Production of Chikungunya Virus Propagated In Aedes Albopictus Cells Compared with the Production of This Virus Propagated in Vero Cells

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Abstract

Chikungunya virus (CHIKV), the cause of chikungunya fever, is a member of genus Alphavirus and family Togaviridae. Chikungunya fever is a re-emerging infectious disease that is endemic to Africa and Asia. The classic clinical symptoms after infection by CHIKV are sudden febrile illness, headache, rash, myalgia, althalgia, edema of the extremities, and gastrointestinal complaints. Moreover, severe dermatological lesions, lethal hepatitis and encephalitis can occur in newborns and old people. CHIKV has been shown to infect a wide variety of cell lines with differential pathogenicity. This study worked with a CHIKV isolated from recent outbreak Thailand (2008-2009) and aimed to compare production of CHIKV propagated in mosquito Aedes albopictus C6/36 cells, which are representative of one of the main natural vectors, and in African green monkey kidney (Vero) cells. CHIKV propagation, microscopic and standard plaque assay were applied for this research. The results showed different patterns of infection. The growth curve of CHIKV in C6/36 cells illustrated that the highest viral titer of CHIKV in C6/36 cells was produced on day 2 post infection. Cytopathic effect (CPE) was utilized to assay productivity in Vero cells. Optimal collection occurred when Vero cells were undergoing 30-40% CPE. While reduced virus titer was observed when CPE reached 50-70%. Virus titer in Vero cells was depend upon the degree of CPE, amount of input virus, time of virus collection and cell type for virus propagation. The results of this studies are primary information and useful for further developing CHIKV studies.

Keywords: Chikungunya virus, Virus propagation, C6/36 cells, Vero cells

Introduction

Chikungunya virus (CHIKV), an arbovirus belonging to the genus Alphavirus (Togaviridae family), leads to a disease known as chikungunya fever [1]. The name Chikungunya originated from the Kimakonde language of Mozambique meaning “that which bend up” and referred to the contorted posture of infected patients suffering from severe joint pain [2-4]. Chikungunya fever, a re-emerging infectious disease was first isolated from the serum of a febrile patient in 1952 from the Makonde Plateau in Tanzania, Africa [5]. Subsequently, a rapid increase of the CHIKV infection was reported over the last 50 years due to decreased mosquito control effort, rapid international transportation, travelers and climate change [6]. In Asia CHIKV is maintained in an urban cycle in which the virus is transmitted in a mosquito-human-mosquito cycle, with Aedes aegypti and Aedes albopictus mosquitoes being the two main vectors of the virus, while CHIKV in Africa is maintained in a sylvatic
cycle with forest dwelling *Aedes* mosquitoes being reported as the major vectors [7]. CHIKV is transmitted to the humans through the bite of an infected mosquito. Moreover, humans serve as the CHIKV reservoir during epidemic periods, while outside this period the main reservoirs are monkeys, birds and other unidentifiable vertebrates in both cycles [8]. The incubation period of the CHIKV infection is usually 2-4 days on average, with range of 1-12 days. The clinical signs and symptoms are a sudden febrile illness, rash, headache, myalgia, edema of the extremities, gastrointestinal complaints and polyarthalgia (a hallmark of CHIKV infection) that frequently persists for two or more months [9,10]. Moreover, hemorrhagic fever has been recently reported in CHIKV infected patients, while in some cases of newborns and adults (elderly persons) both hemorrhagic encephalitis and meningitis fever, which are indistinguishable with dengue fever [11] have been reported. Mother-to-child transmission has also being reported and this transmission leads to congenital and fetal death.

In Thailand, chikungunya infection was first diagnosed by serology in 1953 and was then subsequently detected around the country [12]. Recently, confirmed cases have been reported in provinces in Middle, North and North-East of Thailand in a major outbreak signifying the re-emergence of chikungunya in Thailand [13]. Nowadays, no vaccine or specific treatment for CHIKV infection is available so the infected patients are treated with supportive treatments.

Therefore, this research aimed to study about CHIKV infection in C6/36 cells and Vero cells, CHIKV propagation, productivity in Vero cells and CHIKV growth to be the primary information for CHIKV studies.

