

Guideline for Disease Control of Aquatic Animals

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Ministry of Agriculture, Forestry and Fisheries

Table of Contents

| | Page |
|---|----------|
| I. Principles of disease control measures | |
| 1. Purpose | 1 |
| 2. Establishment of Disease Control System and Roles of Stakeholders | 1 |
| 3. Measures Against Emerging Diseases | 14 |
| 4. Measures Against Existing Diseases Other than Specified Diseases..... | 15 |
| Exhibit: Requirements for Storage Facilities in Japan for Live Aquatic Animals for Human Consumption That Require No Import Permission | 16 |
| Appendix 1: Guidelines for Quarantine at the Destination for Imported Aquatic Animals | 17 |
| Appendix 2: Guidelines for Surveillance of Specified Diseases | 22 |
| Appendix 3: Guidelines for Sanitation Measures in Aquaculture Farms..... | 25 |
| Appendix Forms 1 to 11..... | 28 to 40 |
| II. Guidelines for Diagnosis | |
| 1. Finfish | |
| (1) Viral Hemorrhagic Septicemia (Excluding type IVa) (VHS)..... | 43 |
| (2) Infection with Salmonid Alphavirus | 47 |
| (3) Epizootic Haematopoietic Necrosis (EHN) | 50 |
| (4) Piscirickettsiosis | 54 |
| (5) Enteric Redmouth Disease..... | 57 |
| (6) Whirling Disease | 60 |
| (7) Spring Viremia of Carp (SVC) | 64 |
| (8) Koi Herpesvirus Disease (KHVD)..... | 70 |
| (9) Glugeosis of Red Sea Bream | 76 |
| 2. Crustaceans | |
| (1) Yellow Head Disease | 80 |
| (2) Necrotising Hepatopancreatitis (NHP) | 83 |

| | |
|--|-----|
| (3) Taura Syndrome (TS) | 86 |
| (4) Infectious Hypodermal and Haematopoietic Necrosis (IHHN) | 89 |
| (5) Acute Hepatopancreatic Necrosis Disease (AHPND) | 92 |
| (6) Infectious Myonecrosis (IMN) | 95 |
| (7) Tetrahedral Baculovirus | 98 |
| (8) Covert Mortality Disease of Shrimp (CMD)..... | 101 |
| (9) Gill-Associated Virus Disease (GAV)..... | 104 |
| (10) Spherical Baculovirus | 107 |
| 3. Mollusks and others | |
| (1) Infection with Abalone Herpesvirus | 111 |
| (2) Pustule Disease of Abalone/Blister Disease of Abalone..... | 115 |
| (3) Infection with Oysteid Herpesvirus 1 Microvariants (Limited to OsHV-1 μ var) | 118 |
| (4) Infection with Perkinsus Qugwadi..... | 121 |
| (5) Soft Tunic Syndrome..... | 124 |

I. Principles of disease control measures

I. Principles of disease control measures

1. Purpose

As the aquatic animals imported to Japan have been diversifying in recent years, new infectious diseases that have a high risk of causing substantial damage to the aquatic fisheries in Japan have been identified in countries around the world. It is essential for us to establish a system to prevent fishery epidemics that prevents the invasion of these diseases into Japan and, if such a disease occurs, to detect the occurrence at an early stage and minimizes the damage by promptly taking measures to prevent the spread of the disease. For this reason, in August 2014, the Fisheries Disease Control Expert Council was established to collect disease related information more broadly from within and outside Japan and to conduct a risk assessment based on scientific knowledge. On the basis of the risk assessment, in January 2016, the Ordinance for Enforcement of the Act on the Protection of Fishery Resources (Ordinance of the Ministry of Agriculture, Forestry and Fisheries No. 44 of 1952; hereinafter referred to as “Ordinance of the Fishery Resources Act”) and the Ordinance for Enforcement of the Sustainable Aquaculture Production Assurance Act (Ordinance of the Ministry of Agriculture, Forestry and Fisheries No. 31 of 1999; hereinafter referred to as “Ordinance of the Sustainable Aquaculture Production Act”) were amended; the aquatic animals and diseases subject to import quarantine listed in the Table under Paragraph 1, Article 1-2 of the Ordinance of the Fishery Resources Act, the period listed in the Table under Article 1-5 of the Ordinance of the Fishery Resources Act, and the aquatic animals and plants and specified diseases listed in the Table under Article 1 of the Ordinance of the Sustainable Aquaculture Production Act were reviewed, while epidemic prevention at the border and epidemic prevention in Japan were reinforced.

This Guideline shows basic principles concerning what needs to be done by all stakeholders under the adjustment of the government to prevent the occurrence of aquatic animal diseases, and if a disease occurs, to contain the disease by ~~to take~~ taking appropriate measures promptly and accurately.

2. Establishment of Disease Control System and Roles of Relevant (Persons)

Stakeholders

- (1) Collecting and sharing Information related of diseases in and outside Japan among Stakeholders

- (i) Establishment of disease control system and roles of stakeholders

All relevant sectors must cooperate with each other in order to prevent the occurrence of diseases that are subject to import quarantine and specified diseases (hereinafter collectively referred to as “Subject Diseases”), or other important diseases in Japan and to take control measures promptly and accurately when such diseases occur; it is essential that the national government, prefectural governments, prefectural fishery research laboratories

that give diagnostic service or instructions to fish farmers concerning fish health, (hereinafter referred to as “local fisheries research laboratory”), fish farmers (meaning fish farmers who engage in aquaculture of the living aquatic animals that are listed in the Table under Article 1 of the Ordinance of the Sustainable Aquaculture Production Act; including persons who produce juveniles aquatic animals for other aquaculture farms or stock enhancement), and researchers. For this purpose, stakeholders must share information related to the occurrence status of emerging diseases and other diseases in and outside Japan that have a high risk of causing significant damage to the fishery industry, prevention of occurrence and control measures conducted by each country, and other information on a daily basis, must increase awareness of fishery epidemic prevention together, and thereby must prepare for correct epidemic prevention. Therefore, we will develop an emergency network and communication system among stakeholders.

(ii) Roles of stakeholders

- (A) The Ministry of Agriculture, Forestry and Fisheries, Food Safety and Consumer Affairs Bureau, Animal Products Safety Division (hereinafter referred to as “Animal Products Safety Division”) shall collect emergency calls and reports related to the occurrence of diseases reported to OIE. by country, shall request the provision of information from governments of relevant countries as necessary while observing the occurrence status of diseases and control measures in each country, shall organize the details of collected information, and shall share the information with stakeholders (the Fisheries Agency, Animal Quarantine Service, prefectural governments, local fisheries research laboratories, National Research and Development Agency, Japan Fisheries Research and Education Agency (hereinafter collectively referred to as “RIA”), Fisheries Disease Control Expert Council) as necessary.
- (B) The Animal Products Safety Division shall cooperate with prefectural governments, shall widely collect the occurrence status of diseases in Japan and other information related to various diseases, and shall share the necessary information between stakeholders.
- (C) Research institutions (National Research and Development Agency, Japan Fisheries Research and Education Agency, universities with experts of aquatic animal diseases) shall strive to collect scientific knowledge related to various diseases, including emerging diseases in and outside Japan that have a high risk of causing significant damage to the fishery industry, and shall share necessary information with the Animal Products Safety Division.

(2) Epidemic Prevention of Diseases Subject to Import Quarantine at the Border

(i) Direction of control measures

In order to prevent diseases subject to import quarantine that are specified by the Ordinance of the Fishery Resources Act from invading into Japan from overseas, an importer of aquatic animals listed in Paragraph 2, items (i) and (ii), Article 1-2 of the Ordinance of the Fishery Resources Act (hereinafter referred to as “Aquatic Animals Subject to Import Quarantine”) is required to obtain import permission from the Minister of Agriculture, Forestry and Fisheries. For this purpose, the importer shall apply for import permission to the Animal Quarantine Service along with an inspection certificate issued by the governmental organization of the exporting country based on import conditions for which the Animal Products Safety Division agreed with the governmental organization of the exporting country in advance; and the Animal Quarantine Service shall inspect for import Aquatic Animals Subject to Import Quarantine when they arrive in Japan to confirm that there is no possibility that said animals are infected with a disease subject to import quarantine and to confirm that any aetiological agents of diseases subject to import quarantine would not be widely spread and shall take control measures appropriately as needed.

In addition, aquatic animals listed in the Table of Paragraph 1, Article 1-2 of the Ordinance of the Fishery Resources Act that are alive and edible (excluding those stored in “water areas used for public purposes or the facilities that directly drain into them” as indicated in the Appendix) (hereinafter referred to as “Living Edible Aquatic Animals”) are not Aquatic Animals Subject to Import Quarantine; however, water, . used for transportation during import shall be appropriately handled in terms of fishery epidemic prevention so that it will not be directly discarded or drained into the water areas used for public purposes and the residue of said aquatic animals shall not be used for food for aquaculture.

(ii) Roles of stakeholders

- (A) The Animal Products Safety Division shall discuss with the governmental organization of the exporting country, shall agree with import conditions that indicate requirements necessary for exporting Aquatic Animals Subject to Import Quarantine to Japan, and shall notify the Animal Quarantine Service of the inspection certificate format based on the import conditions. In addition, import conditions and inspection certificate format shall be revised as necessary after discussion with the governmental organization of the exporting country.
- (B) The Animal Quarantine Service shall implement import permission procedures for Aquatic Animals Subject to Import Quarantine based on Operation of the Act on the Protection of Fishery Resources (19 Food Safety and Consumer Affairs Bureau No. 3823 as of September 19, 2007) that is specified separately, and shall give administrative orders and conduct detailed examinations. based on the provisions of Paragraph 1, Article 13-3 of the Act on the Protection of Fishery

Resources (Act No. 313 of 1951; hereinafter referred to as “Fishery Resources Act”) as necessary.

- (C) The Animal Products Safety Division shall disseminate to importers of aquatic animals as specified by Paragraph 2, item (i) Article 1-2 of the Ordinance of the Fishery Resources Act that are alive and imported for use in aquaculture (excluding aquatic animals that are directly sold at stores or sold for ornamental purposes after import) (hereinafter referred to as “Aquatic Animals Subject to Quarantine at the Destination”), business entities related to aquaculture, and other business entities (hereinafter collectively referred to as “Importer”) that quarantine at the destination based on Appendix 1: Guidelines for Quarantine at the Destination for Imported Aquatic Animals of this Guideline shall be enacted after granting import permission.

Importers shall cooperate with the prefectural government that has jurisdiction over the Animal Quarantine Service and aquaculture farmer at destination based on Appendix 1 of this Guideline in order to facilitate quarantine at the destination.

- (D) The prefectural government that has jurisdiction over aquaculture farms that are the destinations of Aquatic Animals Subject to Quarantine at the Destination shall conduct quarantine at the destination based on Appendix 1: Guidelines for Quarantining Imported Aquatic Animals of this Guideline for said imported animals. In addition, the Animal Quarantine Service responsible for import permission procedures shall provide reference information for implementing quarantine at the destination to the prefectural government that has jurisdiction over aquaculture farms at the destination based on Appendix 1 of this Guideline.
- (E) The supervisor of an aquaculture farm that becomes a site for quarantine at the destination shall observe the health of the subject aquatic animals and take other measures based on the Appendix 1: Guidelines for Quarantine at the Destination for Imported Aquatic Animals of this Guideline under the instructions of the local fisheries research laboratory which has jurisdiction, and shall cooperate with inspection conducted by the local fisheries research laboratory as necessary.
- (F) The Importer shall collect information related to the occurrence status of diseases in the exporting country on a daily basis and shall pay full attention particularly to the occurrence status of infectious diseases that have not invaded Japan and to the pandemic prevention system of aquaculture farms in the exporting country when importing aquatic animals.
- (G) The Animal Products Safety Division shall instruct Importers of Living Edible Aquatic Animals concerning the treatment of transporting said aquatic animals from arrival in Japan until the destination so that they

do not directly discard or drain said aquatic animals or water used for transport into water areas used for public purposes.

(H) The Animal Products Safety Division shall instruct that Importers do not use the residue of (G) as food for aquaculture.

(3) Preventing Occurrence of Diseases, Including Specified Diseases Based on the Ordinance of the Sustainable Aquaculture Production Act, in Farmed Aquatic Animals and Plants

(i) Direction of control measures

It is important to organize the inspection system to diagnose specified diseases as specified by the Ordinance of the Sustainable Aquaculture Production Act promptly and accurately, to check the disease-free status, and to correctly identify the existence of occurrence and trends of occurrence. For this reason, local fisheries research laboratories shall disseminate and increase awareness of hygiene measures, conduct patrols, and give instructions to aquaculture farmers, and aquaculture farmers shall practice basic hygiene measures on a daily basis.

(ii) Roles of stakeholders

(A) The Animal Products Safety Division shall plan the implementation of surveillance of diseases of farmed aquatic animals and plants, including specified diseases based on Appendix 2: Guidelines for Surveillance of Specified Diseases of this Guideline, shall correctly identify the disease-free status, disease occurrence status, and trends of diseases in Japan, shall provide feedback on the obtained results to stakeholders in Japan, and shall share the occurrence status of diseases with relevant countries through reporting to OIE. In addition, details of the specific surveillance conducted shall be reported separately to each prefectural government and other stakeholders by the Animal Products Safety Division.

(B) The Animal Products Safety Division shall support the early detection of specified diseases, the inspection methods contributing to prompt and accurate control measures, and the development of disinfection.

(C) The local fisheries research laboratories shall cooperate with research institutions and promote activities for prompt and accurate diagnosis of specified diseases.

(D) Prefectural governments shall use the Fish Epidemic Prevention Officers and Fish Epidemic Prevention Assistants System to organize the epidemic prevention system in prefectures mainly by local fisheries research laboratories, shall collect information related to the occurrence of specified diseases, and conduct inspections in cooperation with stakeholders, and shall implement the prevention of occurrence, early detection, and prevention of the spread of diseases by giving instructions on hygiene control and raising awareness of aquaculture

farmers. In particular, concerning aquaculture farms that raise animals and plants subject to specified diseases, prefectural governments shall patrol local sites based on the plan and give instructions on hygiene as necessary.

- (E) Concerning facilities engaging in aquaculture as their business in a prefecture, the prefectural government shall strive to identify as much as possible species of farmed animals and plants, locations, amount of aquatic animals and plants rearing and import history, introduction or transfer from the outside of parent fish, juveniles, and juvenile rings.
- (F) Aquaculture farmers shall practice basic hygiene control based on Appendix 3: Guidelines for Sanitation Measures at Aquaculture Farms on a daily basis, and record and store daily records on rearing management, history of import and introduction or transfer from outside of parent fish, juveniles, and juvenile rings, and their health status.
- (G) Aquaculture farmers shall acquire the latest knowledge related to specified diseases and strive to prevent the occurrence of these diseases.

(4) Early Detection, Report, and Notification of Abnormalities of Farmed Aquatic Animals and Plants, and Prompt and Accurate Prevention of the Spread of Disease when it Occurs

(i) Direction of control measures

When an owner or manager specified by the Ordinance of the Sustainable Aquaculture Production Act of farmed aquatic animals or plants notices or suspects the occurrence of a specified disease, said owner or manager shall notify the prefectural governor of the fact without delay. Upon notification, the prefectural government shall request, as necessary, said owner or manager to refrain from transfer of the animals or plants and shall conduct an inspection of said animals and plants. When there is a risk of spreading of the disease, the prefectural government shall give orders the owner or manager to conduct necessary measures, such as movement restriction, incineration or burying of the animals or plants, or disinfection. (ii) Roles of stakeholders

(A) Detection, reporting, and notification of diseases

- (a) When aquaculture farmers find an abnormality in farmed aquatic animals and plants that they possess or control, they shall immediately consult or notify the prefectural governments or local fisheries research laboratories that have jurisdiction. In addition, when judging “abnormality,” high mortality or continued death, suspicion of a specified disease, or a specific clinical sign that is different from known diseases shall be identified in reference to II. Guidelines for Diagnosis of this Guideline.
- (b) When a local fisheries research laboratory receives a notification of abnormality from an aquaculture farmer, the local fisheries research laboratory shall notify also the Fisheries Cooperative Associations in

order to handle the abnormality promptly, shall strive to remind the stakeholders, and shall interview them on an outline of the occurrence status of abnormalities with farmed aquatic animals and plants (Appendix Form 1). As a temporary measure, the local fisheries research laboratory shall obtain the cooperation of the Fish Epidemic Prevention Assistants and promptly instruct aquaculture farmers to refrain from transfer and give instructions for other measures necessary to prevent the spread of diseases.

(c) When local fisheries research laboratorys judge that the abnormal farmed aquatic animals and plants are infected or suspected to be infected with a specified disease, they shall instruct aquaculture farmers to notify prefectural governments of that fact in writing (Appendix Form 2) or orally based on the provisions of Paragraph 1, Article 7-2 of the Sustainable Aquaculture Production Assurance Act (Act No. 51 of 1999; hereinafter referred to as “Sustainable Aquaculture Production Act”) and shall continue to give instructions necessary to prevent the spread of diseases. When the notification by aquaculture farmers is made orally, the prefectural government that received the notification shall record the notification matters correctly (Appendix Form 3).

(B) On-site inspection at aquaculture farms

(a) When prefectural governments receive consultation, report, or notification from aquaculture farmers, the prefectural governments shall check the outline of the occurrence status of the abnormality (Appendix Form 1) by means of interviews or documents. If they judge that there is a suspicion of specified diseases, they shall promptly dispatch Fish Epidemic Prevention Officers and other persons who have expertise and experience related to fishery epidemic prevention to the local site.

(b) The dispatched Fish Epidemic Prevention Officers shall conduct an on-site inspection at the local site and confirm the health status of said farmed aquatic animals and plants, shall question the aquaculture farmer, shall check observation records and rearing management records of farmed aquatic animals and plants as set forth in Appendix 3: Guidelines for Sanitation Measures at Aquaculture Farms of this Guideline, check necessary materials, including introduction records of parent fishes, juveniles, juvenile rings from outside the farm and other necessary materials, and shall collect samples for conducting detailed examinations of specified diseases as necessary. In this case, if the aquaculture farmer fails to cooperate with the on-site-inspection, the prefectural governor may order said aquaculture farmer to undergo examinations based on Article 7-2, Paragraph 2 of the Sustainable Aquaculture Production Act (Appendix Form 4).

- (c) The prefectural governments shall request the owner or manager to refrain from transfer of said farmed aquatic animals and plants and take other control measures for the period until the results of detailed examination are obtained.
 - (d) Persons who conduct on-site inspections shall include a Fish Epidemic Prevention Officer and other persons who have expertise related to specified diseases and the on-site inspection shall be conducted by multiple persons as much as possible in consideration of quality, quantity, and other matters related to the operation. When conducting an on-site inspection, disinfection and other necessary measures to prevent the spread of aetiological agents shall be conducted thoroughly and actions shall be taken carefully. In addition, the affairs of said on-site inspection correspond to “Dispositions and Administrative Guidance rendered on the scene by police officials, coast guard officers, or other personnel invested expressly by Acts with the authority to safeguard the public interest, where situations implicating a risk to public health, environmental protection, the prevention of epidemics, public safety, and other public interests arise or are likely to arise” as set forth in Paragraph 1, item (xiii), Article 3 of the Administrative Procedure Act (Act No. 88 of 1993). Therefore, provisions of the Administrative Procedure Act are not applied; however, in consideration of the plenary power of the affairs of on-site inspections, when conducting an inspection, the persons who conduct the on-site inspection are required to carry and present personal identification based on Article 10, Paragraph 2 of the Sustainable Aquaculture Production Act and to take actions carefully, such as fully explaining the purpose of the inspection.
 - (e) When the occurrence of a specified disease is confirmed, in order to take measures to prevent the spread of the disease and other measures promptly and accurately, dispatched Fish Epidemic Prevention Officers shall comprehensively consider the water system related to the aquaculture farm where the on-site inspection is conducted and rearing conditions of animals and plants in facilities related to aquaculture in the surrounding area, shall select places that may be contaminated with aetiological agents for the specified disease as epidemiologically related places, and shall promptly check the health status of aquatic animals and conduct on-site inspections and examinations as necessary in cooperation with relevant aquaculture farmers, departments, and bureaus.
- (C) Reporting when an abnormality occurs.
- (a) When a prefectural government receives a notification from an aquaculture farmer that aquatic animals and plants are infected or suspected to be infected with a specified disease, the prefectural government shall promptly report the necessary epidemiological

information (Appendix Form 5) to the Animal Products Safety Division and relevant prefectural governments.

- (b) When it is found that aquatic animals and plants in the aquaculture farm where an abnormality is detected have been transferred to aquaculture farms in other prefectures, the Animal Products Safety Division shall consult with relevant prefectural governments concerning actions for epidemic prevention.

(D) Inspection of Specified Disease

- (a) local fisheries research laboratories shall examine whether the farmed aquatic animals and plants infected with the specified disease or not as an initial diagnosis based on II. Guidelines for Diagnosis of this Guideline. In this case, the RIA shall provide technical support to local fisheries research laboratories as necessary and shall conduct confirmatory diagnosis as to whether the farmed aquatic animals and plants infected with the specified disease or not (however, concerning koi herpesvirus disease (KHV) and soft tunic syndrome, only persons who passed the diagnosis skill test for KHV and soft tunic syndrome conducted by the RIA can conduct a confirmatory diagnosis in lieu of RIA). When a local fisheries research laboratory requests a confirmatory diagnosis from the RIA, samples shall be sent after confirming the sampling method, sampling tissues, the number of samples, storage temperature, and other matters necessary for correct examination, and the necessary information shall be indicated in Appendix Form 6 and attached to the samples. In addition, when sending samples, dissipation shall be thoroughly prevented in full consideration of the possibility of containing the aetiological agent of specified diseases. As soon as the results of the requested examination are obtained, the RIA shall promptly report the results to the prefectural government that requested the examination.
- (b) In cases where it is confirmed positive as a result of (a) above, the prefectural government shall promptly report the examination results to the Animal Products Safety Division and relevant prefectural governments (Appendix Form 7) and shall discuss actions for specific measures to prevent spreading as set forth in (E) through (H) in cooperation with stakeholders.
- (c) In cases where it is confirmed negative as a result of (a) above, the prefectural government shall promptly report the examination results to the Animal Products Safety Division and stakeholders (Appendix Form 8) and shall release measures for epidemic prevention, such as refraining from transfer. When the prefectural government orders inspection of said aquaculture, farmer based on Paragraph 2, Article 7-2 of the Sustainable Aquaculture Production Act, the prefectural government shall issue a certificate of inspection of the governor (Appendix Form No. 1 of the Ordinance of the Sustainable Aquaculture

Production Act) at the request of the aquaculture farmer.

(E) Movement restriction or inhibition

When a prefectural government finds that a specified disease is likely to spread, the prefectural government shall order a restriction or inhibition of movement on farmed aquatic animals and plants to the extent necessary to prevent the spread of the specified disease after consultation with stakeholders, to persons who own or manage farmed aquatic animals and plants that are infected or are suspected to be infected with said specified disease or are likely to be infected with said specified disease (Appendix Form 9). The term “farmed aquatic animals and plants that are likely to be infected with a specified disease” set forth in Article 8, Paragraph 1, item (iii) of the Sustainable Aquaculture Production Act shall refer to farmed aquatic animals and plants that are susceptible to said specified disease and that are located in the region designated by the prefectural government and may be exposed to aetiological agents, such as farmed aquatic animals and plants that are located in the same water system as the neighborhood of the aquaculture farm where the disease occurred.

(F) Inspection, injection, bathing or other medication

The prefectural government shall order measures to be taken to prevent spreading special diseases by conducting effective inspection, injection, bathing or other drug administration to the farmed aquatic animals and plants to the extent necessary to prevent the spread of specified diseases after consultation with stakeholders, to persons who own or manage farmed aquatic animals and plants that are infected with or are likely to be infected with said specified disease based on the provisions of Paragraph 1, Article 9-2 of the Sustainable Aquaculture Production Act. In this case, when an aquaculture farmer with which said measures were taken requests it, a certificate of injection (bathing or other drug administration) (Appendix Form No. 2 of the Ordinance of the Sustainable Aquaculture Production Act) pursuant to the provisions of Article 9-3 of the Sustainable Aquaculture Production Act must be issued.

(G) Slaughter and incineration of dead bodies and contaminated items

A prefectural government shall order the disposal of farmed aquatic animals and plants by incineration, burying, and another method that inactivates the infectious of the specified disease to the extent necessary to prevent the spread of the specified diseases after consultation with stakeholders, to persons who own or manage farmed aquatic animals and plants that are infected or are suspected to be infected with said specified disease. Prefectural governments and local fisheries research laboratories shall fully use the information that they collect on daily basis, shall discuss with the subject aquaculture farmers and shall examine necessary control measures. In particular, concerning “another method capable of

inactivating of the aetiological agent of the specified disease” set forth in Paragraph 1, item (ii), Article 8 of the Sustainable Aquaculture Production Act, this shall be discussed carefully with the Animal Products Safety Division and RIA.

The aquaculture farmer shall voluntarily dispose of farmed aquatic animals and plants by incineration, burying, or by another method capable of inactivating of the aetiological agent of the specified disease at the actual site or the adjacent sites in principle under the supervision of the prefectural government to conduct preventive measures against spreading of the specified disease effectively and accurately. When transporting said farmed aquatic animals and plants to the disposal site, attention shall be paid so that the aetiological agent does not spread, such as using closed containers. In addition, when selecting a disposal site, attention shall be paid to the following points: a) in cases of burying, the relationship with soil quality, groundwater, and water source; b) in cases of incineration, prevention of fire; c) in cases of using chemicals, appropriate use; d) in cases of using another method, ensure inactivating of aetiological agents; and e) treatment as waste.

