

Report from
Meeting on
Sampling and diagnostic procedures for the
surveillance and confirmation of KHV
disease
Copenhagen
February 25-26th 2014

Contents

Participants:.....	3
Introduction:.....	3
Meeting Agenda	4
Technical scientific opinions for each point of the agenda	5
Introduction - background.....	5
1- Sampling procedures	5
2- Susceptible species.....	5
3- Molecular techniques for surveillance	6
4- Molecular techniques for diagnostics (clinical symptoms/ suspicion).....	6
5- Cultivation.....	6
6- Serology	6
7- Other issues concerning KHV	6
8- The Manual.....	7
9- Recommendation for further development.....	7
References	7
Annex 1.....	9
I. Aetiology of KHVD	11
II. Procedures for diagnosis and confirmation of KHVD	11
II.1. Preparation of samples from fish	11
II.2. Agent detection and identification by PCR based methods.....	11
II.3. Cell cultivation	12
III. Procedures for surveillance of KHVD.....	13
III.1. Preparation of samples from fish	13
III.2. Surveillance for KHVD by PCR based methods.....	13
III.3. Serology	14
IV. Acronyms and abbreviations.....	15
V. References.....	15
Annex 2.....	16

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Introduction:

According to the [Council Directive 2006/88/EC](#) additional legislation should be implemented describing sampling and diagnostic procedures for the diseases listed in Annex IV Part 2 of the Directive. The Sampling plans and the diagnostic methods for the detection and confirmation of VHS and IHN are laid down in the [Commission Decision 2001/183/EC](#) and for ISA in [Commission Decision 2003/466/EC](#). But as KHV was only included as a non-exotic disease at the implementation of the Council Directive no earlier decisions were made for this disease. A preliminary version was given at the web page of the EURL Fish, this version was based on research and meeting activities reported in [“Report of the workshop “KHV PCR diagnosis and surveillance” 12-13 November 2009, Central Veterinary Institute, Lelystad, The Netherlands”](#) and made by a KHV expert working group under the EPIZONE network. But as significant new knowledge based on new research appeared in the recent years the EURL asked the Commission the permission to organize an expert meeting in order to settle an hopefully agree on common new recommendations for implementation in a Commission Decision.

The 2 day meeting was held at the premises of the EURL at Frederiksberg, Denmark and 3 of the top experts in the field of KHV from Germany, Netherlands and UK, respectively, were invited to participate. They accepted all 3.

The meeting was busy and held in a good atmosphere and at the end we finalized both versions of the surveillance and sampling methods and of the diagnostic methods.

Significant changes from the former versions were accepted and recommended for inclusion in the final decision. Among changes are

- The splitting of sampling and diagnostic tests for diagnostic and surveillance purposes, respectively.
- Inclusion of real-time PCR as the method of choice for surveillance.
- Specification on how to define a CyHV-3 strain.

Meeting Agenda

Agenda		
12.30-13.30	Welcome and Lunch	
Tuesday 25.2 13.30	Introduction - background	1) Manual for surveillance 2) Manual for diagnostics
14.00-15.00 Core elements 1	Sampling procedures	<ul style="list-style-type: none"> • Tissue (specify which part) • Lethal/non-lethal sampling • Stress test- mandatory? (welfare aspect not to forget) • Preservation (RNAlater/ cell culture medium or ?) • Pooling
15-15.30 Core elements 2	Susceptible species	If <i>Cyprinus carpio</i> present should other species be tested?
15.30-17.00 Core elements 3	Molecular techniques for surveillance	<ul style="list-style-type: none"> • Generic PCR • Sequencing? • qPCR ? • Conventional PCR • Sensitivity • Specificity • Robustness and reproducibility • Vaccinated fish?
15.30-17.00 Core elements 4	Molecular techniques for diagnostics (clinical symptoms/ suspicion)	<ul style="list-style-type: none"> • E.g. conventional PCR • qPCR • Vaccinated fish
Wednesday 26.2 9.00 9.30	Wrap up from Tuesday	
9.30-10.30 Core elements 5	Cultivation	<ul style="list-style-type: none"> • Should it be included in manual? • If so how • Sampling and preservation procedures • Cell lines • Methodology (co-cultivation)
10.30-11.30 Core elements 6	Serology	<ul style="list-style-type: none"> • To be included? If so • SNT • ELISA • IFAT • Validations, specificity and sensitivity
11.30-12.30 Core elements 7	Other issues concerning KHV	<ul style="list-style-type: none"> • Strain definitions • CyHV-1 • CyHV-2 • CyHV-3 var 1-..
13.30-14.30 Core elements 8	The manual	Agreement shall be obtained and most amendments and changes in cooperated in text
14.30-16.00 Core elements 9	Final Conclusion and recommendations	A report must be submitted to the Commission
16.00	End of workshop	

