Detection of Carp Interstitial Nephritis and Gill Necrosis Virus in Fish Droppings

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Carp interstitial nephritis and gill necrosis virus (CNGV) is an unclassified large DNA virus that morphologically resembles members of the Herpesviridae but contains a large (ca. 280-kbp) linear double-stranded DNA. This virus has also been named koi herpesvirus, koi herpes-like virus, and cyprinid herpesvirus 3. CNGV is the cause of a lethal disease that afflicts common carp and koi. By using immunohistochemistry, molecular analysis, and electron microscopy we previously demonstrated that this virus is present mainly in the intestine and kidney of infected fish. Based on these observations, we postulated that viruses and/or viral components may appear in droppings of infected carp. Here we report that (i) by using PCR we demonstrated that fish droppings contain viral DNA, (ii) fish droppings contain viral antigens which are useful for CNGV diagnosis, and (iii) fish droppings contain active virus which can infect cultured common carp brain cells and induce the disease in naive fish following inoculation. Thus, our findings show that CNGV can be identified by using droppings without taking biopsies or killing fish and that infectious CNGV is present in the stools of sick fish. The possibility that fish droppings preserve viable CNGV during the nonpermissive seasons is discussed.

Common carp (Cyprinus carpio) is a fish species that is widely cultivated for human food; 1.5 million metric tons is harvested annually, principally in China and many other Asian and European countries (www.fao.org). Unlike common carp, the koi subspecies (Cyprinus carpio koi) is a beautiful and colorful fish, and it has become part of a worldwide hobby consisting of keeping the fish in backyard ponds and large display aquaria for personal pleasure and competitive showing. This hobby originated with the Romans in the first century A.D., matured into the present science and art practiced in Japan, and subsequently spread worldwide (1).

A lethal disease encountered in common carp and koi in Israel has also been observed in North America, Europe, and Southeast Asia (2, 10, 16, 21; for a review see reference 7), and this disease causes severe financial losses. The mortality rates have consistently been more than 80% in all ponds (3, 10, 12). Although the disease is highly contagious and extremely virulent, morbidity and mortality are restricted to koi and common carp populations (16). The disease frequently appears during the transient stage in the spring and fall. Thus, it seems that the agent that causes the disease has quite a narrow host range, and its effect in open-air ponds is restricted to specific temperature conditions (18 to 28°C) (3, 17, 20).

This disease is caused by an unclassified virus whose morphology resembles that of herpesviruses, and the double-stranded linear DNA molecule is ca. ~280 kbp long, which is larger than the DNA molecules of other herpesviruses. The viral DNA sequences published so far show that the viral genome is highly divergent, containing only small fragments (16 to 45 bp) that are similar to the genomes of several large DNA viruses. However, amino acid sequences encoded by the viral DNA fragments exhibit similarity primarily to sequences found in members of the Poxviridae, the Herpesviridae, and other large double-stranded DNA viruses (12). Based on these data we temporarily named this virus carp interstitial nephritis and gill necrosis virus (CNGV), although it is also named koi herpesvirus, koi herpes-like virus, and cyprinid herpesvirus 3 (CyHV-3). In recent publications the authors indicate that a number of putative genes have been identified which exhibit significant similarity to genes of CyHV-1 and CyHV-2 and a more distant relationship to genes of ictalurid herpesvirus 1 (7, 22).

CNGV harvested from koi fin cell cultures causes death in 80 to 97% of naive common carp and koi upon bath immersion or injection (10, 16). The virus remains contagious for at least 4 h in water (16, 17) and probably penetrates the fish body through gills, as gills and gill rakers are the first organs affected by the virus (18). Although we previously suggested that CNGV is shed from the gills into the water (18), the mode by which the virus is contagious in ponds is not yet known. CNGV propagates mainly in the intestine and kidney of infected fish, as shown by electron microscopy, by immunohistochemistry, and by using kidney extracts as a source for virus isolation (5, 10, 18, 21). We therefore hypothesized that virus and/or viral proteins may be present in droppings of sick fish. Here we report that fish droppings contain viral proteins and viral DNA, which can be identified by an enzyme-linked immunosorbent assay (ELISA) and PCR. Moreover, the fish feces contain infectious virus, which causes plaques in tissue culture.

