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Citation for published version:

Kembou Tsofack, JE, Zamositano, R, Watted, S, Berkowitz, A, Rosenbluth, E, Mishra, N, Briese, T, Lipkin, WI, Kabuusu, RM, Ferguson, H, Del Pozo, J, Eldar, A & Bacharach, E 2016, 'Detection of Tilapia Lake Virus (TiLV) in Clinical Samples by Culturing and Nested RT-PCR', *Journal of Clinical Microbiology*.
<https://doi.org/10.1128/JCM.01808-16>

Digital Object Identifier (DOI):

[10.1128/JCM.01808-16](https://doi.org/10.1128/JCM.01808-16)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Journal of Clinical Microbiology

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1 **Detection of Tilapia Lake Virus (TiLV) in Clinical Samples by Culturing and**
2 **Nested RT-PCR**

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4

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16

17 Running head: TiLV detection.

18 Key words: virus, TiLV, Tilapia, diagnosis, PCR.

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26

ABSTRACT

27

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

40

41

42

INTRODUCTION

43

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

51

52 Recently, a novel RNA virus termed Tilapia Lake Virus (TiLV) has been identified
53 and recovered from episodes of massive mortalities of wild and pond-cultured tilapia
54 all over Israel (10). High mortalities were also observed in naïve tilapia, exposed to an
55 isolate of TiLV (10). Tilapia mortality, suspected of having a viral etiology, has also
56 been described in Ecuador (11, 12). Although variations in pathological presentation
57 have been described (where lesions were focused in the central nervous system or in
58 the liver, in Israel or Ecuador, respectively), sequencing the whole genome of TiLV

59 revealed that tilapia in the two countries were infected with almost identical viruses
60 (4). This analysis also revealed that this pathogen is a novel orthomyxo-like virus with
61 a 10-segment, negative-sense RNA genome (4). Segment 1 contains an open reading
62 frame (ORF) with weak sequence homology to the polymerase subunit (PB1) of
63 influenza C virus, while the other nine segments showed no homology to other
64 viruses.

65

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

77

78

79

MATERIALS AND METHODS

80

81 **Cell cultures and infection of cells with TiLV.** E-11 (13), TO-2 (14), OmB (15) and
82 TmB (16) cells were grown in Leibovitz (L-15) medium (Gibco, USA), supplemented
83 with 10% inactivated FCS (Gibco), L-glutamine (300 mg/liter), HEPES (pH 7.3; 1%),
84 penicillin (40 U/ml), streptomycin (40 µg/ml), and Nystatin (5 µg/ml). Primary brain
85 cells were prepared and grown as described before (10). For TiLV infections, E-11
86 monolayers in 25cm² flasks (~90% confluence, washed twice with PBS before
87 infection) were incubated with TiLV preparations at 25 °C for 1 h; cells were then
88 washed with PBS and incubated at 25 °C in L-15 medium (supplemented with 10%
89 FCS) and monitored for CPE for up to 14 days. TO-2, OmB and TmB cells were
90 infected with TiLV as described for E-11 cells (see below).

91

92 **Quantification of temperature-dependent TiLV growth.** E-11 cell line and cultures
93 of primary tilapia brain cell (10) (90% confluency in 24-well plates) were infected
94 with TiLV [isolate 4/2011, (10); $10^{3.6}$ TCID₅₀/well] and incubated at 15, 20, 25 or 30
95 °C for up to 19 days. Cells were harvested at the indicated days post-infection and
96 lysed by three freeze-thaw cycles. Total RNA was extracted with peqGOLD Trifast
97 (Peqlab; 30-2010) and levels of TiLV and cellular β -actin RNAs were quantified by
98 quantitative RT-PCR (qRT-PCR). Reverse transcription was performed using Verso
99 1-step RT-PCR ReddyMix kit (Thermo, Lithuania; AB-1454/LD/A); complemented
100 with primers specified below. Quantification was accomplished by real-time PCR
101 using the ABSolute Blue qPCR SYBR Green Rox Mix (Thermo scientific; AB-
102 4163/A) according the manufacturer's instructions with the following specifications:
103 each reaction contained 3 μ l cDNA; TiLV-specific primers [ME1
104 5'GTTGGGCACAAGGCATCCTA3' and clone 7450/150R/ME2
105 5'TATCACGTGCGTACTCGTTCAGT3', 300 nM each, amplifying a 250-bp
106 fragment (10)]; annealing and extension were performed at 60 °C for 1 min. To detect
107 β -actin RNA, we used the primers described in (17) (F β -actin
108 5'GGGTCAGAAAGACAGCTACGTT3' and R β -actin
109 5'CTCAGCTCGTTGTAGAAGGTGT3', amplifying 143 bp fragment). Continuous
110 fluorescence measurements were achieved with StepOne apparatus (Applied
111 Biosystems). Positive and negative controls consisted of TiLV cDNA and no-template
112 control, respectively. Relative quantification (RQ) was calculated according to (18)
113 with the StepOne software (Applied Biosystems).
114

