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Viability of dengue virus in culture stocks is efficiently preserved by storage in diluted forms

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Abstract. Storage of dengue virus (DENV) culture stocks in -80°C is a common laboratory practice to maintain the viability of the virus for long-term usage. However, the efficiency of this method could still be hindered by multiple factors. In our laboratory, we observed a constant and substantial deterioration in the titer of DENV in Vero culture supernatant stored in -80°C. Such incident had badly hampered the laboratory work and prompted an investigation to determine the cause. DENV isolates representing all four serotypes were propagated and the culture supernatants were harvested and stored in aliquots of original stock and 10 fold dilutions (10^{-1} -10^{-4}). DENV titer in these stocks was determined prior to storage and reassessed on the third and sixth month of storage by focus forming unit assay (FFUA). The result demonstrated a constant preservation of titer ranging from 10^4 ffu/ml to 10^5 ffu/ml in the diluted DENV virus culture stocks of 10^{-1}, and 10^{-2} of DENV1-4, a minor reduction of titer from 10^3 ffu/ml to 10^2 ffu/ml at dilution 10^{-3} for DENV4 only and complete deterioration in undiluted culture stock and lower dilution (10^{-4}) within 6 months of storage in -80°C for all serotypes. It is recommended that propagated DENV in Vero cells are stored in 10 fold dilutions as compared to the original form to preserve the titer for long-term usage.

INTRODUCTION

Storage of propagated viruses in laboratories requires critical attention to ensure the viability of the viruses is maintained. DENV is a RNA virus, thus need to be stored at -80°C for long-term usage. However, appropriate storage temperature alone does not safeguard the stability of the viruses. Other external factors such as type of storage tubes (Jarvis *et al*., 1994), repeated freeze and thaw (Greiff *et al*., 1954) and presence of inhibitors in culture media (Peetermans *et al*., 1996) also play role in influencing the stability of propagated virus upon storage. DENV can be easily isolated from patient serum by inoculation into cell lines such as C6/36 or Vero (Phanthanawiboon *et al*., 2014). Even though commercially available culture media is invented to suit the growth of virus in *in-vitro* environment, nonetheless some adverse effect caused by growth-enhancing substances has been reported. The negative effect of ammonia due to degradation of L-glutamine which is an important substance in certain culture media has been reported (Schneider *et al*., 1996).

Deterioration in the virus titer during storage is a serious issue that can manipulate the consistency of experiment results. In our laboratory, we observed a substantial deterioration in the titer which occurred within just three months of storage. This propelled us to screen all DENV culture

stocks in our storage and investigate the cause of the decline in the virus titer upon storage in -80°C.

MATERIALS AND METHOD

Vero cells (ATCC, CCL-81), were grown in a 25 cm^2 tissue culture flask (TPP, Switzerland) and maintained in Minimum Essential Medium with Earle's Balanced Salts (MEM, Hyclone, USA) supplemented with 10% Fetal Bovine Serum (FBS, Biowest, France). Growth condition was constant at 37°C with 5% carbon dioxide (CO_2) until a monolayer of cells is achieved. DENV clinical isolates representing all four serotypes were inoculated and propagated in Vero cells supplemented with 2% FBS in MEM. For each batch of virus propagation, uninfected Vero cells as control was included. The day of virus harvesting was determined when 50% cytopathic effect (CPE) was observed and culture stocks were obtained by overnight freezing at -80°C followed by centrifugation at 610g for 10 minutes.

