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Low Prevalence of Cyprinid Herpesvirus 3 Found in Common Carp (*Cyprinus carpio carpio*) Collected from Nine Locations in the Great Lakes

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ABSTRACT: Cyprinid herpesvirus 3 (CyHV3) is a viral disease of fish first detected in the United States in 1998. Since that time, mortality events in common carp (*Cyprinus carpio carpio*) have occurred in several locations within the Great Lakes basin, but not within the Great Lakes themselves. We sampled 675 carp from 20 sites across the Great Lakes and Lake St. Clair, Michigan, USA, between 19 July and 26 September 2010. We tested the gill and a pooled internal organ sample from each fish for CyHV3 with the use of a quantitative polymerase chain reaction (qPCR) assay. Virus was detected in 18 fish from nine sites in four lakes (Lakes Michigan, Huron, St. Clair, and Ontario). Tissues from these 18 fish were also tested for CyHV3 with the use of the PCR assay recommended by the World Organization for Animal Health; amplification was achieved from two fish and confirmation by sequencing of CyHV3 from one fish collected in Lake St. Clair. The results of this study suggest that CyHV3 is present in the Great Lakes.

Key words: Common carp, cyprinid herpesvirus 3, *Cyprinus carpio*, disease surveillance, koi herpesvirus.

Cyprinid herpesvirus 3 (CyHV3; commonly referred to as koi herpesvirus) was first isolated in the United States and Israel (Hedrick et al., 2000) and has since been identified in Africa, Asia, and Europe (Pokorova et al., 2005). Mortality due to CyHV3 can be high, reaching 80–100% in adult koi (*Cyprinus carpio carpio*; Hedrick et al., 2000). In general, younger fish are more susceptible (more likely to die as a result of CyHV3 exposure; Perelberg et al., 2003). Clinically affected fish typically have dermal hemorrhage and a characteristic gill hyperplasia and necrosis (Pokorova et al., 2005). This virus enters fish through the skin (Costes et al., 2009) and gills (Pokorova et al., 2005).

Detection of CyHV3 in cell culture can be difficult due to virus latency, so molecular methods such as quantitative polymerase chain reaction (qPCR) are frequently used. Despite detection difficulties, surveillance for CyHV3 has been shown to be effective in wild populations. Uchii et al. (2009) detected CyHV3 in 6% of common carp less than 300 mm and 31% of common carp larger than 300 mm in Lake Biwa, Japan. The first reported mortality event in wild fish within the Great Lakes basin attributed to CyHV3 occurred in the Chadakoin River in New York State in 2004 (Grimmitt et al., 2006). Additionally, multiple mortality events over 2 yr were reported in several lakes in Canada, all but one of which occurred within the Great Lakes Basin (Garver et al., 2010). Given the presence of CyHV3 in lakes within the Great Lakes basin, we examined the effectiveness of detecting CyHV3 with the use of molecular virus surveillance methods in the Great Lakes.

All fish were collected from 19 July through 26 September 2010 by boat electrofishing with the use of a Smith-Root model VIa electrofisher (Smith-Root, Vancouver, Washington, USA) in nearshore habitats. At each site ($n=28$; Fig. 1), electrofishing was conducted until 60 common carp were collected or until 4 hr had elapsed. We were unsuccessful at collecting 60 carp from several sites, and at some sites were not able to collect any carp (Fig. 1). The boat used for fish

