CHAPTER 2.3.6.

INFECTION WITH KOI HERPESVIRUS

1. Scope

Infection with koi herpesvirus (KHV) means infection with the pathogenic agent cyprinid herpesvirus-3 (CyHV-3), of the Genus *Cyprinivirus* in the Family *Alloherpesviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

KHV, also known as carp interstitial nephritis and gill necrosis virus (CNGV) (Ilouze *et al.,* 2010), has been classified as cyprinid herpesvirus-3 (CyHV-3) following the nomenclature of other cyprinid herpesviruses: CyHV-1 (carp pox virus, fish papilloma virus) and CyHV-2 (goldfish haematopoietic necrosis virus). Analysis of the complete genome has shown that CyHV-3 is closely related to CyHV-1, CyHV-2, anguillid herpesvirus-1 (AngHV-1) and distantly related to channel catfish virus (Ictalurid herpesvirus: IcHV-1) and Ranid (frog) herpesvirus (RaHV-1) (Waltzek *et al.,* 2005). CyHV-3 was designated the type species of the new *Cyprinivirus* genus within the *Alloherpesviridae* family, that also contains CyHV-1 and CyHV-2. However, the designation KHV has been retained in the *Aquatic Code* and *Aquatic Manual* for reasons of continuity and is used here synonymously with CyHV-3.

The size of the KHV genome is now confirmed as 295 kbp. Virus nucleocapsids have been measured at 100–110 nm in diameter and are surrounded by an envelope (Ilouze *et al.,* 2010). The enveloped virions range in size from 170 to 230 nm in the different infected cell types (Hedrick *et al.,* 2000; Miwa *et al.,* 2007; Miyazaki *et al,* 2008a). Aoki *et al.* (2007) initially described the complete genome sequence of three isolates of KHV and the genome includes 164 open reading frames (ORFs) of which 156 are unique protein-coding genes. They suggested that the finding that 15 KHV genes are homologous with genes in IcHV-1 confirms the proposed place of KHV in the family Herpesviridae. Forty viral proteins and 18 cellular proteins are incorporated into mature virions.

Engelsma *et al.* (2013) detected novel strains of cyprinid herpesvirus closely related to KHV. These strains may represent low or non-pathogenic variants of CyHV-3, but further investigation is required to establish the true genetic relationship between these strains, and KHV.

2.1.2. Survival and stability in processed or stored samples

No information available.

2.1.3. Survival and stability outside the host

Studies in Israel have shown that KHV remains viable in water for at least 4 hours, but less than 21 hours, at water temperatures of 23–25°C (Perelberg *et al.,* 2003). Studies in Japan have shown a significant reduction in the infectious titre of KHV within 3 days in river or pond water or sediment samples at 15°C. However, KHV remained infective for >7 days when kept in environmental water samples that had been sterilised by autoclaving or filtration (Shimizu *et al.,* 2006).

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with KHV according to Chapter 1.5 of *Aquatic Animal Health Code* (*Aquatic Code*) are: all varieties and subspecies of common carp (*Cyprinus carpio*), and common carp hybrids (e.g. *Cyprinus carpio* × *Carassius auratus, Cyprinus carpio* × *Carassius carassius*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is insufficient evidence to fulfil the criteria for listing as susceptible to infection with KHV according to Chapter 1.5 of the *Aquatic Code* are: Goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon idella*) and Crucian carp (*Carassius carassius*).

In addition, pathogen-specific positive PCR and or *in-situ* hybridisation results have been reported in the following organisms, but an active infection has not been demonstrated:

Family	Scientific name	Common name
Acipenseridae	*Acipenser gueldenstaedtii*	Atlantic sturgeon
	Acipenser ruthenus × Huso huso	hybrid sterlet × beluga
	Acipencer oxyrinchus	Russian sturgeon
Cyprinidae	*Leuciscus idus*	blue back ide
	Rutilus rutilus	common roach
	Tinca tinca	Tench
	Hypophthalmichthys molitrix	silver carp
Gammaridae	*Gammarus pulex*	scud (crustacean)
Nemacheilidae	*Barbatula barbatula*	stone loach
Percidae	*Gymnocephalus cernuus*	Eurasian ruffe
	Perca fluviatilis	European perch
Unionidae	*Anodonta cygnea*	swan mussel

2.2.3. Likelihood of infection by species, host life stage, population or sub-populations

For the purposes of Table 4.1, larvae and fry up to approximately 1 g in weight may be considered to be early life stages, fingerlings and grower fish up to 250 g may be considered to be juveniles, and fish above 250 g may be considered to be adults.

All age groups of fish, from juveniles upwards, appear to be susceptible to infection with KHV but, under experimental conditions, 2.5–6 g fish were more susceptible than 230 g fish (Perelberg *et al.,* 2003). Carp larvae appear to be tolerant to infection with KHV.