DENV titer was determined in each passages by Focus Forming Unit Assay (FFUA) as described (Cruz *et al.*, 2007). Briefly, 100 µl of virus stocks was inoculated in 1 x 10^5 Vero cells per well in a 24 well plate (TPP, Switzerland). After incubation for one hour and removal of unabsorbed viruses, each well was overlaid with 1 ml of 1.5% Carboxymethylcellulose (CMC) in 2% MEM. The plate was incubated at 37°C with 5% CO_2. On 72 hours post infection (72 hpi), the CMC was removed and the wells were washed twice with 1 ml of Phosphate buffered saline (PBS). All washing procedure in between each step hereafter were performed using non-sterile 1x PBS. Subsequently, the infected cells were fixed with 300 µl of 4% Paraformaldehyde Phosphate Buffered Saline (Nacalai Tesque, Japan) for 30 minutes and cell permeability was induced for 15 minutes with 300 µl of 1% Nonidet P-40 Substitute (Amresco, USA). Blocking was performed with 300 µl of 3% skim milk (Sigma-Aldrich, USA) for 1 hour followed by incubation with 200 µl of the primary mouse monoclonal antibody against DENV 1-4 envelope protein (GeneTex, Japan) and then with 200 µl of secondary anti-mouse IgG antibody (HRP) (GeneTex, Japan), each at 37°C for 1 hour. In the final step, colour development was performed by adding 200 µl of Peroxidase Stain DAB (Nacalai Tesque, Japan) and incubated for 15 minutes at room temperature. Visible foci were viewed and counted using a Stemi DV4 stereomicrosope (Zeiss, Germany). One focus forming unit is recognized as a cluster of cells stained as light brown under the microscope and virus titer is expressed in ffu/ml.

Next, 5 ml of the harvested DENV culture supernatant with sufficient and known virus titer were stored in -80°C, as undiluted aliquots as well as 10 fold diluted 1 ml aliquots (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) in 1.5 ml centrifuge tubes. Dilutions were made with serum free media (basic MEM without FBS). These culture stocks were assessed for virus titer prior storage and indicated as month zero and reassessed for virus titer on the third and sixth month after storage by FFUA. The FFUA was performed in duplicates for each culture stocks as described earlier.

RESULTS

It was found that DENV titer was well preserved in virus stocks (culture supernatant) stored at particular diluted forms rather than undiluted forms (Table 1). Monitoring of virus titer up to 6 months demonstrated a constant titer in the diluted virus stocks of 10^{-1} and 10^{-2} and a minor reduction at dilution 10^{-3} for all serotypes with exception of 1 log drop in all diluted forms of DENV 4. At dilution 10^{-1} and 10^{-2}, the foci formation for majority of the isolates were too numerous to count indicating higher titer and consistently observed throughout the monitoring period. On the other hand, approximately 99.9% drop in virus titer was observed in the undiluted stocks of DENV 2, DENV 3 and DENV 4 within 3 months of storage and complete deterioration for all serotypes within 6 month of storage. Similarly, up to 100% decline in virus titer

Table 1. Titer of DENV stocks monitored up to 6 months of storage

Isolates	Serotype	VIRUS TITER (FFU/ML)		
		Month 0*	Month 3	Month 6
D1 P6D5	DENV 1	5.5×10^6	1.0×10^4	0
D1 (-1) P6D5		5.5×10^5	4.7×10^5	2.0×10^5
D1 (-2) P6D5		5.5×10^4	4.7×10^4	2.0×10^4
D1 (-3) P6D5		5.5×10^3	4.7×10^3	2.0×10^3
D1 (-4) P6D5		6.9×10^2	1.0×10^1	1.0×10^1
D2 P6D5	DENV 2	9.7×10^5	1.6×10^2	0
D2 (-1) P6D5		9.7×10^4	7.6×10^4	5.7×10^4
D2 (-2) P6D5		9.7×10^3	7.6×10^3	5.7×10^3
D2 (-3) P6D5		9.7×10^2	7.6×10^2	5.7×10^2
D2 (-4) P6D5		2.1×10^2	2.0×10^1	1.0×10^1
D3 P6D5	DENV 3	7.7×10^5	5.0×10^1	0
D3 (-1) P6D5		7.7×10^4	7.1×10^4	2.5×10^4
D3 (-2) P6D5		7.7×10^3	7.1×10^3	2.5×10^3
D3 (-3) P6D5		7.7×10^2	7.1×10^2	2.5×10^2
D3 (-4) P6D5		3.7×10^2	0	0
D4 P5D5	DENV 4	1.8×10^6	2.4×10^2	0
D4 (-1) P5D5		1.8×10^5	3.1×10^4	2.0×10^4
D4 (-2) P5D5		1.8×10^4	3.1×10^3	2.0×10^3
D4 (-3) P5D5		1.8×10^3	3.1×10^2	2.0×10^2
D4 (-4) P5D5		4.7×10^2	1.0×10^1	0

Note: *Month 0 indicates the titer of DENVs' assessed prior to storage.
The initial titers for undiluted stocks for month 0 and (-1) and (-2) dilution for month 0-6 were too numerous to count. Therefore final titer was back-calculated from titer (-3).
P6D5 = passage 6 harvested at day 5; Dilution (-1) to (-4) = dilution 10^{-1} to 10^{-4}.

was detected at higher dilution of 10^{-4} within 6 months of storage. These aforementioned observations were shown in Fig. 1.

