CHAPTER 2.3.6.

**infection with koi herpesvirus**

**1. Scope**

Infection with koi herpesvirus (KHV) means infection with ~~all genotypes of~~ the pathogenic agent cyprinid herpesvirus-3 (CyHV-3), of the Genus *Cyprinivirus* in the Family *Alloherpesviridae*(~~Engelsma~~ *~~et al.,~~* ~~2013; Haramoto~~ *~~et al~~*~~., 2007; Waltzek~~ *~~et al.,~~* ~~2009)~~. ~~However, for familiarity, the abbreviation KHV will be used in this chapter.~~

**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.

~~Early estimates of the genome~~ The size of the KHV genome ~~varied from at least 150 kbp to 277 kbp; the size~~ is now confirmed as 295 kbp. Virus nucleocapsids have been measured at 100–110 nm in diameter and are surrounded by an envelope (~~review:~~ 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 ~~CyHV-3~~ KHV and the genome includes 164 open reading frames (ORFs) ~~as well as~~ 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.

~~The conventional polymerase chain reaction (PCR) developed by~~ 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~~/goldfish~~ 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 ~~polymerase chain reaction (~~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* | Eurasi~~e~~an~~s~~ ruffe |
| *Perca fluviatilis* | European perch |
| ~~Salmonidae~~ | *~~Oncorhynchus mykiss~~* | ~~rainbow trout~~ |
| Unionidae | *Anodonta cygnea* | swan mussel |

**2.2.3. ~~Non-susceptible species~~**

~~Species that have been found non-susceptible to infection with KHV according to Chapter 1.5. of the~~ *~~Aquatic Code~~* ~~are:~~

| **~~Family~~** | **~~Scientific name~~** | **~~Common name~~** |
| --- | --- | --- |
| ~~Agamidae~~ | *~~Intellagama~~**~~lesueurii~~* | ~~Eastern water dragon~~ |
| ~~Ambassidae~~ | *~~Ambassis agassizii~~* | ~~olive perchlet~~ |
| ~~Anguillidae~~ | *~~Anguilla australis~~* | ~~short-finned eel~~ |
| ~~Ariidae~~ | *~~Neoarius graeffei~~* | ~~salmon catfish~~ |
| ~~Chelidae~~ | *~~Emydura macquarii~~* | ~~Macquarie short-necked turtle~~ |
| ~~Clupeidae~~ | *~~Nematalosa erebi~~* | ~~bony bream~~ |
| ~~Eleotridae~~ | *~~Hypseleotris~~* ~~sp.~~ | ~~carp gudgeon~~ |
| ~~Galaxiidae~~ | *~~Galaxias maculatus~~* | ~~common galaxias~~ |
| ~~Limnodynastidae~~ | *~~Limnodynastes tasmaniensis~~* | ~~spotted marsh frogs~~ |
| ~~Melanotaeniidae~~ | *~~Melanotaenia duboulayi~~* | ~~crimson-spotted rainbowfish~~ |
| ~~Mordaciidae~~ | *~~Mordacia mordax~~* | ~~short-headed lamprey ammocoetes~~ |
| ~~Mugilidae~~ | *~~Mugil cephalus~~* | ~~sea mullet~~ |
| ~~Parastacidae~~ | *~~Cherax destructor~~* | ~~common yabby~~ |
| ~~Pelodryadidae~~ | *~~Litoria~~**~~peronii~~* | ~~Peron’s tree frog~~ |
| ~~Percichthyidae~~ | *~~Maccullochella peelii~~* | ~~Murray cod~~ |
| *~~Macquaria ambigua~~* | ~~golden perch~~ |
| ~~Plotosidae~~ | *~~Tandanus tandanus~~* | ~~eel-tailed catfish~~ |
| ~~Retropinna~~ | *~~Retropinna semoni~~* | ~~Australian smelt~~ |
| ~~Terapontidae~~ | *~~Bidyanus bidyanus~~* | ~~silver perch~~ |

**2.2.~~4~~ 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.~~5~~ 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.~~6~~ 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 ~~CyHV-3~~ 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.~~7~~ 6. Vectors**

No species of vector have been demonstrated to transmit KHV to susceptible species. Studies in Japan have however, reported the detection of ~~CyHV-3~~ KHV DNA in plankton samples and, in particular, Rotifera species ~~Plankton samples were collected in 2008 from Iba-naiko, a shallow lagoon connected to Lake Biwa, a favoured carp spawning area~~ (Minamoto *et al*., 2011). ~~Statistical analysis revealed a significant positive correlation between CyHV-3 in plankton and the numbers of Rotifera and the authors suggested that CyHV-3 binds to or is concentrated by the filter feeding behaviour of Rotifera species. In an earlier report of a study in Poland, CyHV-3~~ KHV ~~was~~ 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. ~~The invertebrates were collected from ponds in Southern Poland where outbreaks had occurred in common carp populations over 5 to 6 years. More work is needed to determine how long the infectious virus persists and remains viable in the invertebrates in the absence of the host species.~~

**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), ~~and~~ 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.oie.int/#/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 (~~reviewed by~~ 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 × 103 μ Ws/cm2, temperatures above 50°C for 1 minute and by iodophor (200 mg litre–1) treatment for 30 seconds at 15°C ~~(Kasai~~ *~~et al~~*~~., 2005)~~. 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.