Common carp or varieties, such as koi or ghost (koi × common) carp, are most susceptible and should be preferentially selected for virus detection, followed by any common carp hybrids, such as goldfish × common carp or crucian carp × common carp. Experimental challenges studies by Ito *et al.,* 2014a; 2014b, demonstrated that mortality due to infection with KHV was higher in indigenous Japanese carp (95–100%) compared with domesticated common carp and koi carp, where mortality varied from 30% to 95% and from 35% to 100%, respectively.

2.2.4. Distribution of the pathogen in the host

Gill, kidney, gut and spleen are the organs in which KHV is most abundant during the course of clinical disease (Gilad *et al.,* 2004). In fish surviving experiment challenge by immersion, KHV DNA was more likely to be detected from the caudal fin and brain compared with gill and kidney (Ito *et al*., 2014b).

2.2.5. Aquatic animal reservoirs of infection

There is evidence to indicate that survivors of infection with KHV may become persistently infected with virus and may retain the virus for long periods without expression of clinical signs of infection. The virus has been shown to persist in common carp experimentally infected at a permissive temperature and subsequently maintained at a lower than permissive temperature (Gilad *et al.,* 2003; St-Hilaire *et al.,* 2005). Researchers in Japan conducted a PCR and serological survey of KHV in Lake Biwa in 2006, where episodic outbreaks of infection with KHV had been reported in the 2 years following a major outbreak in 2004. Further analysis of the surviving population showed that 54% of the older carp were seropositive and 31% PCR positive. The maintenance of high levels of antibody to the virus suggests that latent virus may be reactivating periodically in some animals, leading to excretion and a low level of virus circulation in the population, which boosts herd immunity.

2.2.6. Vectors

No species of vector have been demonstrated to transmit KHV to susceptible species. Studies in Japan have however, reported the detection of KHV DNA in plankton samples and, in particular, Rotifera species (Minamoto *et al*., 2011). KHV has also been detected by PCR in swan mussels (*Anodonta cygnea*) and freshwater shrimp (*Gammarus pulex*) (Kielpinski *et al*., 2010) and in migratory wild ducks of the genera *Anas, Mareca, Spatula* and *Oxyura* (Torres-Meza *et al*., 2020) in areas where fish and ducks coexist.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

The clinical signs of infection may become apparent 3–21 days after naïve fish have been introduced to a pond containing infected fish (Bretzinger *et al.,* 1999; Hedrick *et al*., 2000). Morbidity of affected populations can be 100%, and mortality 70–100% (Bretzinger *et al.,* 1999; Haenen *et al.,* 2004). However, in several experiments, differential resistance to infection with KHV among common carp strains was reported (Dixon *et al*., 2009; Ito *et al*., 2014a; Shapira *et al*., 2005). In these reports, the cumulative mortalities of the most resistant strains were approximately 40%. Secondary and concomitant bacterial or parasitic infections are commonly seen in diseased carp and may affect both the mortality rate and clinical signs of infection (Haenen *et al.,* 2004).

2.3.2. Clinical signs, including behavioural changes

During an outbreak of infection with KHV there will be a noticeable increase in mortality in the population. All age groups of fish, except larvae, appear to be susceptible to infection with KHV, although, under experimental infection, younger fish (up to 1 year of age) are more susceptible to infection. Changes to the skin are also commonly observed and include: focal or total loss of epidermis, irregular patches of pale colouration or reddening, excessive or reduced mucous secretion (on skin or gills) and sandpaper-like skin texture. Other clinical signs include endophthalmia (sunken eyes), haemorrhages on the skin and base of the fins, and fin erosion.

Fish become lethargic, separate from the shoal and gather at the water inlet or sides of a pond and gasp at the surface of the water. Some fish may experience loss of equilibrium and disorientation, but others may show signs of hyperactivity.

2.3.3. Gross pathology

There are no pathognomic gross lesions. However, the most consistent gross pathology is seen in the gills, which can vary in extent from pale necrotic patches to extensive discolouration, severe necrosis and inflammation. Internal lesions are variable in occurrence and often absent in cases of sudden mortality. Other gross pathologies that have been reported include adhesions in the abdominal cavity, with or without abnormal colouration of internal organs (lighter or darker). The kidney or liver may be enlarged, and they may also exhibit petechial haemorrhages. Co-infections, for example with ectoparasites such as gill monogeneans, may alter the observed gross pathology.

2.3.4. Modes of transmission and life cycle

Virus is shed via faeces, urine, gills and skin and the main mode of transmission of KHV is horizontal. Early reports suggested that the gills and the intestine are the major portal of virus entry in carp (Dishon *et al.,* 2005; Gilad *et al.,* 2004; Ilouze *et al.,* 2006; Pikarsky *et al*., 2004).

