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Tilapines are important for the sustainability of ecological systems and serve as the second most important group of farmed fish worldwide. Significant mortality of wild and cultured tilapia has been observed recently in Israel. The etiological agent of this disease, a novel RNA virus, is described here, and procedures allowing its isolation and detection are revealed. The virus, denominated tilapia lake virus (TiLV), was propagated in primary tilapia brain cells or in an E-11 cell line, and it induced a cytopathic effect at 5 to 10 days postinfection. Electron microscopy revealed enveloped icosahedral particles of 55 to 75 nm. Low-passage TiLV, injected intraperitoneally in tilapia, induced a disease resembling the natural disease, which typically presents with lethargy, ocular alterations, and skin erosions, with >80% mortality. Histological changes included congestion of the internal organs (kidneys and brain) with foci of gliosis and perivascular cuffing of lymphocytes in the brain cortex; ocular inflammation included endophthalmitis and cataractous changes of the lens. The cohabitation of healthy and diseased fish demonstrated that the disease is contagious and that mortalities (80 to 100%) occur within a few days. Fish surviving the initial mortality were immune to further TiLV infections, suggesting the mounting of a protective immune response. Screening cDNA libraries identified a TiLV-specific sequence, allowing the design of a PCR-based diagnostic test. This test enables the specific identification of TiLV in tilapines and should help control the spread of this virus worldwide.

Tilapines are the second most important group of farmed fish worldwide, with production of >2.5 million tons annually (1, 2, 3), and they serve as a primary protein source in the developing world. The Sea of Galilee (Kinneret Lake) is a major source of potable water and supports commercial fishing. In recent years, the catch fish quantities have been subjected to a persistent decline. Interestingly, although the lake hosts some 27 species of fish (19 of which are native), encompassing members of the families *Cichlidae*, *Cyprinidae*, *Mugillidae*, and *Claridae*, only a catch cutback of tilapines (*Cichlidae*) is striking. For the main edible fish of the lake, *Sarotherodon* (*Tilapia*) *galilaeus* (St. Peter's fish), annual yields decreased from 316 tons in 2005 to 51, 8, and 45 tons in 2007, 2009, and 2010, respectively (O. Sunin and J. Shapiro, Department of Fisheries, Israel Ministry of Agriculture; personal communication).

Being a grazing fish, *S. galilaeus* contributes to the maintenance of the ecological balance of the lake. Hence, beyond its economic impact, the significant decline of St. Peter's fish populations, as well as the other lake tilapines (such as *Tilapia zilli* [common tilapia], *Oreochromis aureus* [Jordan tilapia], and *Tristamella simonis intermedia*) represents a definite threat to the entire ecosystem. The reasons for the decline have not been thoroughly investigated. The transient increase in tilapia catches during 2011 to 2012 (100 and 160 tons, respectively) probably represents an improvement in fishing technologies (e.g., sonar-guided fishing and angling in growing depths) rather than a true greater recovery of this species, as in 2013, catches dropped again to 140 tons (O. Sunin and J. Shapiro, personal communication).

Starting in the summer of 2009, episodes of massive losses of tilapia were recorded in fish farms all over Israel (R. Falk and N. Froiman, Department of Fisheries, Israel Ministry of Agriculture; personal communication). These outbreaks, observed during the hot seasons (May to October), were distinguished by waves of mortality of tilapia with a wide weight range, spreading from one pond to the other. Interestingly, fish morbidity and mortality remained restricted to tilapia (*Oreochromis niloticus* × *O. aureus* hybrid); several species, reared in community with tilapines (including carp [*Cyprinus carpio*] and Gray mullet [*Mugil cephalus*]), showed no clinical symptoms of the disease found in tilapia, even after long-term cohabitation. Moreover, once the initial wave of mortality ceased, no more outbreaks were recorded in the same pond. No apparent reason for the mortality was identified. Routine monitoring of known parasites, bacterial and viral pathogens, and toxins did not reveal any abnormalities and did not resolve the enigma. Attempts to identify emerging viral pathogens of tilapines in diseased fish, such as the herpes-like tilapia larvae encephalitis virus (TLEV) (4) and the viral nervous necrosis (VNN) betanodavirus (5), were unsuccessful. However, heightened surveillance has led to the recognition, both in open waters and in farm ponds, of weakened fish with black discoloration, skin abrasions, and ocular degeneration. A histological analysis of these fish revealed the presence of augmented melanomacrophage centers (MMCs), denoting an ongoing pathological course (6–8).

To elucidate the cause(s) of these changes, we set up an investigation aiming to (i) culture and identify a probable infectious

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agent and explore its role in triggering disease and (ii) to develop a diagnostic tool for its monitoring.

MATERIALS AND METHODS

Cell cultures. Eight established fish cell lines were used in this study: the CHSE-214 (ATCC CRL 1681) from the Chinook salmon *Oncorhynchus tshawytscha*; BF-2 (ATCC CCL 91), derived from the bluegill *Lepomis micropterus*; BB (ATCC CCL 59), from the brown bullhead *Ictalurus nebulosus*; EPC (ATCC CRL 2872) and KF-1 (13), from the common carp *C. carpio*; (5); RTG-2 (ATCC CCL 55), from the rainbow trout *Salmo gairdneri*; FHM (ATCC CCL 42), from the fat head minnow *Pimephales promelas*; and E-11, from the striped snakehead *Ophicephalus striatus* (generously provided by M. Ucko, Israel Oceanographic and Limnological Research). In addition, we generated a culture of primary tilapia brain cells, developed as previously described (13, 17). Briefly, commercial Nile tilapia (*O. niloticus*) (50 g) were euthanized by anesthetic overdose (600 mg/liter tricaine methanesulfonate [MS-222]; Finquel, USA), and the brains were removed aseptically. The minced brains were manually homogenized and passed through a 100-μm mesh grinder; the cells were then washed and seeded in 12.5-ml sealed flasks (Becton-Dickinson, San Francisco, CA, USA) at 25°C. The initial culture medium contained 80% Leibovitz (L-15) medium (Gibco, USA), 10% inactivated fetal calf serum (FCS) (Gibco), and 10% inactivated tilapia serum medium, supplemented with L-glutamine (300 mg/liter), HEPES (1%), penicillin (100 μg/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). During the first 21 days of incubation, 50% of the media were changed every week. Thereafter, the monolayers were trypsinized and transferred into new 25-ml flasks (Cellstar; Greiner Bio-One, Germany) with a 1:1 mixture of conditioned medium (from old cultures) and fresh medium. The cultures of the primary cells were passaged every other week; after 35 passages, the tilapia serum and the condition media were omitted, and the cells were split (at a ratio of 1:2) every 2 to 3 weeks in regular medium (L-15 with 5% inactivated FCS).

