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Derivation of two tilapia (*Oreochromis niloticus*) cell lines for efficient propagation of Tilapia Lake Virus (TiLV)

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ABSTRACT

Tilapia Lake Virus (TiLV) has been associated with disease outbreaks in cultured tilapia worldwide. In this study, we developed and characterized two cell lines from the brain (*OnlB*) and liver (*OnlL*) of the Nile tilapia, *Oreochromis niloticus* for the efficient propagation of TiLV. Both the cells grew well in Leibovitz's – 15 (L-15) medium supplemented with 20% fetal bovine serum (FBS) and have been sub-cultured more than 45 times. Chromosome analysis of the cells revealed that both lines had normal diploid number (2n = 44). TiLV was isolated from diseased tilapia and continuously propagated for 20 passages in these cell lines. The maximum TiLV titer was $10^{7.3 \pm 0.05}$ and $10^{7.0 \pm 0.96}$ $TCID_{50}$/ml, in *OnlL* and *OnlB* respectively. The TiLV isolate consistently produced the same CPE in all passages. *In vivo* challenge experiments using the TiLV infected cell culture supernatant reproduced symptoms of the disease in healthy tilapia, with mortality commencing 10 days post-infection and we were able to isolate TiLV from the challenged fish The above results suggest both cell lines are highly permissive for propagating TiLV and could be important tools for studying the molecular pathogenesis of TiLV infection.

1. Introduction

Tilapia are the second most farmed finfish species after carps and likely to be the most important cultured fish in the 21st Century (Fitzsimmons, 2000). The global production of tilapia in 2015 is estimated at 6.4 million metric tons (MMT), (FAO, 2017a) and the Nile tilapia, *Oreochromis niloticus* is the 6th most cultured species in the world (Reantaso, 2017). Tilapia are considered to be relatively resistant to a number of diseases encountered in other farmed fishes (Del-Pozo et al., 2017), but the emergence of Tilapia Lake Virus (TiLV) disease, the first major disease epidemic reported in tilapia aquaculture, has put the global tilapia industry at risk (Jansen and Mohan, 2017; FAO, 2017b). Though the disease has been confirmed from seven countries, namely Israel (Eyngor et al., 2014; Bacharach et al., 2016), Ecuador (Ferguson et al., 2014; Del-Pozo et al., 2017), Colombia (Tsofack et al., 2017), Egypt (Fathi et al., 2017; Nicholson et al., 2017), Thailand (Surachetpong et al., 2017), Malaysia (Amal et al., 2018), India (Behera et al., 2018) and Ugandan and Tanzanian parts of Lake Victoria (Mugimba et al., 2018), it is likely to be present in many more countries (Dong et al., 2017a). The disease is usually associated with high mortalities (Ferguson et al., 2014; Eyngor et al., 2014; Dong et al., 2017a; Behera et al., 2018) and is characterized by skin erosions, ocular abnormalities and distended abdomen. Internally, the lesions are mainly localized in liver and brain (Eyngor et al., 2014). In a recent study, Liamnimitr et al. (2018) confirmed that the mucus could be used for nonlethal sampling for the detection of TiLV by RT-qPCR and cell culture. Tattiyapong et al. (2018) developed a rapid and trustworthy RT PCR assay for the detection of TiLV in clinical cases as well in asymptomatic tilapia. Tilapia Tilapinevirus (TiLV) initially called Tilapia Lake Virus classified in the Orthomyxovirus group is now classed into a new unassigned group of its own termed Tilapinevirus (Adams et al., 2017).

Cell lines are essential for isolating viruses and studying virus-host interactions (Crane and Hyatt, 2011). Fish viruses are generally host specific, which makes a cell line derived from a particular fish species more appropriate for studying the viruses reported from that species (Pandey, 2013). Therefore, the establishment of susceptible, homologous and tissues-specific cell lines is considered necessary for isolating viral pathogens. *In vitro* culture of TiLV has been carried out using the E-11 cell line derived from snakehead (Iwamoto et al., 2000) as well as OmB and TmB cell lines derived from *O. mossambicus* (Gardell et al., 2014; Lewis and Marks, 1985). However, no cell lines from Nile tilapia are available for the continuous propagation of TiLV. Keeping

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the main target organs of TiLV in consideration, we have developed two cell lines from the liver and brain of *O. niloticus*. Both the cell lines are highly permissive for isolation as well as continuous propagation of TiLV, and will be useful for the development of strategies for the prevention and control of TiLV.

2. Materials and methods

2.1. Primary cell culture

Naive Nile tilapias, *O. niloticus*, were maintained in the laboratory in aerated aquaria. A primary culture from liver and brain (target organs of TiLV) was initiated using the enzymatic model as per Freshney (2005). Briefly, fish were euthanized with an overdose of tricaine methanesulfonate (MS-222, Sigma Aldrich, USA) and dissected using sterilized scissors. Liver and brain tissues were collected aseptically in separate petri plates (Himedia, India) containing 5 ml of phosphate buffer saline (PBS) (Thermo Fisher Scientific, USA) with 2× concentration of antibiotic-antimycotic solution (Sigma Life Sciences, USA). The tissues were washed thrice with PBS by gentle pipetting, cut into small pieces and finally transferred to 15 ml centrifuge tubes containing fresh Leibovitz's L−15 medium (Himedia, India) containing 500 U/ml of collagenase type IV (Gibco by Life technologies, USA). The tubes were incubated at 37 °C in a water bath for 1 h, followed by centrifugation at 500 ×g for 15 min. The supernatant in each tube was discarded and pellet washed twice with fresh PBS. Thereafter, the pellet was resuspended in 7 ml of complete L−15 medium containing 20% Fetal Bovine Serum (FBS) (Gibco by Life technologies, USA), and 1× concentration of antibiotic-antimycotic solution, and seeded in 25 cm^2 cell culture flasks (Thermo Fisher Scientific, Denmark). These were incubated at 28 °C and one third of medium was replaced every four days.