However, a more recent experimental study has demonstrated that the skin covering the fins and body of the carp is the major portal of entry for KHV (Costes *et al.,* 2009). Another study has shown that KHV DNA was detected in two of three fish from the caudal fin and gill, and caudal fin and spleen one day after exposure to sub-clinically infected fish (Ito *et al*., 2014a; 2014b). The virus spreads systemically from main points of entry to the internal organs; high levels of KHV DNA have been detected in kidney, spleen, liver and gut tissue (Dishon *et al.,* 2005; Pikarsky *et al*., 2004). The assembly and morphogenesis of KHV in infected cells is the same as other herpesviruses (Miwa *et al.*, 2007). An ultrastructural examination of experimentally infected carp has provided evidence for immature capsids and mature nucleocapsid assembly in the nucleus and further maturation of the virion in the cytoplasm of infected cells. Hyper-secretion of mucous is very evident in the early stages of infection with KHV and KHV DNA has been

detected at high levels in mucous sampled from experimentally infected carp (Gilad *et al.,* 2004). This is further evidence for active involvement of the skin in viral pathogenesis and an important site of virus shedding. Excretion of virus via urine and faeces may also be an important mechanism for virus shedding; infectious virus has been detected in faeces sampled from infected carp (Dishon *et al.,* 2005; Gilad *et al*., 2004).

2.3.5. Environmental factors

Disease patterns are influenced by water temperature, virulence of the virus, age, population genetics and condition of the fish, population density and stress factors (e.g. transportation, spawning, poor water quality). The disease is temperature dependent, occurring mainly between 16 and 29°C (Haenen *et al.,* 2004; Hedrick *et al*., 2000; Perelberg *et al.,* 2003; Sano *et al.,* 2004). Under experimental conditions, infectious virus was continually shed for a longer period from infected common carp at 16°C than those kept at 23°C or 28°C (Yuasa *et al.,* 2008). However, experimental challenge resulted in high mortality at 28°C but not at 29°C or 30°C, nor at 13°C (Gilad *et al.,* 2004; Ilouze *et al.,* 2010) (optimal temperature range for viral replication may vary with the virus strain).

2.3.6. Geographical distribution

Following the first reports of infection with KHV in Israel and Germany in 1998 and detection of KHV DNA in tissue samples taken during a mass mortality of carp in the UK in 1996, the geographical range of the disease has become extensive and includes most continents, including Europe, Asia, the Middle East, Southern Africa, and North America.

See WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

A safe and effective commercial vaccine is not currently widely available. However, live attenuated virus has been used to vaccinate carp. The vaccine preparation induced antibody against the virus and the duration of the protection was at least 8 months (Ilouze *et al.,* 2010). The vaccine was licensed for emergency use in Israel and has been widely used in carp farms across the country. Various vaccine candidates against KHV have been developed. Results of studies in Japan have shown that oral administration of a liposome-based vaccine containing inactivated KHV was also effective in protecting carp against clinical disease (Ilouze *et al.,* 2010; Miyazaki *et al.,* 2008b). A vaccine candidate based on the double deletion of ORF56 and ORF57 was produced using BAC cloning technology, and the effectiveness of attenuated recombinant vaccines has been demonstrated in experimental challenge experiments (Boutier *et al.*, 2015). The DNA vaccines consisting of plasmids encoding ORF25, ORF81 and ORF 149 showed efficient results under lab conditions (Hu *et al.,* 2020; Zhou *et al.,* 2014a; 2014b;).

2.4.2. Chemotherapy including blocking agents

Chemotherapy is not currently available, however, the antiviral activity of exopolysaccharides against KHV *in vitro* has been reported (Reichert *et al.*, 2017).

2.4.3. Immunostimulation

There is currently no published information on the use of immunostimulants to control infection with KHV in carp. However, it is known to be an area of research interest (Reichert *et al.,* 2017).

2.4.4. Breeding resistant strains

Differential resistance to infection with KHV, but not to virus entry, has been shown among different carp strains (Dixon *et al.,* 2009; Ito *et al*., 2014a; 2014b; Shapira *et al.,* 2005). The progeny of crosses of two strains of domesticated carp and one strain of wild carp were challenged by experimental or natural infection. The lowest survival rate was approximately 8% but the survival rate of the most resistant strain was 60.7% for experimental exposure and 63.5% for natural exposure in ponds (Shapira *et al.,* 2005). In a more recent resistance study, 96 families derived from di-allele crossing of four European/Asian strains of common carp were experimentally challenged with KHV. Survival rates of the five most resistant crosses in the final virus challenge trial ranged from 42.9 to 53.4% (Dixon *et al.,* 2009).