Viruses and virus culture. A total of 25 tilapia lake virus (TiLV) isolates were collected from suspected outbreaks that occurred between May 2011 and June 2013. The isolations were obtained from all Israeli regions where fish are commercially cultured: the coastal shore (2 isolations), the Jordan Valley (comprising the Bet-Shean Valley and the Yizrael Valley; 9 isolations), and Upper and Lower Galilee (3 isolations). In addition, 11 isolations were obtained from various species of wild tilapines from the Sea of Galilee. An outbreak of farmed fish was defined as a sudden and unexplained rise in mortality (≥2% daily) for at least three consecutive days. If two wards were simultaneously affected on the same farm, these were classified as a single outbreak. Therefore, each isolate represents a distinct clinical outbreak. Viruses from wild fish displaying ocular lesions were isolated from commercially caught fish in the Sea of Galilee; each isolate represents a different catch. Fish weighing 20 to 200 g or 40 to 350 g (wild and farmed fish, respectively) were collected during the hot seasons (May to October; water temperature, between 22 and 32°C). To minimize contamination risks, the brains and viscera (kidneys, livers, spleens, and hearts) of the suspected fish were removed aseptically, pooled, and manually homogenized with nine volumes of Hanks' balanced salt solution (HBSS), centrifuged at 3,000 × *g* for 10 min, and the supernatants were filtered through 0.22-μm filters (Sarstedt, Germany). The filtrates were stored at −80°C until use. For infection, monolayers (about 90% confluence) were washed twice with HBSS and incubated with 500 μl of the virus filtrate at 25°C for 1 h, after which the cells were washed with HBSS, supplemented with L-15 medium (2% FCS), and incubated at 25°C. The cultures were observed daily for 21 days for cytopathic effects (CPE). In experiments where the tilapine disease was reproduced by virus injection, we also used a virus named TiLVx2, which was purified by two successive rounds of endpoint dilution assays. This was performed with E-11 cultures, infected with serial dilutions of TiLV (isolate 4/2011; obtained from the brain of a diseased St. Peter's fish that was collected from the Kinneret Lake on June 2011).

Titration of virus. The original virus-containing culture supernatant (isolate 4/2011) was cultured in E-11 cells and serially diluted in 10-fold increments with HBSS; 50 μl from each dilution was inoculated onto E-11 monolayers in 96-well plates. Four wells were used for each diluted sample. The plates were incubated at 25°C and observed daily for CPE. After 7 days, the 50% tissue culture infectious dose ($TCID_{50}$) (ml^{-1}) was calculated by the method of Reed and Muench (18).

Electron microscopy analyses. For examinations of TiLV by transmission electron microscopy, E-11-infected cultures were scraped from the flask, centrifuged (2,000 rpm for 7 min), fixed with 1.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) for 2 h, and then rinsed five times in phosphate buffer (pH 7.2). The pellets, consisting of infected E-11 cells, were postfixed in 1% OsO_4 in phosphate buffer and dehydrated with increasing concentrations of ethanol. The pellets were then washed twice with 100% propylene oxide and treated with propylene oxide-Epon (3:1) for 30 min, followed by propylene oxide-Epon (1:1) for 15 min. Finally, the pellets were embedded in 100% Epon and left overnight. Thin sections (70 to 90 nm) were placed on Formvar-coated copper grids and stained with uranyl acetate, followed by lead citrate, according to the Reynolds method (19). All micrographs were taken with a JEOL 1200-EX electron microscope operating at 60 or 80 kV (Electron Microscopy [EM] Unit, Institute of Biotechnology, Bar-Ilan University, Israel). EM analysis of the negatively stained virion pellets was carried at the EM Unit, Tel Aviv University, exactly as described before (20), with an A JEM 1200-EX transmitting electron microscope (JEOL-USA, Peabody, MA, USA). The virions for this analysis were pelleted by ultracentrifugation through 25% sucrose cushions.

Purification of virus from culture supernatants using sucrose gradient fractionation. Cultured E-11 cells were infected with TiLV (isolate 4/2011), and the culture supernatant was cleared from the cell debris by centrifugation (10 min at 3,000 rpm). The supernatant was layered onto 2 ml of a 30% (wt/vol) sucrose–Tris-EDTA (TE) buffer cushion and centrifuged for 2 h in a T865 rotor at 65,000 rpm (Sorvall Discovery 90SE). The pellet was resuspended in TE buffer and layered onto a sucrose step gradient (11, 21, 22). The gradient consisted of 3-ml layers with sucrose concentrations of 70, 60, 50, 40, 30, 20, and 10% (wt/vol) in TE, from bottom to top. Ultracentrifugation was performed in a TST41.14 rotor for 2 h at 40,000 rpm (Sorvall Discovery 90SE). One-milliliter fractions were taken from the top of the gradient, and the virions were pelleted from each fraction by ultracentrifugation (for 2 h at 65,000 rpm; T865 rotor; Sorvall Discovery 90SE) and resuspended in 1 ml of TE buffer; 100-μl aliquots from each sample were incubated with naive E-11 cells to monitor for CPE. The incubation of cultures with negative controls, consisting of aliquots from fractions of an identical sucrose gradient but with no addition of culture supernatants, resulted in no CPE.

Isolation of nucleic acids from purified virions and cDNA synthesis. Nucleic acids were extracted from purified virion pellets using peqGOLD Trifast for RNA (Peqlab, Germany) or the High Pure PCR template preparation kit for DNA (Roche, Germany). Reverse transcription was performed with the Verso cDNA kit (Thermo, Lithuania), according to the manufacturer's instructions. To identify TiLV-specific sequences, we cleared the supernatants of TiLV (isolate 4/2011)-infected E-11 cultures from the cell debris by centrifugation (for 10 min at 3,000 × *g*), and the purified supernatants were subjected to further purification by ultracentrifugation (for 2 h in a T865 rotor at 65,000 rpm [Sorvall Discovery 90SE]) through a 30% sucrose cushion. The pellet was resuspended in TE, and virions were further purified by sucrose cushions of 40 to 70% (wt/vol). After ultracentrifugation (TST41.14 rotor for 2 h at 40,000 rpm; Sorvall Discovery 90SE), the 40% sucrose fraction was collected, and virions were pelleted by additional ultracentrifugation (TST41.14 rotor for 2 h at 40,000 rpm; Sorvall Discovery 90SE). RNA was extracted from the pellets by guanidine thiocyanate (peqGOLD Trifast; Peqlab). cDNA was generated by reverse transcription and random priming, using the purified RNA as a template. The fragments of this cDNA were isolated by shotgun cloning (23).

