# Host reverse transcriptase helps establish and maintain persistent dengue virus infections in C6/36 insect cells

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**Abstract**

The mechanisms involved in maintenance of persistent Dengue virus-2 (DEN-2) infections in C6/36 cells have not yet been reported. However, the persistence of RNA viruses in *Drosophila melanogaster* was achieved through the combined action of cellular reverse-transcriptase (RT) activity and RNA-mediated interference (RNAi). RT inhibition in C6/36 cells was achieved using tenofovir. Toxicity tests using tenofovir at 0.1, 0.25 and 0.5 mM revealed some cell death at 0.5 mM but only very limited effects at 0.1 mM. Normally, challenging naive *Aedes albopictus* C6/36 cells with Dengue virus-2 (DEN-2) results in relatively mild, transitory cytopathic effects (CPE), and 3-5 split passages result in a return to normal morphology and growth despite the presence of persistent, active infections of DEN-2 in all the cells. Here, it is shown that treatment with tenofovir prior to and after challenge with DEN-2 results instead in severe CPE and death (i.e., failure to establish a persistent infection). In addition, treatment of stable, grossly normal C6/36 cell cultures persistently-infected with DEN-2 induces severe CPE and death. The results indicate a key role of host RT in establishment and maintenance of stable, persistent DEN-2 infections. The underlying mechanisms are unknown, but this cell culture system should provide a good model for further studies to reveal them.

**Key words:** Dengue virus-2 (DEN-2); reverse transcriptase (RT); persistent infection; C6/36 cells; *Aedes albopictus* C6/36 cells;

## INTRODUCTION

Recent research results have proven that insects and crustaceans are capable of a specific, adaptive immune response to viral pathogens based on nucleic acids instead of antibodies (see reviews by (1, 2). This response often leads to active, persistent infections that can last a lifetime but result in no gross or histological signs of disease. Host generated reverse transcriptase (RT) acting on invading viral RNA is a key enzyme in this response and results in the production of viral copy DNA (vcDNA) in both linear (lvcDNA) and circular (cvcDNA) forms that can lead to both cellular and systemic responses based on RNA interference (RNAi) pathways that usually prevent viral disease but not infection.

Our previous work has shown that stable, grossly normal lines of persistently-infected *Aedes albopictus* C6/36 mosquito cell cultures can be produced in the laboratory with the DNA virus *Aedes albopictus* densovirus (*Aal*DNV) and the RNA viruses Dengue virus-2 (DEN-2) and *Japanese encephalitis* virus (JEV) as single, dual or triple infections by serial split-passage (3, 4). However, they underlying mechanisms leading to these persistent infections and their maintenance have not been fully revealed.

Based on the progress described for insects and crustaceans above, we hypothesized that establishment and maintenance of stable, persistent DEN-2 infections in the *Aedes albopictus* cell line (*Aal*-DEN) would be dependent on host generated RT. To test this hypothesis, we used the RT inhibitor tenofovir and predicted that its use would prevent the establishment of a stable *Aal*-DEN infected cell line and that it would also destabilize a previously established stable *Aal*-DEN infected cell line. Tenofovir is in a class of medication called a nucleoside reverse transcriptase inhibitor (NRTI) that functions as a defective adenosine nucleotide that interferences with RT activity.

## MATERIALS AND METHODS

### Insect cell lines and viral inoculum

*Aedes albopictus* C6/36 cells (ATCC, catalogue number CRL-1660) were grown in Leibovitz's (L-15) medium containing 10% heat-inactivated fetal bovine serum (FBS), 10% tryptose phosphate broth (TPB) and 1.2% antibiotic (Penicillin G and Streptomycin). Dengue serotype 2 virus (DEN-2) (NGC strain) used in this work was obtained from the US Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok through the courtesy of

Ananda Nisalax and was stored in 20% fetal bovine serum at -80ºC at the Division of Medical Molecular Biology, Office of Research and Devleopment, Faculty of Medicine, Siriraj Hospital, Mahidol, University, Bangkok. After thawing at room temperature, the stock was used as inoculum for monolayers of naive C6/36 cells in Leibovitz's (L-15) medium containing 1% heat-inactivated fetal bovine serum (FBS), 10% tryptose phosphate broth (TPB) and 1.2% antibiotic (Penicillin G and Streptomycin). At days 5-7 after challenge, the supernatant solution was removed and used as inoculum for subsequent trials.

