*Short Communication*

# The Outbreak of Koi Herpesvirus (KHV) in Koi *(Cyprinus carpio koi)* from Chiang Mai Province, Thailand

**Surachai Pikulkaew[1] Tongkorn Meeyam[2] Wijit Banlunara[3*]**

## Abstract

**Twenty-two sick koi with cumulative mortality showed clinical signs of depression, brachial hemorrhage and necrosis, focal hemorrhage, pale patches and ulcers on the skin. Clinical examination showed the heavy infection by opportunistic organisms such as *Dactylogyrus* sp., *Gyrodactylus* sp. and *Saprolegnia* sp. of the skin, gill and fins. Histopathologically, the gill showed severe acute diffuse necrosis of the branchial epithelial cells and severe diffuse lymphocytic-monocytic interstitial nephritis with necrosis of the tubular epithelial cells were predominantly found in most sick fish. The presence of homogeneous pale basophilic intranuclear viral inclusions was observed. Transmission electron microscopy showed herpesvirus-like particles in the nuclei of gill epithelium. The positive result at 290 base pairs DNA product of KHV was confirmed from the gill samples using the polymerase chain reaction (PCR). In conclusion, this report confirmed the evidence of the outbreak of KHV in koi from Chiang Mai using pathology and molecular method.**

**Keywords :** histopathology, koi herpesvirus, PCR, transmission electron microscopy

[1]Food Animal Clinic, [2]Veterinary Public Health, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, Thailand
[3]Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand
[*]Corresponding author: E-mail : wijit.k@chula.ac.th

Thai J. Vet. Med., 2009. 39(1): 53-58

## บทคัดย่อ

# การระบาดของเชื้อไวรัสเฮอร์ปีส์ปลาคาร์ฟในปลาคาร์ฟในจังหวัดเชียงใหม่ ประเทศไทย

สุรชัย พิกุลแก้ว[1] ทองกร มีแย้ม[2] วิจิตร บรรลุนารา[3*]

**ปลาคาร์ฟ 22 ตัวป่วยและตายด้วยอาการแสดงทางคลินิก ซึม เหงือกมีเลือดออกและเนื้อตายปื้นขาว เลือดออกและแผลหลุมบนผิวหนัง ผลการตรวจทางคลินิก พบการติดเชื้อฉวยโอกาส ได้แก่ ปลิงใสแด็กทิลโลจัยรัสและไจโรแด็กไทรัส และรานำ้ชนิดซาโปเล็กเนียที่ผิวหนัง เหงือก และครีบ จุลพยาธิวิทยาของปลาส่วนใหญ่พบการตายเฉียบพลันของเซลล์เยื่อบุเหงือกอย่างกว้างขวางและไตอักเสบแบบไม่มีหนองอย่างรุนแรง และการตายของเซลล์เยื่อบุท่อไต พบก้อนอินคลูชันของไวรัสชนิดในนิวเคลียสติดสีน้ำเงินจาง กล้องจุลทรรศน์อิเล็กตรอนชนิดแสงผ่านแสดงเชื้อไวรัสรูปร่างเหมือนไวรัสเฮอร์ปีส์ในนิวเคลียสของเหงือก ผลการตรวจโดยปฏิกิริยาลูกโซ่โพลิเมอเรสจากเหงือก ให้ผลบวกต่อเชื้อไวรัสเฮอร์ปีส์ปลาคาร์ฟที่ 290 คู่เบส โดยสรุป รายงานนี้พบหลักฐานยืนยันการระบาดของเชื้อไวรัสเฮอร์ปีส์ปลาคาร์ฟในปลาคาร์ฟในจังหวัดเชียงใหม่ ด้วยวิธีทางพยาธิวิทยาและอณูชีววิทยา**

