Characterization of HHV-6 using an Indian isolate: An in vitro study

By

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A thesis submitted to the
Board of Studies in Life Sciences

In partial fulfilment of requirements
For the Degree of

DOCTOR OF PHILOSOPHY

Of

HOMI BHABHA NATIONAL INSTITUTE

February, 2014

Homi Bhabha National Institute

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We certify that the thesis titled "Characterization of HHV-6 using an Indian isolate: An in vitro study" submitted for the degree of Doctor of Philosophy by Ms. Pallavi Goel is a record of the research carried out by her during the period 2006 to 2013 under our supervision. This work has not formed the basis for the award of any degree, diploma, associateship or fellowship at this or any other institute or university.

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ACKNOWLEDGMENTS

The completion of any academic work is inconceivable without the expert guidance of a mentor. It gives me immense pleasure to express my sincere gratitude to my reverend guide Dr R. Mukhopadhyaya who gave gracious assistance and precious time both at the stage of planning and arrangements of the scheme of experiments to be conducted in lab throughout, as well as at the stage of its final presentation.

I am thankful to Dr. Rajiv Sarin (Director, ACTREC) and Dr. Surekha Zingde (Deputy Director, ACTREC) for providing me an opportunity to work in this institution with an excellent infrastructure and ACTREC fellowship. I am thankful to my doctoral committee members Dr. K. Mohandas (TMH), Dr. N. Shirsat (ACTREC), Dr. R. Mulherkar (ACTREC) & Dr. S. Sharma (TIFR) for their critical analysis of the presented data and expert suggestions that have contributed significantly to the work.

I always felt proud to be a part of the Virology group. All my colleagues Meera, Santhosh, Rohan, Cheryl, Ajit, Hemant & Summer enriched this journey in innumerable ways during the entire period. I would be failing in my duty if I don't make a mention of my great sense of gratitude for the cooperation extended by the laboratory staff in particular Smita, Ganesh & Pralhad.

My batchmates Lalit, Sapna, Polami, Amit R, Amit S, Amit F, Tabish, Atul, Manoj, & Ajit have all together given me numerous memories to cherish. Special thanks are due to my hostel mates especially Dr. Mahima, Rachna Garg, Sanchita, Nidhi, Divya, Amrit & Aparna. I place on record my appreciation to due to my latest dinner group members Priyanka, Manish & Shrikantoo for their help & nice company.

I thank my parents and my brother for supporting me even against their wishes and being always there to encourage me at the right time. I am highly indebted to my husband Dr V.S.Chandhok Advocate for the love, affection, support, encouragement and inspiration given by him at every phase of my life and above all to my mother-in-law for her silent prayers. I thank my late Father-in-law who always blessed me to remain in high spirits. It is to him that I dedicate this thesis. I will always love and remember you wherever you are. Thank you my dear father.

Above all, Thanks to God for giving me the strength and wisdom to complete this work.

CONTENTS

Homi Bhabha National Institute

Ph. D. PROGRAMME

1. Name of the Student: PALLAVI GOEL

2. Name of the Constituent Institution: Tata Memorial Centre

3. Enrolment No.: LIFE 09200604008

4. Title of the Thesis: *Characterization of HHV-6 using an Indian isolate: An in vitro study.*

5. Board of Studies: Life Science

Synopsis

Primary infection with Human Herpesvirus-6 takes place in childhood, accounting for 10% of the febrile diseases during the first 3 yrs of life. Primary infection with HHV-6B is usually asymptomatic and associated with common, self-limited childhood illness roseola infantum [1]. This double-stranded DNA virus exists as two species, with characteristic DNA sequences, distribution, pathogenicity and designated HHV-6A and HHV-6B, the later being ubiquitous and latent throughout the adult life of most healthy individuals. The latent virus can be reactivated in immunodeficient conditions including bone marrow transplantation and AIDS. Several disease associations have been investigated over the years, namely, encephalitis, multiple sclerosis, chronic fatigue syndrome, temporal lobe epilepsy etc. It exhibits high genomic homology to HCMV in genomic organization [2-9]. A B-cell line (designated PJH6) was derived from the PBMC of an individual (donor) with apparent high copy HHV-6. Characterization of this isolate in vitro was undertaken in this study.

Extracellular virus particles were characterized by electron microscopy and analyzed for their infectivity to various cell lines and naïve and stimulated (HHV-6 negative) peripheral blood mononuclear cells. Of late, lot of interests has been generated in studying clinically relevant phenomenon of HHV-6 chromosomal integration (CIHHV-6) [10-13]. Viral integration profile was analyzed for PJH6 cell line, the donor PBMC as well as the parental PBMC samples. Subsequently we explored the biological activities of viral Immediate Early gene-1 (IE-1) in vitro by performing RT-PCR and functional assay. A basic gene therapy vector harboring a viral replication and packaging cassette was derived from our isolate. To expand its potential utility, a convenient detection marker, common restriction enzymes sites and antibiotic selection cassette were also incorporated in the vector. Finally, we also generated a simple and fast biological assay to estimate the titer of infectious virions present in an innoculum. As of now there is no

single step reporter based assay for the same. An HIV-1 LTR driven luciferase based reporter cell line was made and a single step assay was developed for estimating HHV-6B relative concentration in the culture supernatants.

Objectives

- Characterizations of the virus isolate including infectivity.
- Chromosomal integration profile of the isolate & proof of vertical transmission.
- Role of viral IE gene products on activation of select cellular genes.
- Development of a HHV-6 derived basic vector for gene transfer.
- Development of a single step relative viral titer estimation assay.

Materials and Methods

Mammalian cell culture: Adherent cells were cultured as monolayer in DMEM and suspension cells were maintained in RPMI supplemented with 10% FBS and 50 µg/ml of gentamycin. All cultures were maintained at 37^{o}C in humidified cambers with 5% $CO_{2.}$ Suspension cells were maintained at 0.3x10^{6}cells per ml in culture media (CM) and split twice a week in fresh media. Adherent cells were detached from the culture flask using Trypsin-EDTA followed by splitting the cells as per the requirements. For preservation, cells were re-suspended in freezing medium (medium + 10% DMSO) and stored frozen in liquid nitrogen.

Virus production: PJH6 cell line was cultured at 0.3x10^{6} /ml of CM for 72 hr. The cells were pelleted at 400xg for 10 min at room temperature (RT) and cell line supernatant was filtered through 0.45 micron filter to make it cell free. Being a potential HHV-6 producer cell line, the cell culture supernatant was directly used to yield infectious virus particles. However, for further enrichment of the virus stock, the remaining pelleted cells were harvested to obtain cell associated virions. Briefly, cells were resuspended in CM,

subjected to 3 cycles of freeze-thaw, and pelleted at 800xg to remove the cell debri. This lysate was pooled with the cell line supernatant, filtered and used to infect target cells.

Preparation of sample for electron microscopy: Cell free virions were harvested from 80 ml of PJH6 culture supernatant. The virions were concentrated from the filtered supernatant by ultracentrifugation at 16000xg at 4°C for 3 hr. The virus pellet obtained was re-suspended in 200 µl of PBS to obtain a 400 X concentrated virus stock. For EM observation, 50 µl of the concentrated cell free virus was mixed with equal volume of 1 X gluteraldehyde to fix the samples and stored at 4°C until further processing. The samples were processed and imaged in a transmission electron microscope (Tecnai 12 Biotwin, FEI, The Netherlands) at the National Institute of Virology, Pune.

Cell line infection and serial passage of cell free virions: Various lymphocytic cell lines (SupTI, Daudi, and U937) were infected with cell free virus containing supernatant harvested from PJH6 culture. Briefly, $1.0x10^6$ target cells were incubated with 1 ml of viral supernatant for 16 h at 37ºC. Serial passage of virus isolate was carried out by infecting fresh batch of SupTI cells with cell free virus containing supernatant from infected SupT1 cells.

PBMC isolation, culture, and infection: Blood samples were diluted in phosphate buffered saline (PBS) and subjected to ficoll-hypaque density gradient centrifugation. PBMC retrieved were washed thrice in PBS and cultured in RPMI media. For stimulation, 10 µg of phytohemagglutinin (PHA-P) was added to $1x10^6$ cell/ ml and cells were incubated for 72 h. Post stimulation, cells were rewashed and cultured in media containing 20 units/ ml recombinant IL-2. For infection, $1.0x10^6$ naive/ stimulated PBMC were infected with 1 ml of viral supernatant from PJH6 cells. Post infection, cells were washed and cultured in IL-2 containing media as per requirements.

Inverse PCR (IPCR) and sequencing: Genomic DNA derived from HHV-6 positive PBMC samples was digested with MboI at 1U/ µg of DNA for 6 h, 8 h, 10 h, 12 h, and 14 h individually. Post digestion, all the samples were diluted in water to 1 ng/µl. 100 ng of digested DNA from each sample was split into 20 ng aliquots and self ligated with 1U of T4 DNA per sample for 14 h at 15ºC. Subsequently, the spilt reactions were pooled, and the ligated DNA was precipitated in ethanol and sodium acetate. These were used as templates for performing inverse PCR. The amplified fragments were checked for expected size, gel purified and cloned into T/A vector. The clones were sequenced from termini and the data were subjected to BLAST analysis in order to identify the virus-chromosome junctions for individual samples.

Genomic DNA/RNA extraction from mammalian cells: Cells were washed in PBS, lysed in Tris-NaCl buffer containing 1 mM EDTA, 0.4% SDS and 0.2 mg/ml of Proteinase K. DNA was extracted by phenol-chloroform method and precipitated using sodium acetate in absolute ethanol. RNA was isolated from PBS washed cells using TRIzol reagent. The lysate was extracted once with chloroform and then RNA was precipitated in isopropanol. To remove the contaminating genomic DNA from RNA, samples were treated with RNase-free DNase I.

cDNA preparation RT-PCR and densitometry: First strand cDNA synthesis was carried out from 1 µg of total RNA for each sample using random hexamer primers, oligo $(dT)_{18}$ primers and Reverse Transcriptase. The reaction was carried out for 1 h, 42ºC in the presence of RiboLock RNase inhibitor. 100 ng of cDNA per sample was used as template for each RT-PCR reaction. A reaction control was kept each time to rule out the presence of genomic DNA contamination. Equal volumes of PCR products were resolved on agarose gels and the acquired images were analyzed by densitometry using Image J software.

Preparation of ultra competent cells: A single colony of freshly grown E.coli strain DH5α MCR was inoculated into 250 ml of enriched media (SOB) and grown at 18°C with constant shaking till the $O.D._{600}$ reached between 0.3 -0.5. The culture was transferred to chilled centrifuge tubes and pelleted at 4°C. The pellet was resuspended in 80 ml of bacterial transformation buffer (TB) and incubated on ice for 10 min. The cells were pelleted again and resuspended in 18.6 ml of TB with 7% DMSO and split into sterile aliquots of 200 µl, which were snap frozen in liquid nitrogen and stored at -80ºC till use.

Bacterial transformation: A single aliquot of ultra competent cells was thawed on ice and mixed gently either with 100 ng of plasmid DNA or the ligation reaction mixture. Cells were incubated on ice for 30 min, given a heat shock pulse for 55 sec at 42ºC and snap chilled in ice. Cells were then revived by addition of enriched media (SOC) followed by incubation at 37ºC for 45 min with constant shaking. Cells were subsequently plated onto LB agar plates with the appropriate selection markers.

Plasmid DNA mini-preparation: Bacterial cultures were harvested for plasmid preparation after 14-16 h of growth. 1- 5 ml of the culture was pelleted at 12000xg at RT and resuspended in 100 µl of resuspension buffer. 200 µl of lysis buffer was added, mixed gently by inversion, and incubated at RT for 5 min. 150 µl of neutralization buffer was added, mixed gently, and incubated in ice for 5 min. The lysate was spun at 12000xg at RT for 10 min and the supernatant was transferred to a fresh tube. Plasmid DNA was precipitated by addition of 1 ml of chilled absolute ethanol and sodium acetate at $1/10^{th}$ volume of the supernatant. The mixture was incubated at -20ºC for 30 min and DNA was pelleted at 12000xg at 4ºC for 30 min. The pellet obtained was washed once with 500 µl of 70% ethanol, air-dried at RT and resuspended in appropriate volume of Tris-EDTA buffer.

Polymerase Chain Reaction (PCR): PCR reaction mixture was first prepared by addition of appropriate buffer, $MgCl_2$, dNTPs, primers, and Taq DNA polymerase. DNA template was then added at the required concentration and the individual reactions were subjected to required cycles of denaturation, annealing, and extension under standardized temperatures and time durations. PCR reaction mixture was prepared separately in a no DNA room in a PCR work station.

Agarose gel electrophoresis & purification of DNA: Agarose gels were prepared in Tris-Borate-EDTA (TBE) buffer and the electrophoresis was carried out under standardized voltage and current conditions. The resolved DNA fragments were visualized by staining the gels with ethidium bromide and exposure to long wavelength UV light using trans-illuminator. The images were acquired using an automated Gel Documentation system. For purification of the required DNA fragments, the appropriate band of interest was first excised from the gel under UV light exposure and then purified using commercially available DNA extraction kits. Alternatively, low melt agarose gels were prepared and the DNA was purified from the excised gel pieces using phenol chloroform extraction method followed by ethanol precipitation.

Cloning: Inserts were usually prepared by restriction enzyme (RE) digestion of the parent vector harboring the insert and purification of the released fragment. The target vectors were prepared by digestion of the suitable vector with appropriate RE that would generate compatible DNA ends for ligation and purification of the vector backbone. The concentrations of final vector and insert preparations were estimated and the ligation reaction was set up, usually maintaining the vector insert molar ratio of 1:3. For generation of blunt ends, either Klenow fragment or Mung bean nuclease was used as per requirements. PCR fragments were primarily cloned into the T/A vector pTZ57R (pTZ).

Promoter-Reporter plasmid constructs. Oligonucleotide primers with specific restriction sites were designed to amplify promoter regions encompassing select nucleotide (nt) sequences of *c-fos*, hsp-70 and *c-myc* by PCR. Amplified fragments were checked for expected size, gel purified and cloned in pTZ and appropriate sequence configuration for each fragment was ascertained by sequencing at both the termini. The promoter fragments were sub-cloned upstream to Luciferase encoding gene of pGL3-basic plasmid. Briefly, pTZ.*c-fos* was digested with BglII and SacI, while pTZ.hsp-70 and pTZ.*c-myc* were digested with KpnI and BglII and the respective inserts were cloned at compatible sites of pGL3-basic vector thus generating p*c-fos*.Luc, phsp-70.Luc and p*c-myc*.Luc respectively.

Generation of HHV-6 IE1 expression construct: 3.2 kb HHV-6B IE-1 coding sequence was PCR amplified using oligonucleotide primers designed on HHV-6B Z29 isolate nt sequence and cloned in pTZ, followed by sequencing from both termini. IE-1 was subsequently released with SmaI/ XbaI digestion and sub-cloned in-frame with the EGFP gene of pEGFP-N2 vector at compatible ends.

Luciferase reporter assays. HEK-293 cells were cultured in 96 well flat bottom plate at a density of $2x10^4$ cells per well in 100 µl medium and transfected with 320 ng of DNA (160 ng of reporter and 160 ng of effector plasmid DNA) using Lipofectamine. Luciferase reporter activity was measure after 48 h by lysis of cells in commercially available assay reagent following manufacturer's instructions and luminescence signal was detected using a micro plate reader. GFP fluorescence was also measured simultaneously using the same microplate reader for all reactions towards normalization.

Transfections: For adherent cells, cells from fresh passage were plated a day before transfection and replenished with fresh media 4 h prior to transfection. Transfection was performed by standard calcium phosphate method or using Lipofectamine as per

requirements. For suspension cells, plating was done on the same day of transfection, and Lipofectamine 2000 was used and the cells were processed according to the manufacturer's instructions.

Western blotting: The protein concentration for each sample was estimated by modified Peterson's Lowry method, equal amounts of proteins were resolved on appropriate SDS-PAGE and electrotransferred onto PVDF membrane. Membrane was blocked with 5% non fat milk or 3% BSA for 1 h at RT and then probed overnight at 4ºC with the appropriate primary antibody dilution. Blot was washed thrice with Tris-NaCl-Tween buffer (TBST), probed with secondary antibody for 2 h at RT, washed again and developed using enhanced chemiluminiscent detection system.