115 **Quantification of TiLV growth by end-point dilution assays.** E-11, TmB or OmB
116 cell lines were cultured in 96-well plates in 100 μ l/well of Leibovitz (L-15) medium
117 (Gibco, USA), supplemented with 10% inactivated fetal calf serum (FCS, Gibco,
118 USA). Serial dilutions of TiLV were prepared in the above serum-supplemented
119 medium and 100 μ l from each dilution were added to each well (~ 80% confluency).
120 Altogether, 10 wells of each cell line were infected for each dilution. The
121 development of CPE was monitored on a daily basis through 14 days post-infection,
122 when cultures were washed with PBS and stained with crystal
123 violet/formaldehyde/methanol solution. TCID₅₀ values were calculated by the method
124 of Reed and Muench (19).
125

126 **RT- PCR and quantitative PCR (qPCR).** To establish a control for building a RT-
127 PCR for detection of TiLV sequences, a 491 bp long PCR fragment, derived from
128 TiLV clone 7450 (GenBank Accession No. KJ605629), was amplified with primers
129 'Nested ext-1' (5'TATGCAGTACTTTCCCTGCC3') and 'Nested ext-2'
130 (5'TTGCTCTGAGCAAGAGTACC3') (10). The resulting fragment was cloned into
131 pJET1.2/blunt (Thermo Fisher Scientific), which was purified, serially diluted and
132 used (at various known concentrations) as a template for PCR. For these reactions, the
133 following pairs of primers were used: Nested ext-1 and Nested ext-2 (amplifying the
134 491 bp fragment in a reaction called 'external PCR'); 'ME1'
135 (5'GTTGGGCACAAGGCATCCTA3') and '7450/150R/ME2'
136 (5'TATCACGTGCGTACTCGTTCAGT3') (10), amplifying a 250 bp fragment,
137 embedded in the above sequence (in a reaction called 'internal PCR'); or combination
138 of these two pairs in a nested PCR reaction. For the external or internal PCR reactions
139 (15 µl each), the Verso 1-step RT-PCR ReddyMix kit (Thermo, Lithuania; AB-
140 1454/LD/A) was used with 200 nM (final concentration) of each of the above primers
141 and without the enhancer (DNase). Amplification steps included: 1 cycle of 50 °C, 15
142 min (to mimic the reverse transcription step); 1 cycle of 95 °C, 2 min; 25 cycles of 95
143 °C, 60 s / 60°C, 60 s / 72 °C, 60 s and 1 cycle of 72 °C, 7 min. For nested PCR, 3 µl
144 of the external reaction were re-amplified by a PCR reaction (total of 15 µl) of 2X
145 ReddyMix PCR Master Mix (Thermo Scientific; AB-0575/DC/LD/A), using primers
146 ME1 and 7450/150R/ME2 (each at a final concentration of 200 nM). Amplification
147 steps were as above but without the 50 °C, 15 min step. PCR products were separated
148 in a 1% agarose gel by electrophoresis.

149
150 The above external and internal PCR reactions of the plasmid dilutions were also
151 quantified by qPCR, using the Fast SYBR Green Master Mix (Applied Biosystems,
152 4385612) and the cognate set of primers described above (final concentration of 500
153 nM of each primer, per reaction). To quantify the nested PCR by qPCR, the external
154 PCR reaction was performed with the Verso 1-step RT-PCR ReddyMix kit, as
155 described above; 1 µl of this reaction was then re-amplified with the Fast SYBR
156 Green Master Mix, using primers ME1 and 7450/150R/ME2 (500 nM each). For all
157 qPCR reactions the following steps were used: 1 cycle of 95°C, 20 s; 40 cycles of
158 95°C, 3 s / 60°C, 30 s. Fluorescence was monitored with StepOnePlus apparatus
159 (Applied Biosystems). Ct values were calculated by the StepOne software.

