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ISSN 0792 - 156X

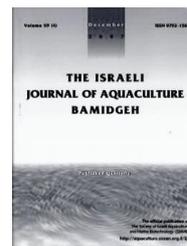
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Histopathology and Detection of Cyprinid Herpesvirus Infection in Two Koi Farms in Tianjin City (China)

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Key words: cyprinid herpesvirus 3; histopathology; *Cyprinus carpio* koi

Abstract

Koi herpesvirus (KHV) is the etiologic agent of koi herpesvirus disease (KHVD) causing mass mortalities in common carp (*Cyprinus carpio* *carpio*), koi (*Cyprinus carpio* *koi*), and ghost carp (*Cyprinus carpio* *goi*) populations. In this study, we report new outbreaks of the koi herpesvirus (KHV) disease in a koi carp farm in Tianjin city, northern China. From June to September 2017, severe mortalities of koi juvenile and broodstock occurred in ornamental fish, and infected koi carp which showed signs of lethargy and loss of appetite, and clinical signs including sunken eyes, gill necrosis, and excessive secretion of mucus. Histopathologically, the gills showed hyperplasia and degeneration of epithelial cells and fusion of the lamellae, and foci necrosis was found in gills, liver, kidney, and spleen. In addition, KHV and Carp edema virus (CEV) were detected in the diseased koi fish by diagnostic PCR tests for the viruses. The results showed that the nucleotide sequences of thymidine kinase (TK), DNA polymerase (SphI-5), and four open reading frame (ORF 25, ORF 56, ORF 72, ORF 81) genes confirmed the highest identity with other KHV strains such as KHV-I, KHV-U and KHV-J, but the CEV was negative. The phylogenetic tree showed KHV-TJ1708 and KHV-TJ1709 clustered with the Asian strain KHV-J (AP008984). Results of the present study confirmed the prevalence of Carp herpes disease (KHVD) in China koi populations by means of clinical examination, histopathology and molecular technique. These data will provide a reference for diagnosis, quarantine, and surveillance of KHV in China.

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Introduction

Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), is a member of the *Cyprinivirus* genus in the family *Alloherpesviridae*, which also includes *Cyprinid herpesvirus 1* (CyHV-1), *Cyprinid herpesvirus 2* (CyHV-2), and *Anguillid herpesvirus 1* (AnHV-1) (Yuasa et al., 2009, Davison et al., 2009). It is a serious epidemic threat to koi and common carp *Cyprinus carpio* globally in terms of koi breeding, and common carp production in captive and wild populations (Kielinski et al., 2010; Gotesman et al., 2013). Due to its impact on the aquaculture species, it is a notifiable disease in the list of OIE Aquatic Animal Health Code (OIE, 2009), the European Union (EU) directive 2006/88/EC. Koi herpesvirus infections were initially recorded in Germany in 1997 (Bretzinger et al., 1999). It was identified later in koi and common carp in the USA and Israel (Hedrick et al., 2000). Infected fish developed lethargic behavior and a lack of appetite, and showed the appearance of white patches, skin hemorrhages, sunken eyes, enlargement of the spleen and kidney, and gill necrosis (Hedrick et al., 2000, Hedrick et al., 2005). After its initial outbreak in Germany, the disease was reported from other Europe countries (Gotesman et al., 2013), North America (Garver et al., 2010), and several Asian countries including Indonesia (Sunarto et al., 2011), Japan (Sano et al., 2004), South Korea (Gomez et al., 2011), Thailand (Pikulkaew et al., 2009), Iran (Rahmati-Holasoo et al., 2016) and China (Tu et al., 2004, Cheng et al., 2011). To date, KHVD has occurred in more than 30 countries or regions worldwide (Toplak et al., 2011), resulting in tremendous economic impact. To date it remains a threat to the common carp and ornamental koi industry as well as other populations (OIE, 2009). In China, the first clinical outbreak of KHVD was reported in imported koi in Guangdong province in April 2002 (Liu et al., 2002). Subsequently, in December KHV was confirmed in Taiwan (Shih et al., 2007, Zhou et al., 2014). Currently, mortality events caused by the third *Cyprinid herpesvirus* CyHV-3 have been reported in Guangdong, Taiwan (Tu et al., 2004, Cheng et al., 2011), Hainan (Zhu et al., 2011), Liaoning and Jilin province (Gao et al., 2017).