**คำสำคัญ :** จุลพยาธิวิทยา ไวรัสเฮอร์ปีส์ปลาคาร์ฟ ปฏิกิริยาลูกโซ่โพลิเมอเรส กล้องจุลทรรศน์อิเล็กตรอนชนิดแสงผ่าน

[1]คลินิกสัตว์บริโภค, [2]สัตวแพทย์สาธารณสุข คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ จ.เชียงใหม่ 50100
[3]ภาควิชาพยาธิวิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัยมหาวิทยาลัย กรุงเทพฯ 10330 ประเทศไทย
[*]ผู้รับผิดชอบบทความ: E-mail : wijit.k@chula.ac.th

## Introduction

Koi herpesvirus (Cyprinid herpesvirus-3, CyHV-3 or KHV) is a serious viral infection in common carp and fancy carp or koi. The cumulative mortality can approach 90-100% within 2 to 3 weeks when the outbreak occurs in commercial populations (Bondad-Reantaso, 2004; Gunimaladevi et al., 2004; Hedrick et al., 2004; Perelberg et al., 2005). It is caused by unclassified and emerging herpesvirus. The outbreak or detection of the pathogen has been reported in Israel, England, Italy, The Netherlands, Germany, Indonesia and Japan (Gunimaladevi et al., 2004). The clinical sign of KHV are the following lethargy, fatigue, gasping movements in shallow water, gill necrosis, sunken eyes, pale patches on skin, and increased mucus secretion (Gilad et al., 2004; Hedrick et al., 2004; Shapira et al., 2005). Because of the high mortality rate and the high infectivity rate, this disease causes serious economic losses in ornamental fish farming. This study represents the first report of KHV infection in koi from northern part of Thailand using histopathology, electron microscopy and the polymerase chain reaction (PCR).

## Materials and Methods

**Clinical examination:** This commercial fish farm is located in Mae Jo, Chiang Mai province. There are 12 typically earthen ponds with capacity of 790 gallon. Water quality assessment was performed every weeks by using commercial test kits. The stocking density for 200-400 g koi is between 5 and 8 fish per gallon. Twenty-two sick koi were transferred from earthen pond to the Faculty of Veterinary Medicine of Chiang Mai University in September 2006. Their chief complaints were depression and skin problem. We recorded clinical signs and examined on external parasite by skin, fin and gill biopsies.

**Histopathological and electron microscopic examination:** Seven dead koi were necropsied. The selected tissues were placed in 10% buffered formalin and allowed to fix for 24 h. Fixed tissues were routinely processed for histopathology in paraffin embedding and 4-μm thickness tissue sections were stained with hematoxylin and eosin (H&E). The selected gill section was stained with Feulgen method for the detection of the

DNA viral inclusions. The formalin-fixed paraffin-embedded gill was digested and deparaffinized in xylene, rehydrated and then immersed in 0.1M phosphate buffer (PB). Tissues were refixed in 2% glutaraldehyde in PB, post-fixed in 1% osmium tetroxide and embedded epoxy-resin. Ultrathin sections were double-stained with uranyl acetate and lead acetate and examined using a transmission electron microscope (JEM2100, Japan)

**Detection of viral DNA by PCR assay:** PCR for the detection of KHV was done according to Gray et al. (2002). Twenty five grams of gill tissue from twenty sick koi were extracted by using commercial DNA extraction kits (QIAGEN®,CA, USA). The SphI-5 primer set (5'-GACACCACATCTGCAAGGAG-3' and 5'-GACA CATGTTACAATGGTGGC-3') was used for 0.4 μM of each primer to amplify a 290 bp product. The amplification was performed with an initial step of 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 3 min and a final extension was at 72°C for 7 min. The PCR-amplified products patterns of samples were compared with the plasmid of KHV (a gift from Inland Aquatic Animal Health Research Institute; AAHIR) as templates following 3% agarose gel electrophoresis at 5 V/cm in 1xTBE buffer using Minicell electrophoresis (Biorad, NY, USA). The resulting band pattern was visualized and recorded using Geldoc 2000 (Biorad).