Flow cytometry: Cells were washed and resuspended in PBS at $0.5x10^6$ cells/ ml and all the samples were acquired for GFP expression and analyzed using Cell Quest software. For cell sorting, samples were prepared in CM at a high cell density and collected in media with 50% serum and double antibiotic concentration.

Generation of HHV-6 amplicon vector: Vector components were first assembled individually in different plasmids by PCR through successive cloning steps and finally combined into the pGL3-basic vector. Origin of replication segment (ori) was PCR amplified from PJH6 genomic DNA, cloned in T/A vector, and sequenced from the termini. Complete packaging and cleavage signal sequences were incorporated downstream to the ori by consecutive 3 rounds of PCR with primers having overlapping ends. The complete ori-pac sequence was then cloned again in pTZ and sequenced.

Transgene expression cassette assembly [LTR-GFP-pA]: LTR promoter was derived from HIV-1 lab isolate GT50 genomic DNA by PCR, and cloned into pTZ. LTR was combined with GFP by cloning in pEGFP-N2 vector at NdeI/ PstI sites. Further, entire LTR-GFP was PCR amplified together and sub-cloned into pGL-3 basic vector at

XbaI/NcoI sites by replacing Luciferase sequence upstream to pA to generate pGL-3-LTR-GFP-pA.

Selection marker (neomycin) expression cassette [CMV-Neo-pA]: Neomycin was released from pTZ backbone and cloned downstream to CMV promoter in pCDNA3.1 (+) at PmeI/ NheI sites. CMV-Neo fragment was then excised by BgllI/ NheI and cloned into pGL3- basic at BglII/ XbaI sites, replacing Luciferase and generating pCMV-Neo-pA.

Amplicon vector assembly: CMV-Neo-pA cassette was released by SalI/ BglII digestion and cloned in pGL-3 LTR-GFP-pA at BamHI/ SalI sites. Further, ori-pac fragment excised from pTZ by BamHI digestion was also incorporated in the construct at BglII by blunt end cloning, thus generating pGL-3.ori-pac.LTR-GFP-pA.CMV-Neo-pA.

Functionality of amplicon vector and generation of stable cell line: PJH6 cells were transfected with the amplicon vector using Lipofectamine. 7 days post transfection cell line supernatant was collected, filtered, and used for transduction of target cells. 3-5 days post transduction, cells were observed for GFP expression by flow cytometry and fluorescence microscopy. Transgene expressing cells were either sorted on the basis of GFP fluorescence or alternatively selected by G418 at 600µg/ ml for enrichment of transduced cells and generation of stable cell lines.

Generation of LV construct for Indicator cells: A plasmid construct having LTR-Luciferase-IRES-GFP expression cassette and a Neo cassette (obtained from Dr. Debshis Mitra, NCCS, Pune) was digested with HpaI and incorporated into the lab generated lentiviral vector at SmaI site in the MCS. Clone obtained in the reverse orientation with respect to the transcription from 5'LTR was used as the transducing construct.

Establishment of an Indicator cell line: The lentiviral vector was co-transfected along with the helper constructs into the packaging cell line 293T for generation of

pseudovirions. Supernatant was collected from the transfected cells 72 h and 96 h post transfection, pooled filtered, and concentrated (50X) by ultracentrifugation. HEK 293 cells were transduced with the concentrated vector supernatant in presence of 8 µg/ml polybrene. 16 h post transduction, cells were washed with DPBS and replenished with fresh media. 48 h post transduction, cells were selected at 600 µg/ ml G418. Post selection, cells were maintained in media without G418. The stable cell line generated was characterized for the presence of transgene expression cassette and lentiviral vector by PCR and several freeze downs of the selected cells were made at early passage.

PEG precipitation of viral proteins: Cell free culture supernatant split into 1 ml aliquots was mixed with 0.5 ml of cold 30% PEG solution and incubated overnight on ice at 4ºC. The suspension was centrifuged at 16000 RPM for 45 min at 4ºC. The supernatant was aspirated off and the pellet was resuspended in 20 µl of culture media by vortexing. Finally, all the aliquots were pooled and used for transductions as per requirements.

Concentration of virus particles: Cell free culture supernatant was cushioned with $1/10^{th}$ volume of 15% sucrose solution and subjected to centrifugation at 20,000 RPM, 4ºC for 2 h. The pelleted virus particles were resuspended in the required volume of media and stored frozen at -80ºC till use.

Virus titer assay: Indicator cells were seeded in 96 well plates at 5000 cells in 100 µl of DMEM per well. 14 h post cell seeding, cells were transduced by replacing the media with the appropriate virus dilutions, keeping the culture volume equal in all the wells. After 16 h, viral supernatant was replaced with fresh 100 µl DMEM in all the wells and simultaneously harvested for luciferase assay.

Luciferase assay: At the time of harvesting cells for assay, total volume of culture media per well was kept 100 µl per well. 100 µl of Luciferase assay was added to each well and

incubated at RT for 5 min. The lysate was mixed gently and 100 µl per well was acquired for luminescence signal using a microplate reader.

Results

Virus morphology documentation and infectivity study

As a primary step towards the characterization of the viral isolate, EM studies were performed on the virus particles shed in culture supernatant. The virus particles exhibited characteristic herpesvirus morphological features, thus confirming their identity as a member of herpesvirus family. Infectivity of the extracellular virus particles was tested by infecting human PBMC and various lymphocytic cell lines. The isolate could successfully infect all the target cells as confirmed by HHV-6B specific nested PCR. The PBMC could be readily infected even in the absence of a mitogen stimulus. Further, serial passage of cell free virus particles in SupT1 cells indicated that the isolate was capable of imparting productive infection.

Chromosomal integration profile of the isolate

Genomic DNA derived from the PJH6 and donor PBMC and donor parents was processed for inverse PCR. Sequencing of the cloned PCR amplicons and BLAST analysis indicated the presence of chromosomal integration in all the samples. The site of integration in PJH6 cell line, donor PBMC, as well as that of PBMC from father was found to be identical, chromosome 12q14, thus indicating an event of vertical transmission from father to son. Although integration was also observed in the mother's genome, the site was different.

Role of viral IE-1 gene product on activation of cellular genes

All the target promoters exhibited an increase in their activity in presence of IE-1, as ascertained by the luciferase assay, thus indicating in-vitro transactivation potential of the viral gene. In order to validate this outcome as a generalized phenomenon exhibited

by the viral IE class of genes, SupTI cells were infected with cell free virus particles and gene expression of a panel of putative target genes was analyzed by RT-PCR followed by densitometry. 8 h post infection hsp-70 showed most significant levels of up-regulation while c-fos showed marginal up-regulation. Interestingly, the expression levels of c-myc remained unchanged. All the experiments were performed thrice and on different days.

Development of a HHV-6 derived basic vector for gene transfer

According to the principle, upon transfection, the amplicon units get replicated and packaged as pseudovirions in the presence of helper virions. The same was accomplished by transfection into PJH6 cells, which resulted in generation of a mixture of wild type and pseudovirions and were used to transduce various cell lines including HEK 293, Daoy, SupTI, and U937. GFP transgene expression was monitored after 3 to 5 days in order to estimate the efficiency of transduction. All the cell lines were successfully transduced and exhibited appreciable levels of transgene expression, although the efficiency of transduction remained low. However, this problem was overcome by selection with G-418 in addition to cell sorting on the basis of GFP expression to obtain positive cell populations. The selected cells retained high levels of transgene expression even after several passages, thus validating the functionality and stability of the amplicon vector.

Generation of a simple viral titer estimation assay using a novel in vitro transactivation system

Based upon the reported fact that HHV-6 IE class of genes potentially trasnactivate the HIV-1 LTR promoter, a simple bioassay was designed as an attempt to estimate the infectious viral titer in inoculums. An LTR-Luciferase expression plasmid was incorporated into a lentiviral platform and an indicator HEK293 cell line was generated

by transduction and selection of cells harboring the reporter cassette. Using concentrated cell free virus preparations obtained either by ultracentrifugation or PEG precipitation, the reporter gene expression from the indicator cell was assayed that showed a positive correlation with serial viral dilutions, thus, verifying the functionality of the reporter assay design.

Discussion

Since HHV-6 discovery in 1986, several isolates have been reported worldwide. Most of these isolates are propagated by infection to fresh cell lines or stimulated PBMC each time. HHV-6B positive B-lymphocytic cell line was earlier derived in our lab and was used to generate infective virus particles. Characterization of the same was carried out in an attempt to establish it as a valuable in vitro tool for indefinite virus production for research purpose. The EM study primarily confirmed the isolate identity as a definite member of herpesvirus family, while the infectivity studies on various cell lines elucidated the potential of the cell line to generate infectious virus particles. Also, PBMC were found highly permissive to viral infection even in the absence of any mitogenic stimulus. Additionally, the nature of infection generated was proved to be productive, which further makes the isolate versatile for varying studies.

Among herpesvirus family members, HHV-6 and EBV are capable of genomic integration, a rare phenomenon whose underlying mechanistic principles are not clear yet. As the previous reports have described the main route of acquisition of CIHHV-6 is through vertical transmission, we ascertained the same in our case by performing inverse PCR, and sequencing of PBMC derived genomic DNA from father, mother, and donor samples. Our results confirmed an event of CIHHV-6 transmission from father to son.

Herpesviruses exhibit a characteristic cascade system of gene regulation in which the IE proteins play a major regulatory role. Previous reports have described the nuclear

localization and heterologous transactivation potential of IE-1 gene. In this study, we further characterized IE-1 regulatory role and observed that IE-1 significantly up-regulates c-fos, c-myc and hsp-70 promoters. Our results are similar to that reported earlier for HCMV IE-1, thus, elucidating another aspect of functional similarity within the closely associated members of the herpesvirus family [14]. However, the scenario was different in case of wild type infection where only hsp-70 and c-fos showed considerable up-regulation at transcriptional levels, while c-myc expression levels remained unchanged. This is in contrast to the HCMV study where up-regulation was observed both in case of IE-1 transfection and virus infection at early time points.

Herpesvirus amplicon vectors derived from HHV-6 was developed relatively fast due to the substantial experience gathered from previously derived HSV-1 amplicons [15]. Owing to their cell tropism, transgene reiterations coupled with presence of less than 1.5 kb of viral DNA sequences, they are considered attractive as non–integrating gene delivery vectors. Our report describes generation of an HHV-6 based amplicon vector derived from the lab isolate. The functionality of the vector proved that our isolate indeed follows the same principle of replication as for other members of the family. Nevertheless, in addition to carrying out basic research studies, our isolate as well as PJH6 cell line can be used for generation of an improved gene transfer vector.

In an attempt to harness the transactivation potential of viral IE class of genes, we designed an indicator cell assay for estimating the infectious viral titer. Towards this end, an indicator cell line harboring HIV-1 promoter linked to luciferase gene cassette, delivered through a lentiviral vector, was generated [16, 17]. Specificity of the assay indicated that the assay can be used to get a semi-quantitative measure of viral titer. Thus, we report a robust and sensitive method that is technically simple, quick in comparison to existing cell culture based methods, uses very small amount of reagents

and suitable for virus inhibitory drug screening or serum neutralizing antibody titer estimation apart from being a useful research tool for selection of infective inoculums [18].

This study thus reports characterization of an infectious HHV-6B isolate including demonstration of an uncommon integration site, activation of HSP-70 by viral IE gene product, preliminary demonstration of making a HHV-6 amplicon vector for gene transfer and development of a simple relative HHV-6B titer assay

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CHAPTER 1

INTRODUCTION

The history of Herpes viruses dates to the ancient Greek times where physicians like Hippocrates described the cutaneous spreading of herpes simplex lesions. The word "*herpes*," which means "to creep or crawl," was used in reference to the spreading nature of the herpetic skin lesions. However, the origin of herpes virus in human history remains unknown and the virus has been prevalent ever since anyone could diagnose fever blisters. Herpes viruses are common inhabitants of animal kingdom and nearly 100 distinct herpesviruses have been isolated from a variety of species which include non-human primates, cat, dog, snakes, rabbit, mouse, rat, frog, and birds (1). To date, nine herpes virus types are known to infect man frequently and constitute the Human Herpesvirus family. Virus family members were initially named after the clinical symptoms, for instance, herpes simplex virus and herpes Zoster virus, or after their discoverers, such as Epstein Barr virus or based on their pathology namely, cytomegalovirus. However, according to the International Committee on Taxonomy of Viruses (ICTV) endorsed nomenclature, the Human herpesvirus family was subdivided into 3 subfamilies designated as alpha, beta, and gamma. Individual members were clubbed into these sub-families based on their biological properties (Table 1) (2).

Sub-family	*Members*	*Common name*
Alpha	Human herpesvirus-1	Herpes Simplex Virus type-1 (HSV-1)
	Human herpesvirus-2	Herpes Simplex Virus type-2 (HSV-2)
	Human herpesvirus-3	Varicella-zoster Virus (VZV)
Beta	Human herpesvirus-5	Cytomegalovirus (HCMV/CMV)
	Human herpesvirus-6A	(HHV-6A)
	Human herpesvirus-6B	(HHV-6B)
	Human herpesvirus-7	(HHV-7)
Gamma	Human herpesvirus-4	Epstein-Barr Virus (EBV)
	Human herpesvirus-8	Kaposi's Sarcoma associated Virus (KHSV)

Table 1: **The Human Herpesvirus family members**

Disease associations of human herpesviruses

In addition to causing acute diseases, they are capable of establishing a latent infection and reactivating under a variety of stimuli, a feature common to all herpesviruses. HSV-1 & HSV-2 are widespread in distributions, that cause fever blisters and genital sores respectively, and occasionally central nervous infections. They show a worldwide distribution and exhibit unique biological properties such as neurovirulence, establishment of latency in the nerve ganglia and the potential to reactivate equally in both immunocompetent as well as immunocompromised individuals, though the reactivations are more common and severe in the latter cases. HSV-1 is transmitted chiefly through saliva, whereas HSV-2 is transmitted either sexually or from mother to child via the genital tract during birth (3). Primary infection with VZV causes chicken-pox in children, and this virus is the most infectious of the herpesviruses that can potentially spread via inhalation of an aerosol of nasopharyngeal secretions from a patient. Once the clinical symptoms resolve, VZV latently persists lifelong in the nervous system of the infected person, with reported 10-20% of cases where it reactivates producing herpesvirus zoster (Shingles), a manifestation generally detected in elderly immunocompromised individuals (4). EBV was discovered almost half century ago from Burkitt's lymphoma derived cells. It is highly prevalent in developing countries, particularly in equatorial Africa and infects most of the children in early years of life generating an asymptomatic primary infection followed by lifelong dormant infection. However, if the infection is delayed until adolescence, it usually presents as infectious mononucleosis (5). A very rare event in a few carriers of this virus is the emergence of Burkitt's lymphoma and nasopharyngeal carcinoma. EBV infection is also implicated in development of autoimmune diseases and post transplant lymphoproliferative disorders. Human B lymphocytes once infected with EBV can be

propagated indefinitely in culture. This property of EBV has been extensively exploited for generating biological material for functional and molecular studies (6). CMV infection was initially documented more than a century ago, originally described as presence of typical cytomegalic cells in parotid glands of infants. However, technological limitations held the successful isolation of the virus for almost 50 years till 1957 (7). Like other herpesviruses, it is widespread in the human population and about 50% of adults are carriers of CMV. CMV infection has been implicated as a major cause of hearing loss and mental retardation in cases of congenital infection and can cause life-threatening complications in infants & in immunocompromised individuals. The viral reactivation is a major concern for transplant recipients and AIDS patients (8). Although CMV is not yet recognized as an oncogenic virus, some evidences suggest a possible role of CMV infection in malignant diseases from different cancer entities (9). HHV-7 is another ubiquitous virus that latently infects over 90% of the human population by the age of 3. HHV-7 infections have been associated with exanthem subitum, hepatitis, multiple sclerosis (MS), and transplant complications (10). HHV-8 is commonly known as the Kaposi's sarcoma-associated herpesvirus as it was initially detected in Kaposi's sarcoma (KS) tissues from an AIDS patient. It is an oncogenic virus that is distinguishable from other herpesviruses as it exhibits a limited and an uneven distribution in the human population. It is also implicated in pathogenesis of other malignancies such as multicentric Castleman's disease and primary effusion lymphoma. Nearly, 30% of the viral genes encode novel proteins not found in other human herpesviruses; most of these proteins are responsible in KS pathogenesis (11).

