CHAPTER 2.3.7.

INFECTION WITH KOI HERPESVIRUS

1. Scope

Infection with koi herpesvirus means infection with the pathogenic agent koi herpesvirus (KHV) of the Genus *Cyprinivirus* and Family *Alloherpesviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The aetiological agent is koi herpesvirus (KHV) in the family *Alloherpesviridae* (Haramoto et al., 2007; Waltzek et al., 2009) although prior to taxonomic classification, it was also known as carp interstitial nephritis and gill necrosis virus (CNGV) (Ilouze et al., 2011). Waltzek et al., 2005provided evidence to support the classification of the virus as a herpesvirus, and named it 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). Sequence analysis of part of the genome has shown that KHV is closely related to CyHV-1 and CyHV-2, and distantly related to channel catfish virus (Ictalurid herpesvirus: IcHV-1) and Ranid (frog) herpesvirus (RaHV-1) (Waltzek et al., 2005). Aoki et al., 2007described the complete genome sequence of KHV and identified 156 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 (Michel et al., 2010). Recently, CyHV-3 was designated the type species of the new *Cyprinivirus* genus within the *Alloherpesviridae* family, that also contains CyHV-1 and CyHV-2. Early estimates of the genome size of KHV varied from at least 150 kbp to 277 kbp but 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 (Ilouze et al., 2011).

Comparisons of the genomes of KHV isolates from different geographical areas by restriction enzyme analysis (Haenen et al., 2004) or nucleotide sequence analysis (Sano et al., 2004) have shown them to be practically identical. Likewise, the polypeptides of KHV isolates from different geographical areas were similar, although one isolate from Israel had two additional polypeptides (Gilad et al., 2003). Aoki et al., 2007compared the complete genome sequences of three KHV strains isolated from Japan, Israel and the United States of America (USA). The genomes were found to be highly similar to each other at the sequence level (>99%), with the Israel and USA strains more closely related to each other than either is to the Japan strain. The three isolates were interpreted as having arisen as two lineages (J and U/I) from a wild-type parent. However, further studies in Japan suggest that the lineages were independently brought to those regions and caused KHV epidemics (Ilouze et al., 2011). A more recent study in France has identified a third intermediate between the J and U/I lineages and suggested that the three lineages of CyHV-3 have been introduced into Europe since 2001 via imported koi carp (Bigarré et al., 2009). More recently, a further intermediate lineage has been discovered that may have emerged in Indonesia (Sunarto et al., 2011).

2.1.2. Survival outside the host

Studies in Israel have shown that KHV remains active in water for at least 4 hours, but not for 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 environmental water or sediment samples at 15°C. However, the infectivity remained for >7 days when KHV was exposed to similar water samples that had been sterilised by autoclaving or filtration (Shimizu et al., 2006). The study also presented evidence for the presence of bacterial strains in the water with anti-viral activity. More recently, the detection of KHV DNA in river water samples at temperatures of 9–11°C has been reported, 4 months before an outbreak of KHVD in a river (Haramoto et al., 2007). However, persistence of the virus may have been aided by the presence of animate vectors and detection of DNA may not always be indicative of the presence of infectious virus.

2.1.3. Stability of the agent

The virus is inactivated by UV radiation and temperatures above 50°C for 1 minute. 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).

2.1.4. Life cycle

In early reports investigators suggested that the gills are the major portal of virus entry in carp (Dishon et al., 2005; Gilad et al., 2004; 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). There is then a systemic spread of the virus from the skin and gills to the internal organs and 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 has been described as the same as other herpesviruses. 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 mucus is very evident in the early stages of KHV infection and KHV DNA has been detected at high levels in mucus 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. High levels of KHV DNA have been detected in gut and kidney tissues and infectious virus has been detected in faeces sampled from infected carp (Dishon et al., 2005; Gilad et al., 2004).

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 the *Aquatic Animal Health Code* (*Aquatic Code*) include: All varieties and subspecies of common carp (*Cyprinus carpio*), and common carp hybrids (e.g. *Cyprinus carpio × Carassius auratus*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the *Aquatic Code* include: Goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon idella*) and Syberian crucian carp (*Carassius auratus*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but an infection has not been demonstrated: Atlantic sturgeon (*Acipenser oxyrinchus*), blue back ide (*Leuciscus idus*), common roach (*Rutilus rutilus*), Euraseas ruffe (*Gymnocephalus cernuus*), European perch (*Perca fluviatilis*), hybrid sterlet × beluga (*Acipenser ruthenus × Huso huso*), rainbow trout (*Oncorhynchus mykiss*), Russian sturgeon (*Acipencer gueldenstaedtii*), scud (crustacean) (*Gammarus pulex*), silver carp (*Hypophthalmichthys militrix*), stone loach (*Barbatula barbatula*), swan mussel (*Anodonta cygnea*) and tench (*Tinca tinca*).

2.2.3. Susceptible stages of the host

All age groups of fish, from juveniles upwards, appear to be susceptible to KHVD (Bretzinger et al., 1999; Sano et al., 2004) but, under experimental conditions, 2.5–6 g fish were more susceptible than 230 g fish (Perelberg et al., 2003). Carp larvae are resistant to KHV infection but the same carp were susceptible to infection on maturation (Ito et al., 2007).

