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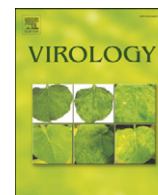

Viral encephalitis of tilapia larvae: Primary characterization of a novel herpes-like virus

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ABSTRACT

We report here an outbreak of an acute disease that caused high mortality rate in laboratory-reared tilapia larvae. The disease was initially observed in inbred gynogenetic line of blue tilapia larvae (*Oreochromis aureus*) and could be transmitted to larvae of other tilapia species. Based on the clinical manifestation (a whirling syndrome), we refer to the disease as viral encephalitis of tilapia larvae. The disease-associated DNA virus is described and accordingly designated tilapia larvae encephalitis virus (TLEV). A primary morphological, biophysical and molecular characterization of TLEV is presented. By virtue of these properties, the newly discovered virus is a herpes-like virus. Phylogenetic analysis, albeit limited, confirms this assumption and places TLEV within the family of *Herpesviridae* and distantly from the families *Alloherpesviridae* and *Iridoviridae*. By using PCR with virus-specific primers, diseased larvae and adult TLEV carriers were also identified in tilapia delivered from external hatcheries.

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Introduction

Tilapias are one of the most important food fishes in the world. Native to Africa and the Middle East, they are undemanding and can be easily bred in captivity under a wide variety of water and climate conditions. They have also been suggested as more disease resistant than other fishes. These exclusive features make tilapia ideal aquaculture species and explain why they have become one of the most important domesticated fishes around the world. Of the three species with recognized aquaculture potential that are generally being used in a large-scale commercial culture of tilapia – *Oreochromis niloticus*, *Oreochromis mossambica* and *Oreochromis aureus* – the Nile tilapia (*O. niloticus*), is by far the most cultured species in fish farming (Fitzsimmons, 2003; Lim and Webster, 2006).

Just two decades ago, it was generally believed that there were very few commercially significant diseases in tilapia aquaculture. This is no longer true. Emergence of new severe tilapia diseases is most likely related to the global intensification of aquaculture. Tilapias are being reared at higher densities than ever before, and more tilapia is being cultured in recirculating systems every year. Although tilapia perform exceptionally well in recirculation systems, so do pathogens. With an ever increasing pathological threat, tilapia health management becomes a highly essential concern (Watanabe et al., 2002; Gupta and Acosta, 2004).

The clinically significant tilapia pathogens fall into the general categories of viruses, bacteria, and protozoa. So far, a limited number of viruses have been reported and classified as etiological agents of tilapia diseases. The lymphocystis virus that affects a variety of marine and freshwater fishes, including tilapias such as *Oreochromis* and *Haplochromis* (Paperna, 1973), is a linear double-stranded DNA virus belonging to the iridovirus family. Bohle iridovirus was reported as the etiological agent of the 'spinning tilapia syndrome' in *O. mossambicus* (Ariel and Owens, 1997). Infectious pancreatic necrosis, a highly contagious systemic disease of young fish held under intensive rearing conditions, is induced by a virus having a bisegmented double-stranded RNA genome and belonging to the family *Birnaviridae*. This aquatic birnavirus was reported to be pathogenic for tilapia (Hedrick et al., 1983; Mangunwiryo and Agius, 1987; ShaoWen et al., 2003). Nodavirus, a member of the family *Nodaviridae*, has a bipartite single-stranded positive-sense RNA genome and is the causative agent of nodavirosis, viral encephalopathy and retinopathy (the VER disease, also called viral nervous necrosis, VNN). The disease causes high mortality rates in the larval stages and produces significant economic losses in the larval culture of a great number of marine fish species; nodavirus was also isolated from tilapia species *O. mossambicus* and *O. niloticus* (Skliris and Richards, 1999; Lio-Po and Penaranda, 2004).

We report here an outbreak of a novel disease characterized by a whirling syndrome and high mortality rates in laboratory-reared tilapia larvae. We designated the disease as viral encephalitis of tilapia larvae. By virtue of morphological features, biophysical and molecular

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characteristics of the pathogen involved in the etiology of this disease, we suggest the causative agent is a herpes-like virus.

Results and discussion

The disease

An unexpected disease struck gynogenetic blue tilapia larvae reared in our laboratory. The disease was manifested by a whirling syndrome (a spiral swimming behaviour) followed by massive mortality of the affected fish. The diseased larvae originated from some distinct mothers, suggesting the vertical transmission of the disease. In most cases, the whirling syndrome was preceded by dark pigmentation that started from fins and speedily spread all over the skin. These specific symptoms of morbidity appeared on days 4–6 at the feeding stage and were followed by rapid onset of mortality with very low survival rate (2–8%). Two peaks of mortality were recorded in blue tilapia larvae (Fig. 1) with statistically different ($p<0.05$) peak timings; the first one (24–26 days post-fertilization) can be definitely associated with vertical transmission of the disease, and the second one (32–34 days post-fertilization) might be attributed to the horizontally transmitted infection.

To assess the possibility of horizontal inter-species transmission of the disease, healthy red tilapia larvae were cohabitated with morbid blue tilapia larvae thus enabling clear distinction of the two species by color. A few days after the first peak mortality in blue larvae, the whirling syndrome and ensuing mortality were evident in red larvae as well. Compared to blue larvae, cumulative mortality rate in red larvae was distinctly lower; 15–30% of red tilapia survived the infection vs. only 2–8% of blue tilapia survivals. A single peak of mortality in red larvae (30–32 days post-fertilization) partly overlapped with the second peak of mortality in blue larvae (32–34 days post-fertilization) with insignificant difference ($p>0.05$) in peak timings (Fig. 1). Both peaks can be reasonably attributed to horizontal transmission of the disease.

Interestingly, the descendents produced by red tilapia female survivors displayed the same two-peak mortality pattern characteristic of blue tilapia (not shown). A similar phenomenon was observed in two other species of tilapia larvae, *O. niloticus* and *S. galilaeus*, i.e., cohabitation with morbid blue tilapia larvae caused single-peak mortality, and the two-peak mortality pattern was evidenced in the offspring of the survived females.