In addition, in cases of using infected aquatic animals as feed ingredients, Appendix: Precautions for Instructions and Confirmation for Use of Fish Infected with Koi Herpesvirus Disease (KHV) for Raw Materials for Feed of “Implementation of Survey for Use of Fish Infected with Koi Herpesvirus Disease (KHV) for Raw Materials for Feed” (notification of the Ministry of Agriculture, Forestry and Fisheries, Food Safety and Consumer Affairs Bureau, Animal Products Safety Division, 15 Food Safety and Consumer Affairs Bureau No. 3615 as of November 25, 2003) shall be observed. When using them as raw materials for processing other than raw materials for feed, their treatment shall be examined in accordance with said precautions.

(H) Disinfection

When a prefectural government finds that a specified disease is likely to spread, the prefectural government shall order the persons who own or manage the farmed aquatic animals and plants that are infected with or are suspected to be infected with the specified disease or that are likely to be infected with the specified disease to disinfect fishing nets, fish reserves and any other articles listed in items of Article 5 of the Ordinance of the Sustainable Aquaculture Production Act on which the aetiological agent of a specified disease is deposited or is likely to have been deposited, to boil or soak in antiseptic solution as appropriate the apparatuses and clothes that may be exposed to the aetiological agent by paying attention to prevent spread of the aetiological agent, and to disinfect breeding water and is drained from facilities where the diseases occurred in principle.

(I) Precautions for taking measures to prevent spread of specified diseases

When taking measures to prevent the spread of specified diseases, it is

necessary to explain that these measures are necessary for fishery epidemic prevention and to state that appeals pursuant to the Administrative Complaint Review Act (Act No. 68 of 2014) shall not be filed concerning the obligation imposed on the owner. In addition, the order to take measures to prevent the spread of said specified disease shall correspond to the adverse disposition under the Administrative Procedure Act; however, since it corresponds to “when, for the public interest, it is necessary to render Adverse Dispositions urgently, and procedures for statements of opinion prescribed in the preceding paragraph cannot therefore be implemented in a timely manner” as set forth in Paragraph 2, item (i), Article 13 of the Act, it is not required to undergo procedures for statements of opinion. However, in consideration of the fact that the provisions of “Presentation of Grounds for Adverse Dispositions” set forth in Article 14 of the Act are applied and the plenary power of said affairs, it is necessary to implement the measures carefully by fully explaining to the person to whom the order is given, the purpose of the measures beforehand.

(J) Report of control measures

When control measures set forth in (E) through (H) are implemented based on the provisions of Paragraph 2, Article 8 of the Sustainable Aquaculture Production Act (including cases where it is applied *mutatis mutandis* pursuant to Paragraph 2, Article 9-2, of said Act), it shall be promptly reported to the Animal Products Safety Division and relevant prefectural governments (Appendix Form 10).

(K) Press release

The Animal Products Safety Division and prefectural government shall announce the occurrence of a specified disease, the measures to prevent its spread.

(L) Release from restriction on movement and other restrictions

After the control measures set forth in (G) and (H) are complete, the prefectural government shall discuss the requirements, time, and other matters related to release from the restrictions set forth in (E) with aquaculture farmers and stakeholders or with the Animal Products Safety Division as necessary in consideration of the characteristics of the specified disease and spread status, release from restrictions, and notify the relevant prefectural governments of that fact.

(M) Cause investigation of occurrence

- (a) When the occurrence of a specified disease (excluding one that has been occurred in Japan) or other disease is detected, the Animal Products Safety Division, prefectural governments, and local fisheries research laboratories shall immediately collect epidemiological information related to the aquaculture farm where the disease occurred, transfer of aquatic animals, and conduct exhaustive research on the use status of water and feed, transfer of articles, distribution of susceptible animals,

weather conditions in cooperation with the RIA.

- (b) The Animal Products Safety Division shall establish an epidemiological research team that consists of members of the Fisheries Disease Control Expert Council and other experts as necessary so that the epidemiological research set forth in (a) above can be implemented promptly and accurately. Said team shall provide the necessary advice and instructions and shall analyze and compile cause investigations of occurrence of the disease based on the results of the research.

(N) Securing personnel necessary for control measures

- (a) The prefectural government shall establish a plan for personnel necessary for implementation of control measures, implementation of restriction on movement, and epidemiological research at the aquaculture farm where the disease occurred, obtain the cooperation of the relevant institutions and organizations, and promptly secure the necessary personnel.

- (b) Concerning (a) above, when it is difficult to take control measures at the aquaculture farm, to conduct research on surrounding aquaculture farms by the prefectural government, the prefectural government shall discuss with the Animal Products Safety Division a request to dispatch officials from the Ministry of Agriculture, Forestry and Fisheries, other prefectural governments.

(O) Valuation of aquatic animals

The valuation amount of aquatic animals that were disposed of by the measures to prevent spreading of the specified disease shall be determined in accordance with the “Operation of Measures to Prevent the Spread of Specified Diseases” (Attachment 5-1 of the Procedures for Payment of Grants for Food Safety and Consumer Affairs Measures (Notification of the Ministry of Agriculture, Forestry and Fisheries, Food Safety and Consumer Affairs Bureau, 16 Food Safety and Consumer Affairs Bureau No. 10272 as of April 1, 2005)).

(5) Promotion of Development of Research and Inspection Methods Related to Diseases and Training Personnel Responsible for Control Measures

(i) Direction of control measures

The epidemic prevention system shall be strengthened by promoting the development of research on diseases, high accuracy diagnosis, inspection methods and by training personnel who assume core roles in control measures at the working level.

(ii) Roles of stakeholders

- (A) The RIA shall be responsible for final diagnosis of specified diseases, shall cooperate closely with universities and other research institutions, and shall promote research and technology development that contributes to preventing the occurrence of diseases, diagnosis of diseases, and

prevention of the spread of diseases. In addition, a person who is authorized by the RIA may conduct definitive diagnosis in lieu of the RIA.

(B) The Animal Products Safety Division shall support research and technology development contributing to the prevention of the occurrence of diseases, diagnosis of diseases, and prevention of the spread of diseases.

(C) The Animal Products Safety Division shall support training of Fish Epidemic Prevention Assistants, certified persons who engage in fish epidemic prevention, and other personnel who assume core roles in control measures at the working level.

(6) Implementation of Risk Assessment

(i) Direction of control measures

Risk assessment of diseases based on scientific knowledge shall be implemented continuously and the necessity of risk management measures based on the risk assessment results shall be examined.

(ii) Roles of stakeholders

The Animal Products Safety Division shall organize a Fisheries Disease Control Expert Council that consists of experts in fishery epidemic prevention and aquatic industries as necessary and shall assess risks based on the collected scientific knowledge. When it is judged to be necessary to take risk management measures, Subject Diseases, Aquatic Animals Subject to Import Quarantine, aquatic animals subject to specified diseases shall be reviewed, this Guideline shall be reviewed, and new guidelines shall be established as necessary.

3. Measures Against Emerging Diseases

(1) Direction of Control Measures

A new disease refers to a disease of farmed aquatic animals and plants that has obviously different symptoms from known infectious diseases. When a new disease occurs, it is necessary to detect the disease at an early stage, to take measures to prevent spread of the disease, to identify aetiological agents, and to identify characteristics of the disease. Therefore, stakeholders shall handle these in cooperation with each other.

(2) Roles of Stakeholders

(i) When the occurrence of a new disease is suspected based on a report by an aquaculture farmer or patrol, investigation, or on-site inspection by a local fisheries research laboratory, the prefectural government shall confirm the details of Appendix Form 11 by holding an interview without delay.

(ii) When an occurrence of an emerging disease is suspected by (i) above, the prefectural government shall promptly report the fact of (i) to the

Animal Products Safety Division and communicate it to the RIA and request diagnosis.

Materials of the diagnosis shall be sent in accordance with 2. (4), (ii), (E), (a) of this Guideline.

(iii) The RIA shall conduct diagnosis for (ii) above and report on the progress and results of the diagnosis to said prefectural government and the Animal Products Safety Division.

(iv) The prefectural government shall discuss with the Animal Products Safety Division and RIA the provisional actions for the period until characteristics of the new disease are confirmed and shall give instructions on the provisional control measures to said aquaculture farmer and stakeholders.

(V) When the prefectural government confirms that an emerging disease has occurred based on the results of the diagnosis conducted by the RIA, the prefectural government shall report that fact to the Animal Products Safety Division without delay in accordance with Appendix Form No. 11.

(vi) The Animal Products Safety Division shall request other prefectural governments to conduct surveys as necessary in order to identify the occurrence status of said new disease outside the prefecture.

(vii) The announcement of the new disease shall be made after holding discussions with the Animal Products Safety Division and the prefectural government.

(viii) When the Animal Products Safety Division judges that the characteristics of the new disease are the same as the specified disease, the Animal Products Safety Division shall consider whether to include the new disease in the specified diseases.

4. Measures Against Established Existing Diseases Other than Specified Diseases

Concerning diseases for which the occurrence has been detected in Japan and which pose an obstacle to sustainable aquaculture production by occurring in many places, appropriate measures need to be taken as much as possible even if it is outside the specified diseases so that the disease will not spread. The prefectural governments shall proactively implement on-site inspections based on Article 10 of the Sustainable Aquaculture Production Act and reports and interviews based on Article 11 of said Act, and thereby strive to identify occurrence status of diseases continuously and accurately in regions and to provide instructions related to effective preventive measures against occurrence and measures to minimize damages based on the identification.

Concerning diseases that require special care, control measures shall be specified separately, and then necessary measures shall be taken.

Exhibit

Exhibit: Requirements for Storage Facilities in Japan for Edible Living Aquatic Live aquatic animals for human consumption that require no import permission

The living aquatic animals listed in the Table of Article 1-2, Paragraph (1) of the Ordinance for Enforcement of the Act on the Protection of Fishery Resources (Ordinance of the Ministry of Agriculture, Forestry and Fisheries No. 44 of 1952) that are used for food, excluding aquatic animals stored in water areas used for public purposes or in facilities that drain water directly to these water areas (hereinafter collective referred to as “Living Edible Aquatic Animals”) and that fail to correspond to “living aquatic animals (in cases of those used for food, limited to those stored in water areas that are used for public purposes or in facilities that drain water directly into the water areas)” set forth in Paragraph 2, item (i), Article 1-2, of said Ordinance shall refer to those that meet the following requirements and shall not require obtaining import permission from the Minister of Agriculture, Forestry and Fisheries based on the Act on the Protection of Fishery Resources (Act No. 313 of 1952).

In addition, inquiries of Importers related to this case shall be handled by the Ministry of Agriculture, Forestry and Fisheries, Food Safety and Consumer Affairs Bureau, Animal Products Safety Division (hereinafter referred to as the “Animal Products Safety Division”). The Animal Products Safety Division shall confirm the necessity of obtaining import permission before import, shall provide the results to the Importer, and shall share the results with the Animal Quarantine Service.

Description

1. Concerning drainage treatment of breeding water that is used at facilities in cases of storing Living Edible Aquatic Animals temporarily after import, the facilities shall correspond to any of the following requirements.
 - (1) It has a structure where the full amount of the breeding water will not be drained into water areas that are used for public purposes (sea, lakes and marshes, or river), but drained into the sewage system.
 - (2) It has a structure where the full amount of breeding water is drained after being processed by a method that can sterilize the aetiological agents of a disease or diseases subject to import quarantine.
2. The supervisor for temporary storage of Living Edible Aquatic Animals after import shall cooperate with the following matters.
 - (1) The supervisor shall cooperate with on-site inspections surveys and instructions conducted by the Animal Products Safety Division as necessary.
 - (2) When the structure of the storage facilities can no longer meet the requirements set forth in 1. above, the supervisor shall promptly communicate with the Animal Products Safety Division.

Appendix 1: Guidelines for Quarantine at the Destination for Imported Aquatic Animals

<Purpose>

These Guidelines shall aim to monitor continuously after import the health status and transfer (hereinafter referred to as “Quarantine at the Destination”) of aquatic animals that are imported for use in aquaculture, and in cases where a disease, including a new disease occurs due to changes to the rearing condition to detect the disease at an early stage and to take measures promptly to prevent the spread of the disease.

1. Aquatic Animals Subject to Quarantine at the Destination

Aquatic animals that are listed in the Table of Article 1-2, paragraph 1 of the Ordinance for Enforcement of the Act on the Protection of Fishery Resources (Ordinance of the Ministry of Agriculture, Forestry and Fisheries No. 44 of 1952; hereinafter referred to as the Ordinance of the Fishery Resources Act) and that are imported for use in aquaculture and are living (excluding aquatic animals that are directly sold at stores. for ornamental purposes after import permission is obtained) (hereinafter referred to as “Aquatic Animals Subject to Quarantine at the Destination”).

2. Site and Period of Quarantine at the Destination

(1) Requirements for aquaculture establishments where Quarantine at the Destination is implemented (hereinafter referred to as “Site of Quarantine at the Destination”).

- (i) In cases of inland water aquaculture, even if a disease occurs at the aquaculture establishment at the destination, it is preferable to be in an aquaculture establishment where it is possible to breed aquatic animals in isolation so that the disease does not spread to existing aquatic animals.

- (ii) In cases of marine aquaculture, it is preferable to be in an aquaculture establishment where it is possible to breed aquatic animals in isolation from existing aquatic animals at the destination.

(2) Precautions for Determining the Site of Quarantine at the Destination

The department responsible for fishery epidemic prevention of the prefectural government that has jurisdiction over the aquaculture farm at the destination (hereinafter referred to as “Prefectural Government Supervising Quarantine at the Destination”) shall determine the Site of Quarantine at the Destination in cooperation with the prefectural fishery research laboratories that conducts

examinations and provide instructions related to fishery epidemic prevention (hereinafter referred to as “local fisheries research laboratories”) based on documents submitted by the importer (applicant) of Aquatic Animals Subject to Quarantine at Destination to the Animal Quarantine Service when filing an application for import permission set forth in 3. (1), after adjustment of 2. (1) with the supervisor of the Site of Quarantine at the Destination before said aquatic animals are delivered.

(3) Period of Quarantine at the Destination

The period of Quarantine at the Destination shall be approximately six months after the Aquatic Animals Subject to Quarantine at the Destination arrive at the Site of Quarantine at the Destination in principle; in cases of conducting controlled breeding by the control order based on, Paragraph 1, Article 13-3 of the Act on the Protection of Fishery Resources (Act No. 313 of 1951) (hereinafter referred to as “Control Order”), including said period (hereinafter the period shall be referred to as “Controlled Breeding Period”).

3. Roles of Animal Quarantine Service

The Animal Quarantine Service shall share information with the Prefectural Government Supervising Quarantine at the Destination concerning the following matters.

- (1) The Animal Quarantine Service shall request the importer (applicant) of Aquatic Animals Subject to Quarantine at the Destination to send a copy of necessary documents submitted at the filing of an application for import permission (application form for import permission, certificate of inspection issued by the governmental organization of the exporting country) to the Prefectural Government Supervising Quarantine at the Destination of said aquatic animals; and shall share basic information related to said aquatic animals (exporting country, type of aquatic animals, amount, loading date and site, transportation method, arrival date and site, results of detailed examination at the exporting country) with the Prefectural Government Supervising Quarantine at the Destination in advance.
- (2) The Animal Quarantine Service shall send the results of visual inspection to be conducted at arrival of said aquatic animals in Japan and certificate of import permission (copy) to the Prefectural Government Supervising Quarantine at the Destination and shall share the information.
- (3) When controlled breeding is conducted based on the Control Order after import permission is granted, the Animal Quarantine Service shall send health observation records of Aquatic Animals Subject to Quarantine at the Destination during the Controlled Breeding Period, the results of detailed examinations to be conducted as necessary, and other information to be used as a reference when conducting Quarantine at the Destination after completion of

controlled breeding, and shall share the information.

4. Roles of Importer (Applicant)

The importer (applicant) of Aquatic Animals Subject to Quarantine at the Destination shall file an application for import permission of Aquatic Animals Subject to Quarantine at the Destination set forth in 3. (1) by the date that is specified separately by the Animal Quarantine Service and shall cooperate with the Animal Quarantine Service and Prefectural Government Supervising Quarantine at the Destination for the following.

- (1) The importer (applicant) shall give advice to the supervisor of the Site of Quarantine at the Destination to adjust with the Prefectural Government Supervising Quarantine at the Destination and assigned local fisheries research laboratory in advance when determining the Site of Quarantine at the Destination set forth in 2. (1), before filing an application for import permission of the Aquatic Animals Subject to Quarantine at the Destination set forth in 3. (1).
- (2) When submitting an application form for import permission to the Animal Quarantine Service, a copy of documents necessary for application of import permission (application form for import permission, certification of inspection issued by the governmental organization of exporting country) shall be sent to the Prefectural Government Supervising Quarantine at the Destination of aquatic animals and shall share it with the assigned local fisheries research laboratory.
- (3) When transporting Aquatic Animals Subject to Quarantine at the Destination to the Site of Quarantine at the Destination, the importer (applicant) shall request cooperation from the person responsible for transportation so that the person does not directly drain water used for transportation into water areas used for public purposes.

5. Roles of Prefectural Governments

The Prefectural Government Supervising Quarantine at the Destination shall share information with the assigned local fisheries research laboratory and shall handle the following in cooperation with said local fisheries research laboratory.

- (1) Identifying Health Status by Visual Observation and Implementing an Examination
 - (i) After the Aquatic Animals Subject to Quarantine at the Destination arrive at the Site of Quarantine at the Destination, a local fisheries research laboratory of the Prefectural Government Supervising Quarantine at the Destination shall check the health status of said aquatic animals as promptly as possible by visiting the Site of Quarantine at the Destination or by reporting via phone by the supervisor of the Site of Quarantine at the Destination, and shall record the breeding water temperature, rearing managements (breeding density, feeding, drug administration, vaccination, disinfection/cleaning), environment (water quality, red tide), and shall retain the records for at least three years.

- (ii) During the period of Quarantine at the Destination, a local fisheries research laboratory of the Prefectural Government Supervising Quarantine at the Destination shall check the health status of Aquatic Animals Subject to Quarantine at the Destination by periodical (approximately monthly) report of the supervisor of the Site of Quarantine at the Destination or by on-site inspections inspection at the Site of Quarantine at the Destination, and shall record breeding water temperature, rearing managements (breeding density, feeding, drug administration, vaccination, disinfection/cleaning), environment (water quality, red tide), and shall retain the records for at least three years.
 - (iii) When the local fisheries research laboratory that is responsible for the Prefectural Government Supervising Quarantine at the Destination judges Aquatic Animals Subject to Quarantine at the Destination suspected to be infected with a specified disease, the local fisheries research laboratory shall promptly report to the Prefectural Government Supervising Quarantine at the Destination, discuss a reaction in cooperation with each other, conduct on-site inspection of the Site of Quarantine at the Destination and confirm the aquatic animal health status. In addition, the Prefectural Government Supervising Quarantine at the Destination shall conduct detailed diagnoses as necessary and promptly report to the Animal Products Safety Division based on this Guideline 2 (4).
 - (iv) The local fisheries research laboratory that is responsible for the Prefectural Government Supervising Quarantine at the Destination shall provide instructions to the supervisor of the Site of Quarantine at the Destination based on Appendix 3: Guidelines for Sanitation Measures at Aquaculture Farms of the Outline of Fishery Control Measures.
- (2) Refrain from Movement of Aquatic Animals Subject to Quarantine at the Destination during the Period of Quarantine at the Destination
- (i) The assigned local fisheries research laboratory of the Prefectural Government Supervising Quarantine at the Destination shall request the supervisor of the Site of Quarantine at the Destination to breed Aquatic Animals Subject to Quarantine at the Destination during the period of Quarantine at the Destination only at the Site of Quarantine at the Destination in principle and refrain from movement to outside the Site of Quarantine at the Destination as much as possible excluding cases of shipping them as food.
 - (ii) In cases where Aquatic Animals Subject to Quarantine at the Destination must be transferred during the period of Quarantine at the Destination, the prefectural government that has jurisdiction over the Site of Quarantine at the Destination at the transfer destination of said aquatic animals shall continue to conduct Quarantine at the Destination and the prefectural government that has jurisdiction over the Site of Quarantine at the Destination at the transfer destination shall record the health status of said aquatic animals as well as the transfer record and

shall retain them for at least three years.

6. Roles of the Supervisor of the Site of Quarantine at the Destination

The supervisor of the Site of Quarantine at the Destination shall implement the following under the instructions of the assigned local fisheries research laboratories of the Prefectural Government Supervising Quarantine at the Destination.

- (1) When the Prefectural Government Supervising Quarantine at the Destination determines the Site of Quarantine at the Destination set forth in 2. (2), the supervisor shall cooperate with the assigned local fisheries research laboratory so that the decision is made based on the requirements set forth in 2. (1).
- (2) During the period of Quarantine at the Destination, the supervisor shall observe the health of said subject aquatic animals, record it including rearing conditions (breeding water temperature, breeding density, feeding, drug administration, vaccination conditions, disinfection/cleaning conditions, water quality, red tide), and retain them for at least three years. When an abnormality is detected, the supervisor shall promptly report it to the assigned local fisheries research laboratories of the Prefectural Government Supervising Quarantine at the Destination.
- (3) The supervisor shall cooperate with on-site inspections at the Site of Quarantine at the Destination or sampling necessary for detailed diagnoses to be conducted as necessary by the assigned local fisheries research laboratory of the Prefectural Government Supervising Quarantine at the Destination and shall cooperate with them when it is requested to present the records set forth in (2).
- (4) The supervisor shall refrain from movement of said subject aquatic animals to outside said site during the period of Quarantine at the Destination. However, in cases where it is necessary to transfer said aquatic animals, the supervisor shall move them after adjusting the transfer based on the requirements set forth in 5. (2) with the assigned local fisheries research laboratory of the Prefectural Government Supervising Quarantine at the Destination (in cases where the Prefectural Government Supervising Quarantine at the Destination changes due to the movement, after obtaining the approval of the prefectural government that has jurisdiction over the aquaculture farm at the destination).

Guidelines for Surveillance of Specified Diseases

<Purpose>

These Guidelines shall indicate the basic idea of disease surveys related to specified diseases.

1. Definitions

The term “Surveillance” as used in these Guidelines shall refer to a means to examine specified diseases and other diseases important for aquatic and aquaculture industries in Japan in order to identify disease-free status, and occurrence conditions and trend of diseases in Japan using a uniform method, and to collect, analyze, and assess the obtained information continuously.

2. Surveillance Area and Subject Diseases

Surveillance shall be conducted for specified diseases, diseases on the OIE list, and other diseases that are considered to be important throughout Japan in comprehensive consideration of occurrence status, geographical distribution, and other necessary matters for each disease.

When conducting the Surveillance, the Ministry of Agriculture, Forestry and Fisheries, Food Safety and Consumer Affairs Bureau, Animal Products Safety Division (hereinafter referred to as “Animal Products Safety Division”) shall plan the purpose, subject aquatic animal types, scope, inspection method, judgment, and other detailed matters in cooperation with the National Research and Development Agency Japan Fisheries Research and Education Agency, National Research Institute of Aquaculture (hereinafter referred to as “RIA”), and other research institutions and experts of diseases related to aquatic animals, and shall separately notify the department responsible for fishery epidemic prevention of prefectural governments (hereinafter referred to as “Prefectural Government”) of it every fiscal year.

In addition, the Animal Products Safety Division shall cooperate with RIA, and strive to proactively collect information on diseases overseas on daily basis. When a risk of invasion of a disease into Japan increases and it is judged necessary to conduct new Surveillance, the Animal Products Safety Division shall notify stakeholders separately of the details of implementation of the Surveillance.

3. Procedures for Surveillance

Surveillance shall be implemented smoothly by paying attention to the basic matters set forth in the following (1) through (6).

(1) Establishment of Surveillance Objectives

The Animal Products Safety Division shall clearly present to persons related to implementation of the Surveillance (aquaculture farmers, Prefectural Governments, RIA, and other research institutions) the objective of identifying disease-free status, occurrence conditions, trends of specified diseases for each disease when conducting Surveillance.

(2) Establishment of Surveillance Plan and Precautions

It is important to pay attention to the following points when establishing and implementing the Surveillance Plan.

- (i) Overall structure of Surveillance (procedures) shall be as simple as possible and shall be easy-to-implement.
- (ii) Surveillance shall be implemented in a flexible manner and it shall be improved by collecting opinions of the persons involved in the Surveillance and by reflecting them.
- (iii) Roles and importance of stakeholders and organization when collecting data and providing information so that persons involved in the Surveillance understand it.
- (iv) Types of data to be collected, definitions of diagnostic criteria, and other information shall be defined.
- (v) A sampling method where the results of the Surveillance become representative of the population shall be selected in order to prevent errors between Prefectural Governments.
- (vi) Measures shall be taken so that actions at each step from collection of data and implementation of measures shall be performed promptly.
- (vii) In order to identify temporal distribution, data shall be collected based on the plan depending on the characteristics of a disease.

(3) Attitude for Conducting Inspections

It is important that Surveillance be inspected by paying attention to the following basic matters for each disease and by the method based on II. Guidelines for Diagnosis of these Guidelines or scientific knowledge.

- (i) When setting a Surveillance area, occurrence conditions of the disease in and outside Japan, transmissibility of diseases, rearing conditions of aquatic animals shall be fully considered; the area conforming to the objective of the inspection shall be selected; and overall conditions of the subject population shall be identified as much as possible.
- (ii) Inspection shall be implemented by selecting a sampling method and inspection method appropriate for the objective of the Surveillance.
- (iii) Sampling time for the inspection shall be the time with the highest possibility that aquatic animals have been exposed to the aetiological agent, in consideration of occurrence temperature, rearing conditions in adjacent areas, transfer history of aquatic animals for each disease.

(4) Reporting and Tallying Inspection Results

The person responsible for Surveillance shall compile inspections results of the Surveillance and shall report it to the Animal Products Safety Division in accordance with the reporting method notified separately in each fiscal year.

(5) Analysis and Assessment of Talled Results

The Animal Products Safety Division shall cooperate with the RIA and experts on diseases related to aquatic animals, conduct analysis and assessment scientifically and objectively, and use them for epidemiological consideration of the diseases, assessment of current control measures, selection of future control measures, establishment of occurrence forecasting method in order to reinforce control measures, and other purposes.

