Diagnostics and vaccines for containment of TiLV - a novel RNA virus lethal to Tilapia

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Project award year: 2016
Three year research project
✓ List the original objectives, as defined in the approved proposal, and any revisions made at the beginning or during the course of the project.

Both this proposal (IS-4903-16C) and the feasibility project (IS-4583-13) that it continues, dealt with the identification of a novel virus that we named tilapia lake virus (TiLV), which causes high mortality to tilapia (and thus severely affects the tilapia aquaculture), and with the development of means (diagnostics and vaccination) to control the spread of this pathogen. The original objectives of the current proposal were: (1) Development of diagnostics for TiLV (culturing, PCR and ELISA assays). These objectives have been achieved as detailed in the Achievements document. (2) Development of vaccines against TiLV (attenuated strains, inactivated vaccine and DNA vaccine). We generated attenuated strains that in preliminary experiments protected tilapia from a challenge. Accordingly, the other suggested vaccination methods were not pursued. Yet, we identified one to two proteins that may serve as the basis for DNA vaccine. In addition, we constructed efficient tilapia expression plasmids (described in the Achievements document). (3) Testing tilapia species for their sensitivity to TiLV infection. We tested wild and farmed tilapia species, infected with TiLV, and characterized pathological signs (see in the Achievements document).

✓ Background to the topic.

Tilapia are an important global food source due to their omnivorous diet, tolerance for high-density aquaculture, and relative disease resistance. Tilapia aquaculture has been threatened by mass die-offs in farmed fish in Israel and other parts of the world. We identified the etiological agent of these disease outbreaks as a novel virus, with almost no homology to any other known viruses. This identification allowed us to develop diagnostic and preventing means to combat and contain TiLV infections.

✓ Major conclusions, solutions, achievements.

The infectious agent implicated in global mass tilapia die-offs poses a threat to the global tilapia industry, which not only provides inexpensive dietary protein but also is a major employer in the developing world. The major achievement of this project is the characterization of the causative agent as a novel virus - named by us tilapia lake virus (TiLV). We also decoded its complete genomic and protein sequences, enabling TiLV detection, containment, and vaccine development.

✓ Implications, both scientific and agricultural.

The discovery of TiLV has major agricultural implications: tilapia are an important global food source and according to the recent report of the Food and Agriculture Organization (FAO) of the United Nations, more than 140 countries are farming tilapia nowadays. For 2018, the economic impact of worldwide trade in tilapia is estimated at more than $12 billion U.S. dollars (USD) and is expected to reach more than $25 billion dollars in 2028. Along recent years in the US, tilapia consumption (imports and domestic) ranged between ~500 to ~600 mt of live weight equivalent. Moreover, developing countries around the world are significant tilapia producers. To date, the discovery of TiLV by us, has led to the identification of this pathogen in Israel and in the United States, and in at least 14 additional countries, across four continents. Hence, TiLV imposes a grave threat to the global tilapia industry and to food security. Accordingly, several important organizations, including the World Organization for Animal Health (OIE), the FAO, the Network of Aquaculture Centres in Asia-Pacific (NACA), and the CGIAR Research Program on Fish Agri-food Systems, have issued TiLV-related warnings. The discovery of TiLV by us and the means that we have developed to combat this virus should greatly contribute to the containment of this pathogen, worldwide, and should greatly benefit global aquaculture industry. TiLV discovery has also significant scientific implications: our success to provide sequences of TiLV complete genome and proteins, clearly demonstrated that TiLV has almost no homology to any other known viruses. Although some of its features suggest that it is an ‘orthomyxo-like’ pathogen, it is such a distinct virus that it has been assigned as a new species - *Tilapia tilapinevirus*. At least two important issues emerge from this notion: 1. what exactly the functions are of TiLV's novel proteins. This issue has a direct relevance for the development of means of detection and vaccination. 2. Are there additional viruses that cause significant illness in animals and that TiLV is their prototype? If such viruses exist, TiLV research should greatly contribute to their research too.
Summary Sheet

Publication Summary

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Training Summary

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Contribution of Collaboration: whether and how project objectives were promoted as a result of the cooperation

The synergistic work, as well as the different expertise of the three labs involved, have led to the successful achievements of the main goals of this project. Tilapia aquaculture has been threatened by mass die-offs in farmed and wild fish. Dr. Avi Eldar (Kimron Veterinary Institute, Israel) has suspected that these outbreaks originated from viral infections and together with Prof. Eran Bacharach (Tel Aviv University, Israel), established culture systems that allowed the isolation, propagation and purification of the virus (which they named ‘tilapia lake virus - TiLV’) from diseased fish. They further established an experimental system that demonstrated that the virus is the causative agent for the tilapia disease (the Bacharach lab established conditions to purify the virus and the Eldar lab found conditions for disease induction in fish, with the purified virus). These labs also sequenced a small portion of TiLV’s complex RNA genome. In collaboration with Prof. W. Ian Lipkin (Columbia University, USA), the sequence of the complete viral genome, has been determined, revealing that TiLV is a novel, emerging virus with almost no homology to other known viruses. With purified preparations (Eldar and Bacharach labs), the segmented nature of the genomic RNA was revealed (Lipkin lab). The Lipkin lab has predicted the open reading frames (ORFs) of TiLV proteins and the Bacharach lab performed mass-spectrometry analyses to confirm these predictions. Lipkin lab cloned portion of these ORFs into tilapia expression plasmids, made by the Bacharach lab. Lipkin lab also provided sequence comparison analyses among different TiLV isolates, establishing the (high) degree of conservation that exist for strains of this virus. The sequences and their conservation have provide the basis for all of the current studies that track the global spread of the virus. Furthermore, decoding the complete genome sequence (Lipkin Lab) allowed the Bacharach lab to elicit antibodies against specific viral peptides and proteins. The sequences, generated by the Lipkin lab, were also useful in generating specific probes for in situ hybridization studies (made by the three labs). Such probes assisted pathology examinations of tissues, dissected from disease fish (Lipkin and Eldar labs). In addition, Eldar lab performed classical pathology examinations of different infected tilapia species. Cold-adaptation of TiLV strains (Bacharach lab) were tested as possible attenuated vaccine strains by the Eldar lab.
Achievements (three pages maximum, 1.5 spaced, font not smaller than 12), in lay terms the major achievements accomplished in the project to date.

✓ Significance of main scientific achievements or innovations.

Achievements for the 1st aim - Development of diagnostics for TiLV:

• TiLV Culturing. We have screened for tilapia cell lines that efficiently support TiLV replication and identified OmB and TmB cell lines (in addition to the non-tilapia, E-11 cell line reported by us in the past). OmB, like E-11 cells, provided a convenient way to monitor CPE, since infected cultures completely detached. In addition, we defined the optimal culturing conditions (including the optimal temperature - 25 °C - for TiLV replication in cultured cells), that should allow the efficient culturing of the virus from diseased fish.

• RT-PCR assays. Briefly, we have developed sensitive conditions for RT-PCR and quantitative RT-PCR (qRT-PCR) assays to detect low amounts of TiLV RNA in organs of diseased fish and in infected cultures. The basis for these assays was the determination of TiLV’s genome sequence, made by us. Essentially, almost all additional RT-PCR-based methods, reported by other research groups, were developed, based on our published sequences.

• ELISA assays. Although the development of a complete ELISA assay has not been achieved yet, the important core reagents for such assays were developed. Specifically, we have generated several kinds of primary antibodies. These include both polyclonal (rabbit) and monoclonal (mouse) antibodies against TiLV-specific peptides. These peptides were generated from one of TiLV’s proteins that according to our biochemical analyses proved to protrude from the virion or surface and from the surface of infected cells. In addition, we generated polyclonal antibodies against yet another TiLV protein that our bioinformatics analyses indicated for its high antigenicity. Moreover, our molecular analyses infer that a high number of copies of this protein are expressed in infected cells and are packaged inside the virions. Indeed, detection of this protein (and thus TiLV) by immunoblotting proved to be very sensitive. The above antibodies were also used successfully to detect TiLV in infected cells, using fluorescence microscopy. Of note, high throughput sequencing (HTS) of field isolates of TiLV from South America and Israel demonstrated the relative high conversation of TiLV proteins between the two isolates.
Detection of TiLV RNA by *in situ* hybridization. Using Segment 5-derived probes with either positive or negative polarity to detect genomic or mRNA/antigenomic RNA, respectively, we were able to detect TiLV-infected cells, both *in vitro* and in tissues (liver) derived from infected fish.

**Achievements for the 2nd aim: Development of vaccines against TiLV:**

- **Attenuated strains.** We generated cold-adapted, attenuated strains that in preliminary experiments protected tilapia from a challenge (thus, we did not pursue other suggested vaccination methods, although we generated the basis for DNA vaccine - see below). Specifically, we first purified TiLV (by endpoint dilutions and infection of OmB/TmB cells) from the Snakehead Retrovirus (SnRV; chronically produced by the E-11 cells, which initially were used to isolate TiLV from diseased fish). We then adapted the virus to grow in non-physiological temperatures (18°C). This procedure resulted in cold-adapted TiLV strains, characterized by their lower titers and CPE development (of infected cultures) at 25 °C, compared to 18°C. We tested vaccine efficacy and virulence of the attenuated viral strains in preliminary disease induction experiments. For example, a group of Nile tilapia (60 fish) was immersed in water, containing candidate cold-adapted vaccine strain. One month later, 14 fish from this group, were challenged with a virulent TiLV. Surviving levels were 85.7%, for the vaccinated group, as opposed to only 28.5% in a control group, made of challenged, unvaccinated fish. Furthermore, additional 18 vaccinated fish were challenged with TiLV virulent strain, seven months post-vaccination. We recorded no death in this group, in sharp contrast to an extensive death (60%) in a control group (20 naïve fish, challenged with virulent TiLV). These preliminary results provide evidence that vaccination against TiLV is doable, and that the tested strains may serve as efficient vaccine strains.