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were rinsed twice with wash buffer (PBS with 0.05% Tween 20 [PBST]) and carbonate-bicarbonate buffer, pH 9.6) and incubated at 4°C overnight. The plates were stored at 70°C until it was used. Atten- tuated CNGV was developed as previously described (17).

**Fish infection.** Infection was carried out by intraperitoneal injection of fingerlings (10 g) with 0.2 ml of a virus suspension containing 200 PFU as previously described (18).

**Sample preparation.** Fish droppings were collected in tubes from the bottoms of the tanks (sediment). The water was discarded from the tubes, and samples were suspended in phosphate-buffered saline (PBS) (1 part sample to 5 to 10 parts PBS, wt/wt). Alternatively, naive and sick fingerlings were anesthetized, and their hind intestines and intestinal secretions were transferred into 0.5 ml of PBS. Specimens were divided into aliquots and stored at −70°C until they were used. Culture media or the stool supernatants were diluted in PBST, added to wells, and incubated for 1 h at room temperature. The plates were washed four times with wash buffer. The second antibody (100 μl biotinylated rabbit anti-CNGV immunoglobulin G [IgG] diluted 1:1,000 in blocking buffer) was added to each well, and the plates were incubated at room temperature for 1 h and then washed four times as described above. Horseradish peroxidase-conjugated streptavidin (1 μg/ml in 100 μl/well of 3% cold fish gelatin in PBST) was incubated for 30 min at room temperature. The wells were washed four times, and 100 μl of 3,3’,5’,5’-tetramethylbenzidine reagent was added to each well, followed by incubation for 15 to 20 min. The reactions were halted by adding 50 μl of 0.5 M H2SO4, and the results were then determined with an ELISA reader at 450 nm.

**Indirect immunofluorescence microscopy.** Koi fin (KF) cells infected with CyHV-1 and rabbit anti-CyHV-1 serum were generously provided by R. P. Hedrick (School of Veterinary Medicine, University of California, Davis). The following staining procedure was used. Fixed cells were incubated for 20 min at room temperature in PBS containing 1% bovine serum albumin (Sigma). Rabbit anti-CyHV-1 serum was diluted 1:50 and rabbit anti-CyHV-1 was diluted 1:10,000, both with PBS containing 1% bovine serum albumin. Cells were washed twice with PBS and stored at −70°C until they were used for infection, ELISA, or PCR tests. For tissue culture experiments samples were also passed through a 0.45-μm Millipore filter.

**ELISA.** Anti-CNGV serum was generated by immunizing a rabbit, as previously described by Ronen et al. (20) and Pikarsky et al. (18). In order to reduce nonspecific background, the antiserum was absorbed in a mixture of Fc and koi fish powder prepared from muscles and kidneys of healthy fish, as described by Harlow and Lane (8). Microwells (catalog no. 439454; Nunc) were precoated with 100 μl of rabbit anti-CNGV serum diluted 1:1,000 in coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6) and incubated at 4°C overnight. The plates were rinsed twice with wash buffer (PBS with 0.05% Tween 20 [PBST]) and blocked with 200 μl/blocking buffer (5% nonfat dry milk in PBST) for 4 h at room temperature. Following two rinses with 400 μl wash buffer, the plates were left to dry and stored at 4°C until they were used. Culture media or the stool supernatants were diluted in PBST, added to wells, and incubated for 1 h at room temperature. The plates were washed four times with wash buffer. The second antibody (100 μl biotinylated rabbit anti-CNGV immunoglobulin G [IgG] diluted 1:1,000 in blocking buffer) was added to each well, and the plates were incubated at room temperature for 1 h and then washed four times as described above. Horseradish peroxidase-conjugated streptavidin (1 μg/ml in 100 μl/well of 3% cold fish gelatin in PBST) was incubated for 30 min at room temperature. The wells were washed four times, and 100 μl of 3,3’,5’,5’-tetramethylbenzidine reagent was added to each well, followed by incubation for 15 to 20 min. The reactions were halted by adding 50 μl of 0.5 M H2SO4, and the results were then determined with an ELISA reader at 450 nm.