(6) Returning the Information Back to the Prefectural Governments and Provision of Information to International Institutions.

The Animal Products Safety Division shall return the compiled assessment results to the Prefectural Government and other stakeholders and shall provide information to countries through OIE as necessary.

Guidelines for Sanitation Measures in Aquaculture Farms

<Purpose>

These Guidelines aim to present basic sanitary management that aquaculture farmers should voluntarily practice on a daily basis so that the occurrence of diseases related to farmed aquatic animals and plants can be prevented and that the occurrence can be detected at an early stage if it occurs and measures to prevent spreading of the disease can be taken promptly, and to have all stakeholders engage in fishery epidemic prevention together.

1. Collection of Information Related to Fishery Epidemic Prevention

Aquaculture farmers shall participate in seminars held by the department responsible for fishery epidemic prevention of the prefectural governments (hereinafter referred to as “Prefectural Government”), and shall consult information related to fishery epidemic prevention that are posted on websites of the Ministry of Agriculture, Forestry and Fisheries, Prefectural Governments, and research institutions, and thereby shall deepen knowledge of characteristic symptoms and knowledge of prevention concerning specified diseases of farmed aquatic animals and plants provided for by the Ordinance for Enforcement of the Sustainable Aquaculture Production Assurance Act, shall confirm occurrence conditions of diseases in and outside Japan, and shall strive to prevent epidemics themselves.

2. Fundamental Activities for Prevention of Diseases

(1) General Hygiene Control

Aquaculture farmers shall identify sanitation of the sea and river surrounding the aquaculture farm, shall pay attention to water quality of breeding water and breeding density that may induce various diseases, shall conduct hygiene control thoroughly, such as cleaning and disinfection of preserve, aquaculture establishments, aquaculture equipment, and storage facilities, shall appropriately collect and dispose of fish that are dead or present clinical symptoms in association with infectious disease without leaving it, and thereby shall strive to organize a hygienic environment.

(2) Introduction of Parent Fish and Juveniles from Outside

- (i) When introducing parent fish and juveniles from outside, aquaculture farmers shall check rearing conditions at the provider (introduction history, occurrence condition of diseases, hygiene managements) in advance, shall strive to breed them by isolating (in cases of marine aquaculture, by segmenting) from existing breeding aquatic animals for a specified period, shall record the following information, and shall retain the records for at least 3 years. In addition, when the

Prefectural Government requests inspection of the introduction records or to conduct on-site inspections, aquaculture farmers shall cooperate with them.

- (A) Date and time of introduction
- (B) Name and address of the provider
- (C) Species, growth stage (egg, larvae, fry, young fish, age in months), amount
- (D) Health condition of aquatic animals, environment (breeding water temperature), disposition of disinfection

- (ii) In cases where parent fish and juveniles are imported and the aquatic animals corresponds to “aquatic animals subject to diseases” subject to import quarantine based on the Act on the Protection of Fishery Resources, the aquaculture farmer shall cooperate with the inspection at the destination conducted by the Prefectural Government based on the Appendix 1: Guidelines for Quarantine at the Destination for Imported Aquatic Animals of the Outline of Fishery Control Measures.

(3) Use and Storage of Food

Aquaculture farmers shall use feed after identifying feed ingredients (species), country of origin, name, lot number, manufacturer (or distributor), and distribution route, and shall store them at an appropriate site and temperature in terms of fishery epidemic prevention.

3. Health Status of Aquatic Animals and Actions for Abnormality

(1) Health status and rearing conditions of aquatic animals and plants

Aquaculture farmers shall observe the health status of aquatic animals and plants on a daily basis, record the following information, and retain the records for at least 3 years. In addition, when the Prefectural Government requests inspection of the introduction records or to conduct on-site inspections, aquaculture farmers shall cooperate with them.

- (i) Health status of aquatic animals and plants, existence of abnormality
- (ii) Breeding water temperature and other breeding environment conditions
- (iii) Number of deaths
- (iv) Transfer and release of aquaculture farm (specific location)
- (v) Disease inspection history (sampling site, amount, inspection site, inspection items, inspection results)
- (vi) On-site inspections record by the Prefectural Government (date, findings)

(2) Actions when abnormality is detected with aquatic animals and plants

In cases where the health status of aquatic animals and plants are different from usual or where there is a suspicion of a disease, such as that the number of deaths increases, aquaculture farmers shall promptly communicate it to and consult with the Prefectural Government (the nearest fishery research laboratory).

4. Appropriate Use of Fisheries Medicine

Aquaculture farmers shall purchase fisheries medicines (in particular, vaccines, and antimicrobial agents) under instructions of competent leading institutions, such as a fishery research laboratory and livestock hygiene service center, shall use them in compliance with approved dosage and administration, precautions, and use period and washout period, shall record the use conditions, and shall retain them for at least 2 years.

In addition, for the use of fisheries medicine, please refer to the “Use of Fisheries Medicine” (issued by the Ministry of Agriculture, Forestry and Fisheries, Fisheries Medicine, Animal Products Safety Division).

Appendix Form 1

Outline of Occurrence Conditions of Abnormality
with Farmed Aquatic Animals and Plants

Report date: MONTH, DAY, YEAR

Name:

| | | | |
|-------------------|-------------------|-------------------------|--|
| Aquaculture farm: | | Name of representative: | |
| Location | Phone number: () | | |

1. Breeding and Production

| Species | Manager | Lab or | Owner: persons; Employees: persons |
|--|---|--|---------------------------------------|
| (Example) Rainbow trout Char Yamame (type of trout) Carp Tiger shrimp Other | (Example) Breeding conditions - Production of egg - Release to market - Production of fish - Purchase of eggs - Purchase of fry - Purchase of adult fish | | |
| | | Release conditions - Release of fresh fish - Release of live fish - Other | |

2. Breeding Pond (Total area: m²)

| | Area: m ² | Number of areas | Total area: m ² | Occurrence of diseases |
|----------------------|----------------------|-----------------|----------------------------|------------------------|
| Pond for fry | | | | |
| Pond for adult fish | | | | |
| Pond for parent fish | | | | |
| Preserve pond | | | | |

3. Water to be Used

| Type | Water amount | Water temperature | Use | Existence of aetiological agent contamination |
|------|--------------|-------------------|-----|---|
| | | | | Yes / No / Not sure |

* Type: Select from spring water, ground water, river water, sea water, or other.

4. Overview Diagram

Water incoming route: Blue

Type of water (i)

Water drain route: Red

Type of water (ii)

Epidemic prevention (quarantine) facility: Green

Type of water (iii)

Occurrence site: ◎

| |
|--|
| |
|--|

5. Breeding and Production Status Table (Fiscal Year)

| Item Species | Eggs (Unit: 10,000) | | Fry (Unit: 10,000) | | Parent fish (Unit: 1,000) | Remarks (Provider of hatching eggs and juveniles) |
|-----------------|------------------------|------------|-----------------------|------------|------------------------------|--|
| | Homegrown | Introduced | Homegrown | Introduced | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |

6. Production Amount and Release Amount (Fiscal Year)

| Item Species | Eggs (Unit: 10,000) | | Fry (Unit: 10,000) | | Fish (ton) | Remarks (Shipping to) (Purchased from) |
|-----------------|------------------------|---------|-----------------------|---------|------------|--|
| | Homegrown | Release | Homegrown | Release | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |

7. Instruction Records

| <u>MONTH, DAY,</u> <u>YEAR</u> | Description |
|-----------------------------------|-------------|
| | |
| | |
| | |
| | |

Appendix Form 2

Notification of Occurrence or Suspected Occurrence of Specified Disease

MONTH, DAY, YEAR

To: Governor of PREFECTURE

(Notifier) Address:

Name:

I hereby give notification as stated below pursuant to the provisions of Paragraph (1), Article 7-2 of the Sustainable Aquaculture Production Assurance Act.

Description

1. Name and address of owner and manager of farmed aquatic animals and plants

| | Name | Address (postal code, street address, prefecture) |
|---------|------|---|
| Owner | | |
| Manager | | |

2. Specified disease which the farmed aquatic animals and plants are infected with or are suspected to be infected with

3. Species of farmed aquatic animals and plants

4. Location of farmed aquatic animals and plants

5. Date and time when it is found that the farmed aquatic animals and plants are infected with or are suspected to be infected with a specified disease, and conditions at the detection

6. Other information to be referred

Materials necessary for estimating invasion route (provider of juveniles, shipping destination, drug administration record, changes in mortality, breeding progress)

Appendix Form 3

**Notification of Occurrence or Suspected Occurrence of Specified Disease
(for recording)**

MONTH, DAY, YEAR

(Person who accepted oral notification) Department:

Name:

I hereby record that I received oral notification of occurrence or suspected occurrence of a specified disease as stated below pursuant to the provisions of Paragraph (1) of Article 7-2 of the Sustainable Aquaculture Production Assurance Act.

Description

1. Name and address of owner and manager of farmed aquatic animals and plants

| | Name | Address (postal code, street address, prefecture) |
|---------|------|---|
| Owner | | |
| Manager | | |

2. Specified disease from which the farmed aquatic animals and plants suffer or are suspected to suffer

3. Species of farmed aquatic animals and plants

4. Location of farmed aquatic animals and plants

5. Date and time when it is found that the farmed aquatic animals and plants are infected with or are suspected to be infected with a specified disease, and conditions at the detection

6. Other information to be referred

Materials necessary for estimating invasion route (provider of juveniles, shipping destination, drug administration record, changes in mortality, breeding progress)

Appendix Form 4

Inspection Order for Notification of Specified Disease

Number
MONTH, DAY, YEAR

To: NAME

NAME, governor

I hereby order those named below to receive the following inspection pursuant to the provisions of Paragraph (2) of Article 7-2 of the Sustainable Aquaculture Production Assurance Act.

Description

1. Name and address of owner and manager of farmed aquatic animals and plants

| | Name | Address (postal code, street address, prefecture) |
|---------|------|---|
| Owner | | |
| Manager | | |

2. Specified disease which the farmed aquatic animals and plants are infected with or are suspected to be infected with

3. Species of farmed aquatic animals and plants

4. Location of farmed aquatic animals and plants

5. Inspection (method)

6. Other necessary matters

Remarks

1. If you are dissatisfied with this order, you can request an examination from the governor, NAME, within three (3) months from the day following the day when you come to know that this order is issued pursuant to the Administrative Complaint Review Act (Act No. 68 of 2014).
2. The action for revocation of this order may be filed against the governor, NAME, within six (6) months from the day following the day when you come to know that this order is issued pursuant to the Administrative Case Litigation Act (Act No. 139 of 1962).
3. If you breach this order, specified penal provisions will apply.

Appendix Form 5

Report (Warning) of Suspected Occurrence of Specified Disease

Number

MONTH, DAY, YEAR

To: Fish and Fishery Products Safety Office (Competent Fishery industry Department of Relevant Prefecture)

Competent fishery industry department of PREFECTURE

I hereby report as stated below that there is a report on the detection of farmed aquatic animals that are suspected to be infected with a specified disease.

Description

1. Name and address of owner and manager of farmed aquatic animals and plants

| | Name | Address (postal code, street address, prefecture) |
|---------|------|---|
| Owner | | |
| Manager | | |

2. Specified disease from which the farmed aquatic animals and plants suffer or are suspected to suffer

3. Species of farmed aquatic animals and plants

4. Location of farmed aquatic animals and plants

5. Date and time when it is found that the farmed aquatic animals and plants are infected with or are suspected to be infected with a specified disease, and conditions at the detection

6. Other information to be referred

Materials necessary for estimating invasion route (provider of juveniles, shipping destination, drug administration record, changes in mortality, breeding progress)

Appendix Form 6

Attached Records for Sending Diagnosis Materials

(PREFECTURE)

| | |
|--|----------------|
| Name of owner of farmed aquatic animals and plants: Address: | |
| Species of farmed aquatic animals and plants: Amount: | |
| Breeding conditions: Content of feed: Water temperature: | |
| Diagnosis materials: Type of materials: | Sampling date |
| Sent date: | Sending method |
| Scope of occurrence, occurrence period: | |
| Cause of occurrence (estimation): | |
| Mortality conditions: | |
| Other information to be referred: | |

Name of sampling and recording person: Job title: _____ Name: _____

Appendix Form 7

Report (Warning) of Occurrence of Specified Disease

Number
MONTH, DAY, YEAR

To: Minister of Agriculture, Forestry and Fisheries (governor of relevant prefecture)

Governor of PREFECTURE [Seal]

I hereby report (warn) as stated below pursuant to the provisions of Article 7-2, Paragraph (3) of the Sustainable Aquaculture Production Assurance Act.

Description

1. Name and address of owner and manager of farmed aquatic animals and plants

| | Name | Address (postal code, prefecture, street address) |
|---------------|------|---|
| Owner | | |
| Administrator | | |

2. Specified disease which the farmed aquatic animals and plants are infected with or are suspected to be infected with

3. Species of farmed aquatic animals and plants

4. Location of farmed aquatic animals and plants

5. Date and time when it is found that the farmed aquatic animals and plants suffer from or are suspected to suffer from a specified disease, and conditions at the detection

6. Other information to be referred

Materials necessary for estimating invasion route (provider of juveniles, shipping destination, drug administration record, changes in mortality, breeding progress)

* When notifying (warning) relevant prefectures, it shall be addressed to the governor of the relevant prefecture.

Appendix Form 8

Report (Warning) when Diagnosis Results of the Specified Disease was Negative

Number
MONTH, DAY, YEAR

To: Fish and Fishery Products Safety Office
(Competent Fishery industry Department of Relevant Prefecture)

Competent fishery industry department of PREFECTURE

We hereby notify that we obtained negative results for the diagnosis of farmed aquatic animals and plants that are suspected to be infected with the specified diseases and notified as of DATE (NUMBER).

Appendix Form 9

Order to Take Actions to Prevent the Spread of Disease

To: NAME

Number
MONTH, DAY, YEAR

NAME, governor [Seal]

I hereby order that you take the following actions based on the provisions of Paragraph 1 of Article 8 of the Sustainable Aquaculture Production Assurance Act.

Description

1. Name and address of owner and manager of farmed aquatic animals and plants

| | Name | Address (postal code, prefecture, street address) |
|---------|------|---|
| Owner | | |
| Manager | | |

2. Specified disease which the farmed aquatic animals and plants suffer from or are suspected to be infected with

3. Species of farmed aquatic animals and plants

4. Location of farmed aquatic animals and plants

5 Details of actions to prevent the spread of diseases (method)

6. Other necessary matters

Remarks

1. It is not allowed to file a complaint against this order pursuant to the Administrative Complaint Review Act (Act No. 68 of 2014).
2. The action for revocation of this order may be filed against the governor, NAME, within six (6) months from the day following the day when you come to know that this order is issued pursuant to the Administrative Case Litigation Act (Act No. 139 of 1962).
3. If you breach this order, specified penal provisions will apply.

Appendix Form 10

Report (Warning) of Actions for a Specified Disease

Number
MONTH, DAY, YEAR

To: Minister of Agriculture, Forestry and Fisheries (governor of relevant prefecture)

Governor of PREFECTURE [Seal]

I hereby report (warn) as stated below pursuant to the provisions of Paragraph 2 of Article 8 of the Sustainable Aquaculture Production Assurance Act.

Description

1. Specified disease and occurrence location (Enter city, town, village.)
2. Species of farmed aquatic animals and plants
3. Issuance date of the order
4. Details of the order
5. Progress of said actions and results of the actions
6. Other necessary matters

* When notifying (warning) relevant prefectures, it shall be addressed to the governor of the relevant prefecture.

Notification of Occurrence of a New Disease

Number
MONTH, DAY, YEAR

To: Minister of Agriculture, Forestry and Fisheries (governor of relevant prefecture)

Governor of PREFECTURE [Seal]

I hereby report the occurrence of a new disease as stated below.

Description

1. Name and address of owner and manager of farmed aquatic animals and plants

| | Name | Address (postal code, prefecture, street address) |
|---------|------|---|
| Owner | | |
| Manager | | |

2. Symptoms

3. Species of farmed aquatic animals and plants

4. Location where the disease occurred

5. Date and time when it is found that the farmed aquatic animals and plants suffer or are suspected to suffer from new disease, and conditions at the detection

6. Actions that have been taken or that are scheduled to be taken

7. Other information to be referred

Materials necessary for estimating invasion route (provider of juveniles, shipping destination, drug administration record, changes in mortality, breeding progress)

II. Guidelines for Diagnosis

1. Finfish

(1) Viral haemorrhagic septicaemia (excluding type IVa)(VHS)

| Responsible organization | Diagnosis flow chart |
|--|--|
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] B --- C["(3) Anatomical observation"] C --- D["(4) (i) (a) Virus isolation by cell culture"] C --- E["(4) (i) (c) RT-PCR with tissues"] D --- F["(-)"] D --- G["(+, ±)"] G --- H["(4) (i) (b) Indirect immunofluorescence"] H --- I["(-)"] H --- J["(+, ±)"] H --- K["(+)"] E --- L["(+, ±)"] E --- M["(-)"] </pre> |
| National Research Institute of Aquaculture | <pre> graph TD N["(4) (i) (a) Virus isolation by cell culture"] --- O["(4) (ii) (a) Virus isolation by cell culture"] O --- P["(+)"] O --- Q["(-)"] P --- R["(4) (ii) (b) Indirect immunofluorescence and (4) (ii) (c) Viral genome sequencing"] R --- S["(+)"] R --- T["(-)"] </pre> |
| Diagnosis | <p style="text-align: center;">- - - + - - - -</p> |
| Other | <p>Although virus isolation is the preferable method of diagnosis for the disease, the initial diagnosis can be conducted with RT-PCR ((4) (i) (c)), if the fish have clinical signs of disease and type IVa has never been reported for that species of fish in Japan.</p> |

Disease: Viral haemorrhagic septicaemia (excluding type IVa)(VHS)

Aetiological agent: Viral hemorrhagic septicemia virus (Rhabdoviridae, novirhabdovirus)

(1) Epidemiological research (excluding type IVa)

(i) Host range: Over 30 fish species, including rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), Atlantic salmon (*S. Salar*), brook trout (*Salvelinus fontinalis*), pike, turbot (*Scophthalmus aquosus*), Greenland halibut (*Reinhardtius hippoglossoides*), musky, bluegill (*Lepomis macrochirus*), smallmouth bass (*Micropterus dolomieu*).

(ii) Geographical distribution

Types Ia, Ib, Id, Ie, and II: The European continent and surrounding waters

Type III: Surrounding waters of Europe and Flemish Cap (Atlantic Ocean off Canada)

Type IVb: Great Lakes in North America

Type IVc: Estuarine or brackish waters of the Atlantic coast of Canada

(Diseases due to types I or Ic have not been reported recently.)

(iii) The fish have been imported from one of the aforementioned areas or the animals have a history of contacting with the fish imported from the area

(iv) The fish farm has a history of introduction of fish from the one of the aforementioned areas.

(v) Juveniles are more susceptible than adults to the disease with severe clinical symptoms.

(vi) The disease tends to occur from winter to spring when water temperature is 7 to 15 °C.

(2) Clinical observation

(i) Darkening of the skin

(ii) Swollen abdomen or bulging eyes (exophthalmia)

(iii) Hemorrhage in the eyes, skin, gills, and base of fins.

(iv) Anemia

(3) Anatomical findings

(i) Hemorrhage is observed widely in the peritoneum, mesentery, and visceral adipose tissue.

(ii) Hyperemia, swelling, and discoloration are observed in the kidney and liver.

(iii) Petechial hemorrhage is observed in the skeletal muscle.

(4) Diagnosis

(i) Initial diagnosis: (a) Virus isolation

Cell line: FHM or BF-2 cells

Inoculum: Either the kidney, spleen, or ovarian fluid is appropriate at the peak of the disease, whereas the heart or brain should be added to the material when the disease is subsiding.

Culture method: Culture for 7 to 10 days at around 15°C.

Results: CPE characterized by rounding of cells.

(b) Indirect immunofluorescence

Virus culture supernatant that is isolated in (4) (i) (a) shall be inoculated

into newly prepared FHM or BF-2 cells; the virus infected cells shall be fluorescently stained by using VHS virus specific antibodies (IP5B11 (OIE)) and type IVa VHS virus specific antibodies (VHS-10 (Ito *et al.*, 2010, 2012)). Whether the type is IVa or another type should be judged by the combination of the results. In other words, when samples to be identified are positive to IP5B11 and VHS-10 antibody, it is judged as type IVa VHS virus (which is the existing type in Japan) and the diagnosis is accordingly negative. On the other hand, when the samples are positive to IP5B11 antibody and negative to VHS-10 antibody, the virus is judged as one of the types other than IVa (thus the virus is a specified disease) and the diagnosis is positive.

(c) RT-PCR (reverse transcription-polymerase chain reaction method: using a commercial kit)

Material: RNA extracted either from the kidney, spleen, heart, brain, or ovarian fluid.

Primers:

Forward primer: 5'-GGG-GAC-CCC-AGA-CTG-T-3'

Reverse primer: 5'-TCT-CTG-TCA-CCT-TGA-TCC-3'

Reverse transcription: 50°C for 30 minutes.

PCR reaction: Denature at 94°C for 2 minutes, and then conduct 35 cycles of incubations at 94°C for 30 seconds, at 52°C for 30 seconds, and at 68°C for 60 seconds. Subsequently, the reaction is held at 68°C for 7 minutes.

Amplicon size: 811 bp

(ii) Confirmatory diagnosis: (a) Virus isolation

The same as the initial diagnosis.

(b) Indirect immunofluorescence

The same as the initial diagnosis.

(c) Viral genome sequencing

Material: RNA extracted either from virus culture supernatant or from the kidney, spleen, heart, brain, or ovarian fluid of fish.

Method and diagnosis: After amplification of G-gene of VHS virus by RT-PCR, analyze the total sequence of the gene, and determine the type on the basis of G-gene sequences on the database.

(5) Histopathology

(i) Necrosis is observed in the urinary system and hematopoietic tissue of the kidney.

(ii) Necrosis is observed in the liver, spleen, and pancreas.

(6) Differential diagnosis

Since it is difficult to distinguish type IVa from other types of VHSV by clinical observation,

it is preferable to conduct virus isolation with cell culture and subsequent antibody assays using antibodies that have different specificity to different types. In terms of sensitivity, it is preferable to use virus culture supernatant obtained in (4) (i) (a) as the sample for the indirect immunofluorescence of (4) (i) (b). However, in cases where prompt diagnosis is needed, a presumptive initial diagnosis can be conducted by using samples (such as homogenates of organs) used for (4) (i) (a).

(7) Disinfection

- (i) Disinfection with 30% propanol (PBS (-)) for 30 seconds or disinfection with 20 % propanol for 2 minutes (artificial sea water)
- (ii) Disinfection with 2.5% phenol (PBS (-)) for 5 minutes (Artificial seawater)
- (iii) Disinfection with 0.1% cresol (PBS (-)) for 5 minutes or disinfection with 0.25% cresol for 15 minutes (Artificial seawater)

(8) Other

- (i) In cases of conducting an inspection of fish for which symptoms are unclear, such as surveillance, it is necessary to conduct virus isolation with cell culture.
- (ii) For the maximum sensitivity, it is desirable to newly inoculate the virus supernatant that is isolated in (4) (i) (a) into cell culture to obtain samples for the fluorescent antibody assays of (4) (i) (b); however, in cases where prompt diagnosis is needed, the infected cells obtained from (4) (i) (a) can be directly used for fluorescent antibody assays.
- (iii) When the results of the immunofluorescence assay at the initial diagnosis are unclear, virus isolation in cell culture shall be implemented again at the confirmatory diagnosis ((4) (ii) (a) Virus isolation in cell culture). Even if obvious CPE is not observed as a result, protocols of (4) (ii) (b) and (c) should be conducted.

Disease: Infection with salmonid alphavirus

Aetiological agent: Salmonid alphavirus (SAV; Togavirus, Alphavirus)

(1) Epidemiological research

(i) Host range: Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), brown trout (*S. Trutta*) (experimental infection)

* All life stages should be considered as susceptible to SAV.

(ii) Geographical distribution: European countries

(iii) Carrier: wild flatfish

(iv) Vector: Salmon louse (*Lepeophtheirus salmonis*)

(v) Water temperature of outbreaks: 8-15°C (Acute). Below 8°C (Chronic).

(2) Clinical observation

(i) Loss of appetite.

(ii) Lethargic swimming

(iii) Erosion and ulceration of the skin

(iv) Poor growth may be observed in the surviving fish.

(3) Anatomical Findings

(i) Yellow mucoid substance is observed in the gut of the fish that lost appetite.

(ii) Petechial hemorrhage of the pyloric caeca

(iii) Reddening of the pancreatic region

(4) Diagnosis

(i) Initial diagnosis: RT-PCR

Materials: RNA extracted from the heart or kidney

Primer:

E2F: 5'-CCG-TTG-CGG-CCA-CAC-TGG-ATG-3'

E2R: 5'-CCT-CAT-AGG-TGA-TCG-ACG-GCA-G-3'

Amplicon size: 516 bp

Reverse transcription at 55°C for 30 minutes

Reaction: Denature at 95°C for 2 minutes, then conduct 40 cycles of incubations at 94°C for 15 seconds, at 60°C for 30 seconds, and at 72°C for 50 seconds. Finally incubate at 72°C for 2 minutes.

(ii) Confirmatory diagnosis: (a) RT-PCR

The same RT-PCR indicated in (i).

(b) Sequence analysis of the PCR amplicon

Materials: PCR amplicon of (ii) (a)

Diagnosis: Confirm that the nucleotide sequence of the PCR amplicon shows a high identity with partial sequence of SAV E2 protein gene that is registered on the database.