Shotgun cloning by random priming. Shotgun cloning was performed as described by Nehls and Boehm (23). The purified cDNA (~10 ng; see above) was double primed with MluI(N)6 primer (GGAACTCAA TGCACGCGTNNNNNN) using ReddyMix PCR master mix (Thermo, Lithuania). The primed products were amplified by PCR with MluI primer (GGAACTCAATGCACGCGT) and cloned into the pJET1.2/blunt vector (CloneJET; Fermentas/Thermo, Lithuania), which was transformed into *Escherichia coli* strain HIT-DH5α cells (Real Biotech, Taiwan). Ampicillin-resistant transformants, grown at 37°C on LB agar plates containing 100 μg/ml ampicillin, were picked and grown overnight in 5 ml of LB supplemented with 100 μg/ml ampicillin. Plasmid DNA was isolated using the HiYield plasmid minikit (RBC, Taiwan). The inserts were amplified by PCR using the pJET1.2-derived primers, separated by electrophoresis in a 1.0% gel, placed in 1× Tris-acetate-EDTA (TAE) buffer at 80 V for 1.5 h, stained with ethidium bromide, excised, and gel purified using the GeneJET gel extraction and DNA cleanup micro kit (Thermo, Lithuania). Single fragments were sequenced by Hy Laboratories (Israel) using ABI 3730. The sequences were analyzed for homologies to nucleotide sequences in the GenBank database using the nucleotide Basic Local Alignment Search Tool (BLASTn) and the Vector NTI 6 (InforMax, Inc.) software. Further searches of protein databases were done by BLASTx. The internal primers from each sequenced clone were tested for PCR amplification of the TiLV genome. The primers derived from clone 7450 specifically amplified the cognate sequence from TiLV-infected cultures, in reverse transcription-PCRs (RT-PCRs).

Rapid amplification of cDNA ends. To extend the sequence of clone 7450 obtained by shotgun cloning, 3′ and 5′ rapid amplification of cDNA ends (RACE) reactions were carried out as described before (24, 25), using total RNA that was extracted from TiLV-infected E11 cells by EZ-RNA reagent (Biological Industries). Briefly, for 3′ RACE, cDNA was generated using primer Q_T (CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAA GCTTTTTTTTTTTTTTTTTVN) and the SuperScript III first-strand synthesis system for RT-PCR (catalog no. 18080-051; Invitrogen), according to the manufacturer's instructions. The cDNA was amplified with clone 7450/150R primer (TATCACGTGCGTACTCGTTCAGT) that was derived from an internal sequence of the shotgun fragment, and with Q_0 primer (CCAGTGAGCAGAGTGACG, derived from Q_T primer), using *Ex Taq* enzyme (catalog no. RR001A; TaKaRa). The resulting PCR products were diluted 1:20 and were subjected to a second PCR with the nested primers Q_I (GAGGACTCGAGCTCAAGC, derived from Q_T primer) and E11-inf-R (AAGTTCTCTTGCCTCTTGG, derived from the sequence of the shotgun fragment). For 5′ RACE, cDNA was generated as above but with primer clone 7450/150F (CACCCAGACTTGCGGACATA). Poly(A) tails were added to the cDNA using terminal transferase (catalog no. 3333566; Roche), according to the manufacturer's instructions. The tailed cDNA was amplified by PCR using primer E-11-inf-F (TCCAAGGAAAC AGCTGAGC, derived from the sequence of the shotgun fragment), together with a mixture of the Q_0 and Q_T primers. The resulting PCR products were diluted 1:20 and subjected to a second nested-PCR using the E11-inf-F-in (GAGGCAATATGGATTCTTCG) and Q_I primers.

RT-PCR. Samples from the brain, heart, head kidney, spleen, and liver were taken from clinical cases of suspected TiLV outbreaks, pooled, and directly frozen at −80°C. Total RNA was purified using peqGOLD Trifast (Peqlab, Germany), according to the manufacturer's instructions, followed by reverse transcription and amplification (Verso 1-step RT-PCR ReddyMix kit; Thermo, Lithuania). The random primers of the kit were substituted with the external specific primer ME1 (GTTGGGCACAAGG CATCCTA) and clone 7450/150R (TATCACGTGCGTACTCGTTCA GT). Cycling was performed at 50°C for 15 min (reverse transcription), 95°C for 2 min (enzyme inactivation), and 35 cycles at 95°C for 30 s, 56°C for 60 s, and 72°C for 60 s; the reaction was terminated by 72°C for 7 min. The PCR products were resolved in 1% agarose gels in 0.5× TAE buffer (40 mM Tris-acetate and 1 mM EDTA).

Nuclease sensitivity assays. The supernatant (9 ml) of a TiLV-infected E-11 culture was collected, and virions were purified and pelleted through a 25% (wt/vol) sucrose cushion, using ultracentrifugation (107,000 × *g* at 4°C for 2 h). A supernatant of uninfected E-11 culture was used as a control. To digest free nucleic acids, the pellets were resuspended in 300 μl of 1× DNase buffer (10 mM Tris-HCl [pH 7.5], 2.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$) and were supplemented with 33 μg of RNase A (Sigma R4642) and 1 U DNase (Baseline-ZERO DNase). The samples were incubated for 40 min at room temperature, after which each reaction mixture was diluted in 9 ml of Leibovitz (L-15) medium, supplemented with 5% FCS, and virions were pelleted as described above. To release nuclease-protected nucleic acids from the virions and to digest possible leftovers of RNase A and DNase I, the pellets were resuspended in 150 μl of proteinase K buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM EDTA, 1% SDS), supplemented with 100 μg/ml proteinase K (Roche), and the proteins were digested for 30 min at 37°C. The nucleic acids were extracted by phenol-chloroform–isoamyl alcohol (CIP) and were precipitated with ethanol, 0.3 M sodium acetate (pH 5.2), and glycogen as a carrier. The nucleic acids were resuspended in 20 μl of buffer (10 mM Tris-HCl [pH 8.3], 10 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 60 mM NaCl), and 3 μl was added to 100 μl of RNase I buffer (100 mM NaCl, 50 mM Tris-HCl [pH 7.9], 10 mM $MgCl_2$, 1 mM DTT) with or without 50 units of RNase I (catalog no. M0243S; NEB). Digestion was carried out for 5 min at 37°C, and the nucleic acids were CIP extracted and precipitated as described above. The nucleic acids were resuspended in 20 μl of reverse transcription reaction mixture; a reaction without reverse transcriptase was also assembled to ensure the absence of protected DNA. The resulting cDNA was amplified with TiLV-specific primers (Nested ext-1 [TATGCAGTAC TTTCCCTGCC] and Nested ext-2 [TTGCTCTGAGCAAGAGTACC]) or with snakehead retrovirus (SnRV)-specific primers (Snakehead gag-pol fw [CAGATCACTGATCGATGC] and Snakehead gag-pol rev [GTCTGAAAGGTAAGGTGG]). The amplified products (491 and 284 bp for TiLV and SnRV, respectively) were separated by electrophoresis in 1% agarose gels.

Ether and chloroform sensitivity assays. TiLV sensitivity assays for ether and chloroform were performed as described before (15, 26).