**PCR.** DNA was extracted from cultured cells and intestine and stool samples with phenol and precipitated with ethyl alcohol. DNA pellets were suspended in ultrapure water (Biological Industries, Kibbutz Beit Haemek, Israel).

The following primers, derived from viral DNA amplicons (accession no. AF289888), were used for amplification of viral DNA fragments by PCR that yielded a 701-bp product: forward primer AP1 (5’-CCCCATGAGCCTGTAGG ACGCCG-3’) and reverse primer AP2 (5’-GCACCCCGCAGTGGTCCTTTGCG- 3’). DNA preparations (20 to 50 ng) were used as templates in reaction mixtures containing 0.5 μM forward primer, 0.5 μM reverse primer, 1 mM MgCl2, each of deoxynucleoside triphosphate at a concentration of 0.1 mM, 1 U of FastStart Taq
DNA polymerase (Roche), and 10× reaction buffer containing 500 mM Tris-HCl, 100 mM KCl, 50 mM (NH₄)₂SO₄ (pH 8.3) (Roche). Cycling was performed as follows: 10 min at 95°C and then 30 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 45 s. PCR products were resolved on a 1% (wt/vol) agarose gel in 0.5× TAE (40 mM Tris-acetate, 1 mM EDTA). Positive PCR products were extracted using a QIAquick gel extraction kit (QIAGEN) according to the manufacturer’s instructions and were cloned into the pGEM-T Easy vector system (Promega). Sequencing of the inserted viral DNA fragments was performed for both strands using the plasmid-derived M13F and M13R primers. Sequencing was performed by the dideoxynucleotide terminator cycle sequencing method using a Prism BigDye Ready Reaction terminator cycle sequencing kit (PE Applied Biosystems). The reaction and cycling conditions were chosen by using the manufacturer’s protocol. Sequencing reactions were performed with an ABI 3700 DNA analyzer (PE Applied Biosystems) at the Center for Genomic Technologies, Hebrew University, Jerusalem, Israel.

RESULTS

Identification of CNGV DNA in excrement of infected fish. Previous results (3, 5, 12, 16, 18) demonstrated that CNGV is present in kidneys and intestines of infected fish. We asked whether viral DNA is present in the hind intestine and excreta of infected fish. Excrement samples collected from fish infected by bathing (18) were positive for CNGV as early as 5 to 7 days p.i. (not shown). In order to synchronize the infection, fish were infected with CNGV, and three fish were sacrificed daily; the sacrificed fish were dissected, and their hind intestines and intestinal secretions were collected. The DNA was extracted from all these samples and assessed by PCR. The detection limit of the PCR was 40 fg of viral DNA/reaction mixture (not shown). Viral DNA was identified in the intestine as early as 4 days p.i. (Fig. 1B), which verified our previous electron microscopy results (16). Figure 1A clearly shows that viral DNA appeared in the intestinal secretions on day 5 p.i. These results demonstrate that viral components are secreted from the intestine into the feces and suggest that fish excrement can be used for CNGV diagnosis. We decided to examine the possibility of using ELISA as an appropriate diagnostic tool for CNGV infection.

Detection of CNGV by ELISA. In order to optimize antigen capture by ELISA, we first used tissue culture media harvested from CNGV-infected and uninfected CCB cultures. ELISA microwells were coated with rabbit anti-CNGV serum diluted 1:1,000, and virus harvested from tissue cultures was applied to the plate. Figure 2 shows that the optical density increased in proportion to the virus concentration and that our assay was capable of detecting concentrations of CNGV as low as 1 to 8 PFU/ml.