2.4.5. Inactivation methods

The virus is inactivated by UV radiation at a dose of 4.0×10^3 µ Ws/cm^2, temperatures above 50°C for 1 minute and by iodophor (200 mg litre^{-1}) treatment for 30 seconds at 15°C. The following disinfectants are also effective for inactivation: iodophor at 200 mg litre^{-1} for 20 minutes, benzalkonium chloride at 60 mg litre^{-1} for 20 minutes, ethyl alcohol at 30% for 20 minutes and sodium hypochlorite at 200 mg litre^{-1} for 30 seconds, all at 15°C (Kasai *et al.,* 2005).

2.4.6. Disinfection of eggs and larvae

Disinfection of the surface of the eggs can be achieved by iodophor treatment (Kasai *et al.,* 2005). There are no publications on the disinfection of larvae.

2.4.7. General husbandry

Biosecurity measures should include ensuring that new introductions of fish are from disease-free sources and installation of a quarantine system where new fish can be held with sentinel fish at permissive temperatures for infection with KHV. The fish should be quarantined for a minimum of 4 weeks to 2 months before transfer to the main site and mixing with naïve fish. Hygiene measures on site should include disinfection of eggs, regular disinfection of ponds, chemical disinfection of farm equipment, careful handling of fish to avoid stress and safe disposal of dead fish.

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

Clinical inspections should be carried out during a period when the water temperature is conducive to development of clinical disease, i.e. above 16°C (see Section 2.3.5). All production units (ponds, tanks, net-cages, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. If moribund fish or fish showing clinical signs are sampled, the probability of detecting KHV is higher than if randomly selected, apparently healthy fish are sampled.

For the purposes of disease surveillance, fish to be sampled are selected as follows:

i) The most susceptible species should be sampled preferentially (see Section 2.2.3). Other susceptible species listed in Section 2.2.1 should be sampled proportionally.

ii) Risk-based criteria should be employed to preferentially sample lots or populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or replacement with stocks of unknown disease status) or where there is evidence of poor water quality or husbandry. Younger fish up to 1 year are more susceptible to clinical disease and are recommended for sampling. If more than one water source is used for fish production, fish from all water sources should be included in the sample.

iii) If weak, abnormally behaving or freshly dead (not decomposed) fish are present, such fish should be selected. If such fish are not present, the fish selected should include normal appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample.

For disease outbreak investigations, moribund fish or fish exhibiting clinical signs of infection with KHV should be collected. Ideally fish should be collected while alive, however recently dead fish can also be selected for diagnostic testing. It should be noted however, that there will be a significant risk of contamination with environmental bacteria if the animals have been dead for some time.

3.2. Selection of organs or tissues

When testing clinically affected fish by PCR methods, and particularly if virus isolation is to be attempted, it is recommended to sample gill, kidney, and spleen tissues. The virus is most abundant in these tissues during the course of overt infection and high levels of virus have also been detected in encephalon (brain) and intestine (gut) tissue (Dishon *et al.,* 2005; Gilad *et al.,* 2004). Moreover, KHV DNA was detected with high probability from the encephalon of the surviving fish at 120 days post-infection (Ito *et al.,* 2014a). When testing subclinical, apparently healthy, fish by PCR methods, it is recommended to also include intestine (gut) and encephalon in a

separate sample. In addition, KHV DNA was detected in the caudal and pectoral fin of all sampled dead fish from the field. As fins can be easily collected using tweezers and scissors, the fins are a suitable organ for PCR detection of KHV in clinically affected fish (Ito *et al.*, 2014a; 2014b).

3.3. Samples or tissues not suitable for pathogen detection

Fish carcasses showing very advanced signs of tissue decomposition are not suitable for testing by any method.

3.4. Non-lethal sampling

While some research has been carried out on the use of non-lethal sampling during the first few days after experimental challenge (Monaghan *et al.*, 2015), due to the lack of formal validation non-lethal sampling is currently not recommended for the detection of KHV.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0.

3.5.1. Samples for pathogen isolation

The success of pathogen isolation depends strongly on the quality of samples (which is influenced by time since collection and time in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. To avoid degradation of samples, use alternative storage methods only after consultation with the receiving laboratory.

3.5.2. Preservation of samples for molecular detection

Tissue samples for PCR testing should be preserved in 80–100% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health and will ensure that the ethanol does not fall to below 70% The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen, but repeated freezing and thawing should be avoided.

3.5.3. Samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Standard sample collection, preservation and processing methods for histological techniques can be found in Section 2.2. of Chapter 2.3.0. General information (diseases of fish).

3.5.4. Samples for electron microscopy

Samples for electron microscopy are not routinely required and are collected only when it is considered beneficial to facilitate further diagnostic investigation. A 2 mm cubed section from each of the appropriate organs described in section 3.2 should be fixed in glutaraldehyde; the recommended ratio of fixative to tissue is 10:1.

3.5.5. Samples for other tests

Not applicable.

