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Detection of Koi Herpesvirus (KHV) in *Cyprinius carpio* (Koi) Stocks using Enzyme-Linked Immunosorbent Assay (ELISA)

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ABSTRACT

Koi herpesvirus (KHV), which is also known as Cyprinid herpesvirus 3 (CyHV-3) infection, is an OIE (international des epizootis) listed disease that caused high losses in common and koi carp in Indonesia and Japan in 2002 and 2003. Since the mid of 2006, the polymerase chain reaction (PCR) has been used in Malaysia for surveillance of koi fingerlings to detect virus nucleic acid, but it has been found to produce unreliable results. Following this, an alternative enzyme-link immunosorbent assay (ELISA) technique for the detection of antibody against KHV was used to find evidence of KHV infection in koi carp stocks on farms that had been sampled for the PCR. For this purpose, a total of 245 serum samples from koi carp stocks were collected and tested for the antibody to KHV by the ELISA at the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) laboratory, Weymouth, UK. Two hundred and eight samples were found to be negative but 37 others were either definitely positive or close to borderline positive and all were retested. The final results showed that 222 (90%) samples were confirmed as negative and 19 (8%) others were definitely positive. Meanwhile, four samples (1.6%) were positive at dilutions of 1:400 or 1:200, but cross reactions with CyHV-1 (causing herpesviral epidermal hyperplasia) could have occurred at those dilutions. Three of the samples were the only positive fish at two sites, but the fourth sample came from a site at which there were 4 definite positive samples (from 20 fish sampled). Thus this study confirmed that Malaysian koi stocks have previously been exposed to KHV. With the lack of bio-security measures and awareness, there was a high probability that the koi carp had been exposed to KHV, leading to subclinical infections and some fish might possibly have become carriers of the virus. Hence, further surveillance needs to be conducted to determine the true situation of the KHV infection in Malaysia.

Keywords: Koi herpesvirus, *Cyprinius carpio*, ELISA

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INTRODUCTION

Koi herpesvirus disease (KHVD) (Hedrick *et al*., 2004), cyprinid herpesvirus-3 (CyHV-3) (Thomas *et al*., 2005), which is also known as carp interstitial nephritis gill necrosis virus (CNGV) (Ronen *et al*., 2003; Arnon *et al*., 2005), is a unique virus which can cause rapid and widespread disease of koi and carps (Ilouze *et al*., 2006). The disease was first detected in Israel and USA in 1998. This disease was then transferred to other countries such as Poland, England, Germany and the Netherlands (Antychowicz *et al.*, 2005). In Israel, for instance, the annual lost was estimated to be $3 million. In Asia, the first KHVD outbreak was detected in Indonesia in April 2002, with an estimated loss of about US$5.5 million (Melba, 2004). The virus then hit Thailand in 2004 during a Koi competition. In Malaysia, KHV was first detected in imported koi broodstocks in 2005 and in juvenile koi at the beginning of 2006 (Nor-Mahya & Azila, 2006). Following this incidence, besides the regular detection of carrier status reported from time to time by AVA Singapore (Azila, pers. comm), NaFisH in collaboration with Perak State Fisheries (PPN), Perak Aquaculture Association, Aquaculture Division (DOF, Putrajaya) and KLIA Fisheries Diagnostic Lab started the monitoring and screening programmes for KHV from July 2006 until December 2008. The technique used for this purpose was polymerase chain reaction (PCR). During this survey, no mass mortality of koi or carps was reported by farmers; however, some of the samples have shown positive detection by commercial kit. As a precaution, positive samples were then retested and confirmed by other labs, whereby the results were usually contradictory.

The PCR is known to have high sensitivity-specificity and able to detect a minimum amount of viral DNA. However, it has some limitation factors, such as the number of fish sampled from the population, the number of DNA copies or virus particles present in the fish, the amount of tissues sampled from the fish and the distribution of virus/DNA in the fish (St-Hillaire *et al*., 2009). Due to the unreliable PCR results, together with the lack of standard protocols for sampling, the EU commission suggested that further screening of broodstocks should be done using enzyme-linked immunosorbent assay (ELISA) to explore whether or not the koi stocks had been exposed to this particular virus. This is in accordance with the EU audit in April 2008 which found big floss in the detection and containment of KHV in Malaysia. Thus, screening of broodstocks might reveal a clearer picture of the occurrence of KHV in Malaysian koi farms.