- **DNA vaccine.** We have generated an efficient tilapia expression vector that highly expresses cloned genes (such as specific TiLV proteins), under the control of tilapia β-actin promoter. We further improved it by cloning an internal ribosome entry site (IRES), derived from the zebrafish connexin IRES, allowing polycistronic expression (of two proteins). Importantly, we identified two of the TiLV proteins that may serve as efficient antigens based on different characteristics: the first protein protrudes from membranes of infected cells and virions (we
demonstrated it by biochemical assays and fluorescence microscopy). The second protein has high antigenicity score (using bioinformatics) and exists in multiple copies in infected cells and virions (based on our molecular analyses). Regardless of their potential as antigens, we currently perform functional studies of these proteins to elucidate their biological functions.

Achievements for the 3rd aim: Testing tilapia species for their sensitivity to TiLV infection. We tested wild and farmed tilapia species, infected with TiLV, and characterized their pathological signs. We performed comparative pathology of TiLV lesions in the livers of three wild tilapines [Jordan tilapia, St. Peter’s fish (Sarotherodon (Tilapia) galilaeus) and common tilapia] and in two farmed species [Nile tilapia and commercial hybrid tilapia (O. niloticus × O. aureus)]. The most severe lesions were recorded in the hybrid tilapia (>80% of the normal hepatic parenchyma were replaced by foci of coagulative necrosis, pyknotic hypereosinophilic cells, macrophages and lymphocytes, multinucleated syncytial cells and scattered swollen hepatocytes). In common tilapia, 30-50% of the hepatic parenchyma was effaced. The Jordan Tilapia was affected to a lesser extent, with only scattered single hepatocytes exhibiting single cell necrosis and rare Inflammatory infiltrates. Interestingly, livers of Nile tilapia exhibited no necrosis and no inflammation, despite the presence of large numbers of multinucleated syncytial cells. No lesions were recorded for infected St. Peter’s fish. Our findings suggest that the presence of viral syncytia is probably not correlated with disease severity, since for Nile tilapia, which, consistently presents syncytial hepatitis, mortalities are lower than those of the common and Jordan tilapia. It will be interesting to establish if infected St. Peter’s fish, that showed relative good physical conditions and presented no hepatic lesions, act as carriers for TiLV.

✓ Agricultural and/or economic impacts of the research findings, if known. Tilapia is the second most important finfish aquaculture species worldwide. According to a recent FAO report, more than 140 countries are farming tilapia nowadays, with annual (estimated for 2018) worldwide trade of >$12 billion. We identified TiLV as the etiological agent for global mass die-offs of tilapia, which threaten the global food security and tilapia industry. In this research, we developed diagnostics and vaccination means for containment of this pathogen, worldwide. These should greatly benefit global aquaculture industry.
Changes to original research Plan

Because of the preliminary success with TiLV attenuated strains, we focused on the development of attenuated vaccine strains and not on TiLV inactivation.
### Publications for Project IS-4903-16C

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Identification of a Novel RNA Virus Lethal to Tilapia

Marina Eyngor, Rachel Zamostiano, Japhette Esther Kembou Tsofack, Asaf Berkowitz, Hillel Bercovier, Simon Tinman, Menachem Lev, Avshalom Hurvitz, Marco Galeotti, Eran Bacharach and Avi Eldar

Published Ahead of Print 17 September 2014.

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Tilapias 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 tilapiines and should help control the spread of this virus worldwide.

Tilapias 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, Mugilidae, and Claridae, only a catch cutback of tilapiines (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 2003 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 tilapiines (such as Tilapia zilli [common tilapia], Oreochromis aureus [Jordan tilapia], and Tristamella simonis intermedius) 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 tilapiines (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 tilapiines 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...
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 macroperatus; BB (ATCC CCL 59), from the brown bullhead Ictalurus nebulosus; EPC (ATCC CRL 2872) and KF-1 (13), from the common carp Cyprinus carpio; and E-11, from the striped snakehead Ophicephalus pumilus. 

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 homogenized and passed through a 100-μm mesh grinder; the cell suspensions 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 (medium supplemented with 10% glucose (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 conditioned medium 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 tilapiines 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 tilapia disease was reproducible by virus infection, 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 (isolated 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 (TCID50) (μl−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% O₃O₄ 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 out at the EM Unit, Tel Aviv University, exactly as described before (20), with an A TEM 1200-EX transmitting electron microscope (JEOL-USA, Peabody, MA, USA). The viros 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 ( Sorval 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 ( Sorval Discovery 90SE). One-milliliter fractions were collected from the top of the gradient, and the viros were pelleted from each fraction by ultracentrifugation (for 2 h at 65,000 rpm; T865 rotor; Sorval Discovery 90SE) and resuspended in 1 ml of TE buffer; 100-μl aliquots from each sample were incubated with naïve 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 viros and cDNA synthesis.** Nuclear acids were extracted from purified virion pellets using pegGOLD 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; Sorval Discovery 90SE) through a 30% sucrose cushion. The pellet was resuspended in TE, and viros were further purified by sucrose cushions of 40 to 70% (wt/vol). After ultracentrifugation (TST41.14 rotor for 2 h at 40,000 rpm; Sorval Discovery 90SE), the 40% sucrose fraction was collected, and viros were pelleted by additional ultracentrifugation (TST41.14 rotor for 2 h at 40,000 rpm; Sorval Discovery 90SE). RNA was extracted from the pellets by guanidine thiocyanate (pegGOLD 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 MuHl(N6) primer (GGAACCTCAAG TGCAAGGCTGNNNNNN) using ReddyMix PCR master mix (Thermo, Lithuania). The primed products were amplified by PCR with MuHl primer (GGCACTGAGTGAGACTGCTGGG) and cloned into the pETL2.2 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 pETL2.2-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 mini 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 (BLAST). Further searches of protein databases were done with the Basic Local Alignment Search Tool (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 Q1 (CCAGTGAGCAAGGACTGCTGGCAGTCAAG GCTTTTTTTTTTTTTTTTTTTTN and the SuperScript III first-strand synthesis system for RT-PCR (catalog no. 18080-045; Invitrogen), according to the manufacturer’s instructions. The cDNA was amplified with clone 7450/150R primer (TATACGGGGTGTAGCTGTCAGT) that was derived from an internal sequence of the shotgun fragment, and with Q1 primer (CCAGTGAGCAAGGACTGCTGGCAGTCAAG GCTTTTTTTTTTTTTTTTTTTTN) and the SuperScript III first-strand synthesis system for RT-PCR (catalog no. 18080-045; Invitrogen), according to the manufacturer’s instructions. The tailed cDNA was amplified by PCR using primer E1-11 inf-F (TCCAAAGGAAAC AGCTGAGC, derived from the sequence of the fragment) together with a mixture of the Q1 and Q2 primers. The resulting PCR products were diluted 1:20 and were subjected to a second PCR with the nested primers Q1 (GGCACTGAGTGAGACTGCTGGG, derived from Q1 primer) and E11-inf-R (AAGTTCTCTTGGCCTCTTGGG, derived from the sequence of the shotgun fragment). For 5′ RACE, cDNA was generated as above but with primer clone 7450/150F (CACCAGACTGTTGCGACATG). Poly(A) tails were added to the cDNA using terminal transferase (catalog no. 3336566; Roche), according to the manufacturer’s instructions. The tailed cDNA was amplified by PCR using primer E1-11 inf-F (TCCAAAGGAAAC AGCTGAGC, derived from the sequence of the fragment) together with a mixture of the Q1 and Q2 primers. The resulting PCR products were diluted 1:20 and were subjected to a second nested-PCR using the E11-inf-F (GAGCAATATGGTACCTG) and Q3 primers.

RT-PCR. Samples from the brain, head, kidney, spleen, liver, 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 (GGTGGCGAACAGG CACCTCTA) and clone 7450/150R (TATACGGGGTGTAGCTGTCAGT). 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, 55°C for 30 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).

Nucleic acid 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 uninfect ed 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 MgCl2, and 0.5 mM CaCl2) and were supplemented with 33 µg of RNase A (Sigma R6462) 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 nucleic-acid-protected nucleic acids from the virions and to digest possible leftovers of RNAase 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 MgCl2, 1 mM diethiothreitol [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 MgCl2, 1 mM DTT) with or without 50 units of RNAse I (catalog no. M02435; 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 TTTCTTGTGC] and Nested ext-2 [TGTGGTGAGGCAAGATTACC]) or with snakehead retrovirus (SnRV)-specific primers (Snakehead gag-pol rev [CAGATCAGTCTGATG]) and Snakehead gag-pol rev (GTCTGAAAGGTAAGGTTT). 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 tilapiine 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 (DO > 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 × 106 TCID50 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-infected fish were collected (5 to 7 days postinfection) 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 Israel...
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; Diaphan), 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 with 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 intraocular inoculation, 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 tilapiines from the Sea of Galilee and commercial ponds.** Recently, disease outbreaks in wild and commercial tilapiines 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 phacoclastic induced uveitis or endophthalmitis. There are cataractous changes within the lens characterized by eosinophilic homogenous spherical structures (morganian 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. 1G). 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 tilapiines, 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 post-inoculation, 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 naïve 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...
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...
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.

