ORIGINAL RESEARCH ARTICLE

Effect of Lyophilization on Infectivity and Viral Load of Adenovirus

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Abstract

Freeze drying (Lyophilization) performed at temperature and pressure below the triple point is being practiced for the preservation of virus stocks for longer periods. The present study is aimed to lyophilize adenovirus strain to study its effects on infectivity and viral load. In-house adenovirus reference strain (stock virus) was propagated in Hep-2 cell line in 25cm^2 cell culture flasks. In 24-well plates the serial dilutions of stock virus from 10^{-1} to 10^{-7} (100µl inoculum) was inoculated in each well with Hep-2 cells for $TCID_{50}$ titer and viral DNA was extracted separately to determine viral load by Taqman Real Time PCR. Stock virus was lyophilized in 3 lots and stored at RT (25±2°C) and 4°C separately for 1, 4 and 6 months and subjected to $TCID_{50}$ (for viral infectivity) and viral load assay (for total viral genome copies). Following lyophilisation and storage of adenoviral strains at RT and 4°C separately did not affect significantly on the viral stability, infectivity as well as viral copy number till 4 months. However, storage at RT for 6 months resulted in 1 log reduction in viral copy number. Thus, storage of even lyophilized virus stock would necessitate a temperature of at least 4°C for prolonged periods. The present study could successfully lyophilize adenovirus and retain its infectivity over a period of 6 months when stored at RT and 4°C. No significant difference in the infectivity or $TCID_{50}$ titer was observed in the lyophilized virus as compared to the stock virus. However, the viral load was observed to increase with lyophilization of the virus over 6 months when stored at 4°C which possibly is due to the concentration of the virus on freeze-drying.

Keywords: Adenovirus, lyophilisation, Physical stability, Formulation

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Introduction

The structure and function of organisms change and get lost with time, as in laboratory cultures. Attempts to stop the biological clock have been conjured by ancient and modern minds; and the heart of many such schemes has been experiments with temperature and water content. Whereas refrigeration technology provides a means of slowing the rate of deterioration of perishable goods, the use of much lower temperatures has proved a means of storing living organisms in a state of suspended animation for extended periods.

Adenoviruses (AdV), belonging to family Adenoviridae, are double stranded Deoxyribonucleic acid (DNA) viruses that carry DNA insert of size 7kb. 52 subtypes of adenoviruses are known to infect humans [1]. Adenoviruses are frequent cause of human mucosal surface, infections particularly in pediatric population and can be responsible for ocular and gastrointestinal illnesses in humans. To preserve maximum infectivity for long periods, cell culture-grown adenovirus must be stored frozen at very low temperatures [2]. However, this makes its handling difficult. The removal of water from viable biological material in the frozen state (freeze-drying) provides another means of arresting the biological clock by withholding water, and commencing again by its addition. Lyophilization or freeze-drying is a controllable method of dehydrating labile products by vacuum desiccation [3]. It is considered advantageous to freeze dry viruses and vaccines wherever possible in order to reduce their volume for storage in cold, to enable easy handling and transport, and to enhance their keeping quality [4].

Materials and Methods

Cell Line and Virus Stock Preparation

A confluent monolayer (90%) of the Hep-2 cell lines were grown in 25mm² presterile disposable NUNC tissue culture flask using (full form?)MEM with antibiotics (penicillin, streptomycin) and 10% fetal calf serum (FCS). Adenovirus reference strain isolated from Department of virology was propagated for virus stock preparation in Hep2cell line and used for $TCID_{50}$ assay and viral load.

Virus Titration by Tissue culture infectious dose 50 ($TCID_{50}$) Assay

Hep2 cell monolayer was prepared and virus infection was performed at different dilutions in 10ml sterile

tissue culture tubes. Cell monolayer was observed daily till the cytopathic effect (CPE) appeared, along with the control wells (MEM inoculated) kept under the same condition. $TCID_{50}$ titer was be calculated by using Reed and Muench [5].

