

KOI HERPESVIRUS DISEASE

1. Scope

Koi herpesvirus disease (KHVD) is a herpesvirus infection (18) capable of inducing a contagious and acute viraemia in common carp (*Cyprinus carpio*) and varieties such as koi carp and ghost carp (16).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The aetiological agent is koi herpesvirus (KHV) in the family Herpesviridae (18, 45) although it has also been given the name carp interstitial nephritis and gill necrosis virus (CNGV) (20, 30). Waltzek *et al.* (42) provided 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) (42). Recently, Aoki *et al.* (2) described the complete genome sequence of KHV and identified 156 unique protein-coding genes. They suggest the finding that 15 KHV genes are homologous with genes in IcHV-1 confirms the proposed place of KHV in the family Herpesviridae. Early estimates of the genome size of KHV varied from at least 150 kbp (14) to 277 kbp (20) but the size is now confirmed as 295 kbp (2, 42). Estimates of virion size also vary. Nucleocapsids of negative-stained virus have been measured at 103–112 nm in diameter surrounded by an envelope (18, 20, 40). The nucleocapsids of thin-sectioned virus have been measured at 78–84, 80–110 and 110–120 nm in diameter (5, 6, 18, 28).

Comparisons of the genomes of KHV isolates from different geographical areas by restriction enzyme analysis (12, 16) or nucleotide sequence analysis (32) 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 (11, 12). Aoki *et al.* (2) compared 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, with the Israel and USA strains more closely related to each other than either is to the Japan strain. The three strains were interpreted as having arisen as two lineages (Japan & Israel-USA) from a wild-type parent.

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 (28). 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 (35). 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 (17). 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 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 (22).

2.1.4. Life cycle

In early reports investigators suggested that the gills are the major portal of virus entry in carp (9, 13, 24, 29). 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 (7). 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 (9, 29). 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 (24). 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 (13). 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 (9, 13).

2.2. Host factors

2.2.1. Susceptible host species

Naturally occurring KHV infections have only been recorded from common carp (*Cyprinus carpio carpio*), koi carp (*Cyprinus carpio koi*) and ghost carp (*Cyprinus carpio goi*), and hybrids of these varieties. Goldfish × common carp hybrids, produced by hybridising male goldfish with female carp, have been reported to show some susceptibility to KHV infection. Although mortality rate was low (5%), approximately 50% of these hybrids examined 25 days after intraperitoneal injection with a high dose of KHV possessed viral genomic DNA, as detected by polymerase chain reaction (PCR) (19).

2.2.2. Susceptible stages of the host

All age groups of fish, from juveniles upwards, appear to be susceptible to KHVD (5, 32, 39) but, under experimental conditions, 2.5–6 g fish were more susceptible than 230 g fish (28). A study in Japan has shown that carp larvae (3–4 days post-hatch) are resistant to KHV infection but the same carp suffered 100% mortality when exposed to KHV 2 months later (21).

2.2.3. Species or sub-population predilection (probability of detection)

Common carp or strains, 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.

2.2.4. Target organs and infected tissue

Gill, kidney, and spleen are the organs in which KHV is most abundant during the course of overt infection (13).

2.2.5. Persistent infection with lifelong carriers

It is not known whether, under natural conditions, survivors of KHVD are persistently infected with virus and, if so, whether they shed the virus or for how long the fish retain the virus. Some of these aspects have been investigated in experimentally infected fish where it was shown that virus could persist in common carp infected at a permissive temperature and subsequently maintained at a lower than permissive temperature (36).

2.2.6. 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.

2.2.7. Known or suspected wild aquatic animal carriers

Carp are commonly mixed together in polyculture systems with other fish species, but no signs of disease or mortalities have been observed in these other species during KHVD outbreaks, under normal polyculture conditions (5, 18, 28, 38). However, in contrast to the findings elsewhere, experimental data from Germany suggest the susceptibility of goldfish and grass carp to KHV (15). Recently, KHV 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 (10, 31). More work is needed to determine how long the virus persists in the goldfish and also if carrier fish shed viable virus. However, there is increasing evidence to suggest that goldfish are a potential covert carrier of KHV. Furthermore, if the findings from Germany (15) are confirmed then all cyprinid species would need to be considered as potential carriers of KHV.

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 (44). 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 (41), but other investigators have reported 8–21 days for the disease to be observed in naïve fish (5, 18).

2.3.2. Prevalence

There are no published observations of virus prevalence in either 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 (36, see Section 2.2.5). However, in other trials in the same study (36) the investigators considered none of the fish to be persistently infected, and suggested that only a small percentage of fish would be persistently infected following KHV infection. 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 (13).

2.3.3. Geographical distribution

Following the first reports of KHVD in Israel and Germany (5, 28), the geographical range of the disease has become extensive. The disease has been spread to many countries world wide, 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 22 different countries. In Europe this includes Austria, Belgium, Denmark, France, Italy, Luxembourg, The Netherlands, Poland, Switzerland and the United Kingdom (4, 8, 16, 33). In Asia, China (Hong Kong) (16), Chinese Taipei (40), Indonesia (37), Japan (32), Korea (Rep. of) (6), Malaysia (16, 23, 25), Singapore (in fish imported from Malaysia) and Thailand (in fish imported into Germany, 16). Elsewhere, South Africa (16) and the USA (14, 18, 39) 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% (5, 41), but the latter can be as high as 90 or 100% (5, 40). Secondary and concomitant bacterial and/or parasitic infections are commonly seen in diseased carp and may affect the mortality rate and display of signs (16).