(5) Histopathology

- (i) Severe destruction of the exocrine pancreatic tissue (acute)
- (ii) Significant loss of the exocrine pancreatic tissue and fibroplasia of periacinar tissue (chronic)
- (iii) Myocardial necrosis with strongly eosinophilic cytoplasm and pyknosis of cardiomyocyte
- (iv) Spongiform degeneration of myocardium in the ventricle and atrium
- (v) Hyaline degeneration of lateral muscles and fragmentation of eosinophilic sarcoplasm
- (vi) Eosinophilic interstitial tissue of the kidney

(6) Differential diagnosis

No information.

(7) Disinfection

There is a report that the virus is inactivated by the treatment of 0.5% commercially available chlorinated disinfectant (supplemented with potassium monopersulphate) at 10°C for 5 minutes.

(8) Other

CHSE-214 cells can be used for virus isolation. Depending on the virus strain, however, CPE may appear only after multiple blind passages.

(3) Epizootic haematopoietic necrosis

| Responsible organization | Diagnosis flow chart |
|--|--|
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] A --- C["(3) Anatomical observation"] B --- C C --- D["(4) (i) (a) Virus isolation"] C --- E["(4) (i) (c) PCR-REA from organs"] D --- F["(-)"] D --- G["(+, ±)"] G --- H["(4) (i) (b) PCR-REA from culture supernatant"] H --- I["(-)"] H --- J["(+, ±)"] E --- K["(+, ±)"] E --- L["(-)"] </pre> |
| National Research Institute of Aquaculture | <pre> graph TD M["(4) (ii) (a) Virus isolation"] --- N["(4) (ii) (b) PCR-REA from culture supernatant (or) (4) (ii) (c) PCR-sequencing analysis from culture supernatant"] N --- O["(+)"] N --- P["(-)"] </pre> |
| Results | <p>- - + - -</p> |
| Other | <p>For maximum sensitivity, it is preferable to implement “(4) (i) (a) Virus isolation”, following “(3) Anatomical observation”. When rapid diagnosis is required for clinically diseased fish, however, “(4) (i) (c) PCR-REA from organs” may be adopted.</p> |

Disease: Epizootic haematopoietic necrosis

Aetiological agent: Infectious hematopoietic necrosis virus (Iridovirus, Ranavirus)

(1) Epidemiological research

- (i) Host range: EHN occurs spontaneously in redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*). In addition, silver perch (*Bidyanus bidyanus*) and Atlantic salmon (*Salmo salar*) are also susceptible in transmission experiments.
- (ii) Geographical distribution: Australia
- (iii) The fish in question have been imported from the aforementioned area.
- (iv) The fish farm has a history of introduction of fish from the aforementioned area.
- (v) Redfin perch can be severely infected with the disease from larvae to adults. Rainbow trout is relatively tolerant to the disease and only juveniles are affected.
- (vi) Disease occurs at 11 to 17°C, although redfin perch does not show clinical signs at temperatures of 12°C or lower.

(2) Clinical observation

- Redfin perch

- (i) Ataxia and decreases in the number of respirations are seen.
- (ii) Marked reddening in the brain and nostrils with surrounding area are seen in adult fish.
- (iii) Whitened muscle in the peduncle is observed in fry.
- (iv) Petechial hemorrhage is seen in the base of fins (in particular, the anal fin).
- (v) Congestion is observed in the gills.

- Rainbow trout

- (i) Severe ataxia, body darkening, and anorexia occur.
- (ii) Ulcers are observed on the skin of the latter half of the body.
- (iii) Minor abdominal distention and protrusion of anal area are seen with O+ fish.

(3) Anatomical findings

- Redfin perch

- (i) White spots of 1 to 3 mm in diameter are seen in the liver of adult fish, although it is difficult to find these lesions in fry.
- (ii) Discoloration and swelling of the spleen. The spleen often becomes gelatinous in fry.
- (iii) Hyperemia in a wide range of peritoneum (the swim bladder, and in particular, around the kidney).

- Rainbow trout

- (i) Swelling of the kidney. The surface of the kidney may bulge irregularly.
- (ii) Swelling and discoloration of the spleen.

(4) Diagnosis

(i) Initial diagnosis: (a) Virus isolation

Cells to be used: BF-2 cells

Inoculum: Homogenate of the kidney or other tissues.

Culture condition: at 22°C for 14 days.

Results: Confirm CPE characterized by scattered rounding cells.

(b) and (c) PCR-restriction enzyme analysis.

- PCR

Materials: DNA extracted from culture supernatant used for virus isolation (for b) or DNA from the liver, kidney, or spleen (for c).

Primers: MCP-1 set

M151: 5'-AAC-CCG-GCT-TTC-GGG-CAG-CA-3'

M152: 5'-CGG-GGC-GGG-GTT-GAT-GAG-AT-3'

Amplicon size: 321 bp

Reaction: Denature at 94°C for 3 minutes, then conduct 35 cycles of incubation at 94°C for 30 seconds, at 50°C for 30 seconds, and at 72°C for one minute. Finally, incubate at 72°C for 5 minutes.

- Restriction enzyme analysis

Materials: Aforementioned PCR amplicon

Restriction enzyme: *Pfl* MI

Reaction: Two hours at 37°C with attached buffer

Results: Confirm that the obtained cleavage pattern agrees with the description in the OIE diagnosis manual.

(ii) Confirmatory diagnosis: (a) Virus isolation

The same as the initial diagnosis.

(b) PCR-REA

- PCR

Materials: DNA extracted from the culture supernatant of (4) (ii) (a).

Primers: MCP-2 set

M153: 5'-ATG-AAC-GTC-GCC-CTC-ATC-AC-3'

M154: 5'-CCA-TCG-AGC-CGT-TCA-TGA-TG-3'

Amplicon size: 625 bp

Reaction: Denature at 94°C for 3 minutes, then conduct 35 cycles of incubation at 94°C for 30 seconds, at 50°C for 30 seconds, and at 72°C for one minute. Finally, incubate at 72°C for 5 minutes.

- Restriction enzyme analysis

Materials: Aforementioned PCR amplicon

Restriction enzymes: *Hinc* II, *Acc* I, *Fnu* 4H I

Reaction: Two hours at 37°C with attached buffer

Results: Confirm that the obtained cleavage patterns agree with the descriptions for the enzymes in the OIE diagnosis manual.

(c) PCR and sequence analysis

- PCR

Materials: DNA extracted from the culture supernatant of (4) (ii) (a).

Primers:

Forward primer: 5'-CGCAGTCAAGGCCTTGATGT-3'

Reverse primer: 5'-AAAGACCCGTTTTGCAGCAGCAAAC-3'

Amplicon size: 580 bp

Reaction: Denature at 95°C for 1 minute, then conduct 35 cycles of incubation at 95°C for 1 minute, at 55°C for 1 minute, and at 72°C for 1 minute. Finally incubate at 72°C for 15 minutes.

- Sequence analysis

Materials: Aforementioned PCR amplicon

Results: Confirm the identity of the nucleotide sequence of the amplicon with the sequence of MCP gene of EHNV on the database.

(5) Histopathological observation

- Redfin perch

(i) Necrosis in the hematopoietic tissue of kidney, which is particularly severe in the pronephros.

(ii) Necrotic foci along the arterioles and veins in the liver. Vacuoles and basophilic inclusion bodies are observed in hepatic cells surrounding those areas.

(iii) Varying degree of necrotic lesions in the spleen.

(iv) Minor edema in the myofibrils of whitened skeletal muscle.

- Rainbow trout

(i) Necrosis in the hematopoietic tissue of kidney.

(ii) Necrotic foci along the arterioles and veins in the liver. Vacuoles and basophilic inclusion bodies are observed in hepatic cells surrounding those areas.

(iii) Varying degree of necrotic lesions in the spleen.

(iv) Necrotic foci in the gastrointestinal epithelium.

(6) Differential diagnosis

EHN has similar symptoms, such as darkening body color, petechial hemorrhage in the base of fins, to those of infectious hematopoietic necrosis (IHN), although exophthalmos, abdominal distention, or ascites are not seen with EHN. Since the differentiation from diseases with similar symptoms is sometimes difficult, it is preferable to implement virus isolation following the anatomical observation. Direct PCR from the organs of affected fish may be adopted, however, when the situation of the disease outbreak requires rapid diagnosis.

(7) Disinfection

Ordinary disinfection method for viruses shall be used for disinfection of hands and equipment.

(8) Other

(i) When the amount of aetiological agent is small, virus isolation by cell culture is more sensitive than PCR. Therefore, cell culture should be adopted to detect the virus in fish without clear clinical signs, such as for targeted surveillance.

(ii) Even if obvious CPE is not observed in the confirmatory diagnosis of (4) (ii) (a), PCR should be conducted as described in (4) (ii) (b) or (c).

(4) Piscirickettsiosis

| Responsible organization | Diagnosis flow chart | | |
|--|---|---|---|
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] B --- C["(3) Anatomical observation"] C --- D["(4) (i) (a) Observation of Giemsa-stained smear"] D -- "(+, ±)" --> E["(4) (i) (b) PCR"] D -- "(-)" --> F["(Investigation as unknown disease)"] E -- "(+, ±)" --> G["(4) (ii) PCR"] E -- "(-)" --> F G -- "(+)" --> H["(+)", "Final Result"] G -- "(-)" --> I["(-)", "Final Result"] F --> J["(-)", "Final Result"] </pre> | | |
| National Research Institute of Aquaculture | | | |
| Results | + | - | - |
| Remarks | When an occurrence of the disease is detected in Japan, the bacterial isolation with fish cell lines, should also be conducted to compare the isolate with known <i>Piscirickettsia</i> strains. | | |

Disease: Piscirickettsiosis

Aetiological agent: *Piscirickettsia salmonis* (*Piscirickettsia*, *Piscirickettsia*)

(1) Epidemiological research

- (i) Host range: Salmonid fishes, such as coho salmon (*Oncorhynchus kisutch*), chinook salmon (*O. tshawytscha*), rainbow trout (*O. mykiss*), and Atlantic salmon (*Salmo salar*) can be infected with the causative agent. Coho salmon is especially susceptible to the disease. Other than salmonids, the causative bacterium has been isolated from European sea bass (*Dicentrarchus labrax*).
- (ii) Geographical distribution: Chile, Norway, Ireland, Scotland, Pacific and Atlantic coast in Canada, and the Pacific coast (sea bass) of the U.S.A.
- (iii) The fish in question has been imported from one of the above countries or regions.
- (iv) The fish farm has a history of introducing fish from one of the above countries or regions.
- (v) The disease usually occurs in the fish cultured in the sea. The disease can also occur, although rare, in the fish cultured in freshwater.

(2) Clinical observation

- (i) Whitish lesions or shallow ulcers with hemorrhage can be seen on the skin.
- (ii) Darkening of the body and sluggish swimming.

(3) Anatomical findings

- (i) White/yellow lesions in the liver under the serous membrane are characteristic for this disease. However, only 5-10% of the affected fish exhibit this symptom and many infected fish, even severely affected ones, do not have this lesion.
- (ii) Pale gills, peritonitis, and ascites.
- (iii) Slightly swollen spleen, discoloration and swelling of the kidney.

(4) Diagnosis

- (i) Initial diagnosis: (a) Observation of Giemsa-stained smear

Giemsa-stained smear preparations are prepared from the liver or kidney.

- (b) PCR

Materials: DNA extracted from the tissues where many bacteria are observed in (a).

Primers:

PSAL-F: 5'-AGA-CCT-GAG-GGT-TAA-AGA-GGG-C -3'

PSAL-R: 5'-TCT-CAG-GTT-CGC-TCC-ACA-TC-3'

Amplicon size: 1108 bp

Reaction: Denature at 94°C for 3 minutes, then conduct 38 cycles of incubation at 94°C for 30 seconds, at 65°C for 20 seconds, and at 72°C for one minute and 30 seconds. Finally, incubate at 72°C for 5 minutes.

- (ii) Confirmatory diagnosis: PCR

In addition to the same PCR as the initial diagnosis of (4) (i) (b),

the following PCR is conducted.

Materials: DNA extracted from tissues where many bacteria are observed in (4) (i) (a).

Primers:

RTS1: 5'-TGA-TTT-TAT-TGT-TTA-GTG-AGA-ATG-A -3'

RTS4: 5'-ATG-CAC-TTA-TTC-ACT-TGA-TCA-TA-3'

Amplicon size: 283 bp

Reaction: Denature at 94°C for 2 minutes, then conduct 39 cycles of incubation at 94°C for 30 seconds, at 50°C for 30 seconds, and at 72°C for 30 seconds. Finally, the reaction tube is held at 72°C for 7 minutes.

(5) Histopathological findings

The causative bacterium can be observed in the cytoplasm of host cells in many organs.

(6) Differential diagnosis

The coinfection with bacterial kidney disease (BKD) is frequently found. They can be distinguished from each other by the morphological differences in the bacterial cells in Giemsa-stained preparations or by PCR.

(7) Disinfection

To disinfect facilities, equipment, or hands and fingers, ordinary disinfectants against bacteria, such as iodine, chlorine, alcohol, or invert soap, can be used.

(8) Other

In order to compare the bacterial strains with known *Piscirickettsia* strains, isolation of the bacterium with cell culture is desirable.

(5) Enteric redmouth disease

| Responsible organization | Diagnosis flow chart | | | |
|--|--|---|---|---|
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- J1(()) B["(2) Clinical observations"] --- J1 J1 --- C["(3) Anatomical observation"] C --- D["(4) (i) (a) Bacterial isolation with TS agar"] D --- E["(4) (i) (b) Biochemical analyses"] E --- F(()) F --- G["(+, ±)"] F --- H["(-)"] G --- I["(4) (i) (c) PCR for isolated colonies"] I --- J(()) J --- K["(+, ±)"] J --- L["(-)"] K --- M["(4) (ii) PCR for isolated colonies"] L --- M M --- N(()) N --- O["(+)"] N --- P["(-)"] O --- Q["+"] P --- R["-"] H --- R L --- R </pre> | | | |
| National Research Institute of Aquaculture | | | | |
| Results | + | - | - | - |
| Remarks | | | | |

(Note: Gram stain and cytochrome oxidase test shall be conducted in the biochemical analyses.)

Disease: Enteric redmouth disease

Aetiological agent: *Yersinia ruckeri* (Enterobacteriaceae, *Yersinia*)

(1) Epidemiological research

- (i) Host range: Although most of the salmonid fishes are susceptible to the disease, the frequency of disease outbreaks and damage of rainbow trout (*Oncorhynchus mykiss*) are especially large. Other than salmonids, the causative bacterium has been isolated from various fish species, including eel (*Anguilla anguilla*), goldfish (*Carassius auratus*), carp (*Cyprinus carpio*), largemouth bass (*Micropterus salmoides*), Amur sturgeon (*Acipenser schrencki*), Siberian sturgeon (*Acipenser baerii*), catfish (*Ictalurus punctatus*), and perch (*Perca fluviatilis*).
- (ii) Geographical distribution: U.S.A., Canada, Chile, Venezuela, European countries, Iran, South Africa, Australia, New Zealand, Turkey, and Japan
(In Japan, the disease occurred only once at a hatchery in 2015.)
- (iii) The fish in question has been imported from one of the areas mentioned above.
- (iv) The fish farm has a history of the introduction of fish from one of the areas mentioned above.
- (v) The disease often occurs from spring through summer when water temperature is rising.

(2) Clinical observation

- (i) Lethargy, Skin darkening.
- (ii) Reddening or hemorrhage in the snout, oral cavity, lower jaw, and base of fins.

(3) Anatomical findings

- (i) Hemorrhage in the internal organs, such as the liver, fat, distal part of intestine, and mesentery.
- (ii) Spleen hypertrophy.
- (iii) Presence of yellowish mucus in the distal part of the intestine or in feces.

(4) Diagnosis

- (i) Initial diagnosis: (a) Bacterial isolation with trypticase soy agar

The colony of the causative bacterium, *Yersinia ruckeri* is, round, milky-white, and translucent, with smooth surface and entire margin. The colonies are usually formed within a few days at 22-25°C.

- (b) Biochemical tests for the bacterium: *Y. ruckeri* is negative for gram stain and negative for cytochrome oxidase.

- (c) PCR

Materials: DNA extracted from the colony.

Primers:

YER3: 5'-CGA-GGA-GGA-AGG-GTT-AAG-T-3'

YER4: 5'-AAG-GCA-CCA-AGG-CAT-CTC-T-3'

Amplicon size: 588 bp

Reaction: Denature at 94°C for 5 minutes, then conduct 30 cycles of

incubation at 94°C for 40 seconds, at 60°C for 40 seconds, and at 72°C for one minute. Finally incubate at 72°C for 5 minutes.

(ii) Confirmatory diagnosis: PCR

Materials: DNA extracted from the colony.

Primers:

ruck1: 5'-CAG-CGG-AAA-GTA-GCT-TG-3'

ruck2: 5'-TGT-TCA-GTG-CTA-TTA-ACA-CTT-AA-3'

Amplicon size: 409 bp

Reaction: Denature at 94°C for 5 minutes, then conduct 35 cycles of incubation at 94°C for 30 seconds, at 55°C for 30 seconds, and at 72°C for one minute. Finally incubate at 72°C for 5 minutes.

(5) Histopathological findings

Many short rods are observed in the kidney, spleen, and liver.

(6) Differential diagnosis

Although clinical signs such as darkening of body color, hemorrhage in the base of fins or intestine, and enlarged spleen are also observed in vibriosis or furunculosis, red mouth disease can be distinguished from these by cytochrome oxidase test, or by PCR.

(7) Disinfection

Ordinary disinfection methods against bacteria are applicable, such as iodine, chlorine, alcohol, or invert soap.

(8) Other

N/A

(6) Whirling disease

| Responsible organization | Diagnosis flow chart |
|--|---|
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] A --- B B --- C["(3) Anatomical observation"] C --- D["(4) (i) (a) Observation of digested cartilage"] C --- E["(4) (i) (b) PCR"] D --- F["(-)"] D --- G["(+, ±)"] E --- H["(+, ±)"] E --- I["(-)"] </pre> |
| National Research Institute of Aquaculture | <pre> graph TD J["(4) (ii) (a) Histopathology"] --- K["(-)"] J --- L["(+)"] M["(4) (ii) (b) Nested-PCR"] --- N["(+)"] M --- O["(-)"] </pre> |
| Results | <p>- - + + - -</p> |
| Remarks | <p>Either the observation of digested cartilage or PCR shall be conducted as the initial diagnosis conducted by prefectural government. Either histopathology or nested-PCR shall be conducted as the confirmatory diagnosis conducted by NRIA.</p> |

Disease: Whirling disease

Aetiological agent: *Myxobolus cerebralis* (Cnidaria, Myxosporidia, Bivalvulid, Myxobolidae, Myxobolus)

(1) Epidemiological research

- (i) Host range: Many salmonid fish can become hosts of this parasite. Brown trout (*Salmo trutta*) has relatively lower sensitivity.
- (ii) Geographical distribution: European countries, Russia, and North and South America (however, the disease has not been reported in Canada.)
- (iii) *M. cerebralis* cannot settle in a new location without the presence of tubifex worm (*Tubifex tubifex*), which is the alternate host of the parasite.

(2) Clinical observation

- (i) Skeletal deformity or twisted body.
- (ii) Blackening of caudal peduncle.
- (ii) Swimming in a circular motion.

(3) Anatomical findings

No particular disease signs are reported in the visceral organs.

(4) Diagnosis

- (i) Initial diagnosis: (a) Observation of enzymatically digested cartilage

Materials: Cartilage is sampled after processing fresh head tissue (for large fish, part of the head can be used) in a water bath at 45°C until tissues become soft.

Pepsin digestion: Digest (few hours to overnight) bone tissues until the samples is reduced to the size of sand grains by 0.5% pepsin solution at 37°C while keeping it at pH4 or lower and stirring and obtain pellet after centrifugation at 1200g for 10 minutes.

Trypsin digestion: Digest the aforementioned pellet by stirring in 0.5% trypsin-PBS (pH8.5) for 30 minutes at room temperature, remove large tissue pieces by filtering the digested material through a filter (mesh size should be about 200 micrometers), and obtain pellet after centrifugation at 1200g for 10 minutes. For tissues collected from frozen fish, trypsin concentration should be 0.05%.

Centrifugal concentration by dextrose (D-glucose): This procedure is conducted when spores are not detected although whirling disease is strongly suspected, or when the detection of spores by microscopy is difficult due to the presence of tissue debris. Frozen samples should not be used. Place 55% dextrose in a centrifuge tube to the depth of 5cm, overlay trypsin digested pellet that is suspended in 1 mL PBS on the dextrose.

Centrifuge the tube with 1200g for 30 minutes to obtain pellet.
Observation of spores: Suspend the aforementioned pellet in PBS, the amount of which is 10 times the amount the pellet and observe spores on a glass slide or a hemocytometer.
Observation can also be conducted after simple staining with a dye, such as crystal violet.

(b) PCR

Materials: Cartilage of head collected for PCR or enzymatically digested cartilage prepared in (4) (i) (a).

Primers:

Tr5-16: 5'-GCA-TTG-GTT-TAC-GCT-GAT-GTA-GCG-A-3'

Tr3-16: 5'-GAA-TCG-CCG-AAA-CAA-TCA-TCG-AGC-TA-3'

Amplicon size: 1300 bp

Reaction: Denature at 95°C for 5 minutes, then, conduct 35 cycles of incubation at 95°C for 1 minute, at 65°C for 2 minutes and 30 seconds, and at 72°C for 1 minute and 30 seconds. Finally, the reaction mixture is held at 72°C for 10 minutes.

(ii) Confirmatory diagnosis: (a) Histopathology

Materials: The head fixed in Davidson-solution. The sample include the gills and base of the skull. For a large fish, each tissue should be fixed separately.

Preparation: Tissues are decalcified as necessary, routinely embedded in paraffin for sectioning. The sections are stained with hematoxylin and eosin or with May-Gruenwald-Giemsa stain, and observed by a microscope. and confirm the histopathological features described in (5).

(b) Nested-PCR

i) 1st PCR: The same as the initial diagnosis of (4) (i) (b).

ii) 2nd PCR

Materials: Amplicon of the 1st PCR of (4) (ii) (b), i).

Primers:

Tr5-17:

5'-GCC-CTA-TTA-ACT-AGT-TGG-TAG-TAT-AGA-AGC-3'

Tr3-17:

5'-GGC-ACA-CTA-CTC-CAA-CAC-TGA-ATT-TG-3'

Amplicon size: 415 bp

Reaction: The same as the 1st PCR of (4) (ii) (a), i).

(5) Histopathological findings

Myxozoan spores that can be considered to be *Myxobolus cerebralis* morphologically are observed in the cartilage.

(6) Differential diagnosis

Many Myxozoan species parasitize salmonid fishes other than *M. cerebralis*. They are difficult to identify by the spore morphology alone. No myxozoan parasite proliferates in the cartilage, however, other than *M. cerebralis*.

(7) Disinfection

- (i) Cleaning and eliminating soil and organic compounds.
- (ii) Processing for 10 minutes at 90°C or higher.
- (iii) Drying for 24 hours or longer. Sun-drying is ideal.
- (iv) Disinfection with 500ppm sodium hypochlorite (NaClO) for 10 minutes.
- (v) Disinfection with quaternary ammonium compound (1500ppm) for 10 to 15 minutes.

(8) Other

PCR inspection of tubifex worms or the exposure of susceptible fish fry to the water in question for a specified period are conducted for the monitoring or surveillance of natural waters in the U.S.A.

Disease: Spring viremia of carp

Aetiological agent: Spring viremia of carp virus (Rhabdoviridae, Vesiculovirus)

(1) Epidemiological research

- (i) Host range: Carp (*Cyprinus carpio*), crucian carp (*Carassius* spp.), goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*), spotted silver carp bighead (*H. Nobilis*), and sheatfish (*Silurus glanis*) are known.
- (ii) Geographical distribution: European countries, former Soviet Union, Brazil, U.S.A., Canada, China, Iran, and Egypt.
- (iii) The fish in question has been imported from one of the countries mentioned above.
- (iv) The fish farm has a history of introducing fish from one of the countries mentioned above.
- (v) The disease often occurs in spring when the water temperature is rising (up to 15°C). Although no mortality is seen when the water temperature is over 23°C, the virus may be retained in fish body.

(2) Clinical observation

- (i) Abnormal swimming and decreases in swimming capability.
- (ii) Abdominal distention and exophthalmia.
- (iii) Petechial hemorrhage in gills and body surface.

(3) Anatomical findings

- (i) Accumulation of ascites (transparent or hemorrhagic).
- (ii) Petechial hemorrhage in liver, kidney, heart, intestine, visceral adipose tissue.
- (iii) Spleen hypertrophy.

(4) Diagnosis

- (i) Initial diagnosis: (a) RT-PCR (reverse transcription-polymerase chain reaction method)

Materials: RNA extracted from tissue of kidney

Primer:

exSVCV F: 5'-GGA-TAA-TAT-CGG-CTT-GGA-AAG-C-3'

exSVCV R: 5'-GCC-TAA-ATG-TGT-TGA-TGG-AAC-G-3'

Amplicon size: 470 bp

Reverse transcription: Incubation at 50°C for 30 minutes.

PCR reaction: Denature at 94 °C for 2 minutes, then conduct 34 cycles of incubation at 94°C for 15 seconds, 50°C for 30 seconds at, and at 68°C for one minute. Finally incubate at 68°C for 7 minutes.

- (b) Virus isolation

Cell line to be used: EPC

Inoculum: Homogenate of tissues, such as the kidney.

Virus culture: At 20°C for 7 days. If no CPE develops, the inoculated culture should be sub-cultured for further seven days.

Results: CPE characterized by rounding cells or detachment of cells from the substrate.

(c) RT-PCR (reverse transcription-polymerase chain reaction method)

i) 1st RT-PCR

Materials: RNA extracted from the supernatant of cell culture with CPE.

Primers:

SVCVF1:

5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3'

SVCVR2:

5'-AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH*-ACN*-CAY*-3'

R*: A or G; H*: A or C or T

N*: A, C, G, or T; Y*: C or T

Amplicon size: 714 bp

Reverse transcription: Incubation at 50°C for 30 minutes.

