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Use of RT-qPCR Methodology as an Alternative Tool for Chikungunya Virus Infectivity Evaluation and Virus Replication Understanding

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Abbreviations: CHIKV: Chikungunya Virus; SFV: Semliki Forest Virus; FBS: Fetal Bovine Serum; MOI: Multiplicity of Infection; CPE: Cultures Cytopathic Effects; PFU: Plaque-Forming Unit; CMC: Carboxymetilcellulose; CPE: Cytopathic Effect

Abstract

Background: Chikungunya virus (CHIKV) is the etiological agent of arthropod-borne disease Chikungunya fever and it has been considered a public health problem due to the lack of efficient treatment or licensed vaccine able to prevent CHIKV infection. Quantification of viral replication is an essential mean to characterize the behavior of different viruses *in vitro*. Knowing the replicative profile of a particular viral as CHIKV is extremely relevant for the development of immunobiologicals or therapeutic approaches. In this context, we aimed to follow up CHIKV cellular infection and evaluate its replicative profile in Vero cells.

Methods: Vero cells infected with CHIKV were monitored during 76 h and supernatant samples were collected at seven different time points. CHIKV quantification in the supernatant samples were performed by TaqMan reverse transcription quantitative polymerase chain reaction (RT-qPCR) and by plaque assay.

Results: Results by plaque assay titration reveled an optimal virus replication between 28 and 45 hours post infections (h.p.i.), resulting in a maximum titer of 8.48 log10 PFU/mL. Data obtained by RT-qPCR revealed similar replication profile, with a titer increase of 0.98 and 1.35 log10 viral genome copies/mL at 28 and 45 h.p.i., respectively. The major difference viral titer was 1.80 log10 viral genome copies/mL at 64 h.p.i.

Conclusions: These data indicated that Vero cells are susceptible to CHIKV infection and demonstrated viral capacity to produce a high viral load of infectious particles. In addition, RT-qPCR can be used as a valuable alternative tool for understanding CHIKV replication in cell culture, allowing correlations between the cell-based and the PCR-based assays to untreated viral stocks for CHIKV.

Keywords: Chikungunya Virus; Vero Cells; Replicative Profile; Plaque Assay; Quantitative Reverse-Transcription Polymerase Chain Reactionb

Introduction

Chikungunya virus (CHIKV) is the etiological agent of arthropod-borne disease Chikungunya fever, responsible for acute and chronic diseases characterized by several symptoms as fever, rash and arthralgia in infected individuals [1]. It belongs to *Alphavirus* genus, *Togaviridae* family and to the Semliki Forest virus (SFV) antigenic complex group [2,3]. It was firstly described at southern Tanzania in 1950's [4] and it has been re-emerged in 2000's [5], becoming a massive epidemic worldwide and a serious public health problem due to the lack of efficient treatment or licensed vaccine able to prevent CHIKV infection [6]. Quantification of viral replication is an essential mean to characterize the behavior of several different virus *in vitro* [7-9]. Knowing the replicative profile of a

particular viral antigen is extremely relevant for the development of immunobiologicals or therapeutic approaches. The virus ability to invade the host cell and replicate itself is the most important property of a virus. For quantifying the load of infectious particles, several technics have been developed.

The two most-common methods for quantifying the infectivity of cytopathic viruses are the plaque assay and the 50% tissue culture infective dose (TCID50) assays [10-13]. Although cell-based viral quantification assays are the most common methods for viral diagnosis, they present some disadvantages as time-consuming and they may be difficult to reproduce in different laboratory settings. Molecular approaches have been developed and explored as alternative methods to identify the presence of different viruses. These PCR-based approaches have been crucial for virological studies and will continue to be until a protocol for culturing the target virus can be developed. Quantitative reverse-transcription polymerase chain reaction (RT-qPCR) and reverse-transcription polymerase chain reaction (RT-PCR) are the most commonly used methods for virus detection. These methods actually only identify for the presence of specific amplicon from the virus genome, which is suggestive of virus infection, and does not assure that the detected target was derived from an infectious viral particle. Besides that, viral genomes are usually produced in excess, and many will be never packaged into virions [14].

