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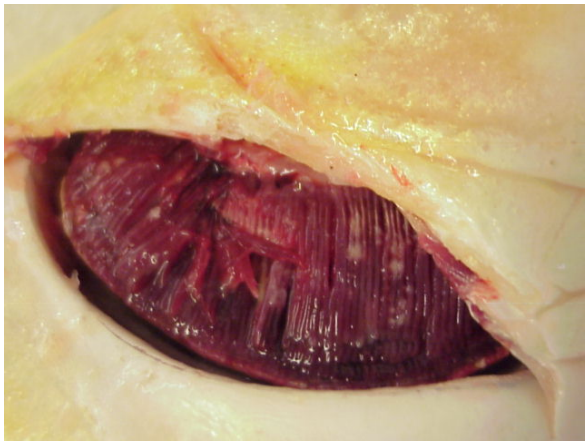
DISEASES

DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF KHV DISEASE



PART 2

DETAILED DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF KHV DISEASE (KHVD)

I. **Detailed diagnostic methods and procedures for the confirmation of the presence of or to rule out the suspicion of KHVD**

When a laboratory examination is required for the purpose of confirmation of the presence of or to rule out the suspicion of KHVD in accordance with Article 57(b) of Directive 2006/88/EC, using the diagnostic methods set out in Section III of Part 2 of Annex I, the detailed diagnostic methods and procedures set out in points I.1 – I.2 of this Part shall apply.

I.1. Preparation of samples from fish

For diagnostic purposes the fish (sent alive or killed and packed separately in sealed aseptic containers) or alternatively frozen organs or organ pieces preserved in 80 % to absolute ethanol or viral transport medium (to be processed within 48 hours after collection) may be used for testing with conventional PCR or qPCR based methods.

For the detection of KHV, gill and kidney shall be collected; in addition spleen, encephalon and intestine may be included in an additional separated sample. In acute cases, tissue material of up to five fish may be pooled.

Furthermore, non-lethal samples such as blood, gill swabs, gill biopsy, mucus scrape may be used in certain cases (namely very valuable fish may be used in the case of the suspicion of the presence of KHV).

I.1.1. DNA extraction

DNA shall be extracted in accordance with standard procedures.

The commercially available DNA extraction kits that produce high quality DNA suitable for use with the PCR protocols referred to in point I.2 may be used.

I.2. Agent detection and identification by Polymerase chain reaction (PCR) based methods

I.2.1. qPCR for KHV detection

For qPCR detection of KHV, the following qPCR assay shall be used:

Forward primer (KHV-86f): 5′- GACGCCGGAGACCTTGTG -3′;

Reverse primer (KHV-163r): 5′- CGGGTTCTTATTTTTGTCCTTGTT -3′;

and probe (KHV-109p): 5′-FAM- CTTCCTCTGCTCGGCGAGCACG -3′.

Cycling conditions: one cycle of 95 °C at 15 minutes, followed by 40 cycles of 94 °C at 15 seconds and 60 °C for 60 seconds. Negative template controls and positive controls shall be included on each plate run. However, other qPCR versions of proven similar efficacy may be used instead.

I.2.2. Conventional PCR for KHV detection

The assay described in this point targeting the Thymine kinase (TK) gene of KHV shall be used. However, other PCR assays with demonstrated similar sensitivities and specificities to the described assay may be used instead.

Forward primer (KHV-TKf): 5′-GGGTTACCTGTAC GAG-3′;

Reverse primer (KHV-TKr): 5′-CACCCAGTAGATTA TGC-3′.

Cycling conditions: one cycle of 95 °C for 5 minutes followed by 35 cycles of 95 °C for 30 seconds, 52 °C for 30 seconds, 72 °C for one minute and one cycle of 72 °C for 10 minutes. Product size should be 409 bp.

The results of the PCR may vary depending on the conditions under which it is performed, namely the thermal protocols might need optimisation, depending on the thermal cycler in use. Furthermore, false-positive results may occur because of false primer annealing or contamination. Negative template controls and positive controls shall be included on each plate run. However, other PCR versions of proven similar efficacy may be used instead.

The first detection in an area shall be confirmed by sequencing or sent to a to a national reference laboratory or to the EU reference laboratory for fish diseases referred to in Annex VI to Directive 2006/88/EC, for immediate identification.

II. **Detailed diagnostic methods and procedures for the surveillance of KHVD**

When sampling and laboratory examination for the purpose of obtaining or maintaining certain health statuses with regard to KHVD as set out in Section I of Part 2 of Annex I are carried out using the diagnostic methods set out in Sections II or III of Part 2 of that Annex, the detailed diagnostic methods and procedures set out in the following points II.1 and II.2 of this Part shall apply.

