THAI AGRICULTURAL STANDARD

TAS 10454-2011

DIAGNOSIS OF KOI HERPESVIRUS (KHV) DISEASE

National Bureau of Agricultural Commodity and Food Standards
Ministry of Agriculture and Cooperatives

DIAGNOSIS OF KOI HERPESVIRUS (KHV) DISEASE

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Koi Herpesvirus Disease (KHVD) is a contagious disease of carps which causes economic loss to the carp raising businesses as ornamental and food. The disease outbreaks were reported in several countries including Thailand causing high morbidity and mortality in carps. KHVD is the disease listed by the World Organization for Animal Health (OIE). Therefore, the Agricultural Standards Committee deems it necessary to establish the Thai Agricultural Standard on Diagnosis of Koi Herpesvirus (KHV) Disease for laboratory to be used as a guideline as well as for carp farm certification.

This standard is based on the following documents:


NOTIFICATION OF THE MINISTRY OF AGRICULTURE AND COOPERATIVES
SUBJECT: THAI AGRICULTURAL STANDARD:
DIAGNOSIS OF KOI HERPESVIRUS (KHV) DISEASE
UNDER THE AGRICULTURAL STANDARDS ACT B.E. 2551 (2008)

Whereas the Agricultural Standards Committee, deems it necessary to establish an agricultural standard for Diagnosis of Koi Herpesvirus (KHV) Disease as a voluntary standard in accordance with the Agricultural Standards Act B.E. 2551 (2008) to promote agricultural commodity to meet its quality and safety standards.

By virtue of Sections 5, 15 and 16 of the Agricultural Standards Act B.E. 2551 (2008), the Minister of Agriculture and Cooperatives hereby issues this Notification on Establishment of Thai Agricultural Standard for the Diagnosis of Koi Herpesvirus (KHV) Disease (TAS 10454-2011), as voluntary standard, details of which are attached herewith.

Notified on 2 September B.E. 2554 (2011)

(Mr. Theera Wongsamut)
Minister of Agriculture and Cooperatives
THAI AGRICULTURAL STANDARD

DIAGNOSIS OF KOI HERPESVIRUS (KHV) DISEASE

1. SCOPE

This agricultural standard provides the laboratory diagnostic methods for Koi herpesvirus (KHV) disease by histopathology, polymerase chain reaction (PCR), and virus isolation.

2. DEFINITIONS

For the purpose of this standard:

2.1 Carp means fish of the family *Cyprinus*, such as fancy carp, common carp and *Cyprinus* hybrids.

2.2 Koi herpesvirus disease (KHVD) means an infectious disease in carp caused by Koi herpesvirus (KHV).

2.3 Diagnosis means investigation processes to identify diseases.

2.4 Presumptive test means laboratory test which is convenient or rapid.

2.5 Confirmatory test means the laboratory tests used to confirm disease diagnosis. The tests have been proved to provide high specificity and sensitivity.

2.6 Specificity means ability of a test to yield a negative result in a group of un-infected samples.

2.7 Sensitivity means ability of a test to yield a positive result in a group of infected samples.

2.8 Positive control set means a test panel containing the standard microbes to be studied for comparison with the unknown samples.

2.9 Negative control set means a test panel that does not contain the standard microbes to be studied for comparison with the unknown samples.

3. DIAGNOSIS

Diagnosis of KHVD aims to screen and confirm sick fish or infected fish that shows no clinical sign or lesion as well as to conduct disease surveillance. In order to obtain effective disease prevention and control measures, epidemiology, clinical signs and pathogenesis of KHVD (Appendix A) should be considered together with the laboratory results.

Each diagnostic procedure provides different efficacy (Appendix B) hence diagnosis of KHVD should not rely on only one diagnostic procedure but a combination of two or more. PCR technique should be used as a confirmatory test.

3.1 SAMPLING

Sampling shall be as described in Appendix C, Table C.1.
3.2 Histopathology

3.2.1 Principle

Histopathology is a microscopic examination of tissue. The preserved tissue is stained with hematoxylin and eosin (H&E) and observed under light microscope.

This method cannot be used as a confirmatory diagnosis because the pathology of disease can be nonspecific. Infected fish may not show pathological lesion of KHVD.

3.2.2 Sample collection and preservation

Collect non-autolysed tissues, e.g. gill, kidney, spleen, liver, pancreas and encephalon from suspected carp and preserve the tissue in 10% formalin buffer. The volume of formalin should be at 10-20 times of the tissue sample. The sample should be stored below 15°C for at least 24 h.

3.2.3 Procedure

Process sample from Section 3.2.2 using histopathological procedure as described in Appendix D.

3.2.4 Interpretation

Inflammation and necrosis of gill tissue are prominent in infected fish. Marked nuclear swelling and margination of chromatin or “signet ring” appearance can be found in branchial epithelial cells and leucocytes including intranuclear inclusion bodies may also be observed.