Extraction of viral DNA

Viral DNA was be extracted from 200µl of tissue culture lysate using RNeasy Minikit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions. In brief, 200µl of AVL Buffer containing 20µl of carrier RNA were dispensed in 1.5 ml of micro centrifuge tube. 200µl of tissue culture fluid was added to the Buffer AVL containing proteinase K into micro centrifuge tube and is mixed by vortexing for 15 sec. The suspension was incubated in the dry temperature bath (56°C) for 10 minutes followed by the centrifugation. After adding 200µl of absolute ethanol, the sample was vortexed for 15sec and centrifuged briefly. Carefully 620µl of the solution was be applied to the QIAamp Mini column with 2ml of collecting tube without wetting the rim. The cap is closed and centrifuged at 6000g (8000rpm) for 1 min. The QIAamp Mini column is placed into the 2ml of clean collecting tube and the tube containing filtrate were discarded. The QIAamp Mini column were opened carefully and 500µl of AW1 Buffer were added to the QIAamp Mini column and centrifuged at 10000g for 1 minute. Followed by the replacement of new 2ml of collection tube and the tube containing filtrate were discarded. 500µl of the AW2 Buffer was added to the column and centrifuged at 20000g for 3 minutes. Followed by the replacement of new 2ml of collection tube and the tube containing filtrate were discarded and centrifuged at full speed for 1 minute. Finally the QIA amp mini column was placed in a Sterile, DNAase, RNAase, free 1.5ml of the micro centrifuge tube. The old collecting tube containing the filtrate was discarded. 50µl of AVE Buffer was added to the QIAamp Mini column and were equilibrated to room temperature for 1 min followed by centrifugation at 8000g for 1 min.

Competent cells preparation, Ligation reaction and Plasmid isolation

DH5-α, a non-pathogenic strain of E.coli was used for the purpose. It were grown overnight in 100ml conical flask containing Luria broth (LB). The overnight bacterial culture was diluted 1/200 to 25 ml of SOC media in 10X culture volume flasks (25 ml in 250 ml flask). Flask containing bacteria was grown to early log phase in shaker incubator. The growth of the culture was measured at an interval of 30 minutes until an OD of 0.4 at 600nm were obtained. The cells were collected by centrifugation at 5000g for 5 min in cold centrifuge (4°C) and were kept on ice in all the further steps. The cells were re suspended in 12 ml of 0.1M ice cold $CaCl_2$ and placed on ice for minimum 30 minutes. The cells were centrifuged at 5000g for 10 min and the pellet was collected. The pellet was further re suspended in 1/10 volume of 0.1M $CaCl_2$. Cells were ready to use as competent cells for the transformation experiment. Ice cold sterile glycerol was added to final concentration of 10% (v/v) with a gentle mixing. Aliquots of 100µl of cells were made and stored at -70°C until further use. The tube containing the pGEM-T easy vector and the control insert DNA tubes were centrifuged briefly to collect the content at the bottom of the tube. The 2X rapid ligation buffer was vigorously vortexed before use. Ligation reaction was prepared and reaction mixture was mixed properly by pipetting and was incubated for 30 min at 4°C for optimal ligation. Two µl of the ligation reaction were added to the eppendorf tube containing 100 µl of the competent cells. The transformation tube containing ligation reaction mixture and competent cells were given heat shock at 42°C for 40-50 second and was immediately returned to the ice bucket. 950µl of the SOC media containing antibiotic was added to the above tube and were incubated in a shaking incubator for 1.5 hrs at 37°C with shaking (~150 rpm). 100 µl of the transformation culture was plated onto duplicate LB/ampicillin/IPTG/X-Gal plates. The plates were incubated overnight at 37°C and were screened for blue and white colonies. The white colonies were the desired insert. To facilitate the blue color development, plates were stored at 4°C (after 37°C overnight incubation). Finally the white colonies, supposed to contain the vector with the desired insert were selected and were grown in 10 ml of LB containing 100 µg/ml Ampicillin, overnight in a shaker incubator at 37°C at speed of 250rpm. The bacterial cells grown in the LB medium by addition of antibiotic were harvested by centrifugation at 6000g for 15 min at 4°C. The cell pellet was re-suspended in 0.3 ml of buffer P1. 300µl of buffer P2 was added and mixed thoroughly and vigorously and were incubated at room temperature (15-25°C) for 5 min. 300µl of chilled buffer P3 was added followed by mixing vigorously inverting the tube (8-10 times) and were incubated on ice for 5 min. Mixture was

