European Union Reference Laboratory for Fish and Crustacean Diseases
NATIONAL INSTITUTE OF AQUATIC RESOURCES, TECHNICAL UNIVERSITY OF DENMARK

DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF Koi Herpes Virus Disease (KHVD) v2023.1

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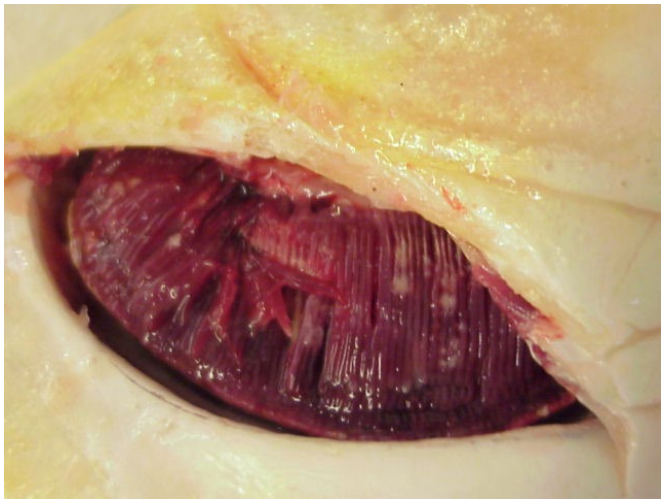

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Contents

Diagnostic procedures for the surveillance and confirmation of KHV disease (KHVD)

I. Aetiology of KHVD

Koi herpesvirus (KHV), which belongs to the family of *Alloherpesviridae* (Aoki et al., 2007),(Waltzek et al., 2009) is the aetiological agent of KHVD. The scientific name is cyprinid herpesvirus 3 (CyHV-3). The scope of the current diagnostic manual defines that KHV isolates are defined as alloherpesviruses with ≥99% identity to the viral DNA polymerase gene and/or the major capsid protein gene of the CyHV-3 strains, KHV/J, KHV/U, and KHV/I (Aoki et al. 2007; Genbank accession numbers AP008984, DQ657948, DQ177346, respectively). Therefore, described strains of cyprinid herpesvirus closely related to koi herpesvirus (Engelsma et al., 2013) are not considered as KHV, and thereby not targeted by these surveillance procedures.

Although no vaccine against KHV is available in Europe, KHV susceptible species can be vaccinated against KHVD with live attenuated virus and may be imported. These animals can also potentially become infected with wild-type virus and spread infection to naïve cyprinids. Furthermore, revirulence of certain live vaccine is possible, as mutation cannot be excluded in live vaccines and this can spread to naïve population (Peeler et al., 2009).

II. Procedures for diagnosis and confirmation of KHVD

II.1. Preparation of samples from fish

Regarding shipment of samples to the laboratory for diagnosing KHVD there are multiple viable options which shall be agreed with the laboratory prior to shipment of the material.

1) Fish can be sent alive or euthanized (<4°C) and packed separately in sealed aseptic containers, in these cases organ sampling will be carried out at the laboratory.
2) Organ pieces, dissected in a sterile manner can be transferred to sterile plastic tubes containing in at least five volumes of DNA/RNA stabilizing reagent (e.g. 70-90% ethanol, RNAlater) according to the manufacturer's recommendation. Samples in DNA/RNA stabilizing reagents can be shipped at room temperature (<25°C). Sample size shall not exceed 5mm in any direction to allow quick penetration and proper stabilization of the viral DNA.
3) Organ pieces, dissected in a sterile manner can be transferred to a tube containing transport media EMEM, these shall be shipped on ice (<4°C).

II.2. Shipment of fish samples

Whole fish may be sent to the laboratory if the temperature requirements referred to in the first paragraph during transportation can be fulfilled. Whole fish may be wrapped up in paper with absorptive capacity and shall finally be shipped in a plastic bag.

Tubes containing fish tissues for (q)PCR analysis shall be placed into insulated containers, such as thick-walled polystyrene boxes, together with sufficient ice or an alternative cooling medium with the similar cooling effect to ensure chilling of the samples during transportation to the laboratory. Freezing of samples should be avoided. However, frozen samples may be accepted in exceptional cases after consultation with the laboratory. The temperature of a sample which is not stabilized in RNA/DNA preservative, during transit must never exceed 10 °C and ice must still be present in the transport box at receipt or one or more freeze blocks must still be partly or completely frozen or a recording of the temperature during the entire transport must be provided (e.g. with a datalogger placed inside the package).

II.3 Sampling procedures

For diagnostic purposes:

In order to confirm the presence of KHV during disease investigation, gill and kidney tissue shall be sampled; in addition skin lesions if present (Moody Nicholas et al., 2022), spleen, encephalon and intestine can be included. In acute disease outbreak with increased mortality, tissue material of up to 5 fish can be pooled.