PCR reaction: Denature at 94°C for 2 minutes, then conduct 35 cycles of incubation at 94°C for 15 seconds, at 55°C for 30 seconds, and at 68°C for one minute. Finally incubate at 68°C for 7 minutes.

ii) 2nd PCR (this is conducted only when 1st PCR is negative)

Materials: Reacted solution of the 1st PCR.

Primers:

SVCVF1: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3'

SVCVR4:

5'-CTG-GGG-TTT-CCN*-CCT-CAA-AGY*-TGY*-3'

R*: A or G

N*: A, C, G, or T; Y*: C or T

Amplicon size: 606 bp

PCR reaction: Denature at 94 °C for 30 seconds, then conduct 35 cycles of incubation at 94°C for 30 seconds, at 55°C for 30 seconds, and at 72°C for 60 seconds. Finally incubate at 72°C for 7 minutes.

(ii) Confirmatory diagnosis: (a) Virus isolation

Inoculum: Homogenate of tissues such as the kidney of fish that are positive for SVCV with RT-PCR, or virus isolated in the initial diagnosis.

Cell line and culture: The same as the initial diagnosis of (4) (i) (b).

(b) RT-PCR (reverse transcription-polymerase chain reaction method)

The same as the initial diagnosis of (4) (i) (c). Second (nested) PCR should be conducted if the 1st PCR is negative.

(c) Nested-PCR

Materials: The product of the 1st PCR or the 2nd PCR of (4) (ii) (b).

Primers:

SVCV nest F: 5'-TGA-AGA-Y*TG-TGT-CAA-TCA-AGT-C-3'

SVCV nest R: 5'-GCG-AR*T-GCA-GAG-AAA-AAG-TG-3'

Y*: C or TR*: A or G
Amplicon size: 369 bp
PCR reaction: Denature at 94°C for 30 seconds, then conduct 25 cycles of incubation, at 94°C for 30 seconds, at 55°C for 30 seconds, and at 72°C for 30 seconds. Finally incubate at 72°C for 7 minutes.

(5) Histopathological finding

- (i) Inflammation and necrosis of blood vessels in liver.
- (ii) Inflammation and multifocal necrosis in the pancreas.
- (iii) Perivascular inflammation in the intestine.

(6) Differential diagnosis

SVCV is discriminated from pike fry rhabdovirus by the nested-PCR indicated in (4) (ii) (c).

(7) Disinfection (See attached quick reference table.)

Ordinary disinfectants against virus can be used for the disinfection of equipment and hands and fingers.

(8) Other

- (i) For the detection of this virus, virus isolation by cell culture is more sensitive than RT-PCR. Therefore, cell culture should be adopted as the method of targeted surveillance in which fish usually show no clear clinical signs.
- (ii) If the result of RT-PCR of (4) (i) (a) is positive, virus isolation in the initial diagnosis can be omitted. However, some SVC genotypes cannot be amplified by this RT-PCR. Therefore, virus isolation should be conducted if the PCR is negative.
- (iii) Whereas the primers for the RT-PCR of (4) (i) (c) i are designed to amplify most of the SVC genotypes, the sensitivity is considerably low. For this reason, amplification may not be confirmed with RNA extracted directly from fish tissues. Therefore, the RNA should be extracted from cell culture in which the virus has proliferated. When the positive band is not confirmed by 1st RT-PCR of (4) (i) (c) i, the 2nd PCR of (4) (i) (c) ii should be conducted using the amplified material of the 1st RT-PCR as the template. When the positive band cannot be confirmed after the 2nd PCR, the diagnosis is negative. When the positive band is confirmed by the 1st RT-PCR of (4) (i) (c) i, it is not necessary to conduct 2nd PCR.
- (iv) (4) (ii) (c) is a nested-PCR using the amplicon of the 1st or 2nd PCR of (4) (ii) (b) as the template. However, the primer set for this PCR is different from the 2nd PCR indicated in (4) (ii) (b) (which is the same as (4) (i) (c)), and designed so that pike fry rhabdovirus, which is closely related to SVCV, will not be detected.
- (v) In the confirmatory diagnosis, even if clear CPE is not observed by cell culture in (4) (ii) (a), the RT-PCR of (4) (ii) (b) should be conducted.

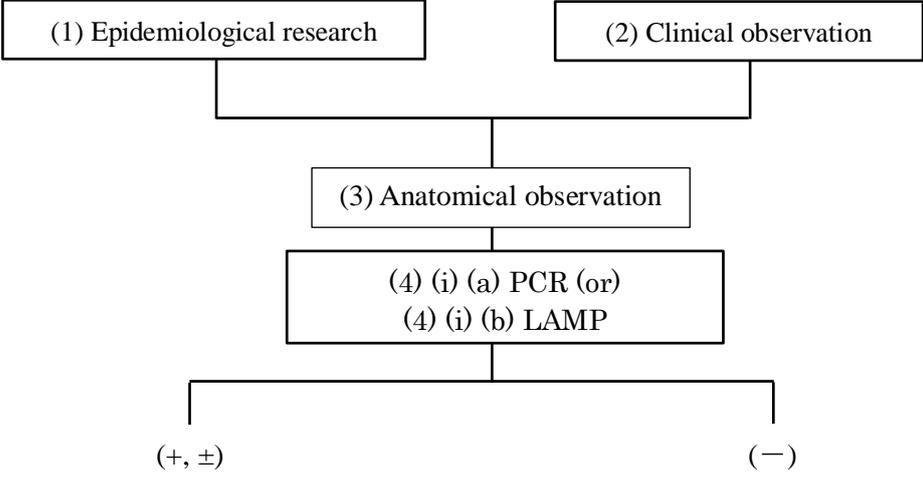
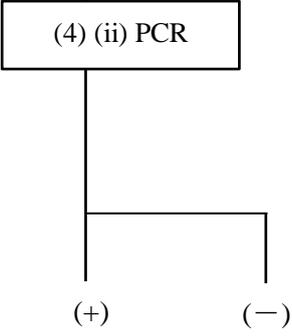
Quick Reference Table of Sterilization Method for Spring Viremia of Carp

| Target of disinfection | Active ingredient | Concentration for use | Renewal of sterilization solution | Ichthyotoxicity | Precautions for use |
|--|--|--|--|---|--|
| Hands | Alkyl toluene | 0.05% (30 seconds) | 2 to 3 days To be changed as soon as possible when the solution becomes dirty. | Yes However, there is no problem as long as solution with a high concentration of the disinfectant does not directly enter the pond. | It is no longer effective when the solution becomes dirty. Ethanol and chlorhexidine gluconate are especially effective when they are sprayed. |
| | Chlorhexidine gluconate | 0.0175% (30 seconds) | | | |
| | Cresol | 0.25% (30 seconds) | | | |
| | Ethanol | 50% (30 seconds) | | | |
| Boots | Alkyl toluene | 0.05% (30 seconds) | 2 to 3 days To be changed as soon as possible when the solution becomes dirty. | Yes | It is no longer effective when the solution becomes dirty. |
| | Chlorhexidine gluconate | 0.0175% (30 seconds) | | Yes | |
| | Cresol | 0.25% (30 seconds) | | Yes | |
| | Available chlorine (bleaching powder, .) | 540ppm (30 seconds) | 2 days in house Everyday outdoors | Extremely strong | *1 |
| Pond (mud bottom) | Available chlorine (bleaching powder, .) | 540ppm (20 minutes) | Whenever used | Extremely strong | *2 |
| Pond (concrete) | Available chlorine (bleaching powder, .) | 540ppm (20 minutes) | Whenever used | Extremely strong | *3 |
| Pond water | Available chlorine (bleaching powder, .) | 540ppm (20 minutes) | Whenever used | Extremely strong | *4 |
| Instruments, equipment, nets, clothing | Benzalkonium chloride | 0.01% (20 minutes) | 2 to 3 days. To be changed as soon as possible when the solution becomes dirty. Disinfectant for nets and clothing shall be discarded each time. | Yes | Nets shall be rinsed with water after disinfection before use. |
| | Alkyl toluene | 0.05% (30 seconds) or 0.035% (20 minutes) | | Yes | |
| | Chlorhexidine gluconate | 0.0175% (30 seconds) or 0.01% (20 minutes) | | Yes | |
| | Cresol | 0.25% (30 seconds) or 0.02% (20 minutes) | | Yes | |
| | Boiling water | 56°C or higher (30 minutes) | | | |
| Vehicles | Alkyl toluene | 0.05% (30 seconds) or 0.035% (20 minutes) | Whenever used | Yes | Spray sterilization solution on non-visible areas, such as the back of tires. |
| | Chlorhexidine gluconate | 0.0175% (30 seconds) or 0.01% (20 minutes) | | Yes | |
| | Cresol | 0.25% (30 seconds) or 0.02% (20 minutes) | | Yes | |

Based on OEI Manual (2015), Kiryu *et al.* (2007) Fish Pathol., 42, 111-113, or literature cited by these documents.

- *1: The disinfectant is no more effective if there is no odor of chlorine. Place a cover on the disinfection bath outdoors in order to prevent light. Since it has strong bleaching power, avoid contact with body or net. According to OIE Manual, disinfection at 540 ppm for 20 minutes is recommended, although it is not practical for the disinfection of boots. Considering the data that 99 to 99.9% of the virus is inactivated by disinfection at 7.6 ppm for 20 minutes (Ahne, W. 1982, Zbl. Vet. Med. B, 29, 457-476), boots that have no dirt with organic compounds on the surface can be disinfected almost completely at 540 ppm for 30 seconds.
- *2: Place water with bleaching powder, for which the available chlorine concentration is 540ppm, so that it covers the mud at the bottom. Residual chlorine will disappear within a few days. Confirm the disappearance of chlorine with commercially available reagents or neutralize chlorine with two times of HYPO (Sodium Thiosulfate Hydrate) to the water containing bleaching powder before discharging. Do not conduct disinfection on rainy days.
- *3: Discharge water from the pond and spray disinfectant by using a watering-pot or other instruments. Check that there is no residual chlorine when discharging water. Do not conduct disinfection on rainy days. For indoor ponds, dissolve HYPO (Sodium Thiosulfate Hydrate) of two times the amount of the bleaching powder into water and spray it to neutralize the chlorine.
- *4: After dissolving bleaching powder in pond water so that it reaches the specified concentration, leave it for a few days, and discharge the water after residual chlorine disappears by sunlight. For indoor ponds, neutralize it with HYPO (Sodium Thiosulfate Hydrate) of two times the amount of the bleaching powder, before discharging the water. Bleaching powder has strong ichthyotoxicity, and water should be discharged after confirming that it is free of residual chlorine with test reagents.

(8) Koi herpesvirus disease

| Responsible organization | Diagnosis flow chart |
|--|--|
| Prefectural government |  <pre> graph TD A["(1) Epidemiological research"] --- B C["(2) Clinical observation"] --- B B --- D["(3) Anatomical observation"] D --- E["(4) (i) (a) PCR (or) (4) (i) (b) LAMP"] E --- F["(+, ±)"] E --- G["(-)"] </pre> |
| National Research Institute of Aquaculture |  <pre> graph TD F["(+, ±)"] --- H["(4) (ii) PCR"] H --- I["(+)"] H --- J["(-)"] </pre> |
| Results | <p style="margin-left: 100px;">+</p> <p style="margin-left: 200px;">-</p> <p style="margin-left: 300px;">-</p> |
| Other | <p>When there is a person whose skill level of PCR for the diagnosis of KHV has been confirmed to be sufficient by the proficiency test provided by the National Research Institute of Aquaculture (NRIA) in the prefectural fisheries research station (prefectural government), the person may carry out confirmatory diagnosis instead of the NRIA.</p> |

Disease: Koi herpesvirus disease

Aetiological agent: Cyprinid herpesvirus 3 (*Alloherpesviridae*, *Cyprinivirus*)

(1) Epidemiological research

- (i) Host range: Carp (*Cyprinus carpio carpio*) and Koi carp (*Cyprinus carpio koi*)
- (ii) Geographical distribution: Israel, European countries, U.S.A., Indonesia, Thailand, Philippines, Taiwan, and Japan
- (iii) The disease occurs at water temperatures of 20 to 25°C.
- (iv) The fish in question has been imported from an area where outbreaks of this disease have been confirmed, or the animal has come in contact with fish imported from such an area.
- (v) The fish farm has a history of the introduction of fish from an area where an outbreak of this disease has been confirmed, or the farm has introduced fish that have a history of contacting with imported fish from such an area.
- (vi) There is a possibility that the water of the fish farm is contaminated with wastewater from such a fish farm described in (iv) or (v).

(2) Clinical observation

- (i) Behavior: Abnormal swimming, such as slow swimming, loss of balance, is observed.
- (ii) External appearances: The most distinctive lesions are discoloration, erosion, focal necrosis, and adhesion of secondary lamellae in the gills. In addition, excess mucus on body surface, congestion or bleeding at the bases of fins, sinking eyeballs are observed.
- (iii) Microscopic examination of body surface: Secondary infections of protozoan parasites, such as *Ichthyobodo*, *Trichodina*, or columnaris bacteria, are often observed.

(3) Anatomical findings

Although there no pathognomonic lesion is known for the disease, adhesion of visceral organs is often observed.

(4) Diagnosis

(i) Initial diagnosis: (a) PCR

Materials: DNA extracted either from the gills, kidney, or spleen.

Primers:

KHV SphI-5 F: 5'-GAC-ACC-ACA-TCT-GCA-AGG-AG-3'

KHV SphI-5 R: 5'-GAC-ACA-TGT-TAC-AAT-GGT-CGC-3'

Amplicon: 292 bp

Reaction: Denature at 94°C for 30 seconds, then conduct 40 cycles of incubations at 94°C for 30 seconds, 63°C for 30 seconds, and 72°C for 30 seconds. Subsequently, the reaction is held at 72°C for 7 minutes.

(b) LAMP

Materials: DNA extracted either from the gills, kidney, and spleen.

Primers:

KHV-FIP:

5'-CCC-AAA-CCC-AAG-AAG-CAG-AAA-CCC-GTT-GCC-TGT-AGC-ATA-GAA-GA-3'

KHV-BIP:

5'-CAC-TCC-TCC-GAT-GGA-GTG-AAA-CTG-CCC-ATG-TGC-AAC-TTT-G-3'

KHV-F3: 5'-CTG-TAT-GCC-CGA-GAG-TGC-3'

KHV-B3: 5'-AAC-TCC-ATC-GCC-GTC-ATG-3'

KHV-LF: 5'-CCC-GCC-GCC-GCA-3'

KHV-LB: 5'-TGG-AAC-TGT-CTG-ATG-AGC-GT-3'

Reaction: Incubation at 65°C for 60 minutes.

Results: Confirm the white turbidity in the reaction solution either by the naked eye.

(ii) Confirmatory diagnosis: PCR

The following PCR shall be conducted in addition to the same PCR for the initial diagnosis.

Materials: DNA extracted either from the gills, kidney, or spleen.

Primers:

KHV TK F: 5'-GGG-TTA-CCT-GTA-CGA-G-3'

KHV TK R: 5'-CAC-CCA-GTA-GAT-TAT-GC-3'

Amplicon: 409 bp

Reaction: Denature at 94°C for 5 minutes, then conduct 40 cycles of incubations at 95°C for 1 minutes, 55°C for 1 minute, and 72°C for 1 minute. Subsequently, the reaction is held at 72°C for 10 minutes.

(5) Histopathological findings

The most characteristic change is hyperplasia, hypertrophy, and focal necrosis of gill epithelial cells. Chromatin margination and pale eosinophilic intranuclear inclusion is seen in the cells of gills or other organs, although this disease cannot be diagnosed by these findings alone.

(6) Differential diagnosis

This disease is often accompanied by the erosion or necrosis of the gills or secondary infections of bacteria, fungi, or protozoa. In particular, it is difficult to distinguish this disease from columnaris disease by clinical inspection and coinfection with *Flavobacterium columnare* is often seen. When adult fish show high mortality and the koi herpesvirus disease is suspected from the clinical signs, the diagnosis with PCR should be conducted.

(7) Disinfection (See attached quick reference table.)

(i) Sterilization of water for aquaculture and drainage

(a) Sterilization

Ultraviolet rays: $4,000\mu\text{W}\cdot\text{sec}/\text{cm}^2$

(b) Sterilization method

- Farming water can be sterilized by 15W UV light at 1L/second.
- Use a conduit-type water flow pathway and suspend a UV light above.
- Water depth shall be 5 cm or less and the distance from the center of the UV light to the bottom of the conduit shall be 10 cm.

(c) Notes

- Pay attention to the life of the UV light and change it frequently.
- Water should be cleaned by a settling tank or other means, since sterilization would be insufficient if the water is muddy or contain any suspended materials.
- For the penetration of ultraviolet rays, avoid disturbing water surface.
- Prevent ultraviolet rays from getting into operators' eyes directly.

(ii) Disinfection of farming ponds

(a) Disinfectant

Chlorine agent with 200ppm of available chlorine (high test bleaching powder, sodium hypochlorite solution)

(b) Disinfection method

- Disinfection should be conducted for 30 minutes to one hour.
- Decrease water depth to 10 cm to 20 cm and spray chlorine agents to achieve the aforementioned concentration.
- A solid chlorine agent is dissolved in water and sprayed.
- The pond wall should be evenly sprayed with a disinfectant at 200 ppm of available chlorine concentration.

(c) Notes

- Chlorine-based disinfectant has strong skin irritancy and corrosivity. Therefore, ensure the use of mask, gloves, eyeglasses, and raincoat during disinfection so that the agent will not touch the body directly.
- When discarding fluid waste after disinfection, ensure to neutralize it with sodium thiosulfate, check residual chlorine by using commercial test kit before discharging the fluid.
- Groundwater shall be directly poured as farming water for aquaculture after disinfection of the pond. Do not use water from a river that may be contaminated with a virus or water from a pond where fish have been reared.

(iii) Disinfection of wastewater

(a) Disinfectant

Chlorine with 3 ppm of available chlorine (high test bleaching powder, sodium hypochlorite solution)

(b) Disinfection method

- Disinfection should be conducted for 30 minutes to one hour.

- Spray chlorine agent to achieve the aforementioned concentration and mix it. The concentration of the chlorine should be prepared so that 15 ppm of chlorine concentration is achieved in pond water, since the chlorine is consumed by organic compounds in the water.

(c) Notes

- Chlorine-based disinfectant has strong skin irritancy and corrosivity. Therefore, ensure the use of mask, gloves, eyeglasses, and raincoat during disinfection so that agents will not touch the body directly.
- When discarding fluid waste after disinfection, ensure to neutralize it with sodium thiosulfate, check residual chlorine by using commercial test kit before discharging the fluid.

(a) Disinfectant

Benzalkonium chloride 0.1% (invert soap)

Chlorine agent at 200 ppm of available chlorine (high test bleaching powder, sodium hypochlorite solution)

(b) Disinfection method

- Soak tools fully in the disinfectant.
- Wash the tools after disinfection and dry them.

(c) Notes

- Change the disinfectant frequently.
- When benzalkonium chloride becomes dirty due to dirt from the hands, it is no longer effective.
- If chlorine-based disinfectant has no odor, it has no effect.

(v) Disinfection of hands and fingers, and small laboratory instruments

(a) Disinfectant

Benzalkonium chloride 0.1% (invert soap)

Ethanol-based disinfectant 70%

(b) Sterilization method

- Soak fingers and instruments fully in the disinfectant bath.
- Wash the instruments after disinfection and dry them.

(c) Notes

- Change the disinfectant frequently.
- It is effective when ethanol is sprayed when using it.
- When benzalkonium chloride becomes dirty due to dirt from the hands, it is no longer effective. Therefore, it is necessary to change it frequently.

(8) Other

N/A

Quick Reference Table of Disinfection Method for Koi Herpesvirus Disease

| Target of disinfection | Active ingredient | Concentration for use | Renewal of disinfectant | Ichthyotoxicity | Precautions for use |
|----------------------------------|--|-----------------------|---|--|--|
| Hands | Benzalkonium chloride (invert soap, .) | 0.1% | 2 to 3 days To be changed as soon as possible when the solution becomes dirty. | Yes However, it is not effective unless thick liquid enters directly into the pond. | It is no longer effective when the solution becomes dirty due to dirt from the hands. It is effective when ethanol is sprayed when using it. |
| | Ethanol | 70% | | | |
| Boots, Instruments and equipment | Benzalkonium chloride (invert soap, .) | 0.1% | 2 to 3 days To be changed as soon as possible when the solution becomes dirty. | Yes | It is no longer effective when the solution becomes dirty due to dirt from the hands. |
| | Available chlorine (bleaching powder, .) | 200ppm | 2 days indoor Everyday outdoors | Extremely strong | There is no effect if there is no odor of chlorine. Place a cover on the sterilization bath outdoors in order to prevent light. Since it has strong bleaching power, avoid contact with hands or net. |
| Pond (mud bottom) | Available chlorine (bleaching powder, .) | 200ppm | | Extremely strong | Place water in the pond to cover the mud at the bottom and set the available chlorine of the water at 200ppm. Residual chlorine will disappear in a few days; however, be sure to confirm that there is no residual chlorine before discharging the water. |
| Pond (concrete) | Available chlorine (bleaching powder, .) | 200ppm | Depends on frequency of use | Extremely strong | Discharge water of the pond and spray disinfectant by using a watering-pot and other instruments. Be strictly careful with downstream outflow (ensure that there is no residual chlorine). Do not conduct disinfection on rainy days. |
| Pond water | Available chlorine (bleaching powder, .) | 3ppm | | Strong | Place disinfectant in the pond to achieve the specified concentration and stir it well. Keep the specified concentration for 30 minutes or longer while measuring chlorine concentration. Residual chlorine will disappear in a few days; however, be sure to confirm that there is no residual chlorine before discharging the water. |
| Nets, clothing | Benzalkonium chloride (invert soap, .) | 0.1% | Disinfectant shall be discarded each time. | Yes | Nets shall be rinsed with water after disinfection before use. |
| | Boiling water | 60 to 100°C | | | Soak in boiling water for 5 minutes. |
| Vehicles | Benzalkonium chloride (invert soap, .) | 0.1% | Depends on frequency of use | Yes | Spray sterilization solution on places which cannot be seen, such as the other side of tires. |

The table is based on the results of one of the research projects “Research on Agriculture, Forestry and Fisheries Using Advanced Technology” funded by Agriculture, Forestry and Fisheries Research Council. (Fish Pathology, Vol. 40, No. 3, pp137-138)

(9) Glugeosis of red sea bream

| | | | |
|--|--|---|---|
| Responsible organization | Diagnosis flow chart | | |
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] A --- B B --- C["(3) Anatomical observation"] C --- D["(4) (i) Observation of spores"] D --- E["(-)"] D --- F["(+, ±)"] E --- G["-"] F --- H["(4) (ii) Sequence analysis"] H --- I["(+)", "(-)"] I --- J["+", "-"] </pre> | | |
| National Research Institute of Aquaculture | <pre> graph TD H["(4) (ii) Sequence analysis"] --- I["(+)", "(-)"] I --- J["+", "-"] </pre> | | |
| Results | - | + | - |
| Remarks | Either fresh or fixed and stained samples can be used for the observation in the initial diagnosis. Sequence analysis is conducted for the PCR amplicon of SSU rDNA in the confirmatory diagnosis. | | |

Disease: Glugeosis of red sea bream

Aetiological agent: *Glugea pagri* (Microsporidia, Glugeidae, *Glugea*)

(1) Epidemiological research

(i) Host range: Red sea bream (*Pagrus major*). Microsporidia generally have high host specificity and there is low possibility that the disease due to the same aetiological agent exists in other fish species.

(ii) Geographical distribution: Daya Bay in Guangdong Province, China.

(2) Clinical inspection

Although distinctive appearances for the disease have not been reported, severely infected fish become sluggish, lose appetite, and eventually die.

(3) Anatomy Findings

White, round, and large xenomas (cysts, which are infected cells that have been enlarged due to numerous spores in the cell) that may develop into a few millimeters are seen adhering to visceral organs.

(4) Diagnosis

(i) Initial diagnosis: Microscopy of squashed fresh xenomas or fixed smear samples of spores.

Materials: fresh xenomas from visceral organs

Sample preparation: Fresh samples are prepared by squashing xenomas to free spores in saline on a glass slide with a coverslip. The smear samples of released spores are fixed with 100% methanol.

Staining and observation: Fresh samples are observed by a microscope at x 1000 with oil-immersion objective in a bright field. Fixed smear samples are observed after staining with Giemsa solution in phosphate buffer (pH 6.8). Confirm the two types (large and small) of spores: long ovoid spores (average length: 7.9µm × average width: 2.9µm) and ovoid spores (average length: 4.4µm × average width: 2.5µm). The number of large spores is smaller and its percentage is low (8.4%).

(ii) Confirmatory diagnosis: Gene sequence analysis

Materials: DNA extracted from spores by a commercial kit.

Primers:

S1: 5'-ATG-AGA-CGT-GAG-AAA-GAG-TGC-TTG-GTA-AA-3'

A1: 5'-CGC-CGA-CCG-CAA-CCT-TGT-TAC-GAC-TT-3'

Amplicon size: 964 bp

Reaction: Denature at 95°C for 5 minutes, then conduct 30 cycles of incubation at 95°C for 30 seconds, at 55°C for 35 seconds, and at 72°C for 2 minutes. Finally incubate at 72°C for 10 minutes.

Sequence analysis: The nucleotide sequence of the PCR product is determined directly by a DNA sequencer. Alternatively, the PCR product is cloned with *Escherichia coli* and the nucleotide sequences of multiple clones are analyzed to determine the

consensus sequence.

Results: Determine the similarity of sequence with the registered sequence (accession No. JX852026) of small subunit ribosomal DNA (SSU rDNA) for *G. pagri* with the BLAST.