2.2. Maintenance, cryopreservation and revival

Cells were dissociated with 0.25% trypsin–EDTA (Gibco, Life Technologies, Canada) and split at a ratio of 1:2 when the primary cultures grew to 80–90% confluence using complete L-15 medium. The cells at different passage levels (every 5–6 passages) were stored in liquid nitrogen. In brief, the harvested cells were centrifuged; the pellet was washed with PBS and then suspended in 1 ml of Recovery™ cell-culture freezing medium (Gibco, Life Technologies, USA) at a density of 10^6 cells ml^{-1}. The cell suspension was aliquoted in cryovials (Thermo Fisher Scientific, Denmark) kept overnight at −80 °C and transferred to liquid nitrogen. Both cell lines were revived and checked for cell viability after 2 months of storage. Briefly, the vials were thawed at 37 °C, mixed drop-wise in 10 ml of complete medium kept in 15 ml centrifuge tubes. Then the tubes were centrifuged at 825 x g at 28 °C and each pellet was washed twice in PBS. Finally, the pellets were resuspended in 7 ml of complete medium and cell viability was determined by trypan blue exclusion test. Subsequently, the cells were seeded in 25 cm^2 flasks and incubated at 28 °C.

2.3. Cell growth studies

The effects of different temperatures and FBS concentration on cell growth were determined with both the cell lines (*OnlL* and *OnlB*) at 20th passage. Briefly, cells were seeded in 6- well tissue culture plates (Thermo Fisher Scientific, Denmark) at a density of 1×10^5 cells $well^{-1}$ and incubated at 15, 20, 28, 30 and 37 °C for 5 days. Every day, cells in the triplicate wells were harvested and counted using a Neubauer hemocytometer. Similarly, the effect of different concentrations of FBS (5, 7.5, 10, 15 and 20%) on cell growth was assessed at 28 °C.

2.4. Immunophenotyping assay

The cells at 25th passage were grown on cover slips placed in 6-well plates (Thermo Fisher Scientific, Denmark) at 28 °C. The cover slips were washed twice with PBS before fixing them in methanol for 30 min at −20 °C and blocked with PBS containing 1% BSA (PBS-A). Thereafter, cells were incubated separately either with mouse anticytokeratin (pan) clone AE1/AE3 antibodies (Sigma Life Sciences, USA) or with mouse anti-fibronectin antibodies (Sigma Life Sciences, USA). PBS-A was used in place of primary antibodies in control. After overnight incubation at 4 °C, the cells were washed with PBS and incubated with rabbit anti-mouse IgG FITC conjugate (Sigma Life Sciences, USA) for 1 h at room temperature. After a final wash with PBS, the cover slips were mounted in buffered glycerol and examined under a fluorescence microscope (Nikon, Japan).

2.5. Chromosome analysis

Chromosome spreads were prepared from *OnlL* and *OnlB* cells at 20th, 32nd and 42nd passage, using a conventional drop-splash technique (Freshney, 2005). After staining with Giemsa for 10 min, chromosomes were observed under a compound microscope and a total of 100 chromosome spreads were counted for each cell line.

2.6. Molecular characterization of the cell line

To authenticate the origin of the cell lines and to check the possibility of cross contamination with cell lines from different species, cytochrome oxidase subunit I (COI) and 16S rRNA genes were amplified from DNA isolated from *OnlL* and *OnlB* cells at 30th passage, using universal primers (Swaminathan et al., 2013). DNA isolated from fin tissue of *O. niloticus* served as a positive control. The PCR products were separated in 1.5% agarose gel and visualized under UV transilluminator (Bio-Rad, USA). Subsequently, the PCR products were sequenced using ABI 3730 DNA analyzer (Applied Biosystems, USA). The resulting DNA sequences for both the fragments were aligned with sequences amplified from fin tissue of *O. niloticus*, and BLAST search was carried out with available sequences in NCBI GenBank.

2.7. Transfection

For transfection studies, both the cells were cultured in a 6-well tissue culture plate at a density of 1×10^5 cells $well^{-1}$ at 35th passage. After 24 h, the sub-confluent monolayers were transfected with 2 μg of pAcGFP1-N1 vector (Clontech, USA) using Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific, USA), following the manufacturer's instructions. After 48 h, the plates were observed under a fluorescence microscope (Olympus, Germany).

2.8. Mycoplasma detection

Mycoplasma contamination was checked using EZdetect™ PCR Kit (HiMedia, India) which is based on amplification of the spacer region between 16S and 23S rRNA genomic DNA sequence. For the test, cells at passage 20 and 36 were grown in L-15 medium without antibiotics for 5 days. Thereafter, the cells were harvested and centrifuged at 200 ×g for 10 min. The supernatant was transferred to micro centrifuge tubes and centrifuged at 15000 ×*g* for 10 min. The pellet was resuspended in 50 μl of 1× TE buffer and heated at 95 °C for 3 min. After centrifugation, 2.5 μl of the supernatant was used for PCR reaction. The PCR mix and PCR cycling conditions were as per manufacturer's instructions. The amplified products were visualized in 1.5% agarose gel.