Specificity of anti-CNGV serum. The possibility of using ELISA as a diagnostic tool for CNGV necessitated analysis of the antigenic cross-reactivity of the anti-CNGV serum with similar viruses. Recently described work of Waltzek et al. (22) showed that there is significant similarity between putative CNGV genes and CyHV-1 and CyHV-2 genes. We therefore tested our anti-CNGV serum with KF cells infected with CyHV-1 5 days p.i. Figure 3 shows that there was no cross-reactivity between the anti-CNGV serum and the CyHV-1-infected cells. Furthermore, no cross-reactivity between the
anti-CyHV-1 serum and CNGV-infected cells was observed (not shown).

**Detection of CNGV in fish excrement by ELISA.** Two excrement samples were collected, one from a sick fish on day 8 following CNGV injection and the other from a naïve mock-infected fish. The samples were treated as described above and tested by ELISA following dilution (Fig. 4). CNGV antigens were found in the excrement collected from the sick fish but not in the excrement collected from the naïve fish. The results also indicate that CNGV antigens were revealed by the ELISA in a sample containing as little as 0.6 μg of total proteins extracted from the stools of sick fish but not in a sample from naïve fish. The specificity of this assay was verified by showing that even 5 μg of protein extracted from the excrement of healthy fish did not react with the anti-CNGV antibodies. These results indicate that ELISA can be used for diagnosis of CNGV infection by detection of viral antigens in fish stools, a procedure which does not require taking biopsies or killing the fish.

**Identification of sick fish by ELISA and PCR.** In order to verify that ELISA and PCR are efficient diagnostic tools for CNGV infection, we assessed whether viral components are present in feces of sick fish exclusively. Excrement samples were collected from naïve carp, naïve tilapia, carp 8 days after infection with CNGV, naturally resistant koi [20] (see Materials and Methods), and immunized koi infected with an attenuated CNGV clone [17] (see Materials and Methods) 20 days p.i. Figure 5A clearly shows that only samples taken from sick carp were positive as determined by ELISA. Naïve carp, naturally resistant immunized fish, and tilapia were all negative according to the ELISA. All the samples which were found to be negative by ELISA did not contain viral DNA as determined by PCR analysis, while the stools of sick carp were positive as determined by both tests (Fig. 5B). The PCR analysis provided further support for the specificity of the ELISA shown in Fig. 4 and 5A.

We then asked how soon after infection the viral components in the fish droppings can be revealed by ELISA. Fish were injected with CNGV, and each fish was kept separately in a tank. Excrement was collected and analyzed in parallel by PCR and ELISA. Figure 6A and B show that viral proteins and viral DNA were revealed in these fish as early as 6 or 7 days p.i. by ELISA and PCR, respectively (also see Fig. 1A).

Our experiments to detect viral components in the intestinal secretions by ELISA failed. Samples applied to the microwells exhibited a very high background, which impeded identification of the viral proteins (not shown), probably due to the presence of mucus and/or proteolytic enzymes. On the other hand, the PCR tests clearly revealed viral DNA in the intestinal secretions as early as 5 days p.i. (Fig. 1A).

**Droppings of sick fish contain infectious virus.** Fifty fish kept at 22°C were infected with CNGV, and three fish were sacrificed daily; the sacrificed fish were dissected, and their intestinal secretions were collected. Samples were prepared as described in Materials and Methods, and filtrates and supernatants were used to infect fresh CCB cultures. Five days p.i. CPE were observed in cultured cells infected with excrement collected on days 7 and 8 p.i. (not shown). To verify that the CPE observed in tissue culture were induced by CNGV, the DNA was extracted from the culture media and tested by PCR for the presence of viral DNA. Figure 7 clearly shows that
cultures infected with fish excrement collected on days 7 and 8 p.i. produced CNGV.

To prove that virus in excrement causes the disease, naïve fish were injected with either pooled intestinal secretions (days 7 to 9) or sediments collected 9 days p.i. Sixty percent of the fish inoculated with the intestinal secretion extracts taken from infected fish died on day 6 after inoculation, and the level of mortality reached 96% 8 days p.i. (Fig. 8). Injection of sediment extracts caused a similar but delayed effect; 96% of the fish died within 10 days p.i. Injection of excrement extracts taken from healthy fish or injection of PBS did not induce the disease (Fig. 8). All the fish injected with sediment or intestinal secretion extracts taken from sick fish died from the disease induced by CNGV, as verified by PCR analysis of DNA extracted from the kidneys (data not shown).