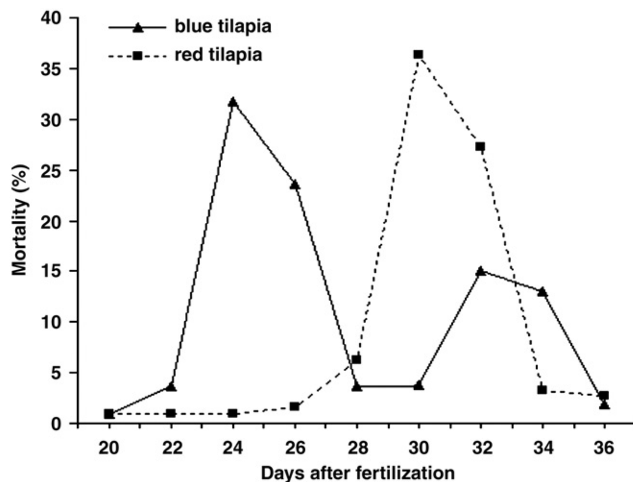

Fig. 1. Evidence of disease transmission and mortality rate in TLEV-affected larvae of blue and red tilapia. Presented is a typical experiment where two groups (250 subjects of swirling syndrome-positive blue larvae and 230 asymptomatic subjects of red larvae) were cohabitated, and mortality rate was followed up until day 36 after fertilization. In this particular experiment, the cumulative mortality rate reached 96% and 80% in blue and red larvae, respectively.

The pathogen

The whirling syndrome implied severe impairment of the central nerve system of the affected fish. Indeed, TEM examination revealed the presence of a putative pathogen, icosahedral hexagonal virus particles, about 100 nm in diameter, with electron dense cores within the cytoplasm of the brain tissue cells of sick larvae (Figs. 2A, B). Such particles were not found in healthy larvae. Viral encephalopathy has been recognized as an ever-increasing challenge in fish aquaculture due to mass mortality of the affected fish (Bovo et al., 1999). RNA viruses involved in these diseases belong to the families *Picornaviridae* (Bloch et al., 1991), *Retroviridae* (Oh et al., 1995) and *Nodaviridae* (Oh et al., 2002). DNA viruses involved represent *Herpesviridae* (McAllister and Herman, 1989) and *Iridoviridae* (Bloch and Larsen, 1993). The morphological characteristics of virus particles found in the brain of diseased larvae were compatible with those of herpesviruses (Roizman, 1982; Van Regenmortel et al., 2000). Based on specificity of the whirling syndrome and detection of the virus in the brain, we designated the causative agent tilapia larvae encephalitis virus (TLEV).

The virus was isolated from sick larvae and purified by isopycnic centrifugation in CsCl gradient. The dominant gradient fraction was found to contain mainly non-enveloped viral capsids. A negative stain TEM image of an isolated particle from this purified fraction is shown in Fig. 2C. The buoyant density of this fraction equaled 1.327 ± 0.005 g/ml (Fig. 3), which is somewhat higher than that usually reported for mature enveloped herpesviruses (1.22–1.28) but might be consistent with that of the capsids. Naked nucleocapsids were frequently observed in herpesviruses-infected cells (Okada et al., 1974, and references therein; Baines and Duffy,

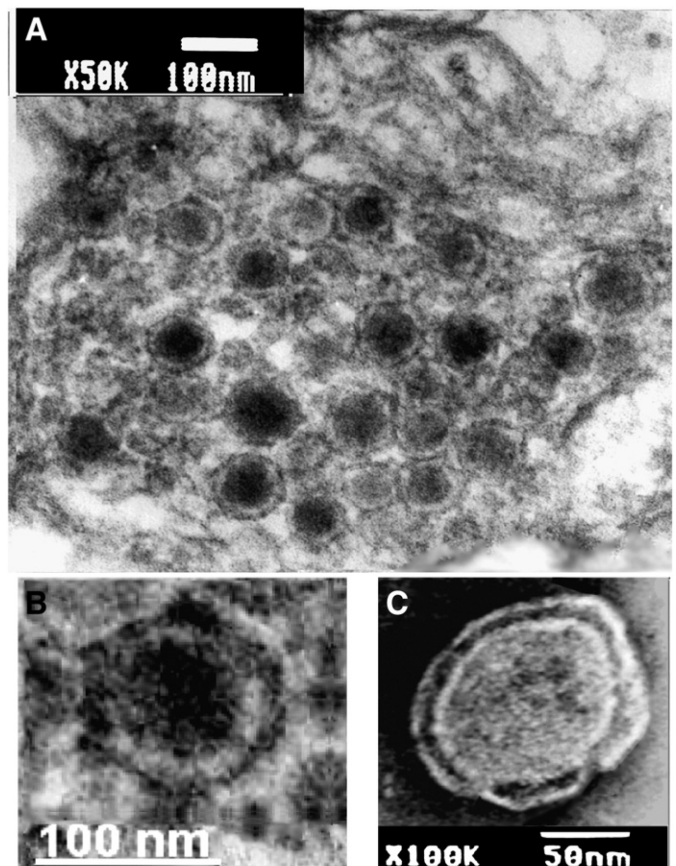

Fig. 2. TEM micrographs of ultrathin sections of TLEV-affected larvae brain tissue (A and magnified in B) and negative stain image of an isolated particle from the purified virus preparation (C). Shown in (A) are endoplasmic reticulum inclusions containing clustered hexagonal particles of about 100 nm diameter.

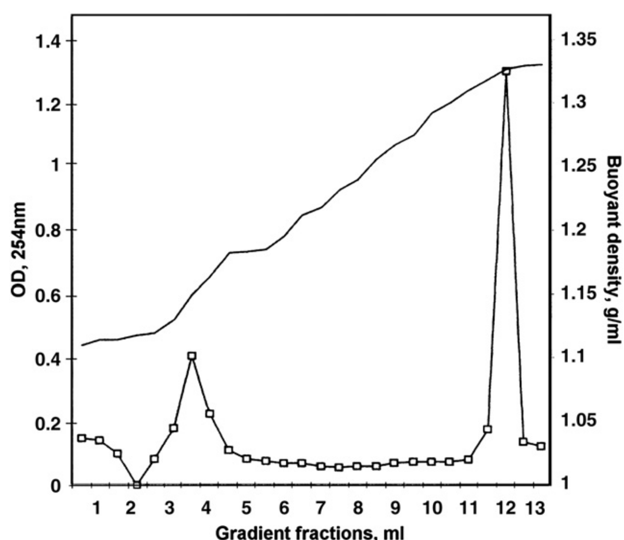

Fig. 3. Isopycnic centrifugation of TLEV in CsCl density gradient. The gradient was fractionated from top to bottom. The upper curve represents buoyant density, and the lower squares-marked curve represents optical density.