3.6. Pooling of samples

Pooling of samples from more than one individual animal for a given purpose should only be recommended where robust supporting data on diagnostic sensitivity and diagnostic specificity have been evaluated and found to be suitable. If the effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, larger fish should be processed and tested individually. Small life stages such as fry can be pooled to obtain the minimum amount of material for virus isolation or molecular detection.

4. Diagnostic methods

The methods currently available for pathogen detection that can be used in i) surveillance of apparently healthy animals, ii) presumptive diagnosis in clinically affected animals and iii) confirmatory diagnostic purposes are listed in Table 4.1. by animal life stage.

Ratings for purposes of use. For each recommended assay a qualitative rating for the purpose of use is provided. The ratings are determined based on multiple performance and operational factors relevant to application of an assay for a defined purpose. These factors include appropriate diagnostic performance characteristics, level of assay validation, availability, cost, timeliness, sample throughput and operability. For a specific purpose of use, assays are rated as:

+++ =	Methods are most suitable with desirable performance and operational characteristics.
++ =	Methods are suitable with acceptable performance and operational characteristics under most circumstances.
+ =	Methods are suitable, but performance or operational characteristics may limit application under some circumstances.
Shaded boxes =	Not appropriate for this purpose.

Validation stage. The validation stage corresponds to the assay development and validation pathway in chapter 1.1.2. The validation stage is specific to each purpose of use. Where available, information on the diagnostic performance of recommended assays is provided in Section 6.3.

OIE Reference Laboratories welcome feedback on diagnostic performance of recommended assays, in particular PCR methods. Of particular interest are any factors affecting expected assay sensitivity (e.g. tissue components inhibiting amplification) or expected specificity (e.g. failure to detect particular genotypes, detection of homologous sequences within the host genome). These issues should be communicated to OIE Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Wet mounts												
Histopathology[3]						++	++	1				
Cell culture						++	++	1				
Real-time PCR	+++	+++	+++	3	+++	+++	+++	3				
Conventional PCR					++	+++	+++	3[5]	++	++	++	3[5]
Conventional nested PCR	+	+	+	NA	++	++	++	NA	+	+	+	NA
Amplicon sequencing[4]									+++	+++	+++	1
In-situ hybridisation												
Bioassay												
LAMP						+++	+++	1				
IFAT						+	+	1				
ELISA												
Other antigen detection methods												
Other method												

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); NA = not available; PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay, respectively.

[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6).

[2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.

[3]Histopathology and cytopathology can be validated if the results from different operators have been statistically compared.

[4]Sequencing of the PCR product.

[5]Bercovier *et al.* (2005) method as modified by Clouthier *et al.* (2017); other conventional PCR assays level 1.

Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

Not relevant.

4.2. Histopathology and cytopathology

Examination of the gills by low-power light microscopy can reveal erosion of primary lamellae, fusion of secondary lamellae, and swelling at the tips of the primary and secondary lamella. The histopathology of the disease is variable and not pathognomonic, but inflammation and necrosis of gill tissues is a consistent feature. Gills also exhibit hyperplasia and hypertrophy of branchial epithelium, and fusion of secondary lamellae and adhesion of gill filaments can be seen. Gill necrosis, ranging from small areas of necrotic epithelial cells of secondary lamellae to complete loss of the lamellae is observed. Branchial epithelial cells and leucocytes may have prominent nuclear swelling, margination of chromatin to give a 'signet ring' appearance, and pale diffuse eosinophilic intranuclear inclusions can be observed. Inflammation, necrosis and nuclear inclusions have also been observed (individually or together) in other organs, particularly the kidney, but also in the spleen, pancreas, liver, brain, gut and oral epithelium.

4.3. Cell culture for virus isolation

The recommended cell lines for KHV detection are*:* CCB and KF-1. Cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

Diagnosis of infection with KHV in clinically affected fish can be achieved by virus isolation in cell culture. However, the virus is isolated in only a limited number of cell lines which can be difficult to handle. Also, 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 (Haenen *et al.,* 2004).

The procedure for virological examination is described in Section 2.3.2. of Chapter 2.3.0 *General information* (diseases of fish).

Confirmatory identification

The most reliable method for confirmatory identification of a virus that has caused CPE is by PCR, followed by sequence analysis of the PCR product. The PCR methods recommended for identification of KHV are the same methods recommended for direct detection in fish tissues. For final confirmation, PCR products of the correct size should be identified as KHV by sequence analysis.

i) Using a suitable DNA extraction kit or reagent, extract DNA from a sample of cell culture material.

ii) Extracted DNA is then amplified using the PCR protocols described below (Section 4.4.2. or 4.4.3). Amplified PCR products may then be sequenced as described in Section 4.4.5

4.4. Nucleic acid amplification

The following controls should be run with each stage of the assay: negative extraction control; positive extraction control; no template PCR control; internal PCR control or positive PCR control. Ideally, the positive extraction control should be distinguishable from viral genomic sequence, thus allowing detection of any cross-contamination leading to false positive results.

