Journal of Academia Vol. 14, Issue 1 (2026) 55 – 63

EFFECTS OF FETAL BOVINE SERUM CONCENTRATION AND VENTED FLASK CONDITIONS ON E-11 CELL GROWTH AND TILAPIA LAKE VIRUS INFECTION

Nur Fadiah Wathiqah Fadzillah [1], Muhamad Afiq Aziz[2], Mohammad Noor Amal Azmai[3], Nurhamimah Zainal Abidin[1*]

[1]*School of Biology, Faculty of Applied Sciences*
Universiti Teknologi MARA (UiTM), Cawangan Negeri Sembilan, Kampus Kuala Pilah, 72000 Kuala Pilah, Negeri Sembilan, Malaysia.
[2] *Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Malaysia.*
[3]*Aquatic Animal Health and Therapeutics Laboratory, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.*

[*]*Corresponding author: nurhamimah@uitm.edu.my*

Abstract

Tilapia Lake Virus (TiLV) is an emerging pathogen threatening global tilapia aquaculture, causing high mortality and significant economic losses. The present study aimed to optimize E-11 cell culture conditions for improved growth and TiLV propagation under different serum and flask ventilation conditions. E-11 cells were cultured in Leibovitz's L-15 medium supplemented with 10%, 15%, and 20% fetal bovine serum (FBS) and maintained at 25°C in a CO_2-free incubator. Cell viability was assessed over four days using the Trypan Blue exclusion assay, while TiLV propagation was evaluated in both seal-cap and vent-cap flasks through microscopic observation of cytopathic effects (CPE). Results showed that E-11 cells supplemented with 15% FBS exhibited the highest viability (85–90%) and optimal proliferation compared to cells grown in 10% or 20% FBS. Morphologically, all cultures displayed similar epithelial-like characteristics with polygonal cell shapes and intact monolayers under the microscope. No significant differences in viability were observed between open- and closed-vented flasks; however, CPE were detected only in infected E-11 cells maintained in vent-cap flasks by 10 days post-infection (dpi), while cells in seal-cap flasks remained morphologically intact. These findings suggest that a 15% FBS concentration and a vented culture environment support both healthy E-11 cell growth and effective TiLV propagation. This optimized culture system enhances the reliability of *in vitro* TiLV studies and supports efforts toward sustainable aquaculture and food security.

Key terms: Aquaculture, Tilapia Lake Virus (TiLV), E-11 cells, Virus propagation, Cytopathic Effect

Article History:- Received: 25 November 2025; Revised: 22 April 2026; Accepted: 29 April 2026; Published: 30 April 2026

DOI: https://doi.org/10.24191/joa.v14i1.9648

Introduction

Cell culture involves the maintenance and propagation of cells in a controlled environment (Aarattuthodi et al., 2022). The aim of *in vitro* cell culture is to sustain cells outside their natural organism while preserving their viability and functional integrity (Segeritz & Vallier, 2017). The selection of an appropriate cell line for culture is primarily determined by the functional characteristics and the specific experimental outcomes required for the cell model (Uysal et al., 2018). Cell culture studies facilitate genetic manipulation and enable studies on differential gene or protein expression (Goswami et al., 2022).

In recent years, numerous fish cell lines from various aquaculture species have been increasingly established (Goswami et al., 2022). These cell lines serve as effective *in vitro* models that mimic *in vivo* conditions, and offer several advantages over mammalian cell culture, including reduced susceptibility to environmental perturbations greater tolerance to hypoxia, adaptability to a wider temperature range,

and easier to maintain for extended periods (Aarattuthodi et al., 2022).