160

161 **TiLV RNA detection by nested RT-PCR.** Total RNA was extracted from cell
162 cultures, or from liver organs (preserved in RNAlater reagent; QIAGEN; 76104), with
163 EZ-RNA Total RNA Isolation Kit (Biological Industries; 20-400-100) according to
164 the manufacturer's instructions. Reverse transcription and first round (external) PCR
165 were performed using Verso 1-step RT-PCR ReddyMix kit (Thermo, Lithuania; AB-
166 1454/LD/A), essentially according to the manufacturer's instructions but with the
167 following modifications: total volume of the reaction was 15 μ l, with primers 'Nested
168 ext-1' and 'Nested ext-2' (see above; 200 nM each). Thermal cycling program
169 included: a cDNA synthesis step (50 °C, 15 min); an inactivation step (95 °C, 2 min);
170 a denaturation step (95 °C, 30 s); 25 cycles of annealing (60 °C, 30 s) - extension (72
171 °C, 1 min); and a final extension step (72 °C, 7 min). 3 μ l from the first round PCR
172 were then subjected to re-amplification by a second (nested) PCR of 2X ReddyMix
173 PCR Master Mix (Thermo Scientific; AB-0575/DC/LD/A), essentially according to
174 the manufacturer's instructions but with the following modifications: total volume of
175 the reaction was 15 μ l, using primers ME1 and 7450/150R/ME2 (see above; 200 nM
176 each). Thermal cycling program included: an initial denaturation step (95 °C, 2 min);
177 35 cycles of denaturation (95 °C, 1 min) - annealing (60 °C, 1 min) - extension (72
178 °C, 1 min); and a final extension step (72 °C, 5 min). PCR products were analyzed by
179 electrophoresis in 1% agarose gels.

180

181 **Amplification of Nervous Necrosis virus (NNV) RNA.** RT-PCR was used to
182 amplify NNV RNA with conditions described above for TiLV RNA, using EZ-RNA
183 Total RNA Isolation Kit and Verso 1-step RT-PCR ReddyMix kit, but with primers
184 F1 (5'GGATTTGGACGTGCGACCAA3') and VR3
185 (5'TGGATCAGGCAGGAAGC3') and annealing temperature of 54 °C. The length of
186 the amplified product is 254 bp (20).

187

188 **Processing of clinical samples.** Brain samples were collected in Israel between 2011
189 and 2013, from pond-raised tilapia (*Oreochromis niloticus* x *Oreochromis aureus*
190 hybrids), suspected to have been infected with TiLV. Brains from two ornamental
191 African cichlids, grown in an ornamental fish breeding farm and which showed
192 symptoms of TiLV infection, were also included in this study. Samples from mid-
193 2012 onwards were processed immediately upon arrival; earlier samples were

194 processed from archived materials (whole fish) stored at -80°C . Negative control fish
195 were collected from fish ponds with no apparent disease.

196

197 Brains were removed aseptically, pooled (2-3 samples from each outbreak; except
198 from the two samples of ornamental fish that were processed separately) and split into
199 two tubes. The first aliquot was used for RNA extraction and subsequent PCR
200 reactions as described above. The second aliquot was utilized for virus isolation and
201 was manually homogenized with 9 volumes of phosphate-buffered saline (PBS)
202 solution and centrifuged at $3000 \times g$ for 10 min; supernatants were filtered through
203 $0.22 \mu\text{m}$ membrane filters (Stardet, Germany) and $200 \mu\text{l}$ were used to infect E-11
204 monolayers as specified below. For Sample No. 12, E-11 cells that were incubated
205 with brain homogenates and that showed no CPE, were freeze-thawed, and $200 \mu\text{l}$ of
206 cleared extract was used to infect naïve E-11 cells. This procedure was repeated once
207 more till a clear CPE was observed.

208

209 Liver samples, diagnosed histopathologically as having lesions typical of syncytial
210 hepatitis (11), were collected from clinically sick fish, from Ecuador and Columbia.
211 Control livers were collected from unexposed, healthy tilapia (*Oreochromis niloticus*),
212 reared in St. George's University, Grenada. All liver samples were preserved in
213 RNAlater reagent (QIAGEN; 76104).

214

215 RESULTS

216

217 **Temperature-dependent viral growth.** Being ectotherms (and cultured at $16-32^{\circ}\text{C}$
218 range) (21), tilapia potentially may be infected over a relatively wide range of
219 temperatures; yet, the effect of temperature on TiLV replication and thus, on its
220 isolation, has not been studied. Hence, TiLV growth at various temperatures was
221 evaluated by infecting monolayers of E-11 and primary tilapia brain cells, with
222 subsequent incubation at various temperatures (15, 20, 25 and 30°C) for up to 19
223 days. Infection was quantified by qRT-PCR reactions, measuring TiLV RNA
224 expression levels, with TiLV specific primers. Viral RNA levels were normalized to
225 cellular β -actin mRNA levels [Relative Quantification (RQ); Fig. 1 and Materials and
226 Methods]. In E-11 cells, the maximum increase in TiLV RNA levels was observed at

227 25 °C at day 9 post-infection (RQ = 1328; Fig. 1A). Higher (30 °C) or lower (20 °C)
228 temperatures resulted in reduced TiLV RNA levels (RQ = 191 and 490, respectively,
229 day 9 post-infection). At 15 °C, TiLV RNA production was dramatically reduced (RQ
230 = 35; day 9 post-infection) and reached maximal levels at day 15 post-infection. In
231 infected primary tilapia brain cells, 25 °C was also the optimal temperature for TiLV
232 replication (Fig. 1B); yet this replication peaked only at day 14 post-infection and
233 reached much lower levels (about 12%), compared to the one in E-11 cells.
234 Altogether, E-11 cells at 25 °C provide optimal conditions for TiLV replication, thus
235 all isolations from clinical samples (see below) were carried out under these
236 conditions.