2006). The mechanism underlying the appearance of de-enveloped herpesvirus capsids in the cytoplasmic matrix is currently still debated (Campadelli-Fiume and Roizman, 2006) ranging between double envelopment model (Stackpole, 1969; Mettenleiter, 2002, 2004; Mettenleiter and Minson, 2006) and impaired nuclear pores model (Leuzinger et al., 2005; Wild et al., 2005). Herpesvirus of green turtles is an example when viral envelopes were not clearly visible while the virus remained infectious (Curry et al., 2000).

The antibodies

The identity of the pathogen in the purified virus and morbid fish was verified by ELISA of anti-virus antibody activity. Anti-TLEV antibodies specifically recognized the virus in diseased larvae and purified virus (Fig. 4A). The peak antibody titers of these specimens did not differ statistically ($p > 0.05$) but each one of them differed significantly ($p < 0.05$) from the low background level in healthy controls.

To assess possible relation between maturation of the immune system and severity of pathogen invasion, the changes in the level of TLEV and tilapia-specific immunoglobulin (Ig) activity have been monitored in parallel during 84 days after fertilization in affected blue tilapia larvae (Fig. 4B). Low albeit distinct levels of the virus and Ig were detected in the very beginning of ontogeny thus implying a direct mother-to-embryo vertical transfer of Ig (Mor and Avtalion, 1990; Avtalion and Mor, 1992; Takemura and Takano, 1997) and TLEV. The profiles of these two traits displayed a similar decrease at the initial stage (until days 5 and 13, respectively).

We have recently assumed (Sinyakov et al., 2006) that the humoral protective mechanism operating at the first stage of pathogen invasion implies an active role of natural antibodies, which neutralize the pathogen until being exhausted. This effect is displayed as initial suppression and results in decreasing levels of natural antibodies and the pathogen. It seems feasible to interpret the decrease we have observed at the initial stage as indicating a similar operation of maternally transferred Ig as protective antibodies and their involvement in virus neutralization until their exhaustion (day 13).

Having reached the lowest values, the two profiles revealed different trends. A slow enhancement of Ig on days 13–20 followed by a fast rise of the antibody activity thereafter (nearly 9-fold increase on day 84 compared to that on day 20) can be reasonably attributed to

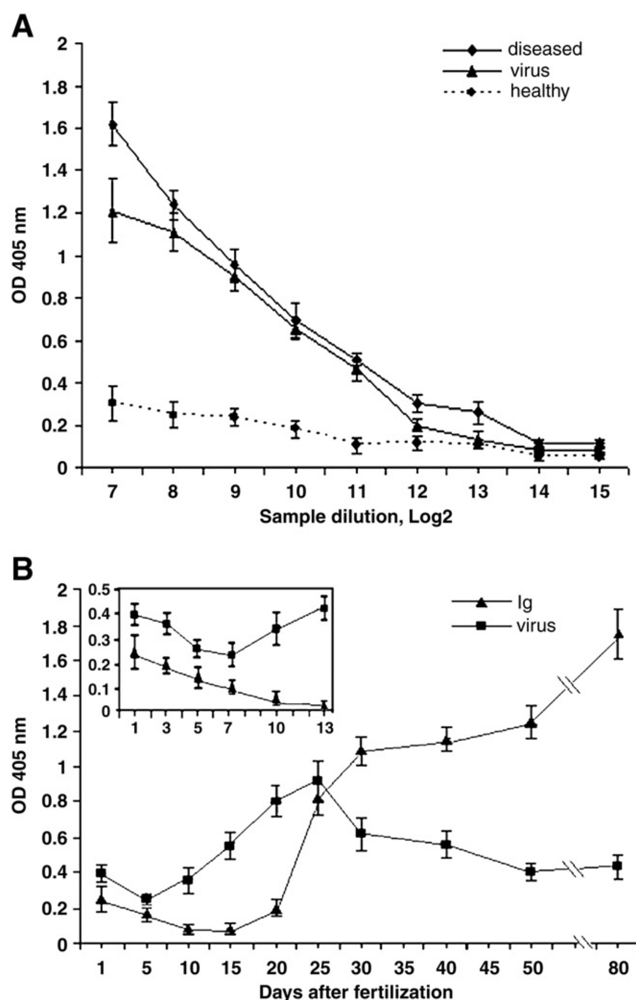

Fig. 4. (A) Sample dilutions (Log_2) of sick larvae extract (marked as 'diseased') and purified virus ('virus') as tested for TLEV presence by ELISA vs. healthy larvae extract ('healthy'). Prior to the assay, the extracts and purified virus were diluted and adjusted to optimal concentrations (18 μg/mL and 0.9 μg/mL, respectively). (B) ELISA dynamics of TLEV ('virus') invasion vs. Ig production in blue tilapia larvae. The inset is a magnified fragment of the first 13 days dynamics; in this period of Ig reduction, Ig represents maternally transferred antibodies. The following period of Ig elevation represents production and accumulation of acquired antibodies.

adaptive immunity, i.e., production and accumulation of acquired antibodies.

The virus overcomes the pressure of mother-originated Ig and natural antibodies and proceeds its invasion until the level of acquired specific antibodies is sufficiently high to take control over the virus and slow down the pathological process (days 24–52). Noteworthy, from this point on, irrespective of the ever-increasing level of acquired antibodies, the virus level remained essentially unchanged until the end of the experiment (day 84) indicating that the survivors turned to be silent virus carriers. This interpretation of the results is in line with previous reports on the Ig profile in tilapia larvae (Takemura, 1993) and protective role of maternally derived immunity in fish (reviewed in Swain and Nayak, 2009; Wang et al., 2009).