Tilapia Lake Virus (TiLV) is an emerging viral pathogen of tilapia, first reported in Israel in 2011 (Piamsomboon & Wongtavatchai, 2021). TiLV infects various species and hybrids of wild and farmed tilapia, including the Nile tilapia (*Oreochromis niloticus*) and affects fish at all developmental stages (Widziolek et al., 2021). TiLV outbreaks have been reported mainly during the hot summer months (May to October) in countries such as Israel, Egypt, and Thailand (Aich et al., 2022). During this period, water temperatures are between 22ºC and 32ºC, and mortality ranges from 20% to 90% depending on regional factors (Widziolek et al., 2021). TiLV is an enveloped fish virus with a linear, negative-sense, single-stranded RNA genome consisting of 10 segments (approximately 10.3 kb). The virions have a diameter of 55 to 100 nm in diameter and belong to the genus *Tilapinevirus* within the *Amnoonviridae* family (Mugimba et al., 2019). However, compared to most other aquaculture viruses, the biological properties of this virus remain poorly understood (Widziolek et al., 2021) .

The selection of a cell line for cell culture is primarily determined by its functional properties and the specific experimental results desired (Lazaro et al., 2024). E-11 cells are clonal of the SSN-1 cell line that derived from *Channa striatus* or commonly known as striped snakehead fish (Aarattuthodi et al., 2022). E-11 cells are adherent cells that grow at a temperature of 25ºC temperature without CO_2. E-11 cells require Leibovitz's L15 medium that supplemented with Fetal Bovine Serum (FBS), penicillin and streptomycin (Pierezan et al., 2020). Study showed that E-11 cells were proven showing high tolerance to TiLV infection and their suitability for various TiLV quantification assays (Tattiyapong et al., 2017). However, the lack of standardised culture conditions may affect the consistency of experimental outcomes. FBS concentration may differ in a range of 2%-20% for E-11 cells or Nile tilapia-derived cells (Raksaseri et al., 2023; Thaganraj et al., 2018, Kembou Tsofack, 2016; Wen, 2016). The present study aims to determine the optimal growth conditions for E11-cells to improve TiLV propagation *in vitro*, providing a foundation for improved diagnostic and virological studies in aquaculture research**.**

Methods

Cell culture

E-11 cells were obtained from Aquatic Animal Health and Therapeutics Laboratory, Institute of Bioscience, Universiti Putra Malaysia (UPM), Malaysia. The cells were maintained in Leibovitz's L15 medium (Elabscience, Texas, USA) supplemented with 10% -15% fetal bovine serum (FBS) (TICO, Netherlands, Europe) and 1% penicillin-streptomycin (Nacalai Tesque, Kyoto, Japan). Cultures were incubated at 25°C in a CO_2-free incubator.

For growth comparison, E-11 cells were seeded at a density of 3.0×10^5 cells per T-25 flask, either vent-cap or seal-cap flasks (Biologix, Shandong, China) (Figure 1). To investigate the effect of FBS concentration on cell proliferation, E11- cells were seeded at a density of 2.1×10^4 cells per well in a 24-well cell culture plate. The cells were maintained in Leibovitz's L-15 medium, 1% penicillin-streptomycin and with different FBS concentrations of 10%, 15% and 20%. Cultures were maintained at 25ºC in an incubator without CO_2. Cells were observed for morphology changes and cell confluency before harvesting at different time points from 24 hours to 96 hours.

Virus Propagation

Tilapia Lake Virus (TiLV) were obtained from Aquatic Animal Health and Therapeutics Laboratory, Institute of Bioscience, Universiti Putra Malaysia. TiLV were propagated in E11-cells using Leibovitz's L-15 medium supplemented with 0.2% FBS and 1% penicillin-streptomycin. Upon reaching confluency, cells were inoculated with 1mL of TiLV at a multiplicity of infection (MOI) of 0.013. Cells were observed daily at different time points under microscope to observe the cytopathic effect (CPE) and the morphological changes of the E-11 cells. For the mock-infected control, E-11 cells underwent the same procedure, except the virus-free medium was used in place of the viral inoculum. This ensured both infected and mock-infected cells were subjected to identical handling conditions, differing only in the presence or absence of TiLV.