Experimental reproduction of the disease and ethical issues. The tilapine species used in this study, *O. niloticus* (strain Chitralada), was grown at a specific-pathogen-free (SPF) facility (UV-treated pathogen-free environment) at a constant temperature of 28°C. The fish were fed a daily regimen of 2% (wt/wt); the water parameters ($O_2 > 5$ ppm, $NH4^+ <$ 1 ppm, NaCl < 1 ppt) were kept constant. All experimentally induced infections were carried out with the field isolate of TiLV (isolate 4/2011, passage 2), which was aliquoted and kept frozen at −80°C. Before use, the virus was thawed and cultured once more (passage 3). For artificial reproduction of the disease, 2.6×10^5 $TCID_{50}$ was injected intraperitoneally (i.p.) (group 1) into each fish (30 to 35 g). All experiments were carried out in triplicate with groups of 30 fish. To prevent waterborne infection, each group of fish was kept in a separate 100-liter aquarium. During the cohabitation trials (group 2), groups of 30 fish were kept in 200-liter aquariums that were divided into three compartments by water-permeable grids, which allowed water (but not fish) circulation throughout the aquarium; a control group was kept in the middle. The fish surviving primary i.p. infection were pooled and 3 weeks after were divided into two groups (each with 15 fish) and infected once again by i.p. injection. The control groups were injected with uninfected (naive) E-11 cultures.

When *in vivo*/*ex vivo* experiments were conducted, the brains of individual TiLV-injected fish were collected (5 to 7 days postinjection) and minced as above. The homogenates (500 μl) were incubated with confluent E-11 cultures. Upon CPE appearance, the supernatants were collected and injected (200 μl) i.p. into naive fish.

The health conditions of the fish were carefully monitored throughout the growing and experiment periods; external signs and mortality rates were monitored twice daily for a total of 21 days. The animal care, experimental handling, and safety regulations conformed to the guidelines established by the Committee on Laboratory Animal Care at the Israeli

Veterinary Services and were conducted under permit 020_b5471_6, issued by the Israeli Committee for Animal Welfare.

Histological analysis. Tissue samples were collected from euthanized naturally infected fish by abdominal incision and were fixed in 10% neutral buffered formalin. The specimens were embedded in paraffin (Paraplast Plus; Diapath), cut by microtome (Reichert-Jung 2050) into serial 5-μm sections, stained with hematoxylin and eosin (H&E) 277 (27), and examined under a light microscope (Leica DMRB). Images were acquired by a Nikon digital light system.

Statistical analysis. The results of the *in vivo* experiments are presented as percentages of the mean mortality rates from three (or two, in case of surviving fish) independent experiments. Each experiment included three experimental groups (three independent repeats) of 30 fish. The experiments with the surviving fish were performed in duplicate (two independent repeats), in which each group was composed of 20 fish. Variability between the experiments (infection by direct intraperitoneal injection, infection by cohabitation, and control fish) was determined by chi-square tests, in which a P value of <0.05 was considered significant.

Culture deposition. TiLV (CNCM accession no. I-4817) was deposited at the Collection Nationale de Cultures de Microorganismes (CNCM) at the Institut Pasteur, Paris, France.

Nucleotide sequence accession number. The GenBank accession number for the extended sequence of clone 7450 is KJ605629.

RESULTS

Geographical distribution and characteristics of diseased tilapines from the Sea of Galilee and commercial ponds. Recently, disease outbreaks in wild and commercial tilapines were detected in the Sea of Galilee and in commercial ponds in Israel, located in the Northern coastal shore, Bet-Shean, Yizrael, the Jordan Valley, and Upper and Lower Galilee. In commercial ponds, this disease resulted in massive mortality (Fig. 1A); the sampling of fish from commercial catches at the Sea of Galilee revealed that all tilapine species are susceptible to the disease, although mass mortalities are not observed. In this case, the diseased fish presented with pronounced ocular lesions (Fig. 1B).

Pathological findings. Gross lesions were characterized mainly by ocular alterations, including opacity of the lens (cataract). In advanced cases, the lesions included ruptured lenses with phacoclastic induced uveitis or endophthalmitis accompanied by the formation of a cyclitic membrane, followed by swelling of the eyeball (buphthalmia), loss of globe integrity with occasional perforated cornea and poring of inspissated content or shrinkage, and loss of ocular functioning (phthisis bulbi) (Fig. 1B). Other lesions included skin erosions (observed in diseased pond-raised tilapines; Fig. 1C), hemorrhages in the leptomeninges (see below), and moderate congestion of the spleen (not shown) and kidney (Fig. 1D).

The histologic lesions of the brain included edema, focal hemorrhages in the leptomeninges, and capillary congestion in both the white and gray matter (Fig. 1E). Foci of gliosis and occasional perivascular cuffs of lymphocytes were detected (Fig. 1F). Some neurons within the telencephalon and particularly in the optic lobes displayed various levels of neuronal degeneration, including cytoplasmic rarefaction and vacuolation and peripherally displaced nuclei (central chromatolysis) (data not shown).

Ocular lesions included an undulated, thin, and frequently coiled and ruptured lenticular capsule, surrounded by circular fibroplasia and fibrosis with multiple synechiae to the iris and ciliary body (posterior synechia) and moderate numbers of eosinophilic granulocytic cells and MMCs. The infiltrate extended into the anterior chamber, iris, vitreous humor, and choroid (endophthalmitis). There are cataractous changes within the lens characterized by eosinophilic homogenous spherical structures (morgagnian globules): markedly enlarged lens epithelial cells with abundant eosinophilic microvacuolated cytoplasm (bladder cells), large lakes of proteinaceous fluid (liquefied lens fibers), mineralization, and flattened elongated cells (fibrous metaplasia) (Fig. 1Ga, compared to normal lens in Fig. 1Gb). The squamous epithelium of the cornea is frequently eroded and ulcerated and infiltrated by moderate numbers of lymphocytes, macrophages, and eosinophilic granulocytic cells, and it is underlined by stromal neovascularization and edema (Fig. 1H).

The hepatic parenchyma displayed occasional randomly distributed foci of hepatocellular swelling and clearing, with cytoplasmic accumulation of granular yellow to brown pigment; the spleen was hyperplastic, with proliferating lymphocytes surrounding the ellipsoids (not shown). MMCs were increased in size and number in both the liver and the spleen (not shown). MMCs are distinctive clusters of pigment-laden cells, commonly seen within the reticuloendothelial supporting matrix of hematopoietic tissues. MMC proliferation is associated with late stages of chronic infection as a response to severe tissue injury in a variety of infections (especially viruses) or poor environmental conditions. Therefore, they are considered indicators of fish population health (6–8).