2.9. Susceptibility of OnlL and OnlB cell lines to tilapia lake virus

The pooled brain and liver tissues from TiLV infected *O. niloticus*

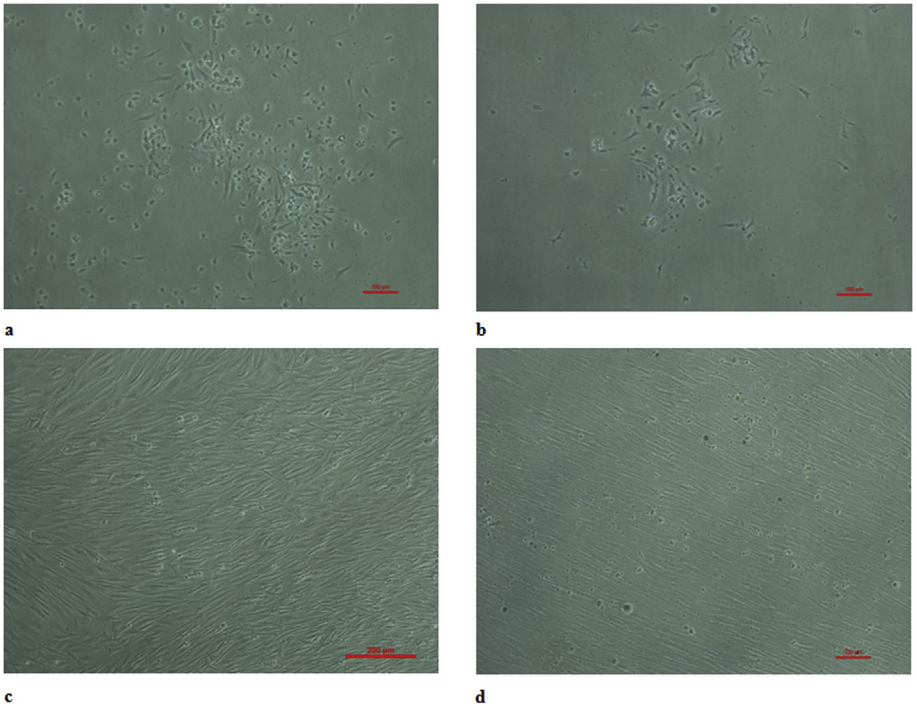

Fig. 1. Phase contrast photomicrographs of Nile tilapia, *Oreochromis niloticus* cells derived from brain and liver. (a) Liver cells dissociated by collagenase Type IV after 6 h of seeding; (b) Brain cells dissociated by collagenase Type IV after 6 h of seeding; (c) Monolayer of *OnlL* cells at passage 25 after 4 days of seeding; (d) Monolayer of *OnlB* cells at passage 25 after 8 days of seeding. Original magnification: ×100.

collected previously from Ernakulam, Kerala (Behera et al., 2018), were homogenized using a pestle and mortar and subjected to alternate freezing at −80 °C and thawing on ice 4 times. The tissue homogenate was centrifuged at 12000 ×*g* for 45 min at 4 °C and supernatant was filtered using 0.22 µm filter (Merck Millipore, Ireland). The filtrate was aliquoted and stored at −80 °C till further use. For virus susceptibility assay, *OnlL* and *OnlB* cells showing 80–90% confluence at 30th passage were inoculated with 250 µl of tissue filtrate and incubated for 1 h at 28 °C. In control flasks, 250 µl of tissue homogenate prepared from liver and brain of tilapia which tested negative in RT-PCR was used. Thereafter, the filtrates were aspirated and fresh L-15 medium with 5% FBS was added to the flasks, which were incubated at 28 °C. The flasks were observed daily for development of cytopathic effects (CPE) under an inverted microscope (Nikon, Japan) for 12 days. The cell culture supernatant from flasks showing CPE was confirmed to be positive for TiLV by RT-PCR following Dong et al. (2017b) and used for serial passaging of the virus in tilapia cell lines.

2.10. Susceptibility of other fish cell lines to TiLV

Eight piscine cell lines developed previously in our laboratory, namely PSF (Swaminathan et al., 2010), RTF (Swaminathan et al., 2012), CFF (Swaminathan et al., 2013), CCKF (Swaminathan et al., 2015), HBF (Swaminathan et al., 2016a), AFF (Swaminathan et al., 2016b), AOF (Oscar, *Astronotus ocellatus* fin, data unpublished) and FtGF (Fantail goldfish, *Carassius auratus* fin, data unpublished) were tested for their susceptibility to TiLV. In brief, 250 µl of tissue culture supernatant from TiLV infected *OnlL* cell line (confirmed positive by RT-PCR) was inoculated in each cell line as above. The cell lines were observed daily for cytopathic effects. The supernatant from cell lines showing CPE was used for serial passaging of the virus in respective cell line.

2.11. Determination of TiLV titer

TiLV titer was determined by end-point dilution on five different susceptible cell lines including *OnlB*, *OnlL*, AFF, CFF and AOF cell lines. Cells were cultured to 80–90% confluence in 96-well tissue culture plates, with 250 µl of L-15 medium supplemented with 2% FBS well^{-1}. Serial dilutions of cell culture supernatant from TiLV infected *OnlB*/*OnlL* cells were prepared in the L-15 medium with 2% FBS, and 100 µl of each dilution was added to the wells in triplicate. The wells were examined for CPE after 7 days and 50% tissue culture infective dose ($TCID_{50}$) ml^{-1} was calculated using the method of Reed and Muench (1938).

2.12. Experimental infection of Tilapia with cell culture propagated TiLV

Apparently healthy tilapia (n = 35, 10–15 cm) were procured from a local fish farm and acclimatized in aquaria for 7 days. The liver and brain tissues from randomly collected tilapia (n = 5) were screened for TiLV using RT-PCR following Dong et al. (2017b) and found to be negative. The remaining fish were divided into two groups, namely control and infected group, each comprising of 15 fish. Following anaesthesia with MS-222, fish in the control group were injected intraperitoneally at random with 100 µl of supernatant from normal *OnlL* or *OnlB* cells whereas, fish in the infected group were injected at random with 100 µl of supernatant from TiLV infected *OnlL* or *OnlB* cells (1×10^6 $TCID_{50}$/fish). Fish were observed daily for development of clinical signs as well as mortality, if any. Three fish from the control as well as infected groups were collected randomly after 7 days of injection and livers and brains were collected for RT-PCR, cell culture infection and also fixed in 10% neutral buffered formalin for histopathological examination.