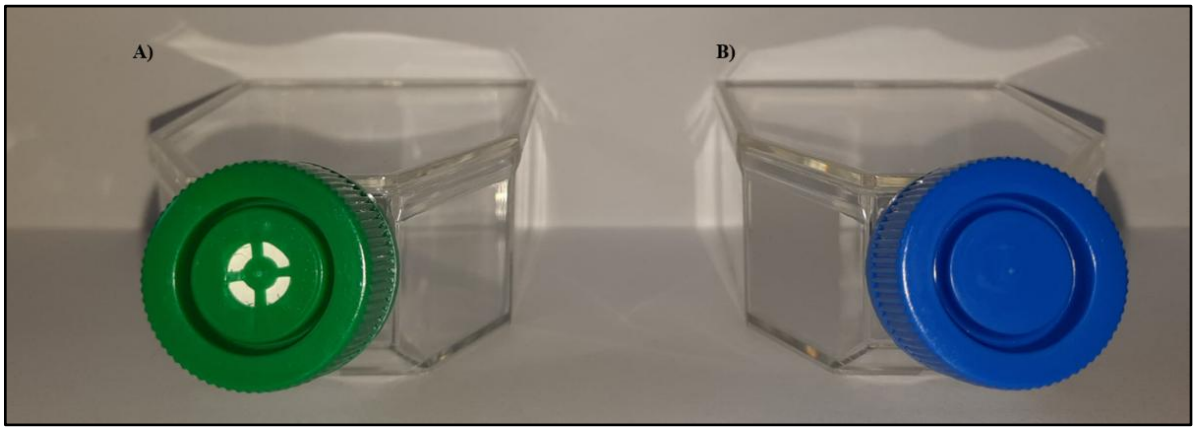

Figure 1. Two type of flask used in this study. (A) vent-cap flask that allows air exchange in the culture environment (B) seal-cap flask that limits air movement within the flask.

Viral RNA extraction and cDNA synthesis

Infected and mock-infected E-11 cells were harvested, and the total RNA was extracted using the GF-1 Viral Nucleic Acid Extraction Kit (Vivantis, Selangor, Malaysia) following to the manufacturer‘s instruction. The purity and concentration of viral RNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). cDNA synthesis was performed by using the RevertAid First Strand cDNA Synthesis Kit (ThermoScientific, USA) referring to the manufacturer’s protocol.

TiLV detection by RT-PCR

The presence of TiLV in E-11 cells was confirmed using nested RT-PCR targeting Segment 3 of the TiLV genome. The first round of PCR reaction consisted of 12.5µL of REDiant (2X) master mix (Axil Scientific, Singapore), 1.5µL of 10 µM of each primer Nested ext-1 (5’-TAT GCA GTA CTT TCC CTG CC-3’) and ext-2 (5’-TTG CTC TGA GCA AGA GTA CC-3’), 2µL cDNA template and nuclease free water up to total volume of 25µL. The second round of fully-nested RT-PCR was conducted in 25µL reaction solution containing 12.5µL REDiant (2X) master mix, 1.5µL of 10µM of each primer Nested ME1 (5’-GTT GGG CAC AAG GCA TCC TA- 3’) and ME2 (5’-TAT CAC GTG CGT ACT CGT TCA GT-3’), 2µL of first PCR product and nuclease free water. The amplification condition consisted of initial denaturation at 94ºC for 2 min, followed by 40 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec, extension at 72ºC for 30 sec and a final extension at 72ºC for 5 min. The PCR product were analysed by gel electrophoresis on a 2% of agarose gel and was visualized under UV light. Samples showing the expected 250 bp amplicon were subjected to Sanger sequencing for TiLV confirmation (Apical Scientific, Serdang, Malaysia).

Trypan blue exclusion assay

E-11 cells were observed and harvested at 24, 48, and 72 hours post-incubation. For harvesting, culture medium was removed and cells were rinsed with 1X PBS (Nacalai Tesque, Kyoto, Japan). Cells were then trypsinised with 1mL of 0.25% trypsin (Sigma-Aldrich, Missouri, USA) and followed by a wash with 3mL of complete growth media. All supernatant were collected and centrifuged at 1,800 rpm for 12 minutes to pellet the cells. For cell viability assessment, equal volumes of cell suspension and trypan blue dyes were mixed and loaded onto a hemacytometer. Live (unstained) and dead (stained) cells were counted under a light microscope to determine cell viability.