Carp infected with carp edema virus (CEV) were found to show similar behaviors such as lethargy, and clinical signs including swollen gills, enophthalmos, and skin lesions. Histological analysis of gills showed a fusion of secondary gill lamellae and an occlusion of the interlamellar space due to the hypertrophy and proliferation of branchial epithelial cells. Infected koi also exhibited necrotic lesions in the gills and in internal organs such as kidney, spleen, liver, and gastrointestinal tract (Hedrick et al. 2000).

From June to September 2017, multiple large mortality events of koi juvenile and broodstock occurred in different districts of Tianjin China. The aim of this report was to identify the occurrence of KHV by clinical examination, histology, and KHV-specific polymerase chain reaction (PCR). Based on the above results, we provide evidence that KHV which has caused mortality of koi is now also present in Tianjin City, China.

Materials and Methods

Sample collection

From June to September 2017, mass mortalities of koi, *Cyprinus carpio*, juvenile and broodstock occurred in an ornamental koi fish farm in Tianjin City, northern China. The diseased fish were collected on three occasions from two commercial ponds. All the koi juveniles were collected from one farm and a koi broodstock from another. Based on the owner's statements, water quality parameters (temperature, pH, and O₂) were within normal ranges. The water temperatures ranged from 20-24°C. The koi broodstock was imported from Japan. Moribund fish were packed in water-filled plastic bags, supplied with oxygen and transported to a laboratory.

Detection of viral DNA by PCR assay

DNA was extracted from 20 mg of tissue (gill, liver, kidney, spleen) using commercial DNA extraction kits (Cell/blood/tissue DNA kit, Tiangen, Beijing, China) according to the manufacturer's instructions (Tiangen, Beijing, China). Two sets of primers were used to detect KHV according to the Quarantine protocol for Koi herpesvirus disease by the General Administration of Quality Supervision, Inspection and Quarantine of P.R.C. The first set designed using the KHV thymidine kinase (TK) gene (AJ535112) was TK-F: 5'-GGGTTACCTGTACGAG-3' and TK-R: 5'-CACCCAGTAGATTATGC-3', with an expected

product of 409 base pairs (bp). The other set was SphI-5-F: 5'-GACACCACATCTGCAAGGAG-3' and SphI-5-R: 5'-GACACATGTTACAATGGTCGC-3' with an expected product of 292 bp as described by Gray et al. (2002). In addition, one more primer used in this study to amplify full length of TK gene was KHV-F: 5'-TGACAAAGTGTGGAGAGGGC-3' and KHV-R: 5'-ATTGGACGCAAAGTCGCTCA-3' designed according to AP008984.1 by NCBI Primer Blast. Four more primers encoding open reading frame (ORF 25, ORF 56, ORF 72, ORF 81) were used for analysis. In addition, nested primers were used to detect CEV designed by Xu et al. (2016). The primers used are shown in Table 1. All primers were synthesized commercially by Genewiz, China.

Table 1. Primer pairs used in this study

Primer	Nucleotide Sequences (5'-3')	Size/bp	T _m (°C)	Reference
TK	F: 5'-GGGTTACCTGTACGAG-3' R: 5'-CACCCAGTAGATTATGC-3'	409	52	Bercovier et al., 2005
SphI-5	F: 5'-GACACCACATCTGCAAGGAG-3' R: 5'-GACACATGTTACAATGGTCGC-3'	292	62	Gray et al., 2002
CEV	F1: 5'-GCTGTTGCAACCATTGAGA-3' R1: 5'-TGAGGTTGCTCCTAATCCT-3'	548	56	Xu et al., 2016
CEV	F2: 5'-GCTGCTGCACTTTTAGGAGG-3' R2: 5'-TGCAAGTTATTTTCGATGCCA-3'	180	58	
KHV	F: 5'-TGACAAAGTGTGGAGAGGGC-3' R: 5'-ATTGGACGCAAAGTCGCTCA-3'	902	56	In this study
ORF25	F: 5'-ATGAGGGCGACCTTCTTCAC-3' R: 5'-TGATTGAGCACGCCGATGTA-3'	332	56	In this study
ORF72	F: 5'-TCGGTTTCACTGACGCTTGA-3' R: 5'-CACAGAGTTCTTGACGGCCT-3'	706	56	In this study
ORF56	F: 5'-TCACCAGCTTGAGCTTCTCG-3' R: 5'-ATCTGTCCCATGTCTCCCA-3'	939	58	In this study
ORF81	F: 5'-TGTCTCAGAACGCCATGTCC-3' R: 5'-TCCGCTACGTTCTGGTAGGA-3'	156	58	In this study

Note: TK: thymidine kinase, SphI-5: DNA polymerase, ORF 25, ORF 56, ORF 72, ORF 81: open reading frame 25, 56, 72, 81 genes

The reaction mix for the PCR contained 10 µl 2 × PCR mix (TIANGEN, CHINA), 2 µl DNA template, 1 µl each primer, and sterile bi-distilled water to a final volume of 25 µl. The mixture was subjected to an initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, an annealing for 30 s and an extension at 72°C for 1 min, followed by a 10 min elongation at 72°C after the final cycle. Five microliters of amplicons were run in 1% agarose gel electrophoresis with ethidium bromide staining. The resulting band pattern was then visualized and photographed under UV illumination.