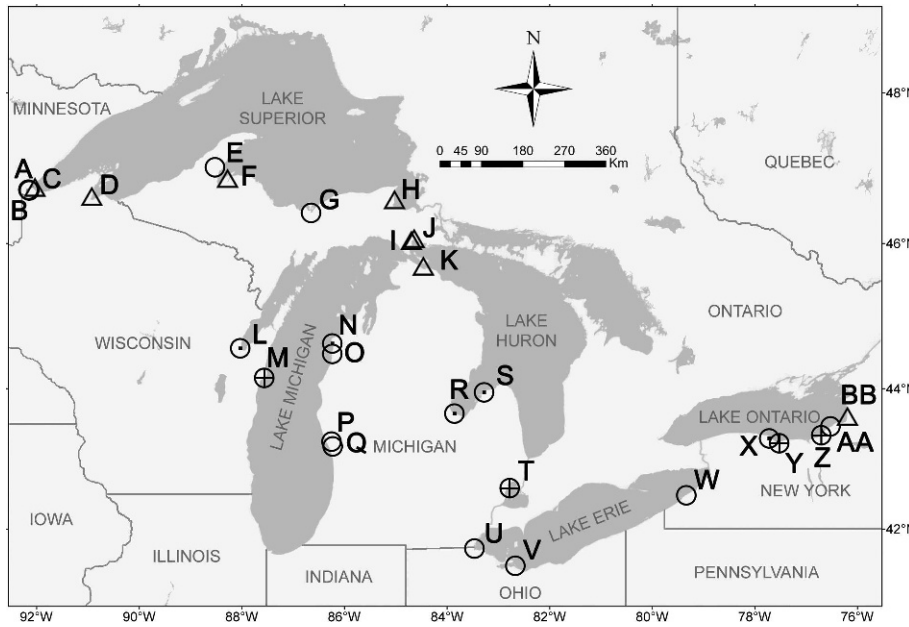

FIGURE 1. Map of sites sampled for common carp (*Cyprinus carpio carpio*). Carp collection was unsuccessful at eight sites (triangles). Carp were collected at 20 sites (circles), but tested positive for CyHV3 at nine sites in only one replicate (circle with dot in center) or in more than one replicate (circle with cross in center) by qPCR. Circles with a blank center represent sites where carp were collected, but none tested positive by qPCR.

collection and all associated gear was disinfected between sites as described in the Wisconsin Boat and Gear Disinfection Protocol (2008).

Upon capture, fish were immediately euthanized with approximately 500 mg/L tricane methanesulfonate (Western Chemical, Ferndale, Washington, USA) buffered 1:1 (w:w) with sodium bicarbonate (Sigma, Carlsbad, California, USA). The length of each fish was measured and two samples of tissue collected: (1) a gill sample and (2) a pooled sample of the liver, kidney, and spleen. All dissection instruments and work areas were disinfected with a 10% solution of household bleach (6% sodium hypochlorite, Clorox, Oakland, California, USA; final solution approximately 10,000 mg/L free chlorine, contact time at least 30 sec) between each fish. A new, sterile scalpel blade was used to dissect each fish. Collected tissues (gill and pooled internal organ sample) were immediately frozen on dry ice for later CyHV3 testing by quantitative PCR (qPCR). Fish carcasses were disposed at local landfills or transported back to Cornell University (Ithaca, New York, USA) for alkaline digestion.

Viral nucleic acids were isolated using MagMax magnetic bead extraction (Life Technologies, Carlsbad, California, USA) and a MagMax-96 viral isolation kit following the protocols described in the kit and the manufacturer's extraction program AM1836_DW_50_V2.

Nucleic acid quantity and purity was assessed with a NanoVue spectrophotometer (GE Healthcare, Piscataway, New Jersey, USA). The qPCR assay used was as described by Gilad et al. (2004). All samples were initially run in duplicate. If CyHV3 was detected in only one replicate, a third replicate was run. For prevalence calculations, any fish where at least one

replicate tested positive for CyHV3 by qPCR was considered a positive fish.

For confirmation under World Organization for Animal Health (OIE) standards, all samples testing positive by qPCR were subjected to the PCR protocol in the OIE Manual of Diagnostic Tests for Aquatic Animals (Way, 2011). Specifically, we used the PCR primers originally described by Bercovier et al. (2005) that amplify a 409 base-pair region of the thymidine kinase (TK) gene of CyHV3 and 5 μL of 10× buffer. Samples with an amplicon visible after ethidium bromide staining of an agarose gel were excised, cleaned up with a QIAquick gel extraction kit (Qiagen, Valencia, California, USA), and sequenced by capillary sequencing.

Data analysis was conducted with the use of OpenEpi (Dean et al., 2009). For apparent prevalence, 95% mid-P exact binomial confidence intervals are reported. For sensitivity and specificity reporting, 95% confidence intervals are reported with the Wilson score.

Carp were collected at 20 of 28 sites (Fig. 1). The mean total length of collected fish was 612 mm (range 235–1,080 mm). The sex ratio of collected fish was balanced between males (n=316) and females (n =344); 15 fish were immature. At least one abnormality was noted at gross necropsy in 548 fish. In the majority of these fish (482), coelomic adhesions were observed. Other gross abnormalities included mottled gills (n=60), external trauma (n=51), external hemorrhage (n=13), friable spleen (n=8), a tumor appearing to originate from the testes (n=5) internal hemorrhage (n=3), pebbled appearance to spleen (n=3), splenomegaly (n=3), pale gills (n=3), abdominal ascites (n=2), pale anterior kidney (n=2), egg bound (n=1), multifocal cystic lesions over body and fins (n=1), and a 1-mm white nodule on gastrointestinal tract (n=1). The tumors observed in five of the collected fish are most likely seminomas, which have been reported in common carp × crucian carp (*Carassius carassius*) hybrids; the presence of these tumors decreases survival during the next spawning (Granado-Lorencio et al., 1987).