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

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<th>TABLE 1 Ether and chloroform sensitivity assays</th>
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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.

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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 with reverse transcriptase and/or RNase I prior to PCR amplification with TiLV-specific primers (Nested ext-1 [TATGCAGTACTTTCCCGC] and Nested ext-2 [TTGCTCTGAGCAAGAGTACC]; amplified product, 284 bp). M, the DNA size marker (B to D).

B 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 (TATCATGCTGATCTCGTTAC) 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 (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 (TATGCAGTACTTTCCCGC) and Nested ext-2 (TTGCTCTGAGCAAGAGTACC). (D) Nucleic 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 [TATGCAGTACTTTCCCGC] and Nested ext-2 [TTGCTCTGAGCAAGAGTACC]; amplified product, 491 bp) or SnRV-specific primers (Snakehead gag-pol fw [CAGATCCTGATCGAGTAC], and Nested ext-2 [TTGCTCTGAGCAAGAGTACC]; amplified product, 284 bp). M, the DNA size marker (B to D).

D 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 postinfection. 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-\( \mu \)m 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 1. 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 pathogen for tilapia. In light of the extensive commercial production of tilapia (1, 2), and seeing as tilapia serve as a primary protein pathogen for tilapia. In light of the extensive commercial production of tilapia (1, 2), and seeing as tilapia serve as a primary protein pathogen for tilapia. In light of the extensive commercial production of tilapia (1, 2), and seeing as tilapia serve as a primary protein pathogen for tilapia. In light of the extensive commercial production of tilapia (1, 2), and seeing as tilapia serve as a primary protein pathogen for tilapia. In light of the extensive commercial production of tilapia (1, 2), and seeing as tilapia serve as a primary protein pathogen for tilapia. In light of the extensive commercial production of tilapia (1, 2), and seeing as tilapia serve as a primary protein 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.

**ACKNOWLEDGMENTS**

We thank M. Gophen of the Galilee Research Institute for his valuable help in the preparation of the manuscript and Laurent Bigarre (ANSES, Plouzané, France) for his valuable help in ruling out known viral pathogens. We also thank Vered Holdengreber of the Electron Microscopy Unit of The George S. Wise Faculty of Life Sciences for the electron micrographs. Finally, we thank H. Shohat for his indispensable help in fish taxonomy and fish netting.

This work was supported by a United States-Israel Binational Agricultural Research & Development Fund (BARD) grant (BARD IS-4583-13) and by the Israel Ministry of Agriculture & Rural Development Chief Scientist Office (grant 847-0389-14). J.E.K.T. is supported by a fellowship from the Manna Center Program in Food Safety and Security at Tel Aviv University.
REFERENCES


Characterization of a Novel Orthomyxo-like Virus Causing Mass Die-Offs of Tilapia

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E.B. and N.M. contributed equally to this article.

ABSTRACT Tilapia are an important global food source due to their omnivorous diet, tolerance for high-density aquaculture, and relative disease resistance. Since 2009, tilapia aquaculture has been threatened by mass die-offs in farmed fish in Israel and Ecuador. Here we report evidence implicating a novel orthomyxo-like virus in these outbreaks. The tilapia lake virus (TiLV) has a 10-segment, negative-sense RNA genome. The largest segment, segment 1, contains an open reading frame with weak sequence homology to the influenza C virus PB1 subunit. The other nine segments showed no homology to other viruses but have conserved, complementary sequences at their 5’ and 3’ termini, consistent with the genome organization found in other orthomyxo-viruses. In situ hybridization indicates TiLV replication and transcription at sites of pathology in the liver and central nervous system of tilapia with disease.

IMPORTANCE The economic impact of worldwide trade in tilapia is estimated at $7.5 billion U.S. dollars (USD) annually. The infectious agent implicated in mass tilapia die-offs in two continents poses a threat to the global tilapia industry, which not only provides inexpensive dietary protein but also is a major employer in the developing world. Here we report characterization of the causative agent as a novel orthomyxo-like virus, tilapia lake virus (TiLV). We also describe complete genomic and protein sequences that will facilitate TiLV detection and containment and enable vaccine development.

Tilapia are increasingly important to domestic and global food security. Comprising more than 100 species, Nile tilapia (Oreochromis niloticus) is the predominant cultured species worldwide (1). Global production is estimated at 4.5 million metric tons with a current value in excess of $7.5 billion U.S. dollars (USD) and is estimated to increase to 7.3 million metric tons by 2030 (2, 3). The largest producers (in order) are China, Egypt, Philippines, Thailand, Indonesia, Laos, Costa Rica, Ecuador, Colombia, and Honduras. The United States is the leading importer, consuming 225,000 metric tons of tilapia annually (4). In addition to their value as an inexpensive source of dietary protein (5, 6), tilapia have utility in algae and mosquito control and habitat maintenance for shrimp farming (7). A wide range of bacteria, fungi, protozoa, and viruses has been described as challenges to tilapia aquaculture (8–10). Bacterial and fungal infections have been addressed through the use of antibiotics or topical treatments. No specific therapy has been described for viral infections of tilapia (11); however, viruses were not implicated as substantive threats until 2009, when massive losses of tilapia, presumed to be due to viral infection, were described in Israel and Ecuador (12, 13). Eyngor and colleagues investigated outbreaks in Israel and reported a syndrome comprising lethargy, endophthalmitis, skin erosions, renal congestion, and encephalitis and demonstrated transmissibility of disease from affected to naïve fish. They cultured a virus from infected fish in E-11 cells (cloned subculture of snakehead fish cell line), demonstrated sensitivity to ether and chloroform, and obtained a sequence specific for disease through cDNA library screening that predicted a 420-amino-acid (aa) open reading frame (ORF) with no apparent homology to any nucleic acid or protein sequence in existing databases. The virus was tentatively named tilapia lake virus lake virus (TiLV); however, no taxonomic assignment was feasible at that time (13). Ferguson and colleagues described a disease in farmed Nile tilapia in South America that differed from that caused by the Israeli virus in that pathology was focused in the liver rather than in the central nervous system (12). No agent was isolated; however, PCR using
primers and probes based on the sequence obtained from the virus isolated in Israel revealed the presence of a similar virus (J. Del Pozo, N. Mishra, R. N. Kabuusu, S. Cheetham Brows, A. Eldar, E. Bacharach, W. L. Lipkin, H. W. Ferguson, unpublished data).

Here, we report comprehensive analysis of the TiLV isolate from Israel. Unbiased high-throughput sequencing (UHTS), Northern hybridization, mass spectrometry (MS), in situ hybridization, and infectivity studies indicate that TiLV is a segmented, negative-sense RNA virus. TiLV contains 10 genome segments, each with an open reading frame (ORF). Nine of the segments have no recognizable homology to other known sequences; one segment predicts a protein with weak homology to the PB1 subunit of influenza C virus, an orthomyxovirus. Our findings suggest that TiLV represents a novel orthomyxo-like virus and confirm that it poses a global threat to tilapiine aquaculture (12, 13).

## RESULTS

### High-throughput sequencing and bioinformatic analysis

RNA extracts of brain from tilapia with disease in Israel were depleted of rRNA and were used as the template for Ion Torrent sequencing. RNA extracted from nuclease-treated, sucrose gradient-purified and concentrated particles from infected E-11 culture cells were used as the template for Illumina sequencing. Reads from two Ion Torrent and two Illumina libraries were taxonomically classified using taxMaps (https://github.com/nygenome/taxmaps) by mapping against the National Center for Biotechnology Information’s (NCBI) nucleotide database, the NCBI RefSeq database (14), the tilapia reference genome sequence (Oreinil.1), and corresponding annotated tilapiine mRNA sequences (15). Unclassified reads (not mapping to any known sequence) were then independently assembled using the VICUNA assembler (16). Contigs from each library were aligned with BLAST (17) against all contigs from the other 3 libraries, retaining hits with an E value of 1e−10 or lower to identify assembled sequences likely to derive from the same segment of the same species of virus in different samples. Single-linkage clustering was used to group together all of the contigs that showed any similarity. We identified 10 contig clusters that contained at least one contig in each of the 4 libraries. Within each cluster, contigs were aligned to each other and manually assembled to generate a maximum-length sequence after inverted tandem duplications at the ends of contigs, likely resulting from amplification artifacts, were removed. Overlapping predicted open reading frames (ORFs) in contigs from different assemblies were used to correct for frameshift errors and to infer the longest possible ORF. Based on a model wherein the genomic segments are anticipated to contain conserved termini, we used a combination of k-mer analysis, read depth analysis, and manual curation to build 5′ and 3′-terminal sequence motifs to refine terminal sequences. Mapping of the initial raw read data against the 10 final consensus sequences with BWA-MEM (17) demonstrated that 99% of the unidentified reads from the Illumina libraries and 87% of unidentified reads from the Ion Torrent libraries mapped to the consensus sequences.