2.2.4. Species or subpopulation predilection (probability of detection)

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 present on the site, such as goldfish × common carp or crucian carp × common carp.

2.2.5. Target organs and infected tissue

Gill, kidney, and spleen are the organs in which KHV is most abundant during the course of overt infection (Gilad et al., 2004).

2.2.6. Persistent infection with lifelong carriers

There is evidence to indicate that survivors of KHVD are persistently infected with virus and may retain the virus for long periods. 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 (St-Hilaire et al., 2005). More recently, evidence for KHV persistence in carp has been presented in a study to determine the distribution of the virus in a wild common carp population. Researchers in Japan conducted a PCR and serological survey of KHV in Lake Biwa in 2006 (Uchii et al., 2009), where episodic outbreaks of KHVD 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 and boosting the immune response.

2.2.7. Vectors

Water is the major abiotic vector. However, animate vectors (e.g. other fish species, parasitic invertebrates and piscivorous birds and mammals) and fomites may also be involved in transmission.

2.2.8. Suspected aquatic animal carriers

There is evidence to indicate that other fish species and some aquatic invertebrates are potential vectors of KHV. The viral DNA has been detected in tissues of healthy goldfish after cohabitation with koi carp experimentally infected with KHV and also in goldfish exposed during natural KHV epizootics in koi (Ilouze et al., 2011). In studies in Germany, KHV has been detected by nested PCR in several different varieties of goldfish (red, lion-head & shubunkin) as well as grass carp (*Ctenopharyngodon idella*), ide (*Leuciscus idus*) and ornamental catfish (*Ancistrus* sp.) (Bergmann et al., 2009). The detection in the goldfish and grass carp was confirmed by *in situ* hybridisation using different primers to those used in the PCR. Also, in a recent study in Poland, KHV was detected by PCR in Russian sturgeon (*Acipenser gueldenstaedtii*) and Atlantic sturgeon (*A. oxyrinchus*) from fish farms in Northern Poland (Kempter et al., 2009). All of the sturgeon samples were taken from farms holding common carp with previous history of KHVD outbreaks. The presence, in sturgeon gill and kidney tissue, of KHV protein and viral genome was confirmed by an indirect fluorescent antibody test and *in situ* hybridisation, respectively.

There is also increasing evidence to indicate that aquatic invertebrates may be KHV vectors. Studies in Japan have reported the detection of KHV DNA in plankton samples and in particular Rotifera species (Minamoto et al., 2010). The plankton samples were collected in 2008 from Iba-naiko, a shallow lagoon connected to Lake Biwa, that is a favoured carp spawning area. Statistical analysis revealed a significant positive correlation between KHV in plankton and the numbers of Rotifera and the authors suggested that KHV binds to and/or is concentrated by the filter feeding behaviour of Rotifera species. In an earlier report of a small study in Poland, KHV was detected in swan mussels (*Anodonta cygnea*) and freshwater shrimp (*Gammarus pulex*) (Kielpinski et al., 2010). The invertebrates were collected from ponds in Southern Poland that had experienced KHVD outbreaks in their common carp populations over 5 or 6 years. More work is needed to determine how long the infectious virus persists in the invertebrates in the absence of the host species and also if the virus remains viable.

Recent studies have provided increasing evidence to indicate that goldfish (*Carassius auratus*) are susceptible to KHV infection. The RNA transcript of the viral thymidine kinase gene has been detected in gill, brain and intestinal tissue from goldfish that had been exposed to KHV by co-habitation with infected koi carp. Goldfish from the same population were then shown to transmit KHV to naïve common carp when water temperature fluctuation was used as a stressor (El-Matbouli & Soliman, 2011). Bergmann et al., 2010a also reported the replication of KHV in goldfish after experimental infection by immersion. KHV DNA and antigen was detected in leucocytes separated from goldfish blood samples by PCR (at 45 days post-infection) and by indirect fluorescent antibody test (at 60 days post-infection).

2.3. Disease pattern

2.3.1. Transmission mechanisms

The mode of transmission of KHV is horizontal but 'egg-associated' transmission (usually called 'vertical' transmission) cannot currently be ruled out. Horizontal transmission may be direct (fish to fish) or vectorial, water being the major abiotic vector. The reservoirs of KHVD are clinically infected fish and covert virus carriers among cultured, feral or wild fish. Virulent virus is shed via faeces, urine, gills and skin mucus. Under experimental conditions, infectious virus was continuously shed for a longer period from infected common carp at 16°C than those at 23°C or 28°C (Yuasa et al., 2008). The disease course can be rapid, particularly

at optimal temperatures (23–25°C), but less rapid at temperatures below 23°C. The disease may manifest itself in 3 days following the addition of naïve fish to a pond containing diseased fish but other investigators have reported 8–21 days for the disease to be observed in naïve fish (Bretzinger et al., 1999; Hedrick et al., 2000).