**Naive C6/36 cells challenged with Dengue virus**

Culture plates (6-well, Costar, Corning) were seeded with C6/36 cells at a density of $10^6$ cells/well and incubated for 24 h at 28ºC to produce confluent monolayers. The cell monolayers were then challenged with DEN-2 at a multiplicity of infection (MOI) of 0.1. After incubation for 2 hours with gentle shaking at room temperature, the medium was removed and fresh medium containing 2% FBS was added for further incubation at 28°C.

**Persistent infection of C6/36 cells with Dengue virus**

Persistent infections of DEN-2 in C6/36 cells were achieved in 6-well plates as previously described (4). Briefly, after 2 days incubation post DEN-2 challenge (acute infections in C6/36 cells), the supernatant solution was removed, and cells were suspended by knocking in L-15 containing 10% FBS at 1:3 dilution and transferred to a new culture well at ½ density for 2-days cultivation (to full confluence) before repeating the decantation, suspension, dilution and transfer process sequentially at 2 day intervals to establish persistently infected cultures. Three replicates were done in the 6 well plates at 2 day intervals. Mock-infected cells were run in parallel to the viral infected cells to serve as negative controls.

**Toxicity test for Tenofovir**

Tenofovir as a nucleoside reverse transcriptase inhibitor in purified chemical powder form was obtained from the Thailand Government Pharmaceutical Organization. Toxicity tests were evaluated by cell viable count. Monolayers of C6/36 cells were treated with tenofovir at the concentrations of 0.1, 0.25 and 0.5 mM in culture media for 24 hours. The concentration used was calculated from the manufacturers recommend dose for human use. After 24 Hours

treatment with tenofovir, culture media were removed and viable cell counts were determined by trypan blue staining and a hemocytometer.

**Inhibition of reverse transcriptase at acute DEN-2 infection**

Monolayers of C6/36 cells were treated with tenofovir at different concentrations as described above for 24 hours. The treated monolayers were then challenged with DEN-2 at a multiplicity of infection (MOI) of 0.1. After incubation for 2 hours with gentle shaking at room temperature, the medium was removed, washed 2 times with PBS and fresh medium containing 10% FBS was added for further incubation at 28°C. After 2 days incubation post DEN-2 challenge (acute infections in C6/36 cells), the supernatant solution was removed and cells were suspended and transferred to a new culture well to establish persistently infected cultures. The morphology of whole culture was observed under light microscope and DEN-2 copy number was measured by real-time RT PCR.

**Inhibition of reverse transcriptase in cultures persistently infected with DEN-2**

The persistently DEN-2 infected cultures were established by serial passage as described above. Monolayers of C6/36 cells persistently infected for 30 passages were treated with various concentrations of tenofovir for 48 hours. The supernatant solution was then removed and cells were suspended and transferred to a new culture well to establish a new passage. The morphology of whole culture was observed under the light microscope and DEN-2 copy number was measured by real-time RT PCR.

**One step real-time RT-PCR**

Culture media without cells from acute or DEN-2 persistently infected cultures were collected for RNA extraction using an RNA extraction kit (Qiagen extraction kit). DEN-2 copy number was counted by one step real time RT-PCR. Primer D2S and D2C were used (Table 1). The standard curve of DEN-2 copy number was prepared by serial dilution of a plasmid containing the dengue virus envelope gene. Each treatment was compared by gene copy number. One step real time RT-PCR was performed according to the directions supplied by the manufacturer (KAPA SyBR fast qPCR). The annealing temperature was 55°C.