**Materials and Methods**

**Cell culture and virus stock**

Chikungunya virus (CHIKV) East Central South African (ECSA) genotype, a Thai isolate E1:226V CHIKV was obtained from a patient in Phang-nga province, Thailand in 2009s and passaged in C6/36 and Vero cells.

Vero cells were cultured in Dulbecco’s modified Eagle medium (DMEM; GibcoTM Invitrogen) supplemented with 5% heat-inactivated fetal bovine serum (FBS; GibcoTM Invitrogen) and 100 units/ml of penicillin/streptomycin (PAA, Pasching, Austria). C6/36 cells were cultured in Minimum Essential (Earle’s Based) supplemented with 10% FBS, 1% L-glutamine and 100 units/ml of penicillin/streptomycin. Vero cells were cultured at 37°C with 5% CO₂, while C6/36 cells were cultured at 28°C.

**CHIKV propagation**

CHIKV was propagated in C6/36 and Vero cells and were cultured in 75 cm² tissue culture flasks and 6-well tissue culture plates, respectively for 24 hrs. Cell culture medium was removed and the cells were washed with phosphate-buffered saline (PBS). Subsequently cells were then replaced with culture medium without FBS containing CHIKV with varying multiplicities of infection (MOIs), individually for 2 hrs with constant agitation every 10 min. Pre-warmed complete medium was then added to a final volume of 15 ml and the cells were further incubated under standard conditions as described above. Supernatant was collected by centrifugation at 400xg for 5 min at room temperature. After removal of cell pellet, the supernatant containing progeny viruses was supplemented with heat-inactivated FBS to a final concentration of 20%. The virus stocks were immediately aliquotted and stored at −80 °C until use. Finally, the viral titers were determined by standard plaque assay on Vero cells.

**CHIKV titration by standard plaque assay**

The infectious viral titer was determined by standard plaque assay on Vero cells [14]. Vero cells were cultured in six-well tissue culture plates under standard conditions until a confluent monolayer was reached. Cells culture medium was removed and the cells were then infected with 200 µl of 10-fold serial diluted virus (10⁻³ - 10⁸) in BA-1 medium and incubated at 37°C, 5% CO₂ for 2 hrs with constant agitation every 10 min. The

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first overlay solution containing the mixture of 2x nutrient solution and autoclaved 1.6%
Seakem LE agarose (1:1) was gently overlaid
and the plates were then left at room
temperature for 45-60 min until overlayer
completely set followed by upside down
incubation at 37°C with 5% CO₂. For CHIKV
propagated in Vero and C6/36 cells, the
second overlay solution containing 0.06%
neutral red and 1.6% Seakem LE agarose was
added into each well on the 3rd and 6th day,
respectively. All experiments were assayed in
duplicate of each sample. On the 4th and 8th
day, the plaques were counted and calculated
in plaque forming unit/ml (pfu/ml).

Statistical analysis
All sample of virus infection were
duplicately analyzed by the GraphPad Prism
program (GraphPad Software). Error bar
represented SEM of duplicates standard
plaque assay.