Statistical analyses

All experiments were performed in triplicate for each condition, including FBS concentration and flask vented type. Data were analysed using GraphPad Prism software version 6.04, www.graphpad.com. Statistical analysis was conducted using a two-sample t-test and results were considered statistically significant with a p-value of < 0.05 (GraphPad Prism software version 6.04, www.graphpad.com).

Result and Discussion

Effect of FBS on E-11 cell growth

E-11 is a cloned derivative for the SSN-1 snakehead fish cell line and is widely used in vitro for fish virus propagation. Published studies generally culture E-11 cells in Leibovitz's L-15 medium in a CO_2-free environment at 24°C to 28°C, supplemented with 5 to 10% FBS (Iwamoto et al., 2000; Thangaraja et al., 2018; Wen, 2016)). Fish cell culture may use a wider range of FBS concentrations, often around 5% to 20% FBS. For example, primary liver and brain cultures of the Nile tilapia *Oreochromis niloticus* were established in L-15 medium containing 20% FBS for efficient propagation of TiLV (Thangaraj aet al., 2018). In other study, the tilapia head kidney cell line THK was shown markedly proliferated in Leibovitz's L-15 medium containing 2% to 15% FBS (Wen, 2016). FBS is important because it provides growth factors, hormones, carrier proteins, lipids, and other essential components that support cell survival and proliferation (Stival et al., 2025). Variation in FBS composition and quality can influence cellular responses and affect the reproducibility of *in vitro* assays. Unoptimized FBS may therefore alter cell physiology, growth and experimental consistency (Stival et al., 2025). Therefore, evaluating multiple FBS concentrations is necessary to define optimal conditions for TiLV infection studies in E-11 cells.

The viability of E-11 cells cultured in Leibovitz's L-15 medium supplemented with different concentrations of fetal bovine serum (FBS) (10%, 15% and 20% FBS) was evaluated over four days (Figure 2A). Viability was measured daily for four days using the trypan blue exclusion assay. Data are presented as mean ± SEM from three independent replicates. Cells grown with 15% FBS consistently exhibited the highest viability throughout the 4-days period, maintaining approximately 85-90% viability up to day 3 before a slight decline on day 4. In contrast, cells supplemented with 10% and 20% FBS showed lower viability, with values ranging between 65-75% during the 4-days period. Statistical analysis revealed no significant differences between the groups; however, E-11 cells cultured with 15% FBS consistently supported better growth trends as compared to those cells supplemented with 10% and 20% FBS. These results also suggest that higher FBS concentrations do not necessarily promote cell growth, with 15% FBS provides the most favorable growth conditions for E-11 cell maintenance and proliferation. Microscopic examination revealed no noticeable differences in cell morphology among the different FBS concentration, as all cultures maintained a typical epithelial-like monolayer morphology throughout the experiment (Figure 2(B)).

