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Koi Herpesvirus: Status of Outbreaks, Diagnosis, Surveillance, and Research

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Abstract

Koi herpesvirus (KHV; Cyprinid herpesvirus 3) is a major threat to common carp and koi carp production worldwide. It is listed by the World Organization for Animal Health (OIE), and has been reported in 26 countries. KHV was designated a Specific Disease by Japanese law in 2003. Japan's National Research Institute of Aquaculture (NRIA) is a reference laboratory for the disease and conducts confirmatory diagnosis. The number of disease occurrences in Japan peaked in 2004, but has been gradually decreasing since then. The disease occurs mostly during spring and autumn when water temperatures are 16-28°C. In general, conventional polymerase chain reaction (PCR) is the most useful method for diagnosing KHV due to its high sensitivity, high specificity, and rapidity. More recently, real time PCR, nested PCR, and loop-mediated isothermal amplification (LAMP) were developed to diagnose the virus. Reverse transcriptase polymerase chain reaction (RT-PCR) targets mRNA of KHV to detect the replicating virus. An enzyme-linked immunosorbent assay (ELISA) detects anti-KHV antibodies in carp serum of surviving infected fish and is expected to be a tool for surveillance of KHV. However, cross reactions with anti-Cyprinid herpesvirus 1 antibodies in the ELISA need to be resolved. A systematic research project on development of diagnostic and prevention technologies for KHV disease was conducted in 2004-2006 by the NRIA in cooperation with Japanese universities and enterprises and SEAFDEC. The fruitful results of the project are included in this review.

Introduction

Koi herpesvirus (KHV; Cyprinid herpesvirus 3) disease is a major threat to common and koi carp production worldwide. It has been reported in 26 countries (Haenen and Olesen, 2007). KHV was first isolated from adult koi and common carp (*Cyprinus carpio*) associated with mass mortality in outbreaks in the United States and Israel in 1998 as a new herpesvirus (Hedrick et al., 2000). The virus was isolated from the gill, kidney, and spleen of affected fish using the koi fin (KF-1) cell line with cytopathic effects (CPE) characterized by severe vacuolation. Its pathogenicity to koi carp and common carp was demonstrated by bath and intraperitoneal injection.

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The common carp brain (CCB) cell line is also useful in isolating the virus (Neukirch and Kunz, 2001).

One problem in the research and diagnosis of KHV was the difficulty in detecting the virus due to low susceptibility of the KF-1 and CCB cell lines to the virus. Thus, KHV-specific polymerase chain assays (PCR) targeting the KHV *Sph* I-5 region (Sph PCR; Gray et al., 2002) and KHV 9/5 region (9/5 PCR; Gilad et al., 2002) were developed and the sensitivity and specificity of these assays were compared with other diagnostic methods. The thermal program of Sph PCR was later improved to obtain higher sensitivity and specificity (improved Sph PCR; Yuasa et al., 2005a), and a new PCR assay targeting thymidine kinase was developed (TK PCR; Bercovier et al., 2005). These methods have sufficient sensitivity to detect the virus in diseased fish and are described in the OIE manual as diagnostic methods for detection of KHV. However, these PCR assays are unable to detect survivors or virus carriers. Nested PCR assays (El-Matbouli et al., 2007), real-time quantitative PCR (Gilad et al., 2004), and loop-mediated isothermal amplification (LAMP; Gunimaladevi et al., 2004; Yoshino et al., 2006) assays were eventually developed with higher sensitivities than conventional PCRs. Even though these new methods are slightly more sensitive than conventional PCRs, their ability to detect virus carriers is unreliable. An enzyme-linked immunosorbent assay (ELISA) was developed in 2005 to detect KHV antibodies in serum of surviving fish exposed to the disease (Adkison et al., 2005).