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 (8, 18, 28, 32, 39, 40). Under experimental conditions the disease has caused high mortality at 28°C (13) but not at 29 or 30°C (20, 27), nor at 13°C (13). 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 (13).

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, attenuated virus has been used to vaccinate carp and protect the fish from virus challenge (27, 30). The vaccine preparation induced antibody against the virus (26), but the duration of the protection is unknown. The vaccine is currently licensed for 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 (43).

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 common carp strains (34) and other studies have suggested an age-related resistance (28). In resistance breeding studies 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% (34).

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 (27, 33, 37, 38). 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 (19). 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 (19).

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 (22).

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 4. Fish 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 (13). 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 (16). Immunodiagnostic methods, similar to those used for the diagnosis of spring viraemia of carp (SVC) (e.g. immunofluorescence [IF] tests or enzyme-linked immunosorbent assays [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 (16).

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 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. Further examination can reveal erosion of primary lamellae, fusion of secondary lamellae, and swelling at the tips of the primary and secondary lamella. 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 pathologies 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

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 (29, 34).

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, 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 (29, 34). 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 (29). 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 (29). A method for direct detection of KHV from kidney imprints by indirect fluorescent antibody test (IFAT) is detailed below.

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) (9).

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 0.5 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 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 0.25 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. These FITC antibodies are most often rabbit or goat antibodies.
- xiv) Rinse four times with PBST.
- xv) Add PBS at 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 microscope with $\times 10$ eye pieces and $\times 20$ – 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 4.3.1.1.2 above 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 4.3.1.1.2 above.

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 (16).

Cell line to be used: KF-1 or CCB

Virus extraction

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

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 cm² 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 (1–1.5ml/5 cm² for cell culture flasks), and incubate at 20°C to 25°C. NOTE: When using multi-well plates, incubation under CO₂ atmosphere will maintain the correct pH during incubation.

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 or HEPES-buffered medium (HEPES = N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) for tightly closed cell culture flasks.
- 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 4.3.1.2.2 below).
- 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.

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.

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 4.3.1.2.3 below).

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 4.3.1.3.1.1.

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) (9).

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.* at the Hebrew University-Hadassah Medical School in Israel (3). The second was developed by Yuasa *et al.* at the National Research Institute of Aquaculture (NRIA), Watarai, Mie, Japan (45) and is an improvement of a published protocol developed by Gray *et al.* (14). If the tissue shows evidence of decomposition then primer sets targeting shorter regions of the genome may need to be used, such as those developed by Hutoran *et al.* (20). Alternatively, existing published primer sets can be modified to target shorter sequences of the KHV genome.

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

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) 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.

PCR

General notes

PCR is prone to false-positive and false-negative results. 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. This will further minimise the risk of false-positive results due to contamination.

Protocol 1 (with Bercovier TK primers)

- i) For each sample, prepare a master mix containing:
- | | |
|----------|--|
| 10 µl | Reaction buffer (×10 conc.) |
| 5 µl | MgCl ₂ (25 mM stock) |
| 0.5 µl | dNTPs (25 mM mix) |
| 0.5 µl | Forward primer (100 pmol µl ⁻¹ stock) |
| 0.5 µl | Reverse primer (100 pmol µl ⁻¹ stock) |
| 0.25 µl | DNA polymerase 500 µ (5 µ/µl) |
| 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 55°C |
| 1 minute at 72°C |
- A final extension step of 10 minutes at 72°C.
- iv) Electrophorese 20 µl volumes of PCR product on a 2% ethidium bromide-stained agarose gel (4% when separating smaller amplification products of <300 bp) at 120 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.

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 MgCl₂ 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 1 µl 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.

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™ 4.0 software, Gene Codes Corporation, Ann Arbor, 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 (1, 26, 30, 36). Antibody has been detected in the serum at 3 weeks after experimental infection and in survivors after 1 year following a natural infection (1, 30, 36).

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 (1). 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	c	d
Direct LM	d	d	c	c	c	d
Histopathology	d	c	c	c	c	c
Isolation in cell culture	d	d	d	d	c	d

Table 5.1. (cont.) Methods for targeted surveillance and diagnosis

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Transmission EM	d	d	d	d	c	c
Antibody-based virus detection assays	d	d	c	c	c	b
DNA Probes – <i>in situ</i>	d	d	c	c	b	c
PCR	d	b	b	b	a	a
Sequence	NA	NA	NA	NA	NA	a
Antibody detection assays (Serology)	d	d	c	b	d	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 currently recommended methods for testing healthy populations of susceptible fish for declaration of freedom from KHV. However, many laboratories are investigating further development of molecular-based methods to increase sensitivity (e.g. real-time and nested PCR) and detect low levels of persistent virus DNA reliably. These assays may well prove suitable for surveillance programmes. 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 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 such as IFAT on tissue imprints or PCR described in Section 4.3.1 above.
- 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.
 - 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);
 - 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 signs 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 described above.

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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: www.oie.int)