primer specificity for some emerging strains, all PCR products generated using protocol 2 should be sequenced to confirm the virus genotype.

Protocol 3 is a multiplex nested RT-PCR protocol that can be used for screening shrimp for any of the seven genotypes of the yellow head complex of viruses. The protocol is as described by Mohr *et al.* (2015) and adapted from Wijegoonawardane *et al.* (2008b). Two primers were designed to each site, one accommodating sequence variations amongst YHV1 isolates and the other variations amongst isolates of the other genotypes (Wijegoonawardane *et al.*, 2008b). It is not known whether this assay will detect the YHV8 genotype recently detected in China (People's Rep. of) (Liu *et al.*, 2014).

Primer sequences

Pathogen / target gene	Primer (5'–3')	Concentration	Cycling parameters ^(a)
Protocol 1 (Wongteerasupaya <i>et al.</i> , 1997; GenBank Accession No.: FJ848675.1 ; amplicon size: 135 bp)			
YHV1 / ORF1b	10F: CCG-CTA-ATT-TCA-AAA-ACT-ACG 144R: AAG-GTG-TTA-TGT-CGA-GGA-AGT	180 nM 180 nM	40 cycles of 94°C/30sec, 58°C/45 sec, 68°C/45 sec,
Protocol 2 (Cowley <i>et al.</i> , 2004; GenBank Accession No.: FJ848675.1)			
YHV1 and GAV / ORF1b	<p>Primary (Amplicon size: 794 bp)</p> <p>GY1: 5GAC-ATC-ACT-CCA-GAC-AAC-ATC-TG GY4: GTG-AAG-TCC-ATG-TGT-GTG-AGA-CG</p> <p>Nested for detection of YHV1 (Amplicon size: 277 bp)</p> <p>GY2: CAT-CTG-TCC-AGA-AGG-CGT-CTA-TGA Y3: ACG-CTC-TGT-GAC-AAG-CAT-GAA-GTT</p> <p>Nested for detection of GAV (Amplicon size: 406 bp)</p> <p>GY2: CAT-CTG-TCC-AGA-AGG-CGT-CTA-TGA G6: GTA-GTA-GAG-ACG-AGT-GAC-ACC-TAT</p>	180 nM 180 nM 360 nM 360 nM 360 nM 360 nM	35 cycles of 95°C/30 sec, 66°C/30 sec, and 68°C/45 sec
Protocol 3 (Wijegoonawardane <i>et al.</i> , 2008b; GenBank Accession No.: FJ848675.1)			
YHV1 to YHV7 / ORF1b	<p>Primary (amplicon size: 359 bp)</p> <p>YC-F1ab pool: ATC-GTC-GTC-AGC-TAC-CGC-AAT-ACT-GC ATC-GTC-GTC-AGY-TAY-CGT-AAC-ACC-GC</p> <p>YC-R1ab pool: TCT-TCR-CGT-GTG-AAC-ACY-TTC-TTR-GC TCT-GCG-TGG-GTG-AAC-ACC-TTC-TTG-GC</p> <p>Nested (amplicon size: 147 bp)</p> <p>YC-F2ab pool: CGC-TTC-CAA-TGT-ATC-TGY-ATG-CAC-CA CGC-TTY-CAR-TGT-ATC-TGC-ATG-CAC-CA</p> <p>YC-R2ab pool: RTC-DGT-GTA-CAT-GTT-TGA-GAG-TTT-GTT GTC-AGT-GTA-CAT-ATT-GGA-GAG-TTT-RTT</p> <p>Mixed base codes: R(AG), Y(CT), M(AC), K(GT), S(GC), W(AT), H(ACT), B(GCT), V(AGC), D(AGT), N(AGCT).</p>	180 nM 180 nM 180 nM 180 nM 180 nM 180 nM 180 nM 180 nM	35 cycles of 94°C/45 sec, 60°C/45 sec, 68°C/45 sec, 35 cycles of 94°C/45 sec, 60°C/45 sec, 72°C/45 sec;

^(a)A denaturation step prior to cycling has not been included.

The Protocol 2 Y3 primer contains a mismatch for GAV but is specific for YHV1. The mismatch can cause false-positives with GAV in the nested PCR of protocol 2 where GAV generates an amplicon of very similar size to the expected size of the YHV1 amplicon. For GAV, the 7th base from left (T) is substituted for C so that the primer sequence for GAV should be 5'-CAT-CTG-CCC-AGA-AGG-CGT-CTA-TGA-3', according to the sequence data of the GAV genome (database accession numbers: NC_010306.1 and AF227196.2).

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

4.4.3. Other nucleic acid amplification methods

Not available.

4.5. Amplicon sequencing

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

4.6. *In-situ* hybridisation

The *in-situ* hybridisation (ISH) protocol of Tang *et al.* (2002) is suitable for detecting YHV1 or GAV (Tang & Lightner, 1999). To preserve viral RNA accessibility, fix tissues sampled from live shrimp in neutral-buffered, modified Davidson's fixative without acetic acid (RF-fixative) (Hasson *et al.*, 1997). To achieve good tissue preservation whilst also preserving RNA accessibility, normal Davidson's fixative can be used as long as the fixation time is limited to 24 hours (maximum of 48 hours). Detailed methods can be found in Tang *et al.* (2002) YHV-infected cells give a blue to purple-black colour against the brown counter stain. Positive controls of YHV-infected tissue and negative controls of uninfected shrimp tissue should be included. The digoxigenin-labelled DNA probe can be prepared by PCR labelling using the following primers:

YHV1051F: 5'-ACA-TCT-GTC-CAG-AAG-GCG-TC-3'
YHV1051R: 5'-GGG-GGT-GTA-GAG-GGA-GAG-AG-3'

4.7. Immunohistochemistry

Not applicable.

4.8. Bioassay

The bioassay procedure is based on that described by Spann *et al.* (1997), but similar procedures have been described by several other authors (e.g. Lu *et al.*, 1994). The bioassay should be conducted in susceptible shrimp that have been determined to be free from YHV complex viruses.

Suspect YHV1-infected samples should be maintained at 4°C or on ice. If necessary, the whole shrimp or the retained cephalothorax may be snap-frozen and stored at -80°C or in liquid nitrogen until required. Viral inoculum should be prepared as described by Spann *et al.* (1997).

Juvenile shrimp of a known susceptible species are injected with viral inoculum. Negative controls (buffer injected) and positive controls (known YHV1 positive material) treatment groups are required. Shrimp should be maintained separately to prevent cross-contamination between treatments. Observe the shrimp and record mortalities for at least 21 days or until the test and positive control groups reach 100% mortality.

Dead shrimp can be processed for PCR and sequence analysis. The surviving shrimp are processed for gross signs, histopathology, PCR and sequence analysis. A positive result is indicated by the detection of gross signs and characteristic histological lesions, and by PCR and amplicon sequence analysis. The negative control shrimp must remain negative for at least 21 days for gross or histological signs of infection with YHV1.

4.9. Antibody- or antigen-based detection methods (ELISA, etc.)

None has been successfully developed.

4.10. Other methods

None at present.

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

Nested Real-time RT-PCR (Protocol 3) is recommended for demonstrating freedom from YHV1 in an apparently healthy populations. Sequencing of any amplified PCR products is required to determine the YHV genotype. Two step PCR negative results are required for YHV1.

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. If a Competent Authority does not have the capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOA Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status⁴

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 YHV1 shall be suspected if the following criterion is met:

- i) Positive result by a recommended conventional RT-PCR detection test
- ii) Positive result by real-time RT-PCR

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with YHV1 is considered to be confirmed if the following criterion is met:

- i) Positive result by real-time RT-PCR and positive result by conventional RT-PCR followed by amplicon sequencing
- ii) A positive result by conventional RT-PCR and identification of YHV1 by sequence analysis of the amplicon from each of two different RT-PCR methods followed by sequence analysis of the amplicons to identify YHV1

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 YHV1 shall be suspected if at least one of the following criteria is met:

- i) Gross pathology or clinical signs consistent with YHV1 infection
- ii) Histopathology consistent with YHV1 infection
- iii) Positive result by real-time RT-PCR
- iv) Positive result by conventional RT-PCR
- v) Positive result by ISH

⁴ For example transboundary commodities.

- vi) Positive result by bioassay

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with YHV1 is considered to be confirmed if the following criterion is met:

- i) Positive result by real-time RT-PCR and positive result by conventional RT-PCR followed by amplicon sequencing
- ii) A positive result from each of two different RT-PCR methods targeting non-overlapping parts of the genome followed by sequence analysis of the amplicons to identify YHV1

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with YHV1 are provided in Tables 6.3.1. and 6.3.2 (no data are currently available for either). This information can be used for the design of surveys for infection with YHV1, 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
YHV1 RT-qPCR	Diagnosis	Infected by co-habitation or feeding	Pleopods	<i>Penaeus monodon</i> , <i>P. merguensis</i>	100% (n=130)	100% (n=130)	Real-time PCR	Validation report

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

6.3.2. For surveillance of apparently healthy animals

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

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

7. References

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* *

NB: There is a WOA Reference Laboratory for infection with yellow head virus genotype 1
(please consult the WOA web site:

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

Please contact the WOA Reference Laboratories for any further information on
infection with yellow head virus genotype 1

NB: FIRST ADOPTED IN 1995 AS YELLOWHEAD DISEASE. MOST RECENT UPDATES ADOPTED IN 2019.

CHAPTER 2.2.X.

INFECTION WITH DECAPOD IRIDESCENT VIRUS 1

1. Scope

Infection with decapod iridescent virus 1 means infection with the pathogenic agent decapod iridescent virus 1 (DIV1), Genus *Decapodiridovirus*, Subfamily *Betairidovirinae*, Family *Iridoviridae*.

2. Disease information

2.1. Agent factors

a. 2.1.1. Aetiological agent

DIV1 is the only species of the genus *Decapodiridovirus* assigned to the subfamily *Betairidovirinae*, family *Iridovirus* (ICTV, 2023). DIV1 is a 150–158 nm, enveloped icosahedral double-stranded DNA virus, with a linear genome of 165 kb composed of 34.6% G + C content and 170–178 putative open reading frames (ORFs) (Li *et al.*, 2017; Qiu *et al.*, 2017; 2018a; Xu *et al.*, 2016). Although *Cherax quadricarinatus* iridovirus (CQIV) (Xu *et al.*, 2016) and shrimp haemocyte iridescent virus (SHIV) (Qiu *et al.*, 2017) have been reported from the redclaw crayfish (*C. quadricarinatus*), and the whiteleg shrimp (*L. vannamei*), respectively, they are classified as different isolates (strains) within the DIV1 species.

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

c. DIV1-infected cephalothoraxes are infectious after homogenisation, centrifugation, filtration and storage at –80°C (Qiu *et al.*, 2022a; Xu *et al.*, 2016).

d. 2.1.3. Survival and stability outside the host

Not available.

For inactivation methods, see Section 2.4.5.

2.2. Host factors

e. 2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with DIV1 according to chapter 1.5. *Aquatic Animal Health Code (Aquatic Code)* are: ~~fleshy prawn (*Penaeus chinensis*), gazami crab (*Portunus trituberculatus*),~~ giant river prawn (*Macrobrachium rosenbergii*), kuruma prawn (*Penaeus japonicus*), Oriental river prawn (*Macrobrachium nipponense*), red claw crayfish (*Cherax quadricarinatus*), red swamp crawfish (*Procambarus clarkii*), ridgetail prawn (*Palaemon carinicauda*), and whiteleg shrimp (*Penaeus vannamei*).

Family	Scientific name	Common name
Cambaridae	<i>Procambarus clarkii</i>	red swamp crawfish
Palaemonidae	<i>Macrobrachium nipponense</i>	Oriental river prawn
	<i>Macrobrachium rosenbergii</i>	giant river prawn
	<i>Palaemon carinicauda</i>	ridgetail prawn
Parastacidae	<i>Cherax quadricarinatus</i>	red claw crayfish
Penaeidae	<i>Penaeus japonicus</i>	kuruma prawn
	<i>Penaeus vannamei</i>	whiteleg shrimp
Portunidae	<i>Portunus trituberculatus</i>	swimming crab

f. 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 DIV1 according to Chapter 1.5 of the *Aquatic Code* are: giant tiger prawn (*Penaeus monodon*).

Family	Scientific name	Common name
Penaeidae	<i>Penaeus chinensis</i>	fleshy prawn
	<i>Penaeus monodon</i>	giant tiger prawn

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: channeled applesnail (*Pomacea canaliculata*), *Helice tientsinensis*, Japanese shore crab (*Hemigrapsus penicillatus*), *Macrobrachium superbum* and *Plexippus paykulli*.

