Video Article

Zika Virus Infectious Cell Culture System and the *In Vitro* Prophylactic Effect of Interferons

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Abstract

Zika Virus (ZIKV) is an emerging pathogen that is linked to fetal developmental abnormalities such as microcephaly, eye defects, and impaired growth. ZIKV is an RNA virus of the *Flaviviridae* family. ZIKV is mainly transmitted by mosquitoes, but can also be spread by maternal to fetal vertical transmission as well as sexual contact. To date, there are no reliable treatment or vaccine options available to protect those infected by the virus. The development of a reproducible, effective Zika virus infectious cell culture system is critical for studying the molecular mechanisms of ZIKV replication as well as drug and vaccine development. In this regard, a protocol describing a mammalian cell-based *in vitro* Zika virus culture system for viral production and growth analysis is reported here. Details on the formation of plaques by Zika virus on a cell monolayer and plaque assay for measuring viral titer are presented. Viral genome replication kinetics and double-stranded RNA genome replicatory intermediates are determined. This culture platform was utilized to screen against a library of a small set of cytokines resulting in the identification of interferon-α (IFN-α), IFN-β and IFN-γ as potent inhibitors of Zika viral growth. In summary, an *in vitro* infectious Zika viral culture system and various virological assays are demonstrated in this study, which has the potential to greatly benefit the research community in elucidating further the mechanisms of viral pathogenesis and the evolution of viral virulence. Antiviral IFN-alpha can further be evaluated as a prophylactic, post-exposure prophylactic, and treatment option for Zika virus infections in high-risk populations, including infected pregnant women.

Video Link

The video component of this article can be found at https://www.jove.com/video/54767/

Introduction

Zika Virus (ZIKV) is an important human pathogen associated with microcephaly and poor pregnancy outcomes[1,4]. ZIKV belongs to the set of medically relevant flaviviruses that can cause neurological defects such as the Dengue, West Nile, and St. Louis encephalitis viruses. The main mode of viral transmission is by the mosquito vector *Aedes aegypti*, and, in addition, sexual transmission has also been reported[5,6]. ZIKV has become a major global health issue due to the expanding geographical distribution of the mosquito vector and its strong correlation with birth defects. ZIKV was first isolated in 1947 from a sentinel rhesus monkey in the Zika forest, Uganda and the first human case was reported in 1952[7,8]. Individuals that become infected with ZIKV present with mild symptoms such as fever, rash, headache, conjunctivitis, and muscle/joint pain. Infected pregnant women can transmit ZIKV to the developing fetus[1]. ZIKV infection has also been linked to Guillain-Barre syndrome, a peripheral nerve auto-immune demyelination disorder[9].

The Zika viral genome consists of a positive sense, single-stranded RNA molecule which is about 10.8 kilobases in length. The genome's structure is organized as 5'NCR-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-2K-NS4B-NS5-3'NCR, with non-coding regions (NCR) flanking a protein-coding region[6]. A single polyprotein (3,419 aa) is translated that is co- and post-translationally cleaved into 10 smaller peptides. Both the 5'NCR and 3'NCR RNA stem-loop structures play a critical part in the commencement of viral genome translation and replication. The structural components of the genome are comprised of the capsid, membrane, and envelope proteins. The non-structural proteins are critical for genome replication.

Currently, Zika viral strains are grouped into three main genotypes: West African, East African, and Asian[6,10-13]. It has been proposed that the East African lineage spread to West Africa and Asia, where it later further evolved[12]. The Asian genotype is responsible for the current outbreaks in the Americas. Zika virus can be cultured in both mosquito and mammalian cells. Primary dermal fibroblasts, immature dendritic cells, cortical neural progenitor cells, and Vero cells are susceptible to Zika viral infection[10,14,15]. Both type I and type II interferons have been shown to restrict ZIKV growth in skin fibroblasts[15]. The objectives of this study are to provide a step-wise, detailed protocol for the production and assaying of the Asian genotype ZIKA viral strain PRVABC59 in a mammalian cell culture system and to demonstrate the utility of this infectious culture system

as a drug development platform. This resource has the potential to greatly benefit the Zika viral and neurological research community to further elucidate its mechanisms of viral pathogenesis and evolution of viral virulence.

Protocol

Note: A schematic outline of the work flow is presented in **Figure 1**.