237

238 **Quantification of TiLV growth in tilapia cell lines.** In our former study (10), eight
239 established fish cell lines were tested for their permissiveness to TiLV infection and
240 only E-11 cells were found suitable for this purpose. We now extended this analysis to
241 three additional tilapia cell lines, derived from ovary [TO-2; (14)], brain [OmB; (15)]
242 and bulbus arteriosus [TmB; (16)]. Initial qualitative analyses revealed that OmB and
243 TmB, but not TO-2, support TiLV replication (data not shown). We next compared
244 the use of the permissive cell lines (E-11, OmB and TmB) in the quantification of
245 TiLV infection by endpoint dilution assays. The cell lines were infected with dilutions
246 of the virus, CPE was monitored for 14 days and TCID₅₀ values were calculated. The
247 results of three independent experiments are shown in Table 1. All three cell lines
248 showed comparable sensitivities to TiLV infection. Yet, E-11 cultures were superior
249 because the CPE development was clearly detected in a relative short time (~4, 6 or 8
250 days post-infection for E-11, TmB or OmB, respectively). OmB cells also provided
251 convenient way to monitor CPE at longer time post-infection (~14 days), as
252 uninfected cells remained attached as monolayers while infected cultures completely
253 detached at this time point. TmB cells were sensitive to TiLV-induced CPE, yet we
254 found that detecting CPE in this line was more difficult compared to the other lines
255 because TmB cells did not support the formation of clear plaques and only a portion
256 of the infected cells detached from the plate over time.

257

258 **Sensitivity and specificity of TiLV detection by PCR.** A sensitive RT-PCR
259 detection method for TiLV is required for rapid and accurate diagnosis of this threat

260 to tilapia aquaculture. We have described TiLV detection by RT-PCR (10); however,
261 assays were not optimized. To optimize this procedure, we first prepared a standard
262 curve for this assay. Specifically, a 491 bp long PCR fragment, derived from TiLV
263 clone 7450 (GenBank Accession No. KJ605629) (10), was cloned into a plasmid and
264 dilutions of the resulting plasmid DNA were used in the following PCR/gel
265 electrophoresis (Fig. 2A) and qPCR (Fig. 2B) reactions. Different sets of primers were
266 used to amplify either the 491 bp fragment ('external PCR'; Fig. 2A, Upper Panel) or
267 an internal 250 bp fragment ('internal PCR'; Fig. 2A, Middle Panel). Additional
268 reaction ('nested PCR'; Fig. 2A, Lower Panel) consisted of the external PCR,
269 combined with the internal PCR (Materials and Methods). This analysis showed that
270 as expected, the external and internal PCR reactions were less sensitive than the
271 nested PCR: the highest dilution in which the TiLV sequence was clearly detected by
272 the external or the internal PCR reactions was 10^{-6} (Fig. 2A, Lane 2, Upper and
273 Middle Panels); relating to detection of $\sim 70,000$ TiLV copies. The nested PCR
274 showed much higher sensitivity, enabling the detection of as low as 7 copies of TiLV
275 sequence (Fig. 2A, Lane 6, Lower Panel). Amplification of the above diluted plasmid
276 DNA by qPCR also demonstrated the higher sensitivity of the nested PCR over the
277 non-nested PCR reactions as much lower threshold cycle (Ct) values were obtained
278 for the former reaction (Fig. 2B). Of note, the detection limit of the nested qPCR (70
279 copies, Fig. 2B), was higher than that of the nested PCR (7 copies, Fig. 2A). These
280 differences likely result from the different reagents and conditions used for these two
281 types of reactions (Materials and Methods).

282

283 The specificity of the developed nested PCR was further demonstrated by the
284 amplification of TiLV sequences from cDNAs that were prepared from TiLV-infected
285 E-11 cells; but not from negative samples composed of cDNAs of NNV-infected, or
286 naïve E-11 cells (Fig. 2C).