Technical scientific opinions for each point of the agenda

Introduction - background

It was agreed that there was a need for establishing two separate chapters, one specifically addressing surveillance and sampling procedures and one describing diagnostic tests.

1- Sampling procedures

For diagnostic purposes:

Gill and kidney tissue shall be sampled; in addition spleen, encephalon and intestine can be included. In acute cases tissue material of up to 5 fish can be pooled. Furthermore, non-lethal samples like blood, gill swabs, gill biopsy, mucus scrape can be used in certain cases (e.g. very valuable fish, suspicion,).

For Surveillance purposes:

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 killed and packed separately in sealed aseptic containers; alternatively frozen organs (target tissues to be collected are gill and kidney) or organ pieces preserved in 80 – 100 % alcohol (e.g. ethanol) or viral transport medium (to be processed within 48 hrs after collection) can be used for testing by PCR based methods.

Pooling samples is not recommended. Larger samples must be homogenized (e.g. mortar and pestle, stomacher) and subsamples retrieved for DNA extraction before clarification, alternatively subsamples can be collected from each tissue included in the sample and placed in “lysis-tubes”.

2- Susceptible species

It was agreed that *Cyprinus carpio* and its hybrids (e.g. common carp x goldfish) have to be collected when present in the farm.

3- Molecular techniques for surveillance

For surveillance a real-time PCR (i.e. Gilad et al.,2004) shall be used as it is the most sensitive and specific test and minimizes the risk of cross-contamination. Real-time PCR 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, M Y, et al, 2013) from the samples or sent to a reference laboratory for confirmation.

4- Molecular techniques for diagnostics (clinical symptoms/ suspicion)

For diagnostics purposes the real-time PCR assay described by (Gilad, O. et al., 2004) shall be used; alternatively the conventional PCR assay described by [Bercovier H. et al., 2005](#) targeting the TK gene of KHV – can be used.

Conventional PCR and real-time PCR assays with demonstrated similar sensitivities and specificities to the described assay may also be used.

5- 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 ([OIE, 2013](#)). Detailed procedures on cell cultivation are given in the OIE Aquatic Manual.

6- Serology

Due to insufficient knowledge of the serological responses of fish to virus infections, the detection of fish antibodies to viruses has not thus far been accepted as a routine screening method for assessing the viral status of fish populations. Validation of some serological techniques for certain fish virus infections could arise in the near future, rendering the use of fish serology more widely acceptable for health screening purposes ([OIE, 2013](#))

7- Other issues concerning KHV

It was agreed that koi herpesvirus (KHV), which belongs to the family of *Alloherpesviridae* ([Aoki, T. et al., 2007](#)),([Waltzek, T. B. et al., 2009](#)) is the aetiological agent of KHVD. The scientific name is cyprinid herpesvirus 3 (CyHV-3), CyHV-3 isolates are defined as alloherpesviruses aligning 100% 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, according to Aoki et al. 2007 (Genbank accession numbers [AP008984](#), [DQ657948](#), [DQ177346](#), respectively). Therefore recently described novel strains of cyprinid herpesvirus closely related to koi herpesvirus are not considered as CyHV-3, and thereby have not to be the targeted by these surveillance procedures.

