Short communication

First Detection of Cyprinid Herpesvirus 3 in Cultured Common Carp in Vietnam

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ABSTRACT—Cyprinid herpesvirus 3 (CyHV-3) is the causative agent of koi herpesvirus disease and poses a significant threat to common and koi carp. Since its first description, the virus has spread worldwide. In 2010 and 2011, mass mortality of common carp was reported in a hatchery and several ponds in northern Vietnam. CyHV-3 was detected in infected tissues by PCR, confirmed by sequencing of the thymidine kinase gene. Duplex PCR demonstrated that the virus belonged to the genetic lineage (I⁺⁺/II[−]) previously described in Indonesia. This suggests CyHV-3 as the causative agent of the disease outbreak and constitute the first report of CyHV-3 in Vietnam.

Key words: Cyprinid herpesvirus 3, outbreak, Vietnam, first detection, genetic lineage

Cyprinid herpesvirus 3 (CyHV-3) is a member of the *Alloherpesviridae* belonging to the genus *Cyprinivirus* (Davison *et al.*, 2013) and the causative agent of koi herpesvirus disease (KHVD) which causes high morbidity in koi and common carp (Hedrick *et al.*, 2000; Gotesman *et al.*, 2013). Clinical and postmortem signs of the disease include hyperplasia and necrosis of the gill epithelium, sunken eyes, skin discoloration, mucus hypersecretion, interstitial nephritis, splenitis and enteritis (Hedrick *et al.*, 2000). While CyHV-3 is often described as specific to common carp, it can infect

other species including members of the *Ancistrus* genus that can act as asymptomatic carriers of the virus (Bergmann *et al.*, 2010). The susceptibility of the goldfish *Carassius auratus*, however remains unclear: while presence of CyHV-3-DNA was detected by PCR (El-Matbouli *et al.*, 2007; Sadler *et al.*, 2008), a recent study did not reproduce the disease in goldfish cohabitating with infected carp and did not detect viral DNA in the fish organs (Yuasa *et al.*, 2013).

Since the first description of the virus from the USA and Israel (Hedrick *et al.*, 2000), CyHV-3 has been detected in many countries, including Asia (Gotesman *et al.*, 2013). In Vietnam, no CyHV-3 was detected in various wild and cultured carps sampled between 2004 and 2007, suggesting that the country was free of CyHV-3 (Lio-Po, 2011).

In the study area, the Son La Province, aquaculture is mainly practiced on a small scale (Steinbronn, 2009) and supplied with fish fry and fingerlings originating from a single hatchery and nursery in Son La City. Farming occurs in earthen ponds and aims either at subsistence or sales on the local market. Polyculture is generally practiced with grass carp *Ctenopharyngodon idella* as the main species alongside common carp, silver carp, bighead carp, mrigal carp, mud carp or Nile tilapia (Steinbronn, 2009; Pucher *et al.*, 2013).

In 2010 and 2011, mass mortality of carp was reported in a hatchery and several farming ponds in northern Vietnam. Here, we present the result from the investigation into the cause of these mass mortalities and report on the first known incidence of CyHV-3 in Vietnam.

With the onset of the rainy season in March 2010, episodes of high morbidity and mortality occurred among common carp in earthen ponds in a polyculture hatchery in Son La City, northern Vietnam at water temperature between 18°C and 25°C. At the beginning of the outbreak, only small common carp in the main hatchery facility in Son La City experienced mortalities. However by April 2010 the brood stock had become affected and approximately 25% of the adult fish died. Notably, fish belonging to other species within the same facility did not exhibit signs of disease. Six common carp (13.5–21.0 cm total length) presenting clinical signs (gill necrosis, sunken eyes and pale patches on the skin) were sampled (Fig. 1). Internally, the liver appeared discoloured and the kidney slightly enlarged. Two grass carp and one silver carp were also sampled to investigate their potential role as reservoir for the disease. Samples were transported on ice to the field laboratory in Yen Chau district for standard post-mortem investigations.

In May 2011, mass mortalities of common carp occurred in seven ponds in Yen Chau district (Son La Province), which is located approximately 150 km from the hatchery. The affected fry originated from the Son

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Fig. 1. Gross clinical signs on an affected carp sampled from the hatchery in northern Vietnam. Note areas of necrosis on the gills (arrows).

La City hatchery and the disease outbreak coincided with changes in the weather and higher temperatures, strong rainfalls and corresponding sudden changes in water quality. Tissues from 16 common carp originating from seven ponds were sampled in this occasion.

All sampled fish were examined clinically and external abnormalities were recorded. Skin, gills and intestinal smears were prepared and examined under light microscopy to investigate the presence of external and internal parasites. Conventional bacteriological investigations were carried out on Columbia agar supplemented with 5% sheep blood (Biomérieux) and nutrient agar (Merck). The plates were incubated at 26°C for up to 5 days. Tissue samples were taken from the skin, gills, hepatopancreas, kidney, spleen and brain. Part of the samples was frozen and the rest of the organs were pooled for each fish, fixed in 70% ethanol for DNA extraction. Samples were transported under refrigerated conditions to the Clinical Division of Fish Medicine at the University of Veterinary Medicine (Vienna, Austria).