However, molecular biology testing offers several advantages, including rapid test results assisting in the detection of outbreaks and newly emerging strains, high sensitivity and specificity, identification of resistant organisms, and quantifiable correlation to disease severity, all of which contribute to timely therapeutic clinical decisions and early infection control interventions [15,16]. Moreover, since plaque assays for frozen-thawed sera can produce false negative results for some virus, knowing the correlation between the cell-based and the PCR-based assays ensures the reliability of PCR data for viremia in non-clinical and clinical studies, especially when the analysis of fresh samples is not possible [17]. To improve the relevance of these PCR-based viral quantitation methods, RT-qPCR variants involving genome encapsulation have been developed [11]. However, the correlations between the cell-based assays and the PCR-based assays have not been studied in untreated viral stocks for CHIKV. In this study, we evaluate the correlations between these cell-based and PCR-based assays to determine if PCR-based assays can be used to estimate the infectivity of CHIKV infection.

Materials and Methods

Cell Line

Vero cells, a lineage derived of African green kidney of *Cercopithecus aethiops* (ATCC CCL-81), were cultured at 37°C in 199 Earle medium supplemented with 40 µg/mL gentamycin sulfate and 5% fetal bovine serum (FBS), and adjusted to 4 mM L-glutamine (all Gibco®BRL, Invitrogen, Life Technologies, Paisley, U.K.) until viral kinetics and plaque assays.

Virus Propagation

The CHIKV strain was kindly provided by Dr. Ricardo Lourenço (Oswaldo Cruz Institute, FIOCRUZ) from clinical sample. CHIKV was cultured in Vero cells in 199 Earle medium at 37°C in a humidified atmosphere containing 5% CO_2, in aseptic conditions, at multiplicity of infection (MOI) 0.01 as previously determined by our group (data not shown). In the second day post infection (d.p.i.), the total volume of cultures supernatant was adjusted to 8% (w/v) D-sorbitol (SigmaAldrich, Darmstadt, Germany), submitted to 0.22 µm sterile filters (Stericup® filter, Merk Millipore, Burlington, Massachusetts, EUA) and stored at -80°C until the moment of use for viral kinetics.

Viral Kinetics

Vero cells were seeded in sterile flasks at concentration of 6 x 10^4 cells/cm^2 and cultured by 24 h in 199 Earle medium supplemented with 40 µg/mL gentamycin sulfate and 5% fetal bovine serum (FBS), and adjusted to 4 mM L-glutamine (all Gibco®BRL, Invitrogen, Life Technologies, Paisley, U.K.) at 37°C with 5% CO_2. After 24 h, cultures were infected with CHIKV at MOI 0.01 in 199 Earle medium in absence of FBS for 1 h in reduced volume, with gently agitation, for viral adsorption. After adsorption time, the inoculum was removed by aspiration and substituted by 2% FBS-199 Earle medium. One flask was not infected and it was used as mock control. Cultures cytopathic effects (CPE) were monitored by optical microscope to demonstrate the extension of infection. During 76 hours of infection, supernatant cultures samples were collected at seven time points (4, 21, 28, 45, 52, 69 and 76 hours post infection (h.p.i.)) and they were stored at -80°C until processing. CHIKV quantification in the supernatant samples was performed by TaqMan quantitative reverse-transcription polymerase chain reaction (RT-qPCR) and by plaque assay.

Determination of Plaque-Forming Unit (PFU)

Vero cells were cultured at 6-well plates at the concentration of 6 x 10^4 cells/cm^2 and maintained by 24 h as described above. On the next day, medium was removed by aspiration and serial supernatant samples dilutions, perfomed previously, were added to 6-well plates monolayers in 0% FBS-199 Earle medium. Cultures were incubated at 37°C with 5% CO_2 for 1 h, under gentle agitation, for virus adsorption. Then, the inoculum was removed, and monolayers were overlaid in 199 Earle medium supplemented Containing 2% carboxymetilcellulose (CMC) and incubated at 37°C with 5% CO_2 for 2 days. The assay was performed in duplicate. After incubation time, cultures were fixed with 5% formaldehyde for at least 2 h and then stained with 0.04% crystal violet for 30 min to visualize and count plaques.