8- The Manual

The final draft for the manual “**Diagnostic procedures for the surveillance and confirmation of KHV disease**” and recommendations on sampling “**SURVEILLANCE AND DIAGNOSTIC METHODS FOR KHVD**” are attached as annexes.

9- Recommendation for further development

The meeting demonstrated to be fruitful bringing together skills and competences on this fish disease from different parts of Europe. Two major issues were raised during the meeting and need specific efforts to be solved:

- **Serology procedures (i.e. ELISA, IFAT and seroneutralization).** These techniques have, theoretically, great potential to be used for surveillance purposes, including the possibility of increasing the number of fish tested and the likelihood of detecting infected fish during the latency phase that characterize infection with KHV. However, further studies are needed to validate e such assays. As no peer-reviewed scientific paper have been published describing protocols that discriminate serum antibodies raised against KHV or other Cyprinid Herpesvirus (CypHV-1), diagnostic specificity of the test has to be improved before implementing serology methods for surveillance activities. Furthermore, increased knowledge of the kinetics of the antibody response in infected fish, and the possibility for viral clearance in infected fish should be further investigated, in order to have enough scientific information to establish protocols for the interpretation of assay results.
- **Cyprinid herpes virus variants closely related to CypHV-3.** These variants have been detected in susceptible species, but, so far they have not been associated with clinical signs of disease. Further studies are needed to elucidate if these variants have to be considered an emerging disease in Europe that requires a specific surveillance program. In addition the risk of these seemingly non- or low virulent viruses mutating into high virulent strain should be assessed.

The experts involved in this working group suggest that specific research activities are conducted to address these issues.

References

1. OIE Manual of Diagnostic Tests for Aquatic Animals, (2013).
2. Aoki, T., Hirono, I., Kurokawa, K., Fukuda, H., Nahary, R., Eldar, A., Davison, A. J., Waltzek, T. B., Bercovier, H., and Hedrick, R. P. (2007). Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide. *J. Virol.* 81(10): 5058-5065.

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5. Engelsma, M. Y., Way, K., Dodge, M. J., Voorbergen-Laarman, M., Panzarin, V., Abbadi, M., El-Matbouli, M., Frank, S. H., Kahns, S., and Stone, D. M. (12-12-2013). Detection of novel strains of cyprinid herpesvirus closely related to koi herpesvirus. *Dis. Aquat. Organ* 107(2): 113-120.
6. Gilad, O., Yun, S., Zagmutt-Vergara, F. J., Leutenegger, C. M., Bercovier, H., and Hedrick, R. P. (8-9-2004). Concentrations of a Koi herpesvirus (KHV) in tissues of experimentally infected *Cyprinus carpio koi* as assessed by real-time TaqMan PCR. *Dis. Aquat. Organ* 60(3): 179-187.
7. Waltzek, T. B., Kelley, G. O., Alfaro, M. E., Kurobe, T., Davison, A. J., and Hedrick, R. P. (27-4-2009). Phylogenetic relationships in the family Alloherpesviridae. *Dis. Aquat. Organ* 84(3): 179-194.



**Diagnostic procedures for the
surveillance and confirmation of KHV
disease**



Diagnostic procedures for the surveillance and confirmation of KHV disease

Contents

I. Aetiology of KHVD	11
II. Procedures for diagnosis and confirmation of KHVD	11
II.1. Preparation of samples from fish	11
II.2. Agent detection and identification by PCR based methods	11
II.3. Cell cultivation	12
III. Procedures for surveillance of KHVD.....	13
III.1. Preparation of samples from fish	13
III.2. Surveillance for KHVD by PCR based methods	13
III.3. Serology	14
IV. Acronyms and abbreviations.....	15
V. References.....	15

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

I. Aetiology of KHVD

For the purpose of these diagnostic procedures KHVD is a disease capable of inducing an acute and severe viraemia in common carp and koi (*Cyprinus carpio*). The causative agent is koi herpesvirus (KHV), which belongs to the family of *Alloherpesviridae* (Aoki, T. et al., 2007), (Waltzek, T. B. et al., 2009). The scientific name is cyprinid herpesvirus 3 (CyHV-3).

