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R E S E A R C H A R T I C L E

Dengue virus serotype 2 proliferation using different cell substrates

*Gangadharam Poli[1], Suresh Kumar Chitta[1], Madhusudana Pulaganti[3], Prasad Reddy S Hari[4], CM Anuradha[1,2]**

[1]Department of Bio-Chemistry, Sri Krishnadevaraya University, Anantapuramu, Andhrapradesh, India- 515003.
[2] Dept of Biotechnology, Sri Krishnadevaraya University, Anantapuramu, Andhrapradesh, India- 515003.
[3]Multidisciplinary Research Unit, SV Medical College, Tirupati, Andhrapradesh, India-517507
[4]Dept of Biotechnology, Koneru Lakshmaiah, Education Foundation, Guntur, Andhrapradesh, India- 522502

A B S T R A C T

Dengue viruses are infectious agents that can replicates only within a living host cells. The advanced cell culture techniques are more appropriate methods to cultivate any viruses in laboratory conditions. By using these cell culture methods, viruses can be produced certainly in high titres. In biotechnological applications, adherent and monolayer cell cultures are using extensively to grow the viruses. Based on the literature, the viruses are non-susceptible to some kind of cell substrates, which results in poor viral growth. Dengue virus serotype-2 (DENV-2) susceptibility is studied with Vero, CHO and HeLa cell lines. These cell lines are widely used in different viral products manufacturing and analytical methods for viral quantification assays. Based on the observed cytopathic effect on monolayer confluency and obtained viral harvest titers, it is concluded that Vero cell substrate is more susceptible to dengue virus (DENV-2) than the other two cell lines.

Keywords: Infectious disease, Virology, Cell cultures, Dengue virus, Cell susceptibility, Cell permissively

A R T I C L E I N F O

Corresponding Author
CM Anuradha
Assistant Profesor
Dept of Biotechnology, Sri Krishnadevaraya University, Anantapuramu, Andhrapradesh, India- 515003.
MS-ID: IJMPR4466

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C O N T E N T S

1. Introduction

Dengue is a viral disease, it is transmitted to humans through the bite of infected mosquitos mainly *Aides aegypti* and, to a lesser extent, *Ae. Albopictus*. Dengue virus (DENV) is a single-stranded positive-sense RNA virus, belongs to the family Flaviviridae, genus Flavivirus. Dengue has 4 serotypes, namely DENV-1, DENV-2, DENV-3, and DENV-4 that can develop dengue infections. One of the most common arbovirus infection causing dengue fever or dengue hemorrhagic fever incidences are increased dramatically in all over the globe and half of the

world human population now at high risk (Maria G. Guzman et al. 2015; Duane J. Gubler 1998).

Once dengue virus enter into the body, over the period of 2-7 days start showing the symptoms of high fever, severe headache, backache, joints pains, The most common symptoms of dengue are high fever for 2–7 days, severe headache, backache, joint pains, nausea and vomiting, eye pain and rash. Many cases observed dengue fever, further develops a severe and fatal form of disease called dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) symptoms include frontal headache, retro-orbital pain, myalgic's,arthralgia's, hemorrhagic manifestations, rash, and low white blood cell count. Many testing methods are available to confirm the dengue infection in humans, mainly antibody tests (IgM and IgG), PCR method and complete blood count (CBC). The effective vector control measures using current tools may help in prevention and control of epidemics of dengue virus infections (WHO S.No-60; Alhaeli A et al. 2016).

Viruses are pathogenic, obligate intracellular organisms. In general, all viruses' uses host cell machinery for synthesis of new viral particles. New technologies and scientific advancements in genetic engineering methods brought many approaches for viral detection, isolation and cultivation including PCR, immune assays, cell cultures methods etc. For dengue virus screening studies, novel cell culture techniques are considered more appropriate and sensitive. Numerous primary cell lineages and established cell lines are accessible for conducting viral in-vitro studies (Ali Hematian et al. 2015; Diane S. Leland et al. 2007).