(5) Histopathological findings

Xenomas are formed in various places on the serosa of visceral organs and often invade into submucosa or smooth muscle layers. Cell reaction characterized by the accumulation of eosinophilic granular cells (EGC) is seen particularly in the lamina propria of the intestine.

(6) Differential diagnosis

The aetiological agent, *G. pagri*, is characterized by having two types (large and small) of spores. *G. vincentiae* from Southern Cardinalfish (*Vincentia conspersa*), a marine fish in Australia, is also reported to have two spore types (large and small). However, the size of the small spore of *G. pagri* is smaller and other structures or size of polar tubes in the spores are also different. In addition, *G. pagri* can be distinguished from *G. hetwigi*, which is morphologically most similar to *G. pagri*, by smaller nuclei and the presence of two spore types (large and small). The observation of the structural difference in the spore requires electron microscopy. Another microsporidian, *Pleistophora pagri* is known to infect fish of genus *Pagrus*, although *G. pagri* is easily distinguished from this species by the fact that *G. pagri* has large spores.

(7) Disinfection

No disinfection method is reported.

(8) Other

N/A

2. Crustaceans

(1) Yellow head disease (YHD)

| Responsible organization | Diagnosis flow chart | | |
|--|--|---|---|
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] A --- B B --- C["(3) Anatomical observation"] C --- D["(4) (i) RT-PCR"] D --- E["(-)"] D --- F["(+, ±)"] </pre> | | |
| National Research Institute of Aquaculture | <pre> graph TD G["(4) (ii) Nested-PCR and sequence analysis"] --- H["(+)", "(-)"] </pre> | | |
| Diagnosis | - | + | - |
| Remarks | Histopathological observation should also be conducted, when a suspected case occurs in Japan. | | |

Disease: Yellow head disease

Aetiological agent: Yellow head virus (YHV genotype 1) (Roniviridae, Okavirus)

(1) Epidemiological research

- (i) Host range: Natural infection is reported mainly in black tiger shrimp (*Penaeus monodon*), and also in kuruma shrimp (*Marsupenaeus japonicus*), banana prawns (*Fenneropenaeus merguensis*), northern white shrimp (*P. setiferus*), offshore greasyback prawns (*M. Ensis*). Many penaeid shrimp, such as white leg shrimp (*Litopenaeus vannamei*), blue shrimp (*L. stylirostris*), brown shrimp (*Farfantepenaeus aztecus*), show susceptibility in experimental infections. Although experiments suggest a wide range of susceptible host species, extra caution is required for the transfer of black tiger shrimp (*P. monodon*), in which spontaneous disease occurrences are reported.
- (ii) Geographical distribution: China, India, Indonesia, Malaysia, Philippines, Sri Lanka, Taiwan, Thailand, and Vietnam
- (iii) Susceptible stages of the host: Post larva (PL) 15 or older shrimp tend to be affected by YHV.

(2) Clinical observation

- (i) Discoloration is seen in the whole body.
- (ii) Exceptionally active feeding for a few days followed by an abrupt cessation of feeding.
- (iii) Shrimp may congregate at pond edges near the surface.
- (iv) The cephalothorax of some shrimp becomes pale yellow, which is caused by the yellow discoloration of the underlying hepatopancreas.

(3) Anatomical findings

The body of severely affected shrimp appears yellowish white, with the cephalothorax being often swelled. The Gills, and also sometimes hepatopancreas, become white or light yellow.

(4) Diagnosis

(i) Initial diagnosis: RT-PCR

Materials: RNA extracted from fresh gills or lymphoid organs

Primers:

GY1: 5'-GAC-ATC-ACT-CCA-GAC-AAC-ATC-TG-3'

GY4: 5'-GTG-AAG-TCC-ATG-TGT-GTG-AGA-CG-3'

Amplicon size: 794 bp

Reaction: One-step RT-PCR is carried out with a commercial kit. Conduct Reverse transcription at 50 °C for 30 minutes. Denature at 94 °C for 2 minutes, then conduct 35 cycles of incubation at 95 °C for 30 seconds, at 66 °C for 30 seconds, and at 68 °C for 45 seconds. Finally incubate at 68 °C for 7 minutes.

(ii) Confirmatory diagnosis: Nested- PCR

i) 1st PCR: The same as the initial diagnosis described in (4) (i).

ii) 2nd PCR

Materials: The amplicon of the 1st PCR in (4) (ii), i)

Primers:

GY2: 5'-CAT-CTG-TCC-AGA-AGG-CGT-CTA-TGA-3'

Y3: 5'-ACG-CTC-TGT-GAC-AAG-CAT-GAA-GTT-3'

Amplicon size: 277 bp

Reaction: Denature for at 95 °C for 15 minutes, then conduct 35 cycles of incubation at 95 °C for 30 seconds, at 66 °C for 30 seconds, and at 72 °C for 45 seconds. Finally incubate at 72 °C for 7 minutes. Conduct sequence analysis of amplicon and confirm the virus genotype.

(5) Histopathological findings

Severe necrosis is scattered in tissues, and pyknosis and karyorrhexis are seen in cell nuclei. Many globular cytoplasmic inclusions with diameter of 2µm or smaller are seen particularly in the lymphoid organs, hematopoietic tissues, gills, subcutaneous tissues, muscle, intestine, gonads, and other organs of ectodermal or mesodermal origin. The inclusion bodies are strongly basophilic and homogeneously stained.

(6) Differential diagnosis

YHV is classified in family Roniviridae, and genus Okavirus. Eight genotypes are known in yellow head complex virus group. YHV is the genotype 1 and aetiological agent of yellow head disease. Gill-associated virus (GAV), which is the genotype 2, and four other genotypes (3 to 6) are generally detected in healthy black tiger shrimp in East Asia, Asia and Australia, though they have almost no relationship with the yellow head disease. Aetiological agent of genotype 7 is unknown. Genotype 8 has been detected in diseased shrimp. YHV and GAV are similar viruses, but they can be distinguished by RT-PCR.

Histopathological findings of YHD are similar to those of Taura syndrome (TS), but they can also be distinguished by RT-PCR.

(7) Disinfection

(i) YHV can be inactivated by heat treatment at 60°C for 15 minutes.

(i) YHV can be inactivated by chlorination of 30ppm (0.03mg/mL).

(8) Other

No effective prevention and treatment methods are known.

(2) Necrotising hepatopancreatitis (NHP)

| | | | |
|--|---|---|---|
| Responsible organization | Diagnosis flow chart | | |
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] A --- B B --- C["(3) Anatomical observation"] C --- D["(4) (i) PCR"] D --- E["(+, ±)"] D --- F["(-)"] E --- G["(4) (ii) Real-time PCR"] G --- H["(+)", "(-)"] H --- I["(+)", "-"] F --- I I --- J["+", "-"] </pre> | | |
| National Research Institute of Aquaculture | | | |
| Results | + | - | - |
| Remarks | | | |

Disease: Necrotising hepatopancreatitis (NHP)

Aetiological agent: Necrotising hepatobacterium (NHPB) or Rickettsial-like organism (RLO)
(undifferentiated bacteria of Proteobacteria, Alphaproteobacteria)

(1) Epidemiological research

- (i) The disease is caused by *Candidatus* Hepatobacter penaei, which is an intracellular bacterium. Isolation and in vitro culture of this bacterium have not been succeeded.
- (ii) Host range: Most of the shrimp species of genus *Penaeus* (*Penaeus*, *Farfantepenaeus*, *Litopenaeus*, *Marsupenaeus*) can probably be infected with the causative agent. Spontaneous infection has been reported in white leg shrimp (vannamei shrimps) (*Litopenaeus vannamei*), blue shrimp (*L. stylirostris*), and Northern brown shrimp (*F. aztecus*). The infection in *L. vannamei* is especially severe. *L. setiferus* is likely to be less susceptible than *L. vannamei*.
- (iii) Geographical distribution: Belize, Venezuela, Brazil, Columbia, Costa Rica, Ecuador, El Salvador, Guatemala, Honduras, Mexico, Panama, Peru, and the U.S.A.
- (iv) Infections are reported in both juveniles and brood stock of *L. vannamei*.
- (v) The disease tends to occur when the salinity changes or a high-water temperature at around 30°C continues.

(2) Clinical observation

- (i) Sluggish movement, darkening of uropods or pleopods by development of chromatophore, and heavy contamination of shells with periphyton.
- (ii) Anorexia, poor growth, emaciation, and softening of shells.
- (iii) Secondary infections with bacteria on the surface of shells.

(3) Anatomical findings

- (i) Atrophy of the digestive gland (hepatopancreas).
- (ii) Darkened gills.

(4) Diagnosis

- (i) Initial diagnosis: PCR

Materials: DNA extracted from the hepatopancreas.

Primers:

NHPF2: 5'-CGT-TGG-AGG-TTC-GTC-CTT-CAG-T-3'

NHPR2: 5'-GCC-ATG-AGG-ACC-TGA-CAT-CAT-C-3'

Amplicon size: 379 bp

Reaction: Denature at 95 °C for 5 minutes, then conduct 35 cycles of incubation at 95 °C for 30 seconds, at 60 °C for 30 seconds, and at 72 °C for 30 seconds. Finally incubate at 60 °C for 1 minute and at 72 °C for 2 minutes.

(ii) Confirmatory diagnosis: Real-time PCR

Materials: DNA extracted from the hepatopancreas.

Primers:

NHP1300F: 5'-CGTTCACGGGCCTTGACAC-3'

NHP1366R: 5'-GCTCATCGCCTTAAAGAAAAGATAA-3'

TaqMan probe

NHP: 5'-FAM-CCGCCCCGTCAAGCCATGGAA-TAMRA-3'

Amplicon size: 67 bp

Reaction: Denature at 95 °C for 3 minutes, then conduct 40 cycles of incubation at 95 °C for 15 seconds and at 60 °C for one minute.

(5) Histopathological findings

The disease is easily diagnosed by regular Hematoxylin-Eosin stain, excluding early phase of infection. Depending on the progress of the disease, the following histopathological changes are observed in hepatopancreas.

- (i) The acute phase of the disease is characterized by atrophied hepatopancreas, moderate atrophy of the epithelium of the gut caecum, presence of bacterial cells, and infiltration of hemocytes and encapsulation of the hepatopancreatic tubules against bacterial infection, and necrosis and detachment of the epithelium of the hepatopancreatic tubules.
- (ii) In the transitional phase, infiltration of hemocytes occurs according to the necrosis and sloughing of the tubular epithelium of hepatopancreas. The epithelium is markedly atrophied, resulting in the formation of large edematous areas in the hepatopancreas. The cell height of the epithelial cells is low and lipid droplets in the cytoplasm are markedly decreased in size and number. Masses of bacteria are seen. There are hemocyte nodules containing masses of bacteria in the center.
- (iii) In the chronic phase, nodules and edematous areas decline in abundance and severity and are replaced by infiltrated hemocytes. The number of hypertrophied cells with masses of bacteria in the cytoplasm has decreased markedly and fibrosis and melanin deposition are observed in the necrotic areas of the hepatopancreas.

(6) Differential diagnosis

No information is available.

(7) Disinfection

The causative bacterium is probably inactivated by chlorine agent or other ordinary disinfectants, though there is no literature information.

(8) Other

There are no reports regarding drug therapy, administration of immunostimulant, selective breeding, or other measures to control the disease.

(3) Taura Syndrome (TS)

| Responsible organization | Diagnosis flow chart | | |
|--|--|---|---|
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] A --- B B --- C["(3) Anatomical observation"] C --- D["(4) (i) RT-PCR"] D --- E["(+, ±)"] D --- F["(-)"] E --- G["(4) (ii) RT-PCR"] G --- H["(+)", "(-)"] F --- I["(-)"] H --- J["+", "-"] I --- J </pre> | | |
| National Research Institute of Aquaculture | | | |
| Results | + | - | - |
| Remarks | Histopathological observation should also be conducted when the shrimp exhibit clinical disease. | | |

Disease: Taura Syndrome (TS)

Aetiological agent: Taura syndrome virus (TSV) (Dicistroviridae, Aparavirus)

(1) Epidemiological research

(i) Host range: The major natural host is the white leg shrimp (Vannamei shrimp) (*Litopenaeus vannamei*). Although spontaneous infection is reported in blue shrimp (*L. stylirostris*), susceptibility of the species is low.

The experimental infections suggest the following species can be hosts of this virus: black tiger shrimp (*Penaeus monodon*), tiger shrimp (*Marsupenaeus japonicus*), fleshy shrimp (*Fenneropenaeus chinensis*), Northern white shrimp (*P. Setiferus*), Southern white shrimp (*P. schmitti*), Northern brown shrimp (*P. aztecus*), and Northern pink shrimp (*P. duorarum*).

(ii) Geographical distribution: Latin American countries, the U.S.A., Indonesia, Thailand, Malaysia, China, and Taiwan.

(iii) The shrimp in question have been imported from one of the aforementioned areas.

(iv) The shrimp farm in question has a history of introduction of shrimp from the one of the aforementioned areas.

(v) Although the disease occurs from post larva to adult shrimp, juveniles to immature shrimp are particularly susceptible to the disease.

(2) Clinical observation

(i) Clinical signs are obvious in white leg shrimp juveniles from 0.05g to 5g; the infection causes acute disease and high mortality.

(ii) Clinical signs are classified according to three disease stages: the acute phase, the transition (recovery) phase, and the chronic phase.

(iii) In the acute phase and transition phase, overall body color changes to pale red; red color of the tail fan and pleopods are especially noticeable. Focal epithelial necrosis is seen in the appendages.

(iv) Shrimp with clear symptoms of the acute phase of the disease typically have soft shells and empty gut.

(v) In the shrimp that have survived the acute phase to the transition phase of the disease, many irregularly shaped, melanised cuticular lesions appear. Such shrimp may have soft cuticles and show expansion of red chromatophores.

(vi) In the shrimp that have survived the transition phase, the infection become persistent, which is the chronic phase of the infection. The chronic phase is characterized by suspended mortality, recovery of normal behavior, and loss of melanised lesions that are visible by naked eye.

(3) Anatomy findings

No marked changes are observed.

(4) Diagnosis

(i) Initial diagnosis: (a) RT-PCR (reverse transcription-polymerase chain reaction: using a commercial kit)

Materials: RNA extracted from hemolymph.

Primers:

TSV 9195: 5'-TCA-ATG-AGA-GCT-TGG-TCC-3'

TSV 9992: 5'-AAG-TAG-ACA-GCC-GCG-CTT-3'

Amplicon size: 213 bp

Reverse transcription: at 60°C for 30 minutes

PCR reaction: Denature at 94°C for 2 minutes, then conduct 40 cycles of incubation at 94°C for 45 seconds, and at 60°C for 45 seconds. Finally incubate at 60°C for 7 minutes.

(ii) Confirmatory diagnosis: RT-PCR

The following PCR shall be conducted in addition to the aforementioned RT-PCR.

Materials: RNA extracted from hemolymph.

Primers:

TSV55P1: 5'-GGC-GTA-GTG-AGT-AAT-GTA-GC-3'

TSV55P2: 5'-CTT-CAG-TGA-CCA-CGG-TAT-AG-3'

Amplicon size: 1303 bp

Reverse transcription: at 50°C 45 minutes.

PCR reaction: Denature at 95°C for 5 minutes, then conduct 40 cycles of incubation at 95°C 60 seconds, at 60°C for 45 seconds, and at 72°C for 90 seconds. Finally, the reaction mixture is held at 72°C for 5 minutes.

(5) Histopathological findings

- (i) In the acute phase, focal or zonal necrosis of cuticular epithelium is observed in all appendages, gills, hindgut, esophagus, stomach, and body surface.
- (ii) Pale basophilic or eosinophilic spherical debris (diameter: 1-20 µm) is often observed in necrotic cells.
- (iii) In the transitional phase, conspicuous infiltration and accumulation of hemocytes occur in the lesion, and they cause melanin deposition, forming irregular black spots. However, these lesions can be formed by other causes.
- (iv) In the chronic phase, histopathological changes that are seen in the acute phase disappear and no marked lesions are observed.

(6) Differential diagnosis

No information is available.

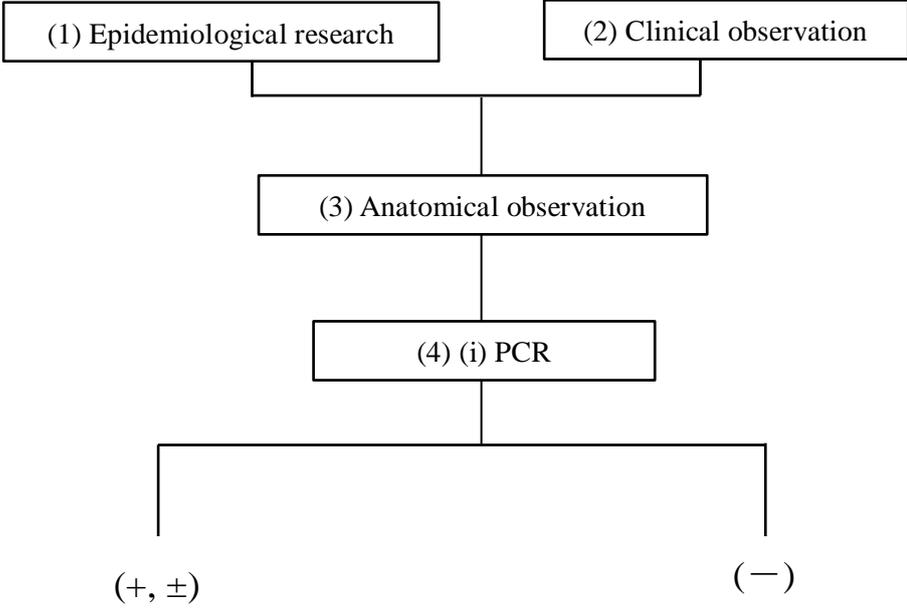
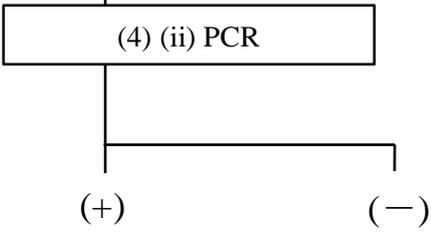
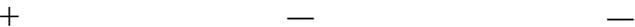
(7) Disinfection

The virus is probably inactivated by chlorine agents or other ordinary disinfectants, although there is no literature information.

(8) Other

In order to prevent vertical transmission, eggs should be washed thoroughly with clean seawater so that eggs and larvae are not contaminated by feces or other materials excreted or shed from brood stock.

(4) Infectious hypodermal and haematopoietic necrosis (IHHN)

| Responsible organization | Diagnosis flow chart |
|--|---|
| Prefectural government |  <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] A --- B B --- C["(3) Anatomical observation"] C --- D["(4) (i) PCR"] D --- E["(+, ±)"] D --- F["(-)"] </pre> |
| National Research Institute of Aquaculture |  <pre> graph TD G["(4) (ii) PCR"] --- H["(+)", "(-)"] </pre> |
| Results |  |
| Remarks | When a suspected case occurs in Japan, histopathological observation should also be conducted. |

Disease: Infectious hypodermal and haematopoietic necrosis (IHHN)

Aetiological agent: Infectious hypodermal and hematopoietic necrosis virus (IHHNV)
(Parvoviridae, *Aedes aegypti* densovirus)

(1) Epidemiological research

- (i) Host range: Spontaneous outbreaks are reported mainly in blue shrimp (*Litopenaeus stylirostris*), white leg shrimp (vannamei shrimp) (*L. Vannamei*), and black tiger shrimp (*Penaeus monodon*). Many shrimp species are known to be susceptible to the virus by experimental infections.
- (ii) Geographical distribution: South-east coast of the U.S.A., Central and South American countries, Hawaii, Guam, Tahiti, New Caledonia, Singapore, Thailand, Malaysia, Philippines, Indonesia, Myanmar, Iran, and Australia.
- (iii) The animals in question were imported from one of the above areas.
- (iv) The shrimp farm has a history of the introduction of shrimp from one of the above areas.
- (v) The disease often occurs in juvenile shrimp.

(2) Clinical observation

- Blue shrimp

- (i) Infected shrimp swim slowly close to the water surface and sink slowly to the bottom. Diseased animals repeat these behaviors until death.
- (ii) Although there are few marked external clinical signs, white or yellowish-brown spots may be seen on the cuticular epidermis (particularly at the junction of abdominal segments), giving such shrimp a mottled appearance.
- (iii) Moribund shrimp is bluish in colour, with opaque abdominal musculature in some cases.

- White leg shrimp (Vannamei shrimp)

- (i) IHHNV causes typical chronic disease in this species.
- (ii) Bent rostrum, wrinkled antenna, deformed integument, and other malformations are observed. These malformations are called RDS (Runt deformity syndrome).
- (iii) In the group showing RDS, the size of the shrimp varies and, very small specimens are often seen.

(3) Anatomical Findings

The infected shrimp does not present obvious signs of infection.

(4) Diagnosis

(i) Initial diagnosis: PCR

Materials: DNA extracted from tissues or hemolymph, including cuticular epithelium of gills.

Primer:

389F: 5'-CGG-AAC-ACA-ACC-CGA-CTT-TA-3'

389R: 5'-GGC-CAA-GAC-CAA-AAT-ACG-AA-3'

Amplicon size: 389 bp

Reaction: Denature at 95°C for 5 minutes, then conduct 35 cycles of

incubations at 95°C for 30 seconds, at 55°C for 30 seconds, and at 72°C for one minute. Finally incubate at 72°C for 7 minutes.

(ii) Confirmatory diagnosis: PCR

The following method shall be conducted in addition to the PCR in the initial diagnosis.

Materials: DNA extracted from tissues or hemolymph, including cuticular epithelium of gills, .

Primer:

392F: 5'-GGG-CGA-ACC-AGA-ATC-ACT-TA-3'

392R: 5'-ATC-CGG-AGG-AAT-CTG-ATG-TG-3'

Amplicon size: 392 bp

Reaction: Denature at 95°C for 5 minutes, then conduct 35 cycles of incubations at 95°C for 30 seconds, at 55°C for 30 seconds, and at 72°C for one minute. Finally incubate at 72°C for 7 minutes.

(5) Histopathological findings

(i) Hypertrophied nuclei with margined chromatin are seen in the cells of ectodermal (cuticular epithelium, foregut and hindgut epithelium, nerve cord, ganglion) and mesodermal tissues (hematopoietic organ, antennal gland cell epithelium, lymphoid organ).

(ii) Eosinophilic Cowdry A-type inclusion body is seen in the nucleus of affected cells.

(6) Differential diagnosis

There is no information.

(7) Disinfection

Chlorine or iodine disinfectant is effective for the disinfection of ponds or other rearing facilities.

(8) Other

N/A

(5) Acute Hepatopancreatic Necrosis Disease (AHPND)
(Early Mortality Syndrome (EMS))

| | | | |
|--|--|---|---|
| Responsible organization | Diagnosis flow chart | | |
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] A --- B B --- C["(3) Anatomical observation"] C --- D["(4) (i) Nested-PCR"] D --- E["(-)"] D --- F["(+, ±)"] E --- G["-"] F --- H["(4) (ii) Nested-PCR and sequence analysis"] H --- I["(+)", "(-)"] I --- J["-"] I --- K["-"] </pre> | | |
| National Research Institute of Aquaculture | <pre> graph TD H["(4) (ii) Nested-PCR and sequence analysis"] --- I["(+)", "(-)"] I --- J["(+)", "(-)"] J --- K["-"] J --- L["-"] </pre> | | |
| Results | - | + | - |
| Remarks | Although the confirmatory diagnosis is conducted by Nested-PCR and sequence analysis of the amplicon, histopathological observation should also be conducted when a suspected case occurs in Japan. | | |

Disease: Acute Hepatopancreatic Necrosis Disease (AHPND)
(Early Mortality Syndrome (EMS))

Aetiological agent : *Vibrio parahaemolyticus* (Vibrionaceae, *Vibrio*)

(1) Epidemiological research

- (i) *Vibrio* bacteria that are the cause of this disease are gram-negative indigenous bacterium. Aetiological agent bacteria are strains that produce toxins derived from plasmid DNA. The bacteria that carry this plasmid or toxin gene can cause the disease.
- (ii) Host range: White leg shrimp (Vannamei shrimp) (*Litopenaeus vannamei*), black tiger shrimp (*Penaeus monodon*), and fleshy shrimp (*Fenneropenaeus chinensis*)
- (iii) Geographical distribution: China, Malaysia, Thailand, Vietnam, and Mexico
- (iv) Susceptible stages of the host: Typical signs of AHPND begin within 10–30 days after stocking of post larvae into a newly prepared pond.

(2) Clinical observation

- (i) Hepatopancreas often becomes pale to white.
- (ii) Shells often softens and gut content is only partially observed or there is no gut content.
- (iii) Moribund shrimp sink to the bottom.

(3) Anatomical findings

- (i) Atrophied and hardened hepatopancreas.
- (ii) Black spots or streaks are sometimes visible in the hepatopancreas.

(4) Diagnosis

- (i) Initial diagnosis: Nested- PCR

Materials: DNA extracted from hepatopancreas.

(1st PCR)

Primers:

AP4-F1: 5'- ATGAGTAACAATATAAAACATGAAAC -3'

AP4-R1: 5'- ACGATTTTCGACGTTCCCAA -3'

Amplicon size: 1269 bp

Reaction: Denature at 94 °C for 2 minutes, then conduct 30 cycles of incubation at 94 °C for 30 seconds, at 55 °C for 30 seconds, and at 72 °C for 90 seconds. Finally incubate at 72 °C for 2 minutes.

(2nd PCR)

Primers:

AP4-F2: 5'- TTGAGAATACGGGACGTGGG -3'

AP4-R2: 5'- GTTAGTCATGTGAGCACCTTC -3'

Amplicon size: 230 bp

Reaction: Denature at 94 °C for 2 minutes, then conduct 25 cycles of incubation at 94 °C for 20 seconds, at 55 °C for 20 seconds, and at 72 °C for 20 seconds.