### Characterization of TiLV genome

PCR primers were designed and used to amplify fragments representing all 10 contigs from RNA extracts from infected fish and purified virus particles. 5′ and 3′ rapid amplification of cDNA ends (RACE) was used to recover terminal sequences in all 10 clusters. Based on similarity in terminal sequences in the individual clusters, we concluded that the clusters represented 10 viral genomic segments and henceforth will refer to them as segments 1 to 10 (GenBank accession no. KU751814 to KU751823) (Table 1). Segment 1 is the largest at 1.641 kb. Segments 2 to 10 are 1.471, 1.371, 1.250, 1.099, 1.044, 0.777, 0.657, 0.548, and 0.465 kb, respectively. Segment 1 predicted a protein with weak homology to the PB1 subunit of influenza C virus (~17% amino acid identity, 37% segment coverage) (see Fig. S1 in the supplemental material). The other nine segments had no homology to other sequences in the GenBank nucleotide and protein databases with mega blast/BLASTx/tBLASTn or tBLASTx. Based on weak homology with PB1 motifs (18–23), we compared the segment 1 ORF with polymerase subunit PB1 recovered from specific members of the Orthomyxoviridae family. Segment 1 putative protein showed low homology to four motifs conserved in RNA-dependent RNA and RNA-dependent DNA polymerases (Fig. 1A).

The nucleotide sequences of the 5′ and 3′ noncoding regions of all 10 segments were aligned using Geneious software 6.8.1 (Fig. 1B). Thirteen nucleotides at the 5′ termini and 13 nucleotides at the 3′ termini were similar in all segments, an organization similar to that observed for influenza viruses (24, 25). Ten of 13 nucleotides on the 5′ termini and 7 of 13 at the 3′ termini had 100% conservation; the remaining nucleotides were conserved in segments 5 to 9. Nucleotide sequences showed complementary features at the 5′ and 3′ termini. Predicted molecular and biochemical features for individual viral segments are provided in Table 1. Mass spectrometry of virus isolated from E-11 cells confirmed the ORFs and coding peptides predicted for segments 2 to 10. No protein sequence was detected that represented segment 1 (Table 1; see Table S1 in the supplemental material). ORFs pre-

### TABLE I Genomic characterization of 10 segments of TiLV isolated from tilapia in Israel

<table>
<thead>
<tr>
<th>Segment no.</th>
<th>Segment length (nt)</th>
<th>GenBank accession no.</th>
<th>Predicted protein length (aa)</th>
<th>Protein mol mass (kDa)</th>
<th>pI</th>
<th>Identification by MS&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>457</td>
<td>51.227</td>
<td>9.64</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
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<td>KU751816</td>
<td>419</td>
<td>47.708</td>
<td>7.99</td>
<td>+</td>
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<td>38.625</td>
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<td>+</td>
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<tr>
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<td>4.45</td>
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</tr>
</tbody>
</table>

<sup>a</sup> Table S1 in the supplemental material describes peptides identified by mass spectrometry.
dicted from segment 5 and segment 6 contained signal peptide cleavage sites between aa 25/26 and 30/31, respectively.

High-throughput sequencing and alignment analyses of a TiLV PCR-positive sample from a fish in Ecuador showed 97.20 to 99.00% nucleotide identity and 98.70 to 100% amino acid identity to the corresponding coding region of nucleotide sequences obtained with samples from Israel (Table 2).

Northern hybridization experiments indicate that TiLV is a segmented RNA virus. The segmented nature of TiLV and the sizes of specific segments were confirmed in Northern hybridization analyses of extracts from E-11 cells infected with TiLV (brain isolate) (Fig. 2A) and from liver of infected fish (Fig. 2B), using 10 segment-specific, discrete probes.

In situ hybridization. To investigate the presence of TiLV RNA in diseased fish, we applied in situ hybridization techniques to both liver and brain samples with TiLV-specific probes. In the brain, hybridization signals for segment 1 genomic RNA (Fig. 2A, arrowheads) and mRNA (Fig. 2B, arrowheads) were confined to the leptomeninges, mostly adjacent to blood vessels. Similar results were obtained for genomic RNA and mRNA of segment 5 (data not shown). No hybridization signal was detected in sections of brains of uninfected healthy controls (naive fish) of approximately the same age collected from a different breeding facility in Israel (see Fig. S2A in the supplemental material). In the liver, hybridization signal for segment 3 mRNA was detected in hepatocytes. Many nuclei were clustered, suggesting the formation of multinucleated cells (Fig. 3C and D). To image infected cells with high resolution, E-11 cultures were infected with TiLV, hybridized with segment 3-derived probes to detect viral mRNA, and imaged. TiLV mRNA was detected in both the nucleus and cytoplasm of multiple cells (Fig. 3E and F). No viral mRNA was detected in noninfected control cells (see Fig. S2B).

TiLV has a negative-sense genome. Based on sequence and RNA hybridization analysis, we postulated that TiLV has a negative-sense genome. Accordingly, a deproteinized genome should not be infectious. RNA was purified from TiLV virion pellets or from TiLV-infected E-11 cells and was transfected into naive E-11 cells. For positive controls, naive E-11 cells were transfected with nervous necrosis virus (NNV [a positive-sense, segmented RNA virus of fish]) RNA extracts from NNV-infected cells or from NNV virions. No cytopathic effect (CPE) was observed following transfection of deproteinized TiLV RNA extracted from cells or virions (Fig. 4A and D), similar to the lack of cytopathic effect upon transfection of control RNA extracted from naive E-11 cell culture (Fig. 4C), from pellets of the culture supernatant (Fig. 4F), or in mock-transfected cells (Fig. 4G). In contrast, transfection of deproteinized NNV RNA, extracted from infected cells (Fig. 4B) or from virions (Fig. 4E), resulted in cytopathic effect in E-11 cells.

FIG 1 Genomic characterization of the segments of TiLV isolated from tilapia in Israel. (A) TiLV segment 1 putative protein shows weak homology to motifs conserved in RNA-dependent polymerases. Sequence comparison of TiLV’s segment 1 predicted protein with motifs I to IV, conserved in polymerases of influenza virus strains C/J/J50 (Inf C) (19), A/WSN/33 (Inf A) (34), and B/Lev/40 (Inf B) (35), vesicular stomatitis virus (VSV) (20), human immunodeficiency virus (HIV) (21), and poliovirus (Polio) (22). The relative motif positions are also shown. Invariant sequences in each motif are in boldface and underlined. TiLV sequences that show identity to one of the influenza virus sequences are highlighted in yellow. (B) Genomic segments of TiLV show conserved and homologous features at 5’ and 3’ termini.
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DISCUSSION

This study was undertaken to characterize the molecular biology, pathogenesis, and partial geographic distribution of TiLV, a novel virus recently implicated in large die-offs of tilapia in Israel and Ecuador (Fig. 3G) (12, 13). Efforts to classify TiLV through classical sequence homology analyses of sequences obtained from infected fish and cultured cells had failed; thus, we pursued Northern hybridization, mass spectrometry, and in situ hybridization to identify and correlate the presence of candidate viral genes and proteins. Results presented here indicate that TiLV is an RNA virus with a genome comprised of 10 unique segments. The largest segment has minimal homology to the PB1 segment of the orthomyxovirus influenza virus C. It also contains the major polymerase motifs (19). We speculate therefore that segment 1 encodes the polymerase of TiLV. The other 9 segments have no apparent homology to other known viral sequences; however, the presence of complementary sequences at their termini and identification of proteins in extracts of infected cells that correlate with the ORFs they carry provide evidence that they represent gene segments of TiLV. Sequence analyses provide no insight into their functions in the TiLV life cycle. In situ hybridization experiments indicated a nuclear site for transcription.

It is likely that TiLV will ultimately be classified as representing a new genus of the family Orthomyxoviridae. As noted above, the 5’ and 3’ noncoding termini of TiLV include 13 nucleotides that are similar in all segments. This organization resembles that observed for the influenza, Thogoto, and infectious salmon anemia orthomyxoviruses (24–27) and enables base pairing and formation of secondary structures important for replication, transcription, and packaging of viral RNA. In addition, all of the 5’ ends of TiLV genomic RNA segments contain a short, uninterrupted uridine stretch (3 to 5 bases long). This feature is reminiscent of the stretch of 5 to 7 uridine residues found in other orthomyxoviruses, on which the viral polymerase “stutters” while generating poly(A) tails (28, 29).

The presence of viral nucleic acid at sites of pathology in brain and liver together with the observation that virus propagated in cell culture is capable of inducing disease in naive fish implicates TiLV as the causative agent of outbreaks of both viral encephalitis and syncytial hepatitis in tilapiines in Israel and Ecuador. The fact that TiLV has been detected in association with disease in two geographically disparate sites underscores that TiLV poses a global threat to tilapiine aquaculture. We have no evidence that TiLV can infect other species; however, the genetic dissimilarity of TiLV to other viruses suggests the potential for discovery of related viruses as TiLV sequences are employed for phylogenetic analysis.