The causative virus was designated koi herpesvirus (KHV) based on a morphological characteristic: its icosahedral capsid has a diameter of approximately 108 nm, similar to herpesviruses (Hedrick et al., 2000). However, the molecular size of the genome of the isolated virus was 270-290 kbp, larger than in viruses of the Herpesviridae family (Hutoran et al., 2005). Based on this, Hutoran et al. (2005) suggested that the virus may represent an as yet unclassified species. On the other hand, approximately 80% nucleotide homology was established between Cyprinid herpesvirus 1 (CyHV-1), Cyprinid herpesvirus 2 (CyHV-2), and KHV (Way et al., 2004). Waltzek et al. (2005) identified four genes cording for a helicase, an intercapsomeric triplex protein, DNA polymerase, and major capsid protein in KVH, as well as in CyHV-1, CyHV-2, and Ictalurid herpesvirus 1 (IcHV-1). Sequence analyses showed that KHV is closely related to CyHV-1 and CyHV-2, and distantly related to IcHV-1 (Waltzek et al., 2005). The full genome sequences of KHV were published by Aoki et al. (2007). Confirmation of 15 KHV genes resulted in clear homologues in the distantly related IcHV-1, classifying KHV in the Herpesviridae family, although genome size is not currently considered a criterion for classification of virus families. Electron microscopy of the viral morphogenesis also supported the inclusion of KHV in Herpesviridae (Miwa et al., 2007).

Natural infections of KHV causing mortality have been recorded only in common carp, koi carp, ghost carp, and hybrids of these strains, all *C. carpio* species. Host species of KHV are generally thought to be restricted to *C. carpio* spp., although experimental data suggest that goldfish and grass carp are also susceptible to KHV (Bergman et al., 2005). Experimentally, goldfish x common carp hybrids showed moderate sensitivity to KHV (Hedrick et al., 2006). In naturally occurring KHV disease, mortality tends to be observed in large *C. carpio* (Sano et al., 2004). Under experimental conditions, however, young fish of 2.5 g and 6 g are more sensitive to the virus than adults of 230 g (Perelberg et al., 2003).

The disease is temperature dependent, occurring in the wild at 16-25°C (Hedrick et al., 2000; Perelberg et al., 2003; Sano et al., 2004). Under experimental conditions, mortality due to the disease is observed at 16-28°C, but not at 13°C, 29°C, or 30°C. The virus can be maintained in experimentally infected carp kept at 12°C for 200 days, and the fish start to transmit the virus when water temperature was changed to 22°C (St-Hilaire et al., 2005).

KHV disease is listed by the World Organization for Animal Health (OIE) and was designated a 'specific disease' by Japanese law in 2003. The National Research Institute of Aquaculture (NRIA), Fisheries Research Agency, is one of the reference laboratories for the disease. NRIA

conducts confirmatory diagnosis of the disease and conducted a systematic research project 'Development of Diagnostic and Prevention Technologies for KHV Disease' in 2004-2006 in cooperation with Japanese universities and enterprises, and SEAFDEC.

This report describes KHV outbreaks in Asia, especially in Japan and Indonesia, and the fruitful results of Japanese research on KHV disease.

Outbreaks of KHV in Asia

Eight countries are KHV-positive or suspected as positive (Table 1; Haenen and Olesen, 2007). Mass mortality due to KHV was first reported in Indonesia in 2002, but the source of KHV was suspected to be koi imported from Hong Kong (Sunarto and Rukyani, 2005). Before that, mass mortality of cultured common carp associated with a herpes-like virus infection occurred in Korea in 1998, but the causative virus was not identified (Choi et al., 2004). KHV disease occurred in Taiwan in 2002 (Tu et al., 2004), Japan in 2003 (Sano et al., 2004), and Thailand in 2004 (Tandavanitj et al., 2005). In Asia, except for Japan, the disease has been limited to carp farms or private ponds. On the other hand, outbreaks of the disease in Japan frequently occurred in the wild population, which may be due to the large number of wild carp.

Outbreaks and spread of KHV disease in Japan. Mass mortality of cultured common carp in floating net cages at Kasumigaura Lake was diagnosed as KHV by the NRIA in October 2003 (Sano et al., 2004). Since carp production in Kasumigaura Lake represented over half of Japan's total production, the impact of the disease was huge. The amount of fish killed reached about 1,200 tons, equivalent to 20% of the annual production. After the disease was diagnosed as KHV, the movement of carp was prohibited by Japanese law and all surviving fish were destroyed (Takashima et al., 2005). Unfortunately, however, infected fish had already been transferred to carp farms and game fishing facilities elsewhere in Japan.