2.3.2. Prevalence

There are limited published observations of virus prevalence in wild or farmed populations of carp. There is evidence from experimental trials of virus persistence in common carp infected at a permissive temperature and subsequently maintained at a lower than permissive temperature (St-Hilaire et al., 2005; see Section 2.2.6). Analysis of blood serum samples from the study showed that a proportion of the carp (at least 10–25%) developed high antibody titres and the immunological response was detectable for several months (St-Hilaire et al., 2009). In other studies, viral DNA was detected in carp by PCR assay, in the absence of disease, at 13°C and it is possible that infected fish surviving at low temperatures may act as reservoirs of the virus (Gilad et al., 2004). In wild populations that have survived a KHV outbreak there is evidence for a high prevalence of seropositive carp. In the PCR and antibody survey of KHV in Lake Biwa in 2006, further analysis of the surviving carp population showed that 54% of the older carp were seropositive and 31% PCR positive (Uchii et al., 2009). As part of a KHV distribution survey of England and Wales, four sites experiencing clinical outbreaks of KHV in 2006 and having no introductions of fish since that time were revisited in 2007 and tested for the presence of KHV antibody by enzyme-linked immunosorbent assays (ELISA) (Taylor et al., 2010). Three of these sites produced positive results and showed 85–93% sero-prevalence in the samples of surviving carp population. The fourth site tested negative.

2.3.3. Geographical distribution

Following the first reports of KHVD 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 (Bretzinger et al., 1999; Perelberg et al., 2003), the geographical range of the disease has become extensive. The disease has been spread to many countries worldwide, predominantly through the trade in koi carp, before the current knowledge of the disease and means to detect it were available. It is now known to occur in, or has been recorded in fish imported into, at least 28 different countries. In Europe KHV has been detected in many countries across the continent (Bergmann et al., 2006; Haenen et al., 2004; Novotny et al., 2010). Most recently KHVD outbreaks have been reported to the OIE from Romania, Slovenia, Spain and Sweden. In Asia, China (Hong Kong), Chinese Taipei, Indonesia, Japan, Korea (Rep. of), Malaysia, Singapore (in fish imported from Malaysia) and Thailand (Haenen et al., 2004; Ilouze et al., 2011; Pikulkaew et al., 2009; Sano et al., 2004). Elsewhere, South Africa, Canada and the USA (Garver et al., 2010; Haenen et al., 2004; Hedrick et al., 2000) have reported occurrence of KHVD. It is likely that the virus is present in many more countries, but has not yet been identified or reported.

2.3.4. Mortality and morbidity

Morbidity of affected populations can be 100%, and mortality 70–80%, but the latter can be as high as 90 or 100% (Bergmann et al., 2010a; Haenen et al., 2004). Secondary and concomitant bacterial and/or parasitic infections are commonly seen in diseased carp and may affect the mortality rate and display of clinical signs of disease (Haenen et al., 2004).

2.3.5. Environmental factors

Disease patterns are influenced by water temperature, virulence of the virus, age and condition of the fish, population density and stress factors (e.g. transportation, spawning, poor water quality). The disease is temperature dependent, occurring between 16 and 25°C (Haenen et al., 2004; Hedrick et al., 2000; Perelberg et al., 2003; Sano et al., 2004). Under experimental conditions the disease has caused high mortality at 28°C but not at 29 or 30°C, nor at 13°C (Gilad et al., 2004; Ilouze et al., 2011). However, viral DNA was detected in the fish by PCR at 13°C, and it is possible that infected fish surviving at low temperatures may be reservoirs of the virus (Gilad et al., 2004).

2.4. Control and prevention

Methods to control and prevent KHVD should mainly rely on avoiding exposure to the virus coupled with good hygiene and biosecurity practices. This is feasible on small farms supplied by spring or borehole water and a secure system to prevent fish entering the farm via the discharge water.

2.4.1. Vaccination

A safe and effective vaccine is not currently widely available. However, live attenuated virus has been used to vaccinate carp and protect the fish from virus challenge. The vaccine preparation induced antibody against the virus and the duration of the protection was at least 8 months (Ilouze et al., 2011). The vaccine was licensed for emergency use in Israel and has been widely used in carp farms across the country. Results of studies in Japan have shown that oral administration of a liposome-based vaccine containing inactivated KHV was effective in protecting carp against KHV infection (Ilouze et al., 2011).

2.4.2. Chemotherapy

Not applicable.

2.4.3. Immunostimulation

There is currently no published information on the use of immunostimulants to control KHVD in carp. However, it is known to be an area of research interest.

2.4.4. Resistance breeding

Differential resistance to KHVD has been shown among different carp strains. 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 61–64% (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. Restocking with resistant species

Natural outbreaks of KHVD have not been reported in commonly farmed herbivorous carp species, including silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*), and bighead carp (*Aristichthys nobilis*). Herbivorous carp species are often raised in polyculture with common carp, but no signs of disease or mortalities have been observed in these species, either under normal polyculture conditions or following experimental cohabitation with infected fish, or direct exposure to the virus (Ilouze et al., 2011). Common carp hybrids also represent a potential control method to prevent serious losses from KHVD. Studies on a population of hybrid male goldfish × female common carp found them to be resistant to KHVD (Hedrick et al., 2006). These hybrids display rapid growth and have a morphological appearance most similar to their maternal parent. However, KHV DNA was detected by PCR in surviving hybrids suggesting that they are potential virus carriers (Hedrick et al., 2006). In contrast, a Polish study reported mortality of 35–42% in a goldfish × koi carp hybrids and 91–100% in a crucian carp × koi carp hybrids, challenged with KHV by bath immersion (Bergmann et al., 2010b; see Section 2.2.1). There may be a high level of genetic variation among hybrids from different crossings and consequently a variation in resistance to KHV. This will be heavily influenced by the strain of common or koi carp used. Different strains of common carp have been shown to vary in their level of resistance to KHVD (Dixon et al., 2009; Shapira et al., 2005).