II.4 Sample preparation for detection of KHV DNA

For the analysis of fish tissues preserved in DNA/RNA stabilization reagent (e.g. 70-90% ethanol, RNAlater®), subsequent work shall be carried out within the following time scales for samples stored at different temperatures:

- samples stored at 25 °C: one week;
- samples stored at 4 °C: one month;
- samples stored at –20 °C: indefinitely.

Disruption and homogenization of tissues for qPCR-based surveillance, can be performed either on the total amount of tissue (the whole pool) or by aliquots of each tissue of every specimen included in the pool (e.g. by TissueLyser, FastPrep or similar).

II.5. DNA extraction

DNA shall be extracted according to standard procedures. DNA extraction kits are available commercially that will produce high quality DNA suitable for use with the PCR protocols detailed.

III. Agent detection and identification by PCR based methods

III.1. qPCR for KHV detection

The protocol is described in the scientific publication from Gilad et al., 2004.

For KHV, amplification shall be performed using the following primers and probe:

- Forward primer : 5'- GACGCCGGAGACCTTGTG -3' ;
- Reverse primer: 5'- CGGGTTCTTATTTTTGTCCTTGTT -3' ;
- And probe : 5' 6 FAM- CTTCCTCTGCTCGGCGAGCACG – BHQ1 3'

Negative template controls and positive controls both for DNA extraction phase and PCR phase shall be included on each plate run. In order to prevent issues with contamination, it is suggested to include positive controls yielding Ct values close to the limit of detection. Additionally, internal controls can be included to rule out sample degradation and ensure successful DNA extraction procedure. The Gilad et al., 2004 uses the glucokinase gene of the carp genome as internal control but other systems are available (e.g. Hoffman et al., 2006).

- Cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 94°C for 15 seconds, 60°C for 1 minute, adjust if necessary the thermal profile.

qPCR assays with demonstrated similar sensitivities and specificities to the described assay may also be used.

III.2. Conventional PCR for KHV detection

The protocol is described in the scientific publication from Bercovier et al. 2005, targeting the Thymidine Kinase (TK) gene of KHV. This assay is currently a very sensitive, single-round assay used world-wide for the detection of KHV.

For KHV, amplification shall be performed with the following primers (product size 409 bp):

- Forward primer 5'-GGGTTACCTGTAC GAG-3'
- Reverse primer5'-CACCCAGTAGATTA TGC-3'

Negative template controls and positive controls both for purification phase and PCR phase shall be included on each plate run. In order to prevent issues with contamination, it is suggested to include positive controls at low concentration.
Cycling conditions: 95°C for 5 minutes, then 35 cycles with: 95°C x 30 seconds, 52°C x 30 seconds and 72°C x 1 minute. Cycle conditions are kit specific and should be adjusted if necessary.
PCR assays with demonstrated similar sensitivities and specificities to the described assay may also be used (e.g. Engelsma et al. 2013 or Bigarré et al., 2009)
First detection in an area officially declared free for KHVD must be confirmed either by sequencing or sent to a reference laboratory for confirmation.

III.3. Cell cultivation

Diagnosis of KHVD in clinically affected fish can be achieved by virus isolation in cell culture. However, cell culture isolation is not as sensitive as the published PCR-based methods to detect KHV DNA and is not considered to be a reliable diagnostic method for KHV detection (WOAH 2022). Detailed procedures are given in the WOAH Aquatic Manual
(https://www.woah.org/fileadmin/Home/eng/Health_standards/aahm/current/2.3.0_General_info.pdf.)

IV. Procedures for surveillance of KHVD

IV.1. Preparation of samples from fish

For Surveillance/eradication plan purposes:

In order to demonstrate freedom from KHV, gill and/or kidney tissue shall be sampled; in addition spleen, encephalon and intestine can be included. In certain cases, such as investigation of valuable specimens, it is possible to use gill biopsy to obtain samples.

It is optimal to sample fish that have been kept for a prolonged time period at the virus permissive temperature range (2-3 weeks at 20°C to 26°C). There are indications that certain management practices (e.g. netting and/or transport of the fish) can reactivate the virus in fish with a carrier status, thus increasing the chance of KHV detection. In order to cope with production procedures and facilitate Fish Health Inspectors activity it is acceptable to:

a) collect a sub-population at transfer from winter to summer ponds and hold the fish in the same water body of the summer pond until minimum temperature requirements have been obtained.
b) collect samples at harvesting or during other fish handling procedures as part of normal management practices.