Molecular characterization

For primary characterization of the viral genome, genetic material was extracted from the purified viral particles, alternatively treated with RNase-free DNase I or DNase-free RNase A and visualized on a standard 0.8% agarose gel. As can be seen in Fig. 5A, the viral genome was completely digested by DNase I and unaffected by RNase A. Furthermore, when the viral nucleic acid was treated with single-

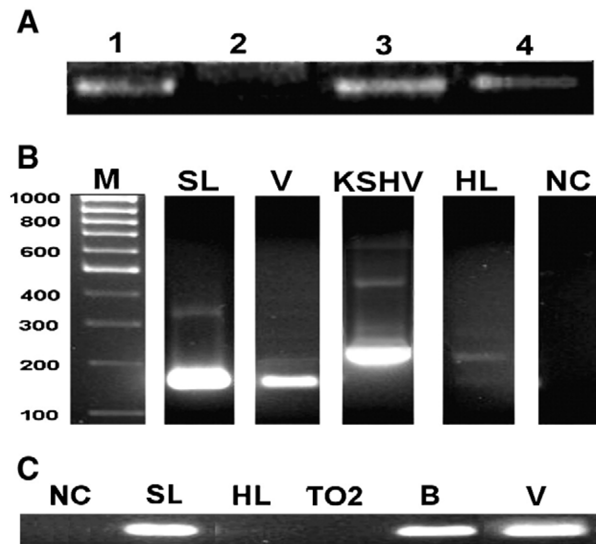

Fig. 5. Molecular characterization of TLEV nucleic acid. (A) Results of endonuclease treatment as revealed by agarose gel electrophoresis. TLEV genetic material was extracted from purified viral particles and treated alternatively with RNase-free DNase I, DNase-free RNase A and the single-strand-specific Mung bean endonuclease. The treated genomes were visualized as ethidium bromide stained signal on a standard 0.8% agarose gel. Presented are electrophoregrams of untreated TLEV nucleic acid (lane 1), and TLEV nucleic acid after alternative treatment with DNase I (lane 2), RNase A (lane 3) and Mung bean nuclease (lane 4). (B) Agarose gel electrophoresis of the ethidium-bromide stained PCR products obtained using degenerate primers for the conserved herpesvirus DNA polymerase and DNA extracted from the following sources: sick larvae (SL) total tissues, TLEV virus (V), cells infected with the Kaposi's sarcoma-associated herpesvirus (KSHV, positive control) and healthy larvae (HL). M, 100 bp DNA molecular weight marker. An expected 221 bp PCR product was a dominating band for KSHV DNA, and 170 bp product was obtained for SL DNA and V DNA. No PCR products were obtained for HL DNA and in the negative control (NC). (C) Agarose gel electrophoresis of TLEV DNA polymerase gene fragment obtained using specific TLEV primers. NC, negative control (without DNA). High molecular weight DNA was extracted from the following sources: sick larvae (SL) total tissues, healthy larvae (HL), tilapia ovary cell line (TO2), brain of sick larvae (B) and purified virus (V). Shown is 172 bp product.

strand-specific Mung bean endonuclease, no effect was observed thus suggesting that the viral genome was double-stranded DNA. Together with TEM analysis, these results matched the biochemical features of herpesviruses genome (Van Regenmortel et al., 2000).

To further verify the assumption that TLEV is a herpes-like virus, a degenerate consensus primer nested PCR technique has been employed to amplify a highly conserved region of the herpesvirus DNA polymerase gene (VanDevanter et al., 1996). This approach has been reported useful to amplify sequences of known herpesviruses and to discover newfound herpesviruses (Rose et al., 1997; Quackenbush et al., 1998; Rovnak et al., 1998; Richman et al., 1999; Ehlers et al., 1999a, b; Rijsewijk et al., 2005). DNA specimens were extracted from sick and healthy larvae and subjected to PCR using degenerate primers directed against the gene encoding herpesvirus DNA polymerase. DNA extracted from cells infected with Kaposi's sarcoma-associated herpesvirus (KSHV) was used as a positive control. Along with a predicted 221-bp band characteristic of KSHV DNA, a single band of approximately 170 bp was visualized for DNA samples of sick larvae. A similar single band resulted for DNA extracted from purified virions that were incubated with DNase I prior to SDS-proteinase K treatment. These results indicated the existence of the conserved herpesvirus DNA polymerase sequence within the TLEV genome that contains possible gaps as compared to the relevant sequence of KSHV. No amplification products were revealed for DNA extracted from healthy larvae and in the negative control (Fig. 5B).

To specifically amplify a region of the TLEV DNA polymerase gene, defined non-degenerate primers were designed (as specified in Methods). The TLEV-specific primers, TLEV-1 and TLEV-2, were located internally relative to the degenerate primer sites (downstream to I-DFA and upstream to I-IYG, respectively) and amplified a 172-bp sequence. DNA specimens isolated from sick larvae and from brain tissue of sick larvae were PCR positive. Furthermore, DNA isolated from purified viral particles was also positive, implying that the genetic material within the isolated viruses contained specifically amplified sequence. In contrast, DNA extracted from healthy larvae grown in isolated containers and from tilapia ovary cell line (TO2) were found negative (Fig. 5C). To determine the nucleotide sequence of the PCR product, it was cloned into pGEM-T Easy Vector System, and the amplicons were sequenced with T7 and SP6 universal promoter primers.

In silico characterization

Alignment of 56 amino acid stretch encoded by the respective fragment of the TLEV DNA polymerase gene is shown in Fig. 6; the relevant fragments of DNA polymerase sequences originated from 17 herpesviruses, 2 alloherpesviruses and 1 iridovirus were used for comparison. The rational to take alloherpesviruses was based on the fact that these viruses represent herpes-like viruses of fish, and it was reasonable to expect their close relation to TLEV. The nucleotide and predicted amino acid sequences of currently known herpes-like viruses of fish were only tenuously related to those of the herpesviruses of mammals and birds. They could not be accommodated within the current taxonomic structure, and thus they formed a distinct lineage, were ascribed to a new genus, temporarily named the 'Ictalurid herpes-like viruses' genus, and later on assigned to a new family, the *Alloherpesviridae* (McGeoch et al., 2006; Doszpoly et al., 2008; Kurobe et al., 2008; Davison et al., 2009). For alignment, we used the sequences originated from two alloherpesviruses, Ictalurid herpesvirus 1 (IcHV-1 or channel catfish virus) and Koi herpesvirus (KHV or CyHV-3, Cyprinid herpesvirus type 3, strain U) associated with devastating losses of wild fish (Aoki et al., 2007). The rational to take an iridovirus was based on the resemblance of morphological features, clinical manifestation and high mortality caused by this virus in fry of tilapia (Ariel and Owens, 1997). In this respect, an iridovirus might also be suspected to be related to TLEV. For alignment, we used the sequence originated from Red sea bream iridovirus, RSIV.

