COMPARISON BETWEEN PLAQUE ASSAY AND TCID$_{50}$ FOR QUANTITATING ZIKA VIRUS

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Abstract
A widespread epidemic of Zika virus (ZIKV) and the increased cases of microcephaly in fetuses of mothers who were infected during pregnancy are a global health concern. To undertake effective research on this virus optimization of techniques to quantitate infectious virus in an experimental setting is required. Initial study showed that Zika virus infection of rhesus monkey LLC-MK2 cells produced significant cytopathic effects, indicating these cells are suitable for use in quantitation methods. This study therefore investigated the utility of the 50% cell culture infectious dose (TCID$_{50}$) endpoint dilution assay, as compared to standard plaque assay using LLC-MK2 cells. In the absence of specific protocols for ZIKV, the conditions previously establish for dengue virus were used. However, when serial dilutions of the virus were used to infect monolayers of LLC-MK2 cells, small and uncountable pinpoint plaques were observed, suggesting this methodology requires significant optimization to be useful for ZIKV. For TCID$_{50}$, cell monolayers were again infected with serial dilutions of ZIKV, and on day 3 post infection cytopathic effects were assessed by microscopy. Clear cytopathic effects were seen in LLC-MK$_2$ cells, with a calculated TCID$_{50}$ of $3.62 \times 10^4$ TCID$_{50}$/ml. The two quantitation methods evaluated here will serve as a base for future experimental investigation into the newly emerging pathogen, Zika virus.

Keywords : Zika virus, titer, plaque assay, TCID$_{50}$

Introduction :
Zika virus (ZIKV) is a mosquito-transmitted virus that belongs to the family Flaviviridae and genus Flavivirus. The viral genome is a positive-sense, single-stranded RNA of 10,794 bases in length that contains 5’ and 3’ untranslated regions flanking a single open reading frame coding one polyprotein that is cleaved into 3 structural proteins which are the capsid, premembrane/ membrane and envelop, and 7 nonstructural proteins which are NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Kuno et al., 2007). Initially, ZIKV was isolated from the blood of a sentinel rhesus monkey placed in the Zika forest of Uganda in 1947. A second isolation from a pool of Aedes africanus mosquito followed at the same site in 1948 (Lanciotti et al., 2008). Two major ZIKV lineages, Asia and Africa, have been identified through phylogenetic analysis. ZIKV infectious cases were reported in Africa, Asia, and large outbreaks in the Pacific. Also, there were 7 cases of acute ZIKV infection in Thai residents and 2 cases reported in travelers returning from Thailand. (Buathong et al., 2015). However, there are no vaccines or therapeutics that are effective against the diseases caused by ZIKV. Before any ZIKV study, the determination of viral titer or infection level is required. Two of the more common approaches for virus quantitation are plaque assay and endpoint dilution.
assays such as tissue culture infective dose (TCID$_{50}$) assay. Both assays rely on the ability of viruses to cause cytopathic effects (CPE) in host cells as a result of infection. The direct quantitative measurement of plaque numbers is formed by various viral dilutions and perform as the number of plaque-forming units (PFU). The TCID$_{50}$ assay is an example of an endpoint dilution assay that determines the point at which 50% of cells in vitro are infected and provides a qualitative measurement of the ratio between uninfected and infected cells (Reed and Muench, 1938). Although these two methods are widely used in virology-based study but the comparison between both methods for ZIKV quantitation has not been studied. In this study, Rhesus monkey kidney (LLC-MK2) cells were infected with various dilution of ZIKV. The ZIKV quantitation was performed by plaque assay and TCID$_{50}$ assay. For plaque assay, infected LLC-MK2 cells were performed in the same condition as Dengue virus and the viral titer was determined on 7 days post-infection (dpi). While, TCID$_{50}$ assay was performed on 3 dpi and the ZIKV titer from both assays was compared to determine the correlation between the results of the two assays.

Methodology:

Cell culture
Rhesus monkey kidney (LLC-MK2) cells were cultured and maintained in Dulbecco’s modified eagle medium (DMEM; Gibco, Invitrogen, Carlsbad, CA) supplemented with 100 units/ml penicillin and 5% heat-inactivated fetal bovine serum (FBS; Gibco, Invitrogen).