In addition, inflammation, necrosis and intranuclear inclusion bodies can be observed, in particular, tissues of kidney, spleen, pancreas, liver, encephalon, intestine and oral epithelium.

3.3 PCR assay

3.3.1 Principle

PCR assay targets specific DNA sequence on KHV genome. Specific primers bind to targeted DNA sequences, and increase the amounts of DNA to detectable level. Amplified DNA is then visualized by electrophoresis.

3.3.2 Sample collection and preservation

(1) Fish of all ages are susceptible to the disease. However, the risk of KHV is found to be higher for carp less than 1 year of age. Sample should be collected from carp that show prominent clinical signs or from carp in the suspected fish population.

(2) Fish should be kept separately and send to the laboratory alive. Details of the sample should be clearly provided (Appendix E).

(3) If live sample cannot be obtained, fish should be humanely euthanized. Put euthanized fish or collect the internal organs, such as gills, liver, spleen, kidney, intestine and encephalon in plastic bag, seal the bag and place in freezer or on ice. Internal organs that are dissected into small pieces can be preserved in 80% -100% ethyl alcohol and should be delivered to the laboratory immediately or within 6 h.
3.3.3 Methods

3.3.3.1 Sample preparation depends on type of sample
(1) For large fish, where target organs can be collected, separate approximately 100 mg - 200 mg/organ and grind thoroughly in sterile distilled water.
(2) For small fish, where target organs cannot be collected, pool the sample to obtain the amount of 100 mg - 200 mg and grind thoroughly in sterile distilled water.

3.3.3.2 DNA extraction from targeted samples can be performed by 2 methods as follows:

**Method 1**

(1) Add 1 ml of sample from Section 3.3.3.1 into 1.5 ml microcentrifuge tube, centrifuge at 15,000 g for 15 min. Remove the supernatant.
(2) Add 150 μl -200 μl of 50X Tris and ethylenediamine tetraacetic acid (TE) buffer and 30 μl lysozyme (20 mg/ml). Place in incubator shaker at 37°C for 40 min.
(3) Add 20 μl of 10% sodium dodecyl sulfate (SDS) and place in incubator at 65°C for 10 min. Add 30 μl proteinase K (10 mg/ml) and place in incubator shaker at 37°C for 24 h.
(4) Add 150 μl -200 μl phenol-chloroform-isooamyl alcohol (25: 24: 1), mix thoroughly and centrifuge at 15,000 g for 15 min. Transfer 200 μl supernatant into a new microcentrifuge tube.
(5) Add 20 μl of 3M ammonium acetate and 1 ml absolute ethanol into the microcentrifuge tube that contains 200 μl supernatant. Store at -20°C for 24 h.
(6) Centrifuge at 15,000 g for 15 min, remove supernatant. Leave the precipitate at room temperature until the ethanol completely evaporates.
(7) Dissolve the precipitate with 100 μl 1X TE buffer or sterile double distilled water to obtain DNA solution for further tests. Keep the solution at -20°C.

**Method 2**

(1) Add 100 μl of sample from Section 3.3.3.1 into 1.5 ml microcentrifuge tube. Add 1 ml DNAzol® reagent, mix thoroughly and place at room temperature for 5 min.
(2) Centrifuge at 10,600 g for 10 min, transfer 1 ml supernatant into a new microcentrifuge tube and add 0.5 ml ethanol. Mix thoroughly and place at room temperature for 5 min.
(3) Centrifuge at 18,000 g for 30 min, remove supernatant and wash the precipitate with 250 μl of 70% ethanol.
(4) Centrifuge at 18,000 g for 5 min, remove supernatant and leave the precipitate at room temperature until the ethanol completely evaporates.
(5) Dissolve the precipitate with 100 μl 1X TE buffer of sterile double distilled water to obtain DNA solution for further tests. Keep the solution at -20°C.
Table 1 Nucleotide sequences of primers
(Sectons 3.3.3.3, 3.3.3.4 and 3.3.3.5)

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide sequences</th>
<th>References</th>
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<tr>
<td>KHV-TKf</td>
<td>5’-GGG-TTA-CCT-GTA-CGA-G-3’</td>
<td>Bercovier et al., 2005</td>
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<tr>
<td>KHV-TKr</td>
<td>5’-CAC-CCA-GTA-GAT-TAT-GC-3’</td>
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<td>Sph I-5 F</td>
<td>5’-GAC-ACC-ACA-TCT-GCA-AGG-AG-3’</td>
<td>Gray et al., 2002</td>
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<tr>
<td>Sph I-5 R</td>
<td>5’-GAC-ACA-TGT-TAC-AAT-GGT-CGC-3’</td>
<td>Modified by Yuasa et al., 2005</td>
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<td>KHV9/5F</td>
<td>5’-GAC-GAC-GCC-GGA-GAC-CTT-GTG-3’</td>
<td>Gilad et al., 2002</td>
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<tr>
<td>KHV9/5R</td>
<td>5’-CAC-AAG-CTC-AGT-CTG-TTC-CTC-AAC-3’</td>
<td></td>
</tr>
<tr>
<td>KHV-1Fn</td>
<td>5’-CTC-GCC-GAG-CAG-AGG-AAG-CGC-3’</td>
<td>Bergmann et al., 2006</td>
</tr>
<tr>
<td>KHV-1Rn</td>
<td>5’-TCA-TGC-TCT-CCG-AGG-CCA-GCG-G-3’</td>
<td></td>
</tr>
</tbody>
</table>