Quantitative Real Time PCR

Briefly, viral RNA was purified from 200 µL of supernatant cells samples using the commercial kit MagMAX CORE (AppliedBiosystems, Hammonton, NJ, USA) following the manufacturer's instructions. The positive strands were reverse-transcribed, amplified and quantified by real-time PCR for part of NSP1 region of the CHIKV genome. For each PCR run, a master mix was prepared on ice using 1× TaqMan Fast Virus 1 Step Master Mix (Applied Biosystems, Hammonton, NJ, USA), 1 µL primers and 0.5 µL probe (500 nM each primer, Forward - 5' AAA GGG CAA RCT YAG CTT CAC 3'and Reverse - 5' GCC TGG GCT CAT CGT TAT TC 3', 250 nM probe 5' FAM- CGC TGT GAY ACA GTG GTT TT 3') (IDT, San José, California, USA). Each run contained six concentrations points (10^7 - 10^2 copies/µL) of a recombinant plasmid (pIDTSMART-AMP) with part of NSP1 sequence standard curve. Five microliters of RNA or standard curve points were added into 15 µL of the PCR master mix. The thermal cycling conditions were an initial step at 50°C for 5 min and 95°C for 20 s, followed by 40 cycles at 95°C for 3 s and at 60°C for 33 s. The unknown samples concentrations were calculated based on fluorometric signal of standard curve. The assay was performed in duplicate and results were achieved taking into account sample dilution factor.

Results

Establishing a correlation between the production of infectious CHIKV particles and the detection of viral genome, RT-qPCR data (viral genome copies/mL) obtained from supernatant samples of different time points were compared to its respective plaque assay results (PFU/mL). In our study, Vero cells were infected with CHIKV at MOI 0.01 and cultures supernatant were collected at seven different time points (4, 21, 28, 45, 52, 69 and 76 h.p.i.). Cytopathic effect was evaluated at each time point, beginning at 28 h.p.i. until 45 h.p.i. when there was almost none adhered cells in the flasks (Figure 1A-N). The results of plaque assay titration from CHIKV-infected supernatants demonstrated a significant increase of PFU and viral genome copies between 4 to 21 h.p.i. At 28 and 45 h.p.i. we observed an optimal virus replication, resulting in a maximum titer of 8.48 log10 PFU/mL (Figure 2). The viral titer decreases from 52 h.p.i., as observed in Figure 2. Data obtained by RT-qPCR revealed similar replication profile to that obtained in plaque assay. When compared to plaque assay, RT-qPCR reached an increased titer of 0.98 and 1.35 log10 viral genome copies/mL at 28 and 45 h.p.i., respectively, with a major difference of 1.80 $\log_{10}$ viral genome copies/mL at 64 h.p.i. (Figure 3).