Selection of cell lines is paramount importance in viral studies, since appropriate cell line and virus combination will allow optimal growth of viruses in the study. In this study, to understand the dengue virus serotype-2 optimal growth in cell lines, a known quantity of virus is inoculated in Vero, CHO and HeLa cell lines of adherent monolayers and infected cells are incubated for up to 7 days. The virus titer is quantified using plague assay, dye staining method (Frank Fenner et al. 1987).

2. Materials and Methods

Cell culture

Cell culture medium plays a vital role in providing appropriate environmental support for optimal cell growth and its function. Eagle's minimal essential medium (MEM), pH 7.0, composed with advanced formulations, which supports the wide range of cell lines cultivation. Medium contains Earle's salts with a bicarbonate/carbonic acid buffering system, 10% heat inactivated FBS, L- glutamine, Glucose, Non-essential amino acids and vitamins as used as regular cell culture applications. Media is included in phenol red as a pH indicator; color may change depending on CO_2 environment. Freshly prepared and filter sterilized media are used in cell cultures passages. Cell cultures of Vero, CHO and Hela cells are adapted to tissue culture flasks (TCF) by using growth medium, cells are maintained sequential sub-culturing when the monolayers reach more than 80% confluence. Trypsin with EDTA (0.25%) used to detach the cells from monolayer surface (Optically clear virgin polystyrene). To achieve faster cell monolayer confluence, actively proliferating cells are seeded with higher cell densities (approximately 0.6 to 1.0 x 10^5 cells/mL) to fresh culture flasks with > 90% viability(Corning® 175cm² U-shaped angled neck with vent cap). Cells were incubated in optimal environment conditions (pH-7.2, CO_2-5%, and temperature 37 °C and humidity-70%) for 3-5 days. To protect the cells from contamination, pre-sterile materials, sterile reagents are used and while handling the cells, good personal hygiene & aseptic conditions are maintained in the biosafety cabinet. Monolayer confluence and viability of cells are observed visually using an inverted phase contrast microscope. The healthy cells shall be attached to the bottom surface of the flask, morphologically cell are elongated in shape and refracting light around their membrane. Monolayer confluence depends on the cell seeding densities, doubling time and incubation hours; confluence is analyzed by checking the ratio of cells occupied the surface area of culture flasks. Cell culture experiments were conducted in the Biosafety level -3 facilities (BSL-3 facility) at Department of Virology, Sri Venkateswara University, Tirupati, India.

Dengue virus (DENV-2) proliferation

Clinically diagnosed patients with acute dengue virus infection (NS1 ELISA kit method) serum samples are collected from Anantapur Hospital, Anantapurumu, Andrapradesh, India. All samples are packed and transported with appropriate procedures in line with standard guidelines of clinical samples handling and management. Further, clinical samples are investigated and viral proliferation studies using cell cultures are conducted in the Biosafety level -3 facility (BSL-3 facility) at Department of Virology, Sri Venkateswara University, Tirupati, India.

Dengue virus serotyping has been done using RT-PCR method andresults found to be Dengue virus serotype-2 (DENV-2). Further, Dengue virus serotype-2 from clinical sample is adopted to cell cultures and propagated in Vero cells, CHO cells, and HeLa cells. The DENV-2 viral inoculum is prepared with concentrations of 0.1% MOI and virus is inoculated to Vero cells, CHO cells, HeLa cells with 80% confluences in individual tissue culture flasks. For virus proliferation and optimum growth, infected cells are incubated for 7 days. During incubation, virus particles synthesized and are released into the cell culture medium supernatant. Host cell debris is separated by filtration and viral harvest is aseptically filtered into sterile containers using sterile grade filters; sterile viral filtrate is stored at -80°C deep freezer. The viral harvest samples are tested for dengue virus titers using plaque assay method.