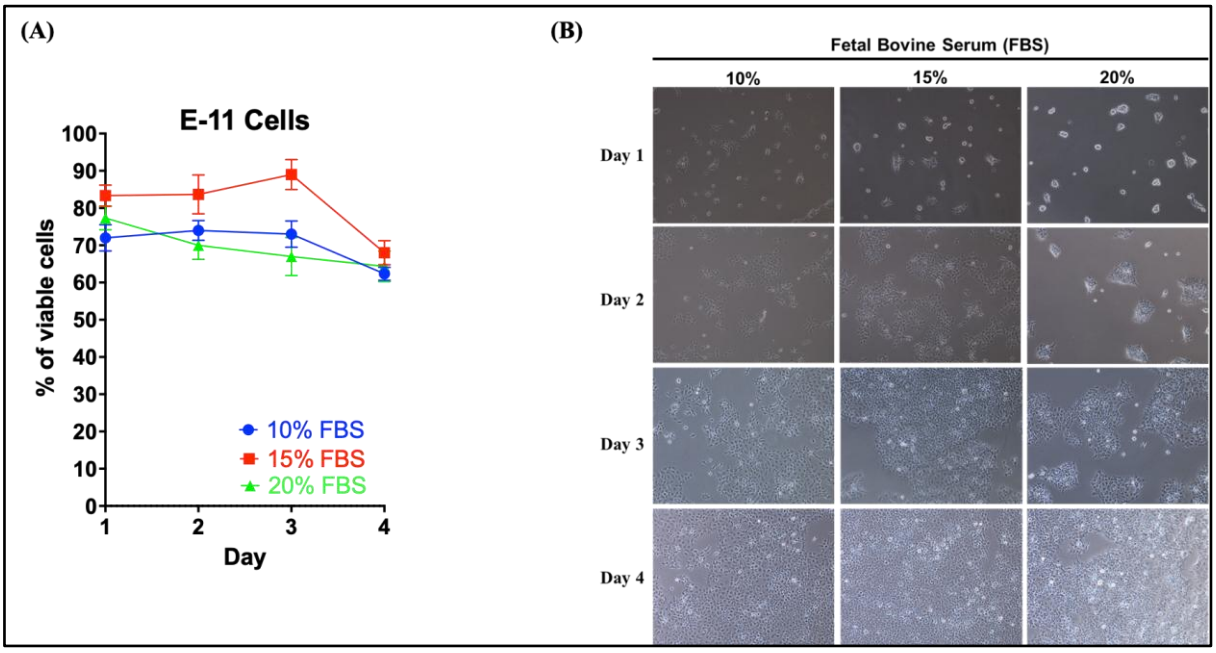

Figure 2. Effect of different concentration of fetal bovine serum (FBS) on E-11 cell viability (A) and morphology under the microscope (B). Data are presented as mean ± SEM from three independent replicates.

Vented effect on E-11 cells

Gas exchange is an important parameter in cell culture as it maintains cellular homeostasis by regulating oxygen availability, removal of metabolic gases and medium pH. In CO_2-free system such as E-11 cell culture, restricted gas exchange may result in accumulation of metabolic by products and medium acidification. All these effects can influence the cell growth, metabolism and susceptibility to viral infection. To evaluate this effect, E-11 cells were cultured in vent-cap and seal-cap T-25 flasks and assessed over four days to determine the influence of gas exchange on the cell growth. Both culture conditions supported high levels of cell viability (>85%) throughout the experiment (Figure 3A). E-11 cells in vent-cap flasks exhibited a slightly higher mean viability (approximately 90%) compared to those in seal-cap flasks (approximately 85%), although the difference was not statistically significant ($p>0.05$). Microscopic observations further revealed no noticeable differences in cell morphology between the two flask types (Figure 3B). These results suggest that both flask types provide suitable environments for E-11 cell maintenance under CO_2-free incubation, with minimal impact of flask ventilation on short-term cell viability.

Notable color differences were observed in the complete growth medium between vent-cap and seal-cap flasks, indicating variations in pH levels. In vent-cap flasks, the medium retained a bright red color, whereas in seal-cap flasks, it gradually shifted to a yellowish-orange tone (Figure 3C). This color variation corresponds to the pH indicator phenol red present in the medium, which turns yellow under acidic conditions and remains red at an optimal pH of approximately 7.4 (Weiskirchen et al., 2023). The observed changes suggest that vent-cap flasks better maintain the physiological pH suitable for E-11 cell growth, while seal-cap flasks experience a slight acidification likely due to limited gas exchange and accumulation of metabolic by-products. Considering the slight acidification observed in seal-cap flasks, prolonged culture under such conditions may potentially influence cellular metabolism and virus propagation efficiency (Leland & Ginocchio, 2017).