2.4.6. Blocking agents

Not applicable.

2.4.7. Disinfection of eggs and larvae

Disinfection of eggs can be achieved by iodophor treatment. KHV has been shown to be inactivated by iodophor at 200 mg litre^{-1} for 30 seconds at 15°C (Kasai et al., 2005).

2.4.8. General husbandry practices

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 KHVD. The fish are then 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 be similar to those recommended for SVC and 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. Sampling

3.1. Selection of individual specimens

All age groups of carp appear to be susceptible to KHVD, although, generally, younger fish up to 1 year are more susceptible to clinical disease and are recommended for sampling. The suitability of selected fish specimens during a suspected KHVD outbreak will depend on the diagnostic test used. Moribund or freshly dead carp displaying typical clinical disease signs are suitable for testing by most of the tests described in Section 4Fish carcasses showing signs of tissue decomposition may only be suitable for testing by PCR-based methods. Likewise, samples taken from apparently healthy fish, in a suspected diseased population, may only be reliably tested by more sensitive PCR-based methods.

3.2. Preservation of samples for submission

Whole fish should be sent to the laboratory alive or killed and packed separately in sealed aseptic containers. However, it is highly preferable and recommended to collect organ samples from the fish immediately after they have been selected at the fish production site. Whole fish or selected organ samples should be sent to the laboratory in refrigerated containers or on ice. The freezing of collected fish or dissected organs should be avoided. However, if frozen fish or organs are received they may only be suitable for testing by PCR-based methods. Small samples of tissue may also be submitted preserved in alcohol (e.g. 80–100% ethanol) for testing by PCR-based methods.

3.3. Pooling of samples

When testing clinically affected fish by PCR-based methods, and particularly if virus isolation is to be attempted, pooling of samples should be avoided or restricted to a maximum of two fish per pool. For health surveillance testing, by PCR-based methods, pooling should be restricted to a maximum of five fish per pool.

3.4. Best organs or tissues

When testing clinically affected fish by PCR-based 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 and intestine (gut) tissue (Dishon et al., 2005; Gilad et al., 2004). When testing sub-clinical, apparently healthy, fish by PCR-based methods, it is recommended to also include intestine (gut) and encephalon.

3.5. Samples/tissues that are not suitable

Fish carcasses showing very advanced signs of tissue decomposition may not be suitable for testing by any methods.

4. Diagnostic methods

Diagnosis of KHVD in clinically affected fish can be achieved by a number of methods. Cell culture isolation of KHV is not currently considered to be as sensitive as the published PCR-based methods for detecting KHV DNA. The virus is isolated in only a limited number of cell lines and these cells can be difficult to handle. Consequently, virus isolation in cell culture is not a reliable diagnostic method for KHVD (Haenen et al., 2004). Immunodiagnostic methods, similar to those used for the diagnosis of spring viraemia of carp (SVC) (e.g. immunofluorescence [IF] tests or ELISAs), may be suitable for rapid identification and diagnosis of KHVD but have not been extensively reported, compared or validated. Until such time as validated tests are available, diagnosis of KHVD should not rely on just one test but a combination of two or three tests (Haenen et al., 2004).

4.1. Field diagnostic methods

4.1.1. Clinical signs

During a KHVD outbreak there will be a noticeable increase in mortality in the population. All age groups of fish appear to be susceptible to KHVD, although, under experimental infection, younger fish up to 1 year old are more susceptible to the disease. On closer examination of individual fish, typical clinical signs include pale discolouration or reddening of the skin, which may also have a rough (sandpaper-like) texture, focal or total loss of epidermis, over- or under-production of mucus on the skin and gills, and pale discolouration of the gills.

Other gross signs include enophthalmia (sunken eyes) and haemorrhages on the skin and base of the fins, and fin erosion.

4.1.2. Behavioural changes

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 they may also show signs of hyperactivity.

4.2. Clinical methods

4.2.1. Gross pathology

There are no pathognomic gross lesions. Final diagnosis must await direct detection of viral DNA or virus isolation and identification. However, the most consistent gross pathology is seen in the gills and this can vary in extent from pale necrotic patches to extensive discolouration, severe necrosis and inflammation. Another commonly observed gross pathology is pale, irregular patches on the skin associated with excess mucus secretion and also under production of mucus where patches of skin have a sandpaper-like texture. Other commonly reported clinical signs include anorexia, enophthalmia (sunken eyes) and superficial haemorrhaging at the base of the fins. Other 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. Presence of gross lesions may also be complicated because diseased fish, particularly common carp, are also infested with ectoparasites, such as *Argulus* sp., *Chilodonella* sp., *Cryptobia* sp., *Dactylogyrus* sp., *Gyrodactylus* sp., *Ichthyobodo* sp., *Ichthyophthirius* sp., *Trichodina* sp. and gill monogeneans, as well as numerous species of bacteria, especially *Flavobacterium columnare* at warmer water temperatures.