Remark: Different primers, PCR conditions (time and temperature), reagents and equipment may be applied only on the occasion that these materials and methods have been published in approved scientific journals and provide equal or higher sensitivity and specificity.

3.3.3.3 Target DNA amplification by the method of Bercovier et al. (2005)

(1) Prepare PCR cocktail as indicated:

- 10X Reaction buffer 10 μl
- 25 mM MgCl₂ 5 μl
- 25 mM dNTPs 0.5 μl
- 1 U DNA polymerase 0.25 μl
- 100 pmol/μl KHV-TKf primer 0.5 μl
- 100 pmol/μl KHV-TKr primer 0.5 μl

Add sterile distilled water to obtain the final volume of 47.5 μl/test

(2) Add 2.5 μl DNA sample into the cocktail microcentrifuge tube. Prepare positive and negative control sets for each diagnostic. Place the tube in thermocycler. If thermocycler is without heated lid, apply 2 drops of sterile mineral oil into each tube in order to prevent evaporation.

(3) Amplify target DNA using thermocycler and set the PCR conditions as follows:

Step 1 Incubate the sample at 94°C for 5 min

Step 2 Incubate the sample at 95°C for 1 min (to initialise denaturation phase)

Incubate the sample at 55°C for 1 min (to initialise annealing phase)

Incubate the sample at 72°C for 1 min (to initialise extension phase)

Repeat step 2 for 40 cycles
Step 3 Incubate the sample at 72°C for 10 min and decrease temperature to 4°C. Maintain sample at 4°C until use.

Separation of DNA band using agarose gel electrophoresis

(1) Add loading dye into PCR product, mix well and then transfer the product into a well of 2% agarose gel, 20 μl per well.
(2) Use 100 bp DNA ladder as a marker.
(3) Electrophorese at 120V for 20 min in TBE (Tris, boric acid, EDTA) solution.
(4) Stain gel with ethidium bromide (0.5 μl/ml) or DNA-staining chemical and visualize the size of PCR product using UV transilluminator.

Interpretation of the results

Positive or negative result may be determined by the size of PCR product band of each sample when compared to a DNA marker, positive/negative control sets as follows:
- If the product band is visible at 409 bp, it defines as positive.
- If the product band is not visible at 409 bp, it defines as negative.

3.3.3.4 Target DNA amplification by the modified method of Gray et al. (2002) and Yuasa et al. (2005)

(1) Prepare PCR cocktail as indicated:
   - 10X Reaction buffer 2 μl
   - 25 mM MgCl₂ 1.6 μl
   - 25 mM dNTPs 1.6 μl
   - 1 U DNA polymerase 0.1 μl
   - 50 pmol/μl Sph I-5 F primer 0.2 μl
   - 50 pmol/μl Sph I-5 R primer 0.2 μl

   Add sterile distilled water to obtain the final volume of 19 μl/test

(2) Add 1 μl DNA sample into the cocktail microcentrifuge tube. Prepare positive and negative control sets for each diagnostic. Place the tube in thermocycler. If thermocycler is without heating lid, apply 2 drops of sterile mineral oil into each tube in order to prevent evaporation.

(3) Amplify target DNA using thermocycler and set the PCR conditions as follow:
   Step 1 Incubate the sample at 94°C for 30 s
   Step 2 Incubate the sample at 94°C for 30 s (to initialise denaturation phase)
       Incubate the sample at 63°C for 30 s (to initialise annealing phase)
       Incubate the sample at 72°C for 30 s (to initialise extension phase)
       Repeat step 2 for 40 cycles
   Step 3 Incubate the sample at 72°C for 7 min and decrease temperature to 4°C. Maintain sample at 4°C until use.


Separation of DNA band using agarose gel electrophoresis

(1) Add 3 μl of 6X loading dye into PCR product, mix well and then transfer the product into a well of 2% agarose gel, 7 μl per well.

(2) Use 100 bp DNA ladder as a marker.

(3) Electrophorese at 100V for 20 min in TBE (Tris, boric acid, EDTA) solution.