**Table 1.** Primers for real-time RT-PCR designed from the envelope region of dengue virus.

| Organism | Gene | Primer name | Sequence (5'-3') | Product size |
|---|---|---|---|---|
| Dengue virus 2 | Envelope | D2S | GTTCGTCTGCAAACACTCCA | 230 bp |
| | | D2C | GTGTTATTTTGATTTCCTTG | |

## RESULTS AND DISCUSSION

### Tenofovir has relatively low toxicity for C6/36 cells

After 24 hours of normal, naïve C6/36 cell treatment with tenofovir at the concentrations of 0.1, 0.25 and 0.5 mM, the number of viable cells decreased and cytopatic effects (CPE) increased in the treatment groups in a dose dependent manner **(Figs. 1 & 2)**. However, the effect of tenofovir was not severe and none of the selected concentrations reached a median lethal dose (LD50) for the C6/36 cell culture.

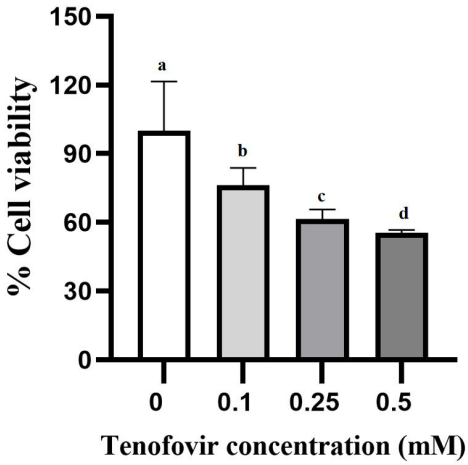


**Figure 1.** Effect of tenofovir on cell viability in C6/36 cells. Cells were incubated with different concentration of tenofovir for 24 h following by cell viability measurement. Baseline cell viability in the control wells not exposed to tenofovir was set at 100%. Data were expressed as percentage of control. Bars with different letters were significantly different ($p<0.05$) from one another by one-way ANOVA

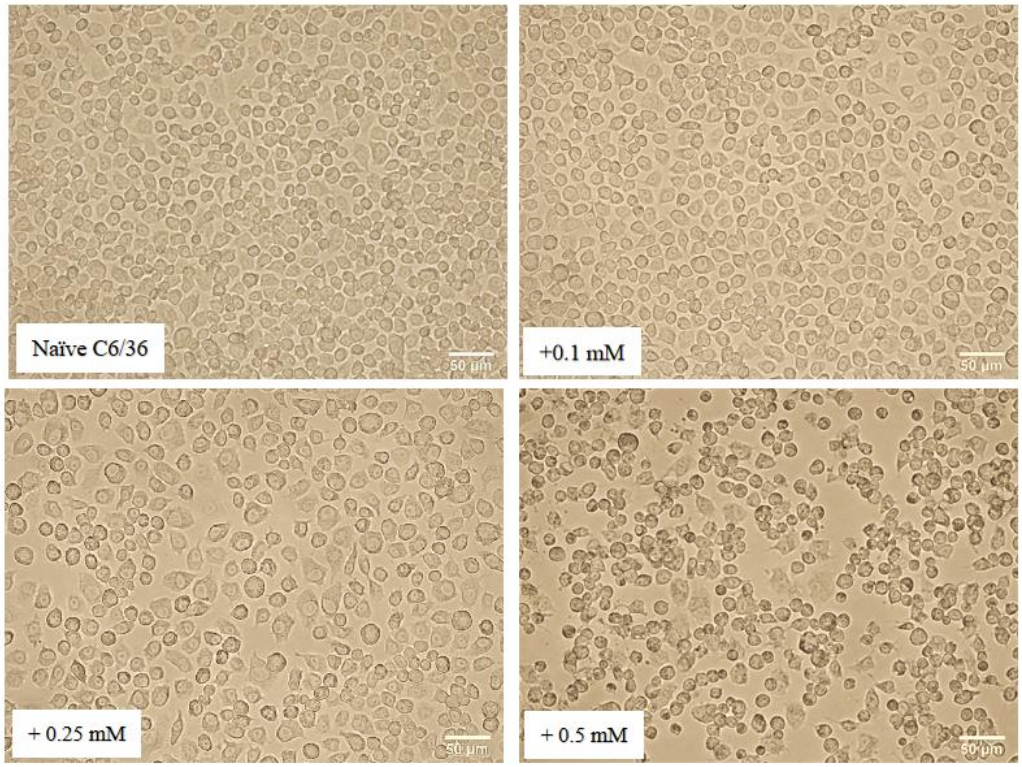


**Figure 2** C6/36 cells morphology after treatment with tenofovir for 24 h (upper left) untreated cells, (upper right) treated with 0.1 mM, (lower left) 0.25 mM and (lower right) 0.5 mM.