Family	Scientific name	Common name
Ampullariidae	<i>Pomacea canaliculata</i>	channeled applesnail
Palaemonidae	<i>Macrobrachium superbum</i>	no common name
Salticidae	<i>Plexippus paykulli</i>	no common name
Varunidae	<i>Helice tientsinensis</i>	no common name
	<i>Hemigrapsus penicillatus</i>	Japanese shore crab

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

All live stages are potentially susceptible to infection; DIV1 has been detected in post-larvae (PL), juvenile and sub-adult stages of shrimp (*Penaeus vannamei*, *P. chinensis*, *Exopalaemon carinicauda*, *Macrobrachium nipponense*, *M. rosenbergii*, crayfish [*Cherax quadricarinatus*, *Procambarus clarkii*] and crab [*Portunus trituberculatus*] as natural infection or by experimental (*per os*) exposure (Chen *et al.*, 2019; Qiu *et al.*, 2018; 2019b; 2020b; 2021b; 2022b). Species with a positive DIV1 polymerase chain reaction (PCR) result, without an active infection include: *Penaeus monodon*, *Pomacea canaliculata*, *Macrobrachium superbum*, *Plexippus paykulli* and *Hemigrapsus penicillatus* (Qiu *et al.*, 2021; 2019a; 2022b; Srisala *et al.*, 2021).

h. 2.2.4. Distribution of the pathogen in the host

The principal target tissues for DIV1 include lymphoid organ, haematopoietic tissues, as well as epithelia and haemocytes in gills, muscle, hepatopancreas, pereopods, pleopods, uropods, and antenna (Qiu *et al.*, 2017; 2019a; 2021a; Sanguanrut *et al.*, 2021).

i. 2.2.5. Aquatic animal reservoirs of infection

There is evidence that crustacean species may become reservoirs of DIV1 infection. DIV1 was detected in non-clinical adult wild giant tiger prawn (*P. monodon*) (Srisala *et al.*, 2021), wild crabs (*Helis tientsinensis*, *Hemigrapsus penicillatus*) in drainage ditches (Qiu *et al.*, 2022a), and *Macrobrachium superbum* in affected shrimp ponds (Qiu *et al.*, 2019a).

Subclinical infection has been reported in gazami crab, *Portunus trituberculatus*, which is widely distributed in environmental waters in Asia and could be a potential source of DIV1 infection on shrimp farms (Qiu *et al.*, 2022a).

j. 2.2.6. Vectors

There are no confirmed vectors of DIV1.

2.3. Disease pattern

k. 2.3.1. Mortality, morbidity and prevalence

Mortality can be high (80–100%) after a natural infection with DIV1 and mostly reported in the adult stage of shrimp (Liao *et al.*, 2022) in shrimp and crayfish species, which has been confirmed by experimental infection through intramuscular injection or oral administration in *P. vannamei*, *Cherax quadricarinatus*, *Procambarus clarkii* and *Macrobrachium rosenbergii* (Qiu *et al.*, 2017; 2019a; Xu *et al.*, 2016). Experimental infection with DIV1 administered orally or by intramuscular injection resulted in 50% and 100% mortality, respectively, in the gazami crab (*Portunus trituberculatus*) (Qiu *et al.*, 2022a).

In pathogenicity studies of crustacean species, mortalities rose more rapidly in *Litopenaeus vannamei* compared with *Cherax quadricarinatus* or *Procambarus clarkii* in experimental infections (Xu *et al.*, 2016).

The prevalence of DIV1 infection was 15.5, 15.2, and 50% in *P. vannamei*, *P. chinensis*, and *M. rosenbergii*, respectively, in a survey of shrimp farms tested in the period 2014 to 2016 (Qiu *et al.*, 2017).

l. 2.3.2. Clinical signs, including behavioural changes

Clinical signs in affected whiteleg shrimp (*P. vannamei*) are reddish bodies, white atrophied hepatopancreas, soft shells and empty stomachs and intestines, while giant freshwater shrimp (*M. rosenbergii*) showed a white discoloration at the base of the rostrum (white head) and hepatopancreatic atrophy (Qiu *et al.*, 2017; 2019a). However, these disease signs are not always distinctive because the course of the disease varies in affected animals.

m. 2.3.3 Gross pathology

See Section 2.3.2.

n. 2.3.4. Modes of transmission and life cycle

Based on experimental and natural infections, DIV1 is thought to be transmitted horizontally by oral routes and contaminated water (Qiu *et al.*, 2017; 2019a; 2022a; Xu *et al.*, 2016).

o. 2.3.5. Environmental factors

Temperature and co-culture play an important role in DIV1 infection. DIV1 has been detected in shrimp and crayfish reared at 16–32°C, but not at temperatures above 32°C in a 2017–2018 survey (Qiu *et al.*, 2018b; 2019b; 2020b; 2021b 2022b). In shrimp farm management, polyculture with different species of crustaceans increases the risk of DIV1 infection in farmed shrimp due to cross-species transmission (Qiu *et al.*, 2019a; 2022a).

p. 2.3.6. Geographical distribution

DIV1 has been reported in farmed shrimp and crayfish in the Asia-Pacific region (Qiu *et al.*, 2017; Xu *et al.*, 2016).

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

2.4. Biosecurity and disease control strategies

q. 2.4.1. Vaccination

Not available.

r. 2.4.2. Chemotherapy including blocking agents

Not available.

s. 2.4.3. Immunostimulation

Not available.

t. 2.4.4. Breeding resistant strains

Not available.

u. 2.4.5. Inactivation methods

Not known.

v. 2.4.6. Disinfection of eggs and larvae

Not available

w. 2.4.7. General husbandry

Biosecurity practices can be used to reduce the risk of DIV1 infection. These includes PCR pre-screening of broodstock and larvae, PCR pre-screening of polychaetes and food organisms for broodstock and larvae,

disinfection of rearing water and farming equipment, controlled stocking density, and avoidance of polyculture with different crustacean species.

Using an experimental protocol of 15-day thermal treatment at 36°C combined with 15-day restoration treatment at 28°C, *P. vannamei* infected by intramuscular injection of DIV1 showed no clinical signs, no DNA replication, no histopathology and in-situ DIG-labelling, loop-mediated DNA amplification (ISDL) results, indicating DIV1 could ~~can~~ be eliminated from challenged shrimp after 36°C treatment (Guo *et al.*, 2022).

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

For diagnosis during a disease outbreak, moribund and apparently healthy crustacean specimens of susceptible species (see Section 2.2.3) from the same ponds, especially in polyculture mode, are selected as samples for identification testing. Apparently healthy or even dead and dried samples from crustacean farms next to the affected farms can be used as sources of materials for examination (Qiu *et al.*, 2019a). For surveillance in apparently healthy populations, all life stages of samples reared at 16–32°C should be suitable for testing (see Section 2.3.5)

Shrimp and crayfish that are 4–7 cm in body length provide the highest detection rate of DIV1 when used for examination (Qiu *et al.*, 2018b ;2019b ;2020b; 2021b ;2022b).

3.2. Selection of organs or tissues

Suitable tissues for testing are lymphoid organ, haematopoietic tissues, muscle, gills, hepatopancreas, pereopods, pleopods, uropods, and antennae (Qiu *et al.*, 2017; 2019a; 2021a; Srisala *et al.*, 2021). Quantitative virus analysis from different tissues of naturally infected *Macrobrachium rosenbergii* showed that muscle and hepatopancreas had lower virus load compared with that of the lymphoid organ, haematopoietic tissues, gills, pereopods, pleopods, uropods and antennae (Qiu *et al.*, 2019a).

3.3. Samples or tissues not suitable for pathogen detection

Autolytic and compound eyes samples are not suitable for PCR-based pathogen detection.

3.4. Non-lethal sampling

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

3.5. Preservation of samples for submission

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

x. 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.

y. 3.5.2. Preservation of samples for molecular detection

Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed, it may be frozen.

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

z. 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.5.3 of Chapter 2.2.0 *General information* (diseases of crustaceans).

aa. 3.5.4. Samples for other tests

Not available

3.6. Pooling of samples

Pooling of samples from more than one individual animal for a given purpose is only 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 shrimp (or other decapod crustaceans) should be processed and tested individually. Small life stages such as larvae or PLs can be pooled to obtain the minimum amount of material for virus isolation or molecular detection.

4. Diagnostic methods

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

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

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

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

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

Shaded boxes = Not appropriate for this purpose.

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

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

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

Method [amend or shade in as relevant]	Surveillance of apparently healthy animals				Presumptive diagnosis of clinically affected animals				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	++	+++	+++	NA	+++	+++	+++	1	+++	+++	+++	1
Conventional PCR	++	++	++	NA	++	++	++	NA				
Conventional nested PCR followed by amplicon sequencing									+	+	+	1
Conventional PCR followed by amplicon sequencing									+++	+++	+++	1
<i>In-situ</i> hybridisation						++	++	1		+++	+++	1
Bioassay					+	+	+	NA				
LAMP	+	+	+	NA	+	+	+	NA				
Quantitative LAMP	++	++	++	NA	++	++	++	1				
Ag-ELISA												
RPA	++	++	++	NA	++	++	++	1				
Other methods ³												

LV = level of validation, refers to the stage of validation in the WOAHP Pathway (chapter 1.1.2); NA = not available;

PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification

Ag-ELISA = antigen enzyme-linked immunosorbent assay; RPA = recombinase polymerase amplification

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 relevant

4.2. Histopathology and cytopathology

Histopathological examination revealed pathognomonic dark eosinophilic cytoplasmic inclusion bodies in the karyopyknotic cells of haemopoietic tissues and lymphoid organs, and in the haemocytes of gills, pereopods and sinus of the hepatopancreas (Qiu *et al.*, 2017; 2019a), as well as cuticular epithelium under the cuticles (Chen *et al.*, 2019).

4.3. Cell culture for isolation

Not available.

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

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

c. 4.4.1. Real-time PCR

Table 4.4.1.1. Primers and probes (sequences) and cycling conditions for DIV1 real-time PCR

Target gene	Primer/probe (5'–3')	Concentration	Cycling parameters ^(a)
Method 1: Qiu <i>et al.</i> , 2018a; GenBank Accession No.: MF599468.1			
ATPase	SHIV-F: AGG-AGA-GGG-AAA-TAA-CGG-GAA-AAC	500 nM	40 cycles of 95°C/100 sec and 60°C/30 sec
	SHIV-R: CGT-CAG-CAT-TTG-GTT-CAT-CCA-TG Probe: FAM-CTG-CCC-ATC-TAA-CAC-CAT-CTC-CCG-CCC-TAMRA	200 nM	
Method 2: Qiu <i>et al.</i> , 2020a; GenBank Accession No.: MF599468.1			
MCP	142F: AAT-CCA-TGC-AAG-GTT-CCT-CAG-G	500 nM	40 cycles of 95°C/10 sec and 60°C/30 sec
	142R: CAA-TCA-ACA-TGT-CGC-GGT-GAA-C Probe: FAM-CCA-TAC-GTG-CTC-GCT-CCG-CTT-CCG-TAMRA	200 nM	
Method 3: Gong <i>et al.</i> , 2021; GenBank Accession No.: MF599468.1			
ATPase	DIV1-F: AGG-AAA-GGA-AAC-GAA-AGA-AAT-TAT-ACC	400 nM	40 cycles of: 95°C/10 sec and 60°C/30 sec
	DIV1-R: GCT-TGA-TCG-GCA-TCC-TTG-A Probe: FAM-CAC-ATG-ATT-TGC-AAC-AAG-CTT-CCA-GCA-TAMRA	200 nM	

^(a)A denaturation step prior to cycling has not been included.

d. 4.4.2. Conventional PCR/nested PCR

Table 4.4.2.1. Primer sequences and cycling conditions for DIV1 PCR and nested PCR

Target gene	Primer (5'–3')	Concentration	Cycling parameters ^(a)
Method 1: Xu <i>et al.</i> , 2016; GenBank Accession No.: ; amplicon size: 103 bp			
MCP	CQIV-MCP-F: GAA-ACT-TTA-TGC-ACA-ATC-TTA-T CQIV-MCP-R: CCA-ATC-ATG-TTG-TCG-TAT-CC	NA	25 cycles of: 94°C/30 sec, 55°C/30 sec and 72°C/30 sec
Method 2: Qiu <i>et al.</i> , 2017; GenBank Accession No.: KY618040; amplicon size: 457 and 129 bp			
ATPase	Primary step: SHIV-F1: GGG-CGG-GAG-ATG-GTG-TTA-GAT SHIV-R1: TCG-TTT-CGG-TAC-GAA-GAT-GTA Nested PCR: SHIV-F2: CGG-GAA-ACG-ATT-CGT-ATT-GGG SHIV-R2: TTG-CTT-GAT-CGG-CAT-CCT-TGA	400 nM 400 nM	Primary and nested steps: 95°C/3 min; 35 cycles of 95°C/30 sec, 59°C/30 sec and 72°C/30 sec

^(a)A denaturation step prior to cycling has not been included.

e. 4.4.3. Other nucleic acid amplification methods

Table 4.4.3 Primers and probes (sequences) for DIV1 LAMP, RPA and qLAMP

Method / Target gene	Primer (5'–3')	Concentration	Cycling parameters ^{(a)/} method
Method 1: Chen <i>et al.</i> , 2019; GenBank Accession No.:xxx			
LAMP/ DNA-directed RNA polymerase II	SHIV-FIP (F1C + F2): TGG-GGT-TTC-ATA-TGG-GCA-AA T-GAT-TTT-AAG-AAT-GGA-AAG-ATC-CTA-TCA-GC SHIV-BIP (B1C + B2): AGG-AGA-AAA-GGT-TGG-ATT-GGT-TAC-TTT-TAC-TTC-TGT-TAC-TGC-GAT-GG SHIV-LF: GAG-AGG-CGT-GCA-ACT-TTC-TG SHIV-LB: TTT-GGC-ATT-GTC-TGC-TAC-AAT-TTC-C SHIV-F3: GAT-GGC-CAT-TCC-TTC-AAA-C SHIV-B3: AAA-ATA-GTC-ATC-CTG-AAA-TCC-T	1600 nM 1600 nM 800 nM 800 nM 200 nM 200 nM	60 cycles of: 60°C 85°C/5 min:
Method 2: Chen <i>et al.</i> , 2020; GenBank Accession No.:xxx			
RPA/ MCP	RPA-F : CAG-ATC-AGA-GCG-CAT-TCG-ATC-CCA-TAG-GCA-CCG-C RPA-R: CGT-AAG-AGA-ACA-TGT-GGT-ATC-CGG-TGA-GTT-CGG-G RPA- Probe: ATA-CGA-ATC-TTC-AGA-TCG-TAT-TCC-CGT-GA(FAM-dT)G(THF)C(BHQ1-dT)GCC-GAT-TAC-TTC-TC (phosphorylation)	400 nM 400 nM 120 nM	40 cycles of: 39°C/45 sec, and 39°C/15 sec:
Method 3: Gong <i>et al.</i> , 2021; GenBank Accession No.:xxx			
qLAMP/ ATPase	F3: GGC-TTG-GTA-TCT-TAT-TCA-GAG-AT B3: ATT-CAC-AAC-ATC-GTC-ACC-AT FIP: CTC-TTG-ATG-GAT-ACA-CTG-ATC-TTC-GGA-GCC-AGA-GAT-TGT-AAC-GG BIP: ATT-CAG-TAT-TCA-AGG-ATT-GGT-TCA-AAA-GTT-CTT-CCA-TCT-ACC-TCT-C LF: TTC-GGT-ACG-AAG-ATG-TAG-C LB: GAA-GAG-TAT-CCT-AAT-ATG-ACC-ATC-C	200 nM 200 nM 1600 nM 1600 nM 800 nM 800 nM	63°C/30 sec 40 cycles of: 63°C/60 sec:

^(a)A denaturation step prior to cycling has not been included.