HHV-6

Clinical associations

The natural history of HHV-6 infection can be represented by 3 stages. The first being primary infection that takes place between 6 and 12 months of age and is known to cause acute febrile illness (12). The species B is implicated in most of the primary infections and has been recognized as the etiologic agent of Roseola infantum (also termed as *exanthem subitum* or the sixth disease), a common childhood disease with skin eruptions that is often benign, self limiting and is observed in subset of patients following primary infection (13). In certain cases, it leads to complications such as encephalitis, febrile seizures, gastrointestinal symptoms, and respiratory distress. However, most of the primary infections are characterized by fever with a self-resolving course (14). Primary infections in adults are rare, and can particularly have fatal consequences in case of immunocompromised cases (14, 15). The second stage of infection can be observed in healthy individuals, where the virus remains latent in the lymphocytes, monocytes, undergoing low level of replication mostly in the salivary glands and is secreted in the saliva. At this stage, although the virus is pathologically quiescent, but frequent shedding in the saliva acts as the source for transmission to uninfected individuals (16). This stage can last for the lifetime of an individual. The third stage is observed relatively infrequently and is represented by complications arising in immunocompromised individuals mostly due to endogenous latent virus reactivation or superinfection in a previously infected individual (14). Moreover, pediatric transplant recipients who are under the age of two are vulnerable to primary infection from an HHV-6 positive donor and have been reported to have fatal consequences (14). The scenario of virus reactivation is of profound clinical significance in transplant recipients where the virus is presumed to induce immunomodulation resulting in a myriad of clinical syndromes such

as drug induced hypersensitivity syndrome, allograft dysfunction, acute cellular rejection, an increased risk of opportunistic infections (17, 18). Moreover, prospective studies have demonstrated that HHV-6 seroconversion was associated with enhanced episodes of CMV reactivation and disease in transplant settings (14).

HHV-6 has also been implicated with the pathogenesis of multiple sclerosis (MS). It has also been observed that an anti HHV-6 IgG titer is positively associated with MS relapse in a dose dependent manner, indicating that either HHV-6 infection or an immune response to HHV-6 antigens may have an effect on the clinical course of MS (14, 19). Apart from this, HHV-6 infections have been associated with encephalitis in immunocompromised as well as immunocompetent conditions, although, the latter is observed relatively rare (20). A number of studies have suggested a possible role of HHV-6B in the pathophysiology of Mesial Temporal Lobe Epilepsy (MTLE), as evidenced by the presence of viral DNA in brain resections from MTLE patients and also the viral tendency to aggregate in the temporal lobe (21). As HHV-6 was initially isolated from patients with lymphoproliferative disorders, the virus has been extensively investigated for its possible role in malignancy. Several studies have reported a link between HHV-6 and nodular sclerosis subtype of Hodgkin's lymphoma (HL) (22). The viral DNA is frequently detected in T-cell non-Hodgkin's lymphoma (NHL). Previous studies from our lab have also reported activation of HHV-6 in HL and NHL (23). HHV-6 has also been postulated to play a role in the development of cervical cancer as well as adult pediatric gliomas (24, 25). Though few reports suggest its role in malignant transforming activities, overall there is still lack of conclusive data linking HHV-6 to human malignancies (14, 26). HHV-6 has been reported to exhibit molecular interactions with viruses such as Human ImmunodeficiencyVirus-1 (HIV-1), EBV and Human Papilloma Virus (HPV) (14). There seems to be a synergistic relationship between HHV-

6 and EBV, considering the facts that while HHV-6 infection activates EBV replication and enhances its transforming potential, on the other hand EBV infection makes the B-cells more susceptible to HHV-6 infection. Few HHV-6 gene products have been shown (in vitro) transactivation of HPV transforming genes. However, there is lack of evidence demonstrating a direct association of HHV-6 in the development of cervical cancer.

Immune response

Both HHV-6 species can infect several types of immune cells with their primary target being CD4+ T lymphocytes that play a major role in generating immune responses. The virus exhibits molecular mimicry as it harbors a number of host acquired genes, whose products, such as virally encoded chemokine, G-protein coupled receptors, can interfere with the normal host defense responses (27). It also modulates the cellular immune response to viruses by up-regulating the production of IFN-α in mononuclear cells, inhibiting the production of IF-ɤ in Peripheral Blood Mononuclear Cells (PBMC), regulating the expression of a panel of interleukins (IL) including IL-Iβ, IL-6, IL-8, IL-10, 1L-12 & IL-18 (14). Other mechanisms of immunomodulation include alteration in the expression of cell-surface molecules for instance, up-regulating the expression of Tumor Necrosis Factor-α family of receptors & IL-2 receptors (14). Interestingly, the expression of the cellular receptor CD46, used for virus entry inside the cells, is down-regulated post infection (27). It up-regulates CD4 levels in continuous T-cell lines and down regulates CD3 expression (14). Additionally, HHV-6 infection exerts an immunosuppressive effect by inhibiting proliferative response of PBMC to mitogens and down regulating IL-2 expression (14).

Transactivation of heterologous genes

HHV-6 also harbors a number of genes that exhibit transactivating potential and this feature can be harnessed to develop an indicator cell line for HHV-6 viral titer assay, as

reporter for other herpesviruses. Some of the reported targets include heterologous promoters for instance, HIV-1 Long Terminal Repeat (LTR, that harbors the viral promoter region) and CD4 (28, 29). The viral genes reported to transactivate HIV-1 LTR include some genes encoded by the Immediate Early locus-A (IE-A) as well as IE-B (IE locus B) loci (14). The region within LTR that bears recognition sequence for transactivation by HHV-6 is located between -103 to -48 base pairs (bp) from the LTR cap site. Extensive studies on the LTR and HHV-6 interaction revealed that this transactivation takes place predominantly through nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) binding sites in the LTR (30).

Antivirals for HHV-6

The therapy includes drugs used to treat its most closely associated member HCMV, as till date no particular drug is specifically approved for the treatment of acute HHV-6 infections. Commonly used drugs include those targeting the viral DNA replication, for instance Foscarnet (pyrophosphate analog), Ganciclovir and Cidofovir (nucleoside analogs) (31). Potential new anti-HHV-6 agents are currently in various stages of development and include drugs targeting all double stranded DNA viruses and even those targeting the malaria parasite (32). Most of them are experimentally available although none have commercial approval.

Background of the present studies

Previous studies from this laboratory reported HHV-6 status in specific immunocompromised conditions. The virus was frequently found activated both in HL and NHL (23). The status was also evaluated in HIV infected mothers and their newborns, and it was found that perinatally co-transmitted HHV-6 was always activated in the neonates born with HIV infection (33). Additionally, while evaluating the cellular distribution of the virus in PBMC samples obtained from immunocompetent healthy

individuals, one individual presented with unusually high amount of the viral DNA showing the presence of single copy of the virus per cell in preliminary observations. Subsequently, an HHV-6 positive B cell line, designated as PJH6, was derived from the PBMC of the said individual by EBV transformation. The present work was aimed at a comprehensive characterization of this new HHV-6 isolate along with development of a simple assay for HHV-6 titration.

Aims and Objectives

- Ultra structure of the isolate and virus infectivity study.
- Chromosomal integration profile of the isolate.
- Role of viral IE gene products on activation of select cellular genes.
- Harnessing the isolate to develop a basic gene transfer vector.
- Development of a single step relative viral titer assay.

CHAPTER 2

REVIEW OF LITERATURE

HHV-6, a member of the β-herpesvirus subfamily, was discovered in 1986 in Dr. Robert Gallo's Lab from refractile cells in cultured leukocytes of immunocompromised patients with lymphoproliferative disorders and HIV-1 infection. First detected in B-cell lymphoma, it was designated as '*human B lymphotropic virus (HBLV)*' (34). However, extensive studies indicated that the virus could also infect other cells of the immune system. It morphologically resembled other viruses of the herpesvirus family, yet it was distinguishable for its biological properties, antigenic features, and host range. Based on these observations it was recognized as a new member of human herpesvirus family, and named HHV-6. Subsequently, several HHV-6 isolates were reported by many laboratories and by early nineties a consensus conference classified these viruses into two distinct but closely related variants, HHV-6A & B (35) and later ICTV conferred species status to each variant. HHV-6A & B show 90% sequence homology and share certain biological properties but differ in having characteristic endonuclease profiles, antigenic specificity, and in vitro growth properties (14, 36). Additionally, their global prevalence also varies between 70-100%, with HHV-6B being more prevalent in comparison to HHV-6A. HHV-6A is predominantly associated with viremic infant infections in African population (14). The age of acquisition of the virus is mostly between 6 to 15 months and primary infections account for almost 20% of the acute fever cases in these infants (14). Transmission occurs mostly through the shedding of virus particles in the saliva, while vertical transmission has also been documented for ~1% of the births. The exact age for seroconversion to the less prevalent species A is not known as yet, but is believed to occur after acquisition of HHV-6B (14).

Ultrastructure, genome organization, and replication

All herpesviruses have a complex yet similar ultrastructure that are almost indistinguishable in electron micrographs. Each particle consists of three main structural elements: (i) an icosahedral protein cage termed as the nucleocapsid is 90-110 nanometer (nm) that harbors the viral genome, (ii) a lipid bilayer envelope, which contains numerous glycoproteins and (iii) a tegument which is a protein-filled region that joins the envelope to the capsid. This complete particle is known as the virion and it varies in size from 180-200 nm (14; Fig-1).

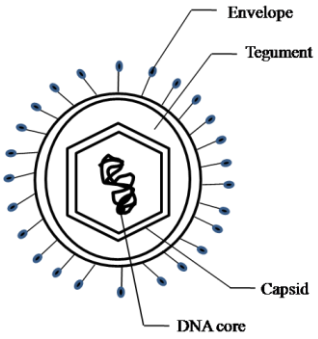
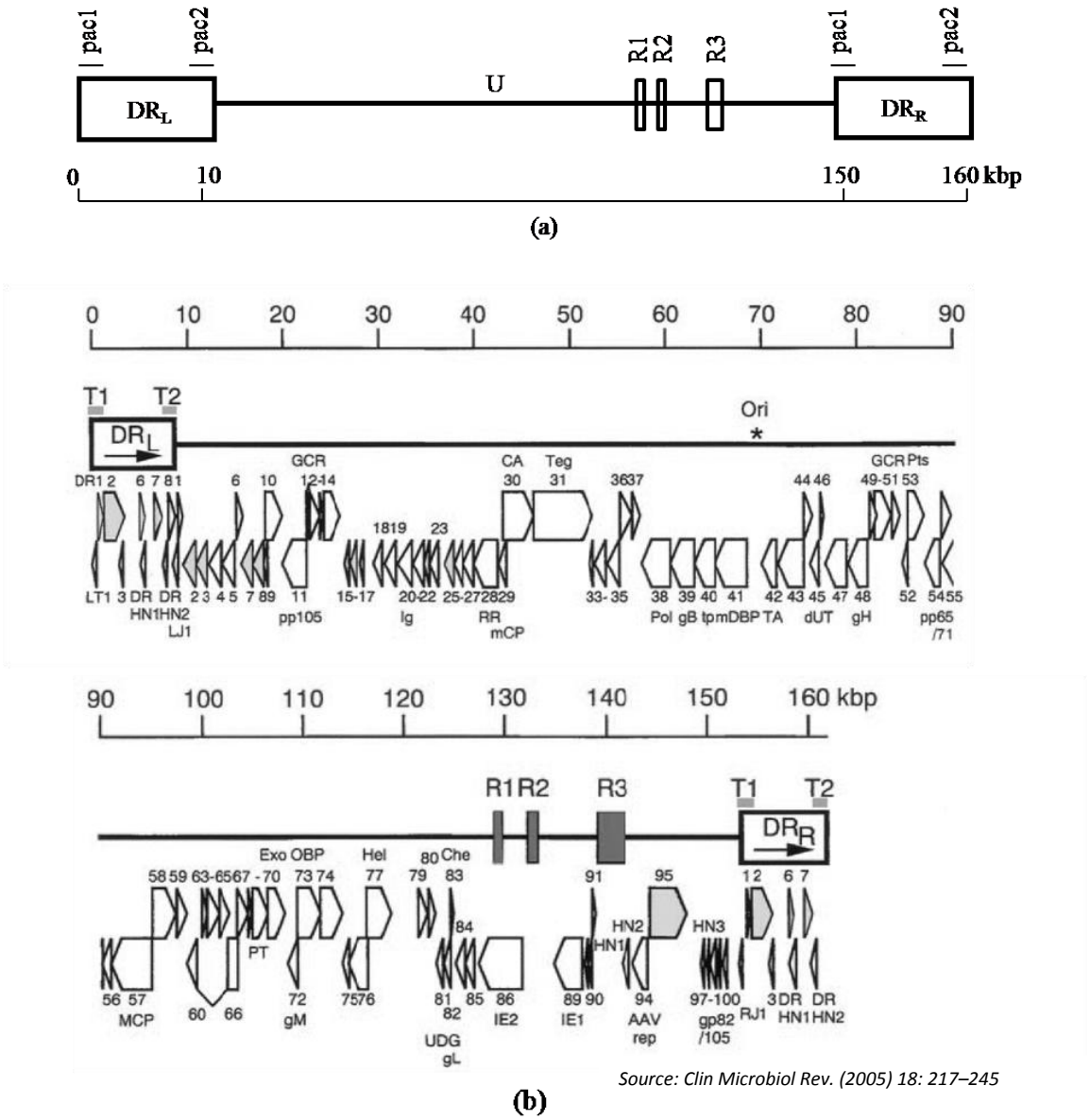


Fig-1. Structural components of a typical herpesvirus particle-schematic.

The herpesvirus genomes are relatively large in size ~105-235 kilobases (kb) and are organized as linear double stranded DNA which has a unique region, flanked by inverted repeats. For HHV-6, the genome length is ~160-162 kb, out of which the U region spans most of the genome length, being ~143-145 kb long and is flanked by ~8-9 kb terminal direct repeats (DRs). The DRs contain the packaging and cleavage motifs towards their both ends, designated as pac-1 and pac-2. Genes in the unique region are designated as U1 to U100 and those in DRs as DR1 to DR7. The overall G + C content of the genome are ~43%. The unique region of HHV-6 genome exhibits maximum similarity to the unique long (U_L) of HCMV genome in comparison with other herpesviruses indicating that it is most closely related to HCMV (37). The genomes are also interrupted by

multiple repeated sequences, varying in number, thus, leading to heterogeneity in genome length up to 10 kb for different isolates of a particular virus (Fig-2).

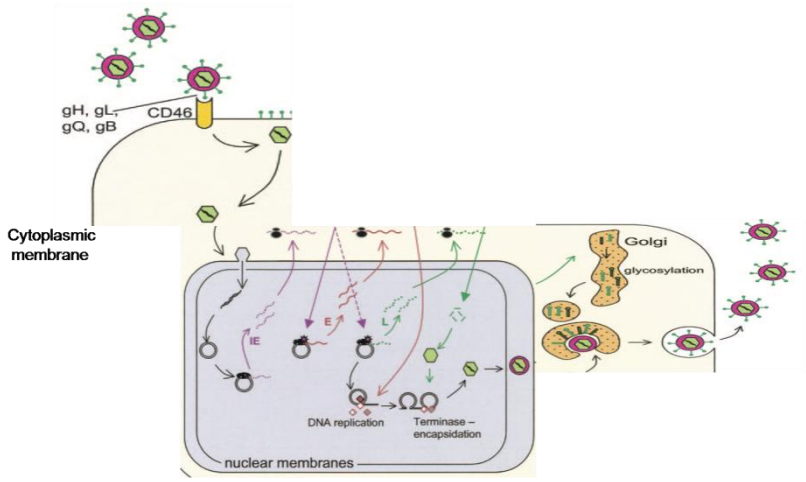


Source: Clin Microbiol Rev. (2005) 18: 217–245

Fig-2. Schematic representation of genomic organization of HHV-6B.
(a) Simplified line diagram of the viral genome showing three intermediate repeat sequences as R1, R2 & R3, and U denotes the unique region, DR_L & DR_R denote the direct repeats, while the pac1 & pac2 represent the packaging sites; (b) Protein coding regions are represented by open arrows and origin of lytic replication (Ori) is indicated by an asterisk.