Isolation of the etiological agent from infected specimens. To culture potential pathogens from diseased tilapines, the organs of fish with the characteristics described above were pooled, homogenized, and incubated with eight different cell lines (see Materials and Methods). No known pathogen was identified, and only the established E-11 cell line and the primary tilapia brain cells consistently showed CPE upon incubation with the abovementioned homogenates. In E-11 cells, CPE became visible 5 to 7 days postinoculation, with the appearance of cytoplasmic vacuoles and plaque formation (Fig. 2A), which rapidly progressed to an almost-complete disintegration of the cell monolayer (at 9 to 10 days postinoculation). The CPE in primary tilapia brain cells was characterized by conversion of the typical elongated cells into swollen, rounded, and granulated cells, which were clearly observed at 10 to 12 days postinoculation (Fig. 2B), leading to vast monolayer detachment (days 14 to 19) but without plaque formation. The control mock-infected E-11 and primary tilapia brain cultures did not show any CPE (Fig. 2C and D, respectively). Notably, similar results were obtained when the supernatants of the cultures with CPE were used to inoculate naive cultures (tested for up to 18 passages) and when the supernatants, or the abovementioned homogenates, were filtered through 0.22-μm filters. In addition, the number of plaques induced by the agent was directly related to its dilution, yielding a one-hit curve (data not shown). A single infectious unit is therefore sufficient to produce a plaque. These results strongly suggest that the described CPE was due to the presence of an infectious agent, likely a virus. The CPE-causing agent was recovered from 25 samples, collected from all Israeli regions where fish are cultured (detailed in Materials and Methods).

Morphological features of virus-like particles. Further support for viral infection in E-11 cultures showing CPE came from EM examination of thin sections of these cells. This analysis revealed the presence of sparse electron-dense particles (diameter, 55 to 75 nm), enclosed in the intracytoplasmic membrane (Fig. 2E) or within the cytoplasm (Fig. 2F). No such particles were found in the healthy control cell cultures. Of note, these particles

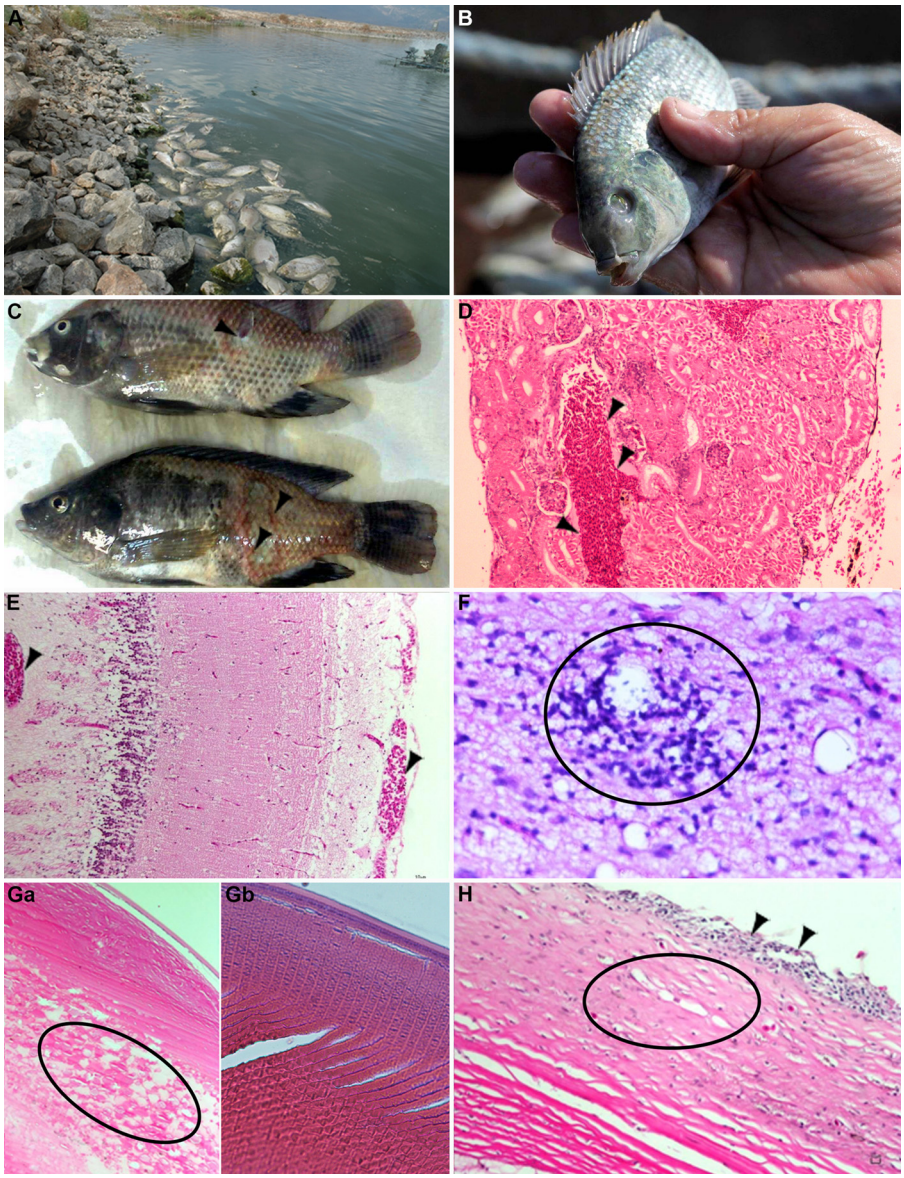

FIG 1 Characteristics of tilapia disease and pathological findings. Tilapia disease in commercial hybrid tilapia (*O. niloticus* × *O. aureus* hybrid) (A and C to E) and in wild tilapia (*S. galilaeus*) from the Sea of Galilee (B and F to H). (A) Tilapia disease outbreak in a commercial pond results in massive mortality (August 2013; courtesy of Nathan Wajsbrut). (B) Diseased tilapia demonstrating shrinkage of the eye and loss of ocular functioning (phthisis bulbi). (C) Gross pathology of skin includes multifocal to coalescing dermal erosions and ulcers (arrowheads; courtesy of Nathan Wajsbrut). (D) Kidney and interstitium. The arrowheads mark a dilated vein packed with large numbers of red blood cells (congestion). Hematoxylin and eosin (H&E) stain ×10. (E) Brain and cortex. The arrowheads mark dilated blood vessels packed with large numbers of red blood cells within the leptomeninges and gray and white matter. H&E stain ×10 was used. (F) Brain and cortex. Perivascular cuffs of lymphocytes (encircled). H&E stain ×40 was used. (Ga) Lens. Cataractous changes characterized by formation of eosinophilic spherical structures (morgagnian globules) accompanied by degeneration of crystalline fibers (encircled). H&E stain ×10 was used. (Gb) Control lens from healthy fish. H&E stain ×10 was used. (H) Eye and cornea. Loss of integrity of the overlying squamous epithelium with inflammatory infiltrate (arrowheads) and multiple capillaries within the stroma (neovascularization; encircled). The collagen fibers within the superficial stroma are smudged and are stained pale eosinophilic (corneal edema). H&E stain ×10 was used.

do not originate from the snakehead retrovirus (SnRV) that is expressed in E-11, since assemblies of this C-type retrovirus are larger and are generated only at the plasma membrane; moreover, SnRV nascent virions were not visualized by EM in this specific cell line (although SnRV sequences can be amplified by PCR) (16). Pellets, purified from the supernatants of infected E-11 cultures by ultracentrifugation through 25% sucrose cushions, were negatively stained and examined by EM. This analysis revealed virion-like structures (approximately 75 to 80 nm) surrounded by a readily detected thick coat (Fig. 2G). Such virions were abundant and were not detected in the control pellets prepared from naive E-11 cells (not shown).