1. Cells

1. Use Vero cells for Zika virus production and analysis of viral replication cycle.
2. Prepare complete growth media containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 units/ml), and 10 mM HEPES.
3. Culture Vero cells with the specified complete growth medium at 37 °C with 5% CO_2.

2. Zika Virus Production

1. Harvest the Vero cells at 80% cell density using 0.25% trypsin in T-75 flask and count the cells.
    1. Aspirate the media from the T-75 culture flask and rinse the cells with 2 ml phosphate buffered saline (PBS).
    2. Add 2 ml of 0.25% trypsin and incubate the flask at 37 °C for 5 min.
    3. Add 8 ml of serum containing growth medium to inactivate trypsin. Pipet up and down to suspend cells and transfer the cells to a 15 ml tube.
    4. Remove 10 µl of cells and mix with equal amount of 0.4% trypan blue. Load the prepared mix to the cell counting chamber slide and obtain viable cell counts using an automated cell counter.
2. Seed a total of 7 million cells in a 30 ml volume into a T-160 flask.
3. The next day, prepare an appropriate multiplicity of infection (0.01 to 0.1 MOI) of Zika virus inoculum in a 10 ml serum free culture media per flask.
4. Remove the spent media from the T-160 flask and then add the freshly prepared viral inoculum (10 ml).
5. Incubate the inoculated flasks in 37 °C with 5% CO_2 for 4-6 hr. Spread the inoculum by gently tilting the flasks sideways at every hr.
6. At the completion of incubation, replace the inoculum with warm serum-supplemented growth media (30 ml) for each flask. Then continue the viral culture for next 96 hr.
7. Verify the progression of infection by observing the appearance of viral plaques on the cell monolayer using a phase contrast microscope and take images (**Figure 2**) as needed at 40X and 200X magnifications.

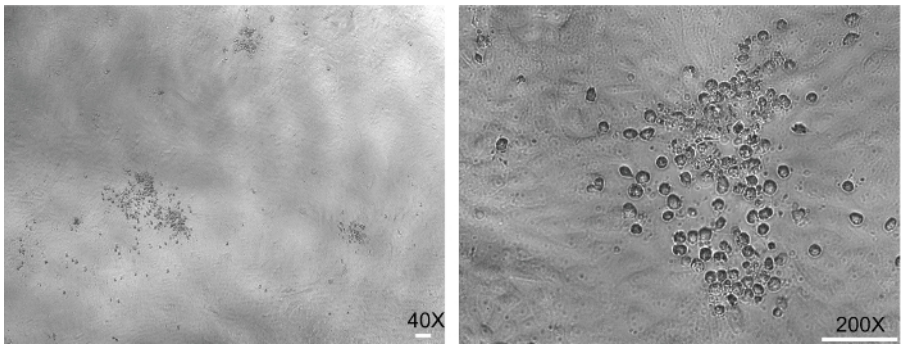

Figure 2: Plaques formed by Zika virus on a monolayer of Vero cells. Bright field images of various magnifications show Zika viral plaques at 48 hpi. Note the presence of rounded cell foci on the monolayer. (Scale bar = 50 µm) Please click here to view a larger version of this figure.

8. Harvest cell culture supernatants at the 96 hr time point and centrifuge the supernatant at 300 x g for 10 min at 4 °C to remove cell debris.
9. Carefully remove the supernatant without disturbing pelleted debris and transfer to 15 ml tubes. Ultracentrifugation at 24,000 x g can be performed to concentrate the viral particles (optional).
10. Store the viral culture supernatants in multiple aliquots at -80 °C.

3. Measuring Zika Virus Titer by Plaque Assay

1. Seed naive Vero cells at 1 x 10^5 cells per well in 2 ml volume using a 12-well plate.
2. The following day, prepare 10-fold serial dilutions of viral culture supernatants collected from the T-160 flask using serum-free media. Remove the spent media from each well and add 400 µl of prepared inoculum onto Vero cells in triplicate.
3. Incubate the inoculated flasks in 37 °C with 5% CO_2 for 4-6 hr. Spread the inoculum by gently tilting the plate sideways at every one hr.
4. At the end of incubation, replace the inoculum with serum supplemented media (2 ml per well).
5. At 48 hr post-inoculation, count the viral foci using a phase contrast microscope. Calculate the viral titer as plaque forming units (PFU) per ml. See **Figure 3** for plaque assay.
6. Fix and stain the cells in 4% formaldehyde and 0.1% crystal violet solution prepared in 20% ethanol for clear visualization of plaques.