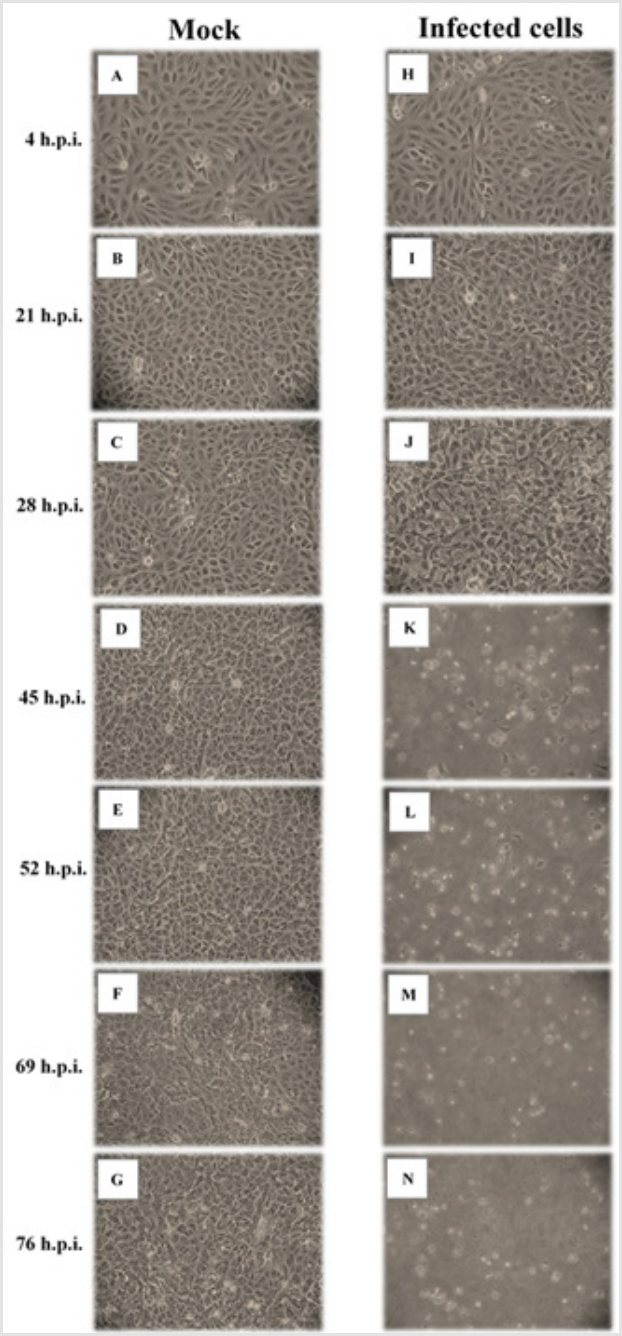

Figure 1: Chikungunya virus infection kinetic in Vero cells. Cells were infected at MOI 0.01 and monitored by optical microscope until 76 h.p.i. At indicated time points, mock (A-G) and infected cells (H-N) supernatants were collected follow by plaque and RT-qPCR assays performing.