4.5. Amplicon sequencing

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

4.6. *In-situ* hybridisation

In-situ hybridisation has been applied to paraffin sections to determine the specific location of DIV1 in target tissues by either DIG-labelled oligonucleotide probe or DIG-labelling-loop-mediated DNA amplification (ISDL) (Chen *et al.*, 2019; ~~Xu *et al.*, 2016~~; Sanguanrut *et al.*, 2021). ISDL is the preferred method to use because it is highly sensitive through simultaneous pathogen DNA amplification and labelling techniques, compared with routine probe-based *in-situ* hybridisation.

4.7. Immunohistochemistry

Not available.

4.8. Bioassay

Bioassay has application in presumptive diagnosis, but cost, accuracy, labour, timing, or other factors limit its application (Qiu *et al.*, 2017; Xu *et al.*, 2016).

4.9. Antibody- or antigen-based detection methods (ELISA, etc.)

Not available.

4.10. Other methods

Not available.

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

Any of the real-time PCR assays is recommended for surveillance to demonstrate freedom in apparently health populations.

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. If a Competent Authority does not have the capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAHP Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status⁵

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.

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

The presence of infection with DIV1 shall be suspected if at least one of the following criteria is met:

- i) Positive result by real-time PCR
- ii) Positive result by conventional PCR,
- iii) Positive result by LAMP
- iv) Positive result by RPA

⁵ For example transboundary commodities.

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

The presence of infection with DIV1 is considered to be confirmed if at least one of the following criteria is met:

- i) Positive result by real-time PCR followed by conventional PCR and amplicon sequencing.
- ii) Positive result by real-time PCR followed by conventional nested PCR and amplicon sequencing.
- iii) ~~A positive result from each of two different real-time PCR methods~~

6.2 Clinically affected animals

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

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

The presence of infection with DIV1 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 real-time PCR
- iii) Positive result by conventional PCR
- iv) Positive result by LAMP
- v) Positive result by RPA
- vi) Histopathological changes consistent with the presence of the pathogen or the disease
- vii) Positive result by *in-situ* hybridisation

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

The presence of infection with DIV1 is considered to be confirmed if at least at least one of the following criteria is met:

- i) Positive result by real-time PCR and positive result by conventional PCR followed by amplicon sequencing
- ii) Positive result by real-time PCR and positive result by conventional nested PCR and amplicon sequencing
- iii) Positive result by real-time PCR and positive result by *in-situ* hybridisation
- iv) ~~A positive result from each of two different real-time PCR methods~~

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with DIV1 are provided in Tables 6.3.1. and 6.3.2 (no data are currently available for either). 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.

j. 6.3.1. For presumptive diagnosis of clinically affected animals

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

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

k.6.3.2. For surveillance of apparently healthy animals

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

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DSe = diagnostic sensitivity, DSp = diagnostic specificity, n = number of ~~samples~~ animals used in the validation study

PCR: = polymerase chain reaction.

7. References

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* *

NB: There is a WOAHP Reference Laboratory for infection with decapod iridescent virus 1
(please consult the WOAHP web site:
<https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).
Please contact the WOAHP Reference Laboratories for any further information on
infection with decapod iridescent virus 1

NB: FIRST ADOPTED IN 20XX.

SECTION 2.4.
DISEASES OF MOLLUSCS

CHAPTER 2.4.0.

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. surveillance of apparently healthy animals, presumptive diagnosis of clinically affected animals or confirmatory diagnosis of a suspect result from surveillance or presumptive diagnosis). See individual disease chapters in this *Aquatic Manual* for specific details of sample requirements.

1.2. Specifications according to mollusc populations

For details of animals to sample for a specific listed disease, see the relevant disease chapter in this *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 WOA *Aquatic Code* Chapter 1.4. *Aquatic animal disease surveillance*.

The following factors should be considered when selecting animals to be sampled:

- i) for apparently healthy populations, susceptible species should be sampled proportionately or following risk-based criteria for targeted selection of lots, epidemiological units or populations with a history of abnormal mortality or potential exposure events (e.g. stocking with animals of unknown disease status);
- ii) If weak, abnormally behaving or freshly dead (not decomposed) animals are present, such animals should be selected. If such animals are not present, animals should be selected in such a way that all epidemiological units of the farm or waterbody are proportionately represented in the sample;
- iii) if more than one water source is used for production, animals from all water sources should be included in the sample.

1.3. Specifications according to clinical status

In addition to sampling of target tissues, other organs showing macroscopic abnormalities or lesions should also be sampled. For disease outbreaks, at least ten diseased or moribund molluscs should be sampled for testing. Parallel samples ($n > 10$) from apparently normal animals in the same production region should also be collected. Collection of dead specimens during disease outbreaks should be avoided when possible, but recently dead samples may be suitable for some diagnostic assays provided the animals are not decomposed. Disease-specific recommendations are provided in Section 3 *Sample selection, sample collection, transportation and handling* of the individual chapters.

1.4. Specifications according to mollusc size

For the WOA-listed diseases it is 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.

1.4.1. For the listed parasites

Juveniles below 1.5 cm: sample the entire animal but remove the shell when possible or proceed with a decalcification protocol. When animals are too small for individual analyses, analyses can be performed on pools of several animals.

Juveniles 1.5–3 cm: sample the entire mollusc and cut in half sagittally. Keep one half of the animal for histological analyses and the other half for molecular analyses.

Molluscs over 3 cm: take a cross-section of the body, passing through the mantle, gills, digestive gland and gonads for histological analyses. Keep the remaining tissues for molecular analyses.

1.4.2. For infection with *Xenohaliotis californiensis*

For abalone ≥ 20 mm, excise several 3–5 mm cross sections containing posterior oesophagus (postoesophagus), digestive gland, and foot muscle.

1.4.3. For abalone herpesvirus infections

Sample as outlined in Section 1.4.2 above with the addition of a cross section of the head to obtain the cerebral ganglion and removal of several sections of the foot and adductor muscle complex including one section 0.25–1.0 cm (distance depends on abalone maximum length) posterior to the head to obtain the pedal ganglion. In addition, a longitudinal section from the anterior pedal ganglion to the posterior portion of the pedal musculature should be taken.

2. General processing of samples

Sampled molluscs should be delivered alive to the diagnostic laboratory. The laboratory should be informed of the estimated time of arrival of the sample so the required materials to process the molluscs can be prepared before receipt of the samples.

Mollusc samples should be packed appropriately in order to keep them alive. Required samples should be shipped as soon as possible after collection from the water. Unless otherwise specified, moribund animals should be sent on ice (but not frozen) to reduce sample decomposition.

For samples that cannot be delivered live to the diagnostic laboratory, specimens should be fixed on site as recommended in the following sections of this chapter or the relevant disease chapters of this *Aquatic Manual*. While this may be suitable for subsequent histology, transmission electron microscopy examination or PCR analyses for example, other techniques, such as fresh smears, tissue imprints, routine bacteriology, mycology or Ray's fluid thioglycollate culture of *Perkinsus* spp., cannot be performed on such samples. Diagnostic needs and sample requirements should be discussed with the diagnostic laboratory prior to collection of the sample.

2.1. Macroscopic examination

The gross observation of molluscs should target, as far as possible, animal behaviour, shell surface, inner shell and soft tissues.

It is often difficult to observe the behaviour of molluscs in open systems. However, observation of molluscs in certain rearing facilities, such as broodstock in tanks and larvae in hatcheries, can provide useful indications of disease-related behavioural changes. If signs are noted (e.g. pre-settlement of larvae on the bottom, food accumulation in tanks, signs of weakening, etc.), samples may be examined for gross signs, including observation under a dissecting microscope for abnormalities and deformities, fouling organisms, and fixed for further processing as recommended below. For adults and juveniles, signs of weakening may include gaping, accumulation of sand, mud and debris in the mantle and on the gills, mantle retraction away from the edge of the shell, decreased activity (scallop swimming, clam burrowing, abalone grazing), etc. The righting reflex of abalone after being inverted does not occur in weakened animals, and it is a good indicator of weakness. Mortality in open systems should be monitored for patterns of losses, and samples should be collected for further analysis. Environmental factors, pre- and post-mortality, should be recorded.

Even under culture conditions, the shells of molluscs may not be clean and fouling organisms are normal colonists of mollusc shell surfaces. Organisms such as barnacles, limpets, sponges, polychaete worms, bivalve larvae, tunicates, bryozoans, etc., do not normally threaten the health of molluscs. Culture systems, such as suspension and shallow water culture, can even increase exposure to fouling organisms and shells may become covered by other animals and plants. This can affect health directly by impeding shell opening and closing or indirectly through competition for food resources. Signs of weakening associated with heavy fouling should be a cause for concern rather than fouling itself. Shell damage by boring organisms, such as sponges and polychaete worms, are usually benign, but under certain conditions may reach proportions that make the shell brittle or pierce through to the soft tissues. This degree of shell damage can weaken the mollusc and render it susceptible to pathogen infections. Shell deformities (shape, holes in the surface), fragility, breakage or repair should be noted, but may not be indicative of a disease concern. Burrowing epibionts may cause deformities and weaken the shell(s). Abnormal coloration and smell may indicate a possible soft-tissue infection that may need to be examined at a laboratory.

The molluscs should be opened carefully so as not to damage the soft tissues, in particular the mantle, gills, heart and digestive gland. The presence of fouling organisms on the inner shell surface is a clear indication of weakness. The inner surface of the shell is usually smooth and clean because of mantle and gill action. Perforation of the inner surface may occur but can be sealed off by the deposition of additional conchiolin and nacre. This may result in formation of mud- or water-filled blisters. Blisters may also form over superficial irritants such as foreign bodies. The degree of shell perforation can be determined by holding the shell up to a strong light. Where abnormalities occurring within the matrix of the shell warrant further investigation, freshly collected specimens can be brought intact to the laboratory or fixed for subsequent decalcification, as required. The appearance of the soft tissues is frequently indicative of the physiological condition of the animal. Soft tissues should be examined for the presence of abscess lesions, pustules, tissue discoloration, pearls, oedema, overall transparency or wateriness, gill deformities, etc., and, when found in association with weak or dying animals. Abnormalities and lesions of the tissues should be noted and recorded, as well as any shell deformities, shell-boring organisms and conspicuous mantle inhabitants. Levels of tissue damage should be recorded and samples of affected and unaffected animals collected for laboratory examination as soon as possible.

2.2. Virological examination

See Chapter 2.4.1. Infection with abalone herpesvirus for specific details.

2.3. Bacteriological examination

See Chapter 2.4.7. Infection with *Xenohaliotis californiensis* for specific details.

2.4. Parasitic (protists) examination

See Chapters 2.4.2 to 2.4.6. Infections with listed protists for specific details.

2.5. Fungal examination

Not applicable for currently listed diseases.

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

1. Mollusc viruses

1.1. Mollusc cell lines

Not applicable. There are currently no confirmed or documented mollusc cell lines suitable for virus isolation.

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 \[liftonline.org\]](http://ICTV.liftonline.org) for latest information).

1.3.2. Virus production for experimental purposes

As no cell lines are known that can be used to produce mollusc virus stocks, infection of known susceptible host species (which are free of infection with the pathogenic agent in question) is the preferred method for virus production for experimental purposes or for the production of positive control material.

1.3.3. Virus preservation and storage

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

2. Mollusc bacteria

Not applicable. There is currently no developed procedure to cultivate *Xenohaliotis californiensis*.

3. Mollusc parasites (protists)

3.1. Culture media

See Chapters 2.4.5 Infection with *Perkinsus marinus* and 2.4.6 Infection with *Perkinsus olseni* for details.

3.2. Storage of cultures

Perkinsus spp. cultures in the exponential phase of growth can be pelleted by centrifugation and cryopreserved by resuspending the pellet in 40% DMEM Ham's F-12 (1:1) culture medium with 10% glycerol and 50% FBS and freezing them using standard procedures.

4. Mollusc fungi

4.1. Culture media

Not applicable for currently listed diseases.

4.2. Storage of cultures

Not applicable for currently listed diseases.