Sensitivity of the infectious agent to ether or chloroform. The abovementioned EM analyses may suggest that the infectious agent, isolated from diseased tilapia, is an enveloped virus. To test this, we exposed the virions in the supernatants of E-11-infected

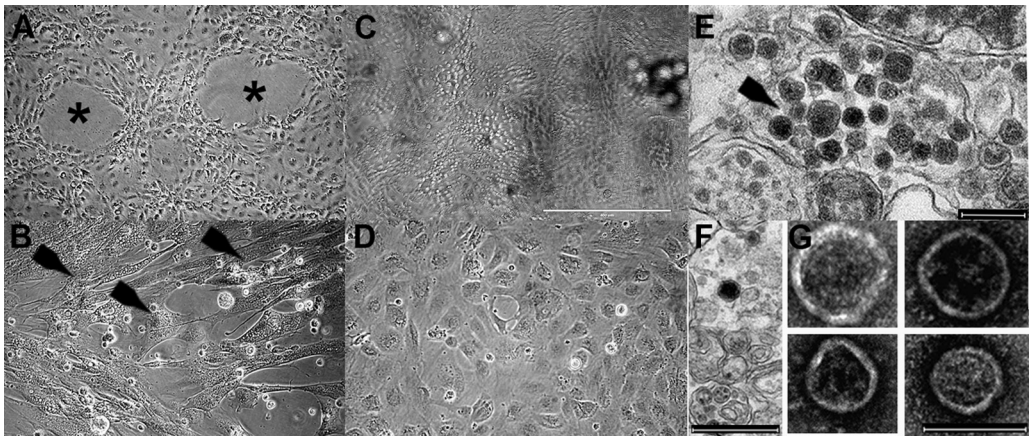

FIG 2 CPE induction in infected cultures and EM analyses. (A) E-11 infected cells. CPE (at day 5 postinoculation). Plaque formation and vacuolated cells at the rims of the plaques. The centers of two plaques are marked with asterisks. (B) Infected primary tilapia brain cells. CPE (at day 10 postinoculation). Conversion of the typical elongated cells into swollen, rounded, and granulated cells (marked with arrows). Mock-infected E-11 (C) or primary tilapia brain (D) cells. (E and F) Transmission EM of thin sections of infected E-11 cells revealed the presence of cytoplasmic particles (diameter, 55 to 60 nm) aggregated (E; marked with an arrow) or not (F) within a membrane. Scale bars, 200 and 500 nm for (E) and (F), respectively. (G) EM of negatively stained virions, pelleted from infected E11 culture supernatants. Scale bar, 100 nm.

cells to either ether or chloroform and measured the effect of these treatments on infectivity. Table 1 summarizes the results of two and three repeats of the ether and chloroform sensitivity assays, respectively. A reduction in the infectivity of approximately three (chloroform) to five (ether) orders of magnitude was observed, demonstrating the sensitivity of the agent to these solvents; this suggested that the infectious agent is indeed enveloped by a lipid membrane.

Initial molecular characterization of tilapine virus. So far, we demonstrated that an infectious agent can be isolated from diseased fish and can be propagated in specific cell cultures. We named this putative disease-causing agent tilapia lake virus (TiLV), as a reference to the site from which it was initially isolated. To purify TiLV, we fractionated TiLV-infected culture supernatants through velocity sucrose step gradients ranging from 10 to 70% sucrose (see Materials and Methods) and found that the CPE-inducing activity was mainly localized to the 30 to 40% sucrose fractions.

To further identify TiLV-specific sequences, we extracted RNA from TiLV virions (purified by ultracentrifugation through sucrose cushions) and used it as a template in a reverse transcription reaction. The fragments of the resulting cDNA were cloned using a shotgun approach (detailed in Materials and Methods). This approach allows the cloning of cDNAs that are present at small amounts, without prior knowledge of their sequences (23). One of these fragments (clone 7450) was subjected to 5′ and 3′ RACE reactions (see Materials and Methods), resulting in the identification of 1,326 bases of a putative TiLV sequence (GenBank accession no. KJ605629), which contained a putative open reading frame (ORF) of 420 amino acids (Fig. 3A). No significant homologies were found in both the nucleic acids and protein sequences of this clone using BLAST searches (10, 28) in the GenBank databases.

TABLE 1 Ether and chloroform sensitivity assays

Treatment	Expt no.	$TCID_{50}$/ml without treatment	$TCID_{50}$/ml plus treatment	Fold reduction	Average fold reduction
Ether	1	$10^{4.49}$	10^{-1}	$10^{5.49}$	1.58×10^5
	2	$10^{4.83}$	10	$10^{3.83}$	
Chloroform	1	$10^{4.63}$	$10^{1.5}$	$10^{3.13}$	0.93×10^3
	2	$10^{3.5}$	$10^{1.5}$	10^2	
	3	$10^{4.63}$	$10^{1.5}$	$10^{3.13}$	

PCR for TiLV detection. To establish a PCR assay for detecting TiLV, total RNA was extracted from the brains, kidneys, hearts, livers, and spleens of moribund fish. In addition, RNA was extracted from TiLV-infected primary tilapia brain or E-11 cultures. These samples were subject to RT-PCR with primers that were derived from clone 7450 (Fig. 3A and B). The PCR assays resulted in the amplification of the expected 250-bp fragment from the brains of TiLV-infected fish (Fig. 3B). The amplification of TiLV was achieved only after a reverse transcription step, even under conditions in which the samples were not treated with DNase (Fig. 3C). This is highly indicative of an RNA genome for TiLV. Importantly, we found consistent amplification in the samples of brain tissues compared to the other organs (data not shown). Amplification was also observed in TiLV-infected primary tilapia brain and E-11 cultures but not in a negative control that included cDNA prepared from the brain of a healthy (naive) fish (Fig. 3B). No amplification was observed in additional negative controls, which included mock-infected primary tilapia brain and E-11 cultures, or E-11 cultures infected with the viral nervous necrosis (VNN) betanodavirus (data not shown). Of note, the absence of amplification in the sample of VNN-infected cells further suggests that clone 7450 represents a sequence derived from TiLV rather than a fish gene that is upregulated upon infection. In all cases, sequencing of the amplified fragments revealed full identity with the expected sequence.