## Results and Discussion

Seventeen koi died within a week. Some of them showed clinical signs of depression, behavioral abnormalities and disorientation before death. Microscopic examination of skin scrapes revealed *Dactylogyrus* sp. (gill flukes), *Gyrodactylus* sp. (skin flukes), *Trichodina* sp., *Chilodonella* sp. and *Saprolegnia* sp. (water moulds). Fin and gill biopsies showed *Dactylogyrus* sp., *Gyrodactylus* sp., *Trichodina* sp. and water mold infection.

The most prominent gross lesions showed congestion, hemorrhage, necrosis and white nodule on the gill (18/20) (Fig. 1). Some fish presented color changes, focal hemorrhage, white patches, scale loss and ulcers on the skin (12/20). Histopathologically, the severe lesions was found in the gill epithelium, spleen, intestine and kidney. The gill was severely acute diffuse necrosis of the branchial epithelial cells and scattered denudating necrotic cells (Fig. 2). The epithelial cell nuclei had enlarged, homogeneous pale basophilic intranuclear inclusion bodies with thick-marginated chromatin of the nuclear wall. Few inclusion bodies present as Cowdry type A form (Fig. 2). The external parasitic sections were also found in the severe lesions of the gill lamella. The skin lesion was ulcerated with necrotic debris and inflammatory cells infiltrated in the subdermis. The kidney showed severe diffuse lymphocytic-monocytic interstitial infiltration and necrosis of the tubular epithelial cells as well as the inclusion bodies in the tubular cells were predominantly found in most sick fish (Fig. 3). Moreover, the inclusion bodies were found in mononuclear cells in the lamina propria and epithelium of small intestine, perivascular sheet cells, myocytes and ganglionic neurons in ovary (Fig. 4).

Electron microscopy revealed a low number of herpesvirus-like particles in the nuclei of gill epithelium (Fig. 5). The morphology was naked particles with the pattern of the dense virus cores in the aggregated nuclear chromatin. The sizes of the naked particles were 100-110 nm in diameter. Some particles were hidden in the rim of the dense marginated nuclear chromatin and budding at the nuclear envelope.

The KHV DNA was detected by PCR assay. A positive result was a 290 base pairs DNA products in 17 of 20 fish (85%) (Fig. 6). The positive samples were compared with DNA of healthy koi and positive (plasmid) control. A minimal concentration of detectable KHV DNA was 0.06 ng/μl.

The clinical finding and the pathology of these fish were similar to the early outbreak of the disease in the USA, Western Europe, Israel and other countries in Asia (Gilad et al., 2002; Bondad-Reantaso, 2004). The acute death of most fish indicated gross lesions still present

