Report from *Ad hoc* Working group of Sampling and diagnostic procedures for the surveillance and confirmation of Koi Herpes Virus Disease (KHVD) 2023

Contents

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Introduction

Koi herpesvirus disease (KHVD) is listed as Category E disease in current Animal Health Law REGULATION (EU) 2016/429 and susceptible and vector species for this disease are described in COMMISSION IMPLEMENTING REGULATION 2022/925. KHVD is listed as category E disease, a listed disease for which there is a need for surveillance within the Union, as referred to in point (e) of Article 9(1) of Regulation (EU) 2016/429;

1) the rules for notification and reporting provided for in Chapter 1 of Part II (Articles 18 to 23)
2) the rules for surveillance provided for in Chapter 2 of Part II (Articles 24 to 30)

With the adoption of Animal Health Law, EU reference laboratories shall upload diagnostic manuals for listed diseases on their website and keep them updated. In the current report, recommendations for laboratory procedures and specific technical scientific questions related to the surveillance and diagnosis of KHVD are provided as consensus of the expert group.

Susceptible species

List of susceptible species is included in COMMISSION IMPLEMENTING REGULATION 2022/925, to date all varieties and subspecies of *Cyprinus carpio*, and *Cyprinus carpio* hybrids e.g. *Cyprinus carpio* × *Carassius auratus*, *Cyprinus carpio* × *Carassius carassius* are listed as susceptible and have to be collected when present in the farm.

Preparation and shipment of samples from fish

Regarding shipment of samples to the laboratory for diagnosing or surveying for 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 case organ sampling is performed at the laboratory
2) Organ pieces, dissected in a sterile manner can be transferred to sterile plastic tubes containing 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 tube containing transport media EMEM, these shall be shipped on ice (<4°C).

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 the samples should be avoided, frozen samples can be accepted in exceptional cases upon dialogue 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).

Sampling procedures

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:

1) 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.
2) 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).

Pooling samples is not recommended, nevertheless pooling 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.

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 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.

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 TissueLyzer, FastPrep or similar).

Molecular techniques for surveillance

For surveillance a qPCR (i.e. Gilad et al., 2004) shall be used as it is the most sensitive and specific test using procedures to minimize the risk of cross-contamination. qPCR assays with demonstrated similar sensitivities and specificities to the described assays may also be used.

If positive samples appear in an area not previously confirmed positive, the test results must be confirmed either by sequencing of a PCR or nested PCR product (i.e. obtained following procedures in Engelsma et al., 2013) from the samples or sent to a reference laboratory for confirmation.

Molecular techniques for diagnostics (clinical symptoms/ suspicion)

For diagnostics purposes the qPCR assay described by (Gilad, O. et al., 2004) shall be used; alternatively the conventional PCR assay described by Bercovier et al., 2005 targeting the TK gene of KHV can be used.

Conventional PCR and qPCR assays with demonstrated similar sensitivities and specificities to the described assay may also be used (e.g. Engelsma et al., 2013 followed by sequencing or Bigarré et al., 2009).

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 KHVD (WOAH 2021). Detailed procedures on cell cultivation are given in the WOAH Aquatic Manual (Section 2.3. Diseases of fish; Chapter 2.3.0. General information; https://www.woah.org/fileadmin/Home/eng/Health_standards/aahm/current/2.3.0_General_info.pdf).

Agent definition

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). For the purpose of the diagnostic procedure in the frame of EU regulations, KHV isolates are defined as alloherpesviruses aligning 99% 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 therefore do not to be targeted by these surveillance procedures.

Despite no vaccine available for KHV in Europe, KHV susceptible species may have been vaccinated against KHVD with live-attenuated virus and could potentially be imported. These animals can potentially be infected with wild-type virus too and spread the infection to naïve cyprinids. Furthermore, reversion to virulence of certain live vaccine is possible since mutation cannot be excluded in live vaccines and this can spread to naïve population (Peeler et al., 2009)

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 $TCID_{50}$ 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 flat bottom 96-well plates containing 24-hour-old monolayer of CCB cells/well 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.

The Manual

The final draft for the manual "**Diagnostic procedures for the surveillance and confirmation of KHV disease"** is available on the website of the EURL for Fish and Crustacean Diseases.

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