5. Techniques

The available diagnostic methods that may be selected for diagnosis of the WOAHL-listed mollusc 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.
- iii) Histology, *in-situ* hybridisation and electron microscopy of fixed specimens.
- iv) Culture methods where applicable.
- v) Molecular methods (including sequencing): Conventional and real-time PCR and LAMP for direct assay with fresh, frozen or ethanol fixed-tissue samples or with extracted DNA.

Bioassays of suspect or subclinical carriers using a highly susceptible host (life stage or species) may also be used as an indicator for the presence of the pathogen.

Pooling of samples from more than one individual animal for a given purpose is only 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 molluscs should be processed and tested individually. However, for eggs, larvae and postlarvae, pooling of individuals may be necessary to obtain sufficient sample material to run a diagnostic assay.

5.1. Gross and clinical signs

Macroscopic examination of gross and clinical signs reveals non-specific signs only (e.g. gaping in bivalves or general weakness of the foot muscle in abalone), and mortality may be caused by several disease agents or physiological problems, such as loss of condition following spawning. To obtain a definitive diagnosis further investigation is required and this can only be determined using a range of other techniques including histology/electron microscopy and molecular techniques such as PCR and gene sequence analysis.

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

Live moribund animals or freshly dead (within minutes) animals provide the optimal tissues for examination. Due to tissue lysis that occurs during the freeze-thaw cycle, frozen samples are not appropriate for histology. Should a delay between animal mortality and sampling occur, it is recommended that animals be stored intact on ice or in a refrigerator.

To obtain a sample that includes all the major tissues, a section should be taken to include digestive gland, gills, gonad, mantle and palps, where possible. For large specimens, it may be necessary to take several sections to include all the important tissues. Tissue preparation for examination by light microscopy involves several steps, including tissue fixation, dehydration, impregnation and embedding of samples, preparation of sections, staining and mounting of slides.

5.3.1. Tissue fixation

Tissue fixation is required to maintain the morphology of the tissues and to prevent post-sampling necrosis. Recommended fixatives used for the study of marine molluscs are Davidson's solution, Carson's solution and 10% formalin in filtered sea water. The ratio of fixative to tissue volume should be at least 10:1 to ensure good fixation.

Davidson's solution:

1 µm filtered sea water	1200 ml
95% Alcohol	1200 ml
35–40% Formaldehyde ⁶	800 ml
Glycerol	400 ml
Glacial acetic acid	10% (add just prior to use)

Carson's solution:

NaH ₂ PO ₄ ·2H ₂ O	23.8 g
Sodium hydroxide (NaOH)	5.2 g
Distilled water	900 ml
40% Formaldehyde ¹	100 ml
Adjust the pH to 7.2–7.4	

10% formalin in filtered sea water solution:

1 µm filtered sea water	900 ml
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⁶ A saturated 37–39% aqueous solution of formaldehyde gas.

35–40% Formaldehyde¹

100 ml

These solutions allow tissue structure to be preserved and different histochemical methods to be used including for *in-situ* hybridisation with DNA probes. Over-fixation (over 24–48 hours) should be avoided. After fixation, the specimens should be transferred to 70% ethyl alcohol, where they can be stored indefinitely. Davidson's solution is normally used because it provides better preservation of the cell nuclei. Carson's solution or 10% formalin in seawater can be used to examine tissues by electron microscopy. As electron microscopy can be a valuable aid in diagnosing or confirming infections in bivalve molluscs, fixing some samples (particularly the smaller ones) with glutaraldehyde, as described in Section B.5.4.1 of this chapter, may be considered, and will provide electron micrographs of the highest quality. It is recommended that a representative portion of the mollusc is fixed in Davidson's solution, while another representative portion is fixed in Carson's solution for further examination to ensure that all tissues/organs are fixed in both fixatives. If neither is available, 10% formalin buffered with filtered seawater will suffice.

For transport and shipping, see *Aquatic Code* Chapter 5.10 *Measures concerning international transport of aquatic animal pathogens and pathological material*.

5.3.2. Dehydration, impregnation and embedding of the samples

The fixed samples are transferred through a series of graded alcohols (70–95% [v/v]) before final dehydration in absolute ethanol. The alcohol contained in the tissues is next eliminated by immersing them in xylene. The tissues are then impregnated with paraffin, which is soluble in xylene, at 60°C. These steps are often carried out automatically using a tissue processing machine. Should processing be delayed, fixed tissues may be stored in 70% ethanol.

Histological blocks are produced by letting the tissues cool in moulds filled with paraffin on a cooling table.

5.3.3. Preparation of the sections

After the blocks have cooled and the paraffin has solidified, histological sections of about 2–5 µm are cut using a microtome. The sections are recovered on histological slides, drained and dried for up to 1 hour at 40–42°C or overnight at room temperature.

5.3.4. Staining and mounting the slides

Before staining, the paraffin is removed from the sections by immersing them in xylene or equivalent clearing solution for 10–20 minutes. This is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10-minute periods each, and they are then rehydrated through a descending series of ethanol baths (for example 95%, 70%, 50%, 30%, 10 minutes each) with a final immersion in tap water for 10 minutes. Different topographical or histochemical staining techniques can then be performed.

When haematoxylin–eosin (H&E) stain is used (haematoxylin or equivalent), nuclear and basophilic structures stain a blue-to-dark-purple colour, the endoplasmic reticulum stains blue, while the cytoplasm takes on a grey colour. The acid dye eosin stains the other structures pink. This staining technique is simple and reproducible and, although it only allows a limited differentiation of cell structures, it is possible to detect any abnormalities in tissue and cellular structure. Other techniques may be applied to demonstrate particular structures or features as required (e.g. trichrome for connective tissue and cytoplasmic granules).

5.4. Transmission electron microscopy methods

Transmission electron microscopy can be used as part of the diagnostic procedures for diseases of molluscs.

Fixation for electron microscopy should be done immediately after the animal has been killed and before fixation for histology. Only samples taken rapidly from live animals will be of any use. The preparation of samples for electron microscopy involves the following steps: tissue fixation, decalcification of the samples (when necessary), dehydration, impregnation and embedding of the samples, preparation and counterstaining of the sections.

5.4.1. Tissue fixation

For tissues that are to be examined by electron microscopy, it is important that the fixation be performed correctly in order to cause as little damage as possible to the ultrastructure. The specimens are cut such that their

dimensions do not exceed 1–2 mm. This small size allows rapid penetration of the various solutions into the tissue sample.

Fixation is carried out directly in 3% glutaraldehyde for 1–4 hours. The samples are washed in buffer three times, then post-fixed in 1% osmic acid (aqueous OsO₄) and washed twice again in buffer. Various formulations of glutaraldehyde fixative and buffers work equally well.

In order to cause as little damage as possible to the ultrastructure, the samples are treated with solutions that have an osmolarity close to that of the tissues. Thus, mollusc tissues are treated with solutions with an osmolarity of approximately 1000 mOsm. The osmolarity of the solutions is adjusted with artificial sea salts or NaCl. Alternatively, the glutaraldehyde can be formulated with 0.22 µm filtered seawater, and filtered seawater used for subsequent washes.

Sodium cacodylate 0.4 M: 8.6 g in 100 ml of distilled water

Sodium chloride 10% in distilled water

Cacodylate buffer, pH 7.4:

1000 mOsm

Sodium cacodylate 50 ml from 0.4 M stock solution

NaCl 20 ml from 10% stock solution

Distilled water 30 ml

Adjust the pH to 7.4

3% Glutaraldehyde:

1000 mOsm

25% glutaraldehyde 2.5 ml

0.4 M sodium cacodylate 5 ml

10% NaCl 3.5 ml

Distilled water 9 ml

1% Osmic acid:

1000 mOsm

4% Osmic acid 1 volume

0.4 M sodium cacodylate 1 volume

NaCl 1 volume from 10% stock solution

Distilled water 1 volume

5% ethylene diamine tetra-acetic acid (EDTA):

Disodium EDTA 5 g

Cacodylate buffer 100 ml

EDTA dissolves when the pH is above 8. When the solution becomes clear adjust the pH to 7.4 by adding concentrated HCl.

If the samples have been previously fixed and stored in Carson's solution, they should be washed several times in a bath of buffer before fixation with 3% glutaraldehyde.

5.4.2. Dehydration, impregnation and embedding of the samples

The samples are dehydrated in successive baths of ethanol: 70% ethanol once, 95% ethanol twice, absolute ethanol three times. The dehydration is completed by two baths of propylene oxide, which allows subsequent impregnation with Epon.

The samples are impregnated progressively. After a first bath in a mixture of polypropylene oxide–Epon (50/50), the samples are placed in a bath of Epon. The longer the incubation, the better the impregnation of the tissues.

Embedding is carried out by placing the samples in moulds filled with Epon resin. A label identifying the sample is included in each block and the blocks are then placed at 60°C (the temperature at which Epon resin polymerises) for 48 hours.

5.4.3. Preparation of the sections and the counterstaining

The blocks are cut to appropriate sizes with a razor blade and, using an ultra-microtome, semi-thin sections (0.5–1 µm) are cut and placed on glass slides. These will be used to monitor the quality of the samples by light microscopy and to locate the areas of interest on the section.

The semi-thin sections are stained at 90–100°C with 1% toluidine blue solution. After drying, the slides are mounted under cover-slips with a drop of synthetic resin and observed using the light microscope.

Ultra-thin sections 80–100 nm thick are placed on mesh copper grids for electron microscopy. Uranyl acetate and lead citrate are used to counterstain the ultra-thin sections.

5.5. Use of molecular techniques for surveillance, 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 pathogen nucleic acids in samples prepared from mollusc tissues. These techniques 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 molluscs and can be undertaken as the standard method. Following real-time PCR-positive results, where possible, conventional PCR with sequence analysis 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. Therefore, each assay and tissue extraction should include a negative control to rule out contamination. False-negative results (positive samples giving a negative result) may lead to unwanted transmission of pathogens and biosecurity failure. Therefore, each assay (and ideally each tissue extraction) should include positive controls to ensure the assay performed correctly. Additionally, mollusc tissues are known to potentially contain PCR inhibitors. It is therefore recommended to check for the presence of PCR inhibitors in DNA extracts to avoid false negative results.

To minimise the risk of contamination, aerosol barrier pipette tips should be used for all sample preparation and PCR steps. Additionally, all PCRs should be prepared in a clean area that is separate from the area where the nucleic acid extraction, amplification 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/cabinets 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 separate from the molecular biology laboratory and reagents.

Nested PCR involves two rounds of PCR and may be used to achieve increased sensitivity and specificity; however, it increases the risk of contamination. Contaminants from previous reactions can carry over and lead to false-positive results. Strict laboratory practices such as separate workspaces, dedicated equipment, and meticulous pipetting techniques are essential to mitigate this risk. In conclusion, nested PCR is not recommended for surveillance but may sometimes be used for confirmative studies.

5.5.1. Sample preparation

Samples should be prepared to preserve the nucleic acid of the pathogen and should be handled and packaged with the greatest care to minimise the potential for cross-contamination amongst the samples or target degradation before the assay can be performed. A water-resistant label, with the appropriate data filled out, should be placed within each package or container for each sample set. Use of household permanent markers should be avoided as their ink dissolves in ethanol and may result in loss of the sample label. Use pencil or histology pens only to label vials or jars.

Some suitable methods for preservation and transport of samples taken for molecular tests are:

- i) *Live, iced specimens or chilled specimens*: for specimens that can be rapidly transported to the laboratory for testing within 24 hours, pack samples in sample bags in an insulated box containing a cold pack and ship to the laboratory. Note: cold packs should not be in direct contact with the animals to avoid freezing some parts of the tissues if histological analyses are also planned on the samples (histology cannot be performed on frozen tissues).
- ii) *Frozen whole specimens*: select live specimens according to the purpose of sampling, quick freeze in the field using crushed dry-ice, or freeze in a field laboratory 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.
- iii) *Alcohol-preserved samples*: 80% analytical grade ethanol (i.e. methanol-free ethanol) can be used to preserve, store, and transport mollusc tissues. Tissues should be fully immersed in ethanol. Shipment can be performed at room temperature.
- iv) *Fixed tissues for in-situ hybridisation*: for this purpose, classic methods for preservation of the tissues for histology are adequate. Davidson's solution is usually a good choice for later use of molecular probes (See Section B.5.3). For DNA, specifically, over-fixation (more than 48 hours) should be avoided.

5.5.2. Preservation of DNA in tissues

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

5.5.3. Nucleic acid extraction

To isolate nucleic acids from tissues preserved in ethanol or other preservative, simply remove the tissue from the fixative or preservative, press the tissues on absorbent paper to remove the excess of ethanol and let the ethanol evaporate, then treat it as fresh or frozen samples. 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, molluscs are fixed and embedded in paraffin, according to the methods described above for histology. Sections are cut at $5\ \mu\text{m}$ thick and placed on aminoalkylsilane-coated slides, which are then dried overnight at room temperature or in an oven at 40°C . The sections are dewaxed 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 could be rehydrated by immersion in a descending 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 ($100\ \mu\text{g ml}^{-1}$) in TE buffer (Tris [50 mM], EDTA [10 mM]), at 37°C for 10–30 minutes in a humid chamber. Slides are dehydrated by immersion in one or several ethanol series and then air-dried. 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. It is also recommended to test non-specific ISH probes (e.g. "universal" 18s probes) on tested samples to check if the material is suitable for ISH analyses.