The scores produced by pairwise alignments (Fig. 6A) were used for *T*-test evaluation of similarity between TLEV and herpesviruses. The similarity between TLEV and alphaherpesviruses was significantly higher ($p<0.05$) than that between TLEV and beta- or gammaherpesviruses, which did not differ. Noteworthy, one of the distinct features of the *Alphaherpesvirinae* subfamily is the capability to induce latent neurotropic infections (Jones, 1998; Roizman and Pellett, 2001; Mettenleiter, 2003; Mettenleiter et al., 2008), which is in line with the brain tissue tropism of TLEV. The highest similarity was revealed between TLEV and the turtle herpesvirus GTHV-Ha (64% identity, 76% similarity). It seems pertinent here to refer to a similar morphology of these two viruses; in turtle herpesvirus, viral envelopes were not clearly seen as well (Curry et al., 2000). At present, the nature of this phenomenon remains obscure.

In the multiple alignment (Fig. 6B), the TLEV-originated sequence revealed the high level of conservation compared to those originated from *Herpesviridae* viruses. At the same time, the iridovirus and alloherpesviruses were evidently unrelated to TLEV. The scores produced by pairwise alignments between TLEV and IcHV and KHV were the lowest (8 and 16, respectively). The score between TLEV and iridovirus RSIV was higher (33) but still much lower compared to *Herpesviridae* viruses (ranged within 41–64). Thus, possible relation of TLEV to alloherpesviruses and iridoviruses can be neglected.

The phylogenetic tree produced by the maximum likelihood-based PhyML program (Fig. 7) was in line with known taxonomic and phylogenetic relationships among herpesviruses; the sequences derived from the viruses belonging to three *Herpesviridae* subfamilies were grouped into separate clusters and placed distantly from those

A

Seq-1	Seq-2	Score	Seq-1	Seq-2	Score
	Alpha			**Beta**	
TLEV	GTHV-Ha	64	TLEV	HCMV	41
TLEV	BHV-1	57	TLEV	HHV-6	50
TLEV	HSV-1	60	TLEV	HHV-7	44
TLEV	PRV	58	TLEV	MCMV	48
TLEV	VZV	62			
TLEV	CanHV-1	53			
TLEV	GalHV-2	57		**Unrelated**	
TLEV	MHV-1	60			
			TLEV	IcHV-1	8
	Gamma		TLEV	RSIV	33
			TLEV	KHV	16
TLEV	AHV-1	50			
TLEV	HVS	48	IcHV-1	RSIV	10
TLEV	EBV	42	IcHV-1	KHV	33
TLEV	EHV-2	44	RSIV	KHV	19
TLEV	KSHV	46			