MATERIALS AND METHODS

Nucleic acid extraction, library preparation, and high-throughput sequencing. Unbiased high-throughput sequencing was performed on Illu-
mina HiSeq 2500 and PGM Ion Torrent platforms. For library preparations, total RNA was extracted from purified virus particles and infected fish brain tissue samples with TRI reagent (Sigma-Aldrich, St. Louis, MO). RNA extractions from TiLV-infected tissues included postextraction DNase I (2 U/µg DNA for 15 min at 37°C; Thermo, Fisher, Waltham, MA) and depletion of rRNA sequences with RiboZero magnetic kits (Illumina, San Diego, CA). High-throughput sequencing was performed in parallel on random-primed cDNA preparations from purified virus and infected brain tissue RNA. Sequencing from purified virus on the Illumina HiSeq 2500 platform (Illumina) resulted in an average of ~200 million reads (100 nucleotides [nt]) per sample. Total RNA was reverse transcribed using SuperScript III (Thermo, Fisher) with random hexamers. The cDNA was RNase H treated prior to second-strand synthesis with Klenow fragment (New England Biolabs, Ipswich, MA). The resulting double-stranded cDNA mix was sheared to an average fragment size of 200 bp using the manufacturer’s standard settings (E210 focused ultrasonicator; Covaris, Woburn, MA). Sheared product was purified (AxyPrep Mag PCR cleanup beads; Axygen/Corning, Corning, NY), and libraries were constructed using KAPA library preparation kits (KAPA, Wilmington, MA) with 6-nt bar code adapters. The quality and quantity of libraries were checked using Bioanalyzer (Agilent, Santa Clara, CA). Samples were demultiplexed using Illumina-supplied CASAVA software and exported as FastQ files. More than 90% of Illumina reads passed the Q30 filter. Demultiplexed FastQ files were assembled to generate reads, contigs, and clusters. Sequencing on the Ion Torrent PGM platform was performed with Ion PGM Sequencing 200 kits on Ion 318 chips (Life Technologies, Carlsbad, CA), yielding on average ~3 million reads per sample, with a mean length of 177 nt. For Ion Torrent, cDNA preparations were sheared (Ion Shear Plus kit; Life Technologies) for an average fragment size of 200 bp and added to Agencourt AMPure XP beads (Beckman Coulter, Brea, CA) for purification. Libraries were prepared with KAPA library preparation/Ion Torrent series kits (KAPA), and emulsion

**FIG 3** Detection of TiLV RNA in brain and liver of infected tilapia and infected E-11 cells by *in situ* hybridization and image of dead tilapia in Israel. (A and B) Brain sections of infected Nile tilapia hybridized with Affymetrix Cy3-conjugated probes (red) of various polarities to TiLV segment 1 to detect genomic RNA (A) or mRNA (B). White arrowheads indicate hybridization signal. (C) Liver sections hybridized with Cy3-conjugated (red) Stellaris probes to segment 3 to detect mRNA. Nuclei are stained with DAPI (blue). (D) Liver section stained with hematoxylin and eosin reveals multinucleated giant cells (asterisk). (E) TiLV-infected E-11 cells hybridized with Quasar 670-conjugated (red) Stellaris probe to segment 3 to detect TiLV mRNA. Nuclei are stained with DAPI (blue). (F) Images of confocal sections of cells in panel E were reconstituted into a 3D image. (G) Dead tilapia at a fish farm in Israel.

**FIG 4** TiLV deproteinized RNA is not infectious. Naive E-11 cell cultures were transfected with deproteinized RNA, extracted from cultured cells ("Cellular RNA" [A to C]) or pellets of culture supernatants ("Virion RNA" [D to F]), from TiLV-infected ("TiLV RNA" [A and D]) or NNV-infected ("NNV RNA" [B and E]) E-11 cells, or from naive E-11 cells ("E-11 RNA" [C and F]). Transfection with no RNA ("Mock" [G]) was also included. Bright-field images of transfected cultures were taken at 8 days posttransfection.
PCR was performed with Ion PGM Template OT2 200 kits (Life Technologies). Ion Torrent reads were demultiplexed and exported as FastQ files by the Ion Torrent PGM software. After bar code and adaptor trimming, length filtering, masking of low-complexity regions, and subtraction of ribosomal and host sequences, reads were mapped as described for Illumina data.

**Mass spectrometry.** TiLV virions were purified by ultracentrifugation through 25% (wt/vol) sucrose cushions. The samples were trypsinned and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on Q Exactive Plus (Thermo Scientific). Peptides were identified by Discoverer software version 1.4, using the Sequest search engine, the Uniprot database, and the putative TiLV proteins as references. All of these analyses were performed at the Smoler Proteomics Center, Technion, Israel.

**Northern hybridization analysis.** Fragments representing each segment were amplified by PCR using primers shown in Table S2 in the supplemental material. Amplified PCR products were purified with QIAquick gel extraction kit (Qiagen, Hilden, Germany) and labeled with biotin with the Deca Label DNA labeling kit (Thermo, Fisher Scientific). RNA was extracted from E-11 cells infected with TiLV from brains of tilapia from Israel and from livers of infected tilapia from Ecuador. RNA extracts were size fractionated by electrophoresis on a denaturing 1.5% agarose gel, transferred to Biobond-Plus nylon transfer membrane (Sigma-Aldrich), and UV cross-linked. Membranes were hybridized overnight in a low-shaker incubator with biotinylated probe combos (1 or 2) at 65°C in 6X SSC (1X SSC is 0.15 M NaCl plus 0.005 M sodium citrate) (3), (31). Biotin-labeled probes were detected with the chemiluminescent niacin acid detection module kit (Thermo, Fisher Scientific). Blots were scanned with a C DiGit chemiluminescence blot scanner (LI COR).

**In situ hybridization.** To detect TiLV RNA, in situ hybridizations were performed with ViewRNA probes (Affymetrix, Santa Clara, CA) and/or Stellaris fluorescent probes (Biosearch Technologies, Novato, CA) in tissue sections and cultured infected or noninfected cells. Tissue samples were collected from euthanized infected or uninfected fish and fixed in 10% neutral buffered formalin. Specimens were embedded in paraffin and serially sectioned (5 μm thick), fixed in 10% formaldehyde (Fisher Scientific), deparaffinized, boiled in pretreatment solution (Affymetrix), and digested with proteinase K (Affymetrix). Sections were hybridized for 3 h at 40°C with custom-designed Quantigene ViewRNA probes (Affymetrix). Bound probes were then amplified per protocol from Affymetrix using PreAmp and Amp molecules. Multiple-label probe oligonucleotides conjugated to alkaline phosphatase (LP-AP type 1) were then added, and Fast Red substrate was used to produce signal (red dots, Cy3 fluorescence). Infected and noninfected cultured cells, grown on 2% gelatin-coated glass coverslips, were fixed with 3.7% formaldehyde. Negative-sense probes, derived from segment 3, labeled with Cy3 or with Quasar 670 fluorophores, and compatible with the Stellaris system, were constructed to detect TiLV mRNA (see Table S3 in the supplemental material). When applied, nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole). Sections were examined by light microscopy (Axio Scope.A1; Carl Zeiss), fluorescence microscopy (Nikon TE2000 or Zeiss Axiosvert 200 M), and confocal microscopy with three-dimensional (3D) image reconstruction (33).

**Infectivity of deproteinized RNA.** Confluent E-11 cell cultures in 25-cm² flasks were infected, or not, with TiLV or NNV (one flask per virus). Cells and culture supernatants were collected upon the appearance of cytopathic effect (CPE) (2 or 3 days postinfection for NNV or TiLV, respectively). Deproteinized RNA was prepared from cells using EZ-RNA extraction kit (Biological Industries, Israel), according to the manufacturer’s instructions. Supernatants were cleared by centrifugation (200 × g at room temperature for 5 min), and virions were pelleted by ultracentrifugation (107,000 × g for 2 h) through a 25% (wt/vol) sucrose cushion. Each pellet was resuspended in 140 μl phosphate-buffered saline (PBS), and deproteinized RNA was extracted using QIAamp viral RNA isolation kit (Qiagen). E-11 cells (80% confluence in 6-well plates) were transfected with the 2.5 μg of cellular deproteinized RNA or with the entire deproteinized virion RNA preparation, using the TransIT-mRNA transfection kit (Mirus Bio LLC, Madison, WI).

**Sequence accession numbers.** TiLV sequences are available at GenBank under the following accession numbers: KU751814, KU751815, KU751816, KU751817, KU751818, KU751820, KU751821, KU751822, KU751823.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mbio.00431-16/-/DCSupplemental.

**Figure S1, PDF file, 0.1 MB.**

**Figure S2, TIF file, 2.7 MB.**

Table S1, XLSX file, 0.01 MB.

Table S2, XLSX file, 0.1 MB.

Table S3, XLSX file, 0.01 MB.

**ACKNOWLEDGMENTS**

We are grateful to Marcelo Ehrlich (Tel Aviv University) and E. Zelinger (Center for Imaging, The Robert H. Smith Faculty of Agriculture, Food and Environment; Hebrew University) for confocal and fluorescence microscopy, Tal Papko and Haim Ashkenazy (Tel Aviv University) for bioinformatics analyses, Tamar Ziv (Technion) for mass spectrometry assays, Milada Mahic (Columbia University) for influenza A virus cultures, Suresh Sharma (Penn State University), Craig Cameron (Penn State University), Katri Basso (Columbia University), and Riccardo Dalla-Favera (Columbia University) for advice on Northern hybridization experiments, and Ellie Kahn for manuscript assistance.

This work was supported by a U.S.-Israel National Agricultural Research and Development Fund grant (BARD IS-4583-13), the Israel Ministry of Agriculture and Rural Development Chief Scientist Office (grant 847-0389-14), NIH AI109761(Center for Research in Diagnostics and Discovery, Center for Excellence in Translational Research), USAID PREDICT, and a fellowship to J.E.K.T. from the Manna Center Program in Food Safety and Security at Tel Aviv University.

**FUNDING INFORMATION**

This work, including the efforts of Japhette Esther Kembou Tsofack, was funded by Manna Center Program in Food Safety and Security at Tel Aviv University. This work, including the efforts of W. Ian Lipkin, was funded by HHS | National Institutes of Health (NIH) AI109761(Center for Research in Diagnostics and Discovery, Center for Excellence in Translational Research), USAID PREDICT, and a fellowship to J.E.K.T. from the Manna Center Program in Food Safety and Security at Tel Aviv University.