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 of the place of origin. The geographical location of the place of origin of samples may be described as the name or location of the site from which the sample has originated, or its geographical co-ordinates. There should also be records that provide information to allow trace-backs on the sample movement from the site of origin to the storage facility or laboratory and within those facilities.

Samples should be accompanied with background information, including the reason for submitting the sample (surveillance, abnormal mortality, abnormal growth, etc.), gross observations and associated environmental parameters, approximate prevalence and patterns of mortality, origin and nature of the molluscs (species, age, whether or not the samples are from local mollusc populations or stocks transferred from another site, date of transfer and source location, etc.). This information should identify possible changes in handling or environmental conditions that could be a factor in mortality in association, or not, with the presence of infectious agents

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.

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

7. Key references for further reading

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NB: FIRST ADOPTED IN 1997. MOST RECENT UPDATES ADOPTED IN 2012.

CHAPTER 2.4.1.

INFECTION WITH ABALONE HERPESVIRUS

1. Scope

Infection with abalone herpesvirus means infection with the pathogenic agent *Aurivirus haliotidmalaco1* (commonly previously known as *Haliotid herpesvirus 1*, and abalone herpesvirus [AbHV-1]) of the genus *Aurivirus* and the Family *Malacoherpesviridae*.

2. Disease information

2.1. Agent factors

a. 2.1.1. Aetiological agent

Aurivirus haliotidmalaco1 AbHV-1 is the aetiological agent of abalone viral ganglioneuritis (AVG); for the purpose of this chapter, the agent will be referred to as AbHV. AVG is a contagious disease of abalone species in Australia (Ellard *et al.*, 2009; Hooper *et al.*, 2007), China (People’s Rep. of) (Gu *et al.*, 2019; Wang *et al.*, 2004) and Chinese Taipei (Chang *et al.*, 2005). Comparison of nucleotide sequences of the Victorian isolate of AbHV-1 and ostreid herpesvirus-1 (Davison *et al.*, 2009; Le Deuff & Renault, 1999) over common coding regions identified similarities ranging from 19% to 53%, indicating that these viruses share a low level of sequence similarity (Savin *et al.*, 2010). AbHV-1 has been assigned as a second member of the *Malacoherpesviridae* (ICTV, 2022). Complete genome sequences of isolates demonstrated that there are at least five genetic variants of AbHV-1 within Australia (Cowley *et al.*, 2012; Corbeil *et al.*, 2016) and one Chinese Taipei strain (Chang *et al.*, 2005). More recent analysis demonstrated that the Chinese strain represents a further variant (Bai *et al.*, 2019b).

Purified AbHV-1 particles (Tan *et al.*, 2008) observed by transmission electron microscopy are enveloped and icosahedral with electron dense cores and 100–110 nm in diameter. The intranuclear location of AbHV-1 particles, their size and ultrastructure are characteristic of members of the *Herpesviridae*. Isopycnic gradient centrifugation (in potassium tartrate and caesium chloride density gradients) indicated a virus particle buoyant density of 1.17–1.18 g ml⁻¹ (Tan *et al.*, 2008).

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

Virus derived from tissue obtained from experimentally infected abalone that had been homogenised in sterile EMEM Gibco containing 10% fetal bovine serum, centrifuged (1500 g for 20 minutes at 4°C), filtered (0.22 µM) and stored as 250 µl aliquots in liquid nitrogen remains infectious for at least 21 months (Corbeil *et al.*, 2012b).

c. 2.1.3. Survival and stability outside the host

Experimental studies (Corbeil *et al.*, 2012b) demonstrated that AbHV-1 remained infectious for up to 5 days when held in seawater at 4°C and for only 1 day at 15°C.

2.2. Host factors

Acute disease was first reported in farmed *Haliotis diversicolor supertexta* in Chinese Taipei (Chang *et al.*, 2005). Subsequently, disease outbreaks occurred in both farmed and wild abalone populations in Australia in all age classes of *H. rubra*, *H. laevigata*, and their hybrids (Hooper *et al.*, 2007). AbHV-1 is also suspected to be the aetiological agent of an epizootic disease that devastated the abalone aquaculture industry in southeastern China (People’s Rep. of) starting in 1999 and continuing through the early 2000s (Gu *et al.*, 2019; Wei *et al.*, 2018; Wu & Zhang, 2016). Interestingly, New Zealand pāua (*H. iris*) was highly resistant to experimental infection (Corbeil *et al.*, 2017).

d. 2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with AbHV-4 according to Chapter 1.5. of the *Aquatic Animal Health Code (Aquatic Code)* are: ~~small abalone (*Haliotis diversicolor*), Greenlip abalone (*Haliotis laevis*), Blacklip abalone (*Haliotis rubra*) and hybrids of Greenlip × Blacklip abalone (*Haliotis laevis* × *Haliotis rubra*).~~

<u>Family</u>	<u>Scientific name</u>	<u>Common name</u>
<u>Haliotidae</u>	<u><i>Haliotis diversicolor</i></u>	<u>small abalone</u>
	<u><i>Haliotis laevis</i></u>	<u>greenlip abalone</u>
	<u><i>Haliotis rubra</i></u>	<u>blacklip abalone</u>
	<u><i>Haliotis laevis</i> × <i>H. rubra</i></u>	<u>hybrid of greenlip × blacklip abalone</u>

e. 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 AbHV-4 according to Chapter 1.5 of the *Aquatic Code* are: ~~none Japanese abalone (*Haliotis discus*) and Rainbow abalone (*Haliotis iris*).~~

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: ~~none.~~

<u>Family</u>	<u>Scientific name</u>	<u>Common name</u>
<u>Haliotidae</u>	<u><i>Haliotis discus</i></u>	<u>Japanese abalone</u>
	<u><i>Haliotis iris</i></u>	<u>rainbow abalone</u>

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

All age classes of *H. diversicolor*, *H. rubra*, *H. laevis*, and hybrids of *H. rubra* × *H. laevis* appear to be highly susceptible to disease (Corbeil 2020; Gu *et al.*, 2019).

g. 2.2.4. Distribution of the pathogen in the host

The major histopathological lesion identified in abalone affected with AVG is ganglioneuritis: inflammation confined to neural tissue. The cerebral, pleuropedal and buccal ganglia can be affected as well as the cerebral commissure and associated peripheral nerves (Bai *et al.*, 2019a; Chang & Handler, 2022; Hooper *et al.*, 2007). The Chinese variant is also able to infect and replicate in haemocytes of *H. diversicolor* (Bai *et al.*, 2020)

h. 2.2.5. Aquatic animal reservoirs of infection

No information available.

i. 2.2.6. Vectors

No information available.

2.3. Disease pattern

Outbreaks of AVG in both farmed and wild abalone populations in Australia are associated with the rapid onset of high mortality rates (up to 90%) in all age classes (Corbeil *et al.*, 2010). Similarly, in Chinese Taipei, during the epizootic in cultured abalone (the water temperature was 16–19°C), both adult and juvenile abalone suffered from the disease, with cumulative mortalities of 70–80%. It was reported that death of all of the abalone in a pond could occur within 3 days of the onset of clinical signs (Chang *et al.*, 2005). A similar disease pattern occurred with experimental infections (Chang *et al.*, 2005; Crane *et al.*, 2009).

j. 2.3.1. Mortality, morbidity and prevalence

In on-farm epizootics in Australia cumulative mortality in all age classes can reach >90%. In experimental trials, 100% mortality can occur within 5 days post-exposure. Most abalone that display gross signs are likely to die within 1–2 days.

In Australia, and similarly in Chinese Taipei, an outbreak of AVG is associated with a rapid rise in mortality rate (up to 90% or more). Affected abalone demonstrating clinical signs (e.g. curling of the foot) are likely to die within 1 day of showing these signs. Ganglioneuritis is observed in sections of neural tissue by light microscopy and confirmation of the presence of AbHV-4 is obtained by real-time PCR or *in-situ* hybridisation (Crane *et al.*, 2016). The precise prevalence of AVG in wild abalone populations in Australian waters is

unknown. The first epidemiological study undertaken in China (People's Rep. of), using real-time PCR (Gu *et al.*, 2019), revealed a detection rate of 27–30% in abalone (*H. diversicolor* and *H. discus hannai*) farms with both healthy and diseased abalone.

k. 2.3.2. Clinical signs, including behavioural changes

AVG outbreaks in both farmed and wild abalone were associated with high mortality rates (up to 90% on farm). Clinically, abalone may demonstrate one or more of the following signs: irregular peripheral concave elevation of the foot; swollen and protruding mouth parts; eversion of the radula; minimal movement of the pedal muscle; excessive mucus production; absence of the marked extension of the foot shown in the righting reflex when healthy abalone are turned onto their backs; reduced pedal adhesion to the substrate. In Tasmania, abalone affected by AVG in processing plants exhibited 'hard foot' or tetany, excessive mucus production, abnormal spawning and 'bloating' (Ellard *et al.*, 2009). These facilities also experienced much lower morbidity and mortality rates than reported on farms or in wild abalone in Victoria, Australia. Similar signs have been reported for an abalone disease epizootic in Chinese Taipei (Chang *et al.*, 2005).

AVG is normally an acute disease, with abalone dying within 1–2 days of demonstrating gross signs of the disease. Wild harvested abalone held in live-holding facilities in Tasmania have previously exhibited slower onset of clinical signs and mortality. Some Tasmanian wild caught abalone have previously tested positive for AVG using real-time PCR without overt clinical or histological signs.

l. 2.3.3 Gross pathology

Abalone that are loosely attached to the substrate owing to weakness or abnormalities of the pedal muscle should be selected for sampling. If this gross pathology is caused by acute AVG, it is likely that these abalone will die within 1–2 days.

m. 2.3.4. Modes of transmission and life cycle

Horizontal transmission (Bai *et al.*, 2019a; Chang *et al.*, 2005; Crane *et al.*, 2009) has been demonstrated experimentally by:

1. exposing healthy abalone to water containing diseased abalone in the same tank without direct contact between the diseased and healthy abalone;
2. placing healthy abalone in water that was previously inhabited by diseased abalone.

In all cases, 100% mortality was observed with a preclinical period of 1–2 days following exposure and then mortality commenced until 100% mortality occurred within 2–5 days post-infection.

n. 2.3.5. Environmental factors

In Australia, the initial outbreak of AVG occurred on a farm during summer 2005/2006 and subsequently appeared to spread to wild populations, which experienced mortality throughout the following year i.e. during all seasons. All experimental infections to date have been carried out in the temperature range 15–18°C. In Chinese Taipei, during the reported epizootic, the water temperature was 16–19°C, and experimental infections were carried out at 17–20°C. In China (People's Rep. of), natural infections were only detected at water temperatures below 23°C (Gu *et al.*, 2019). How temperature affects viral replication and onset of disease has yet to be determined. The possible effects of changes in other environmental factors such as salinity and dissolved oxygen are unknown.

o. 2.3.6. Geographical distribution

Reported in Asia-Pacific.

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

2.4. Biosecurity and disease control strategies

p. 2.4.1. Vaccination

None.

q. 2.4.2. Chemotherapy including blocking agents

No data available.

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- r. 2.4.3. Immunostimulation
No data available.
 - s. 2.4.4. Breeding resistant strains
No data available.
 - t. 2.4.5. Inactivation methods
AbHV-1 was inactivated by treatment with 50 ppm of the iodophor Buffodine® as well as a 1% solution of the non-ionic surfactant Impress®. Calcium hypochlorite (1.5 ppm) treatment also inactivated the virus (Corbeil *et al.*, 2012b).
 - u. 2.4.6. Disinfection of eggs and larvae
No data available.
 - v. 2.4.7. General husbandry
To date, experimental data indicates that AbHV-1 is highly virulent. Practices that could be implemented to reduce the severity of the disease have not been identified. It is interesting to note that, in contrast to the situation in Victoria, Australia, clinical disease has not been reported in wild abalone populations in Tasmania, Australia. Disease outbreaks in processing plants in Tasmania suggest that stress factors may influence expression of subclinical infection.

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

At the first signs of increased numbers of abalone appearing to be weak or behaving abnormally, or sudden onsets of unexplained mortality, live moribund individuals should be selected for sampling. If moribund or freshly dead abalone are not available, samples of overtly normal abalone from all parts of the farm, and representing all age classes, should be selected for sampling.

3.2. Selection of organs or tissues

Neural tissue that includes the cerebral, pleuropedal and buccal ganglia.

3.3. Samples or tissues not suitable for pathogen detection

To date, lesions have not been detected consistently in non-neural tissues.

3.4. Non-lethal sampling

Not available.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.4.0 *General information (diseases of molluscs)*.

w. 3.5.1. Samples for pathogen isolation

The 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.

x. 3.5.2. Preservation of samples for molecular detection

Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.

Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.5.5 of Chapter 2.4.0 *General information* (diseases of molluscs).

y. 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.5.3 of Chapter 2.4.0 *General information* (diseases of molluscs).

z. 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 is only 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 specimens should be processed and tested individually. Small life stages such as larvae can be pooled to obtain the minimum amount of material for molecular detection.

4. Diagnostic methods

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

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

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

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

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

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

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

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

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

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

4.1. Wet mounts

Not applicable.

4.2. Electron microscopy/cytopathology

Transmission electron microscopy is not a routine diagnostic method but can be used to confirm the presence of viral particles in infected ganglia. AbHV-1 particles are icosahedral with electron dense cores and a diameter of 100–110 nm. The intranuclear location of the particles and their ultrastructure are characteristic of members of the *Herpesviridae* (Tan *et al.*, 2008).