3. Results

3.1. Primary cell culture, cryopreservation and revival

The disaggregated cells from liver and brain of tilapia following collagenase treatment adhered to the surface of the flasks by 6 h of seeding (Fig. 1a, b). About 1/3rd of medium in the flasks was replaced with fresh L-15 medium containing 20% FBS every fourth day. A 90–95% confluence was obtained by 10 and 15 days of seeding with liver and brain cells, respectively. Both the cells were subcultured at a split ratio of 1:2 and thereafter, passaged at 7–8 days interval. During

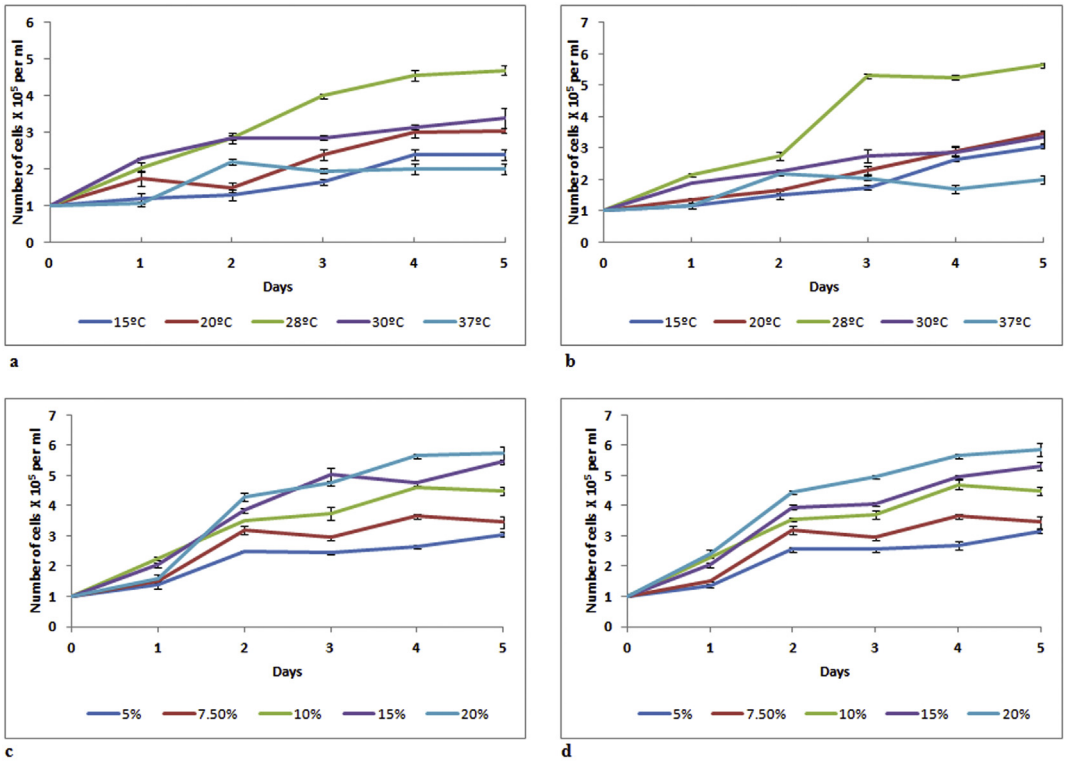

Fig. 2. Effect of temperature and serum concentration on Nile tilapia cell lines growth. (a) *OnlL* cells at different incubation temperatures; (b) *OnlB* cells at different incubation temperatures; (c) *OnlL* cells at selected concentrations of foetal bovine serum; (d) *OnlB* cells at selected concentrations of foetal bovine serum.

the initial passages of both the cells, a heterogeneous population of fibroblast-like and epithelial-like cells was observed. However, a homogeneous population of long and thin fibroblastic cells dominated in cell line from liver after 15 passages, thereafter, passaged at 4–5 days interval (Fig. 1c) and in brain cell line after 18 passages, thereafter, passaged at 8–9 days interval (Fig. 1d). The cell lines have been designated as *Oreochromis niloticus* Liver (*OnlL*) and *Oreochromis niloticus* Brain (*OnlB*), respectively and till date, subcultured for over 45 passages. The *OnlB* and *OnlL* cells showed a viability of more than 75% following revival of cryopreserved cells. These revived cells attached and grew well following seeding at 28 °C in L-15 medium supplemented with 20% FBS and established a monolayer within 14 days. No apparent alterations in morphology were observed in the revived cells from both cell lines.

3.2. Effect of temperature and FBS on cell growth

The optimal conditions for *in vitro* propagation of the two cell lines, *OnlL* and *OnlB* at 20th passage level were determined at five different incubation temperatures ranging from 15 °C to 37 °C (Fig. 2a, b) as well as five different concentrations of FBS ranging from 5 to 20% (Fig. 2c, d). The growth of both *OnlB* and *OnlL* cells was temperature and FBS concentration dependent. No attachment of cells was observed at 37 and 15 °C in both the cell lines and all the seeded cells died. However, at 20 °C, the cells were able to attach and grow in the flasks, but these cells showed slower growth. At 30 °C, the cells proliferated very fast during the first 48 h, but thereafter, the growth and proliferation slowed down, and the cells became enlarged and started dying. Furthermore, culture of both *OnlL* and *OnlB* cells was strongly dependent on the FBS concentration in the medium. Both the cells grew rapidly in L-15 containing 20% FBS in comparison to growth at lower concentrations of FBS. The cells grew well in 10 and 15% FBS and did not show not much difference in their growth rate, but cells in 5 and 7.5% FBS had slower proliferation rate. The optimal temperature and FBS concentration for the growth of *OnlL* and *OnlB* cells were determined to be 28 °C and 20%, respectively.