HHV-6 genome exhibits both interspecies (HHV6-A & B) as well as intraspecies variations. HHV-6B genome encodes nine Open reading frames (ORF) designated as B1 to B9 that are not present in the HHV-6A genome and vice versa. Both exhibit

nucleotide similarity as high as 98% in the conserved regions of the viral genome, while the similarity decreases to almost 31% for certain regions, thereby accounting for most of the biological differences between the two species. The differences amongst different isolates (intraspecies variation) mostly maps to the terminal DR region, particularly for HHV-6B (14). The viral replication cycle comprises of the following steps: (i) virus binding and entry inside the cells, (ii) DNA replication, (iii) maturation involving assembly of structural components, and (iv) viral egress (Fig-3).



Source: Clin Microbiol Rev. (2005) 18: 217–245

Fig-3. Schematic diagram showing successive events of virus entry, replication, maturation, and egress.

(i) *Binding and entry*: The virus enters inside the cells by binding possibly to its cellular receptor CD46, which is a type-I transmembrane glycoprotein, expressed on all nucleated cells (38). For HHV-6A, the viral glycoprotein gH/gL/gQ1/gQ2 complex acts as the ligand for the CD46 receptor, while for HHV-6B, there are conflicting reports about the ligand as well as its cellular receptor. One group has reported that CD46 acts as a receptor, while another has provided evidence for the non- involvement of CD46 in any interaction with the viral gH/gL/gO and gH/gL/gQ complexes identified for HHV-6B, thereby suggesting involvement of yet unidentified cellular receptor (39,40). After binding, fusion of the viral and cellular membranes occurs in the endosomal

compartment, and the nucleocapsid enters inside the cells and is subsequently transported through the cytoplasm to the nuclear pore complex and the viral genome is unpackaged and released into the nucleus (41). (ii) *Replication*: Using the cellular transcriptional and translational machinery, the virus produces its three kinetic classes of viral proteins namely immediate early (IE), early (E) and late (L). The expression of IE genes starts within minutes of viral entry and they are primarily involved in regulating the expression of other genes. The E genes encode proteins involved in the viral DNA replication, which requires a panel of virally encoded proteins (14). Briefly, the viral genome circularizes upon its entry into the nucleus, after which origin binding protein binds to the origin of lytic replication sequence leading to a partial denaturation of viral genome. Subsequently, the helicase-primase complex maintains this gap while the major DNA binding protein stabilizes the replication bubble. Further, viral DNA polymerase initiates the 2^{nd} strand synthesis which is driven by processivity factor. The circular genome is nicked to form rolling-circle intermediate yielding long concatameric strands of the viral DNA that is ultimately cleaved at the packaging sites located towards the ends of the viral genome (14). The organization of the packaging sites is shown below (Fig-4).

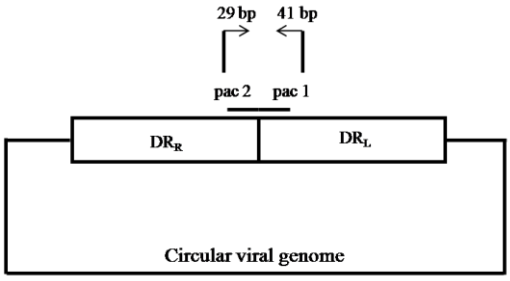


Fig-4. Schematic diagram showing organization of the viral genome upon circularization. *The cleavage-packaging motifs placed adjacent to each other, the cleavage takes place 29 bp away from pac-2 motif and 41 bp away from the pac-1 motif. The arrows point towards the site of cleavage located between the pac sites.*

(iii) *Maturation*: The cleaved unit length genomes are encapsidated yielding nucleocapsids which bud into the perinuclear cisternae thereby acquiring a primary envelope devoid of glycoproteins. In the cytoplasm, the nucleocapsids are first de-enveloped, and then they acquire a tegument followed by secondary spiked envelope. (iv) *Egress*: The glycoproteins are sequentially glycosylated inside the transport vesicles prior to the release of the mature virus. Finally, the enveloped virions are released by the exosomal pathway by fusion with the plasma membrane. One round of complete replication cycle takes about 72 h (14, 41).

Cell tropism

All the isolates reported so far infect activated PBMC and cord blood mononuclear cells (CBMC), which are therefore routinely used to culture the virus. HHV-6 is able to infect a wide variety of cell types in vitro, epithelial cells, haematopoeitic stem cells, natural killer cells, oligodendrocytes, microglia and liver cells, a feature that can possibly be attributed to the ubiquitous nature of the viral receptor, human CD46 (14). However, it is a lymphotropic virus and replicates most efficiently in T-lymphocytes (42). It has been shown to exert cytopathic effects on the CD4+ T-lymphocytes in vitro. HHV-6 has also been shown to productively infect astrocytes and oligodendrocytes, with HHV-6A being more competent to establish a productive lytic infection in comparison to HHV-6B (43). Due to its restricted range of susceptible species, there is a lack of animal model systems for studying the role of HHV-6 human diseases. Recently, a novel marmoset model for HHV-6A & B infection has been established and possibly can be used to study human neurological disorders associated with HHV-6 infection (44). However, most of the virus characterization studies are carried on cell lines that provide an initial insight into the understanding of virus biology.

Detection

The most reliable way to diagnose primary infection is the isolation of virus particles from the cultured PBMC but the method is resource intensive (45). Other techniques available for diagnosis include serological assays that can be carried out by immunofluorescence, enzyme immunoassays, and measurement of neutralizing antibody (45). Since most of us get infected with HHV-6 in early childhood and therefore develop antibodies as a part of natural defense against the viral infection or reactivation, elevated antibody titers rather than just the presence of HHV-6 antibodies in the serum are considered as a sign of active viral infection (46). As the latent virus can also persist at detectable levels in the blood stream of healthy adults, it complicates diagnosis. Previous studies have suggested that the detection of viral DNA in plasma or serum samples is a confirmatory sign of active infection (47). However, this opinion has been negated by a relatively recent observation that the presence of viral DNA in plasma or serum mostly results from the lysis of infected PBMC, and does not indicate the presence complete virus particles (45). Moreover, individuals harboring vertically acquired viral genome always show presence of viral DNA in all of the body fluids, even in the absence of viral replication (45). Thus, the same cannot be used to diagnose active viral infection. For reactivations usually plasma samples are preferred for diagnosis in order to distinguish from latent infections. Reverse transcriptase PCR (RT-PCR) assays are also considered as a very sensitive way for diagnosis of active viral infection. In addition to offering reliability comparable to that achieved by virus isolation, RT-PCR assays are much less labor and cost-effective (45).

Chromosomal integration

Several viruses including retroviruses and adeno-associated viruses, exhibit the phenomenon of chromosomal integration, a condition described by integration of the

viral genome into the host chromosome. This feature is a mandatory event in the life cycles of these viruses. However, the occurrence of this phenomenon is relatively rare among members of the human herpesvirus family, and till date only HSV, EBV, and HHV-6 (both A & B species) have been documented to show integration. In case of HSV, chromosomal insertions of viral sub-genomic fragments, rather than the full-length viral DNA, have been reported in most instances thereby, negating the possibility of infectious virus particle production from the same (48). While for EBV and HHV-6, the entire viral genome gets integrated in the host chromosomes. EBV DNA can be frequently detected in the integrated form upon infection to activated primary B-cells (49). Apart from this, it is found integrated in Burkitt's lymphoma cell lines, and nasopharyngeal carcinoma derived epithelial cells and biopsy specimens (49-51).

Though HHV-6 is ubiquitous, HHV-6A & HHV-6B are the only members of human herpesvirus family for which genomic integration has been observed although only in a minority of individuals, first reported in early nineties (52). The prevalence of chromosomally integrated HHV-6 (CIHHV-6) genotype is ~1% to 3% including children as well as adults, and does not exhibit much variation with regard to different geographic areas (53). CIHHV-6 has been reported to be the primary cause of congenital infection (48). HHV-6 infects most of the human population and consequently the prevalence of integration is much higher than any other human herpesvirus. Since the entire viral genome is integrated into the host germline genome, the same can be inherited from the parents to the offspring. Thus, inheritance of CIHHV-6 takes place in a classical Mendelian manner, and the same can be identified by the presence of identical integration sites in the donor parent and the recipient offspring (54, 55). In one case the offspring inherited 2 integrated copies of the viral genome per cell, one from each of the parents (56).

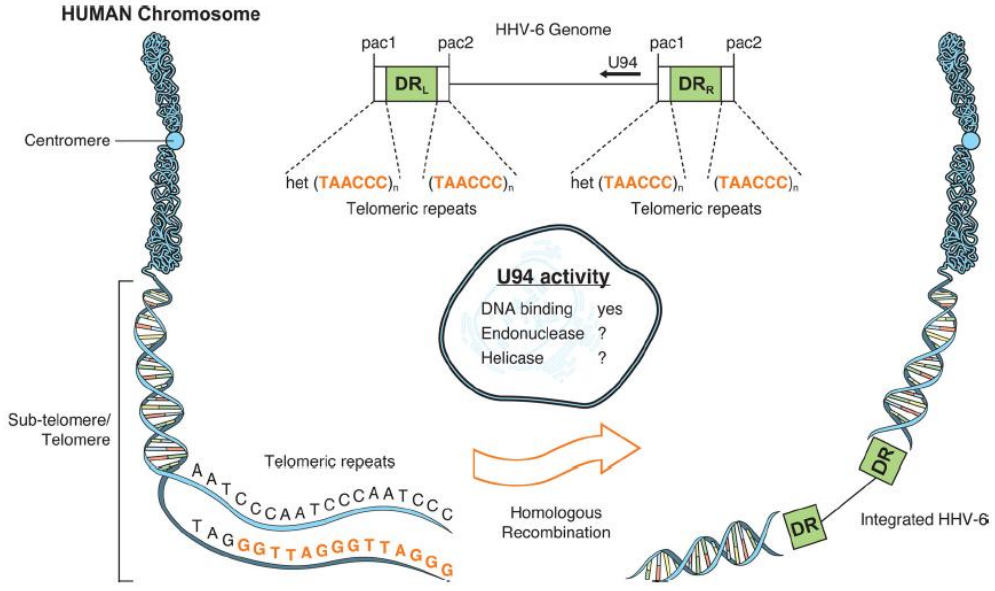
Clinical consequences and detection of CIHHV-6

The cells harboring CIHHV-6 can also be transmitted through hematopoietic stem cell or solid organ transplantation, thus posing a threat to the transplant recipients (57). So far CIHHV-6 phenotype is not linked to any definite pathology but there is a growing concern regarding the medical safety of cells, tissues, and organs from individuals with CIHHV-6 for transplant purposes as the possible clinical problems associated with such samples still remains elusive. Since CIHHV-6 DNA sequences are inherited through the germline, viral DNA is present in every nucleated cell in the body and considerably high levels of the viral DNA can be detected in a range of body fluids including whole blood, serum, plasma, and cerebrospinal fluid (58). Such cases often confound the diagnosis of active HHV-6 infection resulting in unnecessary treatments and antiviral therapies that can potentially lead to serious consequences, especially in immunocompromised conditions (48). Persistent high viral loads can be used to differentiate from those observed during active viral infections as the latter are comparatively lower and exhibit transient viral elevations (59). The hair follicles or nails of the CIHHV-6 positive individuals also harbor detectable levels of HHV-6 DNA, thus PCR testing in these tissues is yet another distinguishing factor from active viral infection and is very useful for diagnosis in cases where obtaining blood samples is complicated (59). The most commonly used method to identify chromosomal integration is FISH, although other complementary methods, for instance inverse PCR followed by sequencing, chromosome specific PCR and Gardella gel analysis have also been used (54, 60) .

Mechanism of chromosomal integration

Both HHV-6A & HHV-6B are capable of chromosomal integration and there seems to be no preferential targeting to any particular chromosome (48,60). The various integration sites identified so far include 1q44, 9q34.3, 10q26, 11p15.5, 17p13.3,

18p11.3, 18q23, 19q13.4, and 22q13.3. Interestingly, most of the integration sites identified so far have been invariably found in the proximity of telomeres. The mechanistic principles behind this phenomenon still remain elusive; however, it has been proposed that the serial TAACCC motifs present at the termini of the viral genome, particularly adjacent to the pac1 sequence, mediates the integration (48). As these viral repeat motifs are identical to the human telomeric repeat sequences (TMR), homologous recombination takes place between them leading to either partial or complete integration of the viral genome (Fig-5). However, the presence of these TMR motifs alone do not seem to be the only factor mediating the entire process since another member of the human herpesvirus family, HHV-7 also harbors these sequences, but has never been reported to integrate in host chromosome. HHV-6 genome uniquely harbors U94 gene that acts as a viral transactivator, which has been reported to inhibit virus replication and block viral gene expression in vitro, thus making it a possible candidate factor favoring virus integration (48).



Source: J.Virol (2010) 84:12100-09

Fig-5. Hypothetical model of the mechanism of the integration of HHV-6 genome in the human chromosome.

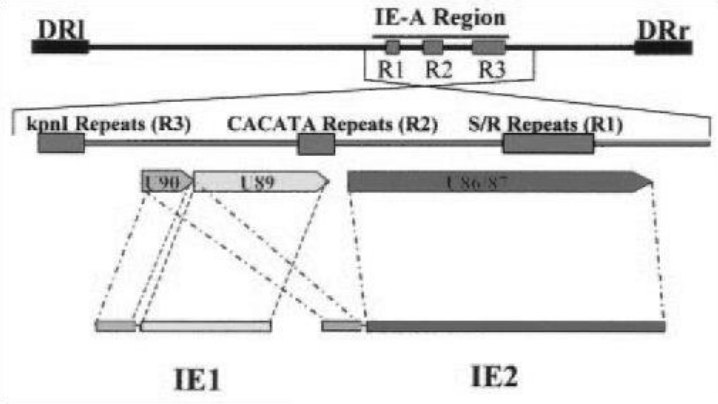
Another report has demonstrated that for herpesviruses including HHV-6 and Marek's disease virus (MDV), the presence of TMR in the viral genome facilitates targeted integration of the viral genome into the telomeric region of the host chromosomes, while if the TMR are absent or mutated, the integration frequency is reduced and occurs elsewhere intra-chromosomally (61).

Reactivation of CIHHV-6

Initially, chromosomal integration was considered as the concluding phenomenon of the HHV-6 lifecycle, although this view has been strongly challenged since it was demonstrated that the integrated viral genome (HHV-6A) is indeed capable of producing virions in vitro (54). In vitro infection of Human Embryonic Kidney 293 (HEK 293) cells with HHV-6 as well as T cells isolated from CIHHV-6 positive patients with Trichostatin A showed a significant increase in the viral copy number post drug treatment in comparison to the untreated cells. This indicated a possible viral reactivation in these cells. The results were further confirmed by co-culturing uninfected Molt-3 cells with the PBMC isolated from the families exhibiting CIHHV-6 in the presence of 12-O-tetradecanoyl-13 acetate (TPA) & Hydrocortisone. This resulted in syncytia formation in the Molt-3 cells and HHV-6 DNA and RNA were also detected in the infected Molt-3 cells, thereby confirming the reactivation of the integrated viral genome (54). Although, there is a lack of sufficient data to conclusively support the hypothesis of viral reactivation, it has been postulated that for individuals presenting with CIHHV-6, treatment or exposure to certain pharmaceutical drugs can potentially lead to the viral reactivation either directly or indirectly and thus needs a careful observation (59). Transplacental passage of the maternal HHV-6 infection has also been reported, suggesting that CIHHV-6 can indeed replicate and account for at least some percentage of congenital infections (62).

HHV-6 Immediate Early genes

The life cycles of all herpesviruses can be characterized by a cascade system of gene regulation that is separable into 3 broad phases termed the IE, E and L phases. As the name suggests, the IE genes are the first genes expressed post virus entry and are master regulators of the viral gene expression. Their transcription initiates within minutes to hours post entry and is mostly dependent on the virion associated proteins (63). The HHV-6 genome contains two major IE loci namely IE-A and IE-B (Fig-2b). The locus B comprises of the ORF U16 to U19, while the locus A comprises of 2 large ORF U89 and U86 (63). The U16/17 spliced gene product, U18 and U19 together are the positional homologues of HCMV UL36-38 genes belonging to a family of transcriptional activators (14, 64). The locus A comprises of two genetic units namely IE1 and IE2, corresponding to ORF U90/89 and U90-86/87 respectively. Both IE-1 and IE-2 are expressed as spliced gene products and both contain an exon derived from U90. This locus also contains 3 intermediate repeats R1, R2 & R3 (14; Fig-6).