Putative transmission routes of the KHV from Kasumigaura Lake are shown in Fig. 1. Epidemiologically, the virus was transmitted from infected carp or KHV-contaminated inlet water to naive carp. For example, KHV-infected fish were brought to a carp farm, the KHV disease occurred in the farm, the farm discharged KHV-contaminated outlet water, and down-stream carp farms were infected by the contaminated water. In another example, a hobbyist may have purchased infected fish from a farm or retailer, the fish transmitted the disease to the private aquarium, the hobbyist released infected fish into the environment. By such transmission routes, KHV was quickly distributed to some areas of Japan.

Table 1. Occurrence of KHV or KHV-like diseases in Asia.

Year	Country	Area of outbreak	Mass mortality	Detection method
1998	Korea	Cage culture in lake	Occurred	Ultrastructural morphology
2001	China (Hong Kong)	?	?	?
2001	Malaysia	?	?	Genome detection by PCR
2002	Indonesia	Cage culture in lake Carp farm	Occurred	Genome detection by PCR
2002	Taiwan	Private pond	Occurred	Genome detection by PCR
2003	Japan	Cage culture in lake Field (river, lake) Carp farm	Occurred	Genome detection by PCR Viral isolation
2004	Thailand	Private pond	Occurred	Genome detection by PCR
2005	Singapore	?	?	Genome detection by PCR

Kasumigaura lake

(carp ⟷ carp)

rivers/channels/
reservoirs/lakes

(carp ⟷ carp)

carp farms

(carp ⟷ carp)

(retailer)

game fishing ponds
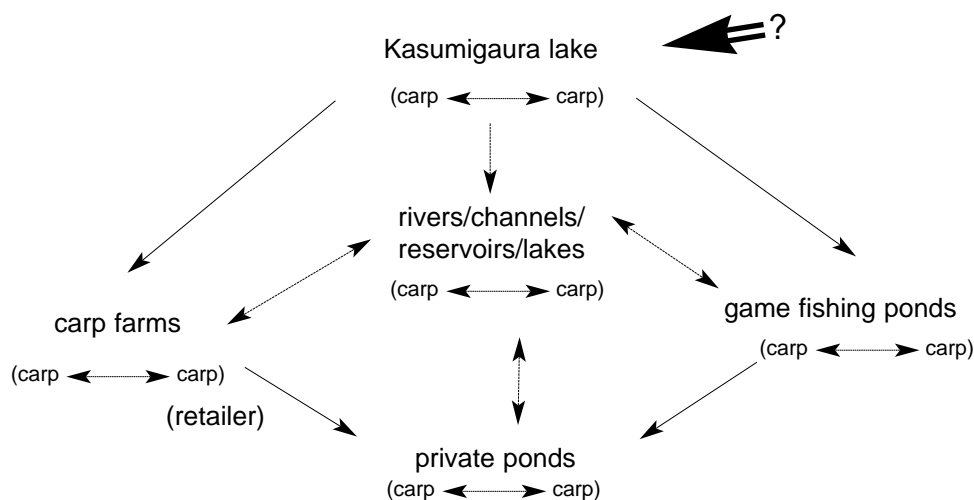
(carp ⟷ carp)

private ponds

(carp ⟷ carp)

Fig. 1. Putative transmission routes of KHV disease among common or koi carp in late 2003. Straight lines indicate virus transmission by transfer of KHV-infected fish; broken lines indicate virus transmission through the water.

The monthly number of cases diagnosed as KHV disease by NRIA in 2004-2006 is given in Fig. 2. There were fewer cases in 2006 than in 2004 and 2005, showing that the virus decreased. For each year, the number of cases increased from May to November, when water temperature was above 16°C. During the winter (December to March), the disease was rarely observed. KHV-infected carp survived for weeks in water of 12°C but died and became the source of infection in cohabiting naive carp after the water temperature increased to 22°C (St-Hilaire et al., 2005). Outbreaks of KHV disease during spring can be explained by survival of this virus in fish at low temperature.