centrifuged at 10,000g in a micro centrifuge for 10 min. The supernatant containing the plasmid DNA were aspirated out carefully. During the mean time QIAGEN-tip 20 was equilibrated by applying 1ml of the buffer QBT, and was allowed to empty the column by gravity flow. The supernatant obtained after centrifugation was applied to the QIAGEN-tip 20 and allowed to enter the resin by gravity flow. The QIAGEN-tip 20 were washed with 2x2 ml of buffer QC, Followed by elution of DNA with 0.8ml of buffer QF. The eluted DNA was precipitated by adding isopropanol at room temperature. (0.56ml per 0.8ml of elution volume). The mixture was be then mixed and centrifuged immediately at ≥ 10,000rpm for 30 min in a micro centrifuge at 4°C and the supernatant was be decanted carefully. The residual DNA pellet were washed with 1ml of 70% ethanol at least thrice by centrifuging the pellet at 10,000rpm for 10 min each. The supernatant was aspirated carefully without disturbing the pellet. The pellet was air dried for 5-10 min, and was re-dissolved in 100µl of TE buffer, pH 8.0.

Virus revival, titration and Viral load in lyophilized adenovirus

Lyophilized virus was revived in sterile conditions using sterile double-distilled water. The virus ampoule was broken with a clean metal rod in one strike at the neck region of the ampoule in the biosafety cabinet. 1ml sterile water were added to the dried virus and mixed by pipetting atleast 20 times. The revived virus was kept at 4°C for 2h so as to acclimatize virus. The virus was aliquoted and further stored at -70°C. To determine the effect of lyophilization on virus titer the lyophilized virus after revival was titrated in Hep-2 cell line and virus titer was determined by $TCID_{50}$ and PFU following the same procedure as described earlier for the stock virus. To determine the effect of lyophilization on adenoviral load the lyophilized adenovirus was be subjected to Real Time PCR as described earlier and the Ct values obtained was extrapolated with standard curve to estimate the viral load.

Statistical Analysis

Statistical analysis of significance was undertaken by a paired sample t-test using SPSS/ QQ Graph Pad Prism software with a value of $P<0.05$ for significance.

Result

Confirmation of Stock Virus

Adenovirus reference strain (kindly obtained from Department of Virology, PGIMER, Chandigarh) was successfully propagated in (Hep-2) cells. Cytopathic effect (CPE) positive cell culture bottles for adenovirus infected cells showed cell grapening, clustering, rounding, clumping and focal dislodging of cells in comparison to control mock infected cells were seen on third to fourth day post inoculation. CPE positive cells were scrapped with a sterile cell scrapper and subjected to Immunofluorescence using polyclonal antisera for AdV. It showed the characteristic intra nuclear brilliant apple green fluorescence in adeno infected cells. Adenovirus PCR was done in virus infected cell line where a fragment of 161bp was amplified from hexon gene of AdV genome and visualized by 2% agarose gel electrophoresis (**Figure 1**).

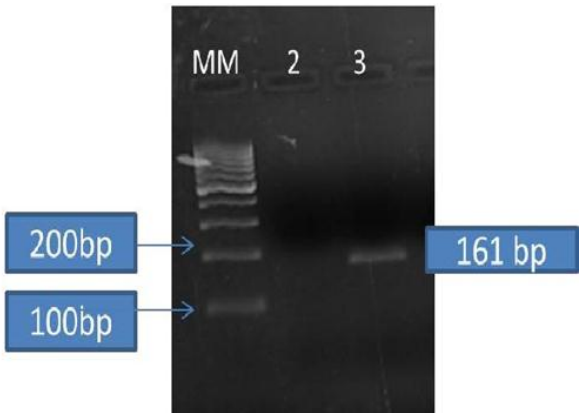

Figure 1: Agarose gel analysis of adenovirus PCR; lane1: 100bp Molecular marker (Fermentas, USA), lane2: negative control, lane3: AdV

These culture bottles were preserved at -80°C deep freezer and subjected to repeated freeze and thaw in three occasions for cell lysis. The supernatant was transferred to a sterile vial and centrifuged aseptically. The clear supernatant occasions for cell lysis. The supernatant was transferred to a sterile vial and centrifuged aseptically. The clear supernatant containing the virus particles was used as positive control for qualitative RT-PCR as well as preparation of standards in Quantitative Real Time RT-PCR.

Comparison of $TCID_{50}$ titer of lyophilized virus stored for different time intervals

The lyophilized virus was stored at room temperature (RT) and 4°C for 1month, 4 months and 6 months. The virus was revived after these fixed intervals of time and infected in Hep-2 to check the infectivity of virus and change in the Tissue culture infectious dose 50 ($TCID_{50}$)

titer. It was observed that lyophilized virus was infective till 6 months and there was no change in the $TCID_{50}$ titer of the lyophilized AdV revived after 1month to 6 months of storage at 4°C. In case of lyophilized AdV stored at RT the $TCID_{50}$ titer was observed to reduce from $10^{-4.5}$ at 1 month to $10^{-3.22}$ at 4 months but it remained constant to $10^{-3.22}$ after 6 months of storage (**Figure 2 and 3**).