ZIKV stock propagation
Zika virus (ZIKV) strain SV0010/15 in this study obtained from Armed Forces Research Institution of Medical Sciences (AFRIMS) and The Development of Disease Control, Ministry of Public Health, Thailand. The viral stock was propagated in the monolayer of Aedes albopictus cell line (C6/36), then incubated at 28°C for 2 h with constant agitation. Fresh culture medium was added and cells were incubated further until cytopathic effects (CPE) occurred. After the presence of CPE, the medium was taken and centrifuged at 1,000xg for 5 min at room temperature. After remove cell debris, heat-inactivated FBS was added to a final concentration of 20% (v/v) FBS. The supernatant was kept at -80°C as a virus stock.

RNA extraction
Viral RNA was extracted from ZIKV stock by using TRI reagent solution. 250 µl of stock ZIKV was mixed with 750 µl of Trizol reagent solution and incubated at room temperature for 5 min. Chloroform was added into the solution with agitation for 15 seconds, then incubated at room temperature for 15 min. After that, the solution was centrifuged at 12,200 x g at 4°C for 5 min to separate solution into 3 phases. The upper phase containing RNA was transferred into a new micro-centrifuge tube and then added isopropanol to precipitate RNA at -30°C for overnight. Next, the solution was centrifuged under the same condition. The RNA pellet was washed with 75% ethanol and centrifuged again. The tube was left at room temperature to dry the pellet, then dissolved by DEPC water and kept at -80°C.

RT-PCR and ZIKV sequencing
cDNA was prepared by incubating 5 µl of viral RNA, 1 µl of reverse ZikaFront primer positions 2479-2499 (5’ TAAGGCCAAGCACATAAGGGA 3’) of the ZIKV genome and 6 µl of MilliQ water at 65°C and 94°C for 5 and 2 min respectively and then rapidly cooling on ice. The reaction mixture was prepared by 5X reaction buffer, RNase out, 10mM dNTP and revertAid reverse transcriptase. The viral RNA solution was then mixed with 8 µl of the mixture solution. cDNA was synthesized in PCR Thermocycler (Veriti) using the following
procedure: 1 cycle at 25°C for 9 min 32 sec, 1 cycle at 55°C for 90 min and 1 cycle at 85°C for 5 min. After reverse transcription, polymerase chain reaction (PCR) was performed by preparing 19 µl of the reaction mixture [MilliQ water 11.6 µl, 5X HF *phusion* buffer (Thermo Scientific), 10 mM dNTP 0.4 µl, forward ZikaFront primer positions 1150-1170 (5' CTA CGT AAT GA GGCA TC 3') 0.6 µl, reverse ZikaFront primer 0.6 µl and *Phusion* DNA polymerase enzyme (Thermo Scientific)] with 1 µl cDNA. Thermocycling program was 1 cycle at 98°C for 30 secs, 35 cycles at 98°C for 10 sec, 60°C for 30 sec, 72°C for 1 min and 1 cycle at 72°C for 5 min. The ZIKV PCR products were visualized on 1% ethidium bromide stained agarose gel. Before sequencing, the PCR products was purified by FavorPrep™ kit (Favorgen). The ZIKV was sequenced by Macrogen Inc. (Korea) and then analyzed the sequence by BLAST analysis with National Center of Biological Information (NCBI) database.

**ZIKV titration by standard plaque assay**

In the absence of specific protocols for ZIKV, the conditions previously establish for dengue virus were used. Plaque titration was performed on LLC-MK2 cells. 200 µl of ten fold serially diluted (10^{-1}-10^{-6}) of ZIKV in BA-1 medium was added in to each well of 6 well plates containing confluent monolayer of LLC-MK2 cells. Then, the plates were incubated at 37°C for 2 h with constant agitation to initiate virus infection to the cells. During this period, the overlay solution was prepared. 2X-nutrient solution (20X Earle’s balanced salt solution, 6.6% yeast extraction, 25 mg of gentamycin sulfate, 6% FBS, 7.5% NaHCO₃ and adjust the volume by MilliQ water) was incubated at 37°C until used. Autoclaved 1.6% (w/v) Seakem LE agarose in water was melted. After 2 h, each well were gently overlaid with 2X-nutrient solution and 0.8% (w/v) Seakem LE agarose in water (1:1). Infect cells were maintained at 37°C, 5%CO₂ for 7 days. On day 7, the cells were fixed by 200 µl of 37% formaldehyde for at least 20 min. The agarose overlay was removed and stained the plaques with 1% crystal violet solution. Next, the crystal violet was rinsed off with water. Finally, the plaques were counted and calculated in plaque forming unit per ml (PFU/ml).