DISCUSSION

The ability to store viruses for long periods of time with minimal loss of viability is critical to ensure sufficient virus stocks are available for subsequent research. The observation from the present study pertinently indicated that DENV stocks propagated from clinical source are best stored in diluted forms rather than original stocks. The pH of the suspension medium is a probable factor that could have contributed to depletion of virus viability. Culture media's pH is commonly near neutral and decreases after cells are grown for few days due to presence of lactate. It is evident that exposure to pH values lower than sub-neutrality induces a change in flavivirus particles and interrupts with membrane fusion, which leads to loss of virus infectivity (Gollins *et al*., 1986; Guirakhoo *et al*., 1993). Another study confirmed 70% loss of DENV2 infectivity when virus were grown in a culture media at pH6.8 compared to pH8 at 4°C (Manning *et al*., 1979). In this study, the virus stock was diluted with fresh media which is at near neutral therefore this will increase the pH of the harvest virus supernatant. Hence, dilutions of the virus stock could help in mitigating the effect of acidic pH on the virus.

Another possible explanation to this observation is that concentration of FBS added to the media for propagation could have also affected the stability of the virus. During the virus infection stage, 2% FBS was added to the media and maintained until the day of virus harvesting. Therefore, the DENV culture stocks also contained some amount of FBS. A previous study observed loss of rotavirus infectivity when virus stock was added with 2% FBS before infection or when FBS was added after host cells were infected with virus. (Graham *et al*., 1980). Hence, when