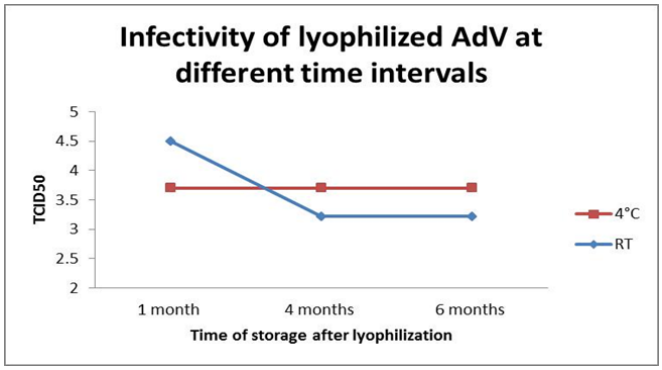

Figure 2: $TCID_{50}$ of lyophilized virus stored at RT and 4°C and revived after 1 month, 4 and 6 months. The value of $TCID_{50}$ on Y- axis corresponds to 10^{-y} of different sample of adenovirus.

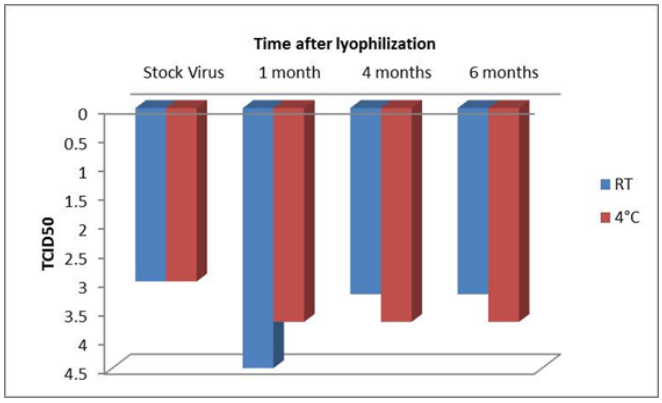

Figure 3: Comparison of $TCID_{50}$ in stock virus and lyophilized virus revived at different time intervals. The value of $TCID_{50}$ on Y- axis corresponds to 10^{-y} of different sample of adenovirus

Comparison of $TCID_{50}$ titer of lyophilized virus stored for different time intervals and stock virus

The $TCID_{50}$ titer of the lyophilized adenovirus stored at RT and 4°C for 1, 4 and 6 months was compared with that of the stock virus. The $TCID_{50}$ titer of lyophilized virus is summarized in **Table 1** whereas the $TCID_{50}$ titer of stock virus was observed to be 10^{-3}.

Table 1: Arrangement of data in calculation of TCID50 titer by Reed and Muench formula

Virus dilution	Infected	Non-Infected	Accumulative Values			
			Infected	Non–infected	Mortality	
					Ratio	Percent
10^{-1}	4	0	14	0	14/14	100
10^{-2}	4	0	10	0	10/10	100
10^{-3}	3	1	6	1	6/7	85.71
10^{-4}	2	2	3	3	3/6	50
10^{-5}	1	3	1	6	1/7	14.28
10^{-6}	0	4	o	10	0/10	0

No significant difference (p, 0.342) was observed in the $TCID_{50}$ titer or the infectivity of the lyophilized virus stored at 4°C for 1, 4 and 6 months as well as AdV stored at RT for 4 and 6 months as compared to the stock virus. The adenoviral load in stock and lyophilized adenovirus was correlated with $TCID_{50}$ titer as described in the **Table 2** using Spearman correlation coefficient. However, no statistical correlation was observed in the viral load and $TCID_{50}$ titer.