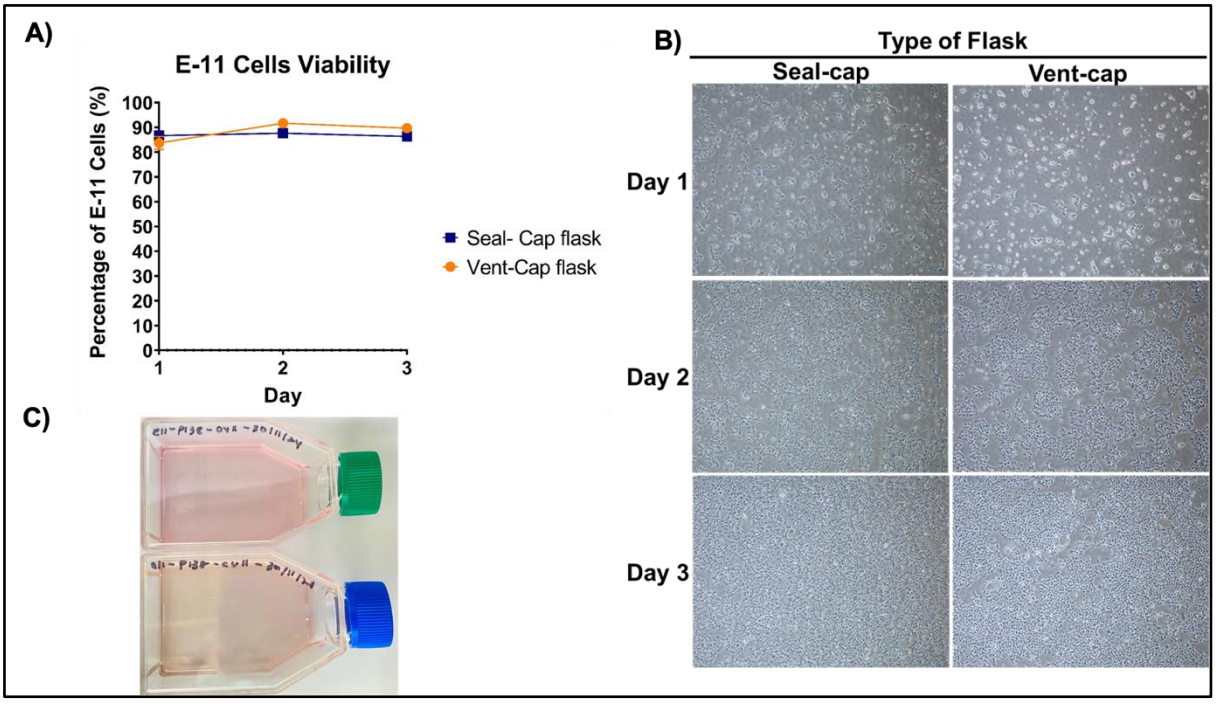

Figure 3. Effect of flask ventilation on E-11 cell viability and culture conditions. (A) Viability of E-11 cells grown in either vent-cap or seal-cap T-25 flasks. Viability was measured daily for three days using the trypan blue exclusion assay. Data are shown as mean ± SEM from three independent experiments. (B) Representative microscopic images showing the morphology of E-11 cells in both flask types across the observation period. (C) Colour changes in the complete growth medium in vent-cap flask (top- green capped flask) and in seal-cap flask (bottom- blue capped flask) which reflect the pH condition of the culture environment.

Cytopathic effects in TiLV-infected E-11 cells

E-11 cells were cultured in seal-cap and vent-cap flasks and observed at 0-, 6-, and 10-days post-infection (dpi) (Figure 4). Mock-infected cells in both flask types remained healthy and confluent throughout the observation period, showing no morphological alterations. Infected cells in the seal-cap flask exhibited no apparent cytopathic effects (CPE) up to 10 dpi, maintaining intact monolayers. In contrast, infected cells in the vent-cap flask began to display distinct CPE by 10 dpi, characterized by cell rounding, detachment, and loss of monolayer integrity. These findings indicate that flask ventilation influences TiLV-induced cytopathic response and E-11 cell morphology. These findings suggest that the ventilation environment influences TiLV-induced cytopathic effects. Since the viruses rely on the host cell's metabolic processes for energy components, limiting the gas exchange in the host cells unable to provide the energy and resources needed for robust viral replication. Therefore, the absence of CPE in seal-cap condition implies that limited gas exchange may inhibit viral replication or delay virus-induced cellular damage, thereby preserving cell viability (Leland & Ginocchio, 2017).