4. Zika Viral Genome Replication Assay

1. Seed naïve Vero cells at 1 x 10^5 cells per well in 2 ml volumes using a 12-well plate. The following day, prepare viral inocula (MOI of 0.01 and 0.1; 400 µl/well) of low and high titer using serum free media in triplicate. For mock infection, use serum free media (400 µl/well) only.
2. Repeat the steps 3.2 to 3.4.
3. At the 48 and 96 hr time points, collect the samples for RNA and immunocytochemistry (ICC).
    1. For harvesting RNA samples, remove the media and then add 400 µl of lysis solution directly to each well and collect the lysates.
    2. For ICC, remove the media and then fix the cells by adding 1 ml of methanol to each well and incubate the plate at 4 °C for 30 min.
    3. From the cell lysate, isolate the total RNA using a RNA isolation kit per the manufacturer's instructions. Quantify the RNA using a spectrophotometer.

4. Perform a two-step reverse transcription quantitative PCR (RT-qPCR) to determine the ZIKV genome content from harvested RNA.
    1. To reverse transcribe the RNA, first set up a 13 µl reaction mix comprised of 5 µg of isolated RNA, 1 µl of dNTPs (10 mM), and 1 µl random hexamer (250 ng) in a 0.2 ml tube. Incubate the tube at 65 °C for 5 min and then place it on ice for one minute. Subsequently, add 1 µl of reverse transcriptase, 4 µl of 5x strand synthesis buffer, 1 µl of 0.1 M dithiothreitol, and 1 µl of RNase inhibitor to the reaction mix. Incubate the tube for an additional 5 min at 25 °C, followed by 60 min at 50 °C and inactivate the reaction by heating to 70 °C for 15 min.
    2. Perform qPCR using 1 µl of the resulting cDNA with 12.5 µl of 2x green dye super mix containing DNA polymerase and 1 µl of 10 mM individual primers specific for Zika virus [Zika virus pan-genotype primers (For: 5'-AARTACACATACCARAACAAAGTGGT-3'; Rev: 5'-TCCRCTCCCYCTYTGGTCTTG-3'), Zika virus Asian genotype PRVABC59 strain primers (5'-AAGTACACATACCAAAACAAAGTGGT-3'; Rev: 5'-TCCGCTCCCCCTTTGGTCTTG-3')], or hepatitis C virus (JFH RTQ F: 5'CTGGGTCCTTTCTTGGATAA-3; JFH RTQ R: 5'CCTATCAGGCAGTACCACA-3'), or cellular housekeeping gene GAPDH (For: 5'-CCACCTTTGACGCTGGG-3'; Rev: 5'-CATACCAGGAAATGAGCTTGACA-3') in a 25 µl reaction volume.
    3. Perform PCR using the run condition 95 °C for 15 sec and 60 °C for 30 sec (40 cycles) in a real-time PCR system.
    4. Use GAPDH expression level based on cycle threshold (Ct) value to normalize the Zika viral genome measurement. Calculate the delta Ct (ΔCt) value of Zika genome compared to that of GAPDH and obtain the $2^{\Delta Ct}$ value. Then, calculate the fold change by taking the ratio of normalized Zika genome contents between infected and uninfected cells at indicated time points. See **Figure 4** for ZIKV genome replication results.

5. Perform ICC using the methanol fixed cells.
    1. Wash the fixed cells three times with 1x PBS and block with ICC blocking buffer (3% goat serum, 3% BSA, 0.1% Triton-x 100 in PBS).
    2. Use mouse monoclonal anti-dsRNA antibody J2 (1 µg/ml) at a dilution of 1:100 in blocking buffer and incubate overnight at 4 °C.
    3. Wash the cells with 1x PBS and add secondary antibody goat anti-mouse IgG-594 (1 µg/ml) at a 1:1,000 dilution in blocking buffer and incubate for one hour at room temperature.
    4. Wash cells with 1x PBS and stain for nuclei using Hoechst dye and observe the cells using a fluorescent microscope at 100X magnification (**Figure 4**).