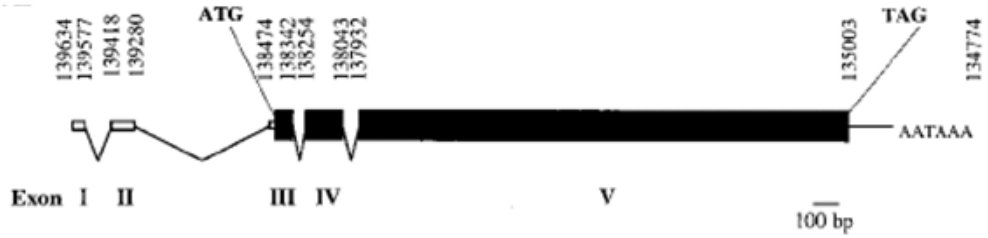
Source: Clin Microbiol Rev. (2005) 18: 217–245

Fig-6. Schematic diagram of the viral IE-A region

R1 is composed of serine and arginine repeats (S/R repeats) that lie within U86/U87, while R2 is composed of CACATA repeats. R3 is located upstream to the IE-A region and is one of the major repetitive elements in the unique region of the viral genome. It is composed of multiple copies of ~103-108 bp units that contain binding sites for

transcription factors for instance NFκB & Activating Protein-2. The R3 repeat region has been suggested to play a role in transcriptional regulation of the IE-A locus (65). Interestingly, the IE locus is the most variable part of the HHV-6 genome with reference to nucleotide identity between HHV-6A & B and shows ~31 % sequence identity in comparison to the overall identity of almost 90-95% for the other ORF. This variation has been speculated to account for some of the biological differences between the two HHV-6 species (14).

For HHV-6B, the IE1 transcript is 3720 nucleotides long, contains 5 exons, of which the ATG start signal is located in the 3rd exon which results in generation of a 3237 nucleotide long ORF that encodes the IE1 protein (63; Fig-7).



Source: J Biol Chem. (2002) 277:19679-19687

Fig-7. Organization of IE1 transcript from HHV-6B.
Structural organization of the mature IE1 transcript showing 5 exons, labeled as I to V from the viral isolate Z29. The numbering indicating the genomic co-ordinates, open boxes represent the non-coding exons while the black boxes represent the coding regions.

4 transcripts of ~1.0 kb, 1.5 kb, 3.7 kb & 4.7 kb in length have been identified for the IE1 gene, out of which only the 3.7 kb transcript is expressed under IE conditions and is described here (66). Kinetic studies performed on freshly infected Molt-3 cells indicated that the transcription of IE1 gene begins immediately after virus entry inside the cells and the 3.7 kb transcripts can be detected as early as 2 hour (h) following infection. The abundance of the transcripts increases up to 12 h post infection, which is followed by a decline in the mRNA levels, until the secondary infections sets in as a result of the release of infectious virions and subsequent infection to fresh cells. This leads to an

increase in the mRNA levels that can be observed at ~ 72-96 h post initial infection (63). The protein encoded by the IE1 gene is 1078 amino acids long and can be detected at 4 h post infection. It is a nuclear protein and has a calculated molecular mass of ~120 kilo Daltons (kDa). The protein is acidic, contains several phosphorylation sites and two Small Ubiquitin-like Modifier (SUMO) protein conjugation sites. IE-2 transcript is composed of 5 exons, out of which the first 4 exons originate upstream of U89 & are very similar to those present in IE1 while, the 5^{th} exon corresponds to U86 (Fig-6). Kinetic studies performed on infected HSB-2 cell line indicated detection of the IE2 transcripts at 4 h following infection and its expression increases over time up to ~96 h, in comparison to IE1 expression that drops after 12 h (67). The IE2 transcript for HHV-6A is shorter in length in comparison to HHV-6B, with the former being 5.5 kb while the latter is 6.3 kb. Studies have also indicated that the IE1 transcript is generated prior to IE2. The protein encoded by IE2 gene is a nuclear protein with a calculated molecular mass of ~180 kDa for HHV-6B and can be detected as early as 8 h post infection. The protein contains several sites for phosphorylation and the C-terminal portion of IE2 harbors the R1 repeat region (67).

Functions of IE1&IE2 proteins

These proteins have been proposed to play a pivotal role in initiation of infection and in establishment of a cellular environment compatible with the lytic phase of infection. In the temporal cascade of viral gene expression, the IE polypeptides trans-regulate the expression of early genes thereby yielding proteins that are mostly involved in the viral DNA replication & metabolism (14). The functions of HHV-6 IE1 are yet to be fully elucidated, except its ability to transactivate heterologous promoters for instance HIV-1 LTR, CD4 (63, 29). IE1 has also been reported to inhibit IFN-β gene expression, thus interfering with the development of innate antiviral response (68, 69). HHV-6 IE2 can

functionally transactivate multiple promoters having varying regulatory elements for instance, HIV-LTR, HHV-6 IE promoter and minimal promoters containing only a TATA box. It has been proposed that IE2 induces transcription of these promoters by interacting with basic cellular machinery, a feature shared by CMV IE2 as well (67, 70-72). Thus, it is evident that although there is a limited amino acid sequence homology between HCMV and its HHV-6 IE positional homologues, many of its functions have remained evolutionally conserved. HCMV has also been documented to regulate the expression of host genes involved in growth regulation and cell cycle control, e.g., c-fos, c-myc and hsp-70 (73). The transactivation is mediated both independently by IE1 & IE2 as well as synergistically in the presence of both proteins. Moreover, HCMV infection was also found to induce the expression of the corresponding endogenous genes at very early time points following infection indicating the involvement of IE genes in the same (73).

Herpesvirus for gene delivery

An efficient transgene delivery to the target cells is important for gene therapy and the same can be accomplished by various methods. One of the most popular methods is the use of viruses or virally derived vectors for gene transfer. A considerable number of virus based vector systems have been generated over the past three decades using retroviruses, adenoviruses, adeno-associated viruses and to a relatively less extent from herpesviruses. The herpesvirus vectors were developed in early eighties, based on the observations that when cultured at high multiplicity of infection, infectious defective particles get spontaneously generated, which are essentially composed of large concatameric genomes having repeat units of relatively limited complexity. This feature was initially explored in HSV to reveal that these defective genomes carried only minimal subset of DNA sequences from the wild-type viral genome, and are comprised

of an origin of DNA replication (HSV-ori) and a cleavage/packaging site (*cis*-acting sequences), which can be efficiently replicated and packaged in the presence of the *trans*-acting wild type virus (74). This led to generation of the first classical HSV-1/2 derived vectors that were comprised of cloned defective genome *cis*-acting elements, which by principle, could indeed replicate in the presence of the helper virus to yield large concatameric genomes and get packaged to deliver their genetic material to target cells. These vectors were termed as 'amplicons' to imply that multiple copies of cloned DNA sequences of interest can be amplified in a head-to-tail arrangement in concatameric defective virus genomes and packaged into virions. These vectors were capable of shuttle delivery of DNA sequences from prokaryotic to eukaryotic cells and could be packaged in different cell lines. Thus, they were considered attractive for gene delivery to the target cells including gene therapy for neurological diseases as HSV is a neurotropic virus (75, 76).

Other than amplicon vectors, yet another gene delivery vector system, the recombinant HSV-1 vectors, was generated by replacing the non-essential viral genes with transgene(s) of interest at different sites in the viral genome. The same can be achieved by two ways, either by deleting the genes required for lytic replication (for instance, the IE genes) while retaining those involved in latency. Alternatively, oncolytic vectors can be made by deleting genes required for replication inside non-dividing cells while retaining those required for replication inside the dividing cells (77, 78). These vectors are thus suitable for cancer therapy and have been used for performing studies on models of neurological diseases (78). In addition to this, these recombinant vectors can also be used as helper viruses for amplicon vectors, thereby providing unique complementary components as per the requirements.

HHV-6 amplicon vector

As the amplicon vectors were relatively easier to generate and was successful for HSV, the same technique was adapted by a number of research groups working on other members of the herpesvirus family. Thus, based on the same aspects of DNA replication and packaging, a number of amplicon vectors were reported, for instance EBV, CMV, HHV-6 & HHV-7 based amplicons (79-82). Amplicon vectors derived from HHV-6 (both A & B) developed relatively fast due to the substantial experience gathered from previously derived HSV-1 amplicons. HHV-6 genome harbors all the essential elements required for generation of amplicon vector as described for HSV. HHV-6 genome has a single origin of replication, it forms concatemers in which the packaging signals are placed adjacent to each other during replication and the characterization of sequences required for genome cleavage and packaging has also been described before (83, 84). Using this preliminary information, the viral origin of replication and cleavage-packaging sequences were incorporated into suitable platforms and used to generate amplicon vectors (81, 85, 86).

The amplicon vector contains the cloned viral DNA replication origin, the packaging signals pac1 and pac2 and the transgene(s). All the three components can be brought together in a plasmid based platform and then transfected into the host cells that support lytic viral life cycle. Subsequently, cells are either infected with the wild type virus (referred to as helper virus) or alternatively, helper virus infected cells can also be transfected with the amplicon plasmids. These transfected as well as transduced cells support the replication of the helper virus as well as the amplicon plasmid units that takes place with the help of factors provided in *trans* by the helper virus. Defective virus genomes having multiple reiterations of the input amplicon plasmids are thereby generated. These concatameric genomes are then cleaved at the cleavage signals to yield

the two genomic termini (86). The cleaved genomes are subsequently packaged as pseudovirions, again using factors contributed by the helper virus. The helper virus replication, packaging, and assembly take place in parallel inside the same cells. As a result, the virions harvested from the packaging cells contain a mixture of the pseudovirions harboring the transgene and the wild type helper virions that can be used to transduce target cells. Following is a pictorial representation of the principle described above (Fig-8).

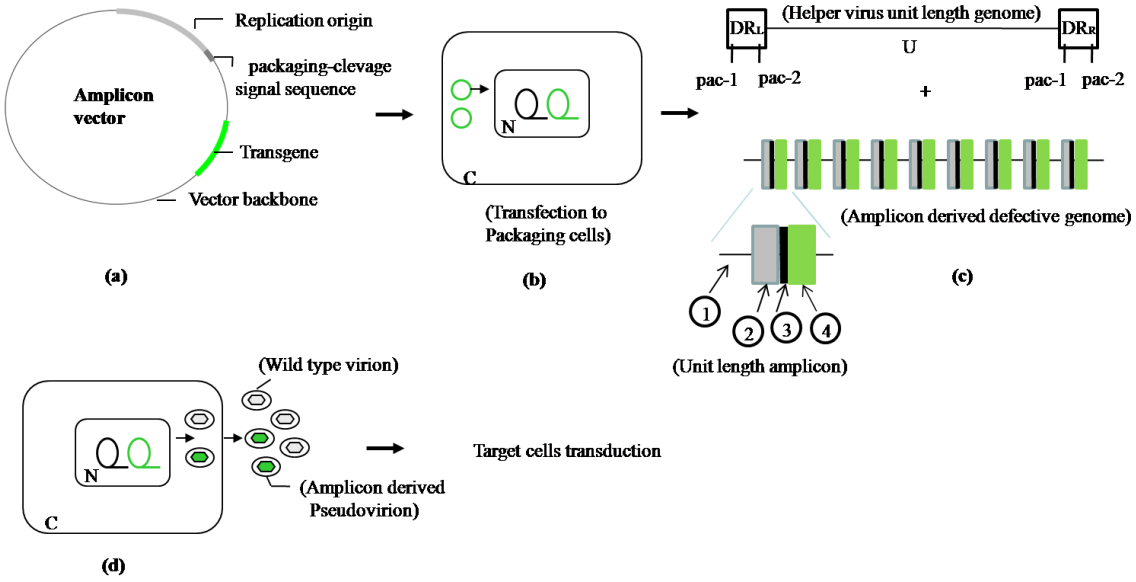


Fig-8. Generation of HHV-6 amplicon vector system.
(a) A basic amplicon vector with its various components; (b) a representative packaging cell transfected with amplicon plasmid showing generation of concatameric intermediates during replication. The genome shown in black represents the helper virus genome while the one shown in green represents the replicating amplicon derived genome; (c) replicated and cleaved unit length helper virus genome and amplicon derived defective virus genome with multiple copies of the amplicon plasmid. The enlarged view shows a single unit of linearized amplicon showing 1: plasmid backbone, 2: replication origin, 3: packaging-cleavage sequence and 4: transgene; (d) cell showing egress of the packaged pseudovirions (green) and helper virions (grey). Abbreviations: C, cellular cytoplasmic compartment; N, the nuclear compartment; U, unique region of viral genome.

HHV-6 amplicons exhibit efficient transgene expression as well as good cell spread. In the presence of helper virus, the vector has been reported to be further transmissible to uninfected cells both as cell associated as well as cell free virions (86). The transgenes

successfully expressed using amplicon vectors include GFP and HSV-1 glycoprotein D (both secreted as well as membrane associated forms).

Amongst all the available gene therapy vector systems, the herpesvirus based vectors exploit most of the advantages of the parent virus. Their most outstanding feature is ability to transfer more than 100 kb of foreign DNA into the nucleus of mammalian cells, no other viral vector systems developed so far has that much loading capacity of foreign DNA. Apart from this, they offer a broad host range with the ability to transduce both dividing and non-dividing cells. Helper free virus systems have also been generated in case of HSV and thus, these vectors do not carry any viral proteins thereby minimizing the chances of any unwanted immune response (87). The amplicon vectors can be employed in basic as well as in applied studies, for instance, exploring functionality of selected foreign transgenes, understanding the mechanisms of virus DNA replication, and packaging & gene therapy approaches to various target cells.

Similarly, EBV derived amplicon system was originally used to analyze viral origin of replication and to explore the immortalizing function of the virus, using EBV as a helper virus (88). Subsequently, first generation packaging cell line, having deletion of EBV packaging signal was developed for virus free packaging of EBV (79, 89). HCMV based amplicons were developed with the aim to exploit its natural tropism for the cells of haematopoeitic lineage (90). Since the development of amplicon vectors more than three decades ago, they have been extensively modified to enhance their safety profile which involved development of helper virus free packaging systems, inclusion of foreign polypeptides in the virus particles to increase the specificity of infection and also to improve the efficiency as well as stability of transgene expression (91). Hybrid vectors have also been created in order optimize the biological properties of the vector DNA for their potential application in clinical gene therapy protocols. For instance, HSV/Adeno

Associated Virus (AAV) hybrid vectors were designed to allow for stable transgene expression in comparison to the basic HSV amplicon vector. Alternatively, HSV/EBV hybrids have also been constructed in order to achieve episomal replication of the amplicon DNA. Additionally, 'tribrid' amplicon vectors have also been successfully generated, for instance those incorporating components from Moloney Murine Leukemia Virus (MMLV) that enable a random genomic integration in dividing cells for e.g., HSV/AAV/MMLV and HSV/EBV/MMLV vectors (91). HSV-1 based vectors have emerged as very promising candidate as an oncolytic virus, as it exerts a moderate pathogenicity in humans, specific antiviral therapy is available to control viral infection. HSV vectors can be produced without wild type virus contamination and the viral genome can be genetically modified to alter cell tropism, express heterologous genes, or delete genes to make it cancer-specific.

Virus titration

Virus titration is an essential step in research and development and essentially involves estimation of the number of infectious virus particles in an innoculum or sample. There are different methods for titration, (i) Measurement of 50% Tissue culture infectious dose ($TCID_{50}$)-the most commonly used end-point dilution assay that involves determination of the dilution of a particular virus stock at which 50% of the cells exhibit cytopathic effects, which is used to mathematically calculate the viral titer; (ii) Plaque assay-based on the ability of virus particles to form plaques on adherent cell monolayers and is expressed as number of plaque forming units/ milliliter (ml) of input virus and each plaque represents one virus particle; (iii) Fluorescent focus assay-immunostaining of the viral antigens in the infected cell monolayers and thus reports the titers as fluorescent focus forming units. This technique has the advantage of being faster in comparison to the $TCID_{50}$ and plaque assays; (iv) Transmission electron microscopy

(TEM)-quantification of the actual number of virus particles inside the cells or any sample based on their morphology; (v) Serological assays-based on quantification of specific viral proteins rather than the actual virus particles. Most commonly used assays include hemagglutination assay (used specifically for influenza viruses), virus neutralization assays, immunostaining & ELISA (92); (vi) Nucleic acid based assays-Quantitative PCR (qPCR) quantifies the viral nucleic acids (DNA/ RNA) in a sample instead of whole virus particles and is considered to be very rapid and a sensitive technique and (vii) Viral enzyme assays-measuring the activity of virally encoded enzymes, for e.g., reverse transcriptase (used routinely for retroviruses) (93) .