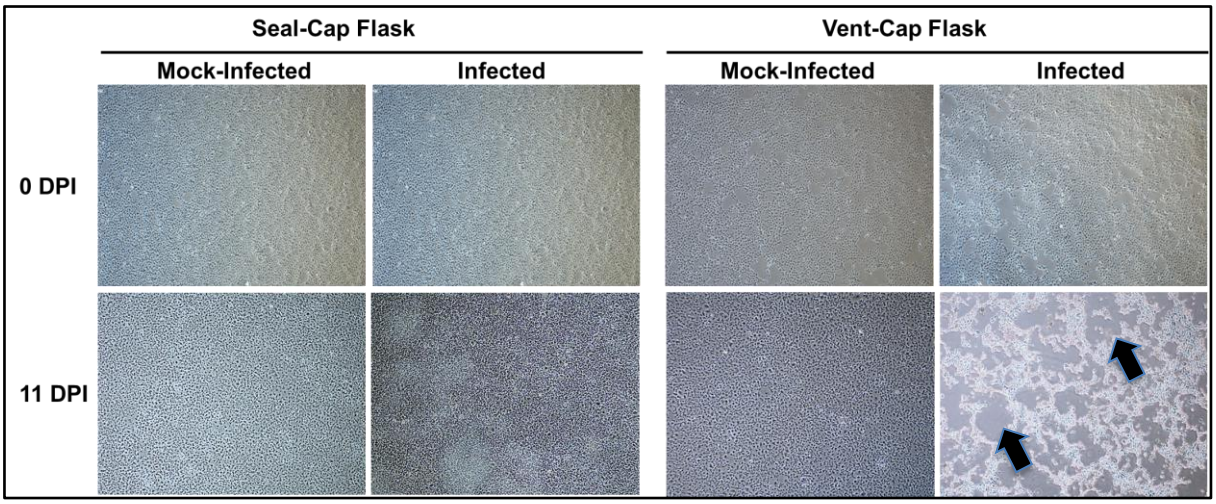

Figure 4. Cytopathic effects of Tilapia Lake Virus in E-11 cells under seal-cap and vent-cap conditions at 0- and 11- days post infection (dpi). Cytopathic changes characterised by rounding, aggregation, detachment and destruction of the monolayer compared to uninfected control (with arrows).

Molecular confirmation of TiLV presence

To confirm the presence of TiLV in infected E-11 cells, total RNA was extracted from infected and mock-infected E-11 cells, followed by cDNA synthesis and nested RT-PCR targeting TiLV segment 3. A distinct amplicon of approximately 250 bp was detected in infected E-11 cells from vent-cap flasks, corresponding to the expected size of the TiLV segment 3 gene. Sanger sequencing of the positive amplicons verified the presence of TiLV as shown in the Figure 5. Phylogenetic analysis revealed that the isolates used in this study has close genetic similarity with previously reported TiLV strains from Malaysia, Hong Kong and Israel. These findings confirm successful TiLV replication under vent-cap conditions, consistent with the observed cytopathic effects.