**RT inhibition increases DEN-2 severity and prevents persistent infections**

When monolayers of naïve C6/36 cells were treated by tenofovir for 24 hours followed by DEN-2 challenge, it was found that the DEN-2 numbers increased with increasing concentration of tenofovir **(Fig 3)**. The highest count was 9.60 $x10^6$ copies/µl at 0.5 mM while that in the untreated group was $3.05x10^6$ copies/µl. The results indicated that cellular reverse transcriptase activity correlated with a reduced level of viral replication.

When we attempted to established DEN-2 persistent infections in C6/36 cell cultures using our normal split passage protocol in the presence and absence of tenofovir, we found differences in morphology and viable cell numbers between the treated and untreated cells from the first passage. The viable cell numbers were lower in the tenofovir treatment group. By passage 3, the number of cells had rapidly declined in the tenofovir treatment groups and cytopathic effects were severe **(Fig 4)**. Attempts to establish persistently infected cells failed

in all the tenofovir treatment groups and only succeeded, as usual, in the untreated control group.

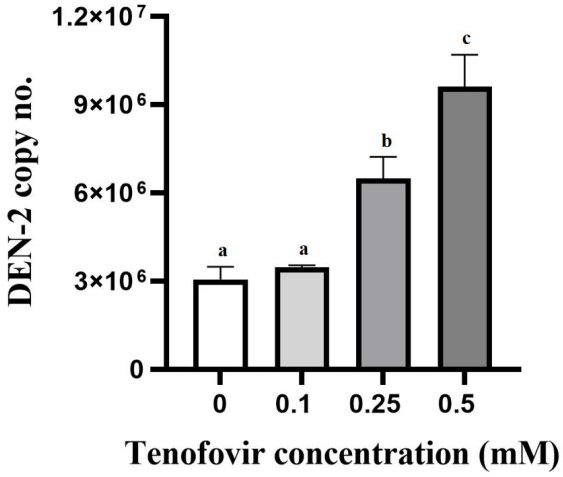


**Figure 3.** DEN-2 copy number in culture supernatant of C6/36 cells treated or not with tenofovir at different concentrations. Bars with different letters were significantly different ($p \leq 0.05$) from one another by ANOVA.