In an attempt to generate an altogether new assay system for virus titration, that would also enable screening of antiviral compounds, a new reporter based cell line for HHV-8 titration was reported in 2003 (94). The report described utilization of the viral replication and transcription activator (Rta) encoded ORF that is a master regulator for the switch from latent to the lytic phase of infection and also a potent transactivator of multiple HHV-8 and cellular promoters. A reporter plasmid was generated that contained the lacZ gene under the control of polyadenylated nuclear RNA promoter, which has been reported to be strongly activated by the viral transactivator, Rta. Using this construct, HEK 293 reporter cell line was generated that gave an enhanced reporter gene expression in response to virus infection. This cell line was used to characterize the factors affecting HHV-8 infectivity. Using the same principle, another reporter cell line was generated to simplify the titration of infectious VZV (95). Luciferase was used as a reporter gene and an up-regulation in its activity in a dose dependent manner was observed upon infection with VZV. The cell line was also used for evaluation of antiviral compounds thus, demonstrating its practical usefulness for treatment of VZV-associated diseases. Based upon the transactivating properties of viral IE class of genes, a similar

reporter cell was also established for a very closely associated member of HHV-6, which is CMV (96). An anti-CMV compound was identified during this study which was further characterized using the same reporter cell line (97). Similar cell lines have already been reported in the past for quantitation of infectious HIV-1 (98).

HHV-6 titer estimation

HHV-6 can infect a variety of cell types but the virus replication is essentially cell type specific. So far the standard $TCID_{50}$ calculation in mononuclear cells has remained the most frequently used method for estimating the infectious viral titer in any given innoculum. Other commonly used techniques including plaque or focus formation assays cannot be employed for due to the fact that this virus mainly establishes a productive infection in T-lymphocytes (99). Although, the $TCID_{50}$ assay has remained a gold standard for titer estimation, the technique has some evident disadvantages for instance it is labor intensive and also requires a large amount of cells and virus samples as a starting material. As the $TCID_{50}$ assay is not suitable for evaluating the neutralizing antibody titer (NT), a relatively convenient assay for HHV-6B was generated using indirect immunofluorescent-antibody (IFA) staining (100). Chemically adhered MT-4 (T-cell line) cell monolayers on spot slides were infected with serial dilutions of their virus preparation and then fixed & stained by IFA technique at ~30-45 h post infection using either sera from exanthem subitum patients or HHV-6 specific monoclonal antibodies. The results correlated well with the $TCID_{50}$ assay, which was performed in parallel on MT-4 cells, using the same dilutions of virus preparation. This new technique was much more rapid in comparison to $TCID_{50}$ assay, required relatively less number of cells as well as virus, and could also be used for performing the NT test. Alternatively, flow cytometry analysis of the infected cells using virus specific antibodies can also be carried out and the same has been reported to be a reliable quantitative method to analyze virus

multiplication in vitro, although overall time required for the completion of the assay is more in comparison to IFA staining described before (101). Real time qPCR has also been reported to a very sensitive, high throughput and a reliable quantitative assay and has been used for virus titration in the infected cells as well as biological samples (102).

CHAPTER 3

MATERIALS & METHODS

SOURCE OF REAGENTS

1] Mammalian cell culture: Dulbecco's Modified Eagle Medium (DMEM), RPMI-1640, Dulbecco's Phospahte-Buffered Saline (DPBS), Opti-Minimum Essential Media (MEM): *Gibco BRL, USA*; Fetal Bovine Serum (FBS): *Gibco BRL, Sigma Aldrich, USA,SAFC Biosciences, Australia*; Sodium Bicarbonate, Ficoll-400, Sodium Diatrizoate, Polyethylene glycol (PEG-8000), Dimethyl sulfoxide (DMSO): *Sigma*; Gentamycin: *Nicholas Piramal, India*; *USA*; Geneticin (G418): *Sigma*, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) buffer (free acid): *HiMedia*, India; Trypsin, Phaseolus vulgaris lectin-P (PHA-P): Difco, USA; Ethylenediamminetetraacetate.$2H_2O$ (EDTA .$2H_2O$): *Gibco BRL, USA*; Interleukin-2 (IL-2): *Roche, Germany*; Lipofectamine-2000: *Invitrogen, USA*, Erythrocin B: *HiMedia*; Sucrose: *USB, USA*; Syringe filters: *MDI,India;* Glutaraldehyde: *Sigma.*

2] Culture plastic wares: *Nunc, Denmark; Corning,BD Falcon, Nalgene, Millipore, Thermo-Fisher, USA; Grenier, Germany*; *Axygen Scientific, USA.*

3] *Bacterial cell culture* Tryptone, Yeast extract, Agar powder: *HiMedia*; Ampicillin, Glycerol, Isopropyl β-D-1-thiogalactopyranoside (IPTG), Ribonuclease A (RNAse A): *Sigma*; Kanamycin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal): *USB*; E.coli DH5αMCR: *Life technologies, USA.*

4] Common salts, buffers, organic reagents, acids: *Sigma, Merck, Fluka, Germany; SRL,Qualigens, India*.

4] DNA extraction, electrophoresis and detection: Agarose, Low melt agarose: *Sigma, Amresco, USA,* Seakem LE, *Switzerland*; Ethidium bromide (EtBr), Bromophenol blue, Xylene cyanol: *USB*; Oligonucleotide primers: *Sigma*; DNA molecular weight markers: *MBI Fermentas, Lithuania*, Proteinase K: *Roche.*

5] Restriction enzymes (RE), DNA modifying enzymes, DNA polymerases: *MBI Fermentas, NEB, USA.*

6] RNA extraction, complementary DNA (cDNA) preparation: TRIzol reagent *Invitrogen*; DEPC: *Sigma*; Oligo (dT)12-18 primer, Random hexamers, dNTPs, Revertaid Reverse Transcriptase enzyme (RT), RNase inhibitor, Deoxyribonuclease I (DNase I): *MBI Fermentas.*

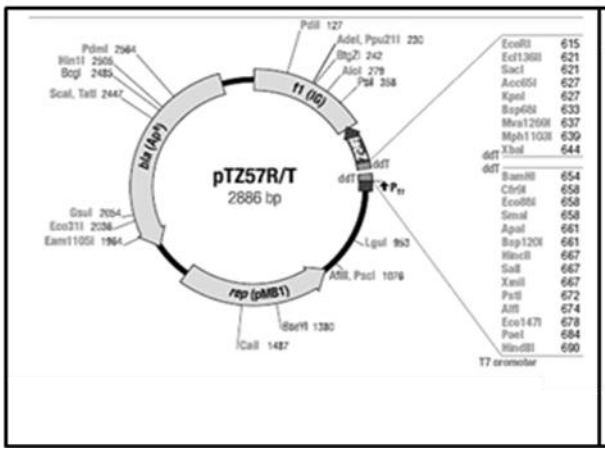
7] Protein-detection, purification: Acrylamide, Bis-acrylamide, 2-Mercaptoetahnol (2-ME), Protease inhibitors, BSA, Tween-20, Sodium dodecyl sulphate (SDS), Ponceau S: *Sigma*; Tetramethylethylenediamine (TEMED), Ammonium Persulfate (APS): *USB*; Polyvinylidene fluoride (PVDF) membrane: *Millipore, India*; Non-fat Dry milk: *Nestle, USA*; Enhanced chemiluminescence (ECL+) detection system, protein molecular weight marker: *GE healthcare, UK*; X-Ray films: *Kodak, USA*; Filter papers: *Whatman, USA*; Folin reagent: *SRL*.

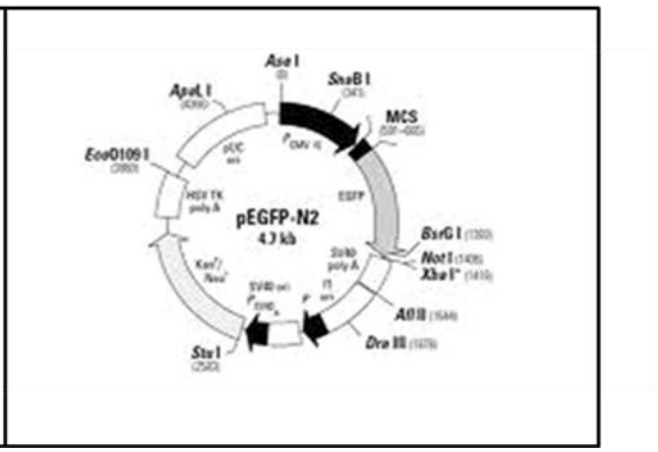
7] Antibodies: Anti-β-Actin (used at dilution of 1:2000), anti-mouse Horseradish peroxidase (HRPO) conjugate (used at a dilution of 1:2000): *Sigma*; anti- Green Fluorescent Protein (GFP, used at a dilution of 1: 15000): *Clontech, USA.*

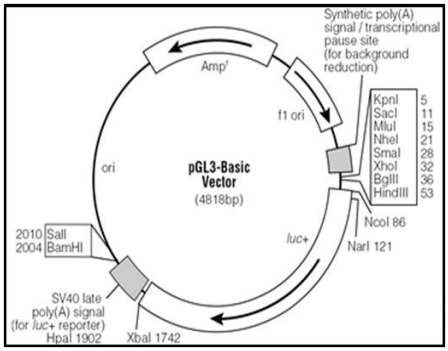
8] Plasmids: pTZ: *MBI Fermentas*; pcDNA 3.1(+): *Invitrogen*; pEGFP-N2: *Clontech*; pGL-3 basic: *Promega*; pIRES2-EGFP: *from Dr.Debashish Mitra.*

9] Kits: Luciferase assay system: *Promega, USA*; DNA purification kits: *Sigma, Invitrogen USA, Machery Nagel, Qiagen, Germany*; plasmid isolation kit: *Qiagen*.

Plasmid map of vectors used







MATERIALS

Mammalian Cell culture

RPMI 1640: Powdered RPMI 1640 and 2 gram (g) of sodium bicarbonate was dissolved in autoclaved deionized water (DW), final volume was adjusted to 1 liter (L) and media was filter sterilized using 0.45/ 0.1 µm filter and stored frozen; *DMEM*: Powdered DMEM, 3.7 g of sodium bicarbonate and 6.51 g of HEPES was dissolved in DW, filter sterilized and stored; *FBS*: Frozen Serum stock was thawed, 50 ml aliquots made aseptically and stored at -20°C; *Antibiotics*: Working concentration of gentamycin was 50 microgram/ ml (µg/ ml) ; *Complete medium (CM)*: 450 ml of sterile RPMI 1640 or DMEM media was supplemented with 50 ml of FBS (final concentration 10%) and antibiotics and stored at 4°C; *Freezing medium*: 1 ml of DMSO was added to 9 ml of CM

and stored at 4°C; IL-2 supplemented medium: 20 units (U) recombinant IL-2 was added per 1 ml of CM.

PHA-P: Lyophilized vial of 50 milligram (mg) was reconstituted with 5 ml of sterile DW, aliquots stored at -20°C and used at a working concentration 10 µg/ ml.

G418: 100 mg/ ml stock solution was prepared by dissolving 1 g powder in 10 ml DW, filter sterilized and stored as 1 ml aliquots at -20°C. Working concentration used was 400-600 µg/ ml of CM.

DPBS: Powdered DPBS was dissolved in DW to a final volume of 1 L, sterile filtered and stored at 4°C.

Phosphate buffered saline (PBS): NaCl-8.0 g, KCl-0.2 g, KH_2PO_4-0.2 g and $Na_2HPO_4.2H_2O$-2.6 g were dissolved in 1000 ml of DW; pH was adjusted to 7.4 and sterilized by autoclaving; *Trypsin–EDTA*: 0.25% Trypsin, 0.02 M EDTA was dissolved in 1X PBS, filter sterilized and stored at 4°C; *Erythrocin B*: 0.4% weight/ volume (w/ v) was dissolved in 1X PBS, filter sterilized and stored at room temperature (RT).

PBMC isolation: Disposable syringes and needles; 0.04 M EDTA: 1.49 g of EDTA was dissolved in DW by adjusting the pH to 8.0, final volume was made to100 ml, filter sterilized and stored at 4ºC. 1 ml of EDTA was used per 10 ml of blood; 1X PBS; lymphocyte separating medium: Ficoll-Hypaque; CM (RPMI 1640).

DNA/ RNA & Polymerase Chain Reaction (PCR)

Genomic DNA extraction: Lysis buffer: 10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA. $2H_2O$. stored at 4°C; 20% SDS: 20 g SDS was dissolved in 60 ml DW by warming at 37°C, final volume made to 100 ml, stored at RT; Proteinase K: 20 mg/ ml stock made in DW, stored as aliquots at -20°C; Phenol: Chloroform: Isoamyl alcohol used in ratio 25:24:1(v/ v); Ethanol; RNase A: 10 mg/ ml in water, stored at -20ºC; TE (pH 8.0); Sodium acetate: 3M (pH 7.4).

RNA extraction & cDNA synthesis: DEPC treated water: 0.01% (v/ v) of DEPC was added to DW, kept on shaker overnight at RT for uniform mixing, autoclaved and stored at -20°C; Trizol reagent; 70% alcohol diluted in DEPC water; Chloroform, Isopropanol, DNAse, 0.5 M EDTA; Oligo (dT)12-18 primer, Random hexamers; Deoxynucleoside triphosphates (dNTPs): 10 mM mix containing dCTP, dGTP, dTTP, dATP; RT enzyme; 5 X reaction buffer; RNAse inhibitor; RNAse free plasticware and autoclaved pipette tips.

Agarose gel electrophoresis: 10 X Gel running buffer: Tris Borate EDTA (TBE): 108 g Tris (0.9 M), 56 g Boric acid (1 M), 40 ml of 0.5 M EDTA (20 mM) was dissolved in DW, volume was adjusted to 1L, stored at RT and used at final concentration of 0.5 X; Agarose powder; Ethidium bromide: 10 mg/ ml; 6 X gel loading dye: 0.25% Xylene cyanol, 0.25% Bromophenol blue, 30% glycerol; DNA markers: λ HindIII, 100 bp, 1 kb.

PCR: Taq DNA polymerase/ Long template PCR Taq, 10X PCR Buffer without $MgCl_2$; 1.5 M $MgCl_2$; 1 mM mix containing dCTP, dGTP, dTTP, dATP; Oligonucleotide primers; Nuclease free water (stock): DW was autoclaved and stored as aliquots at -20°C; thin walled 0.2 ml tubes and face masks.

Transfection

Lipofectamine method: Opti-MEM, Lipofectamine-2000, CM (DMEM/ RPMI) without antibiotics; plasmid DNA suspended in sterile Tris.EDTA (TE): 10 millimolar (mM) Tris, pH 8.0 & 1 mM EDTA, pH 8.0.

N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid-Calcium chloride (*BES-$CaCl_2$) based method:* 2.5 M $CaCl_2$: 3.7 g of $CaCl_2$ was dissolved in 10 ml DW, sterile filtered and stored as 1 ml aliquots at -20^0C; BES buffered saline: 50 mM BES, 280 mM NaCl,1.5 mM $Na_2HPO_4.2H_2O$-1.07 g of BES, 1.6 g of NaCl and 0.027 g of $Na_2HPO4.2H_2O$ were dissolved in DW. The pH of the solution was adjusted to 6.96 with

1 Normal (N) HCl, the final volume made to 100 ml, sterile filtered and stored in aliquots at -20^{0}C; CM (DMEM); plasmid DNA.

Infection, virus concentration

Disposable 0.45 µm syringe filters and syringes; 30% PEG with 0.4 M NaCl: 150 g of PEG and 11.7 g of NaCl was dissolved in DW separately, combined, volume adjusted to 500 ml, filter sterilized and stored at 4ºC; 20% Sucrose: 20 g of sucrose was dissolved in 1 X PBS, volume adjusted to 100 ml, filter sterilized and stored at 4ºC; ultra centrifugation tubes.