Tissue samples (containing pleuropedal ganglion) for examination by electron microscopy should be fixed using 2.5% (v/v) glutaraldehyde and 2–4% (v/v) paraformaldehyde in 0.1 M cacodylate buffer and post-fixed in 1% (w/v) osmium tetroxide, washed in reverse osmosis water (3 × 5 minutes), dehydrated in a graded series of ‘analytical grade’ ethanol (70%, overnight at 4°C; 95%, 20 minutes; 100%, 3 × 20 minutes), infiltrated in 100% Spurr’s resin (overnight) and then embedded in Spurr’s resin.

4.3. Histopathology

Neural tissue (cerebral, pleuropedal and buccal ganglia, branches of the pedal nerve and peripheral nerves) is the prime target and should be sampled, fixed (using 10% formalin) and processed using standard procedures, and stained with haematoxylin and eosin for histological examination as specified in Chapter 2.4.0.

Abalone affected with AVG demonstrate inflammation (increased infiltration by haemocytes) and necrosis confined to neural tissue (cerebral, pleuropedal and buccal ganglia, branches of the pedal nerve and peripheral nerves) as observed in histological sections of neural tissue stained with haematoxylin and eosin and examined by light microscopy (Chang & Handlinger, 2022; Ellard *et al.*, 2009; Hooper *et al.*, 2007).

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section B.5.5 *Molecular methods* of Chapter 2.4.0 *General information* (diseases of molluscs). An 18S rDNA real-time PCR can be used to validate nucleic acid extraction and integrity, and the absence of PCR inhibitors (Crane *et al.*, 2016). Each sample should be tested in duplicate.

Extraction of nucleic acids

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

I. 4.4.1. Real-time PCR

Following validation of the real-time PCR test targeted to ORF49 (Corbeil *et al.*, 2010), the discovery of genotypic variants in Australia not recognised by this test necessitated other real-time PCR tests to be developed based on more conserved regions of the viral genome. Real-time PCR tests targeted to ORF49 and ORF66 have been used extensively in disease investigations and the accumulated data have been used in test validation (Caraguel *et al.*, 2019). For the detection of all genetic variants, the ORF49 and ORF66 real-time PCR tests should be run in parallel, and infection with AbHV can be confirmed by a positive result from either of the two tests. Each of these tests can be multiplexed with an 18S rDNA real-time PCR test, used to validate nucleic acid extraction and integrity, and the absence of PCR inhibitors (Crane *et al.*, 2016).

Primers and probes (sequences)

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters ^(a)
Crane <i>et al.</i> , 2016; GenBank Accession No.: MW412419.1			
AbHV ORF49	ORF49F1: AAC-CCA-CAC-CCA-ATT-TTT-GA ORF49R1: CCC-AAG-GCA-AGT-TTG-TTG-TT 49Prb1: 6FAM-CCG-CTT-TCA-ATC-TGA-TCC-GTG-G-TAMRA	300 nM 300 nM 100 nM	50 cycles of: 95°C/3 sec and 62°C/30 sec
Crane <i>et al.</i> , 2016; GenBank Accession No.: MW412419.1			

AbHV ORF66	ORF66F1: TCC-CGG-ACA-CCA-GTA-AGA-AC ORF66R1: CAA-GGC-TGC-TAT-GCG-TAT-GA 66Prb1: 6FAM-TGG-CCG-TCG-AGA-TGT-CCA-TG-TAMRA	300 nM 300 nM 100 nM	50 cycles of: 95°C/3 sec and 60°C/30 sec
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^(a)A denaturation step prior to cycling has not been included.

m. 4.4.2. Conventional PCR

Conventional PCR may also be used for detection of AbHV-4 in tissue samples. Nucleic acid is extracted as described above. The AbHV1617 PCR has been shown to generate amplicons of various length (522bp to 588bp) depending on the AbHV-4 isolate. Thus, it is potentially useful for epidemiological studies and to confirm positive real-time PCR results (Crane *et al.*, 2016). A second PCR targeting the Taiwanese AbHV-4 DNA polymerase gene has also been developed (Chen *et al.*, 2012). The primer sequences for the two tests are detailed below.

Primer sequences

Pathogen / target gene	Primer (5'–3')	Concentration	Cycling parameters ^(a)
Method 1: Crane <i>et al.</i> , 2016; GenBank Accession No.: MW412419.1 amplicon size: 522–588 bp (depending on genetic variant)			
AbHV	AbHV-16: GGC-TCG-TTC-GGT-CGT-AGA-ATG AbHV-17: TCA-GCG-TGT-ACA-GAT-CCA-TGT-C	360 nM 360 nM	40 cycles of: 94°C/30 sec and 52°C/30 sec
Method 2: Chen <i>et al.</i> , 2012; GenBank Accession No.: HQ317456; amplicon size: 606 bp			
AbHV	40f: TCC-ATC-GAG-ATT-CCC-AGT-TC 146r: ACG-CCA-CCC-TGT-ATA-ACG-AG	400 nM 400 nM	35 cycles of: 94°C/60 sec and 52°C/60 sec

^(a)A denaturation step prior to cycling has not been included.

n. 4.4.3. Other nucleic acid amplification methods

A loop-mediated isothermal amplification assay for rapid and sensitive detection of AbHV-4 has been developed that is 100-fold more sensitive than conventional PCR (Chen *et al.*, 2014) but is not widely used because of false positive and false negative results.

4.5. Amplicon sequencing

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

4.6. *In-situ* hybridisation

In-situ hybridisation localises AbHV-4-infected cells within the neural tissue which, on histological examination, demonstrates ganglioneuritis typified by an inflammatory change with increased cellularity involving mainly haemocytes and glial cells, and cell necrosis in the affected nerves (Mohammad *et al.*, 2011).

The *in-situ* hybridisation (ISH) procedure uses a digoxigenin (DIG)-labelled DNA probe to detect AbHV-4 in formalin-fixed, paraffin-embedded (FFPE) tissue sections and is described in Crane *et al.* (2016).

4.7. Immunohistochemistry

Not applicable.

4.8. Bioassay

A bioassay is not normally required for routine diagnosis. However, when there is a suspect case due to the presence clinical signs and/or histopathology but molecular tests yield negative results, a bioassay (Corbeil *et al.*, 2012a) can be used for confirmation of the presence of a previously unknown genetic variant. Homogenised and clarified neural tissue is used as inoculum and injected (i.m.) into the foot of known uninfected susceptible abalone host species. The inoculated abalone are monitored for clinical signs such as loss of adhesion to the substrate and then samples taken

for histology, molecular analyses and electron microscopy. If presence of a herpesvirus is confirmed by electron microscopy further investigation such as whole genome sequencing should be initiated.

4.9. Antibody- or antigen-based detection methods

None currently available.

4.10. Other methods

None.

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

The real-time PCR assays targeting ORF49 and ORF66 performed in parallel is recommended for surveillance to demonstrate freedom in apparently health populations (Caraguel *et al.*, 2019).

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. If a Competent Authority does not have the capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOH Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status⁷

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.

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

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

- i) Positive result by a real-time PCR
- ii) Positive result by conventional PCR

Histopathological changes consistent with the presence of the pathogen

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of Infection with abalone herpesvirus is considered to be confirmed if one of the following criteria are met:

- i) Positive results by real-time PCR and positive result by conventional PCR followed by sequence analysis of the amplicon
- ii) Positive results by *in-situ* hybridisation and positive result by conventional PCR followed by sequence analysis of the amplicon

6.2 Clinically affected animals

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

⁷ For example transboundary commodities.

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

The presence of infection with abalone herpesvirus 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 a real-time PCR
- iii) Positive result by conventional PCR
- iv) Histopathological changes consistent with the presence of the pathogen or the disease
- v) Positive result of a bioassay

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

The presence of infection with abalone herpesvirus is considered to be confirmed if one of the following criteria are met:

- i) Positive results by real-time PCR and by conventional PCR followed by sequence analysis of the amplicon
- ii) Positive results by *in-situ* hybridisation and by conventional PCR followed by sequence analysis of the amplicon

r. 6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with abalone herpesvirus are provided in Tables 6.3.1. and 6.3.2. This information can be used for the design of surveys for infection with abalone herpesvirus, 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.

s. 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 abalone from the wild and processing plants	Pleuropedal ganglion or pedal nerve cords	<i>Haliotis rubra</i>	100 (48)	100 (48)	Histopathology	Corbeil <i>et al.</i> , 2010
Conventional PCR								
Histopathology								

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

t. 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	Surveillance	Naturally AbHV-1 infected wild and farmed populations; AbHV-1 free populations	Pleuropedal ganglion or pedal nerve cords	<i>Haliotis laevigata</i> ; <i>H. rubra</i> ; <i>H. laevigata</i> x <i>H. rubra</i> hybrids	90.1 (1452)	97.7 (1452)	Histopathology	Caraguel <i>et al.</i> , 2019
Histopathology	Surveillance	Naturally AbHV-1 infected wild	Pleuropedal ganglion or pedal nerve cords	<i>Haliotis laevigata</i> ; <i>H. rubra</i> ;	6.3 (1452)	100 (1452)	real-time PCR	Caraguel <i>et al.</i> , 2019

		and farmed populations; AbHV-1 free populations		<i>H. laevigata</i> x <i>H. rubra</i> hybrids				
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DSe = diagnostic sensitivity, DSp = diagnostic specificity, n = ~~samples~~ number of animals used in the validation study, PCR: = polymerase chain reaction.

7. References

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NB: There is a WOA Reference Laboratory for infection with abalone herpesvirus
(please consult the WOA web site:

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

Please contact WOA Reference Laboratories for any further information on infection with abalone herpesvirus

NB: FIRST ADOPTED IN 2012.

CHAPTER 2.4.4.

INFECTION WITH *MARTEILIA REFRINGENS*

1. Scope

Infection with *Marteilia refringens* means infection with the pathogenic agent *M. refringens* (including O and M types) of the Family *Marteiliidae*.

2. Disease information

2.1. Agent factors

a. 2.1.1. Aetiological agent

Marteilia refringens is a protozoan parasite of the Family *Marteiliidae* (Cavalier-Smith & Chao, 2003; Feist *et al.*, 2009) infecting the digestive system of several bivalve species and inducing physiological disorders and eventual death of the animal (Alderman, 1979; Grizel *et al.*, 1974). Two types of *M. refringens* (Grizel *et al.*, 1974), types O and M, were defined by Le Roux *et al.* (2001). Although more recent results suggest that *M. refringens* should be distinguished from *M. pararefringens* (previously *M. maurini* or *M. refringens* type M) (Kerr *et al.*, 2018), a larger set of samples is required to properly define both species and most available data in the literature do not allow differentiation of *M. refringens* type O (= *M. refringens* in Kerr *et al.*, 2018) or *M. refringens* type M (= *M. pararefringens* in Kerr *et al.*, 2018) to be made.

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

No information available

c. 2.1.3. Survival and stability outside the host

After its release from the European flat oyster (*Ostrea edulis*), *M. refringens* can survive at least 20 days in seawater and faeces. Parasite survival seems improved in faeces compared with seawater (Mérout *et al.*, 2022).

2.2. Host factors

d. 2.2.1. Susceptible host species

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

<u>Family</u>	<u>Scientific name</u>	<u>Common name</u>
<u>Ostreidae</u>	<u><i>Ostrea edulis</i></u>	<u>European flat oyster</u>
	<u><i>Ostrea stentina</i></u>	<u>dwarf oyster</u>
<u>Mytilidae</u>	<u><i>Mytilus edulis</i></u>	<u>blue mussel</u>
	<u><i>Mytilus galloprovincialis</i></u>	<u>Mediterranean mussel</u>
	<u><i>Xenostrobus securis</i></u>	<u>golden mussel</u>
<u>Solenidae</u>	<u><i>Solen marginatus</i></u>	<u>European razor clam</u>
<u>Veneridae</u>	<u><i>Chamelea gallina</i></u>	<u>striped venus clam</u>

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

e. 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 *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*).

Family	Scientific name	Common name
<u>Acartiidae</u>	<u><i>Paracartia latisetosa</i></u>	<u>no common name</u>
<u>Ostreidae</u>	<u><i>Ostrea chilensis</i></u>	<u>Chilean flat oyster</u>
	<u><i>Ostrea denselamellosa</i></u>	<u>Japanese flat oyster</u>

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*).

Family	Scientific name	Common name
<u>Acartiidae</u>	<u><i>Acartia discaudata</i></u>	<u>no common name</u>
<u>Achidiidae</u>	<u><i>Euterpina acutifrons</i></u>	<u>no common name</u>
<u>Centropagidae</u>	<u><i>Centropages typicus</i></u>	<u>no common name</u>
<u>Oithonidae</u>	<u><i>Oithona</i> sp.</u>	<u>no common name</u>
<u>Ostreidae</u>	<u><i>Magallana</i> [syn. <i>Crassostrea</i>] <i>gigas</i></u>	<u>Pacific cupped oyster</u>
	<u><i>Crassostrea corteziensis</i></u>	<u>Cortez oyster</u>
<u>Sididae</u>	<u><i>Penilia avirostris</i></u>	<u>no common name</u>
<u>Veneridae</u>	<u><i>Ruditapes decussatus</i></u>	<u>grooved carpet shell</u>

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

Marteilia refringens usually causes clinical infection in the European flat oyster, *O. edulis* (Berthe *et al.*, 2004; Grizel *et al.*, 1974). In flat oysters and mussels, prevalence and infection intensity are generally higher in individuals 2 years old or older (Audemard *et al.*, 2001; Villalba *et al.*, 1993b).

g. 2.2.4. Distribution of the pathogen in the host

Marteilia refringens infects the digestive tract. Young plasmodia are mainly found in the epithelium of labial palps, oesophagus and the stomach (Grizel *et al.*, 1974). Sporulation takes place in the digestive gland tubules and ducts. Propagules are released into the lumen of the digestive tract and shed into the environment in faeces (Audemard *et al.*, 2002; Berthe *et al.*, 2004; Mérou *et al.*, 2022).

h. 2.2.5. Aquatic animal reservoirs of infection

Infected flat oysters, *O. edulis*, and mussels, *Mytilus edulis* and *M. galloprovincialis*, might not exhibit clinical signs or mortality, however they can release parasite sporangiospores (Arzul *et al.*, 2014; Mérou *et al.*, 2023).

i. 2.2.6. Vectors

None known.