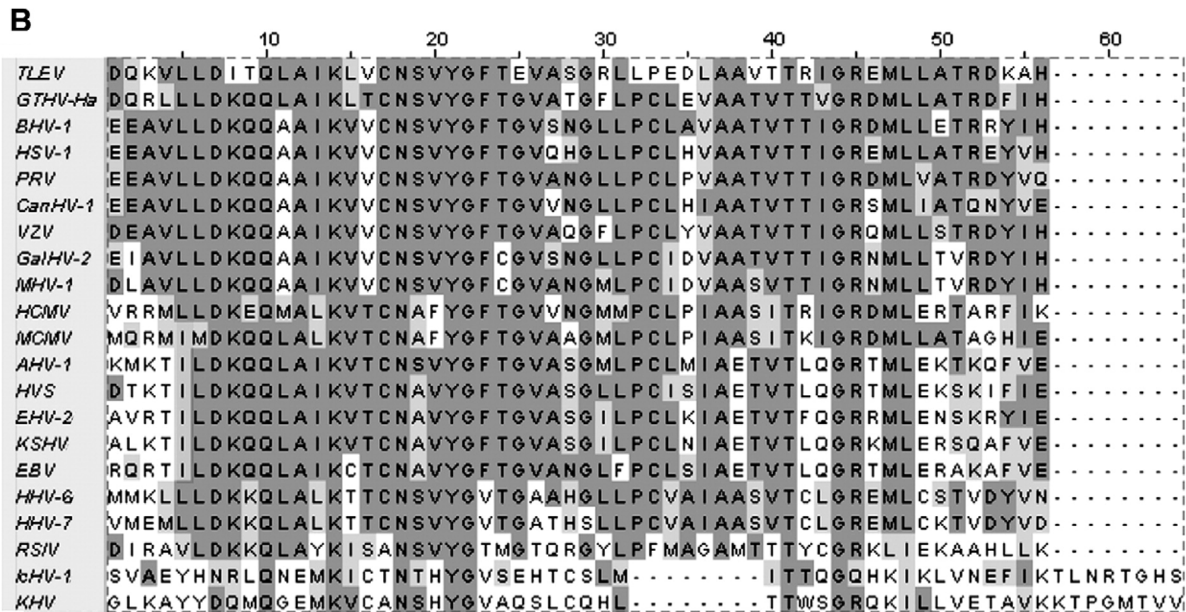

Fig. 6. Sequence alignments. (A) Pairwise alignment scores produced by ClustalW software. Alpha, Gamma and Beta stand for three subfamilies of *Herpesviridae,* viz. *Alpha*-, *Gamma*- and *Betaherpesvirinae*, respectively. (B) Multiple sequence alignment produced by MAFFT program. Shading intensity indicates the level of conservation; the decreasing color intensity corresponds to diminishing conservation of sequence characters ranging from identical (black) to non-matching (white). Compared are 56-amino acid stretches from TLEV DNA polymerase gene and corresponding regions of 17 herpesviruses from the family *Herpesviridae* and three sequences derived from fish viruses from the families *Iridoviridae* and *Alloherpesviridae*. In listing order, 17 viruses of *Herpesviridae* represent eight, five and four viruses accommodated within subfamilies *Alpha*-, *Gamma*- and *Betaherpesvirinae*, respectively. GTHV-Ha (Hawaiian green turtle herpesvirus, accession no. AF035003), BHV-1 (bovine herpesvirus 1, accession no. Z78205), HSV-1 (Herpes simplex virus 1, accession no. X04771), PRV (pseudorabies virus, accession no. L24487), VZV (varicella-zoster virus, accession no. X04370), CanHV-1 (canine herpesvirus, accession no. X89500), GalHV-2 (gallid herpesvirus 2, accession no. L40431), MHV-1 (meleagrid herpesvirus 1, accession no. AF282130), AHV-1 (alcelaphine herpesvirus 1, accession no. AF005370), HVS (saimiriine herpesvirus 2, accession no. X64346), EBV (Epstein-Barr virus, accession no. V01555), EHV-2 (equine herpesvirus 2; accession no. U20824), KSHV (Kaposi's sarcoma-associated herpesvirus, accession no. U93872), HCMV (human cytomegalovirus, a beta human herpesvirus, accession no. M14709), HHV-6 (human herpesvirus 6, accession no. X83413), HHV-7 (human herpesvirus 7, accession no. U43400), and MCMV (murine cytomegalovirus, accession no. M73549). The family of *Iridoviridae* is represented by RSIV (Red sea bream iridovirus, accession no. AB007366). The family of *Alloherpesviridae* is represented by IcHV-1 (Ictalurid herpesvirus 1 or channel catfish virus, accession no. M75136) and KHV (Koi herpesvirus or CyHV-3, Cyprinid herpesvirus type 3, strain U, accession no. DQ657948).

derived from 'alien' iridovirus and two alloherpesviruses. The latter two viruses, KHV and IcHV, were placed close to each other, which is also in line to their interrelation (Waltzek et al., 2005). In accord with the sequence alignment, the phylogenetic analysis also accommodated the TLEV-originated sequence among the relevant sequences of viruses belonging to the family *Herpesviridae*. The phylogram thus verifies our assumption that TLEV appears to belong to the family *Herpesviridae* and is closely related to GTHV. The TLEV-originated

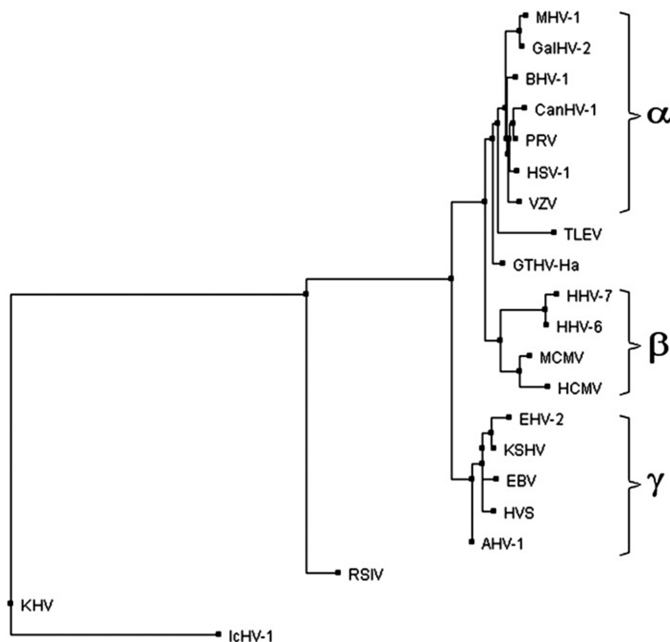

Fig. 7. Maximum likelihood-based PhyML program-produced phylogram of TLEV DNA polymerase sequence fragment and the relevant sequences originated from the viruses listed in Fig. 6 and belonging to families *Herpesviridae*, *Iridoviridae* and *Alloherpesviridae*. The phylogram is based on Fig. 6 sequence alignments. α, β, γ stand for three subfamilies of *Herpesviridae*. The phylogram is a tentative preliminary sketch.

DNA polymerase sequence fragment was deposited in the GenBank database (accession number ID: AY178582). With all that, it must be emphasized that this is the primary characterization of the new virus, and the precise taxonomic placement of TLEV cannot be determined unequivocally until the entire sequence information is available.

At this stage, we can only speculate as to precursor(s) and evolutionary pathways that brought about the emergence of such phenomenon as TLEV. We may consider several options for possible primary host of the new virus. Sequence alignment and phylogenetic analyses, albeit restricted, indicate high similarity of TLEV with alphaherpesviruses that accommodate avian and bovine viruses. The two major sources of blue tilapia in Israel are Lake Hula and Lake Ein Feshkha located north and south from the Sea of Galilee, respectively. These lakes are constituents of the Jordan Rift Valley, which is a part of the Great Rift Valley and a recognised track of seasonal bird migration; so this is the option of an avian primary host. Both of the above lakes had the input from canalization tracks outgoing from some of the nearby poultry and cow farms, which prompts the option of a bovine primary host. It seems also reasonable to suggest that the virus might have been horizontally transmitted from aquatic animals other than fish. The high similarity of TLEV with the turtle herpesvirus might indicate one of the species involved. Both of the above lakes are small shallow water bodies that accommodate multiple populations of turtles, which would explain the relation between the two viruses. It might also be that the families *Herpesviridae* and *Alloherpesviridae* split before fish and other vertebrates split. Then, one would expect both virus lineages to be represented in both host lineages, as long as reciprocal extinction has not occurred. Last but not least, the new virus might be originated from two different primary hosts and thus represent a chimera virus as shown recently for Ostreid herpesvirus 1 (Davison et al., 2005).

Thus, tilapia might be a new host of a virus that has another species as its primary host. The above options indicate which species might be the primary host and explain the closeness of TLEV to herpesviruses of birds, mammals and reptiles and its remoteness from herpesviruses of fish. Having being transferred from a non-fish primary host to tilapia, the newly emerging virus might have undergone significant mutagenesis to cross the interspecies barrier. In natural environment, the virus is in a balanced co-existence with its new host and may be even innocuous for wild tilapia. However, additional genetic changes might occur when the virus of wild fish was transferred to farmed fish. Adaptation of the virus to aquaculture with abundant supply of susceptible hosts might have resulted in drastically increased virulence as suggested for herpesviruses of other intensively cultured species (Nair, 2005; Aoki et al., 2007).