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36. Characterization of Novel Tilapia Orthomyxo-like Virus
Detection of Tilapia Lake Virus in Clinical Samples by Culturing and Nested Reverse Transcription-PCR

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ABSTRACT Tilapia are an important group of farmed fish that serve as a significant protein source worldwide. In recent years, substantial mortality of wild tilapia has been observed in the Sea of Galilee and in commercial ponds in Israel and Ecuador. We have identified the etiological agent of these mass die-offs as a novel orthomyxo-like virus and named it tilapia lake virus (TiLV). Here, we provide the conditions for efficient isolation, culturing, and quantification of the virus, including the use of susceptible fish cell lines. Moreover, we describe a sensitive nested reverse transcription-PCR (RT-PCR) assay allowing the rapid detection of TiLV in fish organs. This assay revealed, for the first time to our knowledge, the presence of TiLV in diseased Colombian tilapia, indicating a wider distribution of this emerging pathogen and stressing the risk that TiLV poses for the global tilapia industry. Overall, the described procedures should provide the tilapia aquaculture industry with important tools for the detection and containment of this pathogen.

KEYWORDS virus, TiLV, tilapia, diagnosis, PCR

Tilapiines, a generic term for edible fish belonging to the family Cichlidae, are fast growers, efficient food converters, and relatively disease resistant. These assets render them most suitable for farming; indeed, tilapiines are one of the most significant groups of farmed fish worldwide and serve as an important protein source, especially in developing countries (1–6). Common ectoparasites and the few bacterial pathogens of tilapiines are well controlled by pharmacotherapy. Few viral diseases have been reported for tilapia, and these are of limited impact (7–9).

Recently, a novel RNA virus termed tilapia lake virus (TiLV) has been identified and recovered from episodes of massive mortalities of wild and pond-cultured tilapia all over Israel (10). High mortalities were also observed in naive tilapia exposed to an isolate of TiLV (10). Tilapia mortality, suspected of having a viral etiology, has also been described in Ecuador (11, 12). Although variations in pathological presentation have been described (where lesions were focused in the central nervous system or in the liver, in Israel or Ecuador, respectively), sequencing the whole genome of TiLV revealed that tilapia in the two countries were infected with almost identical viruses (4). This analysis also revealed that this pathogen is a novel orthomyxo-like virus with a 10-segment negative-sense RNA genome (4). Segment 1 contains an open reading frame (ORF) with weak sequence homology to the polymerase subunit (PB1) of
influenza C virus, while the other nine segments showed no homology to other viruses.

TiLV outbreaks are characterized by high mortalities and economical losses (10, 11), and no vaccines against TiLV are currently available. Thus, there is a great need for the implementation of prompt control measures: culling of infected stocks, setting quarantine, restricting trades, and control of possible vectors. This calls for the development of fast and sensitive detection methods and improved culturing techniques. Here, we show that the presently available reverse transcription-PCR (RT-PCR) assay (10), although highly specific, is of limited sensitivity when applied to clinical samples. Accordingly, we now describe a highly sensitive nested RT-PCR assay for TiLV detection from clinical specimens. In addition, TiLV-sensitive cell lines, other than the reported E-11 cells (10), are described, and the optimal parameters for TiLV culturing are defined.

RESULTS

Temperature-dependent viral growth. Being ectotherms (and cultured at 16 to 32°C range) (13), tilapia may be infected over a relatively wide range of temperatures; yet, the effect of temperature on TiLV replication, and thus on its isolation, has not been studied. Hence, TiLV growth at various temperatures was evaluated by infecting monolayers of E-11 and primary tilapia brain cells, with subsequent incubation at various temperatures (15, 20, 25, and 30°C) for up to 19 days. Infection was quantified by quantitative RT-PCRs (qRT-PCRs), measuring TiLV RNA expression levels, with TiLV-specific primers. Viral RNA levels were normalized to cellular β-actin mRNA levels (relative quantification [RQ]) (see Fig. 1 and Materials and Methods). In E-11 cells, the maximum increase in TiLV RNA levels was observed at 25°C at day 9 postinfection (RQ, 1,328; Fig. 1A). Higher (30°C) or lower (20°C) temperatures resulted in reduced TiLV RNA levels (RQ, 191 and 490, respectively, day 9 postinfection). At 15°C, TiLV RNA production was dramatically reduced (RQ, 35; day 9 postinfection) and reached maximal levels at day 15 postinfection. In infected primary tilapia brain cells, 25°C was also the optimal temperature for TiLV replication (Fig. 1B); yet, this replication peaked only at day 14 postinfection and reached much lower levels (about 12%) compared to the one in E-11 cells. Altogether, E-11 cells at 25°C provide optimal conditions for TiLV replication; thus, all isolations from clinical samples (see below) were carried out under these conditions.

Quantification of TiLV growth in tilapia cell lines. In our former study (10), eight established fish cell lines were tested for their permissiveness to TiLV infection, and only E-11 cells were found to be suitable for this purpose. We now extended this analysis to three additional tilapia cell lines derived from ovary (TO-2 (14), brain (OmB [15]), and bulbus arteriosus (TmB [16]). Initial qualitative analyses revealed that OmB and TmB, but not TO-2, support TiLV replication (data not shown). We next compared the use of the permissive cell lines (E-11, OmB, and TmB) in the quantification of TiLV infection by endpoint dilution assays. The cell lines were infected with dilutions of the virus, cytopathic effect (CPE) was monitored for 14 days, and 50% tissue culture infective dose (TCID_{50}) values were calculated. The results from three independent experiments are

![FIG 1 TiLV replication at different temperatures. E-11 (A) and primary brain (B) cultures in 24-well plates were infected with TiLV and incubated at the indicated temperatures. Total RNA was extracted from infected cells at the indicated time points postinfection, and TiLV and cellular β-actin RNA levels were quantified by qRT-PCR. The results show the relative quantification (RQ) of TiLV RNA levels, relative to β-actin RNA levels (means of duplicates ± standard deviations).]
showed in Table 1. All three cell lines showed comparable sensitivities to TiLV infection. Yet, E-11 cultures were superior because the CPE development was clearly detected in a relative short time (4, 6, or 8 days postinfection for E-11, TmB, or OmB, respectively). OmB cells also provided a convenient way to monitor CPE at a longer time postinfection (14 days), as uninfected cells remained attached as monolayers, while infected cultures completely detached at this time point. TmB cells were sensitive to TiLV-induced CPE, yet we found that detecting CPE in this line was more difficult than for the other lines because TmB cells did not support the formation of clear plaques, and only a portion of the infected cells detached from the plate over time.

**Sensitivity and specificity of TiLV detection by PCR.** A sensitive RT-PCR detection method for TiLV is required for rapid and accurate diagnosis of this threat to tilapine aquaculture. We have described TiLV detection by RT-PCR (10); however, the assays were not optimized. To optimize this procedure, we first prepared a standard curve for this assay. Specifically, a 491-bp-long PCR fragment derived from TiLV clone 7450 (GenBank accession no. KJ605629) (10) was cloned into a plasmid, and dilutions of the resulting plasmid DNA were used in the following PCR/gel electrophoresis (Fig. 2A) and qPCR (Fig. 2B) reactions. Different sets of primers were used to amplify either the 491-bp fragment (“external PCR”; Fig. 2A, top) or an internal 250-bp fragment (“internal PCR”; Fig. 2A, middle). An additional reaction (“nested PCR”; Fig. 2A, bottom) consisted of the external PCR combined with the internal PCR (see Materials and Methods). This analysis showed that as expected, the external and internal PCRs were less sensitive than the nested PCR: the highest dilution in which the TiLV sequence was clearly detected by the external or the internal PCR was $10^{-6}$ (Fig. 2A, lane 2, top and middle), corresponding to the detection of $70,000$ TiLV copies. The nested PCR showed much higher sensitivity, enabling the detection of as few as 7 copies of TiLV sequence (Fig. 2A, lane 6, bottom). Amplification of the above-mentioned diluted plasmid DNA by qPCR also demonstrated the higher sensitivity of the nested PCR over the nonnested PCRs, as much lower threshold cycle ($C_T$) values were obtained for the nested PCR (Fig. 2B). Of note, the detection limit of the nested qPCR (70 copies of TiLV sequence; Fig. 2B) was higher than that of the nested PCR (7 copies of TiLV sequence; Fig. 2A). These differences likely result from the different reagents and conditions used for these two types of reactions (see Materials and Methods).

The specificity of the developed nested PCR was further demonstrated by the amplification of TiLV sequences from cDNAs that were prepared from TiLV-infected E-11 cells, but not from negative samples composed of cDNA of nervous necrosis virus (NNV)-infected or naive E-11 cells (Fig. 2C).

**TiLV detection in diseased tilapia from Israel.** Based on the optimal conditions for TiLV growth and detection defined above, we next set out to isolate TiLV from clinical specimens obtained from 13 different outbreaks between 2011 and 2013 in nine different commercial farms distributed across Israel (Galilee, Jordan Valley, and Mediterranean coastal areas). In all these outbreaks, diseased fish showed typical symptoms related to TiLV infection (10). Brain samples were obtained from commercial pond-raised tilapia for human consumption (Oreochromis niloticus × Oreochromis aureus hybrids; specimens 1 to 11, Table 2) and ornamental African cichlids (specimens 12 and 13, Table 2). Brain samples were chosen for these analyses because this tissue is relatively confined and susceptible to TiLV infection (10). The brains were homogenized

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>TCID$_{50}$ by cell line</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>E-11</td>
</tr>
<tr>
<td>1</td>
<td>$3.2 \times 10^5$</td>
</tr>
<tr>
<td>2</td>
<td>$4 \times 10^5$</td>
</tr>
<tr>
<td>3</td>
<td>$1.6 \times 10^5$</td>
</tr>
</tbody>
</table>

*The same stock of TiLV (grown in E-11 cells) was quantified in three independent endpoint dilution assays. Values are in TCID$_{50}$/ml.
(pools of 2 to 3 brains for each outbreak for samples 1 to 11; samples 12 and 13 each consisted of a single brain) and added to E-11 cells, cultured at 25°C. This procedure resulted in the appearance of CPE at 5 to 6 days postinoculation in 12 out of the 13 cases (Table 2). For specimen 12, two additional passages in E-11 cell cultures were required before CPE became apparent (see Materials and Methods). No CPE was observed for a negative-control group consisting of 15 fish that were collected from ponds showing mortality due to either environmental conditions (low oxygen levels or high ammonia concentrations) or other infectious diseases (i.e., streptococci) (data not shown).