5. Screening Cytokine Library against Zika Virus Infection

1. Seed naive Vero cells at 1 x 10^5 cells per well using a 12-well plate. Once the cells are attached (6 hr post-seeding), add each cytokine at indicated concentrations in biological duplicates (2 ml volume/well). Include vehicle (PBS) alone control.
2. At 12 hr post-treatment, perform Zika viral infection (MOI of 0.1 in 400 µl per well). Include negative control wells without infection (mock).
3. At 4 hr post-infection, replace the viral inoculum with cytokine treated media (2 ml). Incubate the cells at 37 °C for additional 44 hr.
4. At 48 hr post-infection, count viral plaques using a phase contrast microscope and acquire representative images of infected cells at 40X magnification (**Figure 5**).

Representative Results

A Zika viral strain (PRVABC59; GenBank accession number KU501215) of the Asian genotype was utilized in this study[12]. Vero cells at 80% confluency were used for investigating *de novo* Zika viral infection. For viral production and subsequent virological characterization, an early passage (P3) Zika virus was employed. The viral plaques were observed on the second day of infection. Zika viral progenies released from the initially infected cell can spread to neighboring cells, which result in the formation of visible plaques on the monolayer culture (**Figure 2**). The cytopathic effect (CPE) of Zika viral infection is characterized by morphological changes such as rounding and detachment of cells as well as lytic cell death.

For measuring viral titer, virus-containing cell-free supernatant harvested from T-160 flasks was subjected to 10-fold serial dilution and added onto the monolayer of Vero cells in a 12-well plate format (**Figure 3**). The 48 hr post-inoculation time point was used as an endpoint to avoid counting the plaques arising from secondary infection. The wells with 30-100 plaques were chosen for counting to obtain an accurate titer.

RT-qPCR assay was utilized to estimate the genome replication of Zika virus. A pan-genotype primer pair specific for the highly conserved ZIKV NS5 region was included[3,16]. Zika viral strain PRVABC59-specific primers based on the genomic location of pan-genotypic primers (sequences of primers are given in the protocol section 4.4.2) were also tested. Both of the primer pairs showed similar levels of sensitivity in amplifying Zika viral genome from infected cells (**Figure 4**). As a negative control, hepatitis C virus-specific primers were also tested[17]. A significant increase in the ZIKV viral genome content was observed between 48 and 96 hr time points both in the low and high MOI infected cells, suggesting a robust viral infection (**Figure 4**). During Flavivirus genome replication, double-stranded (ds) RNA replicatory intermediates are generated by the formation of base-pairing between sense and anti-sense genomic RNA. An antibody probe specifically for recognizing dsRNA detected viral infected cells (**Figure 4**). Viral dsRNA were localized to the cytoplasm suggesting the genome replication compartments. Immunocytochemistry staining revealed expansion of virus infected cells during the 48 and 96 hr time points.

Using the infectious Zika virus culture system, a small library of cytokines was screened to determine antiviral activity (**Figure 5**). The cells were pre-treated with various cytokines for 12 hr and then infected with Zika virus. Type I interferon, IFN-α, exhibited strong antiviral activity against Zika virus in a broad range of tested doses [10 international units (IU), 100 IU and 1,000 IU]. IFN-β demonstrated a potent inhibitory effect as well. Type II IFN-γ also demonstrated anti-Zika viral activity. Cytokines TNF-α, IL-1 and IL-6 did not inhibit ZIKV at the indicated drug concentrations.