Sample preparation for Electron Microscopy (EM)

DPBS (Calcium, Magnesium free), Gluteraldehyde: 26 X stock (from NIV, Pune) was diluted to 1 X with DPBS and stored at 4ºC.

Lentiviral (LV) transduction

Polybrene (Hexadimethrine bromide): 2 mg/ ml made in DW, filtered, and stored at 4°C.

Bacterial culture, Plasmid Preparations, Transformation

Luria-Bertani medium (LB): Bacto-tryptone 10 g, Yeast Autolysate 5 g, NaCl 10 g dissolved in DW to a final volume of 1 L, autoclaved and stored at RT; *Agar plates*: 35 g of agar powder dissolved per 1 L (1.5%), autoclaved, cooled to 55°C, added antibiotic selection marker as per requirements and poured in 90 mm plates. Upon solidification of agar, plates were stored at 4°C; *Freezing media* 50% Glycerol diluted (v/ v) in water, autoclaved and used at a final concentration of 20%.

Antibiotics: Ampicillin/ Kanamycin 50 mg/ ml stock was prepared in DW, filter sterilized and stored in aliquots at -20°C. Working concentration was 50 µg/ ml; X-gal: 20% (w/ v) stock prepared by dissolving 40 mg/ ml of Dimethylformamide solution and stored in aliquots at -20°C; IPTG: 1 M stock made in water, filter sterilized and stored in aliquots at -20°C.

Ultra-competent cells: 10 mM Pipes, 15 mM $CaCl_2$ and 250 mM KCl were added to DW, pH was adjusted to 6.7 with 5 N KOH to make Transformation buffer (TB). Further, 55 mM $MnCl_2$ was added and final volume was adjusted as required, filter sterilized and stored at 4°C; Super Optimal Broth (SOB): 2% w/ v bacto-tryptone, 0.5% w/ v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$ were added to DW, pH adjusted to 7.0, autoclaved and stored at RT.

Resuspension buffer (solution I): 5 ml of 1 M glucose (50 mM), 2.5 ml of Tris.Cl (pH 8) (25 mM), 2 ml of 0.5 M EDTA (10 mM) added in DW to a final volume of 100 ml, autoclaved and stored at RT; *Lysis buffer (solution II)*: 2 ml of 10 N NaOH (0.2 N), 5 ml of 20% SDS (1%) was added to DW to final volume of 100 ml and stored at RT; *Neutralization buffer (solution III)*: 60 ml of 5 M potassium acetate (3 M), and 11.5% glacial acetic acid was added in DW to final volume of 100 ml, stored at 4°C; RNAse A, TE (pH 8).

Transformation: Ligation reaction/ plasmid DNA in solution, ultra competent cells E.coli (DH5αMCR), SOC broth: 20% Glucose added to SOB media, filter sterilized and stored frozen, LB agar plates with appropriate antibiotics, sterile tooth picks, IPTG and X-gal (in case of blue-white colony screening).

Protein expression & detection

Protein Extraction (from mammalian cells): Lysis buffer: 0.625 ml of 1 M Tris.Cl (pH 6.8), 1 ml of 20% SDS, and volume made to 10 ml with DW; Protease inhibitor (PI) stocks (100 X): Aprotinin, Leupeptin, Pepstatin-1 mg/ ml, PMSF-400 mM.

Protein estimation by Lowry Peterson's modification method: 0.4% Copper sulphate: 0.4 g of $CuSO_4$ was dissolved in 100 ml of DW; 0.8% Sodium Potassium Tartrate: 0.8 g of the powdered salt was dissolved in 100 ml of DW; 20% Sodium bicarbonate: 20 g of sodium bicarbonate was dissolved in 100 ml of DW; Copper-Tartrate-Carbonate reagent

(CTC reagent): made by mixing copper sulphate, tartrate & carbonate stock solutions in the ratio of 1:1:2; 0.8 N NaOH: 3.2 g of NaOH pellets were dissolved into DW to a final volume of 100 ml; 10% SDS: prepared by diluting 20 % SDS stock solution with equal volume of DW; Lowry mix (made fresh each time): made by mixing equal parts of DW, 0.8 N NaOH stock solution, 10 % SDS stock solution & CTC reagent.

Poly Acrylamide Gel Electrophoresis (PAGE): 30% Acrylamide: 29.2 g acrylamide, 0.8 g Bis-acrylamide were dissolved in 60 ml water, final volume was made 100 ml; 1.5 M Tris.Cl (pH 8.8): 18.165 g Tris was dissolved in water, pH adjusted to 8.8 and volume made to 1 L; 1.25 M Tris.Cl (pH 6.8): 15.1375 gm Tris/ L in water with pH adjusted to 6.8; 10 % Ammonium per sulfate (APS) made in water and stored at -20°C; TEMED; 20% SDS; Sample buffer (4 X): 125 mM Tris.cl (pH 6.8), 10% glycerol, 4% SDS, 4% 2ME, 0.02% bromophenol blue; Electrode buffer: 3.025 g Tris (25 mM), 14.4 g glycine (190 mM), 5 ml of 20% SDS/ L in water; protein molecular weight markers; destaining solution: 40 ml water, 50 ml methanol and 10 ml glacial acetic acid were mixed, and stored at RT.

Electroblotting & protein detection: Polyvinylidene Difluoride membrane (PVDF, 0.45 µm pore size); Whatman filter paper; Transfer buffer: 3.03 g Tris (25 mM), 14.4 g glycine (190 mM) 150 ml methanol (15%) was dissolved in DW to a final volume of 1 L; Ponceau S protein staining solution; Tris Buffered Saline (TBS): 20 mM Tris (pH 7.4), 500 mM NaCl; Blocking solution: 3% Bovine Serum Albumin (BSA) or 5% milk in TBS; TBST: 1 ml of Tween was added to 1 L of TBS; primary antibodies: Antibody dilution were made in TBST containing 1% BSA or 3% milk (w/ v); Secondary antibody: dilutions were made in TBST containing 2.5% milk (w/v); Chemiluminescent Substrate, X-ray films and exposure cassette.

METHODS

Genomic DNA extraction from mammalian cells

DNA was extracted by standard method involving disruption and lysis of cells in a hypotonic buffer containing EDTA and SDS, removal of proteins by Proteinase K and organic extraction, followed by recovery of DNA by ethanol precipitation.

1] 1-2x10^6 cells were resuspended in 400 microliter (µl) of lysis buffer, 8 µl of 20% SDS and 4 µl of Proteinase K and incubated at 37ºC in a water-bath overnight (O/ N); 2] The volume of the cell lysate was made up to 500 µl by addition of TE buffer followed by addition of an equal volume of saturated phenol. The suspension was mixed vigorously for 5 minutes (min), centrifuged at 12000xg/ 5 min and the upper phase (aqueous phase) was carefully transferred to a fresh tube; 3] An equal volume of chloroform: isoamyl alcohol mix (24:1) was added, mixed and centrifuged as above. The step was performed twice to remove any residual traces of phenol; 4] The aqueous phase was transferred to a fresh tube containing 1 ml of cold 100% ethanol and 50 µl of sodium acetate. The contents were mixed by gentle inversion and genomic DNA was pelleted by centrifugation at 12000xg at 4ºC/ 30 min; 5] The DNA pellet was washed once with 70% ethanol, semi air dried and resuspended in 50-100 µl of autoclaved DW or TE (pH 8.0) containing RNase. For uniform resuspension, the tube was first tapped gently and then left O/ N at 4ºC. For long-term storage, the DNA was stored at -20ºC. DNA was quantified by standard method of estimating its $OD_{260/\ 280}$ ratios.

RNA isolation from mammalian cells

RNA was isolated by Guanidium thiocynate-phenol-chloroform extraction method using TriZol.

1] Cells rinsed in PBS were suspended in TriZol at 1x10^6 cells per ml. Sample was either processed simultaneously or stored in -80ºC till further use. The frozen solution was

brought to RT, mixed by vigorous pipetting 4-5 times, & incubated again at RT for 5 min for efficient lysis; 2] 200 µl of chloroform was added, mixed by shaking for 5 min, incubated at RT/ 15 min for phase separation, & micro-centrifuged at 12000 RPM/ 15 min/ 4ºC; 3] The aqueous layer was carefully removed and transferred to a fresh tube without disturbing the interface. For RNA precipitation, 500 µl of isopropanol was added, mixed by inversion, incubated at RT/ 5 min, & micro-centrifuged at 12000 RPM/ 15 min / 4ºC; 4] The supernatant was gently removed, 1 ml of 70% ethanol was added to the pellet, the tube was gently inverted & micro-centrifuged at 7500 RPM for 5-10 min/ 4ºC. The supernatant was removed completely, the pellet was semi-air dried and resuspended in 30-50 µl of DEPC water by warming at 65ºC; 5] The tube was cooled to RT, transferred on ice and RNA was quantified by measuring $OD_{260/\ 280}$ ratio. RNA was stored at -80ºC for long term storage.

PCR

The technique was developed by Kary Mullis in 1983 and is currently used as an indispensible technique in most of the medical and biological laboratories for various applications including DNA cloning, sequencing, genetic fingerprinting, diagnostics etc. The method comprises thermal cycling and enzymatic replication of DNA using sequence specific primers and DNA polymerase that enable selective and repeated amplification of the target DNA. PCR reaction mixture was typically prepared by addition of the components described in Table 2.

Components	*Final concentration*
10 X PCR buffer	1 X
1 mM dNTPs	0.1 mM
25 mM $MgCl_2$	1.5-2 mM
Forward primer	10-50 pM
Reverse primer	10-50 pM
Taq DNA polymerase	1 U
Autoclaved DW	to make volume 50 µl

Table 2. PCR reaction components

*[PCR conditions for individual reactions & primer sequences are given in **Appendix**.]*

Individual reactions were routinely subjected to 30 cycles of denaturation, annealing, and extension. PCR reaction mixture was prepared separately in a no DNA room in a PCR work station and complete precautions were taken in order to avoid any PCR related contaminations. Additionally, while setting up HHV-6 specific nested PCR, a paper mask was worn in order to avoid any nasal secretion derived contaminations (103). 500 ng of DNA per sample was used as template for first round of HHV-6 specific nested PCR followed by one tenth reaction volume as template DNA source for the second round. 100 ng DNA from PJH6 was used as a PCR positive control. DNA quality assesment in all PCR reactions was done by amplification of house-keeping β-globin gene PCR using 100 ng of template DNA.

Long PCR: As the name suggest, it is a PCR variant which is more extended than a standard PCR which generally amplifies ~3 kb DNA fragments. However, such large amplifications require proofreading, thus, Thermostable Taq DNA polymerase with a 3'-5' exonuclease activity are used that enable amplification of large genomic DNA fragments which additionally provide higher yields and superior results even with difficult templates. Expand Long Template polymerase was used as per requirements. The Long PCR reaction mix was prepared similarly as standard PCR with minor modifications: 10 mM dNTPs, increased primer concentrations, addition of DMSO, and use of 5U DNA polymerase for each reaction.

Inverse PCR: This technique is used to amplify DNA with only one known sequence and is very useful for determination of insert locations for instance viral/ transposons integration sites in genomic DNA (Fig 9).

1] Genomic DNA derived from HHV-6 positive PBMC samples was RE digested with MboI at 1U/ µg of DNA for 6 h, 8 h, 10 h, 12 h, and 14 h individually; 2] all the samples

were diluted in water to 1 ng/ µl. 100 ng DNA per sample was split into 20 ng aliquots and self ligated with 1U of T4 DNA ligase for 14 h at 15ºC. The spilt reactions were pooled, the ligated DNA was precipitated in ethanol and sodium acetate and used as templates for performing inverse PCR; 4] amplified fragments were checked for expected size, gel purified and cloned into T/ A vector. The clones were sequenced from termini and the data were subjected to Basic Local Alignment Search Tool **(**BLAST) analysis to identify the virus-chromosome junctions for individual samples.

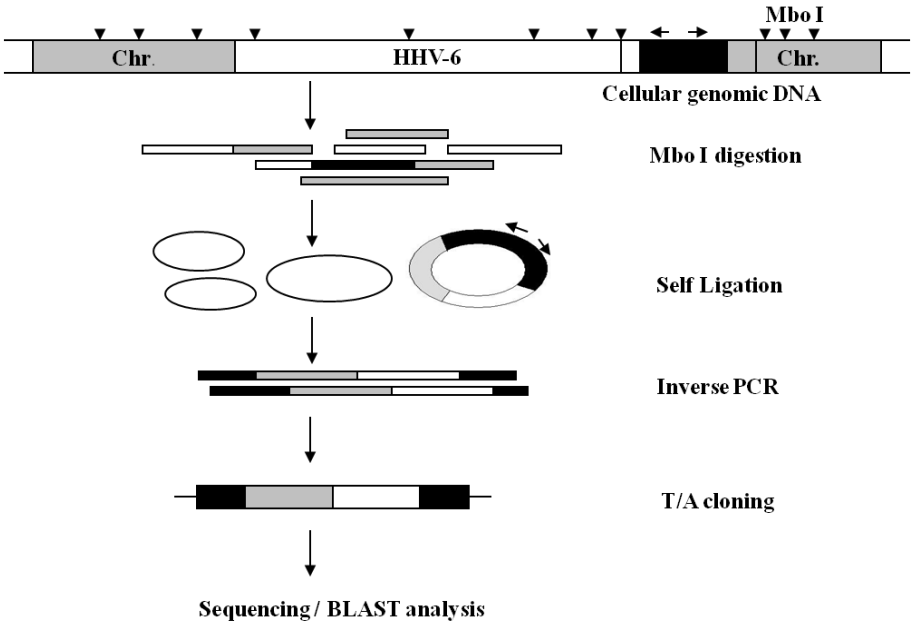


Fig 9. Diagrammatic representation of steps involved in inverse PCR sequencing.

cDNA synthesis, RT-PCR and densitometry

1] RNA was first treated with DNase I, for which the sample was diluted to 100 ng/ µl, reaction buffer (1 X), 0.2 U DNase/ µg of RNA was added, & incubated at 37ºC/ 10 min; 2] Reaction was terminated by addition of 5 mM EDTA followed by heat inactivation at 75ºC/ 10 min. Tube was brought to RT, transferred on ice & RNA was quantified ; 3] cDNA synthesis was carried out in a total volume of 20 µl per reaction. To 1 µg of total RNA, 500 ng oligo $(dT)_{18}$, 200 ng random hexamer primer were added, heated at 70ºC/ 5 min & immediately snap chilled in ice; 4] To this, 1 X reaction buffer, 1 mM dNTP mix,

40 U of RNase inhibitor and 200 U of Reverse Transcriptase was added, mixed gently by tapping and incubated at 25ºC/ 5 min for annealing, followed by reverse transcription at 42ºC for 1 h. Reaction was terminated by heat inactivation at 70ºC/ 10 min. An identical reaction was set up in parallel without the addition of RT as a control; 6] 100 ng of cDNA per sample was used as template for each RT-PCR reaction. GAPDH PCR was performed to assess the quality of RNA preparation. Equal volumes of PCR products were resolved on agarose gels and the acquired images were analyzed by densitometry using Image J software.

Preparation of ultra competent cells

Cell competency is a critical factor for achieving a high recovery of recombinant clones. *E.coli* strain DH5αMCR with a RecA (-) Endo A (-) phenotype was made ultra competent using the following protocol.

1] A single colony of freshly grown host cells was inoculated into 250 ml of SOB & grown at 18ºC with constant shaking (~ 250 RPM) till $O.D._{600}$ reached between 0.3-0.5; 2] The culture was immediately transferred to pre-chilled centrifugation tubes, pelleted down at 4ºC, resuspended in 80 ml of TB and incubated on ice/ 10 min; 3] The cells were pelleted, resuspended in 18.6 ml of TB & DMSO was added drop-wise to a final concentration of 7% and the cells were quickly split into aliquots of 200 µl, which were snap frozen in liquid nitrogen & stored at -80ºC.

Plasmid DNA mini-preparation

Plasmid isolation was routinely carried out by alkaline lysis method that involves bacterial cell lysis under alkaline conditions, followed by neutralization at acidic pH which essentially precipitates out the bacterial chromosomal DNA along with high-molecular weight cellular components, leaving behind plasmid DNA in the suspension that can be precipitated using isopropanol or ethanol.