The positive PCR products were used for sequencing using both forward and reverse primers by Genewiz, China. The BLAST was conducted at National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences were aligned with CLUSTAL W method in MEGA 6.0 software. A phylogenetic tree was rendered based on the neighbor-joining (N-J) method of MEGA 6.0 program, with 1000 bootstrap replicates.

Histopathological and light microscopic examination

Tissues including gills, liver, kidney, and spleen of the naturally infected koi broodstock were dissected and placed in Bouin's fluid and allowed to fix for 8 h. Fixed materials were dehydrated through a graded ethanol series and embedded in paraffin wax. Five micrometres thickness tissue sections were stained with hematoxylin and eosin (H&E) for histopathological analyses under the light microscope with a video camera for taking micrographs (Leica Application Suite V4, Germany).

Results

Outbreaks and clinic signs of diseased fish

Samples were collected from private farms in Tianjin, China from June to September in 2017. The cumulative mortality reached about 80-100% in farming areas where water temperatures were between 20-24°C. All diseased fish showed lethargy and loss of

appetite. In addition, individual diseased koi juveniles in group 1 (6.41 ± 0.1 cm, 6.5 ± 0.1 g) and group 2 (12.5 ± 0.1 cm, 35 ± 0.1 g) usually showed sunken eyes (enophthalmos) and pale gill coloration, even gill necrosis (Fig. 1A, B). Individual diseased koi broodstock in group 3 (65.5 ± 0.1 cm, 5700 ± 1 g) showed excessive mucus production, skin hemorrhages, (Fig. 1C, D) and pale gill coloration (Fig. 1D)



Figure 1. Koi with koi herpesvirus disease (KHVD). (A) Pale gill coloration (arrows) and sunken eyes (arrowhead) are evident in group 1 (B) bilateral enophthalmos (arrowhead) and necrotic gill filaments (arrows) in group 2 (C) skin hemorrhages and (D) the discoloration and necrosis of gill filaments (arrows) and gills covered with a thick mucus layer (*) in group 3

Detection of KHV by PCR method and phylogenetic tree analysis

Results of PCR assay of KHV are shown in Fig. 2. All samples including gills, livers, spleens, and kidneys of the affected juvenile and broodstock koi namely KHV-TJ1708 and KHV-TJ1709 were found positive for KHV-specific DNA sequences and negative for CEV-specific DNA sequence (Fig. 2A, 2B). Specific amplicons, with an expected size, were detected in all samples. The DNA sequence of PCR products (409-bp and 292-bp) were identical and closest in identity (100%) with the CyHV-3 (KHV) TK gene and SphI-5 gene respectively by BLAST search. Specific amplicons with 779-bp and 788-bp from gills of affected koi showed 99.36% and 100% identity to the complete nucleotide sequences of TK genes of KHV-J (AP008984). The phylogenetic tree based on neighbor-joining analyses of the complete nucleotide sequences of TK genes showed KHV-TJ1708 and KHV-TJ1709 clustered with the Asian strain KHV-J (AP008984) (Fig. 3). Four more primers encoding open reading frame (ORF 25, ORF 56, ORF 72, ORF 81) were also amplified and all demonstrated positive PCR results for the genes sequenced from the gills of koi juvenile (Fig. 2C). All the expected fragments were obtained, namely 332-bp of ORF 25 gene product, 939-bp ORF 56, 706-bp ORF 72 and 156-bp ORF 81 in length. In addition, analysis of the deduced amino acid sequence of ORF 25, ORF 56, ORF 72, ORF 81 for homology to known gene sequences in databases via BLASTX was performed and the results showed that KHV-TJ1708 shared high amino acid sequence identities (96.67%, 100%, 99.56% and 100%) to hypothetical protein (BAF48838.1), protein ORF56 (AOO32621.1), capsid triplex subunit 2 (AVL28480.1), and ORF81 (AJK93602.1) from *Cyprinid herpesvirus 3*.