2.3. Disease pattern

j. 2.3.1. Mortality, morbidity and prevalence

Infection is lethal for oysters: a 50–90% mortality rate is usually reported during summer and autumn and is associated with sporulation of the parasite (Grizel, 1985; Grizel *et al.*, 1974). Similarly, morbidity is higher during warmer periods. Mussels are less affected by infection but mortalities up to 40% were reported in impacted areas (Berthe *et al.*, 2004; Villalba *et al.*, 1993b) and naïve mussels presented 100% mortality after being cultured for 6 months in an infected area (Thébault *et al.*, 1999).

Prevalence is highly variable – up to 98% in *O. edulis*. Higher prevalence is expected depending on farming practices and in areas where potential hosts have had more than 1 year of exposure to infection (Berthe *et al.*, 2004; Grizel, 1985). Prevalence usually peaks in summer whereas the parasite is usually absent or found at lower infection intensity in winter and early spring (Audemard *et al.*, 2001; Mérou *et al.*, 2023). An

additional prevalence peak in spring has been reported in several studies (Arzul *et al.*, 2014; Boyer *et al.*, 2013; Carrasco *et al.*, 2007; Mérout *et al.*, 2023).

k. 2.3.2. Clinical signs, including behavioural changes

Clinical signs include dead or gaping molluscs (Grizel, 1985; Grizel *et al.*, 1974) but are not specific for infection with *M. refringens* and could be indicative of other infections.

l. 2.3.3 Gross pathology

Pale digestive gland, thin watery flesh, mantle retraction and reduced growth rate were reported for infected flat oysters (Berthe *et al.*, 2004; Grizel, 1985; Grizel *et al.*, 1974), although these gross signs are not specific for infection with *M. refringens*. Reduced growth rate and inhibition of gonad development were reported for infected mussels (Villalba *et al.*, 1993a).

m. 2.3.4. Modes of transmission and life cycle

Horizontal transmission of *M. refringens* occurs, probably via an intermediate host (Audemard *et al.*, 2002; Carrasco *et al.*, 2008b). The parasite could be experimentally transmitted from *O. edulis* and *M. galloprovincialis* to the copepod *Paracartia grani* (Audemard *et al.*, 2002; Carrasco *et al.*, 2008b). Transmission from *P. grani* to *O. edulis* or *M. galloprovincialis* has not been demonstrated experimentally (Audemard *et al.*, 2002; Carrasco *et al.*, 2008b). In oysters, the early stages of disease occur in the oesophagus, stomach, palps and even gill epithelia. It is thought that initial infection occurs via feeding currents. In mussels, the early stages have been observed in the epithelium of the gills, mantle, stomach and primary digestive tubules (Carrasco *et al.*, 2008a).

The life cycle of *M. refringens* is suspected to be indirect and may include *P. grani* (Audemard *et al.*, 2001; 2002), at least in pond systems. Other species (see Sections 2.2.5 and 2.2.6) might be involved as reservoirs or vectors in the *M. refringens* life cycle but their role has not been demonstrated).

The detection of *M. refringens* DNA in plankton, particularly nanoplankton, and in the benthos, suggests their involvement in the parasite life-cycle including transmission and storage or possible overwintering, respectively (Mérout *et al.*, 2023).

n. 2.3.5. Environmental factors

The threshold temperature for parasite sporulation and transmission is 17°C. This temperature is common in estuaries or bays where prevalence is usually higher in the upper parts of the water column (Audemard *et al.*, 2001; Berthe *et al.*, 2004; Carrasco *et al.*, 2007; Grizel, 1985). Infection with *M. refringens* is seldom observed in open sea waters (Grizel, 1985). High salinity and water renewal could be detrimental to *M. refringens* development and transmission, although these parameters appear to be less significant than temperature (Audemard *et al.*, 2001).

Parasite DNA detection in pelagic compartments was found higher when temperature, salinity and chlorophyll-a were higher (Mérout *et al.*, 2023).

o. 2.3.6. Geographical distribution

Reported in Europe and North Africa.

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

2.4. Biosecurity and disease control strategies

p. 2.4.1. Vaccination

None.

q. 2.4.2. Chemotherapy including blocking agents

None.

r. 2.4.3. Immunostimulation

None.

s. 2.4.4. Breeding resistant strains

None.

t. 2.4.5. Inactivation methods

No data available.

u. 2.4.6. Disinfection of eggs and larvae

No data available.

v. 2.4.7. General husbandry

Stocking at low density or in association with resistant mollusc species, such as *Crassostrea gigas*, has been shown to be effective (Grizel, 1985). Stocking bivalves in deep zones exposed to currents seems to limit the transmission of the parasite. Considering the possible presence of the parasite in the sediment (Mérout *et al.*, 2023), maintaining bivalves at distance from the bottom should limit the number of infected animals.

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

Gaping or freshly dead individuals (2 or more years old) of species referred to in Section 2.2.1., should be sampled preferentially, to increase the chances of finding infected bivalves. For histology, only live (including moribund) bivalves should be sampled.

Sampling of bivalves should be organised when prevalence is known to be at a maximum. When such data are not available in a particular ecosystem, sampling should preferably be carried out when temperature reaches the yearly maximum (Audemard *et al.*, 2001; Carrasco *et al.*, 2007).

3.2. Selection of organs or tissues

A 3–5 mm thick section of tissues including gills and digestive mass is used for diagnosis of *M. refringens* infection by histology and PCR. A piece of digestive gland is preferred for imprints.

3.3. Samples or tissues not suitable for pathogen detection

Tissues other than gills and digestive mass are not suitable.

3.4. Non-lethal sampling

Examination of fresh samples of faeces collected from potentially infected bivalves using light microscopy is possible although this approach has not been validated (See Section 4.1)

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.4.0 *General information (diseases of molluscs)*.

w. 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.

x. 3.5.2. Preservation of samples for molecular detection

Tissue samples for PCR testing should be preserved in 80% (v/v) analytical-grade ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower

grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.

Standard sample collection, preservation and processing methods for histological techniques can be found in Section B.5.5 of Chapter 2.4.0 *General information* (diseases of molluscs).

y. 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.5.3 of Chapter 2.4.0 *General information* (diseases of molluscs).

z. 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 is only 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 specimens should be processed and tested individually. Small life stages such as spat can be pooled to obtain the minimum amount of material for molecular detection.

Performances of diagnostic methods applied on pools have not been evaluated.

4. Diagnostic methods

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

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

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

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

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

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

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV
Wet mounts						+	+	NA				
Tissue imprints		++	++	NA		+++	+++	NA				
Histopathology		++	++	2		+++	+++	NA				
Transmission electron microscopy					+	++	++	NA	+	++	++	NA
Real-time PCR	+++	+++	+++	3	+++	+++	+++	NA	+++	+++	+++	NA
Conventional PCR	++	++	++	2	+++	+++	+++	NA				
Conventional PCR followed by amplicon sequencing									+++	+++	+++	NA
<i>In-situ</i> hybridisation									+	+++	+++	NA
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 WOAHP Pathway (chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively; IFAT = indirect fluorescent antibody test.

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

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

4.1. Wet mounts

Samples to be taken consist of gaping bivalves oysters/mussels or freshly dead bivalves oysters/mussels.

Squash a piece of digestive gland on a glass slide. Observations are then made at ×400 magnification and can potentially show refringent granules in mature sporangia.

Marteilia species are indicated by the presence of large (9–30 µm) spherical bodies containing thick wall structures.

4.2. Imprints

In moderate and advanced infections, digestive gland imprints are prepared.

Samples to be taken consist of fresh, gaping, or freshly dead bivalves.

After drying tissues on absorbent paper, several imprints are made on a glass slide. Slides are air-dried, fixed and stained using a commercially available blood-staining kit, in accordance with the manufacturer's instructions; fixation can be done using methanol or absolute ethanol. After rinsing in tap water and drying, the slides are mounted with a cover-slip using an appropriate synthetic resin. Slides are observed first at ×200 magnification and then under oil immersion at ×1000 magnification.

The observation of cells with a range in size of 5–8 µm diameter in the early stages of development and up to 30–40 µm during sporulation, may indicate infection with *Marteilia refringens*. The cytoplasm stains basophilic, whereas the nucleus stains eosinophilic. Pale halos around large, strongly stained (refringent) granules and, in larger cells, cell-within-cell arrangements are observed. In advanced stages, eight secondary cells can be observed in the primary cells and four spores in each secondary cell (Berthe *et al.*, 2000; 2004; Grizel *et al.*, 1974).

4.3. Histopathology

Samples to be taken consist of live or moribund bivalves.

Sections of tissues that include gills, digestive gland, mantle and gonad should be fixed for a minimum of 24 hours in a recommended fixative followed by standard processing for histology as described in section 5.3 of Chapter 2.4.0 *General information* (diseases of molluscs). Observations are made at increasing magnifications up to ×1000.

Specificity and sensitivity: values of diagnostic sensitivity and specificity for histology were estimated at 70% and 99%, respectively (Thébault *et al.*, 2005).

The observation of cells ranging in size from 4 to 40 µm may be indicative of infection with *Marteilia refringens*. Young stages (uninucleated primary cells) are mainly found in the apical part of the epithelium of labial palps, stomach and sometimes in the digestive tubules. Sporulation involves divisions of cells within cells and generally takes place in the digestive gland tubules and ducts. Refringent granules appear during sporulation but are not observed in early stages. The cytoplasm stains basophilic, whereas the nucleus stains eosinophilic. The granules can range from deep orange to deep red; *M. refringens* can sometimes be observed in other organs including gill and mantle connective tissues (Carrasco *et al.*, 2015; Grizel *et al.*, 1974).

Marteilia refringens is slightly different from other *Marteilia* species including *M. sydneyi* or *M. octospora*. Recognition criteria are mainly based on the number of secondary and tertiary cells (respectively 8 and 4 for *M. refringens*). Although *M. christenseni* and *Eomarteilia granula* display the same number of secondary and tertiary cells as *M. refringens*, they infect different host species in different geographic zones.

4.4. Transmission electron microscopy

A small-sized piece of digestive gland (1–2 mm) should be fixed in an appropriate fixative for at least 1 hour and then processed as described in Section B.5.4 *Transmission electron microscopy methods* of Chapter 2.4.0 *General information* (diseases of molluscs).

The presence of parasites within the epithelia of the digestive gland or the stomach may be indicative of infection with *Marteilia refringens*. Different parasite stages can be observed (Grizel *et al.*, 1974; Longshaw *et al.*, 2001). The first stage (= primary cell) is uninucleated but is often observed presenting a single secondary cell within it. Secondary cells result from a series of divisions within the primary cells and include eight presporangia. These presporangia

(=secondary cells) divide and contain four-spore primordia (= tertiary cells). Spore primordia cleave internally to produce mature spores. Mature spores consist of three sporoplasms, one inside the other, the outermost one containing haplosporosomes.

4.5. Nucleic acid amplification

Samples to be taken consist of tissues of digestive gland and gills from live or freshly dead molluscs.

PCR assays should always include the controls specified in Section B.5.5 *Molecular methods* of Chapter 2.4.0 *General information* (diseases of molluscs). Molluscs are known to potentially contain substances that can inhibit PCR reactions. It is recommended to check for the presence of PCR inhibitors in DNA extracts to avoid false negative results. In case PCR inhibitors are present, DNA samples can be diluted prior to PCR analyses (a 1/10 dilution resolves most cases of PCR inhibition).

Extraction of nucleic acids

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

u. 4.5.1. Real-time PCR

Two multiplex real-time PCR assays targeting the ITS (internal transcribed spacer) gene have been developed for the specific detection and discrimination of *M. refringens* type O and type M (Carrasco *et al.*, 2017; EURL, 2023).

Additionally, a multiplex real-time PCR assay targeting the 18S gene allows the concomitant detection of *M. refringens* and *Bonamia* spp. parasites (Canier *et al.*, 2020). However, validation tests showed that this PCR assay is less specific and also amplifies *M. cochillia* and to a lesser extent *M. sydneyi*.