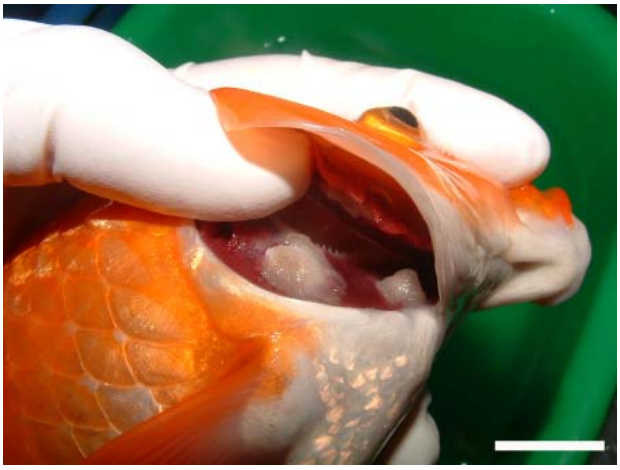

**Figure 1.** The gill of infected koi showing severe congestion and white nodules (bar = 1 cm).

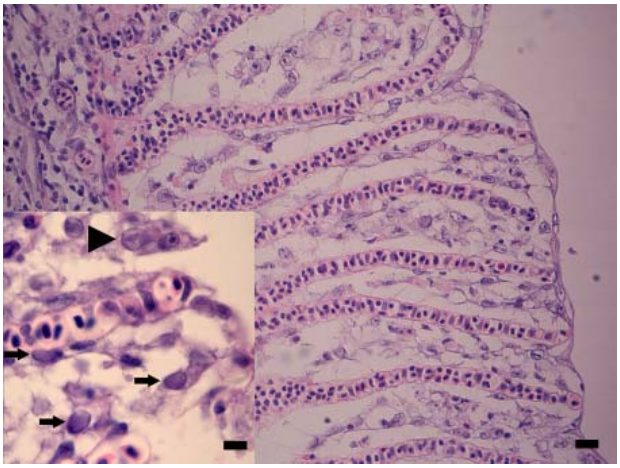

**Figure 2.** Severe acute diffuse necrosis of brachial epithelial cells and denudating necrotic cells of gills (bar = 30 μm). The inset; the nuclei of epithelial cells are enlarging, homogeneous pale basophilic intranuclear inclusion bodies with the thick-marginated chromatin of the nuclear wall (arrows). An inclusion body presents as Cowdry type A form. (arrow head). H&E, bar = 15 μm.

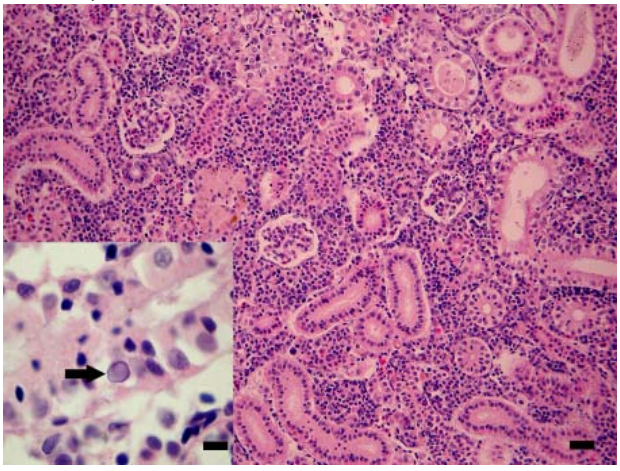

**Figure 3.** Severe lymphocytic-monocytic cells infiltrating in the interstitial tissue of the kidney in a fish (bar = 100 μm). The inset: an inclusion body presenting in the tubular epithelial cells (arrow). H&E, bar = 15 μm.

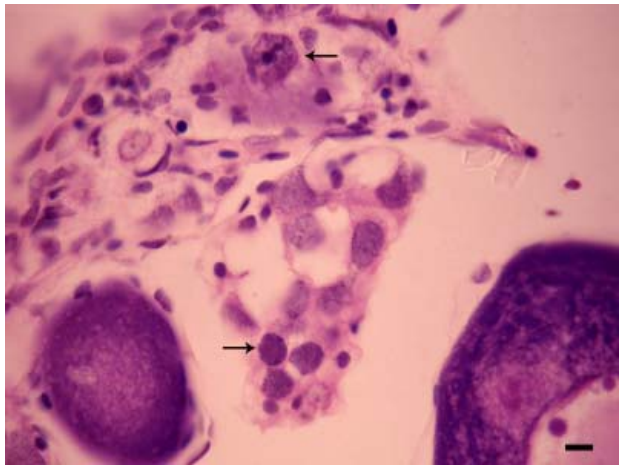

**Figure 4.** The ganglionic-ovarian nerve plexus among the primary oocytes showing the basophilic intranuclear inclusion bodies in the neurons (arrows). H&E, bar = 30 μm.