4.2.2. Clinical chemistry

No published information available.

4.2.3. Microscopic pathology

Further examination of the gills, by low-power 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 can be nonspecific and variable, 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 are commonly observed. Inflammation, necrosis and nuclear inclusions have been observed (individually or together) in other organs, particularly the kidney, but also in the spleen, pancreas, liver, brain, gut and oral epithelium.

4.2.4. Wet mounts

Not applicable.

4.2.5. Smears

KHV has been identified in touch imprints and smears 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).

4.2.6. Electron microscopy/cytopathology

Detection of viral particles by transmission electron microscopy (TEM) examination of tissues from clinically infected carp is not a reliable diagnostic method. Pieces of gill and kidney tissue fixed in glutaraldehyde should be sampled from heavily infected ($>10^6$ virus particles) carp. Best results are obtained from sampling a number of carp in an affected population at different stages of infection. This helps to ensure that some of the tissue samples are from heavily infected individuals.

4.3. Agent detection and identification methods

In this section, not all methods are presented in great detail because there has been no extensive comparison and validation of detection and identification methods for KHV. Where this is the case however, a short description of available published methods is provided. Method recommendations will rely on further testing and validation and further data being obtained, from laboratories that have developed the methods, in order to decide if they are 'fit-for-purpose'.

4.3.1. Direct detection methods

KHV has been identified 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). Virus antigen has also been detected in infected tissues by an immunoperoxidase staining method. The virus antigen was detected at 2 days post-infection in the kidney, and was also observed in the gills and liver (Pikarsky et al., 2004). However, 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.

Immunofluorescence (IF) and *in situ* hybridisation (ISH) methods, performed on separated fish leucocytes, have been used in research applications for detection or identification of KHV. Although these methods have not been thoroughly compared with other techniques, they are non-destructive (non-lethal) techniques and some laboratories may find them useful in a diagnostic setting. 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., 2009and Bergmann et al., 2010a.

ELISA-based methods for direct detection of KHV antigen in infected tissues are under development in a number of laboratories worldwide but no validated methods have been published. Currently, one published ELISA method is available and it was developed in Israel to detect KHV in fish droppings (faeces) (Dishon et al., 2005). 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.

The most commonly used method for detection of KHV directly in fish tissues is using PCR-based assays specific for KHV.

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

Not applicable.

4.3.1.1.2. Smears/Imprints

4.3.1.1.2.1. Indirect fluorescent antibody test on kidney imprints

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 at –20°C, for plastic wells.

v)   Let the fixative act for 15 minutes. A volume of 0.25 ml 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 rinsing steps with 0.01 M PBS solution, pH 7.2, containing 0.05% Tween 20 (PBST), and remove this buffer completely after the last rinsing.

viii)   Prepare a solution of purified antibody or serum 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 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 0.125 ml 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. These FITC antibodies are most often rabbit or goat antibodies.

xiv)   Rinse four times with PBST.

xv)   Add PBS at 0.25 ml 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 incident UV light using a microscope with ×10 eye pieces and ×20–40 objective lenses having numerical apertures of >0.65 and >1.3, respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

4.3.1.1.3. Fixed sections

The method detailed in Section 4.3.1.1.2above is also suitable for detection of KHV antigen in paraffin wax tissue sections fixed in 10% neutral buffered formalin (NBF). 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 in Section 4.3.1.1.2above.

NOTE: For direct detection of viral antigen by IFAT or immunohistochemistry, tissues should be fixed for 24–48 hours in 10% NBF and then the fixative should be replaced with 70% ethanol for prolonged storage.

4.3.1.2. Agent detection, isolation and identification

4.3.1.2.1. Cell culture

Diagnosis of KHVD 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 and these cells 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 KHVD (Haenen et al., 2004).

Cell line to be used: KF-1 or CCB

4.3.1.2.1.1. Virus extraction

Use the procedure described in Chapter 2.3.0., Section A.2.2.2.

4.3.1.2.1.2. Inoculation of cell monolayers

i)   Prior to inoculation of cells organ pool homogenates can be treated with antibiotics as detailed in Chapter 2.3.0., Sections A.2.2.1 and A.2.2.2.

ii)   If cytotoxic effects have been observed after inoculation of antibiotic-treated homogenate, filter at least 1 ml of the 1/10 organ homogenate supernatant through a 0.45 µm disposable cellulose acetate filter unit (or unit fitted with a similar low protein binding filter membrane).

iii) For direct inoculation, transfer an appropriate volume of the antibiotic-treated or filtered homogenate on to 24- to 48-hour-old cell monolayers in tissue culture flasks or multi-well plates. Inoculate at least 5 cm^2 of cell monolayer with 100 µl of the filtered supernatant. Alternatively, make a further tenfold dilution of the filtered supernatant in cell culture medium, buffered at pH 7.6 and supplemented with 2% fetal calf serum (FCS), and allow to adsorb for 0.5–1 hour at 18–22°C.

Then, without withdrawing the inoculate, add the appropriate volume of cell culture medium (0.2–0.3ml cm^{-2} for cell culture flasks), and incubate at 20°C to 25°C.