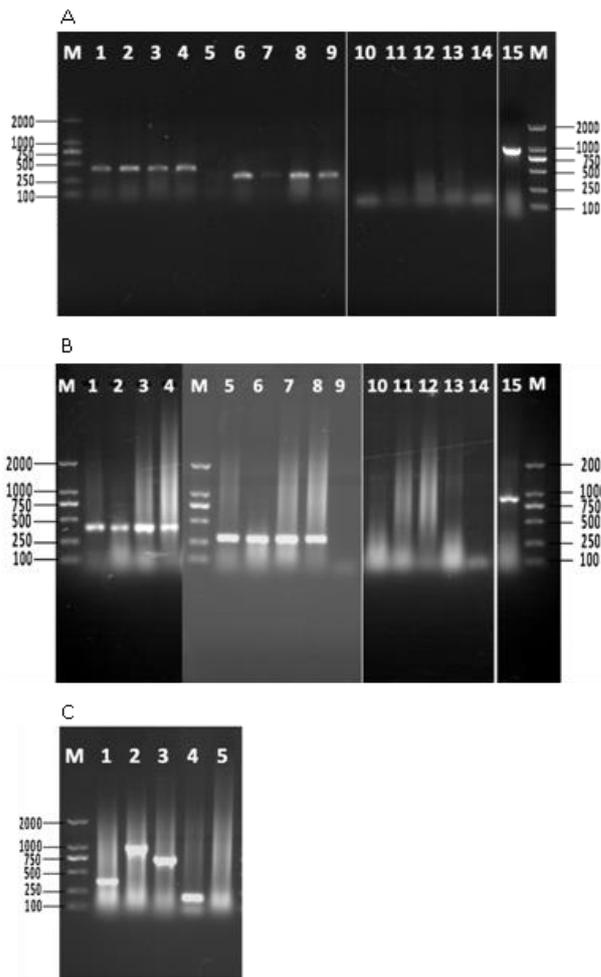


Figure 2. PCR assay of koi herpesvirus (KHV) affected in koi juvenile (A, C) and broodstock (B). A: Lane 1-4: PCR amplification of KHV TK gene of gills, livers, kidney and spleen; Lane 5: negative control (ddH₂O); Lane 6-9: PCR amplification of KHV SphI-5 gene; Lane 10-14: nested PCR assay of carp edema virus (CEV) and Lane 15: PCR amplification of full length of KHV TK gene of gills. B: Lane 1-4: PCR amplification of KHV TK gene of gills, livers, kidney and spleen; Lane 5-8: PCR amplification of KHV SphI-5 gene; Lane 9: negative control (ddH₂O); Lane 10-14: nested PCR assay of carp edema virus (CEV) and lane 15: PCR amplification of full length of KHV TK gene of gills. C: PCR amplification of genes encoding open reading frame of gills: 1: PCR product of ORF25 fragment, 2: PCR product of ORF56 fragment, 3: PCR product of ORF72 fragment, 4: PCR product of ORF81 fragment, 5: negative control. Lanes M: 2000 bp DNA marker. PCR products were analysed on a 1% agarose gel and visualized by ethidium bromide.

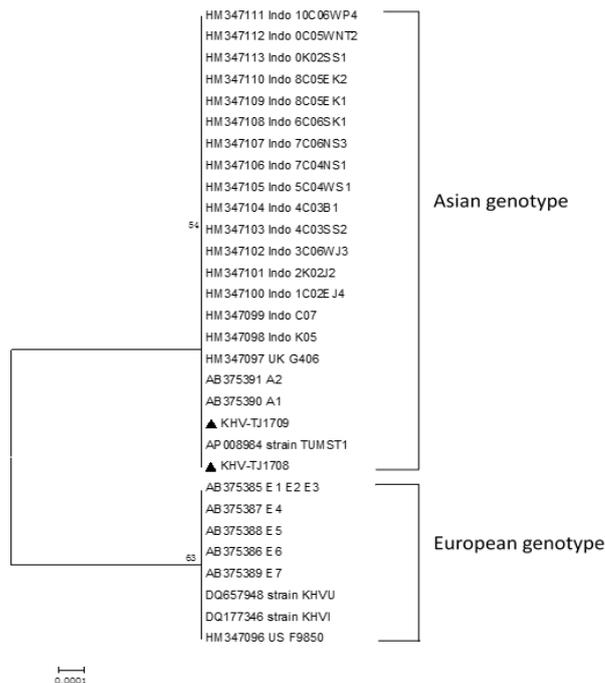


Figure 3. Phylogenetic relationship of two Chinese CyHV-3 isolates with twenty-eight foreign CyHV-3 isolates based on neighbor-joining analyses of the complete nucleotide sequences of TK genes; the tree was generated by the neighbour-joining methods using the MEGA 6.1 program, with 1000 bootstrap replicates. The KHV-TJ1708 and KHV-TJ1709 isolate is indicated by black up-pointing