**ZIKV titration by 50% tissue culture infectious dose (TCID₅₀)**

LLC-MK2 cells were grown in 96-well tissue culture plates under standard condition. When the cells were confluent, the medium was removed and the cells were infected with 50 µl of six different 10-fold dilution (10^{-1}-10^{-6}) of ZIKV stocked virus in DMEM without FBS and incubated for 2 h to initiate virus infection to cells. After 2 h of viral absorption period, 50 µl of DMEM containing 5% FBS was added into each well in the 96-well plates and then incubated at 37°C until required. The cytopathic effect in the wells inoculated with virus dilutions were then observed under an inverted microscope from day 1 to 5 post-infection. The titer was also calculated by the method of Reed and Muench. All experiments were undertaken independently in duplicate.

**ZIKV titration by the enzyme-linked immunosorbent assay (ELISA)**

The preparation of LLC-MK2 cells was similar to TCID₅₀ but incubated cells at 37°C for 7 days. On day 7, the media was removed and washed with 1X PBS for 4 times. Cells were then fixed with 3.7% formaldehyde at room temperature, overnight. To avoid unspecific binding proteins, 5% skim milk was added into each well and incubated at 4°C for 1 h. After that, 5% skim milk was removed and washed with 1XPBS again. Diluted mouse anti-*flavivirus* specific antigen antibody(HB112) at 1:100 in 1X PBS was added and incubated at 4°C, overnight. Next day, primary antibody was removed and washed with 1X Tris-buffer saline with Tween 20 (TBST) for 4 times. Cell were then incubated with the
diluted Horseradish Peroxidase (HRP) conjugated rabbit anti-mouse IgG antibody (1:5500) in 1% skim milk and incubated at 4°C for 1 h. The mixture of 3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate was prepared by peroxidase substrate solution and TMB peroxidase solution (1:1). After 1 h, antibody was removed and cells were then incubated with the mixture of TMB substrate in the dark for 20 minutes. A soluble yellow product presented and the absorbance at OD 450 nm was recorded by the microplate reader.

**Results:**

**Confirmation of ZIKV**

In order to confirm the virus in this study was ZIKV, we extracted RNA and RT-PCR was performed to synthesize cDNA by using forward ZikaFront primer and reverse ZikaFront primer. The results show that ZIKV PCR products size are 1,349 bp (Figure 1). As a further confirmation, the 1,349 bp band were sequenced (Figure 2) and showed 99% nucleotide similarity with ZIKV isolate ESS23-2015 envelope protein gene (KX216638) by BLAST analysis.

![Figure 1](image_url)  
*Figure 1* Agarose gel electrophoresis of PCR products of ZIKV using RT-PCT. The PCR products show a single sharp band at 1,349 bp. M; molecular weight marker (Generuler ladder mix marker, Thermo Fisher Scientific), + ; PCR product of ZIKV, - ; negative control.
Figure 2 The ZIKV sequencing result (A) which was matched with ZIKV isolate ESS23-2015 envelope protein gene (KX216638) with 99% nucleotide similarity (B).

The cytopathic effect of ZIKV-infected cells.

LLC-MK2 cells were infected with ZIKV with various MOI and multiple post-infection incubation periods. Mock LLC-MK2 cells served as the controls without ZIKV infection which were cultured in parallel to the ZIKV-infected cells. From figure 3, the morphology of the ZIKV-infected LLC-MK2 cells showed the dramatic morphological changes, also known as cytopathic effects, including cell rounding, detachment, and formation of large vacuoles in the cytoplasm (Figure 4). These CPE were more obvious when the MOI and post-infection duration were greater comparing to mock-control cells.
**Figure 3** Morphology of the ZIKV-infected cells with varying MOI at multiple post-infection time points. The ZIKV-infected LLC-MK2 cells showed the dramatic morphological changes (the arrow) and the number of cell death increased with higher MOI and longer post-infection duration. Images were taken under inverted microscope with a magnification of x20.

**Figure 4** The morphology changes of the ZIKV-infected LLC-MK2 cells with MOI 1 at 72 h.p.i. (A) which shows significant cytopathic effects, including cell rounding, detachment, and formation of large vacuoles in the cytoplasm comparing with mock-control cells (B).