4.4.1. Sample preparation and extraction of DNA

DNA from infected cells and/or tissues is extracted using a phase-separation method or by use of a commercially available DNA isolation kit used according to the manufacturer's instructions.

4.4.2. Real-time PCR

Real-time PCR assays, such as TaqMan real-time PCR, are favoured by many diagnostic laboratories over conventional PCR, and real-time Taqman PCR is now a common diagnostic procedure that has been shown to detect and quantitatively assess very low copy numbers of target nucleic acid sequences. The most commonly used quantitative assay for detection of KHV is the Gilad Taqman real-time PCR assay (Gilad *et al.,* 2004). It should however, be noted that there is evidence that the published real-time PCR methods, developed for the detection of KHV DNA in fresh tissue samples do not detect novel strains of cyprinid herpesvirus closely related to KHV (Engelsma *et al.,* 2013).

The primer and probe sequences and cycling conditions for the Gilad *et al*. (2004) KHV and an internal housekeeping gene (used as the internal PCR control) real-time PCRs are shown in Table 4.4.2.1.

Table 4.4.2.1. Primer and probe sequences and cycling conditions for the KHV real-time PCR (Gilad et al.*, 2004)*

Target	Primer/probe sequence (5'→3') (concentration)	Cycling conditions	Amplicon size (bp)	Reference
KHV	KHV-86f: GAC-GCC-GGA-GAC-CTT-GTG (400 nM)	1 × 2 minutes @ 50°C 1 × 10 minutes @ 95°C 40 × 15 seconds @ 95°C and 60 seconds @60°C	78	Gilad *et al.* (2004)[1]
	KHV-163r: CGG-GTT-CTT-ATT-TTT-GTC-CTT-GTT (400 nM)			
	KHV-109p: 6FAM-CTT-CCT-CTG-CTC-GGC-GAG-CAC-G-TAMRA (80 nM)			
Glucokinase	CgGluc-162f: ACT-GCG-AGT-GGA-GAC-ACA-TGA-T (400 nM)		69	
	CgGluc-230r: TCA-GGT-GTG-GAG-CGG-ACA-T (400 nM)			
	CgGluc-185p: 6FAM-AAG-CCA-GTG-TCA-AAA-TGC-TGC-CCA-CT-TAMRA (80 nM)			

[1]The Gilad *et al.* (2004) assay was modified slightly by increasing the probe quantity to 100 nM by Clouthier *et al.* (2017).

4.4.3. Conventional PCR

Commonly used conventional PCR methods are shown in Table 4.4.3.1.

Table 4.4.3.1. Primer sequences and cycling conditions for KHV conventional PCR methods

Primer sequence (5'→3') (concentration)	Cycling conditions	Amplicon size (bp)	References
Primary step: CyHVpolfor: CCA-GCA-ACA-TGT-GCG-ACG-G (200 nM) CyHVpolrev: CCG-TAR-TGA-GAG-TTG-GCG-CA (200 nM) Nested PCR: CyHVpolforint: CGA-CGG-VGG-YAT-CAG-CCC (200 nM) CyHVpolrevint: GAG-TTG-GCG-CAY-ACY-TTC-ATC (200 nM)	1 × 2 minutes @ 95°C 40 × 30 seconds @ 95°C, 30 seconds @ 55°C and 45 seconds @ 72°C 1 × 10 minutes @ 72°C	361 339	Engelsma *et al.* (2013)
For: GGG-TTA-CCT-GTA-CGA-G (200 nM) Rev: CAC-CCA-GTA-GAT-TAT-GC (200 nM)	1 × 5 minutes @ 95°C 40 × 60 seconds @ 95°C, 60 seconds @ 55°C and 60 seconds @ 72°C 1 × 10 minutes @ 72°C	409	Bercovier *et al.* (2005)[1] Clouthier *et al.* (2017)

Primer sequence (5' → 3') (concentration)	Cycling conditions	Amplicon size (bp)	References
For: GAC-ACC-ACA-TCT-GCA-AGG-AG (1000 nM) Rev: GAC-ACA-TGT-TAC-AAT-GGT-CGC (1000 nM)	1 × 30 seconds @ 94°C 40 × 30 seconds @ 94°C, 30 seconds @ 63°C and 30 seconds @ 72°C 1 ×7 minutes @ 72°C.	292	Gray *et al.* (2002) Yuasa *et al.* (2005)
For: GAC-GAC-GCC-GGA-GAC-CTT-GTG (300 nM) Rev: CAC-AAG-TTC-AGT-CTG-TTC-CTC-AAC (300 nM)	1 × 5 minutes @95°C 39 ×1 minute @ 94°C, 1 minute @ 68°C and 30 seconds @ 72°C 1 × 7 minutes @ 72°C	484	Gilad *et al.,* (2004)

[1]The annealing temperature and cycling programme described by Bercovier *et al.* (2005) were slightly modified to improve detection limits and the specificity of the assay. See Clouthier *et al*. (2017) for the details.