Fish tissues in ethanol were ground in liquid nitrogen and 25 mg of tissue powder was decanted into a 2-mL micro-centrifuge tube. For each of the pooled fish samples, DNA was extracted using a DNeasy-Blood and Tissue kit (Qiagen), eluted in 100 µL of buffer and stored at -20°C until use.

PCR was applied on each of the 41 genomic templates following the procedure described by Bercovier *et al.* (2005) modified with 39 cycles instead of 35 and an annealing temperature of 55°C instead of 52°C. The amplification product bands (409 bp) were excised from the gel and cloned into pCR4-TOPO vector for sequencing at a commercial facility (LGC Genomics GmbH). The Basic Local Alignment Search Tool (NCBI) was used to compare the insert's sequence with a CyHV-3 thymidine kinase (TK) gene sequence deposited in Gen

Bank (AB375387).

To determine the genetic lineage of the CyHV-3 detected in this study (Isolate Viet-10), a duplex PCR assay was performed using primers oPVP53 and oPVP54 for marker I and primers oPVP55 and oPVP56 for marker II (Bigarré *et al.*, 2009).

Examination showed hyper-secretion of mucus on the skin, dermal lesions, gill necrosis and enlarged kidney in the diseased common carps. Meanwhile, no clinical signs were visible on the grass and silver carp. Parasitological investigations revealed a moderate infestation with *Dactylogyrus*, *Gyrodactylus*, *Trichodina*, *Ichthyophthirius* and *Chilodonella* sp. on the gills and skin, whereas no parasites were found in the intestines. The external parasites detected on the skin and gills of the fish were excluded as the cause of mortalities because the levels of infestation were low. No bacteria could be isolated from any of the internal organs. Histopathologically, necrosis and fusion of the secondary lamellae were observed in the infected fish. The kidney and liver of affected fish showed mild inflammation and hemorrhages.

PCR assays amplified the expected 409 bp fragment from 4 out of 22 common carp samples (in both 2010 and 2011) as well as from one silver carp and one grass carp (Fig. 2). Amplicons were 99% homologous to the CyHV-3 TK gene deposited in GenBank. The reason for this low number of positive samples (only 4 out of 22 common carp displaying clinical signs) is not clear: It is possible that DNA might be degraded in the interval between the sampling and the genomic isolation. Since the clinical signs observed on the diseased common carps were also consistent with KHVD, the results

Fig. 2. Diagnostic PCR for CyHV-3 thymidine kinase gene. The expected amplification product is 409 bp. Lanes numbered 62–68 were common carp, No. 69 (silver carp) and No. 70 (grass carp). No. 65–68 and 69 showed positive results. M: molecular markers, +ve: positive control, -ve: negative control.

suggest CyHV-3 as the causative agent of the disease outbreak. The present study reports the first detection of CyHV-3 in Vietnam. A previous surveillance of CyHV-3 in Vietnam suggested that the country was free of CyHV-3 by 2007 (Lio-Po, 2011). It is likely that the virus was introduced into Vietnam through the trade of fish seed or brood-stock as fish movements.

Both outbreaks occurred at the onset of the hot rainy season when water temperatures rose above 18°C, which is consistent with the fact that the virus is known to cause diseases at temperature ranging from 15–28°C (Gotesman *et al.*, 2013). Moreover, it is likely that the stress associated with the sudden change in water parameters contributed to the CyHV-3 outbreaks. This pattern where disease outbreaks closely follows changes in water conditions at the onset of the rainy season has previously been reported concerning the Vietnamese aquaculture (Pucher *et al.*, 2013).

Although no mortalities were recorded in grass and silver carp, the virus could be detected from fish belonging to both species. This suggests that these fish could act as a symptomatic carrier for the virus, as previously reported (Kempter *et al.*, 2012). Interestingly, Yuasa *et al.* (2013) suggested that, in goldfish, the virus might survive for a few days on the skin and gill surface, however El-Matbouli *et al.* (2007) reported about transmission of the disease from infected goldfish to common carp per exposure. Because the samples in the

present study were pooled as mention above, it cannot be ascertained, which organs were positive.

Because the virus was detected in 2010 at a large hatchery in Son La City and 1 year later in the ponds stocked with fish from this hatchery, it seems plausible that this hatchery spread the virus through farms in the region. Rural hatcheries do not perform quarantine of newly introduced fish and lack diagnostic tools; likely contributing to the spread of infection.

The duplex PCR for marker I and marker II generated two bands of 168 and 278 bp, corresponding to the Asian (allele I⁺) and European lineage (II⁻ allele), respectively (Fig. 3). While Asian genotypes of the virus are genetically distinct from European isolates (Kurita *et al.*, 2009), the virus displayed a mix of patterns with bands corresponding to both the Asian (I⁺) and European lineage (II⁻). The I⁺/II⁻ genotype was previously described in Indonesian isolates and represents a new intermediate genetic lineage (Sunarto *et al.*, 2011). In the future, it would be worthwhile to investigate the lineages of Vietnamese CyHV-3.

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Fig. 3. Duplex PCR illustrating the genetic lineage of the CyHV-3 detected. The 168 and 278 bp bands correspond to the Asian (isolate KHV-TP 30 originating from Taiwan) and European lineage (Isolate 3468/10:1/3, originating from a clinical case in Austria), respectively. Samp 1 and Samp 2: Samples from pooled organs originating from common carp, -veco: negative control, Pos co: positive control (Isolate 3468/10:1/3), Mar: molecular markers.

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