NOTE: When using multi-well plates, incubation under CO_2 atmosphere or addition of HEPES to the cell culture medium (HEPES = N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) will maintain the correct pH during incubation.

4.3.1.2.1.3. Monitoring incubation

i) Follow the course of infection in positive controls and other inoculated cell cultures by daily microscopic examination at ×40–100 magnification for 14 days. The use of a phase-contrast microscope is recommended.

ii) Maintain the pH of the cell culture medium at between 7.3 and 7.6 during incubation. This can be achieved by the addition to the inoculated cell culture medium of sterile bicarbonate buffer for tightly closed cell culture flasks or HEPES-buffered medium for multiwell plates.

iii) If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the tested homogenate supernatants, identification procedures must be undertaken immediately (see Section 4.3.1.2.2below).

iv) If no CPE develops in the inoculated cultures (despite normal progression of CPE in the virus controls), the inoculated cultures should be sub-cultured for a further 14 days. Should the virus control fail to develop CPE, the process should be repeated with fresh susceptible cells and new batches of samples.

4.3.1.2.1.4. Subcultivation procedures

i) Transfer aliquots of cell culture medium from all monolayers inoculated with organ homogenate supernatant onto fresh cell cultures.

ii) Inoculate cell monolayers as described above in Section 4.3.1.2.1; Inoculation of cell monolayers, step iii.

iii) Incubate and monitor as described above in Section 4.3.1.2.1

If no CPE occurs, the test may be declared negative.

4.3.1.2.1.5. Confirmatory identification

The most reliable method for confirmatory identification of a 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 (see section 4.3.1.2.3below).

4.3.1.2.1.6. Confirmation by PCR

i) Extract DNA from the virus culture supernatant using a suitable DNA extraction kit or reagent. An example of extraction of DNA using a salt-based extraction method (DNAzol® reagent) is described below in section 4.3.1.2.3.1.

ii) Extracted DNA is then amplified using the PCR protocols described below in section 4.3.1.2.3.1

Amplified PCR products may then be excised from the gel and sequenced as described in section 4.3.1.2.3

4.3.1.2.2. Antibody-based antigen 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, one published ELISA method is available and was developed in Israel to detect KHV in fish droppings (faeces) (Dishon et al., 2005).

Virus identification methods that rely on the production of KHV-infected cell cultures (e.g. IFAT, immunoperoxidase and serum neutralisation tests) are not recommended. This is because virus growth is slow and unpredictable in the susceptible cell cultures.

4.3.1.2.3. Molecular techniques

Of the published single-round PCR methods, the protocols detailed below are currently considered to be the most sensitive for detection of KHV DNA in fresh tissue samples from clinically diseased carp. The protocols may also allow detection of subclinical levels of virus. The first uses the TK primer set developed by Bercovier et al., 2005 at the Hebrew University-Hadassah Medical School in Israel. The second was developed by Yuasa et al., 2005 at the National Research Institute of Aquaculture (NRIA), Watarai, Mie, Japan and is an improvement of a published protocol. If the tissue shows evidence of decomposition then primer sets targeting shorter regions of the genome may need to be used.

Alternative PCR assays that are favoured by many diagnostic laboratories over conventional PCR, include quantitative PCR assays such as real-time PCR. The most commonly used quantitative assay for detection of KHV is the Gilad Taqman real-time PCR assay (Gilad et al., 2004). 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. Taqman PCR avoids much of the contamination risk inherent to nested PCR assays by minimising the handling of samples through automation during sample preparation and thermal cycling procedures.

The sample preparation protocol detailed below uses a salt-based extraction method (DNAzol® reagent) for extraction of KHV DNA. This is an easy-to-use, short-duration protocol that is also relatively inexpensive compared with some kits. Laboratories that are not familiar with DNAzol® or similar salt-based extraction reagents may find the method less reliable in their hands. However, a number of, salt-based and silica-matrix based, DNA extraction kits are available commercially (popular manufacturers include Roche, Qiagen and Invitrogen) that will produce high quality DNA suitable for use with the PCR protocols detailed.

4.3.1.2.3.1. Direct detection by PCR

4.3.1.2.3.1.1. Sample preparation and extraction of DNA using the DNAzol® reagent

Virus extraction from organ tissues should be carried out using the procedure described in Chapter 2.3.0, Section A.2.2.2.

i)   Add 100 µl of tissue homogenate (1/10 [w/v]) to a 1.5 ml microcentrifuge tube containing 1 ml DNAzol® reagent.

ii) Mix gently by inverting the tube five times and stand at room temperature for 5 minutes, then centrifuge at 10,600 *g* (rcf = relative centrifuge force) for 10 minutes using a microcentrifuge.

iii) Remove 1 ml of the supernatant to a new 1.5 ml microcentrifuge tube containing 0.5 ml of ethanol.

iv) Mix gently by inverting the tube five times and stand at room temperature for 5 minutes, then centrifuge at 18,000 *g* (rcf) for 30 minutes using a microcentrifuge.

v)   Remove the supernatant and rinse the pellet with 250 µl of 70% ethanol in molecular biology grade water.

vi) Spin samples for 5 minutes at 18,000 *g* (rcf).

vii) Remove the ethanol using a pipette and air-dry the pellet by leaving the tubes open on the bench for 5 minutes.

viii) Resuspend the pellet in 50 µl molecular biology grade water, prewarmed to 60°C, and incubate at 60°C for 5 minutes. Samples can be stored at –20°C until required.