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

i) ~~Susceptible species should be sampled proportionally or following~~ 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 ~~for targeted selection of~~ 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.

~~ii)~~ ~~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**

~~Tissue samples for histopathology should be fixed in neutral buffered formalin immediately after collection. To ensure adequate penetration of the fixative the recommended ratio of fixative to tissue is 10:1.~~ 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**

~~Blood samples extracted from the caudal vessel into a vacuum blood collection tube should be centrifuged for the collection of serum or plasma as soon as possible after sampling to avoid lysis of the red blood cells. Serum or plasma samples should be shipped on ice to the laboratory to ensure maintenance of virus infectivity.~~ Not applicable.

**3.6. Pooling of samples**

~~The effect of pooling on diagnostic sensitivity has not been evaluated, therefore, larger fish should be processed and tested individually.~~ 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 ~~or specimens up to 0.5 g,~~ can be pooled to obtain the minimum amount of material for virus isolation or molecular detection.

**4. Diagnostic methods**

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

~~The designations used in the Table indicate:~~

**Ratings ~~against~~ for purposes of use.** For each recommended assay a qualitative rating ~~against~~ 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, ~~successful application by diagnostic laboratories,~~ availability, cost, timeliness, ~~and~~ sample throughput and operability. For a specific purpose of use, assays are rated as:

~~Key:~~

+++ = ~~Most suitable~~ Methods ~~–~~ are most suitable with desirable performance and operational characteristics.

++ = ~~Suitable~~ Method~~(~~s~~)~~ are suitable with acceptable performance and operational characteristics under most circumstances.

+ = ~~Less suitable~~ Methods ~~–~~ are suitable, but performance or operational characteristics may ~~significantly~~ limit application under some circumstances.

Shaded boxes = Not appropriate for this purpose.

~~The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.~~

**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 the 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** | 1. **Surveillance of apparently healthy animals**
 | 1. **Presumptive diagnosis of clinically affected animals**
 | 1. **Confirmatory diagnosis1 of a suspect result from surveillance or presumptive diagnosis**
 |
| --- | --- | --- | --- |
| **Early life stages2** | **Juveniles2** | **Adults** | **LV** | **Early life stages2** | **Juveniles2** | **Adults** | **LV** | **Early life stages2** | **Juveniles2** | **Adults** | **LV** |
| **Wet mounts** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Histopathology3** |  |  |  |  |  | ++ | ++ | 1 |  |  |  |  |
| **Cell ~~or artificial media~~ culture** |  |  |  |  |  | ++ | ++ | 1 |  |  |  |  |
| **Real-time PCR** | +++ | +++ | +++ | ~~1~~ 3 | +++ | +++ | +++ | ~~1~~ 3  |  |  |  |  |
| **Conventional PCR** |  |  |  |  | ++ | +++ | +++ | ~~1~~ 35 | ++ | ++ | ++ | ~~1~~ 35 |
| **Conventional nested PCR** | + ~~+~~ | + ~~+~~ | + ~~+~~ | ~~1~~ NA | ++ ~~+~~ | ++ ~~+~~ | ++ ~~+~~ | ~~1~~ NA | + ~~+~~ | + ~~+~~ | + ~~+~~ | ~~1~~ NA |
| **Amplicon sequencing4** |  |  |  |  |  |  |  |  | +++ | +++ | +++ | 1 |
| ***In-situ* hybridisation** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Bioassay** |  |  |  |  |  |  |  |  |  |  |  |  |
| **LAMP** |  |  |  |  |  | +++ | +++ | 1 |  |  |  |  |
| **IFAT** |  |  |  |  |  | + | + | 1 |  |  |  |  |
| **ELISA** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Other antigen detection methods5** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Other method5** |  |  |  |  |  |  |  |  |  |  |  |  |

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.
1For confirmatory diagnoses, methods need to be carried out in combination (see Section 6).
2Susceptibility of early and juvenile life stages ~~have been defined~~ is described in Section 2.2.3.
3Histopathology and cytopathology can be validated if the results from different operators have been statistically compared.
4Sequencing of the PCR product.