CyHV-3 isolates are defined as alloherpesviruses aligning 100% 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, according to Aoki et al 2007 (Genbank accession numbers [AP008984](#), [DQ657948](#), [DQ177346](#), respectively).

II. Procedures for diagnosis and confirmation of KHVD

II.1. Preparation of samples from fish

For diagnostic purpose the fish can be sent alive or killed and packed separately in sealed aseptic containers or alternatively frozen organs or organ pieces preserved in 80 – 100 % alcohol (e.g. ethanol) or viral transport medium (to be processed within 48 hrs after collection) can be used for testing by PCR based methods.

For KHV detection gill and kidney tissue shall be collected, in addition spleen, encephalon and intestine can be included. In acute cases tissue material of up to 5 fish can be pooled.

Furthermore, non-lethal samples like blood, gill swabs, gill biopsy, mucus scrape can be used in certain cases (e.g. very valuable fish, suspicion etc).

II.1.1. 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.

II.2. Agent detection and identification by PCR based methods

II.2.1. Real-time PCR for KHV detection

For real-time PCR detection of KHV the real-time PCR assay described by (Gilad, O. et al., 2004) shall be used – see table 1.

Primer/probe	Sequence	Cycling conditions	Reference
KHV-86f	5'- GACGCCGGAGACCTTGTG -3'	1 cycle: 50°C for 2 min 95°C for 10 min 40 cycles: 95°C for 15 s 60°C for 60 s	Gilad et al. 2004
KHV-163r	5'- CGGGTTCTTATTTTGTCTTGT -3'		
KHV-109p	5'-FAM- CTTCTCTGCTCGGCGAGCACG -3'		

Table 1. Primers and conditions for the real-time PCR assay targeting the KHV genome. Conditions may vary depending on e.g. buffers, enzymes and thermal cycler used.

In order to assure quality of purified DNA it is recommended to include an internal control. The [Gilad, O. et al., 2004](#) uses the glucokinase gene of the carp genome as internal control but other systems are available (e.g. [Bergmann et al. 2010](#)).

Real-time PCR assays with demonstrated similar sensitivities and specificities to the described assay may also be used.

II.2.2. Conventional PCR for KHV detection

The assay described by [Bercovier, H. et al., 2005](#) targeting the TK gene of KHV – see table 2 below – should be used. This assay is currently a very sensitive world-wide used, single-round assay, for KHV detection. But PCR assays with demonstrated similar sensitivities and specificities to the described assay may also be used.

Primer name	Sequence	Cycling conditions	Product size	Reference
KHV-TKf	5'-GGGTTACCTGTAC GAG-3'	1 cycle: 95°C for 5 min 35 cycles: 95°C for 30 sec 52°C for 30 sec 72°C for 1 min	409 bp	Bercovier et al. 2005
KHV-TKr	5'-CACCCAGTAGATTA TGC-3'	1 cycle: 72°C for 10 min		

Table 2. Primers and conditions for the PCR assay targeting the TK gene of KHV. Conditions may vary depending on e.g. buffers, enzymes, and thermal cycler used. Note on cycling conditions (2013): An annealing temperature of 55°C has been used effectively by many laboratories to amplify KHV with the Bercovier TK primers.

First detection in an area must be confirmed either by sequencing or sent to a reference laboratory for confirmation.

II.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 ([OIE, 2013](#)). Detailed procedures are given in the OIE Aquatic Manual.

III. Procedures for surveillance of KHVD

III.1. Preparation of samples from fish

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 practises (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. If possible samples should be collected 24 hrs after such management practises to enhance the chance of KHV detection ([Bergmann & Kempter 2011](#))

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

For surveillance purpose pooling is not recommended. If pooling is necessary, it is acceptable to pool tissue material from two fish. Larger samples must be homogenized (e.g. mortar and pestle, stomacher) and subsamples retrieved for DNA extraction before clarification. Alternatively, subsamples can be collected from each tissue included in the sample and placed in “lysis-tubes”.

III.1.1. 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. Acceptable tissue medium ratio is 1 + 9 w/v. Minimum 25 mg tissue material must be included in the test.