- (ii) Confirmatory diagnosis: Nested- PCR

The same nested PCR is conducted as in the initial diagnosis, and the sequence of the amplicon is analyzed.

(5) Histopathological findings

The following lesions are reported; however, they are not pathognomonic for this disease.

- (i) Acute progressive degeneration of the hepatopancreas accompanied initially by decrease of R-, B- and F cells followed by a marked reduction of mitotic activity in E-cells
- (ii) The lesion progresses from proximal (mouth side) to distal (anus side) with dysfunction of R-, B-, F-, and lastly E cells. Affected mucosal cells of the hepatopancreas tubule present prominent karyomegaly (enlarged nuclei), encapsulated by haemocytes and disintegrated.
- (iii) Substances released from the disintegrated hepatopancreas cells stimulate bacterial growth, resulting in severe secondary bacterial infection, resulting in complete destruction of the hepatopancreas at the terminal phase of the disease
- (iv) Accompanying the initial sloughing of tubule epithelial cells of the hepatopancreas and the development of a secondary bacterial infection is intense intertubular haemocytic aggregation and haemocyte encapsulation of necrotic tubules and melanisation of the more proximal portions of tubules.

(6) Differential diagnosis

No information available.

(7) Disinfection

- (i) Bacterial growth is decreased by freezing for a few weeks or by heat treatment for 5 minutes at 55°C or one minute at 80°C, although the growth is not much affected by refrigeration.
- (ii) The bacterium loses growth activity after exposure to acidic environment at pH 5 for 15 minutes.
- (iii) The causative bacterium can survive for 9 days in brackish water and for 18 days in seawater.

(8) Other

N/A

(6) Infectious myonecrosis (IMN)

| Responsible organization | Diagnosis flow chart | | |
|--|--|---|---|
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] B --- C["(3) Anatomical observation"] C --- D["(4) (i) RT-PCR"] D -- (-) --> E["-"] D -- (+, ±) --> F["(4) (ii) Nested-PCR"] F -- (+) --> G["+"] F -- (-) --> H["-"] </pre> | | |
| National Research Institute of Aquaculture | | | |
| Results | - | + | - |
| Remarks | Although the confirmatory diagnosis is done by nested-PCR, histopathological examination should also be conducted when a suspected case occurs in Japan. | | |

Disease: Infectious Myonecrosis (IMN)

Disease: Infectious myonecrosis virus (IMNV) (Totiviridae, Totivirus)

(1) Epidemiological research

- (i) Host range: White leg shrimp (vannamei shrimp) (*Litopenaeus vannamei*) (In challenge experiments, infections are confirmed with black tiger shrimp (*Penaeus monodon*) and blue shrimp (*L. stylirostris*).
- (ii) Geographical distribution: North-eastern area of Brazil, Indonesia, and Southeast Asia
- (iii) Susceptible stages of the host: Although the causative agent of this disease infects in all stages of growth, the infection in young and immature white leg shrimp is severe.

(2) Clinical observation

- (i) Clinical disease and high mortality may occur immediately after the stresses such as a sudden change in the water temperature or salinity, or the introduction to net pens.
- (ii) Shrimp may feed actively and the gut may be filled with ingested food just prior to the stress load.
- (iii) White turbidity in lateral muscle due to focal or zonal necrosis (in particular, posterior abdominal segments and tail fan).
- (iv) High mortality may continue for several days after stress imposition.

(3) Anatomical findings

White turbidity in lateral muscle due to focal or zonal necrosis (in particular, distal abdominal segments and tail fan). Hypertrophy (3 to 4 times the size of a normal organ) of lymphoid organ is seen.

(4) Diagnosis method

(i) Initial diagnosis: RT-PCR

Materials: RNA extracted from the hemocytes (hemolymph), muscle tissues, or lymphoid organ.

Primers:

4587F: 5'- CGA-CGC-TGC-TAA-CCA-TAC-AA -3'

4914R: 5'- ACT-CGG-CTG-TTC-GAT-CAA-GT -3'

Amplicon size: 328 bp

Reaction: Conduct reverse transcription at 60 °C for 30 minutes.

Denature at 95°C for 2 minutes, then conduct 39 cycles of incubation at 95 °C for 45 seconds and at 60 °C for 45 seconds.

Finally incubate at 60 °C for 7 minutes.

(ii) Confirmatory diagnosis: Nested- PCR

i) 1st RT-PCR: The same as the initial diagnosis described in (4) (i).

ii) 2nd PCR

Materials: Amplicon of the 1st PCR of (4) (ii), i).

Primers:

4725NF: 5'- GGC-ACA-TGC-TCA-GAG-ACA -3'

4863NR: 5'- AGC-GCT-GAG-TCC-AGT-CTT-G -3'

Amplicon size: 139 bp

Reaction: Denature at 95°C for 2 minutes, then conduct 39 cycles of incubation at 95°C for 30 seconds, at 65°C for 30 seconds, and at 72°C for 30 seconds. Finally incubate at 72°C for 2 minutes.

(5) Histopathological findings

The disease shows the following histological changes, although these are not pathognomonic for this disease.

- (i) Coagulative necrosis in association with the invasion of hemocytes and liquefactive necrosis in skeletal muscle fibers.
- (ii) Skeletal muscle become edematous, and eventually replaced by loose connective tissues.
- (iii) Hypertrophy of the lymphoid organ is observed probably due to the formation of spheroids, which is thought to be a self-defense mechanism.
- (iv) The spheroids mentioned above are sometimes observed in the different organs, such as in gills, heart, antennal glands, or abdominal nerve cord.

(6) Differential diagnosis

White necrotic lesions in muscles of abdominal muscle can be caused by different reasons other than IMNV. Penaeid shrimp affected by covert mortality disease or the giant freshwater prawn (*Macrobrachium rosenbergii*) infected with white tail disease exhibit the similar clinical sign of white muscle, although, these diseases are caused by two different nodaviruses respectively. Therefore, diagnosis should be conducted by PCR.

(7) Disinfection

This virus is probably inactivated by chlorine agents or other ordinary disinfectants, though there is no literature information.

(8) Other

In order to detect IMNV in the shrimp with subclinical infection, the following RT-PCR using RNA extracted from hemolymph collected from live animals can be useful.

Primers: The same as (4) (i) of the initial diagnosis.

Amplicon size: 328 bp

Reaction: Conduct reverse transcription at 55°C for 30 minutes.

Denature at 95 °C for 2 minutes, then conduct 39 cycles of incubation at 95 °C for 15 seconds, at 60 °C for 30 seconds and at 68 °C for 45 seconds. Finally incubate at 68 °C for 2 minutes.

Disease: Tetrahedral Baculovirus

Aetiological agent: *Baculovirus penaei* (BP) (Baculoviridae, Nucleopolyhedrovirus)

(1) Epidemiological research

- (i) This disease is caused by infection with *Baculovirus penaei* (BP).
- (ii) Host range: All penaeid species may be potential hosts
- (iii) Geographical distribution: The Americas and Hawaii
- (iv)
- (v) The shrimp farm of concern has a history of importing shrimp from one of the areas mentioned above.

(2) Clinical observation

Loss of appetite and growth retardation

(3) Anatomical findings

Severely affected animals may present a whitish midgut.

(4) Diagnosis

(i) Initial diagnosis:

- (a) Observation of tetrahedral occlusion bodies in squash preparations of hepatopancreas or midgut with light microscopy

(b) PCR

Materials: DNA extracted from the hepatopancreas and midgut.

Primers:

BPA: 5'-GAT-CTG-CAA-GAG-GAC-AAA-CC-3'

BPF: 5'-TAC-CCT-GCA-TTC-CTT-GTC-GC-3'

Amplicon size: 196 bp

Reaction: Denature at 95 °C for 3 minutes, then conduct 30 cycles of incubations at 94 °C for 30 seconds, at 60 °C for 30 seconds, and at 72 °C for 1 minute. Finally, the reaction mixture is held at 72 °C for 5 minutes.

(ii) Confirmatory diagnosis: PCR

In addition to the PCR for the initial diagnosis, the following PCR should be conducted.

Materials: DNA extracted from the hepatopancreas and midgut.

Primers:

6581: 5'-TGT-AGC-AGC-AGA-GAA-GAG-3'

6582: 5'-CAC-TAA-GCC-TAT-CTC-CAG-3'

Amplicon size: 644 bp

Reaction: Denature at 95 °C for 5 minutes, then conduct 35 cycles of incubations at 95 °C for 30 seconds, at 65 °C for 30 seconds, and at 72 °C for 1 minute. Finally, the reaction mixture is held at 72 °C for 7 minutes.

(5) Histopathological findings

Tetrahedral occlusion bodies are observed in the markedly enlarged nuclei of the epithelial cells of hepatopancreas or midgut. The chromatin of the nuclei is decreased or margined at the nuclear membrane.

(6) Differential diagnosis

Although the histopathological features of this disease are similar to those of baculoviral midgut gland necrosis (BMN), occlusion bodies are not observed in BMN.

(7) Disinfection

The causative virus is probably inactivated by ordinary disinfectants such as chlorine, although there is no published literature to confirm this.

There is a report that *B. penaei* can be inactivated by exposure to a low pH condition (pH3) for 30 minutes, high temperatures (60-90°C) for 10 minutes, cumulative dosage of $7.08 \times 10^6 \mu\text{Wsec/cm}^2$ of ultraviolet irradiation, or desiccation for 48 hours.

(8) Other

N/A

(8) Covert Mortality Disease of Shrimp (CMD)

| Responsible organization | Diagnosis flow chart | | | |
|--|--|---|---|---|
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] A --- C["(3) Anatomical observation"] B --- C C --- D["(4) (i) (a) Histopathology"] C --- E["(4) (i) (b) RT-PCR"] D --- F["(-)"] D --- G["(+, ±)"] E --- H["(+, ±)"] E --- I["(-)"] G --- J["(4) (ii) Nested-PCR"] H --- J J --- K["(+)", "(-)"] </pre> | | | |
| National Research Institute of Aquaculture | | | | |
| Results | — | + | — | — |
| Remarks | Either histopathology or RT-PCR shall be conducted as the initial diagnosis. The confirmatory diagnosis is conducted by the nested- PCR. | | | |

Disease: Covert Mortality Disease of Shrimp (CMD)

Aetiological agent: Covert mortality nodavirus (CMNV) (unclassified virus of Nodaviridae)

(1) Epidemiological research

- (i) Host range: White leg shrimp (vannamei shrimp) (*Litopenaeus vannamei*), kuruma shrimp (*Marsupenaeus japonicas*), and fleshy shrimp (*Fenneropenaeus chinensis*).
- (ii) Geographical distribution: China (The disease has not been reported in other countries.)

(2) Clinical observation

- (i) Atrophy and discoloration of the hepatopancreas and whitish or necrotic muscle in the abdominal segments.
- (ii) Affected shrimp stay on the bottom of ponds and do not swim up to the water surface or into shallow water.

(3) Anatomical findings

- (i) Atrophy or necrosis of the hepatopancreas with discoloration to red, gray, or white.
- (ii) The shell become softened and the stomach and intestine are empty.
- (iii) Whitish or necrotic abdominal muscle.

(4) Diagnosis

(i) Initial diagnosis: (a) Histopathological observation

Materials: The abdominal muscle, hepatopancreas, and lymphoid organ.

Fixation: Tissues are fixed in Davidson's solution, routinely processed, and stained with hematoxylin and eosin.

Results: Confirm coagulative necrosis in the muscle, eosinophilic inclusion bodies in the hepatopancreas and tubular epithelium of the lymphoid organ, and pycnotic nuclei of cells in the muscle and lymphoid organ.

(b) RT-PCR

Materials: RNA extracted from the hepatopancreas, striated muscle, or lymphoid organ that have been collected for RT-PCR.

Primers:

CMNV-7F1: 5'-AAA-TAC-GGC-GAT-GAC-G-3'

CMNV-7R1: 5'-ACG-AAG-TGC-CCA-CAG-AC-3'

Amplicon size: 619 bp

Heat denaturation of RNA: at 65 °C for 5min, followed by rapid cooling

RT-PCR reaction: Reverse transcription at 50 °C for 1hour followed by the incubation at 70 °C for 15 minutes. Denature at 94 °C for 4 minutes, then conduct 35 cycles of incubation at 94 °C for 30 seconds, for at 45 °C 30 seconds, and at 72 °C for 40 seconds. Finally incubate at 72 °C for 7 minutes.

(ii) Confirmatory diagnosis: Nested- PCR

i) 1st PCR: The same as the initial diagnosis described in (i) (b).

ii) 2nd PCR

Materials: The amplicon of the 1st PCR indicated in (4) (ii), i).

Primers:

CMNV-7F2: 5'-CAC-AAC-CGA-GTC-AAA-CC -3'

CMNV-7R2: 5'-GCG-TAA-ACA-GCG-AAG-G-3'

Amplicon size: 165 bp

Reaction: Denature at 94°C for 4 minutes, then conduct 30 cycles of incubation at 94°C for 20 seconds, at 40°C for 20 seconds, and at 72°C for 20 seconds. Finally incubate at 72°C for 7 minutes.

(5) Histopathological finding

Coagulation necrosis of striated muscle, eosinophilic inclusion bodies in the hepatopancreas and tubule epithelium of lymphoid organ, and pyknosis of nuclei in muscles and lymphoid organ.

(6) Differential diagnosis

Diseases showing similar clinical signs are not known.

(7) Disinfection

This virus is probably inactivated by chlorine agents or other regular disinfectants, although there is no literature information.

(8) Other

Virus-like particles of 25nm in diameter without envelope are observed in the hepatopancreas by electron microscopy.

The fluorescent *in situ* hybridization using the following probes has also been developed to detect the causative virus:

CMNV-7Probe-F : 5'-GGC GAT GAC GGC TTGA-3'

CMNV-7Probe-R : 5'-GGC GGT GAG ATG GAT TTT-3'

The database accession number for the gene for RNA dependent RNA polymerase of the virus is; KM112247.

(9) Gill-Associated Virus Disease (GAV)

| Responsible organization | Diagnosis flow chart | | |
|--|--|---|---|
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] A --- B B --- C["(3) Anatomical observation"] C --- D["(4) (i) RT-PCR"] D --- E["(-)"] D --- F["(+, ±)"] </pre> | | |
| National Research Institute of Aquaculture | <pre> graph TD G["(4) (ii) Nested-PCR and sequence analysis"] --- H["(+)", "(-)"] </pre> | | |
| Results | — | + | — |
| Remarks | Histopathological observation should also be performed when suspected case of GAVD is detected in Japan. | | |

Disease: Gill-Associated Virus Disease (GAVD)

Aetiological agent: Gill-associated virus (Yellow head virus genotype 2, genus *Okavirus*, Family *Roniviridae*)

(1) Epidemiological research

- (i) Host range: Naturally occurring infections are reported only in the black tiger shrimp (*Penaeus monodon*), although mortality is reported in brown tiger prawn (*P. esculentus*), banana prawn (*Fenneropenaeus merguensis*), kuruma prawn (*Marsupenaeus japonicus*) in experimental infections.
- (ii) Geographical distribution: Australia, Thailand, and Vietnam. Mortality in the black tiger shrimp caused by this disease is reported from Australia. Although no case of clinical infection of the disease has been reported from Thailand and Vietnam, the virus has been detected from apparently healthy black tiger shrimp in those countries.
- (iii) Susceptible host stages for infection: For the kuruma prawn (*M. japonicus*), animals larger than 20g are less susceptible to the disease than smaller shrimp of 6-13g.
- (iv) Carrier: Black tiger shrimp (*P. monodon*) that are infected with GAV can be carriers of the disease in natural conditions. It is also possible that the brown tiger prawn (*P. esculentus*), banana prawn (*F. merguensis*), and kuruma prawn (*M. japonicus*) become carriers of the disease, since these prawns can be infected with the disease experimentally.

(2) Clinical observation

- (i) Reddening of the body and legs.
- (ii) Swimming near the water surface or at the edge of the pond. Loss of appetite.

(3) Anatomical findings

Discoloration of the gills to pink or yellow.

(4) Diagnosis

(i) Initial diagnosis: RT-PCR

Materials: RNA extracted from fresh gills or lymphoid organs

Primers:

GY1: 5'-GAC-ATC-ACT-CCA-GAC-AAC-ATC-TG-3'

GY4: 5'-GTG-AAG-TCC-ATG-TGT-GTG-AGA-CG-3'

(These primers are generic for YHV (genotype 1) and GAV.)

Amplicon size: 794 bp

Reaction: One-step RT-PCR is carried out with a commercial kit. Conduct Reverse transcription at 50 °C for 30 minutes. Denature at 94 °C for 2 minutes, then conduct 35 cycles of incubation at 95 °C for 30 seconds, at 66 °C for 30 seconds, and at 68 °C for 45 seconds. Finally incubate at 68 °C for 7 minutes.

(ii) Confirmatory diagnosis: Nested- PCR and sequencing

i) 1st PCR: The same as the initial diagnosis method described in (4) (i).

ii) 2nd PCR

Materials: The amplified product of the PCR in (4) (ii) i).

Primers:

GY2: 5'-CAT-CTG-TCC-AGA-AGG-CGT-CTA-TGA-3'

G6: 5'-GTA-GTA-GAG-ACG-AGT-GAC-ACC-TAT-3'

Amplicon size: 406 bp

(Note: In order to match GY2 completely with GAV, the seventh nucleotide of GY2 should be changed from T to C, although there is no report that this mismatch significantly affects the amplification of GAV gene)

Reaction: Denature at 94 °C for 2 minutes, then conduct 35 cycles of incubation at 95 °C for 30 seconds, for 30 seconds, and at 72 °C for 45 seconds. Finally incubate at 66 °C for 7 minutes.

Confirm the genotype by sequencing the amplified product.

(5) Histopathological finding

No detailed report is available for the histopathological features of this disease.

(6) Differential diagnosis

Yellow head complex of viruses (genus *Okavirus*, Family *Roniviridae*) is known to contain 7 genotypes. Among these, gill-associated virus is designated as genotype 2. Yellow head virus is the genotype 1 and the aetiological agent for yellow head disease (YHD). For the black tiger shrimp, animals larger than stage 15 post larva are especially susceptible to YHV (genotype 1). Genotypes 3-6 are often detected in apparently healthy black tiger shrimp in East Africa, Asia and Australia, though they are not related to YHD. Aetiological agentivity of the genotype 7 is not clear. YHV (genotype 1) and GAV are similar, but can be distinguished from each other by the nested RT-PCR described here.

(7) Disinfection

Although no information is available, the virus is probably inactivated by ordinary disinfectants, such as chlorine agents.

(8) Other

No effective measures are known for the prevention or cure of the disease.

(10) Spherical Baculovirus

| Responsible organization | Diagnosis flow chart |
|--|---|
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] B --- C["(3) Anatomical observation"] C --- D["(4) (i) (a) Microscopy of hepatopancreas and midgut"] D --- E["(+, ±)"] D --- F["(-)"] F --- G["(4) (i) (b) PCR"] G --- H["(+, ±)"] G --- I["(-)"] </pre> |
| National Research Institute of Aquaculture | <pre> graph TD E["(+, ±)"] --- J["(4) (ii) PCR"] J --- K["(+)"] J --- L["(-)"] </pre> |
| Results | <div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;">+ +</div> <div style="text-align: center;">- -</div> <div style="text-align: center;">- -</div> </div> |
| Remarks | <p>Either the microscopy of hepatopancreas and midgut or PCR shall be conducted as the initial diagnosis conducted by prefectural government. PCR shall be conducted as the confirmatory diagnosis conducted by NRIA.</p> |

Disease: Spherical Baculovirosis

Aetiological agent: *Penaeus monodon*-type baculovirus (MBV or PemoNPV) (Baculoviridae, Nucleopolyhedrovirus)

(1) Epidemiological research

- (i) The disease is caused by *Penaeus monodon*-type baculovirus (MBV or PemoNPV).
- (ii) Host range: A variety of penaeid shrimp, such as the black tiger shrimp (*Penaeus monodon*) or banana prawns (*P. merguensis*).
- (iii) Geographical distribution: China, Taiwan, and other countries around the Pacific Ocean, Indian Ocean, in the Middle East, around the Mediterranean Sea, in Africa, Hawaii, Tahiti, and the North and South American countries.
- (iv) The shrimp in question has been imported from one of the areas mentioned above.
- (v) The shrimp farm has a history of the introduction of shrimp from one of the areas mentioned above.

(2) Clinical observation

Decreased food consumption and slow growth.

(3) Anatomical findings

White turbidity is often seen in the midgut of severely infected individuals.

(4) Diagnosis method

- (i) Initial diagnosis: (a) Confirm spherical occlusion bodies in squashed preparation of fresh hepatopancreas and midgut with microscopy

(b) PCR

Materials: DNA extracted from hepatopancreas and midgut.

Primers:

261F: 5'-AAT-CCT-AGG-CGA-TCT-TAC-CA-3'

261R: 5'-CGT-TCG-TTG-ATG-AAC-ATC-TC-3'

Amplicon size: 261 bp

Reaction: Denature at 95 °C for 5 minutes, then conduct 35 cycles of incubation at 94°C for 30 seconds, at 60°C for 30 seconds, and at 72 °C for 30 seconds. Finally incubate at 72 °C for 7 minutes.

- (ii) Confirmatory diagnosis: PCR

The following PCR shall be conducted in addition to the PCR for the initial diagnosis.

Materials: DNA extracted from hepatopancreas and midgut.

Primers:

MBV1.4F: 5'-CGA-TTC-CAT-ATC-GGC-CGA-ATA-3'

MBV1.4r: 5'-TTG-GCA-TGC-ACT-CCC-TGA-GAT-3'

Amplicon size: 533 bp

Reaction: Denature at 96 °C for 5 minutes, then conduct 40 cycles of incubation at 94°C for 30 seconds, at 65°C for 30 seconds, and at 72 °C for 30 seconds. Finally incubate for 7 minutes at 72 °C.

(5) Histopathological findings

The epithelial cell nuclei of hepatopancreas or midgut gland are markedly hypertrophied and their chromatin is decreased and marginated. Spherical occlusion bodies are seen in those nuclei.

(6) Differential diagnosis

The histopathological features of this disease are similar to those of Baculovirus Midgut Gland Necrosis (BMN), although no occlusion body is observed in shrimp infected with BMN.

(7) Disinfection

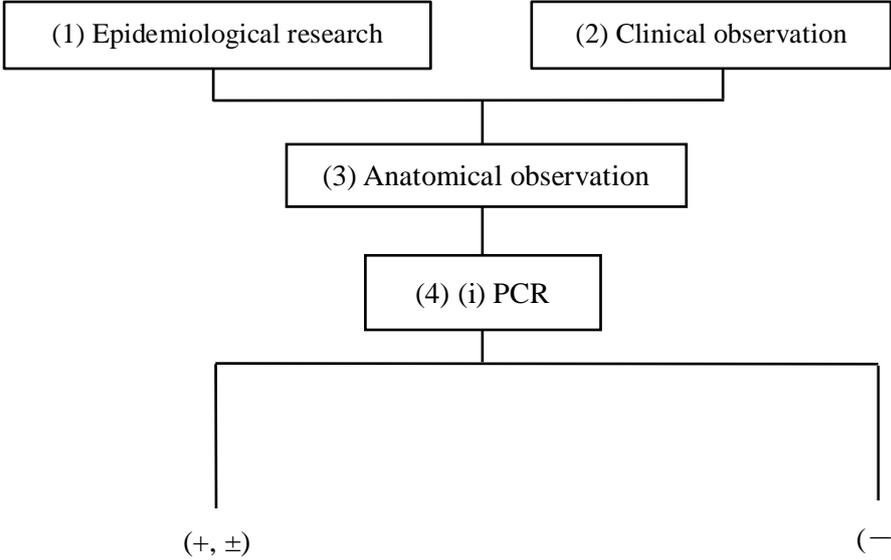
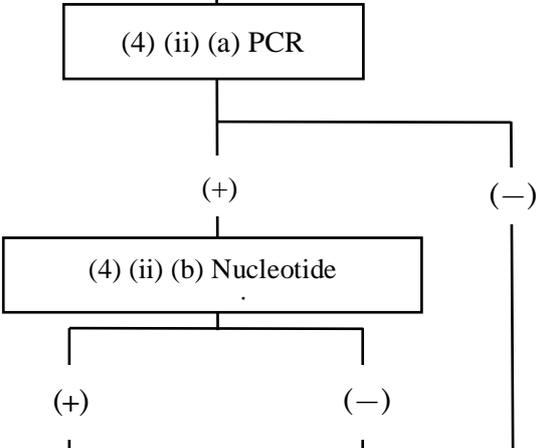
This virus is probably inactivated by chlorine agents or other ordinary disinfectants, although there is no literature information.

(8) Other

In order to avoid vertical infection, eggs should be washed thoroughly with clean seawater to prevent eggs and larva from being contaminated by feces or other materials excreted from adult shrimp.

3. Mollusks and others

(1) Infection with abalone herpesvirus

| Responsible organization | Diagnosis flow chart |
|--|---|
| Prefectural government |  <pre> graph TD A["(1) Epidemiological research"] --- B["(3) Anatomical observation"] C["(2) Clinical observation"] --- B B --- D["(4) (i) PCR"] D --- E["(+, ±)"] D --- F["(-)"] </pre> |
| National Research Institute of Aquaculture |  <pre> graph TD G["(4) (i) (a) PCR"] --- H["(+)", "(-)"] H -- "+" --> I["(4) (ii) (b) Nucleotide"] I --- J["(+)", "(-)"] H -- "-" --> K["(-)"] </pre> |
| Results | <p> + - - - </p> |
| Remarks | <p>Due to polymorphisms (insertion-deletion) in the target region, the PCR generates amplicons of varying lengths (522bp to 588bp) depending on the AbHV isolate. For confirmatory diagnosis, nucleotide sequencing is needed to confirm that the PCR amplicon is derived from AbHV.</p> |

Disease: Infection with abalone herpesvirus

Aetiological agent: Abalone herpesvirus (AbHV) (Malacoherpesviridae, Ostreavirus)

(1) Epidemiological research

(i) Host range:

- *Haliotis diversicolor* (Tokobushi) in Taiwan
- Greenlip abalone (*Haliotis laevigata*), blacklip abalone (*H. rubra*) and the hybrid of these two species
- No clinical changes have been reported from other invertebrates in the epidemic area.
- The disease has not been confirmed in the naive black abalone (*H. discus*) in the cohabitation infection test with the infected tokobushi (*H. diversicolor*).