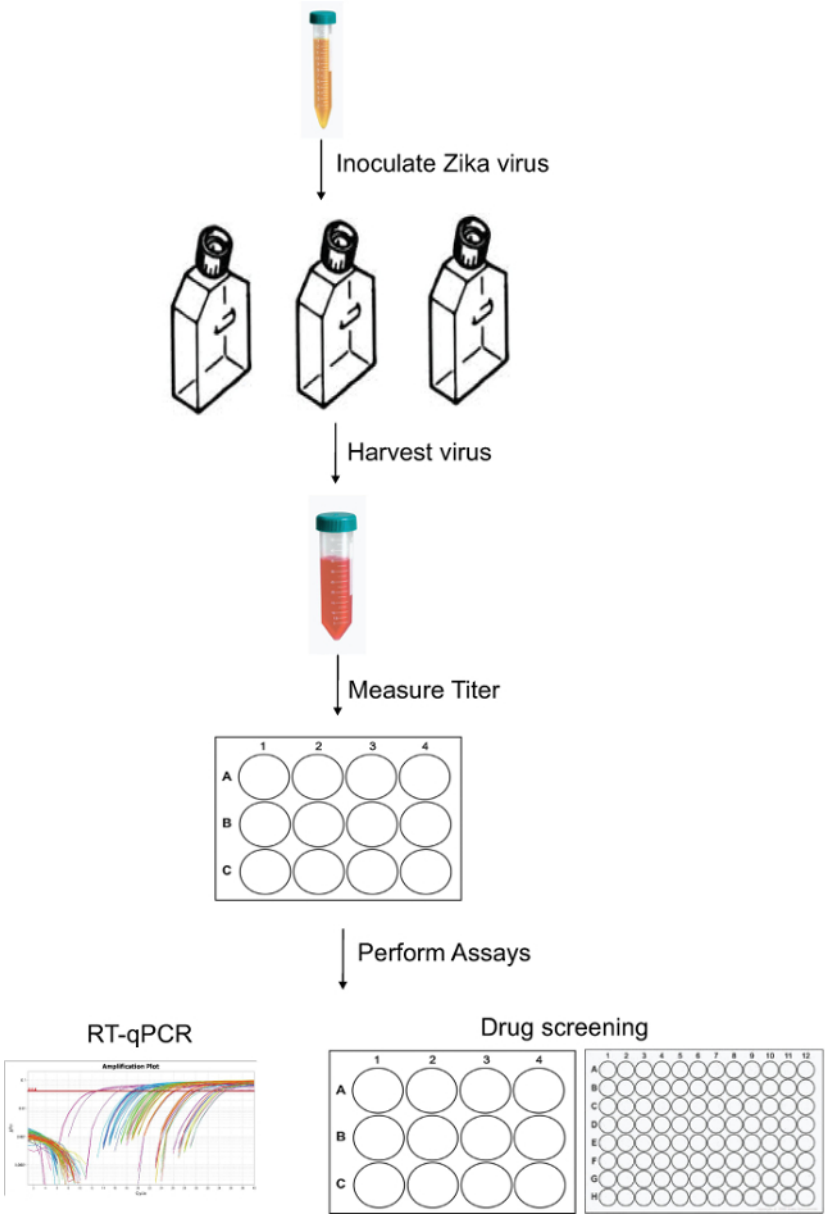

Figure 1: General outline of Zika virus production and assay workflow. For virus production, ZIKV is inoculated onto Vero cells in T-160 tissue culture flasks. At 48 or 96 hpi, the cell-free virus supernatant is harvested and stored at -80 °C for downstream analysis. Virus titer is measured by a limiting dilution assay. The Zika virus inoculum is subsequently used for studying virus replication kinetics and screening drug compounds. Please click here to view a larger version of this figure.