III.2. Surveillance for KHVD by PCR based methods

For surveillance a real-time PCR 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 or sent to a reference laboratory for confirmation.

III.2.1. Real-time PCR for KHV detection

The real-time PCR assay described by [Gilad et al. 2004](#) shall be used – see table 1.

Primer/probe	Sequence	Cycling conditions	Reference
KHV-86f	5'- GACGCCGGAGACCTTGTG -3'	<i>1 cycle:</i> 50°C for 2 min 95°C for 10 min <i>40 cycles:</i> 95°C for 15 s 60°C for 60 s	Gilad et al. 2004
KHV-163r	5'- CGGGTTCTTATTTTTGTCCTTGTT -3'		
KHV-109p	5'-FAM- CTCCTCTGCTCGGCGAGCAG -3'		

Table 1. Primers and conditions for the real time PCR assays targeting the KHV genome. Conditions may vary depending on e.g. buffers, enzymes and thermal cycler used.

In order to assure quality of purified DNA it is recommended to include an internal control. The [Gilad et al. 2004](#) assay uses the glucokinase gene of the carp genome as internal control but other systems are available.

Real-time PCR assays with demonstrated similar sensitivities and specificities to the described assays may also be used.

III.2.2. Conventional PCR for confirmation of KHV detection

For confirmation the generic nested PCR described in [Engelsma, M. Y. et al., 2013](#) can be used followed by sequencing of the amplified product.

Primer name	Sequence	Cycling conditions	Product size	Reference
CyHVpol-forward	5'-CCAGCAACATGTGCGACGG-3'	First round PCR 1 cycle: 95°C 2 minutes 40 cycles: 95°C for 30 sec 55°C for 30 sec 72 °C for 45 sec 1 cycle: 72 °C for 10 min	362 bp	Engelsma et al. 2013
CyHVpol-reverse	5'-CCGTARTGAGAGTTGGCGCA-3'			
CyHVpol-internal forward	5'-CGACGGVGGYATCAGCCC-3'	Second round PCR 1 cycle: 95°C 2 minutes, 40cycles: 95°C for 30 sec 55°C for 30 sec 72°C for 45 sec 1 cycle: 72°C for 10 min	339 bp	
CyHVpol-internal reverse	5'-GAGTTGGCGCAYACYTTCATC-3'			

Table 3. Primers and conditions for the nested PCR assay targeting all cyprinid herpesviruses (CyHV-1, CyHV-2 and CyHV-3). Conditions may vary depending on e.g. buffers, enzymes and thermal cycler used.

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.

III.3. Serology

Due to insufficient knowledge of the serological responses of fish to virus infections, the detection of fish antibodies to viruses has not thus far been accepted as a routine screening method for assessing the viral status of fish populations. Validation of some serological techniques for certain fish virus infections could arise in the near future, rendering the use of fish serology more widely acceptable for health screening purposes ([OIE, 2013](#)).

IV. Acronyms and abbreviations

DNA	DeoxyRibonucleic acid
Gluc	Glucokinase
KHV	Koi Herpes Virus
OIE	Office international des epizooties
PCR	Polymerase Chain Reaction
TK	Thymidine Kinase
Pol	DNA polymerase
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
R	A or G
V	A,C or G
Y	C or T

V. References

1. OIE Manual of Diagnostic Tests for Aquatic Animals, (2013).
2. Aoki, T., Hirono, I., Kurokawa, K., Fukuda, H., Nahary, R., Eldar, A., Davison, A. J., Waltzek, T. B., Bercovier, H., and Hedrick, R. P. (2007). Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide. *J. Virol.* 81(10): 5058-5065.
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Annex 2 PART II

SURVEILLANCE AND DIAGNOSTIC METHODS FOR KHVD

I. Aetiology of KHVD

For the purpose of this Decision, KHVD is a disease capable of inducing an acute and severe viraemia in common carp and koi (*Cyprinus carpio*). The causative agent is koi herpesvirus (KHV), which belongs to the family of *Alloherpesviridae* (Aoki et al. 2007, Waltzek et al, 2009). The scientific name is cyprinid herpesvirus 3 (CyHV-3).