Outbreaks of KHV in Indonesia. In March 2002, mass mortality attributed to KHV disease was observed in a koi farm in Bliter 1, East Java. Prior to the mortality, the farm had introduced
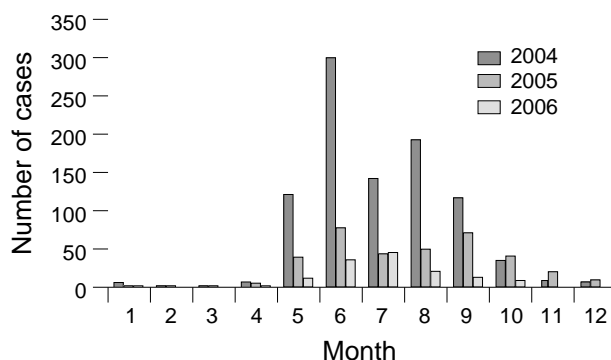
Fig. 2. Cases of KHV disease diagnosed by month in 2004 - 2006 (data from the NRIA).

koi from Hong Kong. The disease spread rapidly throughout Java Island and finally reached Cirata Lake, where production of common carp is the highest in Indonesia, causing huge losses (Sunarto and Rukyani, 2005). In 2003, KHV was transmitted through infected common carp seed or broodstock to Lubuk Lingau City in South Sumatra Province, a famous common carp production area, and spread to South Sumatra, Bengkulu, and Jambi Provinces. In 2004, it spread to Riau, West-Sumatra, and North-Sumatra Provinces (Yuasa, 2007).

The disease was observed in floating net cage culture in lakes (Fig. 3a) and farms using running water from the channel (Fig. 3b), but not in rice field nursery culture (Fig. 3c). We investigated the water temperature at these three sites. The temperature reached above 29°C only in the rice field (Yuasa, 2004). It is likely that mortality in the rice field was inhibited by the high water temperature.

Preventive Measures against Spread of KHV in Japan

Preventive measures against the spread of KHV were conducted according to the Japanese 'Law to Ensure Sustainable Aquaculture Production' whereby managers and employees engaged in aquaculture must notify the prefectural government if they suspect or identify fish infected by KHV (Fig. 4). The prefectural governmental or experimental station that receives the notification can examine the fish and diagnose KHV disease by improved Sph PCR (Yuasa et al., 2005a) or 9/5 LAMP (Yoshino et al., 2006). If positive for KHV, the prefectural government
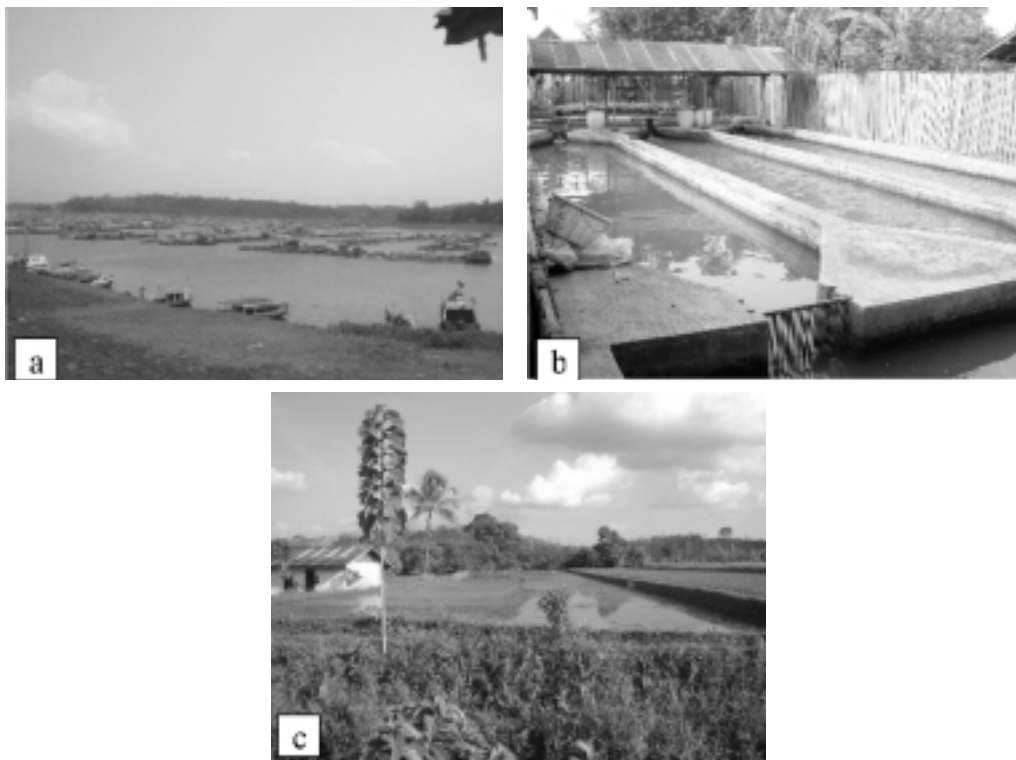