4.3.1.2.3.1.2. PCR

4.3.1.2.3.1.2.1. General notes

PCR is prone to false-positive and false-negative results. False-positive results (negative samples giving a positive reaction), may arise from either product carryover from positive samples or, more commonly, from cross-contamination by PCR products from previous tests. Therefore, each assay and tissue extraction should include a negative control to rule out contamination. To minimise the risk of contamination, aerosol-preventing pipette tips should be used for all sample and PCR preparation steps. Additionally, all PCRs should be prepared in a clean area that is separate from the area where the amplifications and gel electrophoresis are performed. Do not share equipment (e.g. laboratory coats and consumables) between areas and, where possible, restrict access between areas. Contaminating PCR products can be carried on equipment, clothes and paper (e.g.

workbooks). Also, ensure all work-tops and air-flow hoods used for the extractions and PCR set up are regularly cleaned and decontaminated using UV light and bleach. Reagents and consumables should also be systematically decontaminated using UV-light irradiation. To ensure sample integrity, always store the samples (e.g. in a freezer or refrigerator) in a location away from the molecular biology laboratory or area.

4.3.1.2.3.1.2.2. Protocol 1 (with Bercovier TK primers)

i) For each sample, prepare a master mix containing:

10 µl Reaction buffer (×5 conc.)

5 µl MgCl$_2$ (25 mM stock)

0.5 µl dNTPs (25 mM mix)

0.5 µl Forward primer (10 pmol µl^{-1} stock)

0.5 µl Reverse primer (10 pmol µl^{-1} stock)

0.25 µl DNA polymerase 500 µ (5 µ µl^{-1})

30.75 µl Molecular biology grade water
Bercovier TK primers:

Forward = 5'-GGG-TTA-CCT-GTA-CGA-G-3'

Reverse = 5'-CAC-CCA-GTA-GAT-TAT-GC-3'

Product size = 409 bp
For each sample, dispense 47.5 µl into a 0.5 ml thin-walled microcentrifuge tube. Overlay with two drops of mineral oil.

ii) Add 2.5 µl of the extracted DNA. Store the remainder of the DNA at –20°C.

iii) Place tubes in a thermal cycler and perform the following programme:

1 cycle of 5 minutes at 94°C;

40 cycles of: 1 minute at 95°C

1 minute at 52°C (see note below)

1 minute at 72°C

A final extension step of 10 minutes at 72°C.
Note on cycling conditions: An annealing temperature of 55°C has been used effectively by many laboratories to amplify KHV with the Bercovier TK primers.

iv) Visualise the 409 bp PCR amplicon by electrophoresis of the product in a 2% ethidium bromide-stained agarose gel and observe using UV transillumination. An appropriate molecular weight ladder should be included on the gel to determine the size of the product.

v) Products of the correct size should be confirmed as KHV in origin by sequence analysis.

4.3.1.2.3.1.2.3. Protocol 2 (with Gray Sph primers/Yuasa modification)

i)   For each sample, prepare a master mix containing:

2 µl Reaction buffer (×10 conc.)

1.6 µl dNTPs (2.5 mM mix)

0.2 µl Forward primer (50 pmol µl^{-1} stock)

0.2 µl Reverse primer (50 pmol µl^{-1} stock)

0.1 µl DNA polymerase

14.9 µl Molecular biology grade water

   (NOTE: the final concentration of $MgCl_2$ in the master mix is 2 mM)

   Gray Sph primers:

Forward = 5'-GAC-ACC-ACA-TCT-GCA-AGG-AG-3'

Reverse = 5'-GAC-ACA-TGT-TAC-AAT-GGT-CGC-3'

Product size = 292 bp

   For each sample, dispense 19 µl into a 0.2 ml thin walled microcentrifuge tube. Overlay with two drops of mineral oil.

ii)   Add 1µl of extracted DNA.

iii)   Place tubes in a thermal cycler and perform the following programme:

1 cycle of 30 seconds at 94°C;

40 cycles of: 30 seconds at 94°C

30 seconds at 63°C

30 seconds at 72°C

A final extension step of 7 minutes at 72°C.

iv)   Add 3 µl of ×6 loading buffer into each PCR product and electrophorese 7 µl on a 2% ethidium bromide-stained agarose gel at 100 V for 20 minutes and visualise under UV light. An appropriate molecular weight ladder should be included on the gel to determine the size of the product.

v)   Products of the correct size should be confirmed as KHV in origin by sequence analysis.

4.3.1.2.3.1.2.4. Nucleotide sequence analysis of PCR products

PCR products are excised from the gel and purified using a commercial kit for gel purification (e.g. Geneclean®, Q-BIOgene,UK). 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 (e.g. pGEM T, Promega) and both DNA strands are sequenced using the M13 universal primer sets. 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 (e.g. Sequencher^{TM} 4.0 software, Gene Codes Corporation, Ann Arbour, MI, USA). 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.