II. Provisions for programmes to achieve and to maintain certain health statuses with regard to KHVD and to contain KHV infections

II.1. General provisions

When surveillance in wild populations is required according to point 2 of Annex V to Directive 2006/88/EC, the number and geographical distribution of sampling points shall be determined to obtain a reasonable coverage of the Member State, zone or compartment. The sampling points must also be representative of the different ecosystems where the wild susceptible populations are located (i.e. river systems and lakes).

Targeted surveillance should rely on regular monitoring of sites holding susceptible species. Sites should be monitored when water temperatures have reached levels that are permissive for the development of the disease (>17°C) and no sooner than 2 weeks after such temperatures have been reached. Any diseased fish or fish showing abnormal behavior that are found on the site should be sampled and tested using the most sensitive tests available (e.g. PCR).

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 practises (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.

For practical reasons 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 as part of normal management practices. If possible samples should be collected 24 hrs after such management practices to enhance the chance of KHV detection (Bergmann and Kempter 2011).

When farms and/or wild populations have to be health inspected and/or sampled more than once per year, the intervals between the inspections and/or collection of samples shall be as far apart as possible within the season when the water temperature is likely to reach its highest annual points without exceeding the limit of 28°C.

All production units (ponds, tanks, etc.) must be health inspected for the presence of dead, weak or abnormally behaving fish.

Cyprinus carpio and its hybrids (e.g. common carp x goldfish) have to be collected when present in the farm.

Fish to be collected as samples shall be selected as follows:

- If weak, abnormally behaving or freshly dead (not decomposed) fish are present, such fish shall be selected.
- If more than one water source is utilised for fish production, fish representing all water sources shall be included for sampling.
- The fish selected must include fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample.

II.2. Specific provisions to achieve disease free health status (category I) with regard to KHVD

II.2.1 Surveillance programmes

- (a) A Member State, zone or a compartment with a previous category III health status with regard to KHVD may achieve category I with regard to that disease when all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or compartment meets the relevant conditions set out in Annex V to Directive 2006/88/EC and all those farms, and, when required by Annex V, sampling points in wild populations, selected in accordance with the provisions laid down in II.1, have been subject to a four-year surveillance programme:

The farms/sampling points have been health-inspected and sampled for a minimum period of four consecutive years as laid down in Table II.A.

During this four year period the testing of all samples using the diagnostic methods set out in point III.2 produced negative results for KHV and any suspicion of KHVD is ruled out in accordance with the methods set out in points III.3.

- (b) When during the implementation of the programme, infection with KHV is confirmed in a farm included in that programme, and therefore its category II health status has been withdrawn, that farm may immediately regain the category II health status without implementing an eradication programme as described in point II.2.2. when:
- (i) the farm is a continental farm whose health status regarding KHV is independent of the health status regarding that disease of the surrounding natural waters;
 - (ii) the confirmed infected farm is emptied, cleansed, disinfected and fallowed; the duration of the fallowing period shall be at least 6 weeks; and
 - (iii) the confirmed infected farm has been restocked with fish sourced from Member States, zones or compartments with a category I health status with regard to KHVD.

II.2.2 Eradication programmes

II.2.2.1 General requirements

A Member State, zone or a compartment with a previous category V health status with regard to KHVD may achieve category I health status with regard to that disease when all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or the compartment have been subject at least to the following programme:

- (a) The measures laid down in Section 3 of Chapter V of Directive 2006/88/EC have effectively been applied, and in particular a containment area, comprising a protection zone and surveillance zone, is established in the vicinity of the farm(s) officially declared infected with KHV.

The containment area referred to in Article 32 of Directive 2006/88/EC shall be defined on a case-by-case analysis taking into account factors influencing the risks for the spread of the disease to farmed and wild fish, such as: the number, rate and distribution of fish on the farm infected with KHV; distance and density of neighbouring farms; proximity to slaughterhouses; contact farms; species present at the farms; farming practices applied in the affected and neighbouring farms; hydrodynamic conditions and other factors of epizootiological significance identified.