Primers and probes (sequences)

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters ^(a)
Method 1: Carrasco <i>et al.</i> (2017); GenBank Accession No.: MH304865.1			
<i>M. refringens</i> types O and M ITS	Fwd Mare-F: YCA-GGC-GAG-TGC-TCT-CGT-T Rev Mare-R: TGA-TCT-GAT-ATT-ATT-CAG-CTG-TTC-GA Probe Mare-O: CCT-TTC-CCC-GAC-GGC (VIC MGB-NFQ) Probe MareM: GCT-TGC-CCT-ACG-GCC (FAM MGB-NFQ)	400 nM 400 nM 80 nM 80 nM	50 cycles of: 95°C/3 sec and 60°C/30 sec
Method 2: EURL (2023); GenBank Accession No.: MH304863.1			
<i>M. refringens</i> types O and M ITS	Fwd TaqMar-F: GTG-TTC-GGC-ACG-GGT-AGT Rev TaqMar-R: TGA-TCT-GAT-ATT-ATT-CAG-CTG-TTC-G TaqProb-O: GCC-CTT-TCC-CCG-ACG-GCC-G (FAM-BHQ-1) TaqProb-M: GCG-CTT-GCC-CTA-CGG-CCG-TGC (HEX-BHQ-1)	100 nM 300 nM 250 nM 250 nM	40 cycles of: 95°C/30 sec and 60°C/1 min
Method 3: Canier <i>et al.</i> (2020); GenBank Accession No.: MH342044.1			
<i>M. refringens</i> Also amplifies <i>M. cochillia</i> and <i>M. sydneyi</i> 18S	Fwd Mar_18S_F: ACG-ATC-AAA-GTG-AGC-TCG-TG Rev Mar_18S_R: CAG-TTC-CCT-CAC-CCC-TGA-T Probe Mar_18S_IN: GCA-TGG-AAT-CGT-GGA-ACG-GG (FAM-BHQ-1)	400 nM 400 nM 300 nM	40 cycles of: 95°C/15 sec and 60°C/1 min

^(a)A denaturation step prior to cycling has not been included.

v. 4.5.2. Conventional PCR

PCR primers are available that target the ITS1 (internal transcribed spacer) region (Le Roux *et al.*, 2001), 18S gene (Le Roux *et al.*, 1999) and the IGS (rDNA intergenic spacer) region (López-Flores *et al.*, 2004).

Primer sequences

Pathogen/ target gene	Primer (5'–3')	Concentration	Cycling parameters ^(a)
Method 1: Le Roux <i>et al.</i> (2001); GenBank Accession No.: MH329403.1; amplicon size 412 bp			
<i>M. refringens</i> types M and O Also amplifies <i>M. cochillia</i> ITS-1	Fwd Pr4 (M2A): CCG-CAC-ACG-TTC-TTC-ACT-CC Rev Pr5 (M3AS): CTC-GCG-AGT-TTC-GAC-AGA-CG	1000 nM 1000 nM	30 cycles of: 95°C/1 min and 55°C/1 min and 72°/1 min
Method 2: Lopez-Flores <i>et al.</i> (2004) (nested PCR) ; GenBank Accession No.: MH356753.1; amplicon size [525bp & 358 bp]			
<i>M. refringens</i> types M and O Also amplifies <i>M. cochillia</i> and possibly other species IGS	PCR1 Fwd MT1: GCC-AAA-GAC-ACG-CCT-CTA-C Rev MT2: AGC-CTT-GAT-CAC-ACG-CTTT	1000 nM 1000 nM	PCR 1 130 cycles of: 95°C/1 min and 55°C/1 min and 72°/1 min
	PCR2 Fwd MT-1B: CGC-CAC-TAC-GAC-CGT-AGC-CT Rev MT-2B: CGA-TCG-AGT-AAG-TGC-ATG-CA	1000 nM 1000 nM	PCR2 25 cycles of: 95°C/30 sec and 60°C/30 sec and 72°/30 sec
Method 3: Le Roux <i>et al.</i> (1999); GenBank Accession No.: MH342044.1; amplicon size [266bp or 700 bp]			
<i>Marteilia</i> spp. amplifies <i>M. refringens</i> types M and O, <i>M. cochillia</i> , and possibly other species 18S	Fwd SS2: CCG-GTG-CCA-GGT-ATA-TCT-CG (Rev SAS1: TTC-GGG-TGG-TCT-TGA-AAG-GC) Or Rev SAS2: CGA-ACG-CAA-ATT-GCG-CAG-GG	1000 nM 1000 nM 1000 nM	30 cycles of: 95°C/1 min and 55°C/1 min and 72°/1 min

^(a)A denaturation step prior to cycling has not been included.

Note: according to the alignment of available sequences
Rev SAS1 primer sequence should be: TTC-GG-TGG-TCT-TGA-AAG-GC

w. 4.5.3. Other nucleic acid amplification methods

A loop-mediated isothermal amplification (LAMP) assay for the detection of *M. refringens* has been developed, but is not validated (Xie *et al.*, 2012).

4.6. Amplicon sequencing

The size of the PCR amplicon is verified by agarose gel electrophoresis and purified by excision from this gel for sequence analysis. Obtained sequences are compared with published sequences.

Sequencing is recommended as one of the final steps for confirmatory diagnostic. Targeted regions are the SSU rDNA (except 18S PCR SS2/SAS1), ITS1 and IGS (intergenic spacer). Although sequences are available in the public gene banks, it is recommended to refer such cases to the appropriate WOA Reference Laboratory.

4.7. *In-situ* hybridisation

Le Roux *et al.* (1999) developed an ISH genus-specific method targeting the 18S gene. This method allows the detection of all currently known *Marteilia* species. It has been validated against histology for the detection of *M. refringens* (Thébault *et al.*, 2005).

Two other ISH assays have been developed, one targeting the ITS1 (internal transcribed spacer) region (Le Roux *et al.*, 2001) and the other targeting the IGS (intergenic spacer) region (Lopez-Flores *et al.*, 2008a; 2008b). These assays allow the detection of *M. refringens* type O and type M.

Samples to be taken consist of live or gaping molluscs.

Technical procedure:

Reference	Pathogen/target gene	ISH probe	Probe size
Le Roux <i>et al.</i> (1999)	<i>Marteilia</i> spp. 18S	Digoxigenin-labelled PCR product obtained with SS2/SAS1 primers	266 bp
Le Roux <i>et al.</i> (2001)	<i>M. refringens</i> types M and O ITS1	Digoxigenin-labelled PCR product obtained with Pr4/Pr5 primers	412 bp
Lopez-Flores <i>et al.</i> (2004)	<i>M. refringens</i> types M and O IGS	Digoxigenin-labelled PCR product obtained with MT-1B/MT-2B primers	358 bp

The first steps follow the recommendations described in Section B.5.5.4. of Chapter 2.4.0 *General information* (diseases of molluscs). For hybridisation, sections are incubated with 100 µl of hybridisation buffer (4 × SSC [standard saline citrate], 50% formamide, 1 × Denhardt's solution, 250 µg ml⁻¹ yeast tRNA, 10% dextran sulphate) containing approx. 10 ng (2 to 5 µl µl of digoxigenin-labelled probe prepared by conventional PCR as described above (section 4.5.2; Le Roux *et al.*, 1999; 2001, Lopez-Flores *et al.*, 2004; 2008a; 2008b). Sections are covered with *in-situ* plastic cover-slips and placed on a heating block at 94°C for 5 minutes. Slides are then cooled on ice for 1 to 5 minutes before overnight hybridisation at 42°C in a humid chamber. Sections are washed twice for 5 minutes in 2 × SSC at room temperature, and once for 10 minutes in 0.4 × SSC at 42°C. The detection steps are performed according to the manufacturer's instructions. The slides are then rinsed with appropriate buffer. The sections are counter-stained with Bismarck Brown Yellow, rinsed in tap water, immersed in 95% and then 100% ethanol, 30 seconds for each, rinsed in Xylene (10–30 seconds), and cover-slips are applied using an appropriate mounting medium.

Positive/negative controls: inclusion of the following controls is compulsory. 1) Infected host positive control; 2) non-specific ISH (18S) on samples as an internal positive control. 3) No probe ISH negative control; 4) Uninfected host negative control. Positive controls are available on request from the WOAHA Reference Laboratory.

4.8. Immunohistochemistry

Not available.

4.9. Bioassay

Not available.

4.10. Antibody- or antigen-based detection methods (ELISA, etc.)

Not currently available or used for diagnostic purposes but monoclonal antibodies have been developed (Berthe *et al.*, 2004). These antibodies did not cross-react with *M. sydneyi*.

4.11. Other methods

None available.

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

Real-time PCR is recommended for targeted surveillance to declare freedom from infection with *M. refringens*.

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. If a Competent Authority does not have the capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAHA Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free.

6.1. Apparently healthy animals or animals of unknown health status⁸

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link 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.

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

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

- i) Positive result by a recommended molecular detection test
- ii) Visual observation of the pathogen by microscopy

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

The presence of infection with *M. refringens* is considered to be confirmed if the following criterion is met:

- i) positive result by real-time PCR and conventional PCR followed by sequence analysis

6.2 Clinically affected animals

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

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

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

- i) Positive result by wet mounts
- ii) Positive result by tissue imprints
- iii) Positive result by histopathology
- iv) Positive result by real-time PCR
- v) Positive result by conventional PCR

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

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

- i) positive result by real-time-PCR and conventional PCR followed by sequence analysis
- ii) positive result by species-specific ISH and conventional PCR followed by sequence analysis
- iii) Positive result of real-time PCR followed by species-specific ISH

bb. 6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *M. refringens* are provided in Tables 6.3.1. (no data are currently available) and 6.3.2. This information can be used for the design of surveys for infection with *M. refringens*, 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.

cc. 6.3.1. For presumptive diagnosis of clinically affected animals [under study]

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

⁸ For example transboundary commodities.

DSe = diagnostic sensitivity, DSp = diagnostic specificity, n = ~~samples-number of animals~~ used in the validation study.
 dd. 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
Histology	Surveillance	Field samples from France and Netherlands, representative of three different levels of prevalence (free, mild, high)	Section of tissues including visceral mass	<u><i>Ostrea edulis</i></u> Flat oysters	70% (200)	99% (200)	<i>In-situ</i> hybridisation (18S probe) Bayesian analyses	Thébault <i>et al.</i> , 2005
<i>In-situ</i> hybridisation (18S probe)	Surveillance	Field samples from France and Netherlands, representative of three different levels of prevalence (free, mild, high)	Section of tissues including visceral mass	<u><i>Ostrea edulis</i></u> Flat oysters	90% (200)	99% (200)	Histology Bayesian analyses	Thébault <i>et al.</i> , 2005
Real-time PCR (Canier <i>et al.</i> , 2020)	Surveillance	Field samples from the three main producing areas in France, representative of three different levels of prevalence (free, low, high)	Gills and digestive gland tissues	<u><i>Ostrea edulis</i></u> Flat oysters	87,2% (386)	98,4% (386)	Conventional PCR (Le Roux <i>et al.</i> , 2001) Bayesian analyses	Canier <i>et al.</i> , 2020
Conventional PCR (Le Roux <i>et al.</i> , 2001)	Surveillance	Field samples from the three main producing areas in France, representative of three different levels of prevalence (free, low, high)	Gills and digestive gland tissues	<u><i>Ostrea edulis</i></u> Flat oysters	60.7% (386)	99.9% (386)	Real-time PCR (Canier <i>et al.</i> , 2020) Bayesian analyses	Canier <i>et al.</i> , 2020

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

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NB: There is a WOA Reference Laboratory for infection with *Marteilia refringens*
(please consult the WOA web site:

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

Please contact WOA Reference Laboratories for any further information on infection with *Marteilia refringens*

NB: FIRST ADOPTED IN 1995 AS MARTEILIOSIS. MOST RECENT UPDATES ADOPTED IN 2012.

CHAPTER 2.4.5.

INFECTION WITH *PERKINSUS MARINUS*

[...]

2.2. Host factors

2.2.1. Susceptible host species

Eastern oyster, *Crassostrea virginica*; Pacific oyster, *C. gigas*; suminoe oyster, *C. ariakensis*; mangrove oyster, *C. rhizophorae*; Cortez oyster, *C. corteziensis* (Andrews 1996; Calvo *et al.*, 1999; Calvo *et al.*, 2001; Villalba *et al.*, 2004; Cáceres-Martínez *et al.*, 2008); softshell clam, *Mya arenaria*; Baltic macoma, *Macoma balthica* (Dungan *et al.*, 2007).

Species that fulfil the criteria for listing as susceptible to infection with *Perkinsus marinus* according to Chapter 1.5. of the *Aquatic Animal Health Code (Aquatic Code)* are: American cupped oyster (*Crassostrea virginica*), Ariake cupped oyster (*Magallana [Syn. Crassostrea] ariakensis*), Cortez oyster (*Crassostrea corteziensis*) and palmate oyster (*Saccostrea palmula*).

Family	Scientific name	Common name
Ostreidae	<i>Crassostrea corteziensis</i>	Cortez oyster
	<i>Crassostrea virginica</i>	American cupped oyster
	<i>Magallana [syn. Crassostrea] ariakensis</i>	Ariake cupped oyster
	<i>Saccostrea palmula</i>	palmate oyster

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

All stages after settlement are susceptible.

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with *P. marinus* according to Chapter 1.5. of the *Aquatic Code* are: Gasar cupped oyster (*Crassostrea tulipa*), mangrove cupped oyster (*Crassostrea rhizophorae*), and Pacific cupped oyster (*Magallana [Syn. Crassostrea] gigas*).

Family	Scientific name	Common name
Ostreidae	<i>Crassostrea gasar</i>	Gasar cupped oyster
	<i>Crassostrea rhizophorae</i>	mangrove cupped oyster
	<i>Magallana [syn. Crassostrea] gigas</i>	Pacific cupped oyster

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but no active infection has been demonstrated: Columbia black oyster (*Crassostrea columbiensis*), soft shell clam (*Mya arenaria*), and stone oyster (*Striostrea prismatica*).

Family	Scientific name	Common name
Myidae	<i>Mya arenaria</i>	soft shell clam
Ostreidae	<i>Crassostrea columbiensis</i>	Columbia black oyster
	<i>Striostrea prismatica</i>	stone oyster

[...]