(4) Stain gel with ethidium bromide (0.5 μl/ml) or DNA-staining chemical and visualize the size of PCR product using UV transilluminator.

Interpretation of results

Positive or negative result may be determined by the size of PCR product band of each sample when compared to a DNA marker, positive/negative control sets as follows:
- If product band is visible at 292 bp, it defines as positive.
- If product band is not visible at 292 bp, it defines as negative.

3.3.3.5 Target DNA amplification by nested PCR method of Bergmann et al. (2002)

First step PCR

(1) Prepare PCR cocktail as indicated:
- 1X Reaction buffer
- 2 mM MgCl₂
- 400 μM dNTPs
- 1 U DNA polymerase
- 30 pmol/μl KHV9/5F primer
- 30 pmol/μl KHV9/5R primer

(2) Add 70-100 ng DNA sample into the cocktail microcentrifuge tube and add sterile distilled water to obtain the final volume of 50 μl/test. Prepare positive and negative control sets for each diagnostic. Place the tube in thermocycler. If thermocycler is without heated lid, apply 2 drops of sterile mineral oil into each tube in order to prevent evaporation.

(3) Amplify target DNA using thermocycler and set the PCR conditions as follow:

Step 1 Incubate the sample at 95 °C for 5 min
Step 2 Incubate the sample at 94 °C for 1 min (to initialise denaturation phase)
   Incubate the sample at 68 °C for 1 min (to initialise annealing phase)
   Incubate the sample at 72 °C for 30 s (to initialise extension phase)
   Repeat step 2 for 39 cycles
Step 3 Incubate the sample at 72 °C for 7 min and decrease temperature to 4 °C. Maintain sample in 4 °C until use.
Second step of the nested PCR

(1) Prepare PCR cocktail as indicated:
- 1X Reaction buffer
- 2 mM MgCl₂
- 400 μM dNTPs
- 1 U DNA polymerase
- 30 pmol/μl KHV-1Fn primer
- 30 pmol/μl KHV-1Rn primer

(2) Add PCR product from the first-step PCR and add sterile distilled water to obtain the final volume of 50 μl/test. Prepare positive and negative control sets for each diagnostic. Place the tube in thermocycler. If thermocycler is without heated lid, apply 2 drops of sterile mineral oil into each tube in order to prevent evaporation.

(3) Amplify target DNA using thermocycler and set the PCR conditions as follow:
- Incubate the sample at 95°C for 5 min
- Incubate the sample at 94°C for 1 min (to initialise denaturation phase)
- Incubate the sample at 68°C for 1 min (to initialise annealing phase)
- Incubate the sample at 72°C for 30 s (to initialise extension phase)
- Repeat step 2 for 39 cycles
- Incubate the sample at 72°C for 7 min and decrease temperature to 4°C. Maintain sample in 4°C until use.

Separation of DNA band using agarose gel electrophoresis

(1) Add 3 μl of 6X loading dye into PCR product, mix well and then transfer the product into a well of 2% agarose gel, 7 μl per well.
(2) Use 100 bp DNA ladder as a marker.
(3) Electrophoresese at 120V for 20 min in TBE (Tris, boric acid, EDTA) solution.
(4) Stain gel with ethidium bromide (0.5 μl/ml) or DNA-staining chemical and visualize the size of PCR product using UV transilluminator.

Interpretation of results

Positive or negative result may be determined by the size of PCR product band of each sample when compared to a DNA marker, positive/negative control sets as follows:
- If product band is visible at 484 bp and/or 393 bp, it defines as positive.
- If product band is not visible at 484 bp and/or 393 bp, it defines as negative.

3.3.4 Cautions

Separate areas, tools and equipment for steps of DNA extraction and PCR cocktail preparation to prevent contamination. Filtered tip should be used during PCR cocktail preparation.
3.4 Virus isolation

3.4.1 Principle

This technique is to extract from carp tissue, culture virus in cell line and observe cytopathic effect (CPE). However, this virus isolation is not as sensitive as the PCR-based method and considered to be a low sensitivity diagnostic test for KHVD.

3.4.2 Sample collection and preservation

3.4.2.1 Sample collection from different sizes of fish

(1) Newly hatched fish: collect whole fish, remove yolk sac.
(2) 4 cm – 6 cm fish: collect all internal organs, including kidney and encephalon.
(3) Fish longer than 6 cm: collect gill, kidney, spleen, heart or encephalon.
(4) Fully mature fish: collect ovarian fluid, gill and/or internal organs such as kidney, spleen, encephalon and intestine.

3.4.2.2 Place the fish or the organ samples in sterile container and store at 4°C or on ice. Send the sample to the laboratory immediately or within 6 h. If the sample cannot be transferred to the laboratory within the day, the sample should be stored in dry ice or in freezer at -20°C or below and send to the laboratory on the next day.