ELISA is a technique used to detect the presence of KHV antibody in the serum of koi or carps (Adkison *et al*., 2005; Arnon *et al*., 2005; St-Hilaire *et al*., 2009). This immunological based technique is useful in identifying fish that have prior exposure to KHV or fish that have persistent or latent infection, whereby herpesvirus is known to have caused this latent infection (St-Hilaire *et al.*, 2005). ELISA is routinely used in animal disease detection but its usage in aquaculture diseases is still new and thus needs a comprehensive assessment. The advantage of the ELISA is its characteristic of non-lethal sampling procedure, besides its ability to identify positive serum after a long period of exposure. This was demonstrated in the detection of channel catfish virus (CCV) after 6 months of post-exposures (Hedrick *et al*., 1987). Currently, this technique is still under evaluation by the OIE panel and has not been widely used in screening or monitoring the programme of KHV (OIE, 2009). The objective of this surveillance was to provide the information related to the occurrence of KHVD in Malaysia through ELISA, as well as to suggest the control measures of this particular disease in the near future.

MATERIAL AND METHODS

Fish Samples

A total of 245 fish were selected from 15 ponds at koi fish farms in Perak, mainly in the districts of Tronoh and Gopeng. The samples were from the fish aged over 6 months old (14 – 20 cm length),

including the broodstocks. Meanwhile, the source of water for the pond was from the nearby ex-mining pond, with the water temperature normally reached 30°C (between 28 - 2°C) during mid day.

Blood Collection

Fish were anesthetized with clove oil and blood was collected using a 3 cc syringe with a 24 gauge needle. The blood was then transferred into 1.5 mL centrifuge tubes and stored in ice. The blood was allowed to clot for 4 h before centrifuging at 2000 rpm for 20 min to collect the serum. These samples were then kept in -20°C until it was ready for transportation to CEFAS for the ELISA analysis.

Serology

The ELISA plates (Costar®) were coated triplicate with KHV antigens at the concentration of 10 µg/50 µL per well in carbonate buffer, at pH 9.6. The plates were incubated overnight at 20-22°C for 16 h. After washing three times with PBS-Tween 20 (PBST), 50 µL per well koi serum, diluted at 1: 600, was added into each well and it was incubated further for 1 h. The plates were washed three times with PBST and incubated again for 1 h after adding 50 µL mouse anti-carp monoclonal antibody (MAb: Stirling). After three more rounds of washing, 50 µL rabbit anti-mouse conjugate was added into each well, and incubated at 37°C for another hour. After the last washing, the colour reaction was initiated by adding 100 µL of 3,3'5,5'-tetramethyl benzidine (TMB: Sigma), prepared in citrate-buffer and incubated in the dark for 10 min at room temperature. The reaction was stopped by adding 50 µL 1 M H_2SO_4, and the plate was read at 450 nm on iMark microplate reader (BIO-RAD, USA).

RESULTS AND DISCUSSIONS

Out of 245 samples, 37 were either definitely positive or close to borderline positive and all were retested. The final result showed that 222 (90%) samples were confirmed as negative while 19 (8%) others were definitely positive at antibody titre of 1:800 or greater. Meanwhile, four samples (1.6%) were positive at the dilutions of 1:400 or 1:200 but as stated earlier, cross reactions with CyHV-1 (causing herpesviral epidermal hyperplasia) could occur at those dilutions. Three of the samples were the only positive fish at two sites, but the fourth sample came from the site at which there were 4 definite positive samples from 20 fish sampled (*Fig. 1*).

This surveillance finally confirmed that Malaysian koi stocks have previously been exposed to KHV, even though the concerned farmers claimed that they were using local stocks without new introduction or importation of broodstocks in the last 30 years (Azila, pers. comm.). A few data on the import of koi broodstocks between the years of 2000 – 2007 are available. Malaysia took an advanced step to ban the import of koi from Indonesia in 2002, the KHV outbreaks in that country. However, some importation from Japan and the other countries were still continued (Nor-Mahya & Azila, 2006) during these years, and this could have highly contributed to this circumstance.

These imported fish had not been tested for KHV until 2006 with the availability of IQ2000 KHV detection kit in Malaysia. The use of PCR, which is well known for its high specificity and sensitivity, has made this technique the most useful tool for disease diagnosis; however, it does not indicate the state of the disease when dealing with herpesvirus (Morishima, 1999). Herpesvirus is very good in establishing the latent state or carrier status, without any clinical evidence as what has been observed from epidemiological study of KHV conducted by NaFisH in Malaysia (Azila, unpublished data).