Histopathological and microscopic features of naturally diseased koi

Histopathological analysis of gill revealed a fusion of secondary lamellae which resulted from proliferation of the inter-lamellar cellular mass (Fig. 4A). Another significant gill lesion was degeneration of gill epithelium with pyknotic, karyorrhectic, or nuclei intranuclear inclusion even necrosis at the fused lamellae (Fig. 4B).

The most prominent pathological changes in the liver were hydropic degeneration of hepatocytes (Fig. 4C). Furthermore, multifocal necrosis of parenchymal cells with karyolysis and inflammatory infiltrate were also seen in the liver (Fig. 4D).

In the kidney, renal pigmentation and infiltration with inflammatory cells was obviously observed in kidney parenchyma (Fig. 4E). In addition, some of the tubular epithelial cells had a cloudy appearance and slightly separated from basal lamina (Fig. 4E). Necrotic changes of epithelial cells progressing to loss of integrity of renal tubule were presented (Fig. 4F). Interestingly, some cells were observed in renal tubule (Fig. 4F). In the spleen, scattered necrotic splenocytes with eosinophilic inclusions were observed (Fig. 4H) Hemorrhage and expansion of splenic sinus were also observed in the spleen (Fig. 4G).

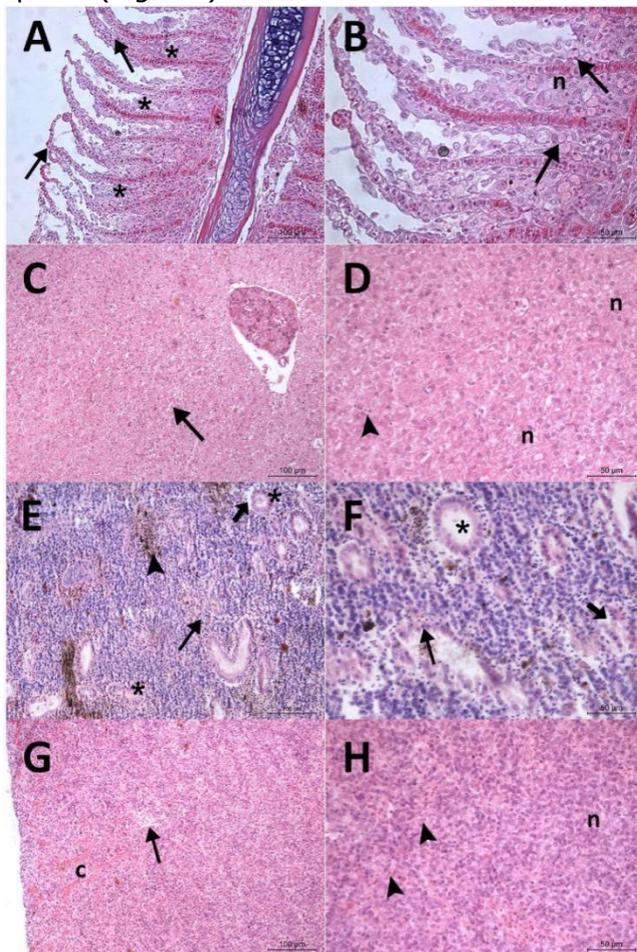


Figure 4. Pathology in koi broodstock analyzed for the presence of koi herpesvirus. A, B: histopathological changes to the gill architecture: A primary gill lamellae with a proliferation of the interlamellar cellular mass (*) and extensive fusion of adjacent secondary lamellae (arrows), B: respiratory epithelial cells exhibit degeneration (arrows) even necrosis (n) at the fused lamellae. C, D: histopathological changes to the liver architecture: C hepatocytes with hydropic degeneration (arrow), D multifocal necrosis of parenchymal cells (n) and inflammatory infiltrate (arrowhead) E, F: histopathological changes to the kidney architecture: E renal pigmentation (arrowhead) and infiltration with inflammatory (thin arrow) in kidney parenchyma, Some of the renal tubular epithelial cells have a cloudy appearance (*) and separated from basal lamina (bold arrow); F necrotic changes of epithelial cells (thin arrow) and loss of integrity of renal tubule (bold arrow) and cells in renal tubule (*). G, H histopathological changes to the kidney architecture: G haemorrhage (c) and expansion of splenic sinus (arrow) H necrotic splenocytes (n) and eosinophilic inclusions (arrowheads). H & E, Scale bars=100 μ m for (A, C, E, G) and 50 μ m for (B, D, F, H)