Alternatively, place fish or the organ samples in cell culture medium or Hanks’ balanced salt solution with added 1,000 µg/ml gentamycin or 800 IU/ml penicillin with 800 µg/ml and 800 µg/ml streptomycin to the medium or solution in order to suppress the growth of contaminated bacteria. Ratio of sample and medium/solution should be at 1:5. Antifungal agents such as Mycostatin® or Fungizone® at the dosage of 400 IU/ml may be added. Fetal bovine serum (FBS) at 5%-10% concentration may also be added in order to preserve the virus Send the sample to the laboratory within 12 h -24 h.

3.4.3 Materials and biological products for virus isolation and identification

3.4.3.1 Cell lines

- Koi fin (KF-1) cell line and Cyprinus carpio brain (CCB) cell line have been proved for the use of KHV culture and isolation.

3.4.3.2 Culture medium

- Eagle’s minimal essential medium (MEM) with Earle’s salt supplemented with 10% FBS, antibiotics (e.g., 100 IU/ml penicillin and 100 µg/ml dihydrostreptomycin) and 2 mM L-glutamine. If fungal contamination is suspected, add 50 IU/ml Mycostatin®. In addition, Leibovitz medium (L15) containing 5% FBS or 10% 4 mM L-glutamine and 50 µg/ml gentamycin may also be used.

- Add 0.02 M N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (HEPES) or sodium bicarbonate and 0.16 M tris-hydroxymethyl aminomethane (Tris) HCl to control pH of the medium.

- For cell growth, culture medium should contain10% FBS and adjust pH between 7.3-7.4. For virus isolation and production, culture medium should contain 2% FBS and adjust pH at 7.6.
3.4.4 Procedure

3.4.4.1 Virus extraction from collected sample

- Virus extraction should be performed at temperature below 15°C. The optimal temperature range is 0°C -10°C.
- Completely decant the medium/solution.
- Homogenise the sample (fish or organs) thoroughly in HBSS, use 9 parts of HBSS per 1 part of sample. Centrifuge at 2,000 g -4,000 g at 2°C -5°C for 15 min and collect supernatant. Add 1 mg/ml gentamycin to the collected supernatant and store at 15°C for 4 h, or at 4°C overnight.
- In case of carp’s ovarian fluid, HBSS with antibiotics should be added not to exceed 5 times of the fluid’s volume to prevent bacterial growth. Centrifuge at 2,000 g - 4,000 g at 2°C -5°C for 15 min, collect supernatant and store at 15°C for 4 h, or at 4°C overnight.

3.4.4.2 Virus culture in cell lines

- Sample used for virus culture shall be treated with antibiotics as indicated in Section 3.4.4.1.
- If cell death occurs due to antibiotic toxicity, prepare sample using filtration technique. Filter at least 1 ml of homogenised sample through 0.45µm cellulose acetate filter unit.
- Transfer approximately 100 µl of antibiotics supplemented sample or filtered sample into tissue culture flask or multi-well plate that contains 24 h -48 h old-cell monolayer. To increase virus attachment, filtered sample may be 10 fold dilution with culture medium pH 7.6, supplemented with 2% FBS and leave at 18°C -22°C for 0.5 h -1 h. Add appropriate amount of culture medium (1 ml -1.5 ml per 5 cm² culture container) and incubate at 20°C -25°C.
- Cell cultured in plate should be kept in carbon dioxide atmospheric condition to maintain pH.
- Observe cytopathic effect (CPE) daily for 14 days, using phase-contrast microscope at 40x-100x magnification.
- If CPE is observed in positive control set but not in the sample, repeat the culture processes for 14 days.

3.4.5 Interpretation of results

(1) Positive result indicated by the observed CPE within 14 days (Appendix F). Other diagnostic techniques such as PCR should be applied to confirm and identify virus type.
(2) Negative result indicated by the absence of CPE at the first culture and after the repeated culture.
Appendix A

Epidemiology, clinical signs and pathogenesis of KHVD

(Section 3)

A.1 Epidemiology

KHVD is an epidemic disease listed in the Aquatic Animal Health Code issued by the World Organisation for Animal Health (OIE). KHVD is listed in the Ministerial Regulation: Additional Animal Epic, B.E. 2547 (2004) under the Animal Epidemic Act, B.E.2499 (1956) and its amendments. KHVD belongs to virus family Herpesviridae, subfamily Cyprinid herpesvirus. KHVD is also known as cyprinid herpesvirus 3 (CyHV-3) or carp interstitial nephritis and gill necrosis virus (CNGV). Genome of KHV is 295 kbp in length and closely related to CyHV-1 (carp pox virus, fish papilloma virus) and CyHV-2 (goldfish haematopoietic necrosis virus).