The cross reaction between CyHv-1 and KHV, that was observed in 4 of the samples tested at the low serum dilutions of either 1:200 or 1:400, might be due to the high similarity between the 2 viruses. CyHV-1 and KHV are herpesviridae and they infect both koi and common carps but the clinical signs are different. CyHv-1 is usually known as carp pox, the

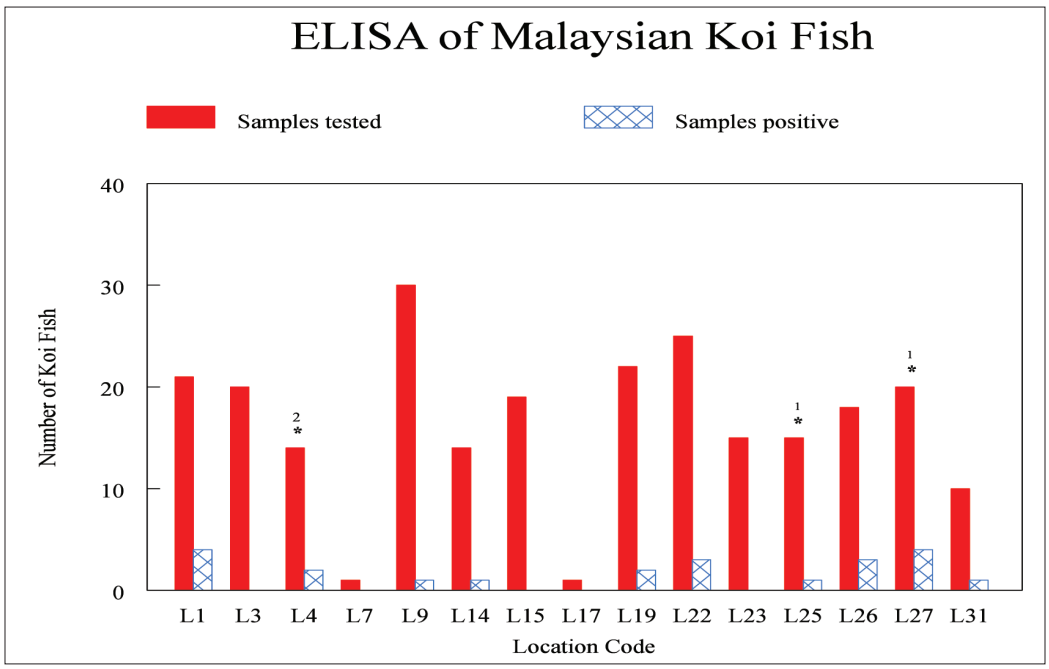

Fig. 1: The number of Malaysian koi fish serum samples retested for ELISA at different dilutions of 1:200, 1:400, 1:800 and 1:1600. At lower dilutions (1:200 & 1:400) cross-reactions with Cyprinid Herpesvirus-1 (CyHV-1) were shown in 4 of the samples (*).

formation of waxy lumps on the skin, fins and lips of fish. However, it is a non-lethal disease, except in small fish and clinical signs usually disappear with the increasing water temperature but the virus can become latent in fish, as what happened in the case of KHV. To some extent, these similarities may complicate the immune system of fish. Following this, Adkison *et al*. (2005) found that cross-reactions between these 2 viruses occurred at 1:50 to 1:500 dilutions. Hence, the researchers suggested higher serum dilutions (i.e. at 1:2500 or greater) should be used to avoid this cross-contamination. In other cases, this cross-reaction was undetectable (St-Hilaire *et al*., 2009), and further testing has to be conducted to clarify this status.

Almost all the farms involved in this study are located in the same area and sharing the same water source and to some extent, the broodstocks. This situation was worsened by the lack of bio-security measures, as well as public awareness towards this disease in the earlier studies. Hence, there was a high probability that these koi carp had been exposed to KHV through sharing of the water source, broodstocks and unsecured trade movement between farms and premises. This KHV, however, did not cause any clinical infections and some fish might possibly just became carriers due to the water temperature that was not at the optimum range for virus to grow and caused problem to the fish (Iida & Sano, 2005). Nonetheless, it is still questionable whether the positive ELISA result is due to latent or persistent infection because as far as author is concerned, no outbreak or clinical signs have been reported in this area yet. Furthermore, a one-time sampling is not conclusive enough to confirm this situation. A series of sampling should therefore be done to determine whether the result is consistent, and PCR specific should be done to KHV as well.