Discussion

Koi herpesvirus (KHV) is the etiologic agent of koi herpesvirus disease (KHVD) causing mass mortalities in common carp (*Cyprinus carpio carpio*), koi (*Cyprinus carpio koi*), and ghost carp (*Cyprinus carpio goi*) populations (Gomez et al., 2011, Toplak et al., 2011). *Cyprinid herpesvirus 3* (CyHV-3) has spread worldwide due to worldwide fish trade (Oh et al. 2001; Sano et al. 2004). In addition, illegal transport and trading of koi has led to rapid spread worldwide (Somga et al., 2010). From June to September 2017, high mortality up to 80–100% of koi occurred in Tianjin, China. Clinical signs of sick fish include sunken eyes, pale gills, and increased mucus production on skin and fins, which in accordance with previous reports (Hedrick et al. 2000; Gotesman et al. 2013). The main target tissues for KHV based on microscopic histopathological analyses of naturally

infected koi broodstock are suspected to be gills, kidney, spleen, and liver, but other tissues including the brain, heart and gut are also involved (Hedrick et al. 2000; Gilad et al. 2004; Miyazaki et al. 2008). The affected koi broodstock exhibited abnormal changes in gill filaments, kidney tubular tissue, spleen, and liver that showed multifocal necrosis and inflammatory infiltrate. In addition, these kinds of pathological changes and abnormalities were also observed and reported in the past in Koi fish affected by KHV infection (Miyazaki et al. 2008).

Diagnosis of a KHV infection relies on the testing of tissues for the presence of KHV-specific DNA sequences by means of polymerase chain reaction (PCR) as isolation of the virus in cell cultures is a difficult and time-consuming method (Haenen et al., 2004, Rahmati-Holasoo et al., 2016). PCR methods have been proven to be practical and rapid techniques for the detection of viral DNA (Gray et al., 2002; Gilad et al., 2004; Bercovier et al., 2005; Rahmati-Holasoo et al., 2016; Luo et al., 2018). A conventional PCR based on primers targeting the thymidine kinase (TK) gene was shown to be specific for KHV and relatively effective in the diagnosis of clinically suspicious cases, which is the golden standard method for KHV diagnosis (Bercovier et al. 2005). There is also a clear genetic distinction between European (Israel and the USA strains) and Asian (Japan strain) genotype isolates. In addition, seven European variants (E1-E7) and two Asian variants (A1 and A2) could be distinguished via sequence analysis of the TK gene (Kurita et al. 2009). In this study, all samples including gill, liver, spleen, and kidney of the affected koi were found positive for TK and SphI-5-specific DNA sequences, with expected length pcr products of 490 and 292 bp. The phylogenetic tree of two Chinese CyHV-3 isolates namely KHV-TJ1708 and KHV-TJ1709 with twenty-eight foreign CyHV-3 isolates showed they clustered with the Asian strain based on the complete nucleotide sequences of TK genes.

In conclusion, the outbreaks of KHVD in Tianjin China koi populations were confirmed by means of clinical examination, histopathology and molecular technique, which can provide a practical reference for epidemiological investigation, diagnosis, quarantine and surveillance of KHV in China.

Acknowledgements

Acknowledgements: This study was supported by the Natural Science Foundation of Tianjin city (No. 16JCZDJC33500, 18JCYBJC29900), National Undergraduate Innovation Training Program (201810061036), Tianjin Agricultural University Innovation Training Program (201810061174), Innovation Team of Tianjin Fisheries Research System (No. ITTFRS2017009), Modern Aqua-ecology and Health Aquaculture Innovation Team of Tianjin (No. TD13-5089), Tianjin agricultural university Key Laboratory of Aqua-ecology platform project (No. J01009030638), Key Scientific Research Project Universities and Colleges in Henan province (No. 17A240001), and Henan Natural Science Foundation (No.182300410021).