The KHVD outbreak was first reported in 1998 in Israel and Germany, and the KHVD aetiology was subsequently confirmed. In Asia, the serious outbreak of KHVD causing significant loss was reported in Indonesia in 2002 as well as in high mortality incidences of common carp in Ibaraki Prefecture was reported in Japan in 2003. KHVD outbreaks were also reported in South Africa, the United States, the European countries, e.g. Austria, Belgium, Denmark, France, Italy, Luxemburg, the Netherlands, Poland, Switzerland and the United Kingdom, Asian countries such as China (Hong Kong), Chinese Taipei, Indonesia, Japan, Korea, Malaysia, Singapore (imported from Malaysia) and Thailand (exported to Germany in 2004). Department of Fisheries, Ministry of Agriculture and Cooperatives has issued the notification of Department of Fisheries: Importation of carp Cyprinus caprio into the Kingdom of Thailand Under the Quarantine system, dated September 30, B.E. 2547 (2003) requiring that all imported carp must be originated from certified fish farms and accompany with aquatic animal health certificate.

A.2 Clinical signs

Carp in all age groups are susceptible to the virus, especially; carp less than 1 year of age are the most susceptible to KHV. Infected carp exhibit signs of anorexia, abnormal swimming, depress, isolate from population, swimming near the edge of the pond or water surface, lose balance and increase respiratory rate. External lesions include change of body color either paler or darker, scale detachment, swollen and pale gills, multifocal gill necrosis, haemorrhage of skin and base of the fin, fin erosion and enopthalmia. Morbidity of affected population may reach 100% and 70%-100% mortality may be observed.

A.3 Pathogenesis

KHV remains active in water for at least 4 h, but not exceed 21 h at water temperature of 23 °C - 25 °C. The main routes of entry for KHV are gill and skin. The virus spreads from gill and skin and infect throughout the internal organs via blood circulation. High virus load can be detected in kidney, spleen, liver and gastrointestinal tract. Infected fish can shed the virus via urine, feces, gill and skin mucus. Water is considered as major abiotic vector for disease transmission. Other fish species, parasitic invertebrates, piscivorous birds, mammals and fomites associated with farm husbandry are also disease vectors. Water temperature also plays important role in KHV pathogenesis. Disease can be found at water temperature of 16°C - 25°C. Under experimental condition, high mortality was observed at water temperature of 28°C; however, the mortality decreased at the temperature 29°C -30°C or 13°C.
Appendix B

Comparison of the efficacy of the diagnostic techniques

(Section 3)

Table B.1 Comparison of the efficacy of the different diagnostic techniques

<table>
<thead>
<tr>
<th>Diagnostic technique</th>
<th>Surveillance</th>
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<th>Presumptive test</th>
<th>Confirmatory test</th>
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<td>Post Larva</td>
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Source: Modified from the Manual of Diagnostic Tests for Aquatic Animals (OIE, 2010)

Remarks

a = recommended due to the high specificity and sensitivity
b = standard technique with good diagnostic sensitivity and specificity
c = can be used in some situations due to limitation of its application
d = not applicable or not suitable
Appendix C

Sampling
(Section 3.1)

Table C.1 Sampling protocol

<table>
<thead>
<tr>
<th>Population</th>
<th>2% Prevalence</th>
<th>5% Prevalence</th>
<th>10% Prevalence</th>
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<td>250</td>
<td>110</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>500</td>
<td>130</td>
<td>55</td>
<td>26</td>
</tr>
<tr>
<td>1,000</td>
<td>140</td>
<td>55</td>
<td>27</td>
</tr>
<tr>
<td>1,500</td>
<td>140</td>
<td>55</td>
<td>27</td>
</tr>
<tr>
<td>2,000</td>
<td>145</td>
<td>60</td>
<td>27</td>
</tr>
<tr>
<td>4,000</td>
<td>145</td>
<td>60</td>
<td>27</td>
</tr>
<tr>
<td>10,000</td>
<td>145</td>
<td>60</td>
<td>27</td>
</tr>
<tr>
<td>100,000 or more</td>
<td>150</td>
<td>60</td>
<td>30</td>
</tr>
</tbody>
</table>

Source: Modified from the Manual of Diagnostic Tests for Aquatic Animals (OIE, 2010)
Appendix D

Sample preparation for histopathology

(Section 3.2.3)

D.1 Sample preparation for histopathology by the paraffin embedding method is as follows:

(1) Immerse tissue from Section 3.2.2 in solutions to a set of order as indicated:
   - Immerse in 70% ethanol for 1 h
   - Immerse in 85% ethanol for 1 h
   - Immerse in 95% ethanol (first bottle) for 1 h
   - Immerse in 95% ethanol (second bottle) for 1 h
   - Immerse in 95% ethanol (third bottle) for 1 h
   - Immerse in 100% ethanol (first bottle) for 1 h
   - Immerse in 100% ethanol (second bottle) for 1 h
   - Immerse in 100% ethanol (third bottle) for 1 h
   - Immerse in xylene or xylene replacement (first bottle) for 1.5 h
   - Immerse in xylene or xylene replacement (second bottle) for 1.5 h

(2) Embed tissue from item (1) in the first bottle of melted paraffin for 2 h and move to the second bottle of melted paraffin and immerse the tissue for 2 h.