4.3.2. Serological methods

The immune status of the fish is an important factor following exposure to KHV, with both nonspecific (interferon) and specific immunity (serum antibodies, cellular immunity) having important roles in herpesvirus infections. Clinical disease dominates at water temperatures of 18°C and above when the host immune response is at its optimum. 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; Ilouze et al., 2011; 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., 2011; St-Hilaire et al., 2005; Taylor et al., 2010).

Serum from koi carp containing antibodies to KHV has been shown to cross-react, at a low level, with CyHV-1, a further indication that these viruses are closely related. Evidence of cross-reacting antibodies was demonstrated in reciprocal 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.

Detection of antibodies may prove to be a valuable method of establishing previous exposure to KHV in apparently healthy fish, and until PCR-based methods have been developed that are able to reliably detect persistent virus in exposed fish, antibody assays may be the only surveillance tools available. However, due to insufficient knowledge of the serological responses of fish to virus infections, the detection of fish antibodies to viruses has not thus far been accepted as a routine screening method for assessing the viral status of fish populations. Validation of some serological techniques for certain fish virus infections could arise in the near future, rendering the use of fish serology more widely acceptable for health screening purposes.

5. Rating of tests against purpose of use

The methods currently available for targeted surveillance and diagnosis of KHV are listed in Table 5.1.The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	c	c	b	d
Direct LM	d	d	c	c	b	d
Histopathology	d	c	c	c	b	c
Isolation in cell culture	d	d	d	d	b	d
Transmission EM	d	d	d	d	b	c
Antibody-based virus detection assays	d	d	c	c	b	b
In situ DNA probes	d	d	c	c	b	b
PCR	d	b	b	b	a	a
Sequence	NA	NA	NA	NA	NA	a
Antibody detection assays (serology)	d	d	c	b	b	d
Bioassay	NA	NA	NA	NA	NA	NA

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; PCR = polymerase chain reaction; NA = Not applicable.

6. Test(s) recommended for targeted surveillance to declare freedom from koi herpesvirus disease

Targeted surveillance should rely on regular monitoring of sites holding susceptible species. Sites should be monitored when water temperatures have reached levels that are permissive for the development of the disease (>17°C) and no sooner than 3 weeks after such temperatures have been reached. Any diseased fish, or fish showing abnormal behaviour, that are found on the site should be sampled and tested using the most sensitive tests available (e.g. PCR). There are no validated methods that are currently recommended for testing healthy populations of susceptible fish for declaration of freedom from KHV. However, many laboratories use more sensitive molecular-based methods, such as real-time and nested PCR, to detect low levels of persistent virus DNA reliably. These assays may well prove suitable for surveillance programmes. There are no published reports of extensive validation of the more sensitive assays but the most commonly used assay is the Gilad Taqman real-time PCR assay (Gilad et al., 2004). This assay is widely acknowledged to be the most sensitive published PCR method available for detection of low-levels of KHV. Alternatively, detection of antibodies may prove to be a valuable method of establishing previous exposure to KHV in apparently healthy fish. Validation of enzyme immunoassays for detection of antibody to KHV could arise in the near future, rendering the use of these assays more widely acceptable for health screening purposes.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

KHV shall be suspected, in a susceptible fish species, if at least one of the following criteria is met:

i)   The presence of typical clinical signs of KHVD in a population of susceptible fish.

ii)   Presentation of typical histopathology in tissue sections consistent with KHVD.

iii)   A typical CPE observed in susceptible cell cultures without identification of the causative agent.

iv)   A single positive result from one of the diagnostic assays ranked as a or b in Table 5.1.

v)   Transfer of live fish from a site where presence of KHV has been confirmed, or is suspected, because of the presence of clinical disease, to sites without suspicion of KHV.

vi)   Other epidemiological links to KHV confirmed sites have been established.

vii)   Antibodies to KHV have been detected.

NOTE: When sites have been designated as suspect under criteria v and vi, testing for KHV should only be attempted if water temperatures have reached levels that are permissive for the development of the disease (>17°C). If water temperatures are below permissive levels then a live sample of the suspect fish may be held at elevated water temperatures (ideally 20–24°C) and tested 14–21 days later.

7.2. Definition of confirmed case

The following criteria should be met for confirmation of KHV:

i)   Mortality, clinical signs and pathological changes consistent with KHV disease (Section 4.2) and detection of KHV by one or more of the following methods:

   a)   Detection of KHV by PCR by the methods described in Section 4.3.1.2.3;

   OR

   b)   Detection of KHV in tissue preparations by means of specific antibodies against KHV (e.g. IFAT on tissue imprints as described in Section 4.3.1.1.2);

   OR

   c)   Isolation and identification of KHV in cell culture from at least one sample from any fish on the site as described in Section 4.3.1.2.1

ii)   In the absence of mortality or clinical disease by one or more of the following methods:

   a)   Detection and confirmation of KHV by PCR by the methods described in Section 4.3.1.2.3;

   b)   Positive results from two separate and different diagnostic assays ranked as a or b in Table 5.1.

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NB: There are OIE Reference Laboratories for Koi herpesvirus disease
(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/scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on Koi herpesvirus disease

NB: FIRST ADOPTED IN 2006; MOST RECENT UPDATES ADOPTED IN 2019.