Gender transmission of the virus

Separate experiments were carried out to assess the role of gender in TLEV transmission and to clear up which of the gonads (ovary or testis) harbor the virus in healthy carrier parents. To this end, DNA was extracted from ovary, testis and brain tissues from five females and five males and tested for the presence of the virus by PCR screening with the use of the virus-specific primers TLEV-1 and TLEV-2. Results indicated that TLEV-specific sequences were present in ovaries of four females and in testes of two males, whereas none of the brain tissues were found virus-positive. This finding reinforced the vertical virus transmission hypothesis and indicated direct involvement of reproductive organs in this process.

For comparative evaluation of the incidence rate of infection in the reproductive organs of tilapia parents, sperm and ovary cells of 37 fish were analyzed prior to fertilization. The gametes were taken from 25 subjects of *O. aureus* (15 males and 10 females) and 12 subjects of *O. niloticus* (six males and six females). In total, 59.5% of the fish were found virus-positive. Within this score, 72% were virus carriers in *O. aureus* and 33.3% were virus-positive in *O. niloticus*. Assessment of gender influence revealed TLEV presence in 61.9% males vs. 56.2% females. Thus, no gender differences were revealed in vulnerability to the virus indicating that both males and females virus carriers are capable of mediating vertical transmission of the disease. On the other hand, susceptibility to the disease was definitely species-dependent.

Finally, batches of eggs laid separately by a healthy virus-carrier (PCR-positive) mother were fertilized with sperm of a virus-free (PCR-negative) male. Virus-positives were found in 57.1% of the randomly picked larvae originated from these eggs.

Concluding remarks

Gender transmission experiments that revealed TLEV healthy carriers in sexually mature asymptomatic fish were carried out with tilapia adults delivered from two unrelated external hatcheries, kibbutz Hamaapil and Gan Shmuel. Also, significant part of tilapia larvae delivered from these sources exhibited the whirling syndrome. Thus, the disease was not restricted to our laboratory but spread outside as well. Noteworthy, there were no specific reports by fish farmers about high mortality of larvae, which might be due to the practice of larvae harvest in the spawning ponds at the age of three weeks, when the TLEV-associated mortality is over (Y. Simon, hatchery manager in kibbutz Sdeh-Eliyahu, personal communication). We believe the disease might be overlooked in a daily routine of natural habitats and aquaculture. High mortality associated with TLEV poses a real threat for tilapia. Systematic PCR screening of brood stocks for TLEV carriers should be considered. TLEV infection in various tilapia species and other fishes and the routes of transmission should be further elucidated.

Methods

Fish

Adult fish, embryos and larvae were used from local stocks of four tilapia species: an inbred meiogynogenetic line of blue tilapia *O. aureus* developed in our laboratory (Don and Avtalion, 1988; Shirak

et al., 2006), *O. niloticus* (originated from Ghana), *Sarotherodon galilaeus* (originated from the Sea of Galilee) and red tilapia (originated from the Philippines and defined as a hybrid of different tilapia species, Galman and Avtalion, 1983).

Mature parental fish were kept in spawning families of two males and six to eight females, which were housed in aquaria of $170 \times 35 \times 35$ cm equipped with aeration and recirculating water system thermoregulated at 28 ± 1 °C (Koiler and Avtalion 1985).

Fertilized eggs were collected from the females' mouth after spawning and grown for 10–12 days in 1 L conic Zuger bottles within a sterile recirculating system equipped with charcoal filter and UV irradiation (Don et al., 1987). The temperature was thermostatically controlled and adjusted at 27 ± 1 °C. At the feeding stage (20 days post hatching), larvae were transferred into aquaria equipped with a similar water recycling system, and water temperature was maintained at 25 ± 1 °C.

Diseased larvae displaying the whirling syndrome were separated at different time intervals after spawning and kept frozen at −20 °C until used.

Virus isolation and purification

Diseased tilapia larvae were homogenized with a glass tissue blender in TNE buffer (10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4), centrifuged at $4000 \times g$ for 20 min, and the supernatant was recentrifuged at $15{,}000 \times g$ for 10 min. The supernatant obtained after the latter centrifugation was pelleted in a Sorvall ultracentrifuge (Sorvall Discovery 90 SE, Hitachi, Tokyo, Japan) in a Beckman SW 41 rotor (Beckman Instruments, Spinco Div., Palo Alto, CA) at $100{,}000 \times g$ for 1 h at 4 °C. The pellet was resuspended in TNE buffer, layered onto a preformed 10–30% (w/w) dextran gradient in TNE buffer and recentrifuged at $90{,}000 \times g$ for 2 h. The virus band was collected, diluted five-fold with TNE buffer and spun down by centrifugation at $100{,}000 \times g$ for 2 h. Protein contents of the purified virus were estimated by a modified Lowry assay (Markwell et al., 1978).

The buoyant density of the virus was measured by isopycnic density centrifugation in preformed gradient of cesium chloride (CsCl). The virus purified in dextran gradient was resuspended in TNE buffer, layered onto a linear 20–40% (w/w) CsCl gradient in TNE buffer and centrifuged at $150{,}000 \times g$ for 20 h at 20 °C. Fractions of 0.5-ml were collected and monitored for absorbance at 254 nm. The buoyant densities of the gradient fractions were calculated from measurements of the refractive index.

Transmission electron microscopy (TEM)

Freshly dissected brain tissue of diseased larvae was fixed in Karnowski solution (4 h at room temperature or 24 h at 4 °C), rinsed (×3) in 0.1 M Sorenson's phosphate buffer and postfixed in 1% osmium tetroxide. The cells were dehydrated in graded alcohol, embedded in Spurrs epoxy matrix (Polysciences Inc., Warrington, PA), sectioned (0.1 μm) with a LKB Ultratome III (LKB, Bromma, Sweden) and stained with uranyl acetate and lead citrate following a routine TEM staining protocol.