References

- Bercovier, H., Fishman, Y., Nahary, R., Sinai, S., Zlotkin, A., Eyngor, M., Gilad, O., Eldar, A. and Hedrick, R. P.**, 2005. Cloning of the koi herpesvirus (KHV) gene encoding thymidine kinase and its use for a highly sensitive PCR based diagnosis. *BMC Microbiol*, 5(1), 13.
- Bretzinger, A., Fischerscherl, T., Oumouna, M., Hoffmann, R. and Truyen, U.**, 1999. Mass mortalities in Koi carp, *Cyprinus carpio*, associated with gill and skin disease. *Bulletin- European Association of Fish Pathologists*, 19(5), 182-185.
- Cheng, L., Chen, C. Y., Tsai, M. A., Wang, P. C., Hsu, J. P., Chern, R. S. and Chen, S. C.**, 2011. Koi herpesvirus epizootic in cultured carp and koi, *Cyprinus carpio* L., in Taiwan. *J Fish Dis*, 34(7), 547-554.
- Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., McGeoch, D. J., Minson, A. C., Pellett, P. E., Roizman, B., Studdert, M. J. and Thiry, E.**, 2009. The order herpesvirales. *Arch Virol*, 154(1), 171-177.

- Gao L, Yu H and Zhou J**, 2017. Identification of Koi herpesvirus disease outbreak under ice in carp cage cultured in baishan, the Yalu River. *J Aquacult.*, 38, 7-11(in Chinese).
- Garver, K. A., Al-Hussinee, L., Hawley, L. M., Schroeder, T., Edes, S., LePage, V., Contador, E., Russell, S., Lord, S. and Stevenson, R. M.**, 2010. Mass mortality associated with koi herpesvirus in wild common carp in Canada. *J Wildl Dis*, 46(4), 1242-1251.
- Gilad, O., Yun, S., Zagmutt-Vergara, F. J., Leutenegger, C. M., Bercovier, H. and Hedrick, R. P.**, 2004. Concentrations of a Koi herpesvirus (KHV) in tissues of experimentally-infected *Cyprinus carpio* koi as assessed by real-time TaqMan PCR. *Dis Aquat Organ*, 60(3), 179-187.
- Gomez, D. K., Joh, S. J., Jang, H., Shin, S. P., Choresca Jr, C. H., Han, J. E., Kim, J. H., Jun, J. W. and Park, S. C.**, 2011. Detection of koi herpesvirus (KHV) from koi (*Cyprinus carpio* koi) broodstock in South Korea. *Aquaculture*, 311(1-4), 42-47.
- Gotesman, M., Kattlun, J., Bergmann, S. M. and El-Matbouli, M.**, 2013. CyHV-3: the third cyprinid herpesvirus. *Dis Aquat Organ*, 105(2), 163-174.
- Gray, W. L., Mullis, L., Lapatra, S. E., Groff, J. M. and Goodwin, A.**, 2002. Detection of koi herpesvirus DNA in tissues of infected fish. *J Fish Dis*, 25(3), 171-178.
- Haenen, O., Way, K., Bergmann, S. and Ariel, E.**, 2004. The emergence of koi herpesvirus and its significance to European aquaculture. *Bulletin of the European Association of Fish Pathologists*, 24(6), 293-307.
- Hedrick, R., Gilad, O., Yun, S., Spangenberg, J., Marty, G., Nordhausen, R., Kebus, M., Bercovier, H. and Eldar, A.**, 2000. A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. *J Aquat Anim Health*, 12(1), 44-57.
- Hedrick, R. P., Gilad, O., Yun, S. C., McDowell, T. S., Waltzek, T. B., Kelley, G. O. and Adkison, M. A.**, 2005. Initial isolation and characterization of a herpes-like virus (KHV) from koi and common carp. *Bulletin of Fisheries Research Agency*, 2(1), V7.
- Kielpinski, M., Kempter, J., Panicz, R., Sadowski, J., Schütze, H., Ohlemeyer, S., & Bergmann, S.M.** (2010). Detection of KHV in Freshwater Mussels and Crustaceans from Ponds with KHV History in Common Carp (*Cyprinus carpio*). [AquacultureHub, Isr. J. Aquacult-Bamidqeh, \(IJA\)](#), 62:1: 28-37
- Kurita, J., Yuasa, K., Ito, T., Sano, M., Hedrick, R. P., Engelsma, M. Y., Haenen, O. L. M., Sunarto, A., Kholidin, E. B. and Hsinyiu, C.**, 2009. Molecular epidemiology of koi herpesvirus. *Fish Pathol.*, 44(2), 59-66.
- Liu, H., SHI, X., GAO, L. and JIANG, Y.**, 2002. Study on the aetiology of koi epizootic disease using the method of nested-polymerase chain reaction assay (nested-PCR). *J Huazhong Agricult Univ*, 21, 414-418.
- Luo K., Yuan H., Xu Q.*, Zhang W.**, 2018. Two Rapid Methods to Identify Three Species of Pathogenic *Vibrio* in *Penaeus vannamei*. [AquacultureHub, Isr. J. Aquacult.-Bamidqeh, \(IJA\)](#) 70.2018.1458]
- Miyazaki, T., Kuzuya, Y., Yasumoto, S., Yasuda, M. and Kobayashi, T.**, 2008. Histopathological and ultrastructural features of Koi herpesvirus (KHV)-infected carp *Cyprinus carpio*, and the morphology and morphogenesis of KHV. *Dis Aquat Organ*, 80(1), 1-11.
- Office International des Epizooties, OIE**, 2009. *Manual of Diagnostic Tests for Aquatic Animals*. Chapter 2.3.6 Koi Herpesvirus Disease.
- Oh, M.-J., Jung, S.-J., Choi, T.-J., Kim, H.-R., Kim, Y.-J., Park, M.-A. and Chun, S.-K.**, 2001. A viral disease occurring in cultured carp *Cyprinus carpio* in Korea. *Fish Pathol.*, 36(3), 147-151.
- Pikulkaew, S., Meeyam, T. and Banlunara, W.**, 2009. The outbreak of koi herpesvirus (KHV) in koi (*Cyprinus carpio* koi) from Chiang Mai province, Thailand. *Thai Vet Med.*, 39(1), 53-58.
- Rahmati-Holasoo, H., Zargar, A., Ahmadvand, S., Shokrpoo, S., Ezhari, S. and Ebrahimzadeh Mousavi, H.**, 2016. First detection of koi herpesvirus from koi, *Cyprinus carpio* L. experiencing mass mortalities in Iran: clinical, histopathological and molecular study. *J Fish Dis*, 39(10), 1153-1163.

