

Koi Herpesvirus-associated mortalities in quarantined koi carp in the Philippines

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Abstract

Illegally imported koi carp were confiscated at the Ninoy Aquino International Airport (NAIA), Manila, Philippines by the Fisheries Quarantine and Inspection Service Officers of the Bureau of Fisheries and Aquatic Resources (BFAR). The confiscated fish were turned over to the BFAR Fish Health Laboratory where they were held for observation at a water temperature of 28°C. After 5 days, some fish were showing abnormal swimming behavior and some had died. The most prominent disease signs in the freshly dead and moribund fish were body ulcerations and pale gills showing white necrotic patches, consistent with the clinical signs of KHV infection. Gills were dissected and fixed in 95% ethanol. All of the samples tested positive for KHV in a 1-step PCR assay.

This paper reports the first case of KHV associated mortalities in illegally imported koi carp confiscated by the Fisheries Quarantine and Inspection Service Officers of BFAR. This highlights the importance of the quarantine and inspection service's role in preventing the illegal entry of fish into the country and the introduction of exotic aquatic diseases.

Introduction

Koi herpesvirus (KHV) is a highly contagious disease that can produce mass mortalities in diseased populations of koi and common carp (*Cyprinus carpio*) causing severe financial losses (Dishon *et al.*, 2005). KHV has recently been classified as *Cyprinid herpesvirus 3* (CyHV-3) of the genus *Ictalurivirus*, family *Alloherpesviridae* in the order *Herpesvirales* (Davison *et al.*, 2009).

The principal diagnostic features of KHV infection include severe branchial necrosis caused by viral replication within the cells

of the branchial epithelium and hepatic parenchyma in the affected fish (Hedrick *et al.*, 2000). Other easily observable diagnostic features of the disease include appetite loss, erratic swimming behavior, discoloration and increased respiratory frequency (Gray *et al.*, 2002). A recent study made by Costes *et al.* (2009) showed that the skin is the major portal of viral entry rather than the gills.

The first outbreak of the disease was reported and isolated in the USA in 1998 following outbreaks in koi and common carp in

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Israel and USA (Hedrick *et al.*, 2000). Since then, many cases have been reported and confirmed all over the world (Pokorova *et al.*, 2005). In Asia, outbreaks occurred in 2002 in Indonesia through importation from China via Hongkong (Sunarto *et al.*, 2005). Also in 2002, mass mortality of carp due to KHV infection was experienced in Taiwan (Tu *et al.*, 2004) and in 2003 in Japan (Sano *et al.*, 2004; Iida and Sano, 2005) and was also recently observed in Thailand (Pikulkaew *et al.*, 2009).

This paper reports the first case of KHV associated mortality in illegally imported koi carp, confiscated at the Ninoy Aquino International Airport (NAIA), Manila, Philippines. This highlights the role of Fisheries Quarantine and Inspection Service of the Bureau of Fisheries and Aquatic Resources (BFAR) in preventing the entry of illegally imported fish stocks into the country, which may bring KHV and other exotic diseases.

Materials and Methods

Fish sample

Forty koi carp placed in an oxygenated polyethylene bag and packed in a styrobox were confiscated at the NAIA and transported in the same condition to the Fish Health Laboratory of the BFAR. The koi carp were held at a water temperature of 28°C in an aquarium supplied with constant aeration. Five days after confiscation, 29 fish died and the remaining 11 eventually died within 14 days. Moribund and recently deceased fish showed symptoms such as necrotic gill filaments, body ulcerations, discolored patches and pale body coloration.

Bacterial and Parasitic Analyses

Kidneys of the moribund fish were aseptically dissected and streaked onto nutrient agar plates for the isolation and determination of the dominant bacteria. Similarly, fresh mounts of the gill filaments were made for the examination of parasites.

Sample Collection for PCR Analysis

Portions of the gills were individually and aseptically dissected, fixed in 95% ethanol and stored at 4°C until used.

DNA Extraction

DNA was extracted from the gill tissues fixed in 95% ethanol using DNAzol Genomic DNA Isolation Reagent (Invitrogen, USA) as described by the manufacturer. Briefly, approximately 50 mg of gill tissues were blotted in tissue paper, washed with TE buffer and homogenized in 1 mL DNAzol, followed by centrifugation for 10 min at 14,800 × g at 4°C. Supernatant was transferred to a new tube and the DNA was precipitated by the addition of 500 µL absolute ethanol. DNA pellets were washed twice in 95% ethanol by centrifugation and air-dried for a few seconds. The dried DNA pellets were suspended in 200 µL of 8 mM NaOH, incubated at 45°C for 15 min and added with 20 µL of TE buffer to adjust the pH to 7.2. DNA was diluted 100-fold in TE buffer and stored at -20°C until use.