Purified virus suspensions were examined by negative staining. Specimens were applied to formvar-coated and carbon-stabilized electron microscopic grids, stained with 2% phosphotungstic acid, and excess stain was removed by gentle touching the edge of the grid with filter paper. Ultrathin sections and negatively stained grids were examined with a JEOL 1200 EX transmission electron microscope (Jeol Ltd., Tokyo, Japan).

Immunological methods

Virus-specific polyclonal antibodies were raised in mice. Female BALB/c mice were immunized with purified virus. Triple immunization, with fortnight intervals, was performed by injection of 50 μg of purified virus protein extract emulsified in Freund's incomplete adjuvant into mouse pad.

The purified virus stock was analyzed by ELISA using mouse anti-TLEV polyclonal antibodies. Nunc Immunoplates II (Nalge Nunc International, Denmark) were coated with the purified virus (100 μl, 0.9 μg/ml) and blocked with 0.4% gelatin (2 h at 37 °C each step). Serial dilutions of mouse anti-TLEV polyclonal serum (linking antibodies), Sigma goat anti-mouse alkaline phosphatase-labeled IgG (resolving antibodies) and Sigma *p*-nitrophenyl phosphate (substrate) were applied consecutively thereafter (1 h at 37 °C each step). The color reaction was measured as absorbance at 405 nm.

TLEV presence and tilapia-specific immunoglobulin (Ig) activity in morbid larvae extracts were monitored by ELISA using rabbit anti-tilapia immunoglobulin as essentially described elsewhere (Mor and Avtalion, 1990; Sinyakov et al., 2002). Briefly, immunoplates were sensitized by coating with the virus and blocked with gelatin. Serial dilutions of tilapia larvae extracts to be tested were added thereafter, allowed to react with rabbit anti-tilapia immunoglobulin and resolved by Sigma goat anti-rabbit alkaline phosphatase-labeled IgG. The color reaction was developed by addition of phosphatase substrate and monitored as absorbance at 405 nm.

Molecular methods

Larvae were homogenized as described elsewhere (Mor and Avtalion, 1988). Cellular DNA was recovered by sodium dodecyl sulfate–proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation (Burleson et al., 1992). Purified virus preparations were incubated with DNase I prior to virus nucleic acid extraction, which followed the same extraction procedure. Identification of the nucleic acid (RNA or DNA) and its strand structure (single- or double-stranded) was carried out with the use of RNase-free DNase I, DNase-free RNase A and Mung bean nuclease. Enzymatic digestions were performed according to the manufacturer's protocol (New England Biolabs, Beverly, MA).

Amplification of a conserved portion of the herpesviral DNA polymerase gene was carried out as described by VanDevanter et al. (1996). The first amplification of 1 μg of tissue-originated DNA or 100 ng of viral template DNA was performed in a total volume of 50 μl with the use of degenerate deoxyinosine-substituted primers, two upstream primers, I-ILK (5′ TCCTGGACAAGCAGCARIYSGCIMTIAA 3′) and I-DFA (5′ GAYTTYGCIAGYYTITAYCC 3′), and one downstream primer I-KG1(5′ GTCTTGCTCACCAGITCIACICCYTT 3′). The second-round amplification on one microliter template of the primary PCR reaction product was performed with deoxyinosine-substituted primers, an upstream primer I-TGV (5′ TGTAACTCGGTGTAYGGTGT 3′), and a downstream primer I-IYG (5′ CACAGAGTCCGTRTCICCR-TAIAT 3′). The 170-bp product of this PCR reaction was used to design a TLEV-specific downstream primer TLEV-1(5′ TCGTGGGCC-TTATCCCGCGT 3′). All reaction mixtures contained 1 μM of each PCR primer, 200 μM of each deoxynucleotide triphosphate, 2 mM $MgCl_2$, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, and 2.5 U of Taq polymerase (Takara Shuzo Co. Ltd., Shiga, Japan). Reaction samples were heated to 94 °C for 3 min and run thereafter in 45 cycles (30 s at 94 °C, 1 min at 46 °C, 1 min at 72 °C), concluding with 7 min at 72 °C for one cycle in a PTC-150 Minicycler (MJ Research Inc., Watertown, MA). 20-μl aliquots from the secondary PCR were electrophoretically separated on 2% agarose gel containing 0.5 μg/ml ethidium bromide (Maniatis et al., 1982).

An additional sequence was obtained by two rounds of PCR amplification. In the first round, the degenerate consensus primers I-DFA and I-KG1 (specified above) were employed with a total of 35 cycles. One microliter of the first-round PCR product was used as a template for the secondary amplification with the I-DFA upstream primer and the TLEV-specific downstream primer TLEV-1

(specified above). This reaction was performed as above, but with a total of 35 cycles and an annealing temperature of 60 °C. This amplification yielded a product that allowed the design and synthesis of a TLEV-specific sense upstream primer TLEV-2 (5′ GAGACCAGAAAGTGCTTCTC 3′).

The PCR products to be sequenced were excised from the gel, purified with the JetSorb gel extraction kit (Genomed, Bad Oeynhausen, Germany), cloned into pGEM-T Easy Vector System (Promega Inc., Madison, WI), and sequenced with T7 and SP6 promoter primers using an Applied Biosystems 373A sequencer (Perkin-Elmer, Foster City, CA). Samples containing 1 μg of tissue-originated purified DNA or 100 ng of viral template DNA were used for PCR amplification with TLEV-specific primers. Amplification and cloning conditions were as described above.

In silico methods

TLEV-originated amino acid sequence was translated from the amplified product excluding the degenerate primed region and assessed by sequence alignment and phylogenetic analyses. Sequences to compare were obtained from GenBank and Swiss-Prot databases. The multiple sequence alignment was performed by MAFFT software version 6 with default parameters (Katoh et al., 2002; Katoh and Toh, 2008). The phylogenetic tree was constructed with the use of the maximum likelihood-based PhyML program (Guindon and Gascuel, 2003). Pairwise alignment scores were produced by the ClustalW software on the EMBL-EBI server (Larkin et al. 2007).

Statistics

The unpaired two-tailed *T*-test was applied for evaluation of differences in peak timings of mortality, between the groups in ELISA antibodies detection, and for discrimination in similarity between TLEV and herpesviruses belonging to subfamilies *Alpha*-, *Beta*-and *Gammaherpesvirinae*.

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