For the establishment of the protection and surveillance zones the following minimum criteria shall apply as regards the geographical demarcation of those zones:

- (i) a protection zone shall be established in the close vicinity of a farm officially declared infected with KHV and shall correspond to the entire water catchment area of the farm officially declared infected with KHV; the competent authority may limit the extension of the zone to parts of the water catchment area provided that the prevention of the spread of KHVD is not compromised.
 - (ii) a surveillance zone shall be established outside the protection zone and shall correspond to an extended area outside the established protection zone.
- (b) All farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the protection zone not officially declared infected with KHV shall be subject to an official investigation comprising at least:
 - (i) The collection of samples for testing of 10 fish, when clinical signs or post-mortem signs consistent with infection with KHV are observed or 30 fish, when clinical or post-mortem signs are not observed.; and
 - (ii) One health inspection; in those farms where the tests referred to in the paragraph above have produced negative results health inspections shall continue with at least one inspection during the season when the water temperature is likely to exceed 17°C until the protection zone is revoked in accordance with Point II.2.2.1(c).
 - (c) All farms officially declared infected with KHV shall be emptied, cleansed, disinfected and fallowed. The duration of the fallowing period shall be at least 6 weeks. When all farms officially declared infected are emptied, at least 3 weeks of synchronised fallowing shall be carried out. This paragraph also applies to new farms officially declared infected during the implementation of the eradication programme.

When fallowing of the officially declared infected farms is carried out, the protection zones shall be converted into surveillance zones.

The competent authority may decide to require the emptying, cleansing, disinfection and fallowing of other farms within the established protection and surveillance zones. The length of the fallowing period shall be determined by the competent authority following a case-by-case risk evaluation.

- (d) All farms officially declared infected and all other farms fallowed within the established protection and surveillance zones must be restocked with fish:
 - (i) sourced from Member States, zones or compartments with a category I health status with regard to KHVD; or
 - (ii) for a transitional period until 31/12/2020, with fish from Member States, zones or compartments with an approved KHV surveillance programme.

Restocking shall only take place when all farms officially declared infected have been emptied, cleansed, disinfected and fallowed in accordance with point II.2.2.1(c).

- (e) All farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or compartment covered by the eradication programme and, when surveillance in wild populations is required, sampling points selected in accordance with the provisions laid down in Point II.1, must subsequently be subject at least to the programme laid down in Point II.2.1.

II.2.2.2 Regaining disease freedom for continental compartments comprising one single farm previously being disease free

A continental compartment comprising one single farm with a previous category I health status with regard to KHVD, whose health status with regard to that disease is independent of the surrounding natural waters, and whose category I status have been withdrawn, may regain it immediately after the competent authority has confirmed that the following conditions are complied with:

- (a) the confirmed infected farm with KHV is emptied, cleansed, disinfected and fallowed; the duration of the fallowing period shall be at least 6 weeks;
- (b) the confirmed infected farm with KHV has been restocked with fish sourced from Member States, zones or compartments with a category I health status with regard to KHV.

II.3. Specific provisions for maintenance of category I status with regard to KHVD

When targeted surveillance is required to maintain category I health status, as laid down in Article 52 of Council Directive 2006/88/EC, all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or compartment must be health inspected and sampled in accordance with Part II Table B, taking into account the risk level of the farm for the contraction of KHVD.

For the purpose of determining the health inspection frequency, category I compartments with regard to KHVD comprising one or more farms, where the health status regarding KHVD is

dependent on the health status of surrounding natural waters, these compartments are considered of high risk for the contraction of KHVD.

In Member States, zones or compartments in which the number of farms is limited and targeted surveillance on these farms does not provide sufficient epidemiological data, the surveillance schemes to maintain the freedom status shall include sampling points selected in accordance with the provisions laid down in Point II.1.

Those sampling points shall be inspected and sampled by rotation of 50% of the sampling points each year. The sampling must be carried out in accordance with Part II Table C. The samples must be selected, prepared and examined as described in point III and the laboratory examinations must be negative as regards to the agent of KHV.