We also exploited the above PCR assay to further test the RNA nature of the TiLV genome (detailed in Materials and Methods). For this, we exposed the virions in the supernatants of TiLV-infected E-11 cultures to DNase I and RNase A to digest nucleic acids that are not protected by virions. The particles were then pelleted

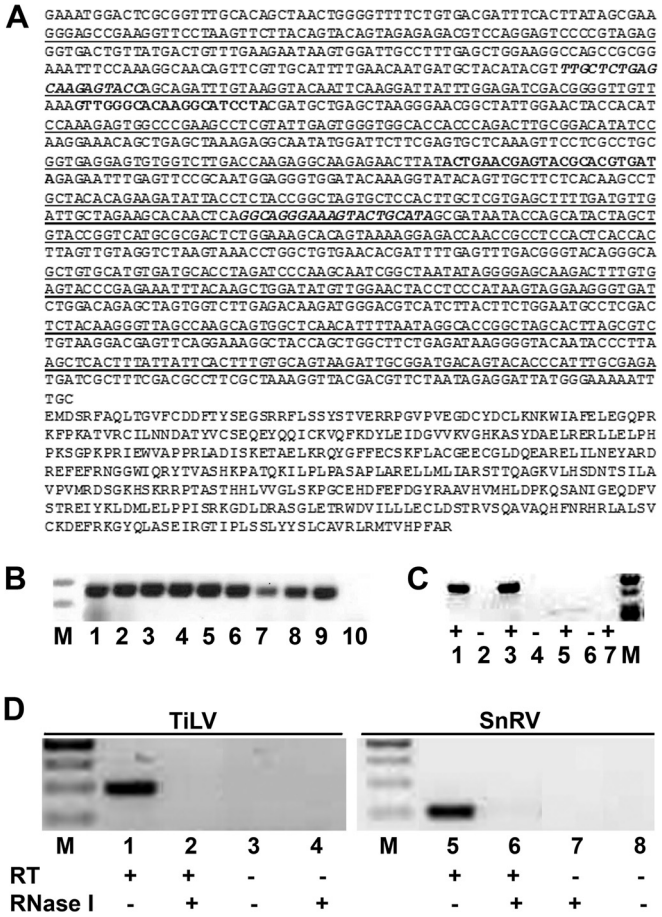

FIG 3 Sequence and PCR detection of TiLV. (A) Shotgun, 5′, and 3′ RACE methodologies were used to clone and sequence a portion of TiLV. Shown are the 1,326 bases of clone 7450 (GenBank accession no. KJ605629), which contains an open reading frame (underlined). The putative translation product (420 amino acids [aa]) of this open frame is shown at the bottom, in a single-letter code. The primer binding sites are shown in bold (for PCR amplification of a 250-bp fragment) or in bold and italic letters (for PCR amplification of a 491-bp fragment). (B) Detection of TiLV by PCR. Total RNA was extracted from brains of TiLV-infected fish (lanes 1 to 7) and a healthy fish (lane 10), as well as from E-11 and primary tilapia brain infected cell cultures (lanes 8 and 9, respectively), and was used as a template for cDNA generation. A 250-bp fragment was amplified with the ME1 (GTTGGGCACAAGGCATCCTA) and clone 7450/150R/ME2 (TATCA CGTGCGTACTCGTTCAGT) primers. (C) Reverse transcription is required for PCR amplification of TiLV. Total RNA was extracted from the supernatant (lanes 1 and 2) or from cell extracts (lanes 3 and 4) of TiLV-infected E-11 culture, or from naive E-11 culture (lanes 5 and 6). The samples were not treated with DNase, and reverse transcription was carried out (+) or not (−) prior to the PCR step. A "no RNA" negative control (lane 7) was also included. A 491-bp fragment was amplified with the primers Nested ext-1 (TATGCAGTACTTTCCCTGCC) and Nested ext-2 (TTGCTCTGAGCAAGAGTACC). (D) Nuclease sensitivity assays. Nuclease-protected nucleic acids were extracted from purified virions and were treated (+) or not (−) with reverse transcriptase and/or RNase I prior to PCR amplification with TiLV-specific primers (Nested ext-1 [TATGCAGTACTTTCC CTGCC] and Nested ext-2 [TTGCTCTGAGCAAGAGTACC]; amplified product, 491 bp) or SnRV-specific primers (Snakehead gag-pol fw [CAGATCACTGA TCGATGC] and Snakehead gag-pol rev [GTCTGAAAGGTAAGGTGG]; amplified product, 284 bp). M, the DNA size marker (B to D).

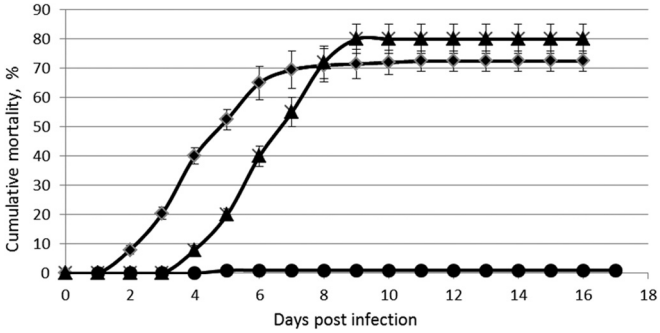

FIG 4 Kinetics of TiLV-induced mortality. Fish were divided into groups of 30 SPF fish. Groups 1 (◆) and 2 (▲) were infected by i.p. injection or cohabitation, respectively. The control group (●) was composed of an identical number of fish inoculated with the supernatants of naive E-11 cultures. Variability between the three experimental groups was determined by chi-square tests, in which a P value of <0.05 was considered significant. Bars represent standard errors.

through sucrose cushions and digested with proteinase K, and the protected deproteinized nucleic acids were purified. These nucleic acids were exposed to RNase I, an enzyme with a preference for single-stranded RNA. The resulting products were reverse transcribed or not and subjected to PCR amplification using TiLV-specific primers. The amplification of TiLV sequences was observed only after a reverse transcription step (Fig. 3D, lanes 1 and 3, similar to the result in Fig. 3C), and only if RNase I was avoided (Fig. 3D, lanes 1 and 2). The single-stranded genomic RNA of SnRV, which was copurified along the TiLV genome, was used as an internal positive control for this assay (Fig. 3D, lanes 5 to 8). These results further indicate that the TiLV genome, encapsidated in the virion, is composed of RNA, likely in single-stranded form.