5~~Specify the test used~~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 ~~or artificial media~~ culture for virus isolation**

The recommended cell lines for KHV detection are*:* CCBandKF-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).

*~~Cell line to be used:~~* ~~KF-1, KFC or CCB.~~

~~Use~~ The procedure for virological examination is described in Section .2.3.2. of Chapter 2.3.0 *General information* (~~on~~ diseases of fish)~~, Section A.2.2.2~~.

*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 ~~(Section 4.3.1.2.3 below)~~. For final confirmation, PCR products of the correct size should be identified as KHV ~~in origin~~ by sequence analysis ~~(Section 4.4.5 below).~~

i) Using a suitable DNA extraction kit or reagent, extract DNA from a sample of ~~the virus culture that includes both cellular and supernatant~~ 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 ~~excised from the gel and~~ sequenced as described in Section ~~4.3.1.2.3~~ 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). ~~However, it should be noted that real-time PCR positive results are presumptive only and should be confirmed by convention PCR and sequence analysis.~~

~~Furthermore,~~ It should however, be noted that there is evidence that the published ~~conventional PCR and~~ real-time PCR methods, developed for the detection of KHV DNA in fresh tissue samples ~~from clinically diseased carp, fail to~~ do not detect novel strains of cyprinid herpesvirus closely related to KHV ~~some KHV variants genotypes in clinically affected fish~~ (Engelsma *et al.,* 2013). ~~Until this is resolved, in geographic locations where these variants may be present it is highly recommended that the assay described by Engelsma~~ *~~et al~~*~~. (2013) is used in place of the current assays; i.e. it is recommended to use using the nested or one-tube semi-nested PCR assay or increasing the cycle number of the single-round assay to detect the virus in apparently healthy carriers.~~

~~The following controls should be run with each assay: negative extraction control; control; no template control; internal PCR control. Ideally, the positive control should be distinguishable from viral genomic sequence, thus allowing detection of any cross-contamination leading to false positive results.~~ The primer and probe sequences and cycling conditions for the Gilad *et al*. (2004) KHV and ~~koi glucokinase~~ 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°C1 × 10 minutes @ 95°C40 × 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) |

1The Gilad *et al.* (2004) ~~(2014)~~ assay was modified slightly by increasing the
probe quantity to 100 nM by Clouthier *et al.* (2017).

**4.4.3. Conventional PCR**

~~Engelsma~~ *~~et al.~~* ~~(2013) reported that the published single-round PCR methods traditionally thought to be the most sensitive for detection of KHV DNA in fresh tissue samples fail to detect some KHV genotypes in clinically affected fish. Therefore, the assay described by Engelsma~~ *~~et al~~*~~. (2013) is highly recommended when detecting KHV variants. By extending the number of cycles to 50 or using the nested second round of amplification the assay may also be suitable to detect virus in sub-clinical carriers. This method and other~~ Commonly used conventional PCR ~~protocols~~ 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°C40 × 30 seconds @ 95°C, 30 seconds @ 55°C and 45 seconds @ 72°C1 × 10 minutes @ 72°C | 361339 | Engelsma *et al*. (2013) |
| For: GGG-TTA-CCT-GTA-CGA-G (200 nM)Rev: CAC-CCA-GTA-GAT-TAT-GC (200 nM) | 1 × ~~15~~ 5 minutes @ ~~94~~ 95°C40 × ~~45~~ 60 seconds @ 95°C, ~~45~~ 60 seconds @ 55°C and 60 seconds @ 72°C1 × ~~7~~ 10 minutes @ 72°C | 409 | Bercovier *et al*. (2005)1Clouthier *et al.* (2017) |
| For: GAC-ACC-ACA-TCT-GCA-AGG-AG (1000 nM)Rev: GAC-ACA-TGT-TAC-AAT-GGT-CGC (1000 nM) | 1 × 30 seconds @ 94°C40 × 30 seconds @ 94°C, 30 seconds @ 63°C and 30 seconds @ 72°C1 ×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°C39 ×1 minute @ 94°C, 1 minute @ 68°C and 30 seconds @ 72°C1 × 7 minutes @ 72°C | 484 | Gilad *et al.,* (2004) |

1The 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) ~~and immunofluorescence (IF) methods~~ performed on separated fish leucocytes, ~~have~~has been used in research applications for detection, confirmation, or identification of KHV. Although this ~~these~~ method~~s~~ ~~have~~has not been thoroughly compared with other techniques and is not included in Table 4.1., ~~they are~~ it is a non-destructive (non-lethal) technique~~s~~ and some laboratories may find ~~them~~ it useful in a research ~~diagnostic~~ setting ~~and for confirmation of PCR results~~. Details of the methods are not given here but detailed protocols for separation of leucocytes from blood and for ~~IF and~~ 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 cm2 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 ~~CyHV-3~~ 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 cm2 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 cm2 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 ~~incident UV light using~~ 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 ~~and these methods may also be suitable for confirmatory identification of KHV~~. Currently, two published ELISA methods are available and ~~was~~ 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~~b~~). 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**