Plaque assay (dye staining)

This assay is used for the enumeration of viral particle in the sample by counting the number of plaques formed on the Vero cell (Pedro Henrique Carneiroet al.2022). Briefly, the Vero cells were grown in T75 cm^2 on serum free

medium (SFM) and the confluent monolayer from the flask is trypsinized and used for preparing the 6-well tissue culture plates. The plates were seed with 3 ml of Vero cells at the density of 80000 cells/ml. The plates were incubated at 36 ±0.5 ˚C for 48 hours. Once the 90% monolayer formed in individual wells of all the plates, cells are ready to be infected.

Virus dilution

The virus (sample and working standard) were diluted in SFM medium in serial tenfold (10^{-1} to10^{-6}) dilution. All the operations performed on wet ice and immediately removed to – 70 ˚C.

Addition of virus to the cells

The 6- well plates were removed from the incubator and labelled properly; the contents of individual wells were decanted in a sterile container in the laminar hood. To the cells, 100 µl of individual dilution of virus sample added and small (500 µl) amount of medium also added to each well to prevent dryness of the cells during adsorption period. The plates were incubated for 75 minutes for virus attachment. After the attachment of the virus to the cells, agarose overlay (Melt 1.8% low melting agarose in oven and cool to 35-37 ˚C add equal amount of SFM medium containing 4mM L- glutamine and mix well) is added to each well to immobilize the movement of virus on the cell monolayer. The plates were sealed and incubated at 36 ± 0.5 ˚C under 5% CO2. The plates were observed daily for the changes in the morphology of the infected cells.

Staining of the plaques

After 5 days of incubation, the plates were removed and 1-ml of formal saline is added to all the wells, left for two hours at room temperature. The agarose was gently removed from all the wells, and the monolayers at the bottom were stained with 1.0% solution of crystal violet. The plaques appear as a clear halo against a blue background. Count the number of plaque at the highest dilution of the virus and calculate the titer as below.

Calculation:

-No of plaques x 10 x dilution factor

-The virus titer of the sample was expressed as ‘no of PFU/ml’.

3. Results and Discussion

The dengue virus infected Vero cells, CHO cells and HeLa cells showed more or less similar kind of cytopathic effect. Post viral infection, infected cells undergo, stress condition due to viral components' synthesis (viral proteins, Nucleic acids and membrane) occurs inside the cells which utilizes intracellular mechanism of host cells. This viral invasion induces the biochemical changes and morphological changes. The first sign of infected cells start with structural changes, multipolar elongated cells turn to round cells. The development of these changes in infected cells during incubation, leads to cell synchronization, slowly cells are detached from the monolayers; these changes are called cytopathic effect (CPE). The increased virus-cell interaction initiates enhanced onset of cytopathic effect due to high multiplicity of viral infection during incubation period (P.N. Sirisena et al. 2016).

Initially, infected cells show very little or no CPE, after 2 days of incubation slowly increases abnormality of cell appearance. At incubation period 5-7 days, it reaches the shift and cell monolayer completely destructed. The 80% of Vero cells become rounded and detached from the monolayer on 5th day, whereas HeLa and CHO cells showed on 6th day. On day-7, almost all the cells are plead out from monolayer and necrotic cells and cell clumps are floated in the media supernatant. Monolayer in control flasks observed 100% cell confluence of elongated cells with clear supernatant medium.

All three viral harvest titres are quantified using plaque assay method; results are found high titers in Vero cells when comparative with CHO and HeLa cells (Pedro Henrique Carneiroet al.2022). These virus titers results also co-relate with the CPE's observed on fifth day, Vero cells showed early CPE comparative with other two cell lines. This is happened due to higher infectivity rate of host cells resulted in early CPE and attained high viral titers even though same concentration of virus inoculum (0.1% MOI) was used. It is also needed to be considered factors are influencing the viral growth in cell cultures which includes variabilities in viral sample stability, process conditions, viral infection process and accuracy of plaque assay method.