Fig. 3. Three types of common carp culture sites carp in Indonesia: (a) floating net cage culture in lakes, (b) pond culture using running water from a channel, and (c) rice field culture for nursing.
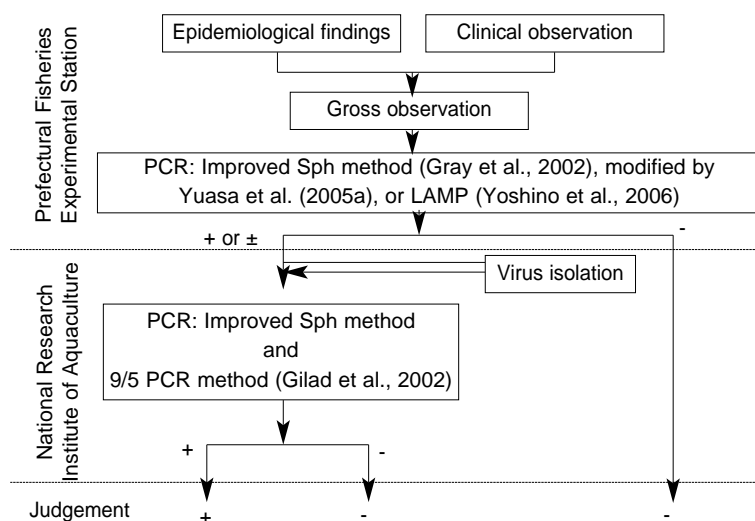
Fig. 4. Inspection for KHV infection in Japan.

can request NRIA to confirm the diagnosis. Under instruction from the Ministry of Agriculture, Forestry and Fisheries (MAFF), NRIA promptly acts on the request using improved Sph PCR and 9/5 PCR (Gilad et al., 2002). If the results of the NRIA tests are also positive, the prefectural government can direct the manager or owner of the fish farm to stop transferring fish from the contaminated facility and to burn or bury them. When outbreaks occur in the wild, the prefectural government can ban fishermen from transferring carp from the affected site to other environments.

Following strict regulation, the number of KHV disease outbreaks decreased from 2004 to 2006 (Table 2). The incidence of KHV disease in rivers and carp farms in 2007 was 4.9% and 11.8%, respectively (Table 3), indicating that the measures used to prevent the spread of the disease were effective.

Research on KHV Disease in Japan

A research project on KHV disease was conducted from 2004 to 2006 under a grant of the Ministry of Agriculture, Forestry and Fisheries (Table 4). Following are the main results.

Kinetics of KHV. KHV was detected by PCR in affected organs including the gills 3-40 days post-infection (dpi) in experimentally-infected fish. The titer of the virus in the gills, fin, scales, kidney, and intestine was higher than in other organs, and peaked at 7-9 dpi (Yuasa et al., 2005b). Common carp exposed to KHV and kept at 16°C died from the infection 21-52 dpi, while those kept at 23°C and 28°C died 5-20 dpi and 7-14 dpi, respectively, indicating that water temperature influences time of mortality (Yuasa et al., 2008). Infected fish kept at 16°C transferred the virus to naive fish for a much longer period than fish kept at 23°C or 28°C, suggesting that infected fish even without obvious disease signs that are reared at low temperatures may be important infectious factors. The KHV genome existed in the brain throughout the experimental period of one year although KHV mRNA was not detected in the brain homogenate of survivors, suggesting that latent KHV infection can be established in the brain (Yuasa et al., 2007a).

Susceptible fish species, strains, and sizes. Seven freshwater fish species habiting in Japan,

Table 2. Frequency and distribution of KHV disease outbreaks in wild and cultured *Cyprinus carpio* in Japan in 2003-2006.

Year	Natural systems (rivers, lakes, reservoirs)	Culture systems (farms, parks, private ponds)	Total
2003	29	65	94
2004	349	561	910
2005	78	230	308
2006	43	139	182

Data from the Ministry of Agriculture, Forestry and Fisheries, Sept. 2007

Table 3. Distribution of KHV in rivers and carp farms in Japan in 2007.

