Journal of Clinical Microbiology®
AMERICAN SOCIETY FOR MICROBIOLOGY

CLINICAL VETERINARY MICROBIOLOGY

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

Received 29 August 2016 **Accepted** 24 November 2016

Accepted manuscript posted online 14 December 2016

Citation Kembou Tsofack JE, Zamostiano R, Watted S, Berkowitz A, Rosenbluth E, Mishra N, Briese T, Lipkin WI, Kabuusu RM, Ferguson H, del Pozo J, Eldar A, Bacharach E. 2017. Detection of Tilapia lake virus in clinical samples by culturing and nested reverse transcription-PCR. J Clin Microbiol 55:759–767. https://doi.org/10.1128/JCM.01808-16.

Editor Brad Fenwick, University of Tennessee

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Tilapines, a generic term for edible fish belonging to the family Cichlidae, are fast growers, efficient food convertors, and relatively disease resistant. These assets render them most suitable for farming; indeed, tilapines 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 tilapines 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

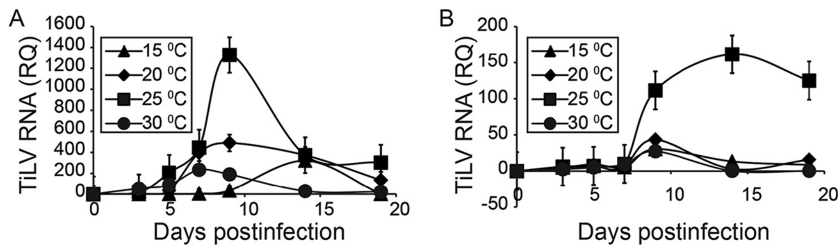

FIG 1 TiLV replication at different temperatures. E-11 (A) and primary brain (B) cultures in 24-well plates were infected with TiLV and incubated at the indicated temperatures. Total RNA was extracted from infected cells at the indicated time points postinfection, and TiLV and cellular β-actin RNA levels 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).

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

TABLE 1 Comparison of $TCID_{50}$ values for three TiLV-susceptible cell lines[a]

Expt no.	$TCID_{50}$ by cell line		
	E-11	TmB	OmB
1	3.2×10^5	5×10^5	1.6×10^5
2	4×10^6	4×10^5	5×10^5
3	1.6×10^5	2×10^5	1.6×10^5

[a]The same stock of TiLV (grown in E-11 cells) was quantified in three independent endpoint dilution assays. Values are in $TCID_{50}$/ml.