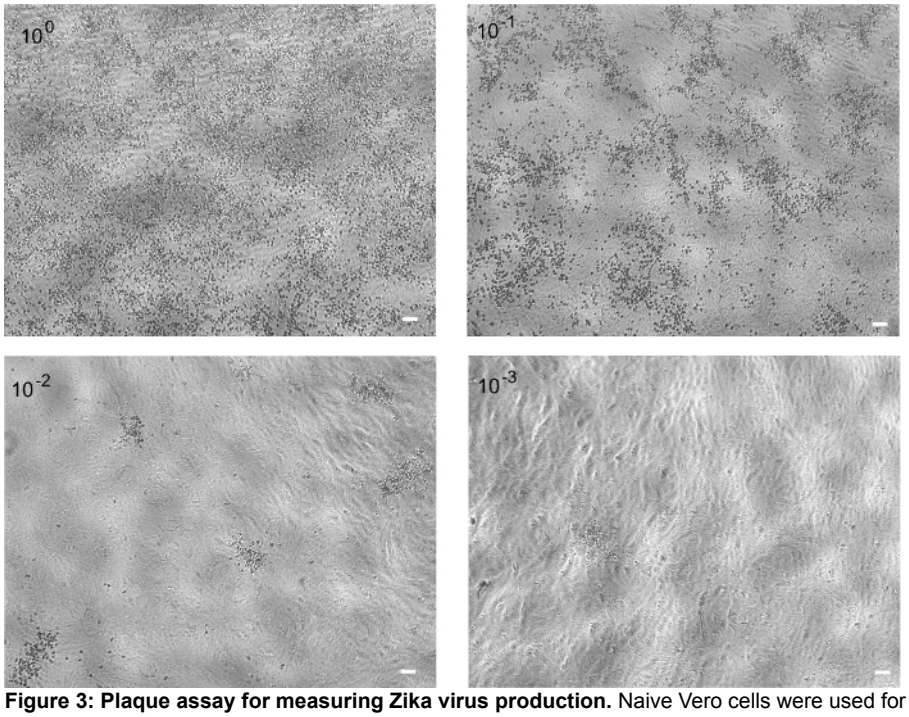

Figure 3: Plaque assay for measuring Zika virus production. Naive Vero cells were used for measuring virus titer. Bright field images depict the density of viral plaques at various dilutions (Scale bar = 50 µm). Note: The 1:100 dilution image shows distinct plaques that can be accurately counted. Please click here to view a larger version of this figure.