	First and second class rivers	Carp farms
No. contaminated	140	171
Total no.	2831	1462
% contaminated	4.9	11.8

Data from the Ministry of Agriculture, Forestry and Fisheries, Sept. 2007

ayu (*Plecoglossus altivelis*), goldfish (*Carassius auratus*), gibel (*Carassius auratus langsdorfii*), common dace (*Tribolodon hakonensis*), common minnow (*Zacco platypus*), dark chub (*Zacco temminckii*), and common carp, were exposed to KHV by immersing the fish in water containing the virus. Except for common carp, no mortality was observed among the exposed fish species (Ito et al., unpubl.). The susceptibility of tilapia (*Oreochromis niloticus*) and catfish (*Pangasius hypophthalmus*) to the virus was examined by cohabiting these two species and naive common carp with infected common carp. In this test, the naive common carp were infected by KHV, manifesting mortalities within 8 days post-cohabitation, however no KHV genome was detected in the exposed tilapia or catfish and none of the experimental fish in these two species died (Yuasa, 2004). The ghost carp, a strain of *C. carpio* indigenous to Europe, shows higher sensitivity to the virus than the Eurasian strain of common carp or koi carp (Ito et al., 2007a). Common carp larvae and juveniles of different sizes were exposed to KHV (Ito et al., 2007b). Juveniles (avg TL: 13.8 and 29.2 mm) had high mortalities due to the virus infection, but larvae (avg TL: 7.5 and 8.7 mm) showed no susceptibility to the virus. The KHV genome was not detected by PCR in any larvae exposed to the virus. The reason for the low susceptibility in larvae is unknown.

Gene analysis of KHV. The nucleotide sequences of three genomic regions (*Sph* I-5, 9/5, and TK) in KHV samples from four Asian countries, three European countries, and the USA were compared (Kurita et al., 2009). Results showed that the sequences of the Asian KHV isolates were almost homogeneous. The European KHV had minor varieties in their sequences, but all were clearly distinct from the Asian KHV. The sequence from the USA was consistently similar to several from the Netherlands. Thus, KHV from European countries and the USA (European type) are genetically unrelated to KHV from Asian countries (Asian type).

Verification of PCR detection methods. The thermal program of Gray's PCR method using the *Sph* I-5 primer set (Sph PCR) was modified to reduce negative factors such as the appearance of non-specific reactions and long reaction time. Further, this improved Sph PCR was more sensitive than the original Sph PCR in detecting the virus (Yuasa et al., 2005a). The sensitivity and specificity of PCR methods for detecting mRNA of KHV were tested in four conventional PCR methods (the original Sph PCR, Gray et al., 2002; the improved Sph PCR, Yuasa et al., 2005a; 9/5 PCR, Gilad et al., 2002; and TK PCR, Bercovier et al., 2005) as well as in nested PCR (Kanchanakhan et al., 2005), real-time TaqMan PCR (Gilad et al., 2004), and the newly

Table 4. Studies included in the Development of Diagnostic and Preventive Measures against Koi Herpesvirus Disease Project, 2004-2006 (funded by the Ministry of Agriculture, Forestry and Fisheries).

1. Pathological and epidemiological studies
   (1) Histopathological studies (Nippon Veterinary and Animal Science Univ.)
   (2) Kinetics of KHV in fish body (NRIA, FRA)
   (3) Susceptibility of various fish species indigenous to Japan (NRIA, FRA)
   (4) Gene analysis of KHV in Japan (NRIA, FRA)
   (5) Comparison of characterization of KHV among Asian isolates (SEAFDEC)

2. Development of new diagnostic/detection methods
   (1) Improvement of PCR (NRIA, FRA)
   (2) Development of rapid diagnostic method (Eiken Chemical)
   (3) Development of other detection method (NRIA, FRA)

3. Development of preventive measures
   (1) Establishment of disinfecting method (Hokkaido Univ.)
   (2) Development of vaccine (Kyoritsu Seiyaku)
   (3) Search for curative measure (Tokyo Univ. of Marine and Technology)

developed RT-PCR (Yuasa et al., 2007b). Results indicated that all the conventional PCRs, except the original Sph PCR, were sufficiently sensitive and specific to diagnose the disease. Sensitivity of the nested PCR and TaqMan PCR were slightly higher than that of the conventional PCRs, although each had a negative factor: a high risk of cross-contamination in the case of the nested PCR and a higher cost in the case of the TaqMan PCR. Compared with the other PCRs, RT-PCR positively correlated with infectivity in tissues of naive carp as detected by bioassay (Yuasa et al., 2007b).