shown 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

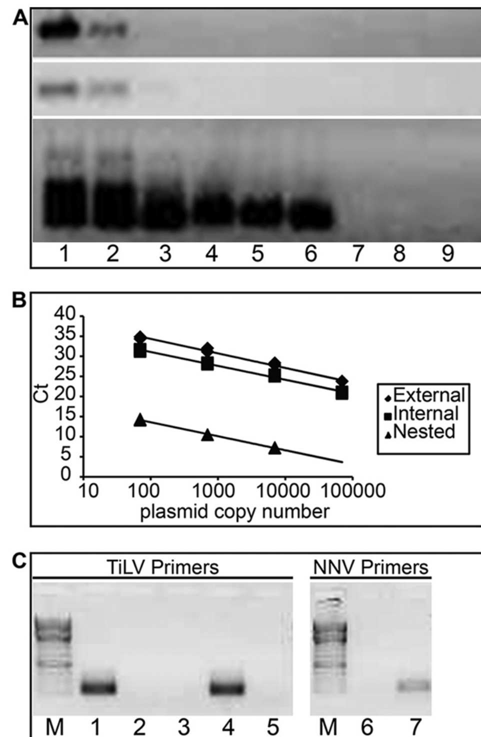

FIG 2 Sensitivity and specificity of PCR, nested PCR, and qPCR in the amplification of TiLV. (A) Ten-fold dilutions of a plasmid containing a 491-bp-long PCR fragment, derived from TiLV clone 7450 (GenBank accession no. KJ605629), were subjected to PCR with primers, amplifying either a 491-bp (top) or 250-bp (middle) fragment. A nested PCR using the above-described two sets of primers was also performed (bottom). Lanes 1 to 9 show the PCR products for the 10^{-5} to 10^{-13} dilution range, respectively, separated on a 1% agarose gel by electrophoresis. The PCR that amplified the 10^{-10} dilution (lane 6) contained 7 copies of the TiLV sequence. (B) qPCRs were also applied to the dilutions and primer pairs described in panel A. The threshold cycle (C_T) values were plotted against calculated TiLV copies, and trendlines were added using Excel software. Reactions were run in triplicate, and only the linear range is shown. (C) cDNAs of TiLV-infected E-11 cells (lane 1), naive E-11 cells (lane 2), or NNV-infected E-11 cells (lane 3) were subjected to nested PCR with TiLV-specific primers as in panel A, to detect TiLV sequences. Amplification of TiLV sequences from the plasmid described in panel A was used as a positive control (lane 4). An amplification reaction with a no-DNA template served as a negative control (lane 5). The absence or presence of NNV sequences in cDNAs, prepared from naive (lane 6) or NNV-infected (lane 7) E-11 cells, respectively, was confirmed by PCR with NNV-specific primers. M, size markers.

(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).

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

		TiLV detection		
Specimen no.	Location	CPE[a]	RT-PCR	Nested RT-PCR
1	Farm 1, Galilee	+	+	+
2	Farm 1, Galilee	+	−	+
3	Farm 2, Jordan Valley	+	−	+
4	Farm 2, Jordan Valley	+	−	+
5	Farm 3, Jordan Valley	+	+	+
6	Farm 4, Jordan Valley	+	+	+
7	Farm 4, Jordan Valley	+	−	+
8	Farm 5, Jordan Valley	+	−	+
9	Farm 6, coastal region	+	−	+
10	Farm 7, Jordan Valley	+	−	+
11	Farm 8, Galilee	+	−	−
12	Farm 9, Jordan Valley	+[b]	−	+
13	Farm 9, Jordan Valley	+	−	+
% positive		100	23	92

[a]CPE was detected in E-11 cells incubated with the brain specimens.
[b]CPE visible only after two additional passages on E-11 cell cultures.

TiLV detection in diseased tilapia from South America. 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

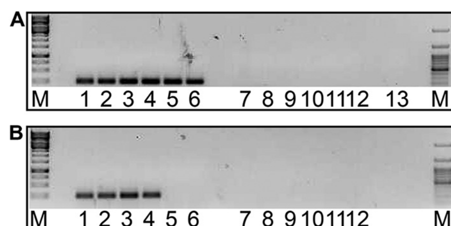

FIG 3 Detection of TiLV RNA in preserved tilapia livers from Ecuador and Columbia. Nested RT-PCR was used to determine the presence or absence of TiLV RNA in liver samples preserved in RNAlater reagent. (A) Samples from Ecuador of diseased (lanes 1 to 6) or healthy fish (lanes 7 to 12). A reaction mixture 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 ($TCID_{50}$) 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 5′ 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), L-glutamine (300 mg/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 $10^{3.6}$ $TCID_{50}$/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 the manufacturer's instructions, with the following specifications: each reaction mixture contained 3 μl of cDNA and TiLV-specific primers (ME1, 5′-GTTGGGCACAAGGCATCCTA-3′; and clone 7450/150R/ME2, 5′-TATCACGTGCGTACTCGTTCAGT-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 (F β-actin, 5′-GGGTCAGAAAGACAGCTACGTT-3′; and R β-actin, 5′-CTCAGCTCGTTGTAGAAGGTGT-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. $TCID_{50}$ 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′-TATGCAGTACTTTCCCTGCC-3′) and Nested ext-2 (5′-TTGCTCTG AGCAAGAGTACC-3′) (10). The resulting fragment was cloned into pJET1.2/blunt (Thermo Fisher Scientific), which was purified, serially diluted, and used (at various known concentrations) as the template for 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′-GTTGGGCACAAGGCATCCT A-3′) and 7450/150R/ME2 (5′-TATCACGTGCGTACTCGTTCAGT-3′) (10), amplifying a 250-bp fragment, embedded in the above-mentioned sequence (in a reaction called internal PCR); or combination of these two pairs in a nested PCR. For the external or internal PCRs (15 μl each), the Verso 1-step RT-PCR ReddyMix kit (catalog no. AB-1454/LD/A; Thermo, Lithuania) was used with 200 nM (final concentration) of each of the above primers and without the enhancer (DNase). The amplification steps included 1 cycle of 50°C for 15 min (to mimic the reverse transcription step); 1 cycle of 95°C for 2 min; 25 cycles of 95°C for 60 s, 60°C for 60 s, and 72°C for 60 s; and 1 cycle of 72°C for 7 min. For the nested PCR, 3 μl of the external reaction was reamplified by a PCR (total of 15 μl) of 2× ReddyMix PCR master mix (catalog no. AB-0575/DC/LD/A; Thermo Scientific), using primers ME1 and 7450/150R/ME2 (each at a final concentration of 200 nM). The amplification steps were as described above but without the 50°C 15-min step. The PCR products were separated in a 1% agarose gel by electrophoresis.