As a consequence, suggestion was made to increase the number of the serum samples to be tested using ELISA to determine the cut-off value that could fit the Malaysian KHV scenario. The studies by Adkison *et al.* (2005) and St.

Hilaire *et al.* (2009) described that the use of koi fish which had been exposed and/or infected by KHV revealed that the detection of the antibody was still detectable at the highest dilution (> 1:1600). In the cases of Malaysia, the status of the samples was unknown and there were assumptions made that the antibody could only be detected at certain value to be optimized later. Therefore, the data gathered may be useful for controlling and prevention of KHV in the future without sacrificing the industry in Malaysia.

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REFERENCES

Adkison, M. A., Gilad, O., & Hedrick, R. P. (2005). An enzyme linked immunosorbent assay (ELISA) for detection of antibodies to the koi herpesvirus (KHV) in the serum of Koi Cyprinus carpio. *Fish Pathol.*, *40*, 53-62

Antychowicz, J., Matras, M., Bergmann, S. M., & Haenen, O. (2005). Epidemiology, pathogenicity and molecular biology of koi herpesvirus isolated in Poland. *Veterinary Research*, *49*(4), 367-373.

Dishon, A., Perelberg, A., Bishara-Shieban, J., Ilouze, M., Davidovich, M., Werker, S., & Kotler, M. (2005). Detection of Carp Interstitial Nephritis and Gill Necrosis Virus in Fish Droppings. *Appl. Environ. Microbiol., 71*(11), 7285-7291.

Hedrick, R. P., Gilad, O., Yun, S. C., McDowell, T. S., Waltzek, T. B., Kelley, G. O., & Adkison, M.A. (2004, March 13). *Initial isolation and characterization of herpes-like virus (KHV) from koi and common carp*. Paper presented at the International Symposium on Koi Herpesvirus Disease-Strategy for Koi Herpesvirus Disease Control, Yokohama, Japan.

Hedrick, R. P., Groff, J. M., McDowell, T., & Wingfield, W. H. (1987). Response of adult channel catfish to waterborne exposure to channel catfish virus. *Prog. Fish-Cult*, *49*, 181-187.

Iida T., & Sano, M. (2005). Koi herpesvirus disease. *Uirusu, 55*, 145-151.

Ilouze, M., Dishon, A., Kahan, T., & Kotler, M. (2006). Cyprinid herpes virus-3 (CyHV-3) bears genes of genetically distant large DNA viruses. *FEBS Letters, 580*(18), 4473-4478.

Melba, G. B. R. (2004). Trans-boundary aquatic animal diseases: Focus on Koi Herpes virus (KHV). *Aquaculture Asia,* 9, 24-28.

Morishima, T. (1999). Progress in diagnosing herpesvirus infections. *Nagoya J. Med. Sci.,* 62, 83-97.

Nor-Mahya, Y., & Azila, A. (2006, June). *Observation of Koi Herpesvirus Disease (KHVD) in ornamental koi carp.* Paper presented at the 4th National Fisheries Symposium, Kuching, Sarawak, Malaysia.

OIE. (2009). Koi herpesvirus disease. Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.3.6. Retrieved from http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/2010/2.3.06_KHVD.pdf

Ronen A., Perelberg, A., Abramowitz, J., Hutoran, M, Tinman, S., Bejerano, I, Steinitz, M., & Kotler, M. (2003). Efficient vaccine against the virus causing a lethal disease in cultured Cyprinus carpio. *Vaccine, 21*(32), 4677-4684.

St-Hilaire, S., Beevers, N., Joiner, C., Hedrick, R. P., & Way, K. (2009). Antibody response of two populations of common carp, Cyprinus carpio L., exposed to koi herpesvirus. *Fish Dis.,* 32, 311-320.

St-Hilaire, S., Beevers, N., Way, K., Le Deuff, R-M., Martin, P., & Joiner, C. (2005). Reactivation of koi herpesvirus infections in common carp (*cyprinus carpio*). *Dis. Aquatic Organisms,* 67, 15-23.

Thomas, B. W., Garry, O. K., David, M. S., Keith, W., Larry, H., Hideo, F., Ikuo, H., Takashi, A., Andrew J. D., & Ronald P.H. (2005). Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family *Herpesviridae. Gen. Virology*, 86, 1659-1667.