287

288 **TiLV detection in diseased tilapia from Israel.** Based on the optimal conditions for
289 TiLV growth and detection defined above, we next set out to isolate TiLV from
290 clinical specimens obtained from 13 different outbreaks between 2011 and 2013 in
291 nine different commercial farms, distributed over Israel (Galilee, Jordan valley and
292 Mediterranean coastal areas). In all these outbreaks, diseased fish showed typical
293 symptoms related to TiLV infection (10). Brain samples were obtained from

294 commercial pond-raised tilapia for human consumption (*Oreochromis niloticus* x
295 *Oreochromis aureus* hybrids; Specimens No. 1-11, Table 2), and ornamental African
296 cichlids (Specimens No. 12 and 13, Table 2). Brain was chosen for these analyses
297 because this tissue is relatively confined and susceptible to TiLV infection (10). The
298 brains were homogenized (pools of 2-3 brains for each outbreak for Samples No. 1-
299 11; Samples No. 12 and 13, each consisted of a single brain) and added to E-11 cells,
300 cultured at 25 °C. This procedure resulted in the appearance of CPE at 5 to 6 days
301 post-inoculation, in 12 out of the 13 cases (Table 2). For Specimen No. 12, two
302 additional passages in E-11 cell cultures were required before CPE became apparent
303 (Materials and Methods). No CPE was observed for a negative control group,
304 consisting of 15 fish that were collected from ponds showing mortalities due to either
305 environmental conditions (low oxygen levels or high ammonia concentrations) or
306 other infectious diseases (i.e. streptococci) (data not shown).

307

308 The above 13 brain tissues were also tested for the presence of TiLV sequences by the
309 internal and nested PCR reactions described above. For this, total RNA was extracted
310 from portions of the brains, reverse transcribed using random primers and PCR
311 amplified with TiLV-specific primers (Materials and Methods). The internal PCR
312 detected TiLV sequences in only three samples (23%), in contrast to the nested PCR
313 that detected the virus in 12 samples (92%, Table 2). The amplification of TiLV
314 sequences was also verified by sequencing the PCR products (data not shown). None
315 of the negative controls scored positive when examined by the nested PCR, further
316 demonstrating the specificity of this assay (data not shown).

317

318 **TiLV detection in diseased tilapia from South America.** To further examine the
319 developed nested RT-PCR, we applied it to detect TiLV RNA in liver samples,
320 preserved in RNAlater reagent, that were taken from South American tilapia, showing
321 signs of syncytial hepatitis (11, 12), or from healthy controls. This test was performed
322 in a blinded way using the following procedure: the presence or absence of TiLV
323 RNA in the samples was tested by RT-PCR (12), in St. George's University, Grenada.
324 The samples were then coded and shipped, preserved in an RNAlater reagent, to Tel
325 Aviv University, where RNA was extracted and nested RT-PCR was performed
326 without knowing the samples' identities. Fig. 3A shows the results of this procedure
327 for Ecuadorian samples: six examined samples scored positive (Lanes 1-6) while six

328 samples scored negative (Lanes 7-12). This fully matched the classification made of
329 the samples, before shipment.

330

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

340

341 Overall, these results demonstrate that the developed nested RT-PCR can be applied
342 for detection of TiLV strains in Israel and South America and suggest that preserved
343 material can be analyzed too. Importantly, these results further show for the first time
344 that TiLV is present also in tilapia farmed in Colombia, and confirm the global
345 distribution of this newly recognized pathogen.

346

347 DISCUSSION

348

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

353

354 Temperature is the first parameter that we examined for optimization of TiLV
355 culturing, since outbreaks of viral diseases of fish are typically temperature-dependent
356 (22). Of note, the temperature at which a disease occurs does not necessarily reflect
357 the optimal temperature for the *in vitro* growth of the cognate pathogen. For example,
358 deadly outbreaks of viral hemorrhagic septicemia (VHS) in farmed Japanese flounder,
359 caused by viral hemorrhagic septicemia virus (VHSV), occurred when water
360 temperatures were between 8 and 15 °C, while the isolated VHSV strain replicated
361 most rapidly at 20 °C (23). Similarly, spring viremia of carp (SVC), caused by

362 spring viremia of carp virus (SVCV), occurs with high mortalities at water
363 temperatures of 10 to 17 °C, while the optimum temperature for the *in vitro*
364 replication of SVCV is 20 °C (24). In the case of TiLV, the broad range of water
365 temperature (~ 24 to 33 °C) that occurs during the hot season (May to October) (10),
366 calls for determination of the optimal temperature for efficient virus growth, *in vitro*.
367 Our results (Fig. 1) clearly demonstrate that 25 °C allows maximal growth of TiLV.