The above-described external and internal PCRs of the plasmid dilutions were also quantified by qPCR using the Fast SYBR green master mix (catalog no. 4385612; Applied Biosystems) and the cognate set of primers described above (final concentration of 500 nM each primer per reaction). To quantify the nested PCR by qPCR, the external PCR was performed with the Verso 1-step RT-PCR ReddyMix kit, as described above; 1 μl of this reaction was then reamplified with the Fast SYBR green master mix using

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). C_T 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 RNA*later* 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′-TGGATCAGGCAGGAAGC-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 (Starsdet, 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 RNA*later* 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.

REFERENCES

1. FAO. 2010. Cultured aquatic species information programme, Oreochromis niloticus (Linnaeus, 1758). Food and Agriculture Organization of the United Nations, Rome, Italy. http://www.fao.org/fishery/culturedspecies/Oreochromis_niloticus/en.
2. FAO. 2010. Species fact sheets: Oreochromis niloticus (Linnaeus, 1758). Food and Agriculture Organization of the United Nations, Rome, Italy. http://www.fao.org/fishery/species/3217/en.
3. FAO. 2014. The state of world fisheries and aquaculture. Food and Agriculture Organization of the United Nations, Rome, Italy. http://www.fao.org/3/a-i3720e/index.html.
4. Bacharach E, Mishra N, Briese T, Zody MC, Kembou Tsofack JE, Zamostiano R, Berkowitz A, Ng J, Nitido A, Corvelo A, Toussaint NC, Abel Nielsen SC, Hornig M, Del Pozo J, Bloom T, Ferguson H, Eldar A, Lipkin WI. 2016. Characterization of a novel orthomyxo-like virus causing mass