Reproduction of tilapine disease by intraperitoneal injection. To test if TiLV can cause disease in tilapines, the supernatants from naive or TiLV-infected E-11 or primary tilapia brain cultures were filtered (0.22 μm), and 200 μl was injected i.p. into naive *Tilapia nilotica* (groups of 30 fish; see Materials and Methods). All of the naive fish that were inoculated with the control supernatants (of naive E-11 cultures) remained asymptomatic. However, 74 to 85% of the fish that were injected with the supernatants of TiLV-infected E-11 or TiLV-infected primary tilapia brain cultures developed clinical disease (lethargy, discoloration, ocular alterations, skin patches, and ulcerations) and died within 10 days (Fig. 4). The same mortality rate was also observed for fish injected with TiLV that was purified by endpoint dilution assay (TiLVx2; see Materials and Methods, and data not shown). Furthermore, the brains from experimentally infected fish were harvested and coincubated with naive E-11 cells; such cultures developed a characteristic CPE. The supernatants of these cultures were then harvested and injected into naive fish, resulting in the appearance of the disease in fish. Overall, this *in vivo*/*ex vivo* passage experiment was serially repeated three times, with a consistent mortality rate of 75 to 85% in each round within 10 days postinjection. This clearly confirmed that TiLV, isolated from infected fish and propagated in E-11 cells, is indeed the etiologic agent of the disease. Importantly, fish that survived the experimentally induced disease (35 fish) were completely immune to disease development upon a challenge consisting of a second i.p. injection (3 to 4 weeks after the first injection). This suggests that fish can mount a protective immune response to TiLV.

Reproduction of tilapine disease by cohabitation. Disease induction by i.p. injection is obviously artificial and does not repre-

sent the natural route of infection. To determine if TiLV is transmissible in a setting resembling natural conditions, a cohabitation experiment was performed in which naive fish were cohabitated with fish experimentally infected with TiLV (see Materials and Methods). These experiments clearly demonstrated that the naive fish developed a lethal disease, with a mortality rate similar to the one obtained by the i.p. route but with slower kinetics (2 to 3 days delay in reaching 50% mortality, $P < 0.05$ [Fig. 4]). These experiments provide proof of the ability of TiLV to spread through a waterborne route.

DISCUSSION

A serious emerging disease in wild populations of tilapine species in the Sea of Galilee, including *S. galilaeus*, *T. zilli*, *O. aureus*, and *T. simonis intermedia*, and in the pond-raised hybrid tilapia *O. niloticus* × *O. aureus* in Israel led to the current studies of infectious etiology. The association of disease outbreaks with seasonality (May to October, when the water has relatively high temperatures) further indicates the involvement of an infectious agent, since water temperature affects the emergence of a wide range of parasitic, bacterial, and viral diseases of fish (14, 29–34).

Here, we report the isolation of a previously undescribed virus, TiLV, from spontaneously diseased fish and the induction of disease in tilapia by this agent. The incubation of extracts from diseased but not healthy tilapines with cultures of fish cells (E-11 and primary tilapia brain cells) resulted in the appearance of CPE in infected cultures. Moreover, the inoculation of supernatants, harvested from these cultures, into naive tilapines resulted in the appearance of disease. TiLV was reisolated in cell cultures from experimentally infected fish, and this agent might induce a similar disease upon inoculation of new naive fish. Furthermore, an experimentally induced disease might be achieved with a purified TiLV obtained by endpoint dilutions. Of note, the signs of the naturally occurring disease (discoloration, skin patches, ocular alterations, and lethargy) were also observed in the experimentally induced disease. The TiLV sequences were amplified from diseased fish and TiLV-infected cell cultures but not from naive fish, mock-infected cultures, or cultures infected by another agent (VNN). Altogether, Koch postulates were fulfilled for this agent.

Several lines of evidence indicate that this infectious agent is a virus. First, the agent was through 0.22-μm filters while retaining its infectivity, ruling out the possibility of infection by microorganisms larger than this filter size (such as bacteria and fungi). Second, the appearance of CPE after serial passages of the agent in cell cultures excludes the possibility of a filterable toxin-induced CPE. Third, virion-like structures were visualized by EM in infected cells and in the supernatants of cultures of these cells. Fourth, CPE activity was demonstrated for relatively dense fractions of sucrose gradients, similar to known assembled virions. Fifth, the encapsidated TiLV genome is made of RNA, as evidenced by the fact that it was amplified by RT-PCR only (and not by PCR) from samples of sick fish and from cell cultures that were inoculated with extracts of such fish, as well as by the fact that this amplification was sensitive to initial digestion with RNase I. RNA genomes are only known to occur for viruses. EM analyses and the sensitivity of TiLV to organic solvents (ether or chloroform) further suggest that TiLV is an enveloped virus.

TiLV-induced disease in tilapines was achieved either by i.p. injections or by cohabitation; the cohabitation mode of transmission demonstrates the ability of TiLV to spread by the waterborne route. It should be noted that in these experiments, relatively high mortality rates were observed for both the i.p. and waterborne routes. This is in line with the extensive mortality observed in commercial ponds but not with the less extensive mortality observed in the Sea of Galilee. The possible explanation for this difference relies in the fact that our experimental system and commercial ponds represent a "closed community," in contrast to the Sea of Galilee, which resembles an "open community." The spread and outcome of diseases in the commercial ponds are usually much more severe than in the Sea of Galilee. Examples for the influence of the environmental conditions on disease progression (33) include piscine rhabdoviruses, in which classical acute hemorrhagic septicemias (35, 36) may change to subacute, chronic, or nervous forms (9, 37–41), and these are seldom difficult to visualize and monitor in open waters (42). Likewise, the influence of harsh husbandry conditions in close community on the severity of the disease was documented for VNN (14, 43).

The existence of fish that survived the TiLV-induced disease strongly suggests that an effective immune response against this pathogen can be mounted. This has important applications for future disease containment strategies. Besides the possibility of vaccine development, the determination of the susceptibility of different tilapia species to TiLV should be considered a measure of disease containment. This notion is based on the well-documented differences in disease resistance among species of the same genus. Examples include a wide range of susceptibilities for viruses infecting salmonids (12, 44–51) or for VNN resistance in Atlantic halibut (*Hippoglossus hippoglossus*) populations (52). Overall, fish that survive TiLV may establish a core of broodstock that can prevent the killing of the whole population. Indeed, survivors of piscine rhabdoviruses that developed adaptive immunity (46, 49, 53, 54) allowed with time the selection of rhabdovirus-resistant strains (44, 45).

This work also provides an initial molecular characterization of TiLV that includes cloning a portion of its genome. Notably, this clone possesses an ORF with no similarities to those of published sequences. This further suggests that TiLV is a new emerging pathogen for tilapia. In light of the extensive commercial production of tilapia (1, 2), and seeing as tilapia serve as a primary protein source in the developing world, it is highly important to diagnose this new pathogen. The amplification of TiLV sequences from diseased fish and TiLV-infected cultures, described in this work, provides the basis for a PCR-based diagnosis, allowing prompt screening, surveillance, epidemiological studies, and disease containment.

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