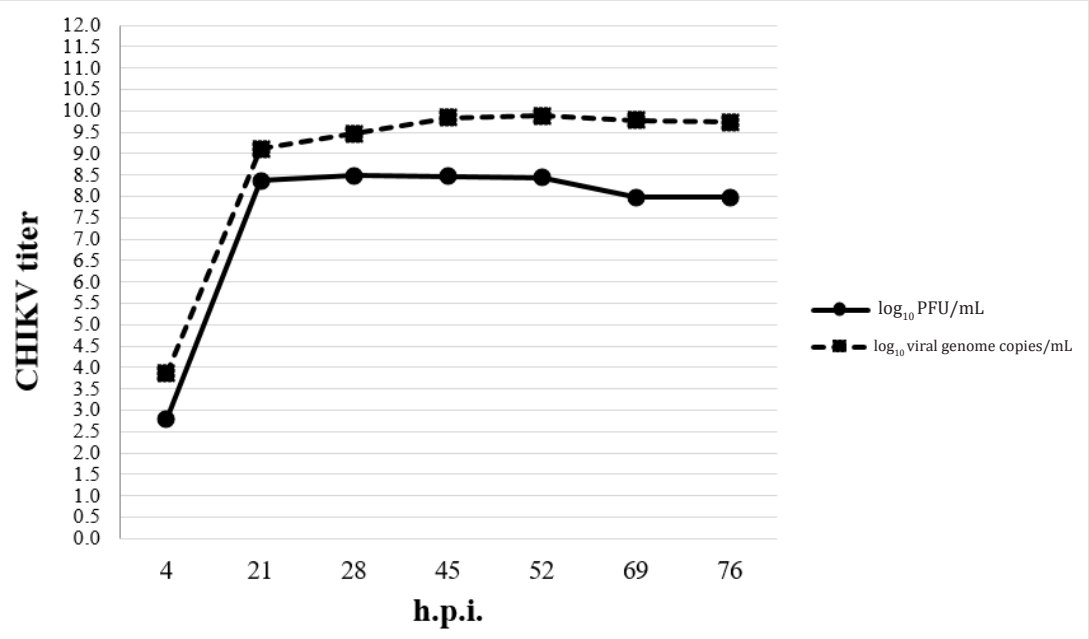

Figure 2: Replication profile of CHIKV in Vero cells. Vero cells were infected with CHIKV at MOI 0.01 and supernatant samples were collected at 4, 21, 28, 45, 52, 69 and 76 h.p.i. Titers obtained by plaque assay (PFU) and RT-qPCR (viral genome copies) were demonstrated in $\log_{10}$/mL.

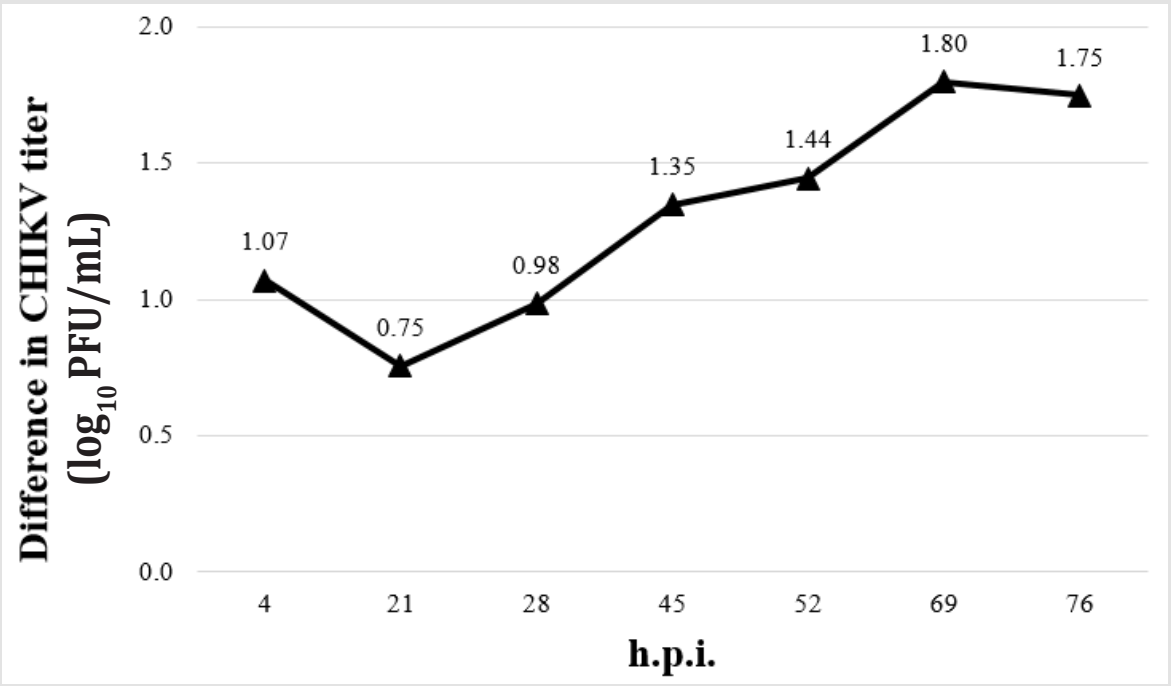

Figure 3: Comparative analysis of CHIKV viral titers. Viral titers difference obtained by RT-qPCR and plaque assay. At 69 h.p.i., titer difference was the highest one comparing both approaches, 1.80 $\log_{10}$/mL.