Results and Discussion
CHIKV was propagated one time in
C6/36 cells, a cell line derived from whole
hatched larva of Aedes albopictus mosquitoes,
which are one of the main vectors of CHIKV
transmission. Then the supernatant containing
progeny viruses was collected as viral stock.
One-hundred μl of CHIKV was used to infect
C6/36 cells again. Supernatant was collected
daily after infection (day 1 to day 8). Subsequently, 100 μl of CHIKV stock of each
day were infected individually in Vero cells,
which are normal kidney epithelial cells from
African green monkey that are commonly cells
for virus propagation. While cytopathic
effects (CPE) in C6/36 cells was poorly
differentiated and difficult to observe under a
microscope. CPE in Vero cells was clearly
showed that CHIKV from C6/36 (2dpi, 3dpi,
5dpi, and 8dpi) that was passaged in Vero
cells induced overt CPE in Vero cells at 2nd, 3rd, 4th and 8th day post infections when
compared with mock-infected cells (Figure 1).
The cells showed vacuoles within the
cytoplasm and they became shrunken. Cells
culture supernatant was collected to determine
the time which showed the highest viral yield
by changed morphology observation. The viral
titer was determined by standard plaque assay.
CHIKV was collected at 2nd, 3rd, 4th and 8th
day post infections and showed viral yield as
10^3, 10^3, 2×10^6 and 7.5×10^5 pfu/ml,
respectively. These results suggested that the
input virus was affected the time for overt
CPE to occur. If the input virus is high, the
CPE will occur more quickly than the cells
containing lower input total virus. The titer of
collected CHIKV at 2nd and 3rd day post
infections showed low titer because these
virus stocks were collected when the CPE of
Vero cells reached to 50-70%. Moreover these
results suggested that CHIKV from C6/36
cells at 2nd and 3rd day post infections
illustrated high titer because CPE were rapidly
observed. While collected CHIKV at 5th and
6th day post infections in C6/36 cells
represented CPE at 4th and 8th day post
infections, respectively. The cells showed
lately CPE because the titers of CHIKV from
C6/36 cells are lower than the input virus. The
virus stocks showed that the highest viral yield
(2×10^6 pfu/ml) was produced on the 4th day
post infection because they were collected
when Vero cells were observed CPE (40%)
thus this stock will be used for further studies.

CHIKV from C6/36 cells was propagated
in Vero cells and the virus titer was then
determined. The results suggested that when
CHIKV was propagated many times through
C6/36 cells, the titer was decreased whereas
plaque morphology in Vero cells that were
infected with CHIKV from C6/36 cells
represented as pinpoint shapes and small sizes
(Figure 2). Thus CHIKV was propagated to
increase titer in Vero cells and the CHIKV
stock from Vero cells was then infected to
examine the growth curve in C6/36 cells. In
long term virus production, C6/36 cells were
infected with CHIKV from CHIKV
propagated in C6/36 cells for 2 times and
passed in Vero cells one time dividing into
two sets of experiment which are prolong
periods (multiplicity of infection (MOI)
0.0025 for 6 days and MOI 0.01 for 8 days)
for revealing the viruses and cell interactions
at different MOI and period of times. CPE of
C6/36 infected cells were hardly observed
when compared with mock-infected cells. To determined the times with the highest viral yield, cells culture medium containing progeny viruses was collected daily after infection and the amount of infectious viruses was quantified by standard plaque assay in Vero cells. Results showed that the long term profile of CHIKV production with the highest yield of virus was produced at 2\textsuperscript{th} day post infection in both of MOI 0.0025 and MOI 0.01(Figure 3). Moreover when the viral load was increased to an MOI 0.01, the peaks of viral titer is mildly higher but it was still increased to the highest yield at 2\textsuperscript{th} day post infection. Furthermore, the viral titer of CHIKV which was propagated in C6/36 cells was increased (Figure 3). While plaque morphology of infected CHIKV in Vero cells that was propagated through Vero cells one time represented as large plaque more than pin point and also showed the higher titer of CHIKV. The highest viral yield around $10^7$ PFU/ml was determined. Interestingly, the growth curve was hard to repeat because the growth curve variation can be occurred by many effects such as input virus and cell counting. Moreover, previous reports showed that the viruses representing small plaque are less virulent than the viruses that showed large plaque and these data are corresponding with our results that represented small plaques as lower titer and large plaques as higher titer [15]. Therefore CHIKV propagated in Vero cells showed the higher titer than CHIKV which were propagated only in C6/36 cells.

**Conclusions**

The growth curve results of CHIKV on C6/36 showed the benefit information for virus stock production, so that the highest yield of virus will be collected at 2\textsuperscript{th} day post infection. The different cell lines generated different patterns of CPE and viral titer in response to viral infection including the different plaque morphology which corresponded to virulent of virus.

Figure 1. The cell morphology under microscopy (Olympus) with 20x magnification.

Figure 2. Plaque formation. The upper and lower panel represented plaque from CHIKV propagated in only C6/36 cells and Vero cells, respectively.
Figure 3. The growth curve of CHIKV passaged in C6/36 cells. Each point represents the average of duplicate and SEM of this experiment is shown in error bars.

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