~~Infected carp produce antibodies against the virus, and ELISA-based tests that reliably detect these antibodies at high serum dilution have been published (Adkison~~ *~~et al.,~~* ~~2005; Bergman~~ *~~et al.,~~* ~~2017a; Ilouze~~ *~~et al.,~~* ~~2010; St-Hilaire~~ *~~et al.,~~* ~~2005). Antibody has been detected in the serum at 3 weeks after experimental infection and in survivors after 1 year following a natural infection (Adkison~~ *~~et al.,~~* ~~2005; Ilouze~~ *~~et al.,~~* ~~2010; St-Hilaire~~ *~~et al.,~~* ~~2005; Taylor~~ *~~et al.,~~* ~~2010).~~

~~Serum from koi containing antibodies to KHV has been shown to cross-react, in low dilutions, with CyHV-1. a further indication that these viruses are closely related. Evidence of cross-reacting antibodies was demonstrated in ELISA and western blot analyses of serum from koi infected with CyHV-1 or KHV (Adkison~~ *~~et al.,~~* ~~2005). Diagnostic virologists should also be aware that fish recently vaccinated against KHV may test positive in antibody detection ELISAs.~~

None published or validated.

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

~~There are no well validated methods that are currently recommended for testing healthy populations of susceptible fish for declaration of freedom from infection with KHV; there is increasing evidence that the published real-time PCR assays may fail to detect all genotypes of KHV. Therefore, conventional nested PCR assays described by Engelsma~~ *~~et al.~~* ~~(2013) which will detect all known KHV genotypes is currently recommended for surveillance to demonstrate freedom in apparently health 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 ~~the KHV variants~~ that were described by Englesma *et al.* (2013). ~~In geographic locations where these variants may be present, the conventional nested PCR published by Englesma~~ *~~et al~~*~~. (2013) should also be considered.~~

**6. Corroborative diagnostic criteria**

This section only addresses the diagnostic test results for detection of infection in the ~~presence~~ absence (6.1) or ~~absence~~ 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**3F3F**[[1]](#footnote-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 ~~and~~ conventional PCR ~~followed by~~ 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.

**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 ~~are~~ 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 ~~and by conventional PCR~~ followed byconventional PCR or conventional nested PCR and sequencing of the amplicon

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

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

iv) Detection of KHV in tissue samples by conventional PCR or conventional nested PCR and ~~followed by~~ 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 the 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~~s~~ 6.3.1. ~~and 6.3.2~~. 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.

~~The diagnostic sensitivity (DSe) and specificity (DSp) of PCR assays, based on an analysis of field collections and experimentally infected carp (Amita~~ *~~et al~~*~~., 2002, Ito~~ *~~et al~~*~~., 2014a; 2014b) demonstrated 94–100% DSe and 100% DSp.~~

**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 PCR1 | Diagnosis | Experimentally infected koi and apparently healthy wild common carp3 | kidney | Common carp & koi (*Cyprinus carpio* L.) | 99 | 93 | None; Bayesian latent class modelling | Clouthier et al., 20174 |
| PCR2 | Diagnosis | Experimentally infected koi and apparently healthy wild common carp3 | kidney | Common carp & koi (*Cyprinus carpio* L.) | 99 | 93 | None; Bayesian latent class modelling | Clouthier et al., 20174 |

DSe: = diagnostic sensitivity, DSp = diagnostic specificity.
1Gilad *et al*. (2004) method as modified by Clouthier *et al.* (2017);
2Bercovier *et al.* (2005) method as modified by Clouthier *et al.* (2017);
3Note that Clouthier *et al.* (2017) reported diagnostic performance for a combined dataset of
clinically affected and apparently healthy populations.
4The 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).

**~~6.3.2. For surveillance of apparently healthy animals~~**

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

~~DSe: = diagnostic sensitivity, DSp = diagnostic specificity.~~

~~1~~~~The diagnostic accuracy study did not include samples that were known to be positive for all KHV genotypes.~~

**7. References**

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**NB:** There are OIE Reference Laboratories for Infection with koi herpesvirus
(see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: <http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/> ).
Please contact the OIE Reference Laboratory for any further information on
Infection with koi herpesvirus

**NB:** First adopted in 2006; Most recent updates adopted in 2019.

1. For example transboundary commodities. [↑](#footnote-ref-1)