3.3. Characterization of tilapia cell lines

The cell cycle of *OnlL* and *OnlB* cells at 20, 35 and 42 passages was arrested in metaphase using colchicine at final concentration of 1 μg ml^{-1} for chromosome analysis. A total of 100 chromosome spreads at metaphase showed a range from 28 to 52 chromosomes for *OnlB* cells and from 30 to 54 for *OnlL* (Fig. 3a) cells with a clear peak at 44. The diploid number of chromosomes (2n = 44), observed for *OnlB* and *OnlL* cell lines (Fig. 3b), was found consistent even at 42nd passage. *OnlL* and *OnlB* cells incubated with mouse anti-fibronectin antibodies showed strong fluorescence in immunophenotyping assay, whereas, no fluorescence was observed in control cells as well as cells incubated with mouse anticytokeratin (pan), clone AE1/AE3 antibodies. The origin of *OnlL* and *OnlB* cell line was confirmed by amplification and sequencing of partial fragments of COI and 16S rRNA genes. The nucleotide sequences from *OnlB* and *OnlL* were identical to sequences amplified from fin of *O. niloticus* and showed maximum similarity (99%) with sequences of *O. niloticus* available on NCBI GenBank. These data confirmed that the origin of the developed *OnlL* and *OnlB* cell lines was from *O. niloticus*. Further, cells transfected with pAcGFP1-N1 vector showed fluorescent signals after 48 h and transfection efficiency of *OnlB* and *OnlL* cells was calculated as *c.* 8000^{-1} cells, indicating their potential to be used for expression of foreign genes. Further, no amplicon was observed in 1.5% agarose gel following amplification of 16S and 23S rRNA intergenic spacer region of *Mycoplasma*, which confirmed that the *OnlL* and *OnlB* cells were free of *Mycoplasma* contamination.

3.4. Susceptibility of OnlL and OnlB cell lines to TiLV and determination of TiLV titer

Following inoculation of filtered tissue homogenate from RT-PCR positive tilapia, CPE was observed in both the cell lines from 3 days post inoculation (dpi), whereas, no CPE was observed in control flasks of

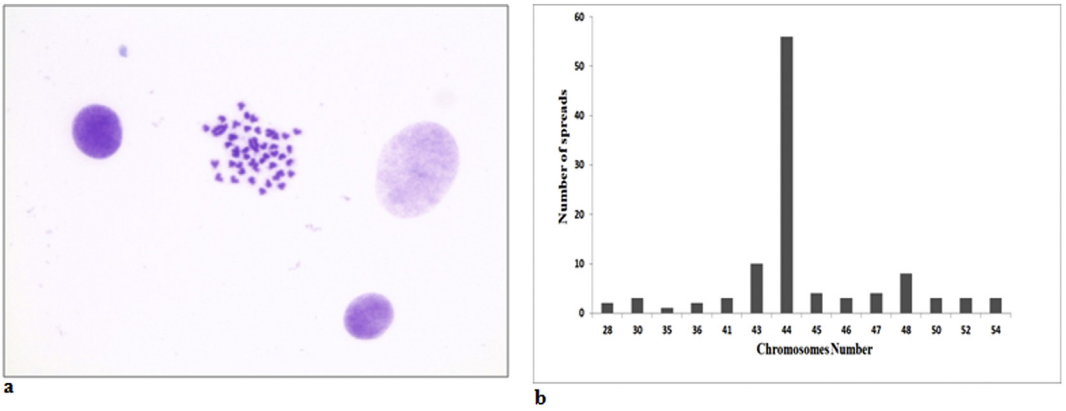

Fig. 3. Chromosomal typing of *OnlL* cell line. (a) Phase-contrast photomicrograph of single cell, chromosomes arrested in metaphase at the 32nd passage; (b) Frequency distribution of chromosomes of 100 cells.

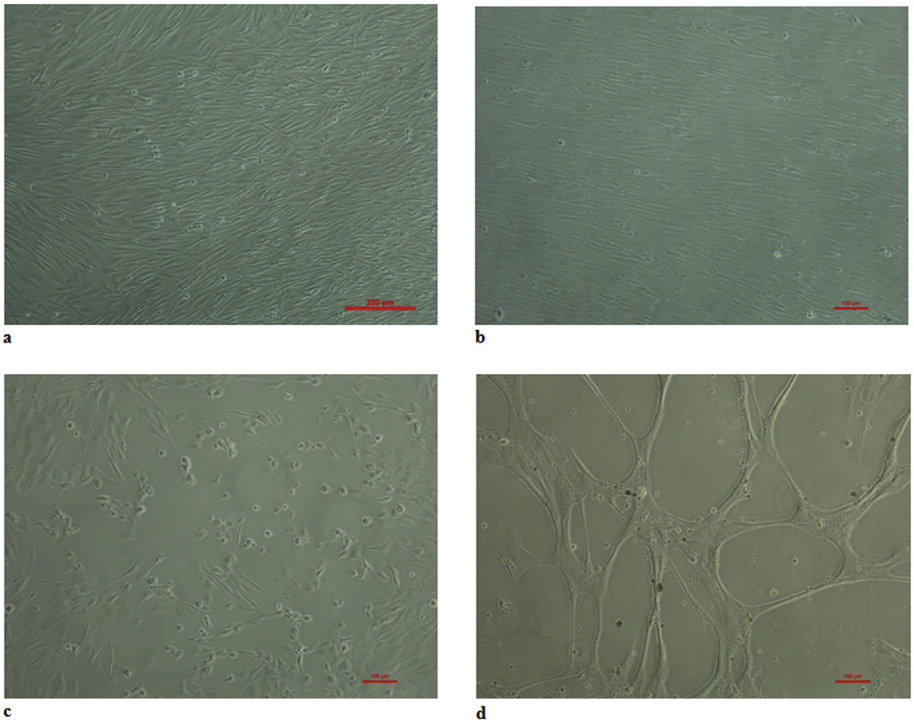

Fig. 4. Various cytopathic effects (CPE) in Nile tilapia, *Oreochromis niloticus* cell lines infected with TiLV. a) Uninoculated *OnlL;* b) Uninoculated *OnlB* cell line; c) *OnlL* cell line infected with TiLV at 4 day post-infection (dpi); d) *OnlB* infected with TiLV at 4 dpi. Original magnification: ×100.