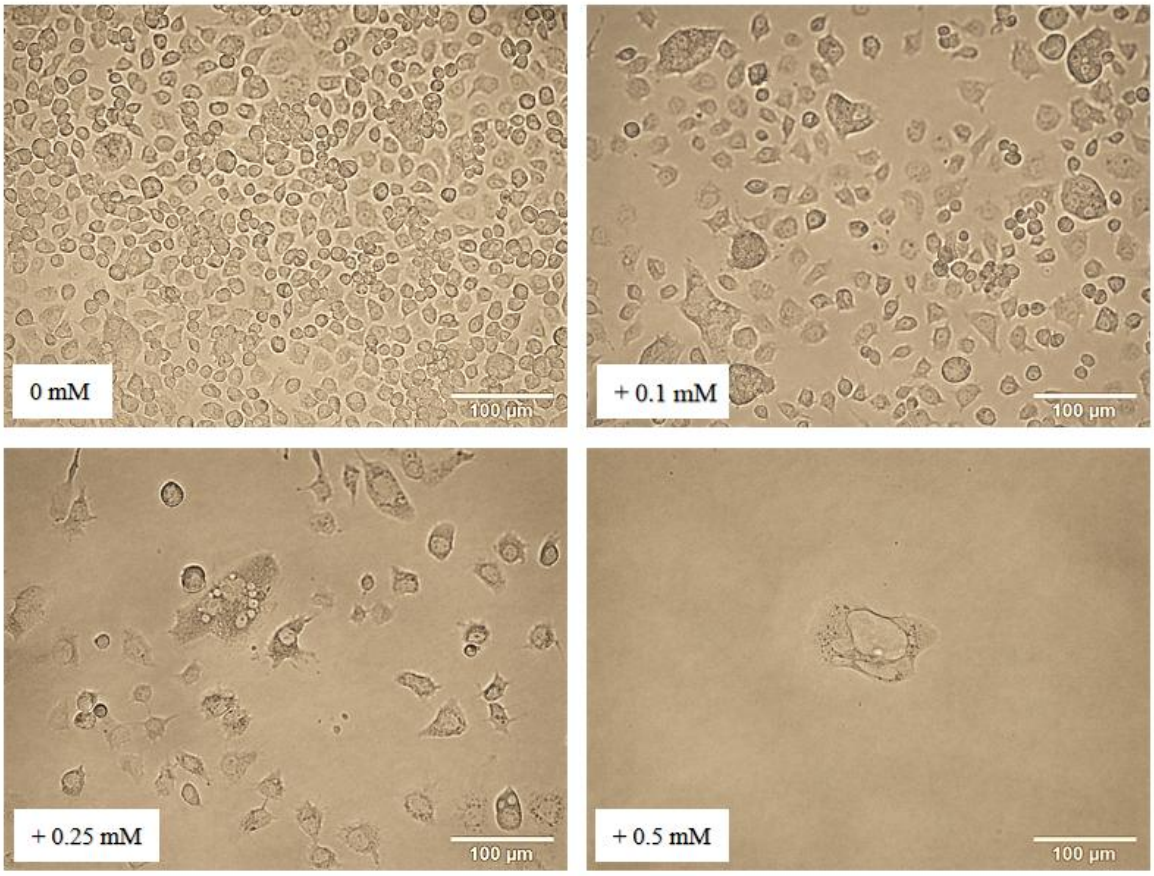


**Figure 4.** Morphology of DEN-2 infected C6/36 cells at passage 3 in media containing different tenofovir concentrations.

**Tenofovir treatment destabilizes persistently infected cell cultures**

Persistent infection of dengue virus in C6/36 cells was established by split-passage until passage 30. The cell morphology in the persistently infected cultures was similar to that in uninfected naive cell cultures. By 48 hours, all treatments exposed to tenofovir showed cytopathic effects with severity dependent on the tenofovir concentration while the untreated DEN-2 infected cultures remained normal (**Fig.5**). In cultures treated with 0.5 mM tenofovir, the cell layer was ruined and there were no surviving cells. The results suggested that reverse transcriptase was an essential element in the maintenance of the persistent DEN-2 infections. Moreover, DEN-2 replication increased in the presence of tenofovir. The DEN-2 copy number with 0.1 mM tenoforvir treatment (7.64 $x10^6$ copy) was higher than that in the untreated persistent infections (3.04 $x10^6$ copy) in same passage (**Fig 6**). The number of surviving cells with 0.25 and 0.5 mM tenoforvir treatment was very low, and this resulted in lower DEN-2 copy numbers than with 0.1 mM tenoforvir.

We can conclude the reverse transcriptase activity correlated with the ability of C6/36 cells to tolerate DEN-2 in persistent infections. Thus, the activity of host reverse transcriptase may be critical in allowing natural mosquitoes to maintain persistent DEN-2 infections.

We have shown that the development and maintenance of stable cultures of C6/36 cell lines persistently infected with DEN-2 is correlated with the activity of host derived RT. Recent reports from insects and crustaceans suggest that RNAi mechanisms may be involved. However, it is known that C6/36 cells lack a functional Dicer 2 (Dcr2) enzyme(5, 6), so if the RNAi pathway is involved in the ability to tolerate both DNA and RNA viurses in persistent infections, then it must be via a mechanism that does not involve Dcr2. Thus, C6/36 cells may provide a good model for further studies on the detailed mechanisms related to this RT requirement and how it prevents the cells from entering a diseased state despite the persistent presence of DEN-2.