(3) Place tissue in embedding mold that contains melted paraffin. Place the block on the embedding mold and fill the mold with melted paraffin. Place the mold on cool tray until paraffin is set. Keep the mold at room temperature.

(4) Cut the tissue embed with microtome to 4 µm -5 µm thick and place on glass slide.

D.2 Sample preparation for histopathology by staining tissue with hematoxylin & eosin stain is as follows:

(1) Remove paraffin on tissue slide (deparaffinize) by immerse the slide in xylene or xylene replacement for 2 times at 2 min each.
(2) Immerse tissue slide in 100% ethanol for 2 times at 2 min each

(3) Wash tissue slide through running water for 2 min -5 min.
(4) Immerse tissue slide in hematoxylin for 3 min.
(5) Wash tissue slide through running water for 5 min -10 min.
(6) Immerse tissue slide in eosin for 3 min.
(7) Wash tissue slide through running water.
(8) Dehydrate tissue slide with 70% ethanol.
(9) Dehydrate tissue slide with 100% ethanol for 2 times at 1-2 min each.
(10) Immerse in alcohol/clearene (50/50) for 1 min -2 min.
(11) Immerse in xylene or xylene replacment for 2 times at 2 min each.
(12) Apply 1 drop of mounting media and cover tissue slide with cover glass.
(13) Observe tissue slide with microscope at 400x magnification.
Appendix E

Sample: Record form for disease diagnosis

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Date</th>
<th>ID number</th>
</tr>
</thead>
</table>

### 1. General information

1.1 Owner’s name: House No. Village No. Sub-district: District: Province: Tel: 

1.2 Species: Amount per container 

#### 1.3 Source of fish

- [ ] Imported
- [ ] Cultured
- [ ] Wild caught
- [ ] Bought from:

#### 1.4 Type and size of rearing system

- [ ] Cement pond size:
- [ ] Earthen pond size:
- [ ] Glass aquaria size:
- [ ] Fiber tank size:
- [ ] Other size:

#### 1.5 Rearing density (Fish/m²)

### 2. Feed management

#### 2.1 Feed type

- [ ] Commercial feed
- [ ] Live feed
- [ ] Supplemented feed
- [ ] Other

#### 2.2 Feeding ratio (%) and frequency (per day)

### 3. Water quality management

#### 3.1 Water source

- [ ] Tap
- [ ] Underground
- [ ] Surface
- [ ] Rain
- [ ] Other

#### 3.2 Water changing rate (per day)

#### 3.3 Management for aeration

- [ ] Yes
- [ ] No

#### 3.4 Water quality

- Temperature
- BOD
- Dissolved Oxygen (DO)
- pH
- Hardness
- Total alkalinity
- Total ammonia (NH₃)
- Nitrite (NO₂)

### 4. Information on infected fish

#### 4.1 General appearances

- [ ] Eyes
- [ ] Gill
- [ ] Skin
- [ ] Pustule(s)
- [ ] Tumor
- [ ] Fin
- [ ] Respiration
- [ ] Swimming, buoyancy
- [ ] Other

#### 4.2 Age

- [ ] 1-15 Days
- [ ] 15-30 Days
- [ ] 1-6 Month(s)
- [ ] 6-12 Months
- [ ] 1-3 Years
- [ ] 3-5 Years
- [ ] Years or above

#### 4.3 Morbidity (%)
4.4 General management, e.g. water changing, medicine/chemical application

5. Diagnostic results

☐ Parasitology Sample No. .................................................................
☐ Parasitology result..............................................................................
Bacteriology Sample No. ...........................................................................
Bacteriology result....................................................................................
Antimicrobial susceptibility test

☐ oxytetracycline    ☐ sulfamethoxazole + Trimethoprim

☐ Mycology Sample No. .................................................................
Mycology result...................................................................................
☐ Pathology Sample No. .................................................................
Pathology result..................................................................................
☐ Virology Sample No. .................................................................
Virology result...................................................................................
Molecular biology Sample No. ..............................................................
☐ PCR result......................................................................................

6. Suggestion......................................................................................

7. Treatment follow-up

☐ Very Effective    ☐ Slightly effective    ☐ Moderately effective
☐ Not effective      ☐ Other..........................

8. Conclusion of diagnoses

..........................................................................................................................
..........................................................................................................................

Officer’s signature..........................................................

(....................................)

Date........Month............Year.............