Discussion

Chikungunya fever is an arthropod-borne disease in which symptoms are shared with other arboviral infections such as those caused by Zika, Dengue [18] and Mayaro virus [19,20]. Despite these pathogenesis similarities, its clinical consequences and treatment are different, demanding a specific detection method for each one of them. The comparative quantitative analysis of virus titers in cell culture using the presented methods revealed some insights into CHIKV particle production. CHIKV RNA genome was quantified from cell culture supernatants, which most certainly contained free RNA genomes from lysed cells resulting of virus replication. Thus, it was observed that there are some prominent

differences in the magnitude of genomes and infectious particle. RT-qPCR method is an alternative tool to estimate vaccine titration, for example, in occasions before CPE or for non-cytophatic viruses. In the present study, we established a correlation between the production of infectious CHIKV particles by plaque assay and the detection of virus genome by RT-qPCR aiming to follow up CHIKV cellular infection and evaluate the replicative profile in Vero cells.

Vero cell line had already been demonstrated to be susceptible to CHIKV infection, presenting high percentage of infected cells in a short time, according to the employed MOI and the virus strain [21,22]. Its ability to induce quick CPE in Vero cells, compared with to other virus, helps to understand about CHIKV virulence and life cycle [23]. Viral kinetics performed in this study allowed observation of a massive CHIKV-infection resulting in high virus load in a short time of infection and using a low MOI. Before 24 h.p.i., it was possible to observe a slight morphological discrepancy in infected cultures, such as a rounded cell shape (Figure 1I), when compared to mock cultures (Figure 1B). At 28 h.p.i., CHIKV-infected cultures presented retraction, event characteristic of CPE, what possibly triggered cells detachment from flasks along with cell lysis caused by viral infection in posterior time points (Figure 1H-N). Despite of this, monolayers were well preserved until this infection time point, but it is not observed from 45 h.p.i. anymore. The intensity of CPE varies according to cell line used in infection. Such as our results, Li et al. (2013) demonstrated that Vero cells presents an intense CPE [24].

In the other hand, arthropod cell lines like C6/36 or Lulo, respectively, presented a moderate or indistinct CPE when infected with CHIKV [25]. A suggestive reason of intense CPE is apoptosis [24]. Initial CHIKV infection results in a wide titer difference between 4 and 21 h.p.i., suggesting an intense viral replication, as detected by both methods (Figure 2). Data obtained by plaque assay demonstrated that maximum titer was obtained between 28 and 45 h.p.i. which is the most suitable time range to collect CHIKV supernatants. This event was associated with intense CPE observed in infected cultures at 45 h.p.i., as observed in Figure 1H-N. Previously data from TCID50 assay demonstrated that Vero E6 cells infected with three different CHIKV strains at MOI 10 reached a maximum titer of 8 log10 on the 4th day post infection [26], while in our results this titer was observed until 52 h.p.i. followed by titer decline. Titer decrease after 52 h.p.i. was also observed in HeLa lineage. This event can be explained by a reduced replication maybe due to the presence of high levels of dead cells, as observed by immunofluorescence [27], besides to the lack of stability of viral particles in the culture medium after so long. Thus, at 28 h.p.i. could be considered the best moment to obtain a considerable pool of infectious viral particles (Figure 2).

Plaque assay titers were compared to its respective RT-qPCR titers at each time point (Figure 2). We could demonstrate that CHIKV replicative profile presents the same growth tendency for both methodologies. However, RT-qPCR titers were higher than that reached by plaque assay, since viral genome from supernatant samples is not only included in virus infectious particles. In the sense, the comparative analysis revealed that titers obtained by PFU and RT-qPCR showed a relevant difference mainly at 45 h.p.i. At 69 h.p.i., titer difference was the higher during entire kinetic (Figure 3) and this can be explained by the loss of infectivity of the viral particles during the final incubation times. According to the previous results, RT-qPCR is a promising approach to monitor active viral replication *in vitro*. In addition, it may be used as a valuable alternative tool for CHIKV replication understanding in cell culture allowing correlations between plaque assays and the RT-qPCR for CHIKV, as demonstrated to other arboviruses studies [28,29]. Although future experiments must be performed, the results presented in this study are promising and extremely relevant for the development of immunobiologicals or therapeutic approach.

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