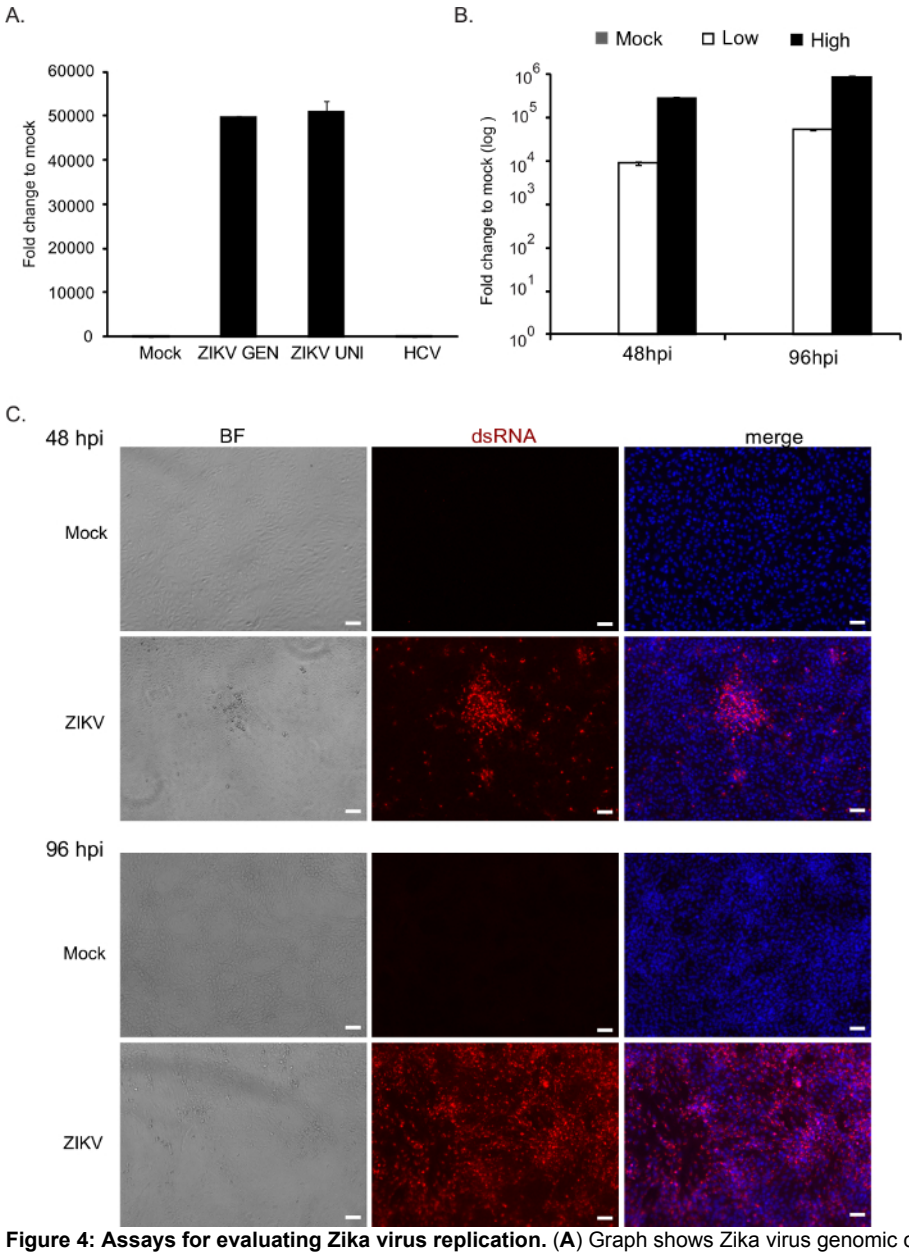

Figure 4: Assays for evaluating Zika virus replication. (**A**) Graph shows Zika virus genomic content measured by RT-qPCR. Both pan-genotype (ZIKV GEN) and PRVABC59-strain (ZIKV UNI) primers showed equal sensitivity and specificity. As expected, hepatitis C viral (HCV) primers did not amplify any product. (**B**) Graph presents the Zika viral growth kinetics at the indicated time points in low and high titer infected cells compared to that of the uninfected mock control. Mean values and standard deviations are given. (**C**) Immunocytochemistry assay for investigating viral replication. Zika virus infected cells were specifically stained with dsRNA antibody. At 48 hpi, infected cells are in few clusters, whereas at 96 hpi most of the cells are infected. Hoechst stain was used for the visualization of nuclei (Scale bar = 50 μm). BF: Bright field image. Please click here to view a larger version of this figure.