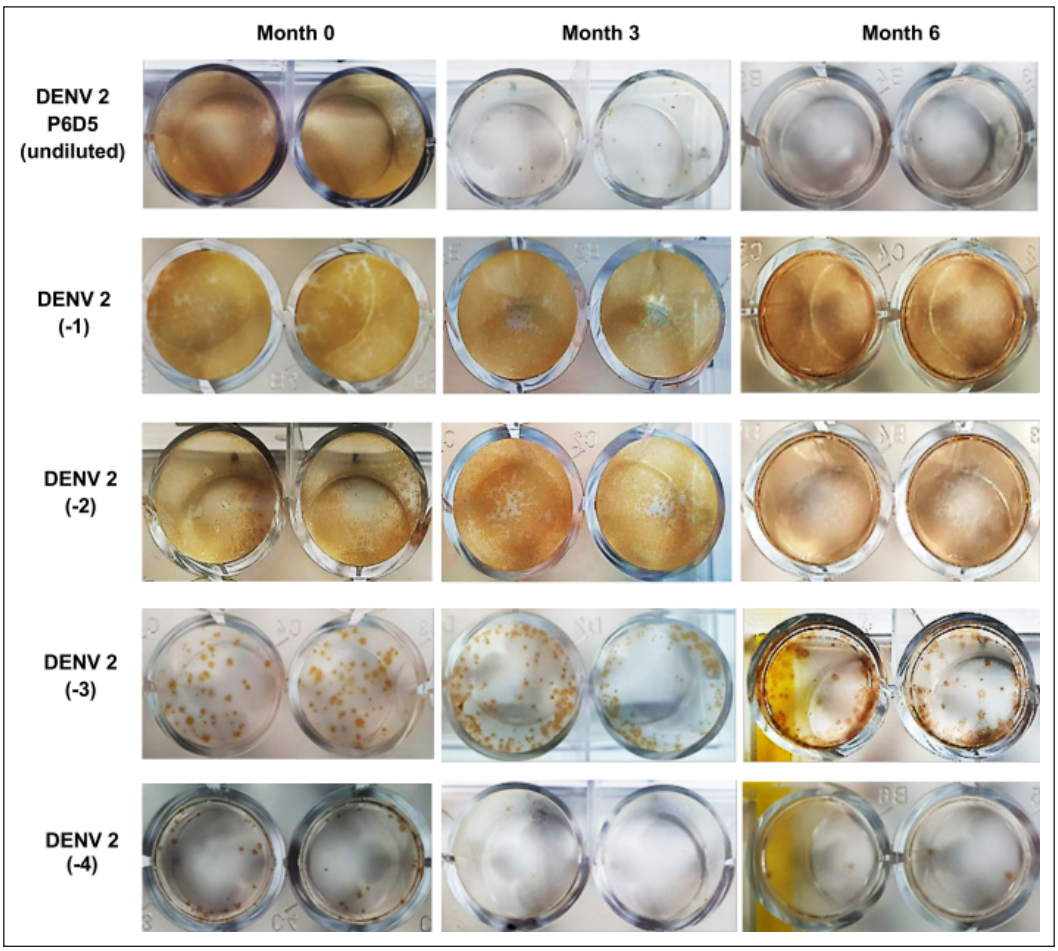

Figure 1. The representative FFUA result indicating DENV 2 titer based on foci count which was monitored up to 6 months of storage. Month 0 indicates the titer of DENVs' assessed prior to storage.

the original stocks were diluted, the concentration of FBS reduced, and the negative effect on viability of virus became nil. This coincides with the observation that infectivity of DENV was preserved in diluted stocks of 10^{-1} till 10^{-3} up to 6 months of storage.

Current study also demonstrated that undiluted viruses kept losing the infectivity throughout the third month, but the effect was pronounced at the sixth month. This shows that FBS may not have immediate effect on the viruses but rather time-dependent. However, viability of the virus in dilution 10^{-4} was also not preserved. This could be due to the low content of viable virus in such dilution hence, the depletion due to the freezing and thawing effect is unavoidable.

As much as the role of FBS in promoting cell growth is acknowledged, however FBS has been reported to inhibit virus entry into the cell, therefore virus stocks are recommended to be diluted prior to infection (Szretter *et al.*, 2006). A study on Hepatitis C virus (HCV) showed that high titer infectious HCV was produced from serum free media compared to media with serum as serum free condition has enhanced viral release (Mathiesen *et al.*, 2014). Another study demonstrated that FBS blocks HCV infection in a dose-dependent manner, however the FBS component responsible to cause such effect was not investigated (Qin *et al.*, 2013). The study interestingly highlighted that FBS inhibits the attachment of HCV to host cells, but it does not affect the post-binding steps

that occur after HCV has entered the cells. This also explains the reason for using the recommended 10% FBS in media for cell growth and a lower percentage of 2% FBS in media after virus infection. We suggest that, FBS does not destroy the virus directly in frozen state, but it has some adverse effect on the pathogenicity of dengue virus at various stages such as virus entry, attachment or even maturation of the virus. One possible mechanism could be that FBS derived proteins may have bound non-specifically, blocking the virus binding to the host cell after being thawed from frozen state and affecting the subsequent replication and release of infectious virus. This could possibly explain our observation of preservation of infectivity of diluted virus in this study. When diluted, the virus infectivity is preserved because the FBS derived proteins or other possible inhibitors are diluted to a concentration much lower which is about 0.2% - 0.002% regardless the concentration of the virus in this study. This implies concentration of virus does not play a role in preserving the infectivity of the virus. In this study, we observed 10^{-1} and 10^{-2} are appropriate dilutions to minimize the adverse effect of FBS derived proteins and allow the attachment of DENV to host receptor.

As a recommendation based on the findings of the present study, DENV stocks are preferably diluted to 10 folds (10^{-1} & 10^{-2}) prior to long-term storage in -80°C. Additionally, washing the cells thoroughly with PBS prior to virus infection is pivotal to remove remnants of FBS from the media which is used to propagate cells.

CONCLUSION

The study described a method for preservation of DENV titer in Vero culture stocks for long-term usage. The elaborated method would benefit as a simple and inexpensive way to solve issues related to deterioration of virus titers in culture stocks.

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