4.4.4. Other nucleic acid amplification methods

A loop-mediated isothermal amplification (LAMP) targeting TK gene has been developed for detection of KHV and shown to be more or equally sensitive as the single-round conventional PCR assays. An assay incorporating DNA hybridisation technology and antigen–antibody reactions in combination with LAMP has also been developed and reported to have improved sensitivity and specificity (Soliman & El-Matbouli, 2010).

4.5. Amplicon sequencing

PCR products are excised from the gel and purified using a commercial kit for gel purification. Single, intense (bright) PCR products, after purification, are sequenced directly in both directions with the primers used in the initial amplification. Alternatively, less intense (faint) PCR products are cloned using a TA cloning vector and both DNA strands are sequenced. The amplification, cloning and sequencing are performed in duplicate to eliminate potential errors introduced by the Taq polymerase. Sequence reactions are then analysed on a Genetic Analyser and the alignments and consensus sequences generated using appropriate computer software. Testing laboratories that have no sequencing facilities are recommended to use commercial companies that offer a sequencing service. Testing laboratories should follow the instructions supplied by the chosen sequencing service for submission of samples.

4.6. *In-situ* hybridisation

In-situ hybridisation (ISH) performed on separated fish leucocytes, has been used in research applications for detection, confirmation, or identification of KHV. Although this method has not been thoroughly compared with other techniques and is not included in Table 4.1., it is a non-destructive (non-lethal) technique and some laboratories may find it useful in a research setting. Details of the methods are not given here but detailed protocols for separation of leucocytes from blood and for ISH can be found in published reports by Bergmann *et al.* (2009; 2010).

4.7. Indirect fluorescent antibody test (IFAT)

KHV can be detected in touch imprints of liver, kidney and brain of infected fish by immunofluorescence (IF). Highest levels of positive IF were seen in the kidney and the virus could be detected by IF on a kidney imprint 1 day post-infection (Pikarsky *et al.,* 2004; Shapira *et al.,* 2005). The detection of KHV by immunostaining must be interpreted with care, as positive-staining cells could result from cross-reaction with serologically related virus (e.g. CyHV-1) or a non-viral protein (Pikarsky *et al.,* 2004).

A method for direct detection of KHV from kidney imprints by indirect fluorescent antibody test (IFAT) is detailed below.

i) Bleed the fish thoroughly.

ii) Make kidney imprints on cleaned glass slides or at the bottom of the wells of a plastic cell culture plate.

iii) Allow the imprint to air-dry for 20 minutes.

iv) Rinse once with 0.01 M phosphate-buffered saline (PBS), pH 7.2, then three times briefly with cold acetone (stored at –20°C) for glass slides or a mixture of 30% acetone/70% ethanol, also stored at –20°C, for plastic wells.

v) Let the fixative act for 15 minutes. A volume of 0.5 ml/2 cm^2 well is adequate for imprints in cell culture plates.

vi) Allow the fixed imprints to air-dry for at least 30 minutes and process immediately or freeze at –20°C.

vii) Rehydrate the dried imprints by four rinses with 0.01 M PBS solution, pH 7.2, containing 0.05% Tween 20 (PBST), and remove this buffer completely after the last rinse.

viii) Prepare a solution of purified antibody or antiserum to KHV in 0.01 M PBS, pH 7.2, containing 0.05% Tween 20 (PBST), at the appropriate dilution (which has been established previously or is given by the reagent supplier).

ix) Block with a solution of 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.

x) Rinse four times with PBST.

xi) Treat the imprints with the antibody solution (prepared at step viii) for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur. A volume of 0.25 ml/2 cm^2 well is adequate for imprints in cell culture plates.

xii) Rinse four times with PBST.

xiii) Treat the imprints for 1 hour at 37°C with a solution of fluorescein isothiocyanate (FITC)-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier.

xiv) Rinse four times with PBST.

xv) Add PBS (0.5 ml/2 cm^2 well) to the treated imprints in cell culture plates and examine immediately or mount the glass slides with cover-slips using glycerol saline at pH 8.5 prior to microscopic observation.

xvi) Examine under a fluorescence microscope. Positive and negative controls must be found to give the expected results prior to any other observation.

Paraffin wax tissue sections fixed in 10% neutral buffered formalin (NBF) are also suitable for detection of KHV antigen by IFAT. However, the deparaffinised sections, rehydrated in PBS, may need to be further treated to reveal antigen that may be masked by over fixation of the tissue. A common treatment is incubation of the sections with 0.1% trypsin in PBS at 37°C for 30 minutes. The sections are then washed in cold PBS before proceeding with steps viii–xvi above. Tissues collected for direct detection by IFAT (or other immunohistochemical staining, e.g. immunoperoxidase) should be fixed for 24–48 hours in 10% NBF and then the fixative should be replaced with 70% ethanol for prolonged storage.