II.1. Preparation of samples from fish

If possible, fish that have been kept for a prolonged time period at the virus permissive temperature range (namely, 2 to 3 weeks at 15 °C to 26 °C) shall be sampled. If possible, samples shall be collected 24 hours but not later than 72 hours after management practices that may reactivate the virus in fish with a carrier status, such as netting or transport, in order to enhance the chance of KHV detection.

For the purpose of the surveillance of KHVD, the fish may be sent alive or killed and packed separately in sealed aseptic containers or alternatively frozen organs or organ pieces preserved in 80 % to 100 % alcohol or viral transport medium (to be processed within 48 hours after collection) may be used for testing PCR based methods. For the surveillance of KHVD, gill and kidney tissue shall be collected.

For the purpose of surveillance of KHVD, pooling shall be avoided where possible. If pooling is necessary, tissue material from a maximum of two fish may be pooled. Larger samples shall be homogenised in mortar and pestle or stomacher, and subsamples retrieved for DNA extraction before clarification. Alternatively, subsamples may be collected from each tissue included in the sample and placed in 'lysis-tubes'.

II.1.1. DNA extraction

DNA shall be extracted in accordance with standard procedures. Commercially available DNA extraction kits that produce high quality DNA suitable for use with the PCR protocols set out in point II.2 may be used.

The acceptable tissue medium ratio shall be 1:9 w/v. 20 to 25 mg tissue material shall be included in the tests.

II.2. Surveillance of KHVD by PCR based methods

For the surveillance of KHV, a qPCR shall be used. If positive samples appear in an area not previously confirmed positive, the test results shall be confirmed either:

(a) by sequencing of a PCR or nested PCR product from the samples.

The obtained clean consensus sequence shall match (by at least 98 %) with these reference sequences.

(b) or alternatively, samples may be sent to a national reference laboratory for confirmation.

II.2.1. qPCR for KHV detection

The qPCR described as follows shall be used:

Forward primer (KHV-86f): 5′- GACGCCGGAGACCTTGTG -3′;

Reverse primer (KHV-163r): 5′- CGGGTTCTTATTTTTGTCCTTGTT -3′;

and probe (KHV-109p): 5′-FAM- CTTCCTCTGCTCGGCGAGCACG -3′.

Cycling conditions: one cycle of 95 °C at 15 minutes, followed by 50 cycles of 94 °C at 15 seconds and 60 °C for 60 seconds.

The results of the qPCR may vary depending on the conditions under which it is performed, namely the thermal protocols might need optimisation, depending on the thermal cycler in use. Furthermore, false-positive results may occur because of false primer annealing or laboratory contamination. Negative template controls and positive controls shall be included on each plate run. However, other qPCR versions of proven similar efficacy may be used instead.

II.2.2. Conventional PCR for confirmation of KHV detection

For confirmation of the presence of infection with KHV, the generic nested PCR described in the following Table 2.1 shall be used followed by sequencing of the amplified product.

Table 2.1

Primers and conditions for the nested PCR assay targeting all cyprinid herpesviruses (CyHV-1, CyHV-2 and CyHV-3)

Primer name	Sequence	Cycling conditions	Product size
CyHVpol-forward	5′-CCAGCAACATGTGCGACGG-3′	First round PCR *1 cycle:* 95 °C 2 minutes *40 cycles:* 95 °C for 30 seconds 55 °C for 30 seconds 72 °C for 45 seconds *1 cycle:* 72 °C for 10 minutes	362 bp
CyHVpol-reverse	5′-CCGTARTGAGAGTTGGCGCA-3′		

Primer name	Sequence	Cycling conditions	Product size
CyHVpol-internal forward	5′-CGACGGVGGYATCAGCCC-3′	Second round PCR *1 cycle:* 95 °C 2 minutes, *40 cycles:* 95 °C for 30 seconds 55 °C for 30 seconds 72 °C for 45 seconds *1 cycle:* 72 °C for 10 minutes	339 bp
CyHVpol-internal reverse	5′-GAGTTGGCGCAYACYTTCATC-3′		

The results of the PCR may vary depending on the conditions under which it is performed, namely the thermal protocols might need optimisation, depending on the thermal cycler in use. Furthermore, false-positive results may occur because of false primer annealing or laboratory contamination. Negative template controls and positive controls shall be included on each plate run. PCR versions of proven similar efficacy may be used instead.

Sequencing may be performed by the laboratory or at external specialised sequencing companies. Sequencing results shall be analysed by aligning the sequences to the known reference sequences of KHV (Gen Bank accession numbers AP008984, DQ657948 and DQ177346). The obtained clean consensus sequence must match at least to 98 % with those reference sequences.