Table 2: Correlation of $TCID_{50}$ and Viral load in lyophilized and stock virus

Stock virus	$TCID_{50}$		Viral load by Real-Time PCR(Copies/ml) in 10^4	
	10^{-3}		1590	
	Stored at 4°C	Stored at RT	Stored at 4°C	Stored at RT
Lyophilized AdV revived after 1 month	$10^{-4.5}$	$10^{-3.7}$	2920	6810
Lyophilized AdV revived after 4 months	$10^{-3.22}$	$10^{-4.5}$	2220	7200
Lyophilized AdV revived after 6 months	$10^{-3.22}$	$10^{-3.7}$	672	2650

Confirmation of successful cloning

Hexon region of the viral genome was cloned in pGEM-T Easy vector (chloramphenicol resistance) and transformed in E coli DH5α. Cloned plasmid containing the AdV hexon insert had a defective LacZ gene and those cells are transformed with this vector formed white colony in ampicillin agar plate using JH7 and KW53 primers (**Figure 4**).

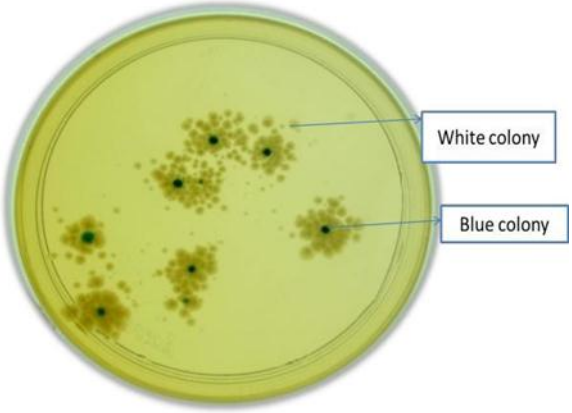

Figure 4: Plate showing Blue (vectors without inserts) and white (vectors with insert) colonies.

White colony from the plate, theoretically containing the desired inserts were picked and subjected to plasmid extraction. The plasmids isolated from the white colonies were subjected to PCR to yield 161bp bands in agarose gel. Polymerase Chain reaction (PCR) resulted in 161 bp amplicon with hexon region specific primer pair (**Figure 5**).

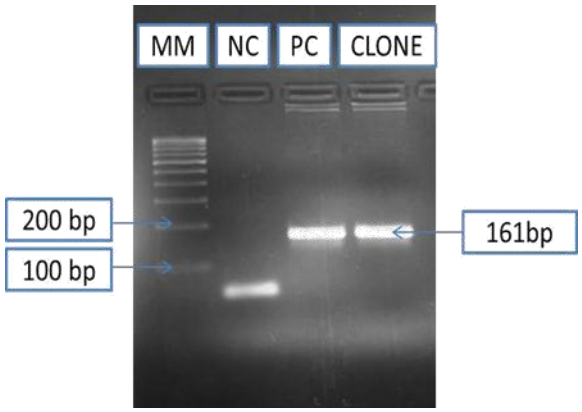

Figure 5: Conformation of successful cloning; lane1: 100 Base pair molecular marker (Fermentas, USA), lane 2: NC and 3: PC & lane 4: 161 bp band using hexon specific primer in AdV clone.

Comparison of Viral load of lyophilized virus stored for different time intervals

The effect of lyophilization on viral load of AdV over a period of time was studied. No significant difference was observed in viral load of AdV stored at 4°C for 1, 4 and 6 months.

However, in lyophilized virus stored at RT a significant difference in viral load was observed between the virus stored for 1month and that stored for 6 months at RT ($P<0.05$) but no difference was observed in viral load of AdV stored at RT for 1 and 4 months (Figure 6).