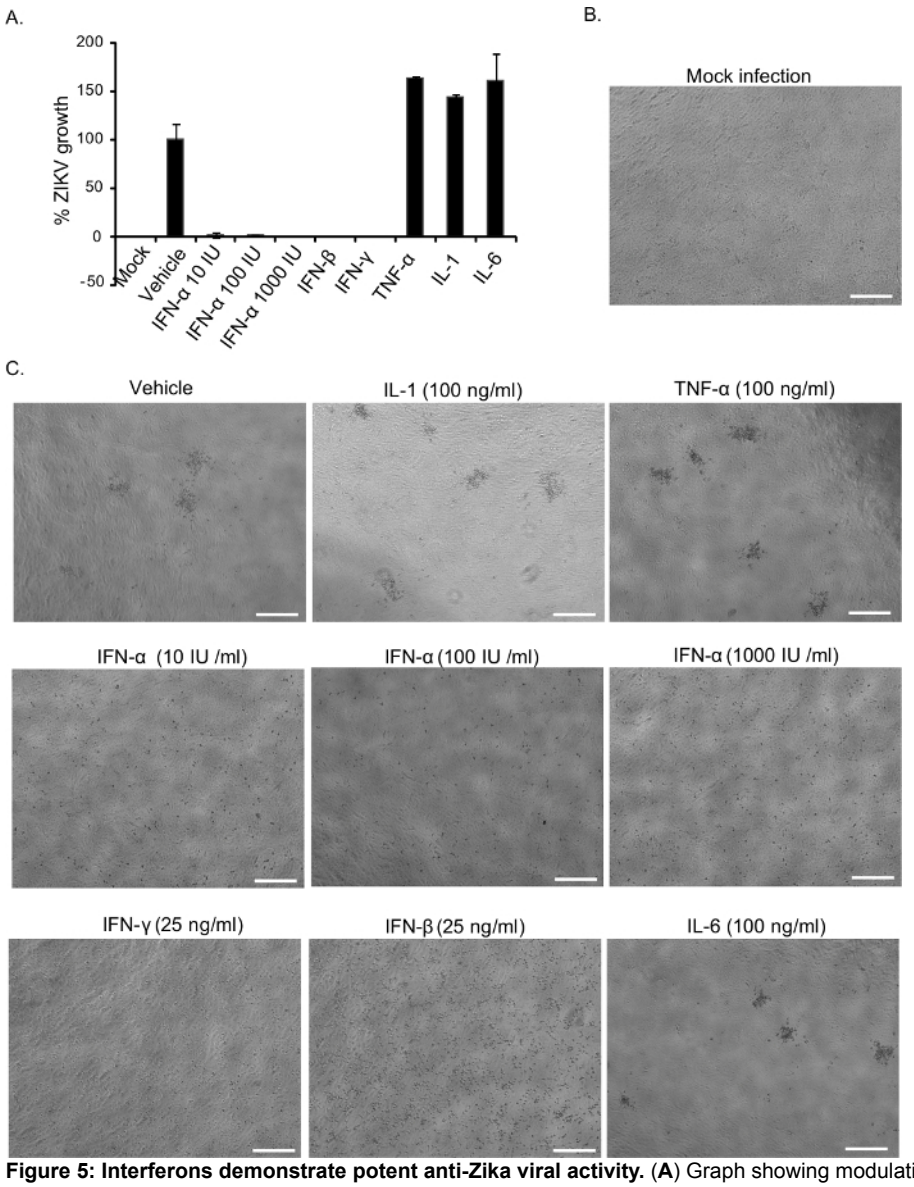

Figure 5: Interferons demonstrate potent anti-Zika viral activity. (**A**) Graph showing modulation of Zika viral growth by various cytokines compared to that of vehicle control. Mean values and standard deviation are shown in the bar graph. Interferons showed statistically significant inhibition of Zika virus replication (p value <0.05). (**B** and **C**) Phase contrast images of Zika virus-infected cells with or without cytokine treatment. Viral plaques formed by infected cells can be seen as foci. Note that interferon-treated wells do not have viral plaques. Mock control without ZIKV infection included as negative control. (Scale bar = 50 µm) Please click here to view a larger version of this figure.

Discussion

Here, a streamlined protocol for culturing Zika virus *in vitro* is presented. Critical steps including, identifying optimum end points for expanding virus culture, measuring titer, and quantifying genome replication were provided. Zika virus is a human pathogen, so, while handling infectious agents, biosafety procedures are to be strictly followed. A monkey kidney cell line, Vero, was used for demonstrating various virological assays. Zika viral replication kinetics may differ in cells of various tissues and species. Additional cell lines can be used as described previously[15]. Zika virus has been isolated from the brain tissues of infected fetuses[3] and has been shown to infect cortical neural progenitor cells[14]. Thus, evaluating the pathophysiology of ZIKV infection in neuronal cells can provide additional cell-specific insights. Differences in the neurovirulent phenotype of African and Asian genotypes can be investigated using this infectious culture system. The viral production procedure described here will also be useful for generating high-titer virus towards *in vivo* pathogenic studies in non-human primate or rodent model systems.

While, this study is limited to the usage of Vero cells, high-titer producing cell lines of human and mosquito origins can be tested. During viral production, if CPE is observed in only 10-30% of cells on day 3, the infected cells can be split in 1:4 density and culturing can be continued for additional 4-5 days. At the end of the 7th day, a complete CPE can be observed.

The efficiency of ZIKV pan-genotype and strain-specific primers were compared and determined that both are sensitive in quantifying Zika viral genome in low and high titer virus infected cells. Furthermore, in this study, an antibody-based probe is verified to identify infected cells. This

dsRNA-specific antibody successfully recognized cytoplasmic viral genomes in the Zika virus-infected cells. These optimized reagents will be a useful resource to dissect various stages of viral replication such as translation, genome replication, assembly and virion morphogenesis.

This *in vitro* infectious cell culture system is applicable to future studies using Zika virus-specific sub-genomic replicons and infectious virus generated from complementary DNA (cDNA) clones. Development of Zika viral infectious cDNA clones will accelerate the functional study of viral genes including NS1, and NS4B by combining genetic and molecular biological techniques. Establishment of Zika virus tagged with a fluorescent or luminescent based reporter gene would be a valuable tool in making further use the cell culture system in high-content screening assays.

Both type I and type II IFNs demonstrated anti-Zika viral activity. IFN-α, IFN-β and IFN-γ can be further evaluated in clinical settings as a prophylactic, post-exposure prophylactic, and treatment against Zika virus infection and associated diseases. High-risk groups including pregnant women positive for Zika virus, can be treated with interferons as a first line of treatment. Interferon-α treatment has been shown to be safe for pregnant women[18,19].

In summary, an infectious cell culture system that can be used to investigate various aspects of Zika viral growth is described and outlined. The protocol and reagents described here are important resources for those who study Zika virus and the neurological research community.

Disclosures

The authors have nothing to disclose.

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