Development and verification of LAMP detection methods. Two LAMP assays were developed in the project. The LAMP method using a primer set designed in the *Sph* I-5 region (Sph LAMP) was more sensitive to the LAMP method using a primer set designed in 9/5 region (Yoshino et al., 2006). Sph LAMP was more sensitive than the improved Sph PCR or TK PCR (Yoshino et al., 2006).

Verification of ELISA detection methods. ELISA can be used to detect the presence of anti-KHV antibodies in the serum of carp (Adkison et al., 2005). This method was verified using serum of naturally and experimentally KHV-infected carp. Healthy common carp were kept in a net-cage set in a lake where KHV disease had occurred. Fish were periodically sampled and tested using the ELISA. Results showed that the level of antibodies was higher in fish that encountered the KHV outbreak than in healthy fish. However, ELISA using purified virus of KHV as antigens had cross reactions with CyHV-1 (Adkison et al., 2005). Thus, further improvement of ELISA is necessary to detect KHV carriers.

Development of preventive measures. The virucidal effects of ultraviolet (UV) irradiation, heat treatment, and disinfectants against KHV were evaluated. Results indicated that UV irradiation, heating above 50°C, and 1 min treatment with disinfectants such as 200 mg/l iodophore, 60 mg/l benzalkonium chloride, 30% ethyl alcohol, and 200 mg/l sodium hypochlorite for 30 s effectively inactivated the virus (Kasai et al., 2005). Viral viability in environmental water was significant-

ly reduced within three days after inoculation, suggesting that KHV can be rapidly inactivated in environmental water (Shimizu et al., 2006).

An inactivated vaccine against KHV disease was developed within the project. Other groups, likewise, suggested the efficacy of oral vaccination with a KHV liposome vaccine against KHV infection in carp (Yasumoto et al., 2006). However, the cost of producing both vaccines were too expensive. It is essential to establish cell lines that are more sensitive to the virus than CCB cells or KF-1 cells for inexpensive mass production of a vaccine. Vaccination with attenuated non-pathogenic KHV was effective in preventing the disease in Israel (Ronen et al., 2003). However, the use of attenuated vaccine is not permitted in Japan.

In Israel, the transfer of KHV-infected fish to a non-permissive temperature of 30°C effectively treated the fish as the fish became resistant to subsequent exposure (Ronen et al., 2003). In our project, it was confirmed that two time-water temperature shifts at 3 d intervals to 30°C for 5 d was effective in reducing morality in KHV-infected fish. However, results also suggested that the treated fish became carriers of the virus (Fukuda et al., 2007). Survival of KHV in CCB cells maintained for 30 days at 30°C was also demonstrated by Dishon et al. (2007), indicating the contamination risk of fish treated in 30°C water.

Summary

KHV has been renamed Cyprinid herpesvirus 3 (CyHV-3), classified in the family Herpesviridae. Only *Cyprinus carpio* species including common carp, koi carp, and ghost carp (juvenile to mature fish), are susceptible to the virus. CCB cells and KF-1 cells are useful for replicating the virus but are not sufficiently sensitive to detect the virus in infected fish. PCR assays including conventional methods and real-time quantitative PCR as well as LAMP assays are sufficiently sensitive for detecting the virus from diseased fish. ELISA assay can detect antibodies in surviving fish that experienced the disease. KHV-infected fish transmit the virus for a longer period at lower temperatures. KHV disease causes systemic infection but exists in the brain longer than in other internal organs of surviving fish. KHV can be inactivated by UV-irradiation, heat treatment above 50°C, and chemical treatment. The attenuated vaccine is effective in preventing the disease, although the risk of reversion to its pathogenic form is a potential threat. Maintaining infected fish in water of 30°C and above helps fish recover from the infection, but the fish can become carriers of the virus.

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