Source: Modified from record sheet used for aquatic animal disease diagnosis, Inland Aquatic Animal Research Institute, Department of Fisheries.
Appendix F

Illustrations of Koi herpesvirus diagnosis

(Section 3)

Figure F.1 Focal necrosis on carp’s gill tissue (yellow arrow)
Courtesy: Ms. Thanida Haetrakul, Veterinary Medical Aquatic animal Research Center, Faculty of Veterinary Science, Chulalongkorn University

Figure F.2 Histopathology of the gill of KHV infected carp. Intranuclear inclusion bodies are found in the infected cells. Hypertrophy and hyperplasia of gill epithelium is presented, causing fusion of gill filaments.
Courtesy: Ms. Varinee Panyawachira, Inland Aquatic Animal Research Institute, Department of Fisheries
Figure F.3 Histopathology of the gill of KHV infected carp. Intranuclear inclusion bodies are found in the infected cells. Hypertrophy and hyperplasia of gill epithelium is presented, causing fusion of gill filaments.

Courtesy: Ms. Varinee Panyawachira, Inland Aquatic Animal Research Institute, Department of Fisheries

Figure F.4 Histopathology of the kidney of KHV infected carp. Excessive amount of protein is found in the cytoplasm of renal epithelium cells. Intranuclear inclusion bodies are abundantly found.

Courtesy: Ms. Varinee Panyawachira, Inland Aquatic Animal Research Institute, Department of Fisheries
Figure F.5 Pathology of KF-1 cell line infected with KHV. Cells are degenerating and detaching which can be observed by the presence of vacuolated cytoplasm. (400x magnification)

Courtesy: Dr. Prapansak Srisapoome, Faculty of Fisheries, Kasetsart University

Figure F.6 PCR result of KHV diagnosis, PCR method is based on Bercovier et al., (2005). DNA band at the size of 409 bp is visible, using KHV-TKf / KHV-TKr primers.

Courtesy: Dr. Tinarat Srisuwan, National Institute of Animal Health, Department of Livestock

<table>
<thead>
<tr>
<th>M</th>
<th>N</th>
<th>S</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight marker 100 bp</td>
<td>KHV negative sample</td>
<td>Sample of KHV infection</td>
<td>KHV positive sample (Band formed at 409 bp)</td>
</tr>
</tbody>
</table>
Figure F.7 PCR result of KHV diagnosis, PCR method is based on Gray et al., (2002) and its modified version by Yuasa et al., (2005). DNA band at the size of 292 bp is visible, using SphI-5 F / SphI-5 R primers. 

Courtesy: Dr. Tinarat Srisuwan, National Institute of Animal Health, Department of Livestock
**Figure F.8** PCR result of KHV diagnosis, PCR method is based on Bergmann et al., (2006). DNA band at the size of 484 and/or 392 bp are visible, using KHV9/5F / KHV9/5R and KHV-1Fn / KHV-1Rn primers.

Courtesy: Dr. Tinarat Srisuwan, National Institute of Animal Health, Department of Livestock
Appendix G

Units

Units and symbols used in this standard and the SI unit (International System of Units or Le Système International d’Unités) approved to be used are:

<table>
<thead>
<tr>
<th>Items</th>
<th>Unit</th>
<th>Unit symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mass</strong></td>
<td>gram</td>
<td>g</td>
</tr>
<tr>
<td></td>
<td>milligram</td>
<td>mg</td>
</tr>
<tr>
<td><strong>Volume</strong></td>
<td>milliliter</td>
<td>ml</td>
</tr>
<tr>
<td></td>
<td>microliter</td>
<td>µl</td>
</tr>
<tr>
<td></td>
<td>cubic centimeter</td>
<td>cm³</td>
</tr>
<tr>
<td><strong>Length</strong></td>
<td>centimeter</td>
<td>cm</td>
</tr>
<tr>
<td></td>
<td>millimeter</td>
<td>mm</td>
</tr>
<tr>
<td></td>
<td>micrometer</td>
<td>µm</td>
</tr>
<tr>
<td><strong>Time</strong></td>
<td>hour</td>
<td>h</td>
</tr>
<tr>
<td></td>
<td>minute</td>
<td>min</td>
</tr>
<tr>
<td></td>
<td>second</td>
<td>s</td>
</tr>
<tr>
<td>Amounts of a chemical substance</td>
<td>mole</td>
<td>mol</td>
</tr>
<tr>
<td>Temperature</td>
<td>degree Celsius</td>
<td>°C</td>
</tr>
<tr>
<td>Concentration</td>
<td>milligram per milliliter</td>
<td>mg/ml</td>
</tr>
<tr>
<td></td>
<td>microgram per milliliter</td>
<td>µg/ml</td>
</tr>
<tr>
<td>Centrifugal force</td>
<td>gravity</td>
<td>g</td>
</tr>
<tr>
<td>Voltage</td>
<td>volt</td>
<td>V</td>
</tr>
</tbody>
</table>