368

369 We also determined TiLV growth in several types of fish cells. When comparing
370 primary tilapia brain cells to E-11 cells, TiLV replication generated much higher viral
371 RNA in the latter cells, despite the fact that E-11 cells are derived from the snakehead
372 fish (*Ophicephalus striatus*) (13, 25) - a freshwater perciform fish (family
373 *Channidae*), which is distant from tilapines (family *Cichlidae*). Our present study
374 identified two additional tilapia cell lines that support TiLV growth: the OmB; (15) or
375 TmB (16) cells, derived from tilapia brain or bulbus arteriosus, respectively. In
376 respect to CPE development, E-11 and OmB were superior compared to TmB.
377 Plaques were readily detected in E-11 cells, whereas TiLV-infected OmB cultures
378 were characterized by almost complete detachment from the plate. Thus, E-11 cells
379 are convenient for plaque assays and OmB cultures are useful in end-point dilution
380 (TCID₅₀) assays. E-11 cells, which are derived from a species distant from the natural
381 host, should also be useful in studies involving TiLV attenuation. Yet, E-11 cells also
382 produce the snakehead retrovirus (SnRV) (13) and this may hamper the development
383 of pure vaccine strains for TiLV. Since OmB and TmB cells are SnRV-free (our
384 unpublished results), these cells should be useful in generating pure TiLV strains.

385

386 We demonstrated that TiLV culturing is a sensitive method for detection of the virus.
387 Yet, this methodology is time consuming and labor intensive; thus, it is inadequate
388 when prompt and accurate control measures are required (i.e. culling of infected
389 stocks). Hence, we developed RT-PCR-based techniques that are fast and sensitive.
390 We demonstrated that the nested RT-PCR protocol, described here, detects only few
391 molecules of TiLV genome and can be applied in detecting TiLV RNA in fresh and
392 preserved organs of diseased fish. The protocol is based on amplification of consensus
393 regions that were identified by analyzing high-throughput sequencing data, obtained
394 from TiLV samples collected in Israel and Ecuador. This analysis revealed high
395 sequence homology between the Israeli and Ecuadorian samples across TiLV genome

396 (4) and thus, all TiLV segments can be used as templates in RT-PCR reactions. The
397 four primers used in our protocol are derived from Segment 3 of the Israeli isolate of
398 TiLV (4, 10). Three primers (Nested ext-1, Nested ext-2 and ME1) fully match
399 sequences of TiLV obtained from 12 Ecuadorian samples. The fourth primer
400 (7450/150R/ME2) fully matches eight of the 12 Ecuadorian samples, but has a single
401 mismatch in its second position, compared to the other four samples (sequences of the
402 latter contain a G instead of an A). This 5' mismatch should not interfere with
403 amplification and the described set of primers readily amplified TiLV sequences from
404 samples obtained from disease outbreaks in both Israel and Ecuador. Moreover, the
405 power of this RT-PCR-based assay was exemplified when it detected TiLV in organs
406 of diseased tilapia, obtained from yet another country - Colombia.

407

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

413

414 ACKNOWLEDGEMENTS

415

416 We thank Prof. Dietmar Kueltz (University of California, Davis) for generously
417 providing the OmB and TmB cell lines, and Prof. Ronit Sarid (Bar-Ilan University)
418 for providing the TO-2 cell line. We are indebted to Dr. Marina Eyngor (Kimron
419 Veterinary Institute) for her technical assistance. This work was supported by a
420 United States-Israel Binational Agricultural Research & Development Fund (BARD)
421 grant (BARD IS-4583-13) and by the Israel Ministry of Agriculture & Rural
422 Development Chief Scientist Office (grant 847-0389-14). J.E.K.T. is supported by a
423 fellowship from the Manna Center Program in Food Safety and Security at Tel Aviv
424 University.