OnlL and *OnlB* cells (Fig. 4a, b). The CPE due to the infection of TiLV in *OnlL* cells included syncytia formation, shrinkage and rounding of cells, and complete destruction of monolayer (Fig. 4c). In *OnlB* cells, plaque formation, increased granularity, elongation of cells followed by rounding and destruction of monolayer were observed (Fig. 4d). The complete destruction of monolayer was observed after 5 and 8 dpi in *OnlL* and *OnlB* cell lines, respectively. In the subsequent passages, CPE due to the TiLV in *OnlL* and *OnlB* cells was observed from 3 to 5 dpi. Both the TiLV infected cells and cell culture supernatants were found positive in RT-PCR and the expected PCR product (415 bp) was sequenced (Fig. 5).

3.5. Susceptibility of other fish cell lines to TiLV

Out of the eight fish cell lines, three cell lines, namely CFF, AFF and AOF supported propagation of TiLV. The CPE was observed in the 3 cell lines at 5–6 dpi and complete monolayer destruction were observed by 12 dpi. However, no CPE was observed after 3rd serial passage in the three cell lines *i.e.* CFF, AFF and AOF. The remaining cell lines including CCKF, FtGF, PSF, HBF and RTF cell lines did not show CPE following inoculation of TiLV. The details of the cytopathic effects and comparison of CPE in the susceptible cell lines is given in Tables 1 and 2, respectively.

3.6. Determination of TiLV titer

The TiLV titer was found to be $10^{7.3}$ and $10^{7.0}$ $TCID_{50}$ ml^{-1} in *OnlL* and *OnlB* cell lines, respectively during initial passages and$10^{6.0}$ and $10^{5.4}$ $TCID_{50}$ ml^{-1} in *OnlL* and *OnlB* cells, respectively, at the 20th passage. The TiLV isolate consistently produced similar CPE in all the passages. However, the yield of virus from CFF, AFF and AOF cell lines was very low, with a maximum titer of $10^{4.0}$ $TCID_{50}$ ml^{-1} (Table 3).

3.7. Experimental infection of Tilapia with cell culture propagated TiLV

Following a challenge with TiLV propagated in *onlL*, the fish started

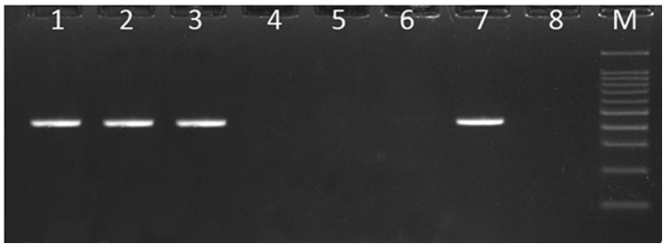

Fig. 5. Detection of TiLV in *OnlL* and *OnlB* cell lines infected with TiLV and experimentally challenged Nile tilapia, *Oreochromis niloticus* by RT-PCR. Lane 1–3; RNA isolated from *OnlL* and *OnlB* cells infected with TiLV and experimentally challenged Nile tilapia with TiLV infected *OnlL* cells respectively, Lane 4–6; RNA isolated from *OnlL* and *OnlB* cells not infected with TiLV and experimentally challenged Nile tilapia with *OnlL* cells not infected with TiLV respectively, Lane 7; Positive control (TiLV RNA extracted from infected tilapia in our previous studies), Lane 8; Negative control (RNA isolated from cell culture supernatant from cells that were not infected with TiLV), Lane M 100 bp DNA ladder.

Table 1
Cytopathic effects (CPE) produced by tilapia lake virus on different piscine cell lines tested in the study.

Cell line	CPE
OnlL	Syncytia formation, cell shrinkage, rounding and complete destruction of monolayer.
OnlB	Plaque formation, cellular granulation, elongation followed by rounding and destruction of monolayer.
CFF	Cell elongation, rounding, detachment and destruction of monolayer.
AOF	Cell rounding and detachment of monolayer.
AFF	Plaque formation, cellular granulation, elongation followed by, rounding and detachment of cells.
CCKF, RTF, PSF, HBF and FtGF	No CPE was observed.

Table 2
Comparison of cytopathic effects (CPE) induced by tilapia lake virus in different cell lines.

dpi	*OnlL*	*OnlB*	CFF	AFF	AOF
2	+	+	–	–	–
3	+++	++	–	–	–
5	**	+++	+	+	–
8	**	**	+++	+++	++
12	**	**	**	**	**

"+", "++", "+++" and "–" refer to 0–25% CPE, 25–50% CPE, 50–75% CPE and no CPE respectively. "**" refers to monolayer destruction; dpi – days post inoculation.

Table 3
Determination of $TCID_{50}\,ml^{-1}$ of tilapia lake virus at different passage levels in the susceptible cell lines.