**Determination of ZIKV titers**

To evaluate the ability of LLC-MK2 cells that to produce viral progeny in vitro by determining viral titers in the ZIKV-infected LLC-MK2 cells, using a standard plaque assay. The plaque morphology of ZIKV-infected LLC-MK2 cells were too small which are difficult to count the plaque number. Roughly counting of plaque number at the dilution $10^{-5}$ of ZIKV stock was $1.5 \times 10^7$ plaque forming units (PFU)/ml on the day 7(Figure 5). Due to the unclear plaque morphology of ZIKV in the standard plaque assay, we further determined the ZIKV titer by TCID$_{50}$ method. The results show a gradual increase of the ZIKV-infected LLC-MK2 cell which was stained with crystal violet with an increase of ZIKV dilution. At the dilution $10^{-1}$, it showed the highest efficiency of ZIKV infection due to the minimal of living cells in the well. To optimize the suitable day for TCID$_{50}$ method, we selected day 3 as the optimal day due to the confluent monolayer of cells and a significant cytopathic effects (CPE) such as cell death and morphological changes can be seen on these day (Figure 6).

**Figure 5** The plaque morphology of ZIKV-infected LLC-MK2 cells from the viral dilution $10^{-1}$ to $10^{-6}$. Too small and uncountable pinpoint plaques were observed in ZIKV-infected LLC-MK2 cells (A) whereas the plaque of DENV-infected LLC-MK2 cells under the same conditions provided the sharp, clear-border and large plaque size.
According to the method of Reed and Muench, the number of wells with and without CPE were counted and the TCID\textsubscript{50}/ml from day 1 to day 5 were calculated. The graph shows that there were no cytopathic effects on day 1. Then, ZIKV titers increased from \(3.7 \times 10^4 \pm SD 1531.6\) TCID\textsubscript{50}/ml on 2 d.p.i. to \(5.7 \times 10^4 \pm SD 8691.4\) TCID\textsubscript{50}/ml on 4 d.p.i.. However, there were more than 50% of infected cells resulting in unable calculation on 5 d.p.i. (Figure 7). Furthermore, ELISA was also performed to optimized the viral titer, the graph shows an increase of the absorbance at OD 450 nm which represented the viral titer from the dilution \(10^{-1}\) to \(10^{-4}\). At the dilution of \(10^{-5}\), the viral titer began to decrease but still presented in the final dilution at \(10^{-6}\) (Figure 8).

**Figure 6** The optimal day for TCID\textsubscript{50} method on 3 d.p.i. was selected due to the confluent monolayer cell and a significant CPE which were cell death and morphology changing.

**Figure 7** The determination of ZIKV titer in ZIKV-infected LLC-MK2 cells by TCID\textsubscript{50} method. On 3d.p.i., the ZIKV titer was \(3.62 \times 10^4 \pm SD 1531.6\) TCID\textsubscript{50}/ml but there were more than 50% of infected cells resulting in unable calculation after 4 d.p.i..
The determination of ZIKV titer in ZIKV-infected LLC-MK2 cells by ELISA method. The ZIKV titer was found from the dilution of $10^{-1}$ to $10^{-6}$.

**Discussion and Conclusion:**

Recently, there are numerous methods for viral titer quantitation but plaque assays continue to represent the gold standard in determining viral concentrations for infectious varions. From the results, ZIKV-infected LLC-MK2 cells provided too small and uncountable pinpoint plaques which can not optimize the viral titers. There are numerous factors in obtaining clear and large plaques such as appropriate host cell selection, proper media and suitable growth conditions for cellular and viral viability, immobilized viral propagation and an accurate determination of the viral incubation period to allow adequate time for distinct and countable plaque formation (5,6). Plaque size, clarity, border definition, and distribution should all be noted as they can provide valuable information on the growth and virulence factors of the virus in question. Therefore, the development of plaque assay for determine the Zika viral titer is required to serve as a base for further experiment.

To overcome the difficulties in ZIKV- uncountable plaques, the TCID$_{50}$ which is the endpoint dilution assay was used to measure the virus titer for the viruses that do not produce plaques. The important role in TCID$_{50}$ assay is the quality of the CPE because the infected cells were difficult to visualize. ZIKV produce significant CPE in infected-LLC-MK2 cells such as cell rounding, detachment and large vacuole formation within cytoplasm comparing to mock-uninfected cells. Also the number of cell death was obvious when the dilution of virus was reduced. Furthermore, the calculation used to determine the TCID$_{50}$ is based on the Reed and Muench formula (4), which is more complicated than the formula that used to determine PFU/ml in the plaque assay. Although the TCID$_{50}$ calculation may be complicated, there are online tools available to facilitate this calculation. In conclusion, despite the differences between the assays discussed above, it is considered that the difference between ZIKV titers obtained from TCID$_{50}$ assay and plaque assay will be a valuable piece of data for use in the future ZIKV research in the current collaborative environment.

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References