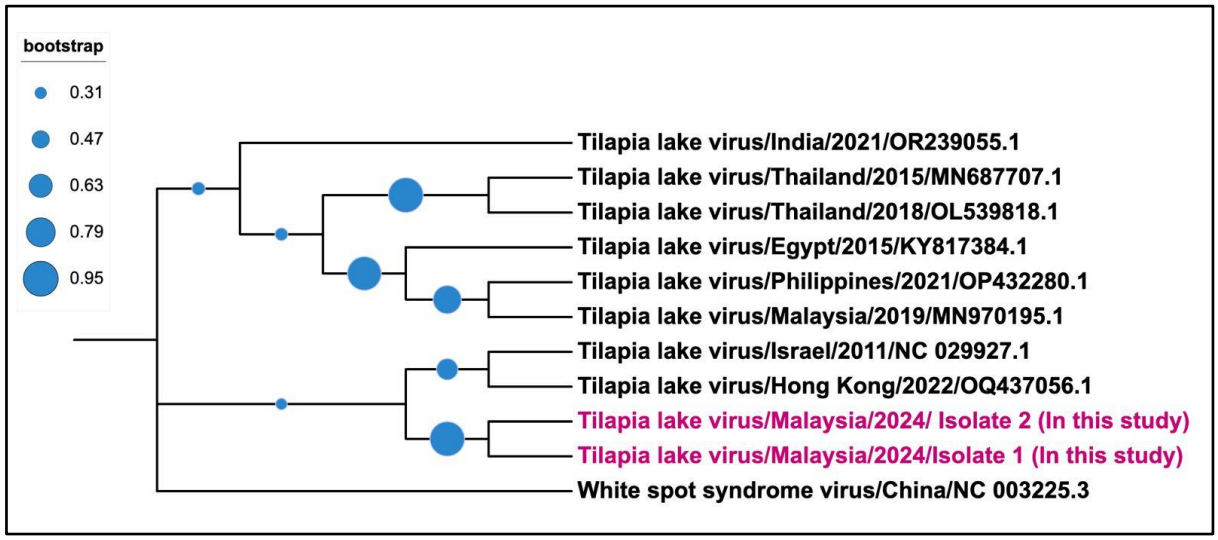

Figure 5: maximum-likelihood phylogenetic tree of Tilapia lake virus (TiLV) segment 3 gene sequences. The tree was constructed using segment 3 TiLV gene sequences obtained from GenBank together with two TiLV isolates generated in this study (shown in magenta). White spot syndrome virus (China/NC_003225.3) was included as an outgroup. Bubble sizes represent bootstrap support values, with larger circles indicating higher evolutionary relationship.

Conclusion

This study successfully established the optimised *in vitro* conditions for E-11 cells, contributing to improved experimental consistency for TiLV-related research. The findings revealed that a 15% FBS concentration supported the most effective proliferation of E-11 cells, ensuring consistent and healthy cell growth. Furthermore, the study demonstrated that flask ventilation plays a significant role in maintaining medium pH. While E-11 cells exhibited comparable growth and morphology under both vent-cap and seal-cap conditions, cytopathic effects and molecular detection of TiLv were observed only in vent-cap flasks. These findings suggest that adequate gas exchange supports conditions permissive for TiLV infection. Overall, this studyhighlights the importance of optimizing culture parameters, specifically serum concentration and ventilation conditions to support E-11 cell culture systems suitable for TiLV infection studies.

Acknowledgement

Author acknowledges the Ministry of Higher Education, Malaysia for funding under the Fundamental Research Grant Scheme (FRGS) to Universiti Teknologi MARA (Project no: FRGS/1/2022/STG01/UITM/02/2) and Higher Institution Centre of Excellence to Institute of Bioscience, Universiti Putra Malaysia (Project no: 5220001).

Author Contribution

NFWF,MAA,NZA,MNAA- Conceptualisation; NFWF,MAA,NZA- designated experiment; NFWF,MAA,NZA- Writing manuscript; NFWF- Conduct the experiment; MAA,NZA- Supervision.

Conflict of Interest

Authors declare no conflict of interest.

Declaration on the Use of Generative AI

The authors declare that generative artificial intelligence (AI), specifically ChatGPT (OpenAI), was used solely to assist in language refinement and rephrasing of the manuscript. The AI tool was not used for data analysis, interpretation, or generation of scientific content. All final content has been carefully reviewed and approved by the authors, who take full responsibility for the accuracy and integrity of the work.

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