No. of Passage	*OnlL*	*OnlB*	CFF	AFF	AOF
1	$10^{7.3}$	$10^{7.0}$	$10^{4.0}$	$10^{3.0}$	$10^{4.0}$
3	$10^{6.9}$	$10^{6.8}$	$10^{2.0}$	$10^{2.0}$	$10^{2.0}$
8	$10^{7.0}$	$10^{6.2}$	–	–	–
15	$10^{6.2}$	$10^{5.6}$	–	–	–
20	$10^{6.0}$	$10^{5.4}$	–	–	–

exhibiting clinical signs at 6–7 dpi, and these included skin discoloration, abdominal distension, protrusion of scales, exophthalmia, and pale liver (Fig. 6a), similar to those observed in naturally TiLV infected fish. The experimentally infected fish started dying at 10 dpi, with cumulative mortality reaching 100% by 12 dpi (Fig. 6b). However, no morbidity or mortality was observed in control group injected with supernatant from tilapia cell line. The histopathological examination of tissues from experimentally challenged tilapia revealed typical syncytial giant cells in liver (Fig. 6c) and congestion of the blood vessels as well as haemorrhages in sections of brain. In TEM analysis, enveloped round or oval shaped mature virus particle, of 60–80 nm diameter, were observed in cytoplasm of liver cells from experimentally infected tilapia (Fig. 6d). Pooled tissue samples (liver and brain) from euthanized tilapia were positive in RT-PCR and produced CPE in *OnlL* and *OnlB* cell lines (data not shown). All tissue samples of control tilapia were negative for TiLV in RT-PCR. The nucleotide sequences of 415 bp fragment of TiLV segment 3 was submitted to NCBI GenBank (Accession No. MF574205).

4. Discussion

Though primary cultures from the brain of *O. niloticus* have been used for isolation of TiLV (Eyngor et al., 2014), no cell lines are available from *O. niloticus*. Therefore, in the present study, we have developed two cell lines from liver and brain of Nile tilapia, which are the main target organs of TiLV (Eyngor et al., 2014) and these cell lines have been designated as *OnlL* and *OnlB*, respectively.

Both the cell lines have been cultured for more than 45 passages and characterized using an array of tests. The cell lines exhibit optimum growth at 28 °C in L-15 medium supplemented with 20% FBS, similar to that observed for most cell lines from tropical fishes (Sood et al., 2015). The modal chromosome number of both the cell lines was 44, which is the same as that reported for *O. niloticus* (Poletto et al., 2010; Mahmoud et al., 2010). The origin of *OnlL* and *OnlB* cell lines from *O. niloticus* was authenticated by partial amplification and sequencing of two mitochondrial genes COI and 16S rRNA, their alignment with sequences of the two genes amplified from fin tissue of Nile tilapia, and BLAST search with available sequences in NCBI GenBank. The amplification and sequencing of mitochondrial genes, namely 12S rRNA, 16S rRNA, 18S rRNA and COI are commonly used for confirming the origin of the cell lines as well as checking any cross contamination with cells of other cell lines (Rougee et al., 2007). Further, the *OnlL* and *OnlB* cells were determined to be fibroblastic origin using biochemical markers of the cytoskeleton. These markers have been employed previously for confirming the type of cells in fish cell lines (Mauger et al., 2009; Chaudhary et al., 2013). In contrast to our findings, cell lines derived from the liver of trout and pilchard typically have epithelial morphology (Ostrander et al., 1995; Lai et al., 2000; Williams et al., 2004). Faisal et al. (1995) reported hepatocyte-like, stellate and spindle-shaped morphology in three cell lines from liver of spot croaker. The cell line from brain of *O. niloticus* in the present study consisted of fibroblast-like cells. Previously, the cell lines developed from brain have been reported to have fibroblast morphology (Ahmed et al., 2009; Lai et al., 2001) as well as epithelioid morphology (Wen et al., 2008; Ku et al., 2009). Successful transfection in both the cell lines using lipofectamine suggested that the cell lines from *O. niloticus* can be used for the expression of foreign genes employing heterologous promoter, as reported earlier (Sood et al., 2015; Swaminathan et al., 2016b).

Following inoculation with filtered tissue homogenate prepared from pooled liver and brain from RT-PCR positive tilapia, the morphological changes in the *OnlL* and *OnlB* cell lines consisted of vacuolation, plaque formation, increased granularity, and rounding of cells followed by detachment of monolayer. Previously, a number of cell lines, namely E-11, OmB, TmB, CFF as well as primary tilapia brain cells have been reported to be susceptible to TiLV (Eyngor et al., 2014; Tsofack et al., 2017; Behera et al., 2018). Moreover, cytopathic effects similar to those observed in the present study have been reported in E-11 cell line and primary tilapia brain cells (Eyngor et al., 2014), whereas, CPE in CFF cell line consisted of elongation of cells followed by rounding and detachment (Behera et al., 2018). In the present study, clear cytopathic effects could be observed in both the cell lines and importantly, similar CPE was observed in both *OnlL* and *OnlB* cell lines