TABLE 1. Contingency table comparing detections of CyHV-3 in a gill biopsy and a pooled internal organ sample of liver, kidney, and spleen for common carp (*Cyprinus carpio carpio*) samples for all detections by qPCR.

	Pool		
	Positive	Negative	Total
Gill			
Positive	3	11	14
Negative	4	656	660
Total	7	667	674

Eighteen fish tested positive by qPCR for CyHV3 at nine sites representing four water bodies (Lakes Michigan, Huron, St. Clair, and Ontario; Fig. 1). Of the 18 fish that tested positive, amplicons using the PCR primers were obtained in two fish, and successful sequencing was obtained from one fish collected in Lake St. Clair. There was 100% nucleotide identity between the sequence from the fish collected in this study (from Lake St. Clair) and all other isolates used (GenBank accession numbers HM347112.1, JN 180630.1, HM347096.1) in an alignment, except for one isolate from the United States (EU932920.1). There was no association between abnormalities and the probability of testing positive in this sample. Virus was detected from seven female, ten male, and one immature common carp. The overall apparent prevalence detected in this study was 2.52% (95% confidence interval [CI]: 1.53, 3.93).

The median quantity detected by qPCR was 12 genome copies (range: 1–819). More positives were detected in the gill biopsy than in the pooled organ sample (Table 1). The three fish where CyHV3 was detected in pool and gill had levels in gill at 10^2 or above, and two of those fish had levels in the pooled organ sample at 10^2 or above. Cohen's unweighted kappa (a measure of concordance between tests) was 0.2757 between gill and a pooled

organ sample (95% CI: 0.2046, 0.3467; Table 1). The sensitivity of testing gill compared to a pooled organ sample was 42.86% (95% CI: 15.82, 74.95; the specificity was 98.35% (95% CI: 97.07, 99.08); the positive predictive value was 21.43% (95% CI: 7.57, 47.59), and the negative predictive value was 99.39% (95% CI: 98.45, 99.76).

Although concordance was fair between gill and a pooled organ sample (Landis and Koch, 1977), this appeared to be due to the very low quantities detected in fish. When detected quantities were 10^2 or higher, CyHV3 was detected with the use of both sample types. Additionally, the high negative predictive value suggests that a negative gill sample is highly predictive of a negative pooled organ sample. More comparison of these sample types is needed; however, the potential for a nonlethal sample is important from a welfare as well as a logistical standpoint. Adult carp are very large, and proper euthanasia and carcass disposal, especially in rural areas, can be problematic.

In addition to the common carp collected, three common carp–goldfish hybrids (*Cyprinus carpio* × *Carassius auratus*) and one triploid grass carp (*Ctenopharyngodon idella*) were sampled. All of these fish tested negative for CyHV3. There are conflicting reports on the susceptibility of carp–goldfish hybrids (Michel et al., 2010) and of grass carp (Perelberg et al., 2003). We did not collect enough of either species to assess the prevalence of CyHV3 adequately. To our knowledge, this is the first report of grass carp in Lake Ontario.

No virus isolation in cell culture was conducted during this study, so we cannot be certain that the viral genomes detected in carp were from infectious virus particles. We were able to confirm detection from one fish with the use of standards established by the OIE. It is not surprising that so few fish were confirmed by OIE methods, because the levels detected by PCR were very low and qPCR has a lower detection limit (1–5 virions; Bergmann et al., 2010) than the OIE PCR used (30 virions; Bercovier et al., 2005).

This study presents evidence that CyHV3 is present in at least three of the five Great Lakes as well as Lake St. Clair. At some sites, particularly in Lake Michigan and Lake Superior, the temperature was below that where a stressful event can reactivate latent CyHV3, which may have decreased our detection in these areas (Eide et al., 2011). The history of this virus in this system is not known, but there have been at least two reports of CyHV3-associated mortality events in smaller lakes within the Great Lakes basin since 2004. Although common carp are not native to the Great Lakes, they currently play an important role in the ecology of nearshore areas of the Great Lakes, and more information is needed on the possible effects of the changes in population dynamics that could be brought on by widespread CyHV3 infection (Matsui et al., 2008).

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