- Sano, M., Ito, T., Kurita, J., Yanai, T., Watanabe, N., Miwa, S. and Iida, T.,** 2004. First detection of koi herpesvirus in cultured common carp *Cyprinus carpio* in Japan. *Fish Pathol.*, 39(3), 165-167.
- Shih, D., Chien, T. U. and Cheng, C. H.,** 2007. The Research of the Transmission Vectors of Koi Herpes Virus (KHV). *Taiwan Vet J*, 33.
- Somga, J., de la Peña, L. D., Sombito, C., Paner, M. G., Suarnaba, V. S., Capulos, G. C., Maria, P. and Po, G.,** 2010. Koi herpesvirus-associated mortalities in quarantined koi carp in the Philippines. *Bull European Assoc Fish Pathologists*, 30(1), 2-7.
- Sunarto, A., McColl, K. A., Crane, M. S., Sumiati, T., Hyatt, A. D., Barnes, A. C. and Walker, P. J.,** 2011. Isolation and characterization of koi herpesvirus (KHV) from Indonesia: identification of a new genetic lineage. *J Fish Dis*, 34(2), 87-101.
- Toplak, I., Grilc Fajfar, A., Hostnik, P. and Jenčič, V.,** 2011. The detection and molecular characterization of koi herpesvirus (KHV) in Slovenia. *Bull European Assoc. Fish Pathologists*, 31(6), 219-226.
- Tu, C., Weng, M.-C., Shiau, J.-R. and Lin, S.-Y.,** 2004. Detection of koi herpesvirus in koi *Cyprinus carpio* in Taiwan. *Fish Pathol.*, 39(2), 109-110.
- Xu L.P, Wang J.B, Zhang W, Pan Y, Cao H, Wang S, Wang X.L, Wang P, Na L.H and Jiang Y.L,** 2016. A Preliminary Study of Carp Edema Virus Disease. *J Inspection and Quarantine*, 26(5):14-16 (in Chinese)
- Yuasa, K., & Sano, M.** (2009). Koi Herpesvirus: Status of Outbreaks, Diagnosis, Surveillance, and Research. [*AquacultureHub-Isr. J. Aquacult.-Bamidgeh \(IJA\)*](#), 61(3):169-179.
- Zhou, J.X., Wang, H., Li, X.W., Zhu, X., Lu, W.L., Zhang, D.M.,** 2014. Construction of KHV-CJ ORF25 DNA vaccine and immune challenge test. *J Fish Dis*, 37, 319-325.
- Zhu X, Li X W, Wang H, LV W.L, Zhou J.X,** 2011. Isolation and identification of koi herpesvirus. *Chinese J Preventive Vet Med.*, 33(5): 340-343 (in Chinese).