4.8. Bioassay

Bioassay is not recommended as a diagnostic procedure.

4.9. Antibody- or antigen-based detection methods

Enzyme-linked immunosorbent assay (ELISA)-based methods for direct detection of KHV antigen in infected tissues are under development in a number of laboratories. Currently, two published ELISA methods are available and were developed in Israel to detect KHV in fish faeces (Dishon *et al.,* 2005) but also after isolation in cell culture using different KHV isolates at different temperatures (Bergmann *et al.* 2017). The ELISA methods developed will have low sensitivity that may be suitable for detection of the high levels of KHV found in clinically diseased fish tissue but not suitable for KHV surveillance in healthy populations.

4.10. Other methods

None published or validated.

5. Test(s) recommended for surveillance to demonstrate disease freedom in apparently healthy populations

Real-time PCR is the recommended test for surveillance in apparently healthy animals to declare freedom from infection with KHV. However, there have been unpublished observations that the method may not detect novel strains of cyprinid herpesvirus closely related to KHV that were described by Engelsma *et al.* (2013).

6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the absence (6.1) or presence of clinical signs (6.2) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision-making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

6.1. Apparently healthy animals or animals of unknown health status [1]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographic proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection shall be suspected if: a positive result has been obtained on at least one animal from at least one of the following diagnostic tests:

i) A positive result from a real-time PCR assay

ii) A positive result from a conventional nested PCR assay.

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with KHV is considered to be confirmed if at least one of the following criteria is met:

i) Detection of KHV in tissue samples by real-time PCR followed by conventional PCR and sequencing of the amplicon

ii) Detection of KHV in tissue samples by real time PCR followed by conventional nested PCR and sequencing of the amplicon

6.2. Clinically affected animals

No clinical signs are pathognomonic for infection with KHV however, they may narrow the range of possible diagnoses.

1 For example transboundary commodities.

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs associated with infection with KHV as described in this chapter, with or without elevated mortality

ii) Histopathological changes consistent with infection with KHV as described in this chapter

iii) KHV typical CPE in cell culture

iv) A positive result by a real-time PCR

v) A positive result by a conventional (single round or nested) PCR

vi) A positive result by LAMP assay

vii) A positive result by IFAT

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection shall be confirmed if at least one of the following criteria is met:

i) KHV isolation in cell culture followed by virus identification by conventional PCR or conventional nested PCR and sequencing of the amplicon

ii) Detection of KHV in tissue samples by real-time PCR followed by conventional PCR or conventional nested PCR and sequencing of the amplicon

iii) A positive result by LAMP assay followed by conventional PCR or conventional nested PCR and sequencing of the amplicon

iv) A positive result by IFAT followed by conventional PCR or conventional nested PCR and sequencing of the amplicon

iv) Detection of KHV in tissue samples by conventional PCR or conventional nested PCR and sequencing of the amplicon

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to OIE.

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with KHV are provided in Table 6.3.1. This information can be used for the design of surveys for infection with KHV, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data are only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2. and the information is available within published diagnostic accuracy studies.

6.3.1. For surveillance of clinically affected apparently healthy animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
Real-time PCR[1]	Diagnosis	Experimentally infected koi and apparently healthy wild common carp[3]	kidney	Common carp & koi (*Cyprinus carpio* L.)	99	93	None; Bayesian latent class modelling	Clouthier *et al.*, 2017[4]

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
PCR[2]	Diagnosis	Experimentally infected koi and apparently healthy wild common carp[3]	kidney	Common carp & koi (*Cyprinus carpio* L.)	99	93	None; Bayesian latent class modelling	Clouthier *et al.*, 2017[4]

DSe: = diagnostic sensitivity, DSp = diagnostic specificity.
[1]Gilad *et al.* (2004) method as modified by Clouthier *et al.* (2017);
[2]Bercovier *et al.* (2005) method as modified by Clouthier *et al.* (2017);
[3]Note that Clouthier *et al.* (2017) reported diagnostic performance for a combined dataset of clinically affected and apparently healthy populations.
[4]The diagnostic accuracy study did not include samples that were known to be positive for the KHV-like CyHV strains reported by Engelsma *et al.* (2013).

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NB: There are OIE Reference Laboratories for infection with koi herpesvirus
(please consult the OIE Web site for the most up-to-date list:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact OIE Reference Laboratories for any further information on
infection with koi herpesvirus

NB: FIRST ADOPTED IN 2006. MOST RECENT UPDATES ADOPTED IN 2022.