Disease freedom may only be maintained as long as all samples using the diagnostic methods set out in point III.2 produces negative results for KHVD and any suspicion of KHVD is ruled out in accordance with the methods set out in points III.3.

II.4. Specific provisions to declare category III health status with regard to KHVD in Member States, compartments or zones with a previous category V health status.

A Member State, zone or a compartment with a previous category V health status with regard to KHVD may declare category III health status with regard to that disease provided that:

- (a) the provisions laid down in points II.2.2.1(a), II.2.2.1(b) and II.2.2.1(c) have been met;
- (b) all farms officially declared infected and all other farms fallowed within the established protection and surveillance zones shall be restocked with fish sourced from Member States, zones or compartments with a category I, II or III health status with regard to KHVD. Restocking shall only take place when all farms officially declared infected have been emptied, cleansed, disinfected and fallowed in accordance with II.2.2.(c); and
- (c) no suspicion or confirmation of KHVD has occurred during the period of two years that follows the completion of the measures of paragraphs (a) and (b).

II.5. Minimum provisions to be implemented in Member States, zones or compartments to contain KHVD

In the case of confirmation of KHVD in a Member State, zone or compartment, competent authorities shall at least take the measures provided for in Article 39 of Directive 2006/88/EC. When establishing a containment area the minimum criteria laid down in II.2.2.1 (a) shall be taken into account.

III. Procedures for surveillance for declaration of KHVD freedom

III.1. Samples

The tissue material to be examined is parts of gill and kidney. Organ pieces from a maximum of 2 fish may be pooled.

III.2. Surveillance methods to achieve or to maintain disease-free status of KHVD

The diagnostic method to achieve or to maintain disease-free status of KHVD is the Real-time PCR.

The protocols for the surveillance methods and the evaluation of their results are given in Annex ... and can be accessed on the following web-site: www.eurl-fish.eu.

IV. Procedures for diagnostic investigation

III.1. Samples

The tissue material to be examined is parts of gill and kidney. Organ pieces from a maximum of 5 fish may be pooled.

III.3. Official investigation and diagnostic methods to rule out and to confirm infection with KHV

When a suspicion of KHVD must be confirmed/ruled out according to Article 28 of Directive 2006/88/EC or other EU legislation, the following inspection, sampling and testing procedure must be complied with.

The official investigation shall include at least one health inspection and one sampling of 10 fish, when clinical signs or post-mortem signs consistent with infection with KHV are observed or 30 fish, when clinical or post-mortem signs are not observed. Samples shall be tested in accordance with the following diagnostic methods:

The presence of the infection with KHV shall be considered as confirmed if KHV is detected by PCR.

Suspicion of KHV can be ruled out, if these tests reveal no further evidence of the presence of KHV.

The protocols for the diagnostic methods and the evaluation of their results are given in Annex ... and can be accessed on the following web-site: www.eurl-fish.eu.

PART II - TABLE A

Surveillance scheme for zones and for compartments for the four year control period which precedes achievement of disease-free status of KHVD

		Number of clinical inspections per year	Number of laboratory examinations per year	Number of fish in the sample
Farms/sampling sites	First two years of the surveillance period	2	1	30
Farms/sampling sites	Second two years of the surveillance period	2	2	30
Maximum number of fish per pool: 2				

PART II - TABLE B

Surveillance schemes for zones or compartments to maintain disease freedom status for KHVD

Risk level	Number of health inspections	Number of fish in the sample
High	1 every year	30
Medium	1 every 2 years	30
Low	1 every 4 years	30
Maximum number of fish per pool: 2		

PART II - TABLE C

Surveillance scheme to maintain KHVD free status in Member States, zones or compartments where the number of farms is limited and targeted surveillance on these farms does not provide sufficient epidemiological data

	Number of clinical inspections per year	Number of laboratory examinations per year	Number of fish in the sample
Sampling points	1 every 2 years	1 every 2 years	30
Maximum number of fish per pool: 2			