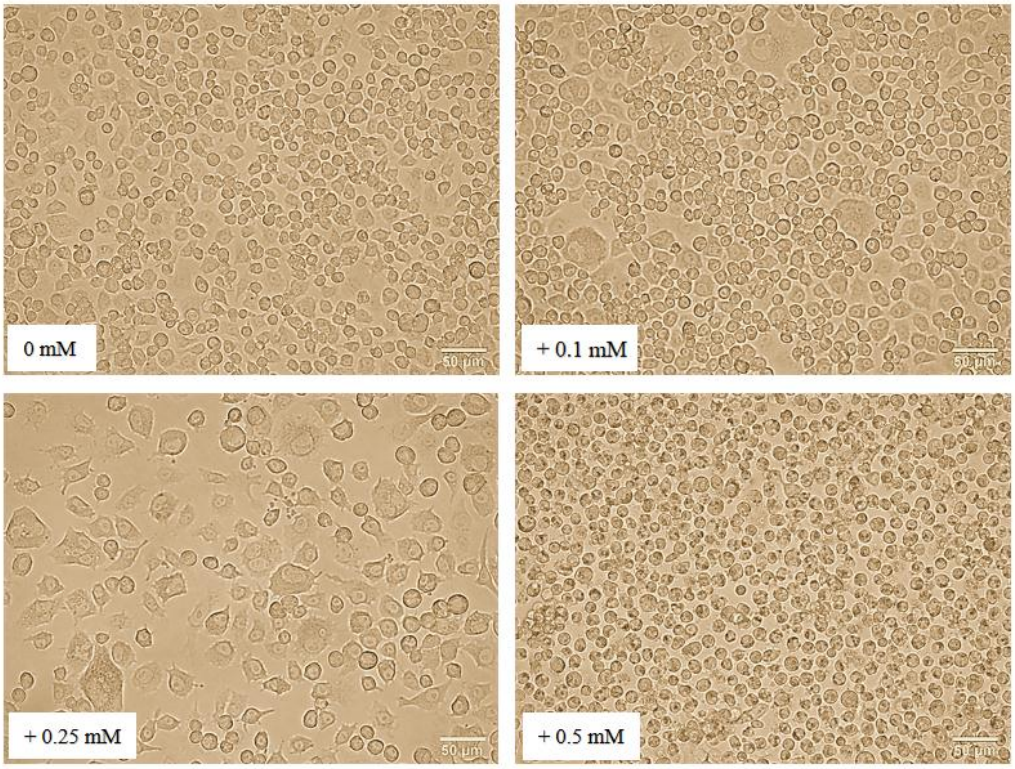


**Figure 5.** Cell morphology at 48 hr after C6/36 cell cultures persistently-infected with DEN-2 for 30 passages were treated or not with tenofovir. Untreated persistently infected cells (upper left) and persistently infected cells treated with 0.1 mM tenofovir (upper right). Persistently infected cells treated with 0.25 mM tenofovir (lower left) and persistently infected cells treated with 0.5 mM tenofovir (lower right).

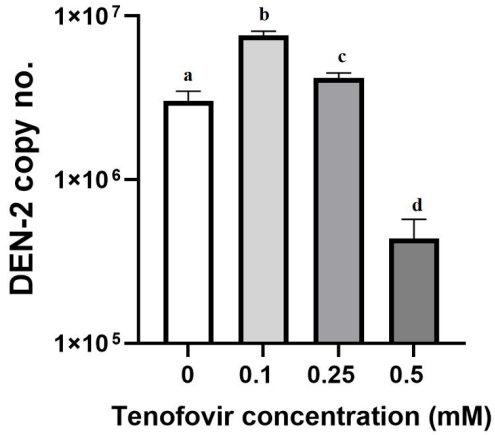


**Figure** 6. Copy numbers of dengue virus in the supernatant from C6/36 cell cultures at passage 30 treated on not with tenofovir at different concentrations. Bars marked with different letters are significantly different ($p \leq 0.05$) by ANOVA.

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