425

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

428

REFERENCES

- 429 1. **FOA**. 2010. Cultured aquatic species information programme, *Oreochromis niloticus*
430 (Linnaeus, 1758). *on* Food and Agriculture Organization of the United Nations, Rome,
431 Italy. http://www.fao.org/fishery/culturedspecies/Oreochromis_niloticus/en.
- 432 2. **FOA**. 2010. Fisheries and Aquaculture Department. Species fact sheets:
433 *Oreochromis niloticus* (Linnaeus, 1758), *on* Food and Agriculture Organization of the
434 United Nations, Rome, Italy. <http://www.fao.org/ffishery/species/3217/en>.
- 435 3. **FOA**. 2014. The state of world fisheries and aquaculture, *on* Food and Agriculture
436 Organization of the United Nations, Rome, Italy. [http://www.fao.org/3/a-](http://www.fao.org/3/a-i3720e/index.html)
437 [i3720e/index.html](http://www.fao.org/3/a-i3720e/index.html).
- 438 4. **Bacharach E, Mishra N, Briese T, Zody MC, Kembou Tsofack JE, Zamostiano R,**
439 **Berkowitz A, Ng J, Nitido A, Corvelo A, Toussaint NC, Abel Nielsen SC, Hornig M,**
440 **Del Pozo J, Bloom T, Ferguson H, Eldar A, Lipkin WI**. 2016. Characterization of a
441 Novel Orthomyxo-like Virus Causing Mass Die-Offs of Tilapia. *MBio* **7**.
- 442 5. **Gomna A**. 2011. The role of tilapia in food security of fishing villages in niger state,
443 nigeria. *African Journal of Food, Agriculture, Nutrition and Development* **11**:5561-
444 5572.
- 445 6. **Cleasby N, Schwarz AM, Phillips M, Paul C, Pant J, Oeta J, Pickering T, Meloty A,**
446 **Laumani M, Kori M**. 2014. The socio-economic context for improving food security
447 through land based aquaculture in Solomon Islands: A pen-urban case study. *Marine*
448 *Policy* **45**:89-97.
- 449 7. **Bigarre L, Cabon J, Baud M, Heimann M, Body A, Liefbrig F, Castric J**. 2009. Outbreak
450 of betanodavirus infection in tilapia, *Oreochromis niloticus* (L.), in fresh water. *J Fish*
451 *Dis* **32**:667-673.
- 452 8. **McGrogan DG, Ostland VE, Byrne PJ, Ferguson HW**. 1998. Systemic disease
453 involving an iridovirus-like agent in cultured tilapia, *Oreochromis niloticus* L. – a case
454 report. *Journal of Fish Diseases* **21**:149-152.
- 455 9. **Shlapobersky M, Sinyakov MS, Katzenellenbogen M, Sarid R, Don J, Avtalion RR**.
456 2010. Viral encephalitis of tilapia larvae: primary characterization of a novel herpes-
457 like virus. *Virology* **399**:239-247.
- 458 10. **Eyngor M, Zamostiano R, Kembou Tsofack JE, Berkowitz A, Bercovier H, Tinman S,**
459 **Lev M, Hurvitz A, Galeotti M, Bacharach E, Eldar A**. 2014. Identification of a novel
460 RNA virus lethal to tilapia. *Journal of Clinical Microbiology* **52**:4137-4146.
- 461 11. **Ferguson HW, Kabuusu R, Beltran S, Reyes E, Lince JA, del Pozo J**. 2014. Syncytial
462 hepatitis of farmed tilapia, *Oreochromis niloticus* (L.): a case report. *J Fish Dis*
463 **37**:583-589.
- 464 12. **Del-Pozo J, Mishra N, Kabuusu R, Cheetham S, Eldar A, Bacharach E, Lipkin WI,**
465 **Ferguson HW**. 2016. Syncytial Hepatitis of Tilapia (*Oreochromis niloticus* L.) is
466 Associated With Orthomyxovirus-Like Virions in Hepatocytes. *Vet Pathol*
467 doi:10.1177/0300985816658100.
- 468 13. **Iwamoto T, Nakai T, Mori K, Arimoto M, Furusawa I**. 2000. Cloning of the fish cell
469 line SSN-1 for piscine nodaviruses. *Dis Aquat Organ* **43**:81-89.
- 470 14. **Chen SN, Shi SC, Ueno Y, Kou GH**. 1983. A cell line derived from Tilapia ovary. *Fish*
471 *Pathology* **18**:13-18.
- 472 15. **Gardell AM, Qin Q, Rice RH, Li J, Kultz D**. 2014. Derivation and osmotolerance
473 characterization of three immortalized tilapia (*Oreochromis mossambicus*) cell lines.
474 *PLoS One* **9**:e95919.
- 475 16. **Lewis DH, Marks JE**. 1985. Microcultures of *Sarotherodon mossambicus* (Peters)
476 cells: their use in detecting fish viruses. *Journal of Fish Diseases* **8**:477-478.

- 477 17. **Hassanin AAI, Kaminishi Y, Osman MMM, Abdel-Wahad ZH, El-Kady MAH, Itakura**
478 **T.** 2009. Development and application of a real-time quantitative PCR assay for
479 determining expression of benzo-a-pyrene- Inducible cytochrome P450 1A in Nile
480 tilapia (*Oreochromis niloticus*). *African Journal of Biotechnology* **8**:6588-6595.
- 481 18. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using real-
482 time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**:402-408.
- 483 19. **Reed LJ, Muench H.** 1938. A simple method of estimating fifty percent endpoints.
484 *The American Journal of Tropical Medicine and Hygiene* **27**:493-497.
- 485 20. **Ucko M, Colorni A, Diamant A.** 2004. Nodavirus infections in Israeli mariculture. *J*
486 *Fish Dis* **27**:459-469.
- 487 21. **El-Sayed A-FM.** 2006. *Tilapia culture*. CABI Pub., Wallingford, UK ; Cambridge, MA.
- 488 22. **Le Morvan C, Troutaud D, Deschaux P.** 1998. Differential effects of temperature on
489 specific and nonspecific immune defences in fish. *J Exp Biol* **201**:165-168.
- 490 23. **Isshik T, Nishizawa T, Kobayashi T, Nagano T, Miyazaki T.** 2001. An outbreak of
491 VHSV (viral hemorrhagic septicemia virus) infection in farmed Japanese flounder
492 *Paralichthys olivaceus* in Japan. *Dis Aquat Organ* **47**:87-99.
- 493 24. **Ahne W, Bjorklund HV, Essbauer S, Fijan N, Kurath G, Winton JR.** 2002. Spring
494 viremia of carp (SVC). *Dis Aquat Organ* **52**:261-272.
- 495 25. **Frerichs GN, Morgan D, Hart D, Skerrow C, Roberts RJ, Onions DE.** 1991.
496 Spontaneously productive C-type retrovirus infection of fish cell lines. *J Gen Virol* **72**
497 **(Pt 10)**:2537-2539.
- 498
- 499