The above-mentioned 13 brain tissues were also tested for the presence of TiLV sequences by the internal and nested PCRs described above. For this, total RNA was extracted from portions of the brains, reverse transcribed using random primers, and PCR amplified with TiLV-specific primers (see Materials and Methods). The internal PCR detected TiLV sequences in only three samples (23%), in contrast to the nested PCR that detected the virus in 12 samples (92%, Table 2). The amplification of TiLV sequences was also verified by sequencing the PCR products (data not shown). None of the negative controls scored positive when examined by the nested PCR, further demonstrating the specificity of this assay (data not shown).
To further examine the developed nested RT-PCR, we applied it to detect TiLV RNA in liver samples preserved in RNA later reagent, which were taken from South American tilapia showing signs of syncytial hepatitis (11, 12), or from healthy controls. This test was performed in a blinded way using the following procedure: the presence or absence of TiLV RNA in the samples was tested by RT-PCR (12) at St. George’s University, Grenada. The samples were then coded and shipped, preserved in an RNA later reagent, to Tel Aviv University, where RNA was extracted and nested RT-PCR was performed without knowing the samples’ identities. Figure 3A shows the results of this procedure for Ecuadorian samples: six examined samples scored positive (lanes 1 to 6), while six samples scored negative (lanes 7 to 12). This fully matched the classification made of the samples before shipment.

Tilapia with syncytial hepatitis were also observed in farms in Colombia, and liver samples were examined for the presence of TiLV RNA, as described above. This analysis revealed that out of the six samples that were scored positive for TiLV, four samples also scored positive after their shipment (Fig. 3B, lanes 1 to 4), while the two other samples scored negative (Fig. 3B, lanes 5 and 6). This discrepancy likely resulted from the degradation of TiLV RNA in these samples. Indeed, attempts to amplify TiLV RNA from these two samples using different sets of primers that were derived from another segment of TiLV genome failed as well (data not shown). For the negative samples, no PCR products were observed (Fig. 3B, lanes 7 to 12).

Overall, these results demonstrate that the developed nested RT-PCR can be applied for detection of TiLV strains in Israel and South America and suggest that preserved material can be analyzed as well. Importantly, these results further show, for the first

### TABLE 2 TiLV detection in clinical specimens by culturing, RT-PCR, and nested RT-PCR

<table>
<thead>
<tr>
<th>Specimen no.</th>
<th>Location</th>
<th>TiLV detection</th>
<th>CPE</th>
<th>RT-PCR</th>
<th>Nested RT-PCR</th>
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<tbody>
<tr>
<td>1</td>
<td>Farm 1, Galilee</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Farm 1, Galilee</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3</td>
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<td>+</td>
<td>−</td>
<td>+</td>
<td></td>
</tr>
<tr>
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<td>Farm 2, Jordan Valley</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td></td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6</td>
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<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Farm 4, Jordan Valley</td>
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</tr>
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<td>−</td>
<td>+</td>
<td></td>
</tr>
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<td>10</td>
<td>Farm 7, Jordan Valley</td>
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<td>−</td>
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</tr>
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<td>11</td>
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<td>+</td>
<td>−</td>
<td>−</td>
<td></td>
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<tr>
<td>12</td>
<td>Farm 9, Jordan Valley</td>
<td>+ b</td>
<td>−</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Farm 9, Jordan Valley</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>% positive</td>
<td></td>
<td>100</td>
<td>23</td>
<td>92</td>
<td></td>
</tr>
</tbody>
</table>

aCPE was detected in E-11 cells incubated with the brain specimens.
bCPE visible only after two additional passages on E-11 cell cultures.

**FIG 3** Detection of TiLV RNA in preserved tilapia livers from Ecuador and Columbia. Nested RT-PCR was used to determine the presence or absence of TiLV RNA in liver samples preserved in RNA later reagent. (A) Samples from Ecuador of diseased (lanes 1 to 6) or healthy fish (lanes 7 to 12). A reaction mixture with no RNA served as a negative control (lane 13). (B) Samples from Columbia of diseased (lanes 1 to 6) or healthy fish (lanes 7 to 12). M, DNA size markers.
time to our knowledge, that TiLV is present also in tilapia farmed in Colombia, and they confirm the global distribution of this newly recognized pathogen.

DISCUSSION

TiLV, a recently identified pathogen, causes recurrent outbreaks in wild and cultured tilapia. These outbreaks are characterized by significant mortality and morbidity, resulting in massive losses to tilapia industry both in Israel and South America (4, 10–12). Thus, efficient methods for TiLV isolation and detection are required.

Temperature is the first parameter that we examined for optimization of TiLV culturing, since outbreaks of viral diseases of fish are typically temperature dependent (17). Of note, the temperature at which a disease occurs does not necessarily reflect the optimal temperature for the in vitro growth of the cognate pathogen. For example, deadly outbreaks of viral hemorrhagic septicemia (VHS) in farmed Japanese flounder, caused by viral hemorrhagic septicemia virus (VHSV), occurred when water temperatures were between 8 and 15°C, while the isolated VHSV strain replicated most rapidly at 20°C (18). Similarly, spring viremia of carp (SVC), caused by spring viremia of carp virus (SVCV), occurs with high mortality at water temperatures of 10 to 17°C, while the optimum temperature for the in vitro replication of SVCV is 20°C (19). In the case of TiLV, the broad range of water temperature (24 to 33°C) that occurs during the hot season (May to October) (10) calls for determination of the optimal temperature for efficient virus growth in vitro. Our results (Fig. 1) clearly demonstrate that 25°C allows maximal growth of TiLV.

We also determined TiLV growth in several types of fish cells. In a comparison of primary tilapia brain cells to E-11 cells, TiLV replication generated much more viral RNA in the E-11 cells, despite the fact that E-11 cells are derived from the snakehead fish (Ophiocephalus striatus) (20, 21), a freshwater perciform fish (family Channidae) which is distant from tilapines (family Cichlidae). Our present study identified two additional tilapia cell lines that support TiLV growth: the OmB (15) and TmB (16) cells, which are derived from tilapia brain and bulbus arteriosus, respectively. With respect to CPE development, E-11 and OmB were superior compared to TmB. Plaques were readily detected in E-11 cells, whereas TiLV-infected OmB cultures were characterized by almost complete detachment from the plate. Thus, E-11 cells are convenient for plaque assays, and OmB cultures are useful in endpoint dilution (TCID<sub>50</sub>) assays. E-11 cells, which are derived from a species distant from the natural host, should also be useful in studies involving TiLV attenuation. Yet, E-11 cells also produce the snakehead retrovirus (SnRV) (20), and this may hamper the development of pure vaccine strains for TiLV. Since OmB and TmB cells are SnRV free (our unpublished data), these cells should be useful in generating pure TiLV strains.

We demonstrated that TiLV culturing is a sensitive method for detecting the virus. Yet, this methodology is time-consuming and labor-intensive; thus, it is inadequate when prompt and accurate control measures are required (i.e., culling of infected stocks). Hence, we developed RT-PCR-based techniques that are fast and sensitive. We demonstrated that the nested RT-PCR protocol described here detects only a few molecules of TiLV genome and can be applied in detecting TiLV RNA in fresh and preserved organs of diseased fish. The protocol is based on the amplification of consensus regions that were identified by analyzing high-throughput sequencing data, obtained from TiLV samples collected in Israel and Ecuador. This analysis revealed high sequence homology between the Israeli and Ecuadorian samples across the TiLV genome (4), and thus, all TiLV segments can be used as templates in RT-PCRs. The four primers used in our protocol are derived from segment 3 of the Israeli isolate of TiLV (4, 10). Three primers (Nested ext-1, Nested ext-2, and ME1) fully match the sequences of TiLV obtained from 12 Ecuadorian samples. The fourth primer (7450/150R/ME2) fully matches eight of the 12 Ecuadorian samples but has a single mismatch in its second position compared to the other four samples (sequences of the four samples contain a G instead of an A). This S′- mismatch should not interfere with amplification, and the described set of primers readily amplified TiLV sequences from samples obtained from
disease outbreaks in both Israel and Ecuador. Moreover, the power of this RT-PCR-based assay was exemplified when it detected TiLV in organs of diseased tilapia, obtained from yet another country: Colombia.

This is the first report, to our knowledge, of TiLV occurrence in Colombian aquaculture, which adds to the reports of TiLV outbreaks in Israel and Ecuador. This substantiates TiLV as an emerging pathogen and highlights the risk that TiLV poses for the global tilapia industry. The methods described here should detect the virus through early onset of TiLV infection, assisting in its containment.