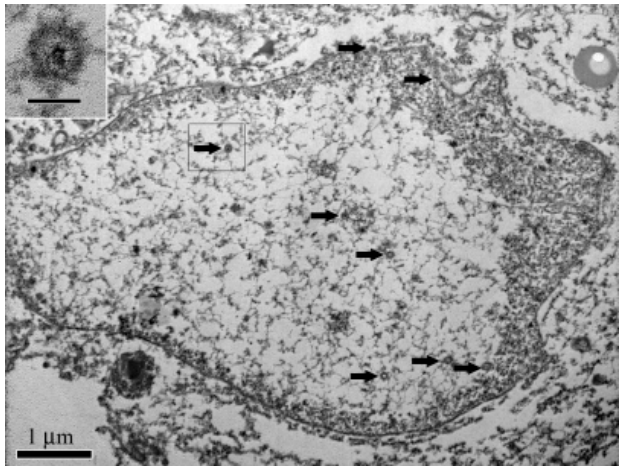


**Figure 5.** Electron micrograph of the naked particles of Herpesvirus (arrow heads) in the aggregated nuclear chromatin of the infected gill epithelium (bar = 1 μm). Inset: demonstrates a higher magnification (selected rectangle line) of a single Herpesviral particle (bar = 100 nm).

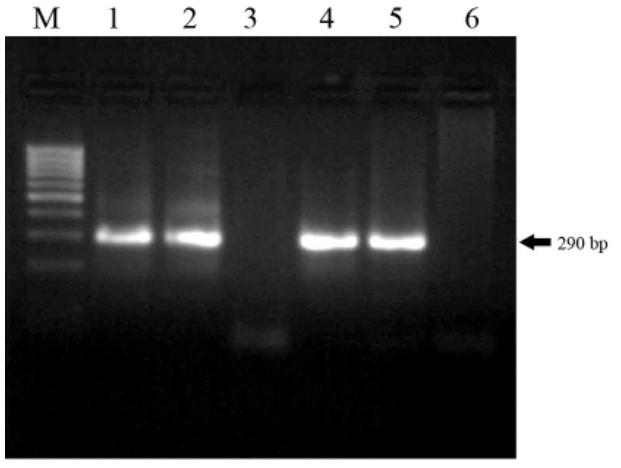


**Figure 6.** Detection of KHV virus DNA products (290 bp) by PCR method. Lane M: 100 bp DNA marker, lane 1-2: gill samples of infected koi, lane 3: sample of healthy koi, lane 4 and 5: positive control (dilution of 1:10 and 1:100) and lane 6: reagent-only negative control.

on the skin. The opportunistic ectoparasites and bacteria usually found on infected fish (Gilad et al., 2004). From our studied, the fungus, protozoa and monogenean were found commonly as opportunistic pathogens; otherwise the bacterial culture should be taken. However, these opportunistic pathogens are usually indicators of poor sanitation or poor water quality. As in other animals, opportunistic infections in fish are associated with immunosuppression (Shapira et al., 2005). So, a routine checking of water and management at the rearing pond are also importance for diagnosis. The most prominent pathological changes were noted in the gills and kidneys. The gill lesions are similar to almost all previously published reports (Hedrick et al., 2000; Gray et al., 2002; Pikarsky et al., 2004). Heavy ectoparasites were found in the gills and skin of these fish. The severity of gill lesions depended on viral pathogenesis and secondary ectoparasitic infections. The gill lesions might be preceded by parasites, bacteria or co-infection with KHV. Many obvious intranuclear inclusion bodies were present in many affected cell types. Typical inclusion bodies are enlarged and homogeneous pale basophilic intranuclear inclusion bodies with the thick-marginated chromatin of the nuclear wall. Interestingly, some typical mammalian herpes viral inclusion bodies are Cowdry type A, which was found in the gill epithelium of our studied. Ultrastructurally, the virus particles were morphologically identical to herpesvirus. However, a low number of the particles was difficultly observed. These data provide evidence that KHV infected the koi. The presence of the viral inclusion bodies in many cells suggest that the virus is a contagious and emerging disease. Pikarsky et al. (2004) revealed that the virus is not restricted to cells of the hematolymphoid lineage by immunohistochemistry. Moreover, the mononuclear cell infection is a cause of the rapid spread of virus in the fish (Pikarsky et al., 2004). Interestingly, the peripheral nervous tissue in these fish was also found the inclusion bodies. Most published reports do not demonstrate the infection in the nervous system, only one experiment of KHV infection showed mild lesions in the brain at 6 day post-inoculation (Pikarsky et al., 2004).