FIGURE LEGENDS

500

501

502 **Figure 1. TiLV replication at different temperatures.** E-11 (A) and primary brain
503 (B) cultures in 24-well plates were infected with TiLV and incubated at the indicated
504 temperatures. Total RNA was extracted from infected cells at the indicated time
505 points postinfection and TiLV and cellular β -actin RNA levels were quantified by
506 qRT-PCR. The results show the relative quantification (RQ) of TiLV RNA levels,
507 relative to β -actin RNA levels (means of duplicates \pm standard deviations).

508 **Figure 2. Sensitivity and specificity of PCR, nested PCR and qPCR in the**
509 **amplification of TiLV.** (A) Ten-fold dilutions of a plasmid containing a 491 bp long
510 PCR fragment, derived from TiLV clone 7450 (GenBank Accession No. KJ605629),
511 were subjected to PCR with primers, amplifying either a 491 bp (Upper Panel) or 250
512 bp (Middle Panel) fragments. A nested PCR, using the above two sets of primers was
513 also performed (Lower Panel). Lanes 1 to 9 show the PCR products for 10^{-5} to 10^{-13}
514 dilutions range, respectively, separated on a 1% agarose gel by electrophoresis. The
515 PCR reaction that amplified the 10^{-10} dilution (Lane 6) contained 7 copies of TiLV
516 sequence. (B) qPCR reactions were also applied to the dilutions and primer pairs
517 described in (A). The values of the threshold cycle (Ct) were plotted against
518 calculated TiLV copies and trendlines were added using Excel software. Reactions
519 were run in triplicates and only the linear range is shown. (C) cDNAs of TiLV-
520 infected E-11 cells (Lane 1), Naïve E-11 cells (Lane 2) or NNV-infected E-11 cells
521 (Lane 3) were subjected to Nested PCR with TiLV-specific primers as in (A), to
522 detect TiLV sequences. Amplification of TiLV sequences from the plasmid described
523 in (A) was used as a positive control (Lane 4). Amplification reaction with no DNA
524 template served as a negative control (Lane 5). The absence or presence of NNV
525 sequences in cDNAs, prepared from naïve (Lane 6) or NNV-infected (Lane 7) E-11
526 cells, respectively, was confirmed by PCR with NNV-specific primers. M, denotes
527 size markers.

528

529 **Figure 3. Detection of TiLV RNA in preserved tilapia livers from Ecuador and**
530 **Columbia.** Nested RT-PCR was used to determine the presence or absence of TiLV
531 RNA in liver samples, preserved in RNAlater reagent. (A) Samples from Ecuador of
532 diseased (Lanes 1-6) or healthy fish (Lanes 7-12). Reaction with no RNA served as a

533 negative control (Lane 13). (B) Samples from Columbia of diseased (Lanes 1-6) or
534 healthy fish (Lanes 7-12). M marks DNA size markers.

535

536

TABLES

537

538 **TABLE 1. ^aComparison of TCID₅₀ values for three TiLV-susceptible cell lines**

Experiment No.	Cell Lines		
	E-11	TmB	OmB
1	3.2x10 ⁵	5x10 ⁵	1.6x10 ⁵
2	4x10 ⁶	4x10 ⁵	5x10 ⁵
3	1.6x10 ⁵	2x10 ⁵	1.6x10 ⁵

539 ^aThe same stock of TiLV (grown in E-11 cells) was quantified in three independent
540 endpoint dilution assays. Values are in TCID₅₀/ml.

541

542 **TABLE 2. TiLV detection in clinical specimens by culturing, RT-PCR and**
543 **nested RT-PCR.**

Specimen No.	Location	^a CPE	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	+	-	+
% Positives		100	23	92

544 ^aCPE was detected in E-11 cells, incubated with the brain specimens.

545 ^bCPE visible only after two additional passages on E-11 cell cultures.