die-offs of tilapia. mBio 7(2):e00431-16. https://doi.org/10.1128/mBio.00431-16.
5. Gomna A. 2011. The role of tilapia in food security of fishing villages in Niger state, Nigeria. Afr J Food Agric Nutr Dev 11:5561–5572.
6. Cleasby N, Schwarz AM, Phillips M, Paul C, Pant J, Oeta J, Pickering T, Meloty A, Laumani M, Kori M. 2014. The socio-economic context for improving food security through land based aquaculture in Solomon Islands: a pen-urban case study. Mar Policy 45:89–97. https://doi.org/10.1016/j.marpol.2013.11.015.
7. Bigarré L, Cabon J, Baud M, Heimann M, Body A, Lieffrig F, Castric J. 2009. Outbreak of betanodavirus infection in tilapia, Oreochromis niloticus (L), in fresh water. J Fish Dis 32:667–673. https://doi.org/10.1111/j.1365-2761.2009.01037.x.
8. McGrogan DG, Ostland VE, Byrne PJ, Ferguson HW. 1998. Systemic disease involving an iridovirus-like agent in cultured tilapia, Oreochromis niloticus L.–a case report. J Fish Dis 21:149–152. https://doi.org/10.1046/j.1365-2761.1998.00082.x.
9. Shlapobersky M, Sinyakov MS, Katzenellenbogen M, Sarid R, Don J, Avtalion RR. 2010. Viral encephalitis of tilapia larvae: primary characterization of a novel herpes-like virus. Virology 399:239–247. https://doi.org/10.1016/j.virol.2010.01.001.
10. Eyngor M, Zamostiano R, Kembou Tsofack JE, Berkowitz A, Bercovier H, Tinman S, Lev M, Hurvitz A, Galeotti M, Bacharach E, Eldar A. 2014. Identification of a novel RNA virus lethal to tilapia. J Clin Microbiol 52:4137–4146. https://doi.org/10.1128/JCM.00827-14.
11. Ferguson HW, Kabuusu R, Beltran S, Reyes E, Lince JA, del Pozo J. 2014. Syncytial hepatitis of farmed tilapia, Oreochromis niloticus (L): a case report. J Fish Dis 37:583–589. https://doi.org/10.1111/jfd.12142.
12. Del-Pozo J, Mishra N, Kabuusu R, Cheetham S, Eldar A, Bacharach E, Lipkin WI, Ferguson HW. 2017. Syncytial hepatitis of tilapia (Oreochromis niloticus L.) is associated with orthomyxovirus-like virions in hepatocytes. Vet Pathol 54:164–170. https://doi.org/10.1177/0300985816658100.
13. El-Sayed A. 2006. Tilapia culture. CABI Publishing, Wallingford, United Kingdom.
14. Chen SN, Shi SC, Ueno Y, Kou GH. 1983. A cell line derived from tilapia ovary. Fish Pathol 18:13–18. https://doi.org/10.3147/jsfp.18.13.
15. Gardell AM, Qin Q, Rice RH, Li J, Kultz D. 2014. Derivation and osmotolerance characterization of three immortalized tilapia (Oreochromis mossambicus) cell lines. PLoS One 9:e95919. https://doi.org/10.1371/journal.pone.0095919.
16. Lewis DH, Marks JE. 1985. Microcultures of Sarotherodon mossambicus (Peters) cells: their use in detecting fish viruses. J Fish Dis 8:477–478. https://doi.org/10.1111/j.1365-2761.1985.tb01283.x.
17. Le Morvan C, Troutaud D, Deschaux P. 1998. Differential effects of temperature on specific and nonspecific immune defences in fish. J Exp Biol 201:165–168.
18. Isshik T, Nishizawa T, Kobayashi T, Nagano T, Miyazaki T. 2001. An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed Japanese flounder Paralichthys olivaceus in Japan. Dis Aquat Organ 47:87–99. https://doi.org/10.3354/dao047087.
19. Ahne W, Bjorklund HV, Essbauer S, Fijan N, Kurath G, Winton JR. 2002. Spring viremia of carp (SVC). Dis Aquat Organ 52:261–272. https://doi.org/10.3354/dao052261.
20. Iwamoto T, Nakai T, Mori K, Arimoto M, Furusawa I. 2000. Cloning of the fish cell line SSN-1 for piscine nodaviruses. Dis Aquat Organ 43:81–89. https://doi.org/10.3354/dao043081.
21. Frerichs GN, Morgan D, Hart D, Skerrow C, Roberts RJ, Onions DE. 1991. Spontaneously productive C-type retrovirus infection of fish cell lines. J Gen Virol 72:2537–2539.
22. Hassanin AAI, Kaminishi Y, Osman MMM, Abdel-Wahad ZH, El-Kady MAH, Itakura T. 2009. Development and application of a real-time quantitative PCR assay for determining expression of benzo-apyrene-inducible cytochrome P_{450} 1A in Nile tilapia (Oreochromis niloticus). Afr J Biotechnol 8:6588–6595.
23. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262.
24. Reed LJ, Muench H. 1938. A simple method of estimating fifty percent endpoints. Am J Trop Med Hyg 27:493–497.
25. Ucko M, Colorni A, Diamant A. 2004. Nodavirus infections in Israeli mariculture. J Fish Dis 27:459–469. https://doi.org/10.1111/j.1365-2761.2004.00565.x.