The gold standard for the diagnosis of KHV is the viral DNA detection. The PCR is a routine quarantine and disease diagnosis for KHV (Gilad et al., 2003 and 2004; Ishioka et al., 2005). This study showed the strong result that gill samples were positive by PCR. The detection of KHV in koi gill tissue by PCR is an effective way to diagnose the KHV infection in koi (Gray et al., 2002; Gilad et al. 2004).

In conclusion, this study is the first report on the evidence of KHV virus in Thailand using the pathological, electron microscopy and PCR methods and on the outbreak of this contagious disease among Thai commercial koi farm.

## Acknowledgements

The authors would like to thank the Faculty of Veterinary Medicine, Chiang Mai University for financial support, the AAHRI, Department of Fisheries, Bangkok for the DNA plasmid.

## References

Bondad-Reantaso, M.G. 2004. Trans-boundary aquatic animal diseases: Focus on Koi herpes virus (KHV). Aquacult. Asia. 9: 24-28.

Gilad, O., Yun, S., Andree, K.B., Adkison, M.A., Zlotkin, A., Bercovier, H., Eldar, A. and Hedrick, R.P. 2002. Initial characteristics of koi herpesvirus and development of polymerase chain reaction assay to detect the virus in koi, *Cyprinus carpio* koi. Dis. Aquat. Organ. 48: 101-108.

Gilad, O., Yun, S., Adkison, M.A., Way, K., Willits, N.H., Bercovier, H. and Hedrick, R.P. 2003. Molecular comparison of isolates of an emerging fish pathogen, koi herpesvirus, and the effect of water temperature on mortality of experimentally infected koi. J. Gen. Virol. 84: 2661-2668.

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. Org. 60: 179-187.

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: 171-178.

Gunimaladevi, I., Kono, T., Venugopal, M.N. and Sakai, M. 2004. Detection of koi herpesvirus in common carp, *Cyprinus carpio* L., by loop-mediated isothermal amplification. J. Fish Dis. 27: 583-589.

Hedrick, R.P., Gilad, O., Yun, S. and Spangenberg, J.V. 2000. A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. J. Aquat. Anim. Health. 12: 44-57.

Ishioka, T., Yoshizumi, M., Izumi, S., Suzuki, K., Suzuki, H., Kozawa, K., Arai, M., Nobusawa, K., Morita, Y., Kato, M., Hoshino, T., Iida, T., Kosuge, K. and Kimura H. 2005. Detection and sequence analysis of DNA polymerase and major envelope protein genes in koi herpesviruses derived from *Cyprinus carpio* in Gunma prefecture, Japan. Vet. Microbiol. 110: 27-33.

Perelberg, A., Ronen, A., Hutoran, M., Smith, Y. and Kotler, M. 2005. Protection of cultured *Cyprinus carpio* against a lethal viral disease by an attenuated virus vaccine. Vaccine. 23: 3396-3403.

Pikarsky, E., Ronen, A., Abramowitz, J., Levavi-Sivan, B., Hutoran, M., Shapira, Y., Steinitz, M., Perelberg, A., Soffer, D. and Kotler, M. 2004. Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. J. Virol. 78: 9544-9551.

Shapira, Y., Magen, Y., Zak, T., Kotler, M., Hulata, G. and Levavi-Sivan, B. 2005. Differential resistance to koi herpes virus (KHV)/carp interstitial nephritis and gill necrosis virus (CNGV) among common carp (*Cyprinus carpio* L.) strains and crossbreds. Aquaculture. 245: 1-11.