DISCUSSION

The aquaculture industry has grown tremendously throughout the world in the last few decades (14, 19). Any intensive bioproduction may experience disease problems, and viral diseases of fish contribute to immense economic losses every year. Because of the popularity of the colorful fish koi, they are intensively exported and imported from continent to continent, without sufficient quality control legislation (11). The measures taken on koi and common carp farms throughout the world in order to reduce the losses caused by CNGV include early detection and isolation of sick fish. However, the carp disease induced by CNGV is very short, and fish die within 6 to 24 days p.i. Early detection of the virus may prevent introduction of sick fish into virus-free zones or even save the contaminated fish if they are incubated in water at temperatures above 30°C for 20 to 25 days (16, 20). Several methods have been used to detect sick fish, and the most common method is simply to identify signs and symptoms of the disease. Since this method selects fish only after they become sick, it is useless in halting...
FIG. 8. Mortality rate for fish exposed to stool extracts containing CNGV. Carp were infected with CNGV, and 8 days p.i. their excreta were collected, processed, and used to infect naïve fish. Naïve fish (30 fish per group; average weight, 17 g) were injected intraperitoneally with extract prepared from secretions taken from intestines of CNGV-infected fish (▲) or from excrement from infected fish collected from the bottom of the tank 4 h after removal of the fish (●). Fish injected with PBS (●) or with extract prepared from excrement of noninfected fish (□) were used as control groups.

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whether the spread of the disease. This is the reason why other methods, such as PCR (4, 5, 6, 12, 18) and ELISA (13), are used worldwide for early detection. Taking biopsies or even blood samples from fish does not ensure that the samples harbor the virus. In addition, such surgical interventions are not favored by hobbyists and retailers.

Previous observations indicated that intestines and kidneys of sick fish harbor quite large quantities of the virus (5, 16, 18). We hypothesized, therefore, that fish droppings may contain viral antigens, which would allow detection by ELISA. Our experiments clearly showed that viral DNA can be detected as early as 4 to 8 days p.i. However, since sick fish do not secrete daily and the amount of excrement varied among samples, it was difficult to monitor the increase in viral antigens in the feces following infection, although it was quite clear that the viral antigen titer increased until day 9 p.i. Using fish droppings collected from the tank bottom or from cloaca do not harm the fish, and the tests are easy and inexpensive. Identification of viral DNA in stools by PCR is also feasible, although it often requires optimization of the Mg²⁺ concentration in the reaction mixture, probably because the stools and/or water in the sample contain various amounts of divalent cations.

Finding viral antigens and viral DNA in fish droppings encouraged us to ask whether feces harbor viable virus. Surprisingly, fish droppings contain quite large amounts of active virus, which cause CPE in CCB cultures and induce the disease following injection of naïve fish. Again, it is difficult to estimate the exact titer of the virus in the stools, for the reasons cited above. However, our results strongly suggest that stools harbor quite large amounts of infectious virus.

The findings presented in this report clearly show that CNGV is excreted by infected fish in the feces and that the virus that diffuses from stools into the water is infectious and efficiently induces the disease. However, the data evoke additional, unsolved questions. How are fish infected with CNGV? Does the diffused virus from stools penetrate the fish body through the gills (18) or via the digestive system? How long does the virus remain active in feces? Previously, we showed that virus harvested from cultured cells remains active for 4 h in water (17), but it is plausible that fish droppings preserve the active virus for a long time, probably during the nonpermissive seasons. Although we failed to find viral DNA and viral proteins in the naturally resistant fish and in the immunized fish, the possibility that infected carp bear latent virus, which could be secreted via the stools, cannot be excluded.

In summary, this report shows that viable virus is secreted into the stools and that fish droppings are useful for early diagnosis of CNGV infection by ELISA and PCR. These methods are efficient for revealing sick fish a few days before death and do not require bleeding and/or surgical intervention, which are not favored by hobbyists and retailers.