Detection of KHV using PCR

DNA samples were submitted to one-step PCR test using the SphI-5 primer pair (Gray *et al.* 2002; Yuasa *et al.* 2005). The SphI-5 primer sequences were 5'-GACACCACATCTGCAAGGAG-3' and 5'-GACACATGTTACAATGGTCGC-3' for the

forward and reverse primers, respectively. PCR reaction was carried out in a 25 µL reaction mixture containing 1X PCR Buffer, 2mM MgCl₂, 0.1 mM each of the dNTPs, 0.1 µM each of the forward and reverse primers and 0.6 U Taq DNA polymerase (Invitrogen, USA). One µl of the diluted DNA was used as template in the PCR reaction. Amplification was performed in a programmable thermal cycler (Eppendorf Mastercycler, Germany) with the following amplification profile: initial denaturation at 94°C for 30 s, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min and extension at 72°C for 30 s, and ending with a final extension at 72°C for 7 min. All PCR products were kept at 4°C until ready for electrophoresis. The PCR products were separated in 2% TAE-agarose gel, stained with ethidium bromide and visualized in a Gel Documentation System (Syngene, GeneGenius, UK). KHV specific primers gave an amplification product of 290 bp.

Results and discussion

Illegally imported koi carp were confiscated at the NAIA, Philippines by the Fisheries Quarantine and Inspection Service Officers of BFAR, in coordination with the Bureau of Customs. The koi carp were bought, by a passenger, at an ornamental fish show in China. The origin of the koi carp is unknown and may have been transported into China specifically for the purpose of the fish show.

Bacterial analysis carried out on the kidneys of the moribund fish revealed that there were no dominant bacteria isolated. Further, fresh gill mounts showed low levels of protozoan parasites. These results suggest that neither

the bacteria nor the parasite were the cause of the mortalities in the confiscated koi carp.

However, the result of the PCR analysis as shown in the agarose gel electrophoresis (Figure 1) confirmed the presence of KHV DNA in all gill tissues examined, suggesting the presence of KHV infection in the illegally imported koi carp. Kurita *et al.* (2009) made a molecular epidemiological study comparing the regions of the KHV genomic DNA for sequence variations on the isolate from this disease occurrence and confirmed that the causative agent was KHV classified as an A1 variant of the Asian genotype. Considering that the onset of clinical signs and that the mortality occurred only five days after confiscation, the koi carp might have been latently infected with KHV. Apparently, stressful conditions encountered during the fish show, as well as during transport might have weakened the immune system of the fish. Consequently, this might have caused the replication of the virus and the manifestations of the clinical signs which lead to mortality. The dead and moribund fish showed symptoms characteristic of KHV infection and consistent with the observations of Hedrick *et al.* (2000) during the KHV outbreaks in the United States and Israel in 1998. According to St-Hilaire *et al.* (2005), KHV can remain dormant and non-infectious for several months but can be reactivated to become pathogenic. Reactivation can occur even in fish populations that had never experienced elevated mortality and that they can subsequently infect other fish cohabitated with them especially at elevated temperatures above 20°C. We speculate that the confiscated koi carp were already latently infected with

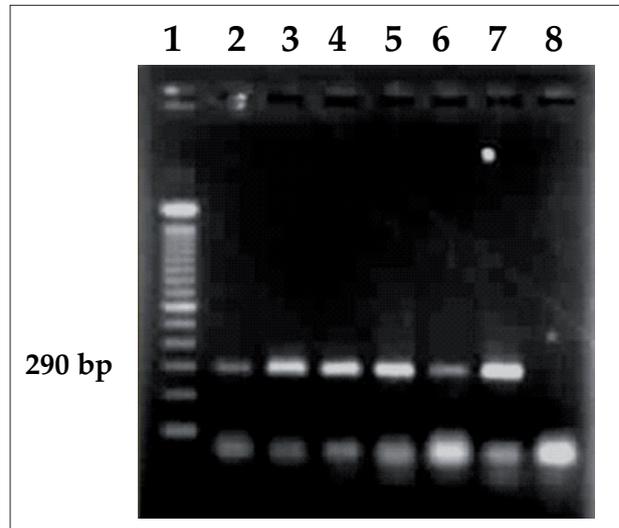


Figure 1. Agarose gel electrophoresis of PCR amplification products: Lane (1): 100 bp DNA marker; (2): Positive control; (3-7): Gill samples of koi carp; (8): Negative control.

KHV, which was subsequently expressed upon exposure to elevated temperatures of 28°C during its quarantine and the stress associated with its transport. Hedrick *et al.* (2000) noted that the clinical signs of KHV can manifest as early as 4 days post exposure to the virus and the infected fish usually dies within 3-4 days upon onset of clinical signs.

This paper reports the first case of KHV associated mortality in illegally imported koi carp confiscated by the Fisheries Quarantine and Inspection Service Officers of BFAR stationed at NAIA. Their role and the strong coordination with the Bureau of Customs personnel involved in the post-border inspection is vital in the prevention of illegal movement of fish which may bring with them exotic diseases, especially when the fish were not subjected to a rigid pre-border health screen. Further, this also aids in the spread of unknown fish diseases that are not yet detectable. It has been known that

inter-regional and international trade in live fish provides a pathway for the movement of pathogens (Hedrick, 1996) and that fish trade including koi shows has contributed to the rapid spread of KHV infection globally (Gilad *et al.*, 2003). So far, there have been no reports of KHV infection in the Philippines as noted by Pokorova *et al.* in 2005. This observation is confirmed by a recent surveillance study made by Lio-Po *et al.* (2009) who reported that to date, no KHV outbreak has been recorded in the Philippines. The KHV-free status in the Philippines might be partly due to the strict implementations of quarantine rules and regulations that have prevented the entry of this exotic pathogen. Continued monitoring, surveillance and strict implementation of quarantine rules are necessary to prevent the entry of exotic pathogens that can affect the economically important plant and animal species.

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