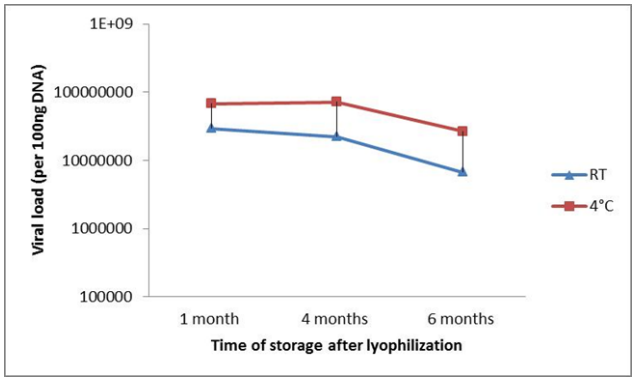

Figure 6: Viral load of lyophilized virus stored at RT and 4°C for 1 to 6 months

Discussion

Freeze-drying will not reverse the damage incurred prior to formulation and care must be exercised when selecting an appropriate cell type or technique used to culture or purify the cell or its extracts prior to freeze-drying. To sustain freeze-drying it is necessary to establish a pressure gradient from a sample (highest pressure), to condenser, and finally vacuum pump (lowest pressure) so that water migrates from the sample as drying progresses. In the present study adenovirus was successfully propagated in Hep-2 cells. The lyophilization cycle was standardized and the virus suspension was lyophilized at temperature below -40°C and under 50m Torr vacuum. The essence of the formulation exercise should be to minimize freeze-drying damage, loss of viability, or activity. This study considers the stability of adenovirus after freeze-drying and storage and also determines its titer and load. The infectivity of the virus was retained when stored for over 6 months at 4°C or RT. We determined the $TCID_{50}$ titer of the adenovirus lyophilized for 1-6 months and observed no significant difference in the infectivity when stored at 4°C or RT. Thus, the cell culture-grown adenovirus could withstand the freeze-drying process, and it should be stored at 4°C and RT to retain maximum infectivity. Many culture collections and gene banks insist on high recovery values prior to a protocol being adopted for regular use; 50% viability post thaw has been accepted in some culture collections as a nominal cutoff for adopting maintenance by cryopreservation alone (McClure et al., 2011). The ability to determine viral titer rapidly and at high accuracy is one of the most important tools desired when working with viruses in the research laboratory [7, 8]. Real-time PCR has been shown to be more sensitive than cell culture based techniques for detection of viruses in clinical specimens; it was therefore interesting to investigate whether real-time PCR technology could also be an important tool for rapid and efficient estimation of viral titer [9]. In the present study the viral load of stock and lyophilized adenovirus was determined and compared with each other. The viral load of the lyophilized virus stored at 4°C was observed to increase as compared to the stock virus whereas that stored at RT for 1 and 4 months the viral load increased slightly but at 6 months the viral load decreased as compared to the stock virus. There is evidence that a cryopreservation method yielding high initial recovery values, maintains viability at that level on prolonged storage. Titer determined using either by plaque forming unit (PFU) or endpoint dilution ($TCID_{50}$) assays, methods that are time-consuming and labor intensive [10]. As a complement to these

established methods of determination of virus titers, we here describe a Real-Time Quantitative PCR assay that enables rapid recording of viral load values that corresponds to titer measurements of viruses. However, the results presented here showed no significant relationship between Real Time PCR v/s $TCID_{50}$. Even though the $TCID_{50}$ technique is time consuming, need expertise but is capable of dealing with the infective/live virus which has the great importance in viral isolation, manufacturing live-attenuated vaccines, viral antigen and viral standards. Real Time PCR which detects both the infective and non-infective viral particles is highly sensitive and rapid, used most popularly in the viral diagnostic labs and has great importance in the early diagnosis and management by of the patient [9]. It should, however, be taken into consideration that viruses causing persistent infection that consequently would give a positive signal in real-time PCR cannot be converted to viral titer, as this would give a false impression that the virus is able to cause cytopathic effect. Our data also demonstrated that using real-time PCR technology is a reliable, rapid and robust method that corresponds to standard quantification methods used to measure viral titers. Thus, highly reproducible over a dynamic range of viral concentrations commonly used in research laboratories and could therefore save time in comparison with the traditional cell culture-based viral titration methods. The reproducibility of quantitative real-time PCR was higher than for $TCID_{50}$, which could be partially explained by the fact that real-time PCR does not require a manual estimation based on visual observations.

Conclusion

The present study could successfully lyophilize adenovirus and retain its infectivity over a period of 6 months when stored at RT and 4°C. No significant difference in the infectivity or $TCID_{50}$ titer was observed in the lyophilized virus as compared to the stock virus. However, the viral load was observed to increase with lyophilization of the virus over 6 months when stored at 4°C which possibly is due to the concentration of the virus on freeze-drying.

Competing Interest

The authors declare no competing interest.

Authors Contribution

Bimlesh Kumar Jha designed the experiment. Bimlesh Kumar Jha, Birendra Prasad Gupta, Prashanna Maharjan, Somila kakshapati and Nabin Narayan Munankarmi performed the cloning and transfection experiment. Bimlesh Kumar Jha, Prashanna Maharjan, Somila kakshapati, Nabin Narayan Munankarmi and Birendra Prasad Gupta performed the virus titration experiment and data analysis. Bimlesh Kumar Jha, Prashanna Maharjan, Somila kakshapati Nabin Narayan Munankarmi and Birendra Prasad Gupta wrote the manuscript.

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