(ii) Geographical distribution: Taiwan (northeastern area), Australia (Victoria and Tasmania).

(iii) The shells in question have been imported from one of the aforementioned areas.

(iv) The abalone farm in question has a history of introducing shells from one of the aforementioned areas.

(v) Disease occurrences:

- In Australia, the disease occurred regardless of the age of the abalone, with mortality rates of over 90%.
- In Taiwan, outbreaks were also confirmed in both adult and juvenile abalones, with 70-80% mortalities (water temperature was 16 to 19°C).
- In farming ponds, as in experimental challenge tests, it is reported that death occurs within 3 days after the clinical symptoms are confirmed.
- In an experimental transmission by intramuscular injections with filtrate of the homogenate of affected abalone tissues, 100% death was recorded within 2 to 5 days after the challenge.

(2) Clinical observation

- (i) Escape behavior from light is not seen.
- (ii) Reduced pedal adhesion to the substrate.
- (iii) There are cases where no marked symptoms appear.

(3) Anatomical Findings

- (i) Mantle recession.
- (ii) Irregular peripheral concave elevation of the foot.
- (iii) Minimal movement of the pedal muscle.
- (iv) Swollen and protruding mouth parts.
- (v) Eversion of the radula.
- (vi) Excessive mucus production.

(4) Diagnosis

(i) Initial diagnosis: PCR

Materials: DNA extracted from the nerve tissue or muscle containing nerve tissues. Tissues are stored in fixative (80% ethanol; 19.75% glycerol; 0.25% β -mercaptoethanol) or 95% ethanol. DNA is extracted from approximately 20 mg of tissue

using a commercially available kit. DNA is eluted at a concentration of < 100 ng / μ l, and PCR reaction is performed with the following primer set.

Primers:

AbHV-16: 5'-GGC-TCG-TTC-GGT-CGT-AGA-ATG-3'

AbHV-17: 5'-TCA-GCG-TGT-ACA-GAT-CCA-TGT-C-3

Amplicon size: 522 to 558bp

Target region of PCR: 40900 - 41457 (GenBank accession No. HM631981)

Reaction: Denature at 95°C for 5 minutes, then conduct 40 cycles of incubations at 94°C for 30 seconds, at 52°C for 30 seconds, and at 74°C for 45 seconds. Finally incubate at 72°C for 7 minutes.

* Mutant strains exist in this virus, which give amplification products of different sizes from 522 bp to 588 bp.

(ii) Confirmatory diagnosis: (a) PCR

Materials: DNA extracted from the nerve tissues or muscle containing the nerve tissues. PCR condition is the same as the PCR in the initial diagnosis.

(b) Nucleotide sequencing

Materials: PCR amplicon from (ii) (a)

Method: Nucleotide sequencing

Results: The nucleotide sequence of the PCR amplicon showing a high level of identity with the target region of the AbHV genome should be confirmed (GenBank Accession No. HM631981).

(5) Histopathological findings

(i) Ganglioneuritis is observed in the head, foot muscle, and mouth region.

(ii) Lesions have not been observed in organs other than nervous tissues.

(iii) Icosahedral viral particles (100–110 nm in diameter) with electron dense core and envelope are observed with transmission electron microscopy

(6) Differential diagnosis

No information is available.

(7) Disinfection

There is no report on disinfection. This virus is probably inactivated by an ordinary disinfectant such as chlorine.

(8) Other

(a) AbHV is highly aetiological agent and, once the disease occurs, there is no method to reduce the damage.

(ii) Horizontal infection via seawater occurs.

(iii) OIE recommends abalone culture in the farms where the high levels of on-farm biosecurity and regional movement restrictions can be implemented. Following an on-farm outbreak, the destruction of infected stock, disinfection of water and equipment, and the use of sentinel abalone to test the presence of the virus are recommended.

(2) Pustule Disease of Abalone/Blister Disease of Abalone
(caused by *Vibrio furnissii* (= *V. fluvialis* biotype II))

| | |
|--|---|
| Responsible organization | Diagnosis flow chart |
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] A --- C["(3) Anatomical observation"] B --- C C --- D["(4) (i) (a) Observation of stamp preparation"] D --- E["(-)"] D --- F["(+, ±)"] F --- G["(4) (i) (b) Bacterial isolation"] G --- H["(+, ±)"] G --- I["(-)"] H --- J["(4) (i) (c) Biochemical tests of the isolate"] J --- K["(-)"] J --- L["(+, ±)"] K --- M["(-)"] L --- N["(4) (ii) (a) Biochemical tests"] I --- O["(-)"] N --- P["(4) (ii) (b) PCR and gene sequence"] P --- Q["(+)"] P --- R["(-)"] Q --- S["(+)"] R --- T["(-)"] E --- M I --- O K --- M T --- O </pre> |
| National Research Institute of Aquaculture | <pre> graph TD N["(4) (ii) (a) Biochemical tests"] --- O["(4) (ii) (b) PCR and gene sequence"] O --- P["(+)"] O --- Q["(-)"] P --- R["(+)"] Q --- S["(-)"] </pre> |
| Results | <p>- - + - -</p> |
| Remarks | |

Disease: Pustule Disease of Abalone/Blister Disease of Abalone
(caused by *Vibrio furnissii* (= *V. fluvialis* biotype II))

Aetiological agent : *Vibrio furnissii* (= *V. fluvialis* II)(Proteobacteria, Vibrionales, Vibrionaceae, *Vibrio*)

(1) Epidemiological research

- (i) Host range: Ezo abalone (*Haliotis discus hannai*) is known as a host. The susceptibility of other abalone species has not been studied.
- (ii) Geographical distribution: Outbreaks of the disease have been reported only in the Dalian area of China, although the causative agent, *Vibrio furnissii*, is known to be distributed in estuarine environment worldwide.

(2) Clinical observation

- (i) Pustules or blisters containing white pus are formed on the foot.
- (ii) As the disease progresses, the abalone stops eating and eventually die. In the infected animals, the bacterium grows in various tissues, such as the mantle, digestive tract, digestive caeca, gonad, and hemolymph.

(3) Anatomical Findings

- (i) Pustules or blisters containing white pus are formed on the foot.

(4) Diagnosis

- (i) Initial diagnosis: (a) Observation of smear preparations of the lesions
Materials: Fresh pustule lesions.
Sample preparation: The contents of pustules are taken from a dissected lesion and smeared on glass slides.
Staining and observation: Stain the preparation with Giemsa stain and observe short rods under a microscope.

(b) Bacterial isolation

- Materials: The contents of pustules are sampled aseptically and diluted with 10 volume of the broth that has the same nutritional formula as the agar plates used in the next step.
- Bacterial isolation: Spread the diluted sample on either TS agar (Tryptic soy agar with 2-3% NaCl), BHI agar (Brain heart infusion agar with 2-3% NaCl), Marine agar, or TCBS agar (Thiosulfate citrate bile salts sucrose agar with 2-3% NaCl). The agar plates are incubated for 2-4 days at 24-37°C.

(c) Biochemical tests of the isolated bacterium

- Materials: Subculture of the predominant colonies on the agar plates of (4) (i) (b).
- Tests: Short rods should be confirmed with Giemsa-stained smear prepared from the isolated bacterial colonies. The bacterium is Gram negative,

sensitive to O129 disk, positive for oxidase and catalase, and show active motility in wet preparation under a microscope.

(ii) Confirmatory diagnosis: (a) Characterization of the isolated bacterium

The same as (4) (i) (c).

(b) PCR and gene sequencing

Materials: The same as (4) (i) (c).

Primers:

F-primer:

5'-ACT-CTT-ATT-TAC-GTC-AAA-GGA-CAG-3'

R-primer: 5'-TCT-TGC-AGC-GCT-TCA-AGA-ATT-TC-3'

Amplicon size; 722 bp

Reaction: Denature at 95 °C for 4 minutes, then conduct 30 cycles of incubation at 95 °C for one minute, at 55 °C for 30 seconds, and at 72 °C for one minute.

Finally incubate at 72 °C for 7 minutes.

Results: The sequence identity of the amplicon should be confirmed with the sequence of *V. furnissii* mreB gene registered in GenBank (accession No. DQ907418.1).

(5) Histopathological findings

Liquefactive necrosis occurs in the connective tissues and muscle in the lesion by marked inflammation. The lesions are first observed in the foot, and later found in the whole soft tissues as the disease progresses. In the final phase of the disease, only hemocytes and bacterial cells remain in the center of a lesion.

(6) Differential diagnosis

There are reports that *Vibrio cambellii*, *V. harveyi*, and *V. carchariae* can cause similar diseases. In addition, other bacteria are sometimes isolated from the foot, although those bacteria do not cause pustules or blisters. The infection with *Perkinsus sp.* is also known to cause yellowish pustules or abscesses on the foot.

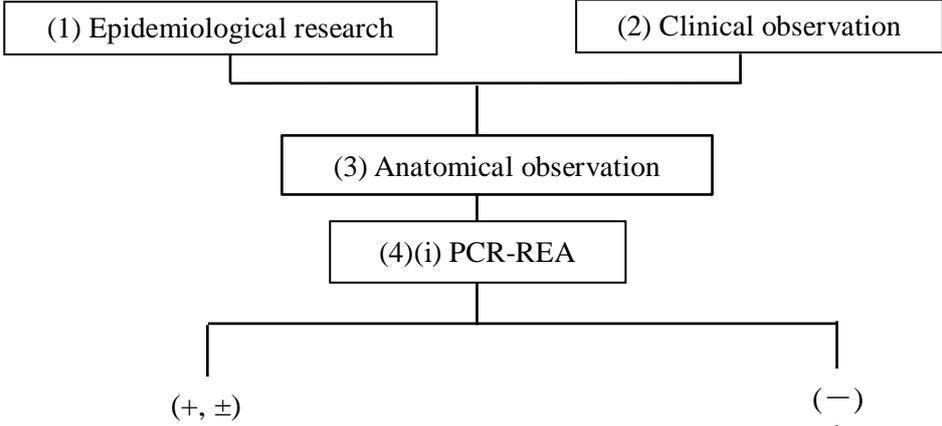
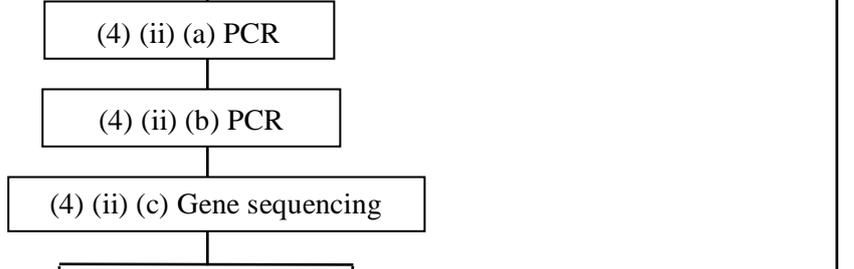
(7) Disinfection

Ordinary disinfectants against bacteria can be used for the disinfection of hands and equipment.

(8) Other

N/A

(3) Infection with Oysteid herpesvirus 1 microvariant (limited to OsHV-1 μ var)

| Responsible organization | Diagnosis flow chart | | |
|--|---|-----|---|
| Prefectural government |  <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] A --- B B --- C["(3) Anatomical observation"] C --- D["(4)(i) PCR-REA"] D --- E["(+, ±)"] D --- F["(-)"] </pre> | | |
| National Research Institute of Aquaculture |  <pre> graph TD E["(+, ±)"] --- G["(4) (ii) (a) PCR"] G --- H["(4) (ii) (b) PCR"] H --- I["(4) (ii) (c) Gene sequencing"] I --- J["(+)"] I --- K["(-)"] </pre> | | |
| Results | (+) | (-) | |
| Remarks | + | - | - |

Disease: Infection with ostreid herpesvirus 1 microvariant (limited to OsHV-1 μ var)

Aetiological agent: Ostreid herpesvirus 1 microvariants (limited to OsHV-1 μ var)
(Malacoherpesviridae, Ostreavirus)

(1) Epidemiological research

- (i) Host range: Pacific oyster, Portuguese oyster
- (ii) Geographical distribution: European countries
- (iii) The disease often occurs in summer when the water temperature is high.

(2) Clinical observation

- (i) Mass mortality is observed within a short period of time.
- (ii) Infected hosts tend to keep their shells open or they may be slow to close the shells.

(3) Anatomical findings

No characteristic signs are observed.

(4) Diagnosis

(i) Initial diagnosis: PCR and restriction enzyme analysis

(a) PCR

Materials: DNA extracted from tissues such as the mantle, gills, or ganglion.

Primers:

C2: 5'- CTC-TTT-ACC-ATG-AAG-ATA-CCC-ACC-3'

C6: 5'- GTG-CAC-GGC-TTA-CCA-TTT-TT-3'

Amplicon size: 700 bp

Reaction: Denature for 10 minutes at 94°C, then conduct 40 cycles of incubation for 30 seconds at 94°C, 30 seconds at 63°C, and for 30 seconds at 72°C. Finally incubate for 7 minutes at 72°C.

(b) Restriction enzyme analysis (REA)

Materials: The amplicon obtained from the PCR above

Restriction enzyme: *Mfe* I

Reaction: Incubation at 37°C for 15 minutes to overnight.

Judgment: The PCR product is digested into two fragments that are approximately 500 bp and 200 bp.

(ii) Confirmatory diagnosis: (a) PCR

The same as the initial diagnosis indicated in (4) (i) (a).

(b) PCR

Materials: DNA extracted from tissues such as the mantle, gills, or ganglion.

Primers:

IA2: 5'-AAT-CCC-CAT-GTT-TCT-TGC-TG-3'

IA1: 5'-CGC-GGT-TCA-TAT-CCA-AAG-TT-3'

Amplicon size: Approximately 600 bp

Reaction: The same as in (a).

(c) Gene sequence analysis

Materials: Amplicon obtained in (4) (ii),(a) and (b).

Method: Gene sequence analysis

Results: The sequence identity of the amplicon is confirmed with the sequence of OsHV-1 μ Var described in Segarra *et al.* (Virus research 153, pp92-99, 2010). In addition, the sequence of the amplicon by C2C6 primer set is registered in GenBank (accession no. HQ842610).

(5) Histopathological finding

Lesions accompanied by enlarged cell nuclei with perinuclear chromatin are observed in the connective tissue.

(6) Differential diagnosis

No information is available.

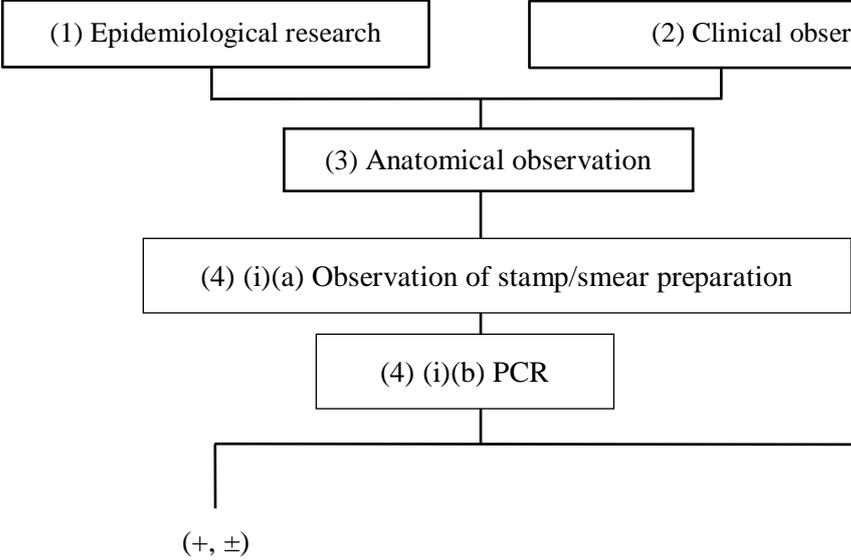
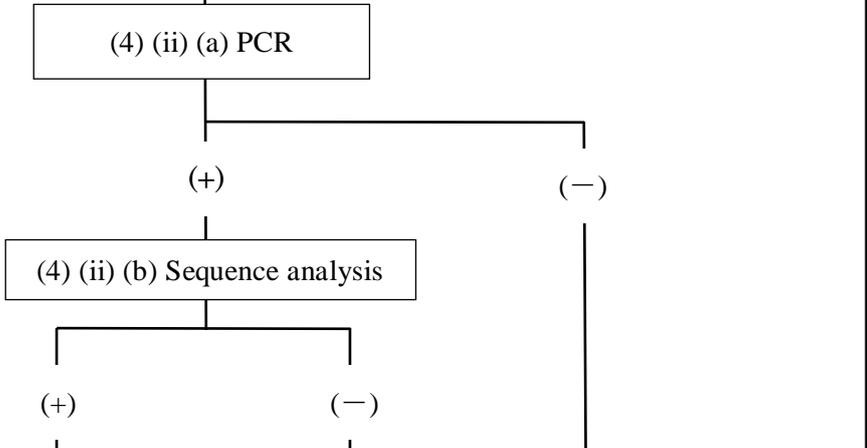
(7) Disinfection

This virus is probably inactivated by chlorine agent or other regular disinfectant, although there is no literature information.

(8) Other

N/A

(4) Infection with *Perkinsus qugwadi*

| Responsible organization | Diagnosis flow chart |
|--|---|
| Prefectural government |  <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] A --- B B --- C["(3) Anatomical observation"] C --- D["(4) (i)(a) Observation of stamp/smear preparation"] D --- E["(4) (i)(b) PCR"] E --- F["(+, ±)"] E --- G["(-)"] </pre> |
| National Research Institute of Aquaculture |  <pre> graph TD H["(4) (ii) (a) PCR"] --- I["(+)"] H --- J["(-)"] I --- K["(4) (ii) (b) Sequence analysis"] K --- L["(+)"] K --- M["(-)"] N["(-)"] --- O["(-)"] </pre> |
| Results | <div style="display: flex; justify-content: space-around; width: 100%;"> + - - - </div> |
| Remarks | Appropriate primers within the gene should be prepared as necessary, to determine the total gene sequence. |

Disease: Infection with *Perkinsus qugwadi*

Aetiological agent: *Perkinsus qugwadi* (Apicomplexa, Perkinsasidea, Perkinsus)

(1) Epidemiological research

- (i) Host range: Yesso scallop (*Mizuhopecten yessoensis*).
- (ii) Geographical distribution: British Columbia, Canada
- (iii) The disease occurred in cultured Yesso scallop that had been introduced from Japan to British Columbia.
- (iv) Susceptible stages of the host: The Yesso scallop can be infected with the disease at any developmental stages.

(2) Clinical observation

- (i) The disease often causes mass mortality.
- (ii) No particular clinical sign is observed in the external appearances of affected scallop.

(3) Anatomical findings

- (i) White pustules up to 5 mm in diameter are formed on the digestive gland.
- (ii) The gonad becomes distended and opaque.

(4) Diagnosis

- (i) Initial diagnosis: (a) Microscopic observation of stamp preparation

Materials: Lesions in the gonad

Observation of zoospores: Stamp/smear preparations prepared from gonad are stained with Wright-Giemsa or other equivalent stains. Observe zoospores of the parasite in the preparations under a microscope.

(b) PCR

Materials: Tissues sample for PCR, such as the gonad, digestive gland, mantle, or gills.

Primers:

PqguF7TC: 5'-CCA CTC TGG TAG TCT TGT CTT C -3'

PQ3R: 5'-AGA ATG GCG ACG CTG ATG AA -3'

Amplicon size: 281 bp

Reaction: Denature at 94°C for 3 minutes, then, conduct 40 cycles of incubation at 94°C for 30 seconds, at 54°C for 30 seconds, and at 72°C for 30 seconds. Finally incubate at 72°C for 10 minutes.

- (ii) Confirmatory diagnosis: (a) PCR

The same PCR as the initial diagnosis of (4) (i) (b).

(c) Sequence analysis (SSU rRNA gene)

Materials: The same extracted DNA used in (4) (ii) (a).

Primers:

Pm18S-1098F: 5'-AGG AAT TGA CGG AAG GGC A -3'

PqITS-22R: 5'-CGC AGT TTA AAT GAA TCG GT -3'

Amplicon size: 1,796 bp

Reaction: Denature at 94°C for 5 minutes, then conduct 30 cycles of incubation for at 94°C 30 seconds, at 54°C for 30 seconds, and at 72°C for 45 seconds. Finally incubate 72°C for 7 minutes at.

Results: Determine nucleotide sequence of the amplicon and confirm the identity of the sequence with that of *Perkinsus qugwadi* (GenBank accession no. AB716689).

(5) Histopathological findings

Trophozoites, tomonts, zoosporangia, or zoospores that can be considered morphologically as *Perkinsus qugwadi* are observed in the tissues, such as the gonad, digestive gland, mantle, or gills.

(6) Differential diagnosis

Infections of the parasites of the genus *Perkinsus* have been reported in other mollusks. However, *Perkinsus qugwadi* can be distinguished from the other *Perkinsus* organisms as it does not develop prezoosporangia in fluid thioglycollate medium and does not show dark blue staining with Lugol's iodine. In addition, *P. qugwadi* can also be distinguished from other *Perkinsus* species by the sequence of SSU rRNA gene.

(7) Disinfection

It is reported that the treatments with N-halamine, freshwater, and UV light are effective to inactivate the other *Perkinsus* parasites.

(8) Other

Building a disease resistant population by selective breeding of surviving scallop from *P. qugwadi* infection is reported to be effective as a preventive measure against the disease.

(5) Soft Tunic Syndrome

| Responsible organization | Diagnosis flow chart | | | | | |
|--|---|---|---|---|---|---|
| Prefectural government | <pre> graph TD A["(1) Epidemiological research"] --- B["(2) Clinical observation"] B --- C["(3) Anatomical observation"] C --- D["(4) (i) (a) PCR"] C --- E["(4) (i) (b) Histopathology"] D --- F["(-)"] D --- G["(+, ±)"] E --- H["(+, ±)"] E --- I["(-)"] G --- J["(4) (ii) (a) PCR"] H --- K["(4) (ii) (b) Histopathology"] J --- L["(+)", "-)"] K --- M["(+)", "-)"] F --- N["-"] L --- O["+"] L --- P["-"] M --- Q["+"] M --- R["-"] I --- S["-"] </pre> | | | | | |
| National Research Institute of Aquaculture | | | | | | |
| Results | - | + | - | + | - | - |
| Remarks | <p>Either (a) or (b) shall be conducted as the initial diagnosis. Either (a) or (b) shall be conducted as the confirmatory diagnosis.</p> | | | | | |

Disease: Soft tunic syndrome

Aetiological agent: *Azmiobodo hoyamushi* (Euglenozoa, Kinetoplastea, Neobodonida, *Azmiobodo*)

(1) Epidemiological research

(i) Host range: Sea-squirt (*Halocynthia roretzi*, edible ascidian) and Stalked sea squirt (*Styela clava*).

(ii) Geographic distribution: Korea and Japan (North of Oshika Peninsula in Miyagi Prefecture and Iwate Prefecture in Japan)

(iii) Susceptible stages: Usually the animals older than one year old are affected.

(iv) Carrier: Infected ascidian can be carries.

(2) Clinical observation

(i) In the early stage of the disease, the tunic softens around the two siphons.

(ii) The whole tunic becomes extremely softened and thin in severely affected animals.

(3) Anatomical findings

No significant change is observed in the internal soft tissues.

(4) Diagnosis method

(i) Initial diagnosis: (a) PCR

Materials: Fresh affected tunic or the flagellate migrated from small pieces of softened tunic into seawater *in vitro*.

Primers:

ProtoHoya 18S-145: 5'-AAG GGG TGC TTC CGA TCC GTG G-3'

ProtoHoya 18S-679r: 5'-AAG GAT GGG ACG GAA CCG ACT GC-3'

Amplicon size: 535 bp

Reaction: Denature at 98°C for 30 seconds, then conduct 40 cycles of incubations at 98°C for 10 seconds and at 72°C for 30 seconds. Finally incubate at 72°C for 5 minutes.

(b) Histopathology

Materials: Pieces of tunic fixed in Davidson's solution.

Preparation: Paraffin sections stained with hematoxylin and eosin.

Observation: Observation of the flagellate cells (10-14 x 2-3µm) that are stained with hematoxylin. One end of the flagellate cell tapers off to a flagellum.

(ii) Confirmatory diagnosis: (a) PCR

The following PCR shall be conducted in addition to the same PCR for the initial diagnosis.

Materials: The same as the section of the initial diagnosis.

Primers:

AhF: 5'-GCC TCT GTG GTT TGC TCC TTC GTG T -3'

AhR: 5'-TAC TGG GCG GCT TGG ATC TCG T-3'

Amplicon size: 642 bp

Reaction: Denature at 95°C for 3 minutes, then conduct 40 cycles of incubations at 95°C for one minute, at 64°C for 34 seconds, and at 72°C for one minute. Finally incubate at 72°C for 5 minutes.

(b) Histopathology (the same as in (i) (b)).

(5) Histopathological finding

(i) The layer of the fiber of softened tunics are usually much disturbed, often with hollow spaces within the matrix.

(ii) Hematoxylin stained flagellate cells are observed in the affected tunic.

(6) Differential diagnosis

No disease with similar clinical signs has been reported.

(7) Disinfection

The *in vitro* 50% effect concentration (EC₅₀) are 10mg/L or less for formalin, hydrogen peroxide, bithionol, chlorine dioxide, or bronopol when the causative agent is treated with one of these drugs for 24 hours.

(8) Other

N/A