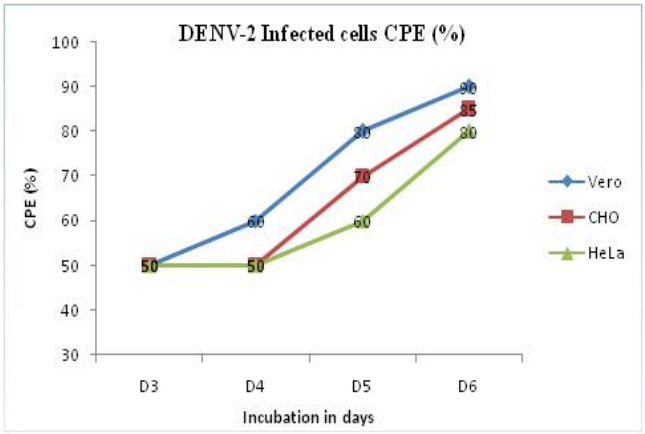

Figure-1: DENV-2 virus infected cells CPE (%) on D3 to D6

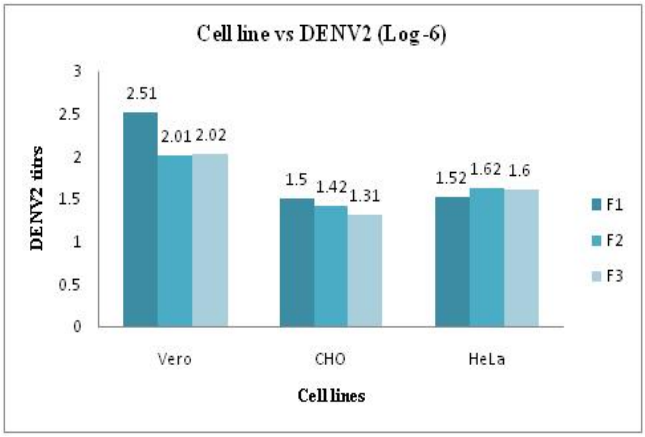

Figure-2: DENV-2 virus titers produced in different cell substrate

Table1.Optimal process condition for cell cultures

Parameters	Limit
pH	7.0–7.4
CO_2	5–10%
Temperature	35–37 ˚C
Humidity	60-78%
Osmolality	280–320 mOsmol/kg

Table.2 Key information of cell lines used in this study

Description	**Vero**	**CHO**	**HeLa**
Origin	Kidney cells	Chinese Ovary	Cervix
Species	African green Monkey	Hamster	Human
Morphology	Epithelial cells	Epithelial cells	Epithelial cells
Culture type	Adherent	Adherent	Adherent
Doubling time (hr)	22-24	14-17 hours	33-35
Incubation	3 days	3 days	3 days
Confluency	80%	80%	80%

Table.3 Cell lines vs DENV2 Log $_6$ titers

	F1	**F2**	**F3**	**CF**
Vero	2.51	2.01	2.02	-ve
CHO	1.5	1.42	1.31	-ve
HeLa	1.52	1.62	1.6	-ve
Average value	2.18	1.41	1.57	NA

Table-4: DENV-2 virus infected cells CPE (%) on D3 to D6

	D3	**D4**	**D5**	**D6**
Vero	LT 50%	60%	80%	90 %
CHO	LT 50%	50%	70%	85%
HeLa	LT 50%	50%	60%	80%

4. Conclusion

Based on the study results, Dengue serotype -2 (DENV-2) virus can be proliferated in Vero cells, CHO cells and HeLa cells. These cell lines are useful for DENV-2 virus isolation from clinical samples containing very low viral titers, which is required for additional viral characterization studies. This experiment is also demonstrated that virus growth susceptibility in cell cultures using with similar process conditions. Further, cell line specific viral infection process and its process variabilities optimization can be will helpful to produce the high viral titers in short time.

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Conflict of Interest

The authors declare no conflict of interest.

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