If possible samples should be collected 24 hours after such management practices to enhance the chance of KHV detection (Bergmann and Kempter 2011). For surveillance purpose the fish can be sent alive or euthanized and packed separately in sealed aseptic containers; alternatively frozen organs (target tissues to be

collected are gill and kidney) or organ pieces preserved in 70-90% ethanol) or viral transport medium (to be processed within 48 hrs after collection) can be used for testing by PCR based methods.

Pooling of samples is not recommended, but pooling of up to two fish is allowed. In order to obtain suitable samples, larger tissue samples must be homogenized (e.g. mortar and pestle, stomacher) and a representative aliquot retrieved for DNA extraction (refer to paragraph **II.1.1. DNA extraction**)

IV.2. Surveillance for KHVD by PCR based methods

For surveillance a qPCR shall be used as it is the most sensitive and specific test and minimizes the risk of cross-contamination.

If positive samples appears in an area not previously confirmed positive the test results must be confirmed either by sequencing of a PCR or nested PCR product from the samples, as described in IV.2.2, or sent to a reference laboratory for confirmation.

IV.2.1. qPCR for KHV detection

The assay described by Gilad et al. 2004 shall be used as described in **III.1. q PCR for KHV detection**

IV.2.2. Conventional PCR for confirmation of KHV detection

For confirmation of KHV the generic nested PCR described in Engelsma, M. Y. et al., 2013 shall be followed by sequencing of the amplified product.'

FIRST ROUND PCR (product size 361 bp)

- Forward primer: 5'-CCAGCAACATGTGCGACGG-3'
- Reverse primer: 5'-CCGTARTGAGAGTTGGCGCA-3'

SECOND ROUND PCR (product size 339 bp)

- Forward primer: 5'-CGACGGVGGYATCAGCCC-3'
- Reverse primer: 5'-GAGTTGGCGCAYACYTTCATC-3'

Cycling conditions for both PCR rounds: *1 cycle:* 95°C 2 minutes, then *40cycles:* 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 45 seconds; then *1 cycle:* 72°C for 10 minutes

Negative template controls and positive controls both for DNA extraction phase and PCR phase shall be included on each run.
The results of the PCR reactions shall be evaluated by agarose gel electrophoresis using appropriate DNA staining (e.g. ethidium bromide). An amplicon of 361 (PCR)/339 bp (nPCR) shall be observed in positive KHV samples and positive controls.
The results of the PCR may vary depending on the conditions under which it is performed, namely the thermal protocols might need optimization, depending on the thermal cycler in use.
Other PCR versions of proven similar efficiency may be used instead.

Sequencing of the PCR template shall be performed for viral genotype identification. The sequencing can be performed by the laboratory or at external specialised sequencing companies. Sequencing results can be analysed by aligning the sequences to the known reference sequences of KHV (Genbank accession numbers AP008984, DQ657948, DQ177346)(Aoki, T. et al., 2007). The obtained clean consensus sequence should match with these reference sequences.

V. Additional identification methods

In case of carriers displaying subclinical latency phase, the CyHV-3 genome can remain difficult to detect by PCR, and in a purpose of surveillance, an alternative serum neutralization (SN) or ELISA method based both on the detection of CyHV-3-specific antibodies can be useful to characterize fish status.

Regarding the seroneutralization test, as described by Cabon et al., 2016, briefly, sera or plasma are heat inactivated at 45°C ±2 for 30 minutes and then twofold serially diluted from 1/40 to 1/5120. An amount of 50 to 150 TCID50 of virus/well is added to each well containing the diluted sera to be tested, and after incubation overnight at 5°C, the mix is transferred to 24-hour-old flat bottom 96-well plates containing CCB cells reaching approximately 80% confluency. Plates are incubated at 24°C for 8 to 10 days and eventually fixed with formaldehyde 3.7% for 1 hour, and stained with crystal violet 1‰ for at least 1 hour, after which any clear CPE can be observed by light microscopy.

Otherwise, ELISA assay, as mentioned by Bergman et al., 2017, also enables to detect KHV specific antibodies, with the advantage to not requiring any cell culture, except for the step of antigen production.

VI. Acronyms and abbreviations

bp	base pair
Ct	cycle threshold
CyHV-3	cyprinid herpesvirus 3
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMEM	Eagle's Minimal Essential Medium
Gluc	Glucokinase
KHV	Koi herpesvirus
KHVD	Koi herpesvirus disease
nPCR	nested Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
Pol	DNA polymerase
qPCR	quantitative Polymerase Chain Reaction
RNA	ribonucleic acid
SN	serum neutralisation
TK	Thymidine Kinase
WOAH	World Organization for Animal Health
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
R	A or G
V	A,C or G
Y	C or T

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