MATERIALS AND METHODS

Cell cultures and infection of cells with TiLV. E-11 (20), TO-2 (14), OmB (15), and TmB (16) cells were grown in Leibovitz (L-15) medium (Gibco, USA), supplemented with 10% inactivated fetal calf serum (FCS; Gibco), 1-glutamine (300 μM/liter), 1% HEPES (pH 7.3), penicillin (40 U/ml), streptomycin (40 μg/ml), and nystatin (5 μg/ml). Primary brain cells were prepared and grown as described before (10). For TiLV infections, E-11 monolayers in 25-cm² flasks (~90% confluence, washed twice with phosphate-buffered saline (PBS) before infection) were incubated with TiLV preparations at 25°C for 1 h; cells were then washed with PBS and incubated at 25°C in L-15 medium (supplemented with 10% FCS) and monitored for CPE for up to 14 days. TO-2, OmB, and TmB cells were infected with TiLV as described for E-11 cells (see below).

Quantification of temperature-dependent TiLV growth. E-11 cell line and cultures of primary tilapia brain cell (10) (90% confluence in 24-well plates) were infected with TiLV (isolate 4/2011 [10] at 10^3.6 TCID₅₀/well) and incubated at 15, 20, 25, or 30°C for up to 19 days. Cells were harvested at the indicated days postinfection and lysed by three freeze-thaw cycles. Total RNA was extracted with peqGOLD TriFast (catalog no. 30-2010; Peqlab), and levels of TiLV and cellular β-actin RNAs were quantified by quantitative RT-PCR (qRT-PCR). Reverse transcription was performed using the Verso 1-step RT-PCR ReddyMix kit (catalog no. AB-1454/LD/A; Thermo, Lithuania) complemented with the primers specified below. Quantification was accomplished by real-time PCR using the ABSolute Blue qPCR SYBR green Rox mix (catalog no. AB-4163/A; Thermo Scientific), according to the manufacturer’s instructions, with the following specifications: each reaction mixture contained 3 μl of cDNA and TiLV-specific primers (ME1, 5'-GTGGGACACAAGGACTTCA-3', and clone 7450/150R/ME2, 5'-TACACGTGCTACTGTTCACT-3', 300 nM each, amplifying a 250-bp fragment [10]), and annealing and extension were performed at 60°C for 1 min. To detect β-actin RNA, we used the primers described in reference 22 (5'-GGGTACGAAAGACGCTACTGT-3', and R β-actin, 5'-CTCGGCTCTGTGTTAGAAAGTG-3', amplifying a 143-bp fragment). Continuous fluorescence measurements were achieved with a StepOne apparatus (Applied Biosystems). Positive and negative controls consisted of TiLV cDNA and a no-template control, respectively. Relative quantification (RQ) was calculated according to reference 23 with the StepOne software (Applied Biosystems).

Quantification of TiLV growth by endpoint dilution assays. E-11, TmB, or OmB cell lines were cultured in 96-well plates in 100 μl/well Leibovitz (L-15) medium (Gibco, USA), supplemented with 10% inactivated fetal calf serum (FCS; Gibco, USA). Serial dilutions of TiLV were prepared in the above-described serum-supplemented medium, and 100 μl from each dilution was added to each well (~80% confluence). Altogether, 10 wells of each cell line were infected for each dilution. The development of CPE was monitored on a daily basis through 14 days postinfection, when cultures were washed with PBS and stained with crystal violet-formaldehyde-methanol solution. TCID₅₀ values were calculated using the method of Reed and Muench (24).

RT-PCR and qPCR. To establish a control for building an RT-PCR for the detection of TiLV sequences, a 491-bp-long PCR fragment derived from TiLV clone 7450 (GenBank accession no. KJ605629) was amplified with primers Nested ext-1 (5'-TATGCAGTACTTTCCCTGCAC-3') and Nested ext-2 (5'-TGGTCTCTGCACAAAGGATC-3') (10). The resulting fragment was cloned into pET1.2/Blunt (Thermo Fisher Scientific) and 1 μl of the plasmid was used, and used at various known concentrations as the template for real-time PCR. For these reactions, the following pairs of primers were used: Nested ext-1 and Nested ext-2 (amplifying the 491-bp fragment in a reaction called external PCR); ME1 (5'-GTGGGACACAAGGACTTCA-3', and clone 7450/150R/ME2, 5'-TATACGTGCTACTGTTCACT-3', 300 nM each, amplifying a 250-bp fragment [10]), and annealing and extension were performed at 60°C for 1 min. To detect β-actin RNA, we used the primers described in reference 22 (5'-GGGTACGAAAGACGCTACTGT-3', and R β-actin, 5'-CTCGGCTCTGTGTTAGAAAGTG-3', amplifying a 143-bp fragment). Continuous fluorescence measurements were achieved with a StepOne apparatus (Applied Biosystems). Positive and negative controls consisted of TiLV cDNA and a no-template control, respectively. Relative quantification (RQ) was calculated according to reference 23 with the StepOne software (Applied Biosystems).
primers ME1 and 7450/150R/ME2 (500 nM each). For all qPCRs, the following steps were used: 1 cycle of 95°C for 20 s, and 40 cycles of 95°C for 3 s and 60°C for 30 s. Fluorescence was monitored with a StepOnePlus apparatus (Applied Biosystems). Ct values were calculated using the StepOne software.

**TILV RNA detection by nested RT-PCR.** Total RNA was extracted from cell cultures or from liver organs (preserved in RNAlater reagent; catalog no. 76104; Qiagen), with EZ-RNA Total RNA isolation kit (catalog no. 20-400-100; Biological Industries), according to the manufacturer’s instructions. Reverse transcription- and first-round (external) PCR were performed using the Verso 1-step RT-PCR ReddyMix kit (catalog no. AB-1454/LD/A; Thermo, Lithuania), essentially according to the manufacturer’s instructions, but with the following modifications: the total volume of the reaction was 15 μl, using primers Nested ext-1 and Nested ext-2 (see above; 200 nM each). The thermal cycling program included a cDNA synthesis step (50°C, 15 min), an inactivation step (95°C, 2 min), a denaturation step (95°C, 30 s), 25 cycles of annealing (60°C, 30 s) and extension (72°C, 1 min), and a final extension step (72°C, 7 min). Three microliters from the first-round PCR was then subjected to reamplification by a second (nested) PCR of 2× ReddyMix PCR master mix (catalog no. AB-0575/DC/LD/A; Thermo Scientific), essentially according to the manufacturer’s instructions, but with the following modifications: the total volume of the reaction was 15 μl, using primers ME1 and 7450/150R/ME2 (see above; 200 nM each). The thermal cycling program included an initial denaturation step (95°C, 2 min); 35 cycles of denaturation (95°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 1 min); and a final extension step (72°C, 5 min). The PCR products were analyzed by electrophoresis in 1% agarose gels.

**Amplification of NNV RNA.** RT-PCR was used to amplify NNV RNA under the conditions described above for TiLV RNA, using the EZ-RNA Total RNA isolation kit and Verso 1-step RT-PCR ReddyMix kit but with primers F1 (5′-GGATTTGGACGTGCGACCAA-3′) and VR3 (5′-TGGATCGGCAGGAAAGC-3′) and an annealing temperature of 54°C. The length of the amplified product is 254 bp (25).

**Processing of clinical samples.** Brain samples were collected in Israel between 2011 and 2013 from pond-raised tilapia (Oreochromis niloticus × Oreochromis aureus hybrids) suspected to have been infected with TiLV. Brains from two ornamental African cichlids, which were grown in an ornamental fish breeding farm and which showed symptoms of TiLV infection, were also included in this study. Samples from mid-2012 onwards were processed immediately upon arrival; earlier samples were processed from archived materials (whole fish) stored at −80°C. Negative-control fish were collected from fish ponds with no apparent disease.

Brains were removed aseptically, pooled (2 to 3 samples from each outbreak, except from the two samples of ornamental fish that were processed separately), and split into two tubes. The first aliquot was used for RNA extraction and subsequent PCRs as described above. The second aliquot was utilized for virus isolation, manually homogenized with 9 volumes of phosphate-buffered saline (PBS) solution, and centrifuged at 3,000 × g for 10 min; supernatants were filtered through 0.22-μm membrane filters (Sartedt, Germany), and 200 μl was used to infect E-11 monolayers as specified below. For sample 12, E-11 cells that were incubated with brain homogenates and that showed no CPE were freeze-thawed, and 200 μl of cleared extract was used to infect naive E-11 cells. This procedure was repeated once more until a clear CPE was observed.

Liver samples diagnosed histopathologically as having lesions typical of syncytial hepatitis (11) were collected from clinically sick fish from Ecuador and Columbia. Control livers were collected from unexposed healthy tilapia (Oreochromis niloticus) reared at St. George’s University, Grenada. All liver samples were preserved in RNAlater reagent (catalog no. 76104; Qiagen).

**ACKNOWLEDGMENTS**

We thank Dietmar Kueltz (University of California, Davis) for generously providing the Omb and TmB cell lines, and Ronit Sarid (Bar-Ilan University) for providing the TO-2 cell line. We are indebted to Marina Eyngor (Kimron Veterinary Institute) for her technical assistance.

This work was supported by a United States-Israel Binational Agricultural Research & Development Fund (BARD) grants (BARD IS-4583-13 and IS-4903-16C) and by the Israel Ministry of Agriculture & Rural Development Chief Scientist Office (grant 847-0389-14). J.E.K.T. is supported by a fellowship from the Manna Center Program in Food Safety and Security at Tel Aviv University.

E.B., A.E., N.M., T.B., and W.I.L. have applied for patents in the fields of TiLV diagnostics and vaccines.

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