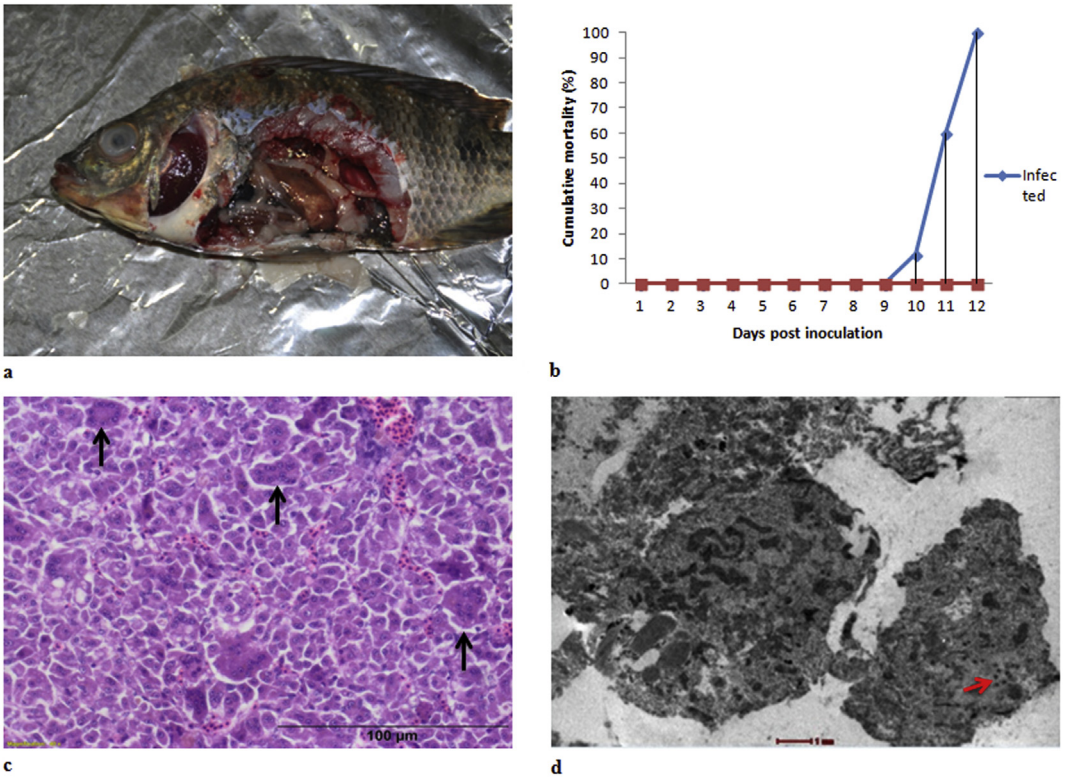

Fig. 6. Experimental challenge of TiLV in Nile tilapia, *Oreochromis niloticus* with TiLV infected *OnlL* cell culture supernatant. a) Post-mortem changes including ascitic fluid and necrotic and pale liver were observed; b) Cumulative mortality curve of the experimentally infected tilapia using TiLV propagated in *onlL* cells; c) Liver section of the experimentally challenged tilapia showing syncytial giant cells (black arrows) and increase in sinusoidal spaces; d) Transmission electron micrograph ultrathin sections of liver tissue from experimentally infected tilapia demonstrating 60–80 nm diameter virions showing a round enveloped viral particle in the cytoplasm of infected cells (red arrow). Scale bar: 500 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

up to 20 passages. In conformity with our findings, Eyngor et al. (2014) also observed similar CPE in E-11 cells for up to 18 passages. However, E-11 cells have been reported to be persistently infected with a retrovirus (Iwamoto et al., 2000) and therefore, may not be suitable for purification of the virus. Moreover, in an earlier study, Tsofack et al. (2017) reported TiLV titer in three cell lines, namely E-11, TmB and OmB, to be 4×10^6, 5×10^5 and 5×10^5 $TCID_{50}\,ml^{-1}$, respectively. However, in our study, TiLV titers were higher and reached $10^{7.3}$ and $10^{7.0}\,TCID_{50}\,ml^{-1}$ in *OnlL* and *OnlB* cell lines, respectively. The other fish cell lines in the present study including CFF, AFF and AOF were permissive to TiLV and supported its propagation up to 3rd serial passage but the virus titer was comparatively less. Moreover, CPE development was clearer and detected in a shorter period of time in *OnlB* and *OnlL* cell lines when compared to CFF, AFF and AOF cells. Therefore, the cell lines developed in the present study are more suited for cell culture based surveillance for TiLV and also purification of the TiLV. The TiLV titer was high in *OnlL and OnlB* cells during 3–8 passages and *OnlL* cells yielded more TiLV when compared to *OnlB* cells. Therefore we recommend *OnlL* over *OnlB* cells for the propagation of TiLV up to 8 passages.

In the present study, no CPE was observed in a number of cell lines including CCKF, RTF, PSF, HBF and FtGF cell lines. In earlier studies, no CPE was observed in CHSE-24, BF-2, BB, EPC, KF-1, RTG-2, FHM and TO-2 cell lines (Eyngor et al., 2014; Tsofack et al., 2017). The above findings are consistent with an earlier report (Lu et al., 1999) that permissive cell lines derived from the same host species are more suitable for *in vitro* propagation of virus as it may be not able to grow on cell line derived from other species.

Importantly, the cell pellet of both the infected cell lines was positive for TiLV in RT-PCR. Further, the injection of culture supernatant in native tilapia could successfully reproduce the disease, similar to that observed in natural outbreaks. TiLV was re-isolated from experimentally infected fish, fulfilling Koch's postulates. Similar to our results, the disease has been reproduced previously using supernatant from infected cell lines, (Behera et al., 2018; Eyngor et al., 2014; Tattiyapong et al., 2017). The gross lesions, namely exophthalmia, abdominal swelling, darkening of body colour and ascitic fluid in affected fish were similar to those reported earlier. In addition, typical syncytial cells in liver, and haemorrhages and congestion in the brain were similar to that reported in naturally and experimentally infected tilapia (Behera et al., 2018; Eyngor et al., 2014; Tattiyapong et al., 2017; Ferguson et al., 2014). Therefore, it can be inferred that both the developed cell lines are highly permissive for TiLV replication and the virus propagated using the two cell lines was virulent. Both the cell lines, *OnlL* and *OnlB* had been deposited at National Repository of Fish Cell Line (NRFC), ICAR-National Bureau of Fish Genetic Resources, India for further dissemination among the researchers in India.

5. Conclusion

Tilapia is an economically important fish among small fish farmers worldwide and the emergence of TiLV threatens their livelihood and food security. Better strategies for control of TiLV and development of vaccines will be helpful to reduce the losses due to this emerging disease. The newly established cell lines, *OnlB* and *OnlL*, will be extremely useful as a sensitive *in vitro* tool for detection and further studies on TiLV.

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