1] Bacterial cultures were harvested for plasmid preparation after 14-16 h of growth. 1- 5 ml culture was pelleted at 12000xg/ RT and cells were resuspended in 100 µl of solution I containing RNase A, by vortexing the tubes vigorously; 2] 200 µl of solution II was added, mixed gently, incubated at RT/ 5 min, 150 µl of solution III was added immediately, mixed and incubated in ice for 5 min; 3] Lysate was spun at 12000xg at RT/ 10 min, supernatant was transferred to a fresh tube and plasmid was precipitated by addition of 1 ml of chilled 100% ethanol and sodium acetate at 1/ 10th volume of the supernatant and incubation at -20ºC/ 30 min; 4] Plasmid DNA was pelleted at 12000xg at 4ºC/ 30 min, pellet obtained was washed once with 500 µl of 70% ethanol, air- dried & resuspended in 20-50 µl of TE (pH 8.0).

Bacterial transformation

The process of introducing foreign plasmids into bacteria for amplification is termed transformation. The bacterial cell membrane is made passively permeable to target DNA by treating cells with $CaCl_2$ in ice cold conditions, followed by heat shock pulse which allows foreign DNA to enter inside cells and then cells are placed on ice again. This method works well for circular plasmids.

1] A single aliquot of ultra competent cells was thawed on ice, 100 ng of plasmid DNA or ligation reaction mixture was added, mixed gently, & incubated on ice for 30 min; 2] Heat shock pulse was given at 42ºC for 55 sec in a water bath and the tubes were immediately snap chilled in ice/ 5min; 3] Cells were revived by addition of enriched media (SOC) followed by incubation at 37ºC/ 45 min with constant shaking and subsequently plated onto LB agar plates with the appropriate selection markers and incubated at 37ºC for 14-16 h. In case of blue-white screening, IPTG and X-gal stock, 40 µl each was spread on the plates prior to plating the cells.

Agarose gel electrophoresis & purification of DNA

DNA fragments ranging from 50 bp to 50 kb in length are resolved in agarose gels and easily visualized by staining with intercalating dye such as EtBr.

1] Gels ranging from 0.8-2% were prepared in 1 X TBE buffer and casted in the pre-set gel-casting trays. 6 X loading dye were added to DNA samples at 1 X concentration, and samples were loaded into the wells of the gel pre-immersed in 0.5 X TBE. DNA marker for reference was also loaded in the gel each time; 2] Electrophoresis was carried out under standardized voltage and current conditions. The resolved DNA fragments were visualized by staining the gels with ethidium bromide (0.5 µg/ ml) and exposure to long wavelength UV light using an automated Gel Documentation system (Alpha Innotech, USA); 3] For purification of the required DNA fragments, the appropriate band of interest was excised from the gel under UV light exposure, immersed in 3 volumes of gel solubilization buffer (w/ v), and incubated at 65ºC till its complete dissolution. The suspension was cooled to RT, passed through the charged column, washed, and eluted in the elution buffer or DW water as per requirements; 2] Alternatively, low melt agarose gels were prepared; the excised gel pieces were immersed in TE melted by heating as above. Upon cooling, mixture was extracted once with phenol, once with chloroform: isoamyl alcohol and DNA was precipitated in ethanol as described before.

Gene Cloning

Incorporation of target DNA molecules (inserts) into vectors (plasmid/ virus) is necessary in order to enable their replication and expression inside the cells. Plasmid DNA is usually treated with RE to generate compatible ends (blunt/ cohesive) where foreign DNA is inserted by ligation. The ligation mixture is introduced into competent cells and the clones are screened by RE digestion or PCR. For performing RE digestions, 10 X reaction buffer (used at 1 X concentration), target DNA, 1 U RE/ µg DNA and

autoclaved DW to make up the volume was combined and incubated at 37ºC for 4 h to O/ N.

1] The RE digested DNA was first purified either by kit or phenol-chloroform extraction and resuspended in 15-16 µl of DW; 2] 1 X Klenow reaction buffer, 0.1 mM dNTPs and 5 U of Klenow fragment was added to a final volume of 20 µl, and incubated at 37ºC/ 10 min. Reaction was terminated by heat inactivation at 70ºC/ 10 min followed by phenol-chloroform extraction; 3] Ratio of vector: insert used was generally 1:3 for sticky ends and up to 1:6 for blunt ends. The purified vector and insert preparations were loaded on agarose gel to confirm their expected size and DNA quality and quantity before ligation; 4] Ligation reaction was set up in a total volume of 5-20 µl depending upon the final DNA concentration of the vector and insert. The following components were added, vector and insert DNA, 1 X ligation buffer, PEG-4000 (in case of blunt-ends), 5U of ligase enzyme was added in the end and incubated at 22ºC for 14-16 h in a water bath; 5] The reaction was terminated by heat inactivation at 70ºC for 10 min and stored at 4ºC till the next step which was usually carried out on same day.

Mammalian cell culture & maintenance of cell lines

The main cell types cultured are either primary cells or cell lines that can be grown for prolonged periods in vitro. All cells were maintained in a cell incubator at 37ºC, humidified environment with 5% CO_2.

1] A freeze down vial of cells was removed from liquid nitrogen facility, quickly thawed in a water bath and cells were immediately transferred to a sterile 50 ml tube to which excess of pre-warmed CM was added drop wise, while gently swirling the tube simultaneously; 2] To check the viability, a small aliquot of the cell suspension was removed, mixed with an equal volume of erythrocin B and loaded onto a hemocytometer for cell counting. The remaining cells were spun at 1000 RPM for 10 min at RT; 3]

Supernatant was discarded, cell pellet was resuspended in CM, transferred into a T-25 flask, and incubated; 4] Suspension cells were routinely cultured at a cell density of 0.3 x10^6cells/ ml of CM (RPMI). Cells were supplemented with fresh media every 3 or 4 days in order to maintain a healthy cell density. For changing media, cells were spun as mentioned before, and cell pellet was appropriately resuspended in fresh medium. 5] Adherent cells were cultured as monolayer in DMEM and split when the cells reached a confluency of about 80-90%. In order to split the cells, first the media was removed from the culture plate, 2 ml of Trypsin per T-25 flask was added over the cells and incubated for 2-5 min till the cells started detaching from the flask; 6] 8 ml CM was immediately added to the flask to neutralize any further trypisinization and the cellular clumps were dislodged by gentle pipetting. Subsequently, cells were transferred to a sterile tube, spun, and given an additional wash with DPBS to remove any residual trypsin. Washed cells were seeded as per requirements; 6] to make freeze downs, cells with >85% viability were washed with excess of DPBS and re-suspended in freezing medium at 1.0x10^6/ ml for adherent cells, 2-5x10^6/ ml for suspension cells and put in cryogenic vials as 1 ml aliquots. Vials were immediately transferred to -80ºC, kept for 12-14 h followed by transfer to liquid nitrogen container.

HHV-6 production

Virus was obtained from PJH6 cell line, an EBV transformed B-lymphocyte cell line established earlier in the laboratory from PBMC of a male individual with high copy HHV-6. Being a potential HHV-6 producer cell line, the cell culture supernatant was directly used as a source of infectious virus particles. However, for further enrichment of the virus stock, at instances the cells were also harvested to obtain cell associated virus. The cultures were handled in a class-II biosafety cabinet with class-III handling

practices. All the virus contaminated materials were treated either with 70% ethanol or 1% sodium hypochlorite made in water before re-use or discard.

1] PJH6 cell were seeded at $0.3x10^6$/ ml of CM (RPMI) for 72 h. The cells were pelleted at 1000 RPM for 10 min at RT and cell free supernatant was filtered through 0.45 µm filter to remove debris for target cell infection immediately or stored at -80ºC (extracellular virus stock); 2] To obtain intracellular virus as well, the remaining pelleted cells were resuspended in CM, subjected to 3 cycles of freeze-thaw, and pelleted at 3000 RPM for 5 min to remove the cell debris. This lysate was pooled with the cell line supernatant, filtered and used to infect target cells.

PBMC isolation from human blood

PBMC was purified from the blood samples using Ficoll-hypaque density gradient centrifugation, which takes the advantage of the differences in densities between various blood components. RBC and granulocytes being denser collect at the bottom of the gradient; in contrast the mononuclear cells and platelets have a lesser density than Ficoll-hypaque and collect on the top of the same layer. Platelets can be separated from mononuclear cells during subsequent washing and centrifugation steps.

1] Blood samples were collected in tubes containing EDTA and diluted with 2 volumes of 1 X PBS. 1 ml of Ficoll-hypaque per 3 ml diluted blood was under layered at the base of each tube and spun at 1500 RPM for 30 min at RT; 2] The Buffy coat was gently collected from the gradient using a Pasteur pipette, transferred to a fresh tube, diluted with excess volume of DPBS and centrifuged at 1200 RPM for 10 min at RT. Pelleted cells were again resuspended in DPBS, viability was checked and cells were spun at 1000 RPM for 10 min at RT; 5] cell pellet was either resuspended in CM for propagation or processed for DNA isolation/ making freeze downs as per requirements.

Infection of PBMC

1] $1x10^6$ freshly isolated PBMC was incubated with 1 ml of viral supernatant for 16 h, washed thrice with DPBS and processed for DNA isolation; 2] for mitogen stimulation, cells were suspended at $1x10^6$/ ml and incubated for 72 h in presence of PHA-P at10 µg/ ml; 3] cells were washed thrice with DPBS and then infected as above. Mock infection was performed in parallel using CM; 4] infected cells were washed thrice with DPBS and cultured for 48 h in CM supplemented with 20 units/ ml recombinant IL-2 and harvested for DNA isolation.

Infection of cell lines

Two lymphocytic cell lines SupTI (T-cell lineage), Daudi (B-cell lineage), and the monocytic cell line U937, were infected with the extracellular virus harvested from PJH6 culture.

1] $1x10^6$ target cells with a viability >90% were pelleted and washed once with DPBS and resuspended directly in 1 ml viral supernatant and incubated for 16 h. Mock infection was carried out in parallel by using CM in place of viral supernatant; 3] for infection by *spinoculation*, cells were spun at 1200xg at 37ºC for 1 h using a swing out rotor (HS-4; Sorvall RC-5B). Subsequently, the cell pellet was dislodged by gentle pipetting and incubated for 16 h in a 35 mm well plate at $1.0x10^6$ cells/ well; 4] cells were spun, supernatant discarded and cells were washed thrice with excess DPBS and incubated in fresh media at $0.3x10^6$/ ml for 48 h. Cells were washed once in PBS and harvested for genomic DNA isolation. Mock infected cells were also processed similarly; 5] for serial infections, infected SupTI cell culture was further expanded by adding fresh media and culture supernatant was collected identically, filtered, and used to infect fresh batch of SupTI cells as mentioned above.

EM

1] $6.0x10^6$ PJH6 cells were cultured to an expanded volume of 80 ml; 2] cells were spun at 1000 RPM for 10 min/ RT, cell free filtered supernatant was ultracentrifuged in a 45.94 rotor (Kontron, Italy) at 16000xg for 3 h/ 4ºC to obtain a virus pellet, which was resuspended in 200 µl PBS (400 X); 3] 50 µl aliquot of the concentrated virus was mixed with an equal volume of 1 X gluteraldehyde, adsorbed on carbon coated grids and negatively stained with phospo-tungstic acid. Virus preparation grids were imaged in a transmission electron microscope (Tecnai 12 Biotwin, FEI, The Netherlands) at National Institute of Virology, Pune.

Concentration of virus

PEG precipitation method. PEG precipitation is inexpensive, relatively simple and a nondestructive way to efficiently concentrate viruses.

1] Cell free culture supernatant was split to 1 ml aliquots, mixed with 0.5 ml of cold PEG solution by inverting the tubes several times and incubated over night on ice; 2] The mixture was micro-centrifuged at 16000 RPM for 45 min at 4ºC. The supernatant was aspirated off and the pellet was resuspended in 20 µl CM by vortexing (50 X stock). All the aliquots were pooled to for future use in target cell infection, as per requirements.

Sucrose centrifugation method.

1] Extracellular and intracellular virus preparations were transferred to centrifugation tubes and 20% sterile sucrose solution (1/ 10^{th} volume of viral supernatant) was under-layered at the bottom of each tube; 2] tubes were spun at 72000xg for 2 h at 4ºC in a Kontron TFT 45.94 rotor (66). The supernatant was carefully aspirated off and the viral pellet was resuspended in CM at 1/ 50^{th} of the starting volume. The stock was stored as aliquots in -80ºC.

Indicator cell line development

An indicator cell line with stably integrated transgene expression assemblies was generated by lentiviral mediated transduction following standardized lab protocol.

1] ~1.5x10^6 293FT cells were seeded a day before transfection in T-25 flask. 4 h prior to transfection, media was replaced with 7 ml fresh CM; 2] A transfection mix was prepared by adding the following components (Table 3).

Transducing vector bearing transgene (pLV)	24 µg
Tat plasmid (pTat)	4 µg
Rev plasmid (pRev)	4 µg
MDG envelope plasmid (pMDG)	8 µg
Gag-Pol-pRRE (pGPΔERRE)Packaging plasmid	16 µg

Table 3. Components of transfection mixture for production of LV particles

20 µl $CaCl_2$ and 200 µl BES was added and cells were transfected as before; 3] 16 h post transfection, media was replaced with 10 ml fresh media. Culture supernatant was collected at 48 h and 72 h post transfection, pooled and ultracentrifuged at 50,000xg at 4ºC for 1.15 h to pellet the virions. The viral pellet was resuspended in 2 ml CM (RPMI) and stored at -80ºC; 4] for transduction, 0.1x10^6 HEK 293 cells were transduced with 1 ml of the concentrated virus suspension (previously thawed on ice and brought to RT) in presence of 8 µg polybrene and incubated for16 h; 5] cells were washed twice with DPBS, 3 ml CM (DMEM) was added and incubated for 48 h. Culture was continued for 3 weeks in presence of G418 at 600 µg/ ml for selection to obtain stable clones. Several freeze downs were made at early passages post selection.

HHV-6 titer assay

1] 50 X concentrated virus stock, made by either PEG precipitation or ultracentrifugation, were serially diluted in CM for titration as follows (Table 4).

S.No.	Virus	CM	Final conc. (%)
(a)	100 µl 50 X stock	0 µl	100
(b)	100 µl of (a)	100 µl	50
(c)	100 µl of (b)	100 µl	25
(d)	100 µl of (c)	100 µl	12.5
(e)	0 µl	100 µl	0 (control)*

Table 4: Dilutions of the viral stock for titration

* *in case of PEG precipitated stock, 5 ml CM was precipitated and pellet resuspended in 100 µl of CM (equivalent to 50 X concentration) and used as a control.*

2] Indicator cells in CM were incubated in 96 well plates at 5000 cells/ well for 14 h when media was replaced with 100 µl of the serially diluted virus preparations (i.e., different virus concentrations) and further incubated for 16 h; 3] virus supernatant was replaced with fresh 100 µl CM in all the wells and immediately processed for luciferase assay following manufacturer's instruction.

Transfection

The term is used for the process by which nucleic acids are introduced into mammalian cells by non-viral methods. Together with the advent of reporter based assay, it has emerged as a powerful tool to study gene function, protein expression, etc. in context of a cell.

Calcium phosphate method. It involves mixing DNA with calcium chloride followed by controlled addition to a buffered saline to generate a precipitate that is dispersed onto the cultured cells, which take up this foreign material by endocytosis. This method was used for transfection of adherent cells.

1] Freshly passaged cells were plated a day before transfection so as to achieve a 30-40% confluency; 2] 4 h prior to transfection, cells were replenished with fresh media and the transfection mix was prepared by adding the following components: 10 µl calcium chloride (2.5 M), DNA 1-3 µg as per requirements and sterile DW to make volume 100

µl; 3] The components were mixed gently, 100 µl of BES buffer was added drop wise and incubated for 45 min at RT to enable complex formation; 4] The reaction mix was added to cells drop wise, the plates were swirled gently for uniform distribution and incubated for 16 h. Culture were replaced with fresh medium, and observed for transgene expression by microscopy or immunoblotting at 48 h since transfection.

Transfection using cationic lipids. This method involves generation of negatively charged liposome-nucleic acid complexes that are usually taken by the cells by endocytosis or fusion with the plasma membrane by the lipid moieties of the liposome. Lipofectamine 2000 was used for transfection of both adherent as well as suspension cells.

1] For adherent cells, cell seeding was done in 96 well plates one day prior to transfection in CM without antibiotics. Transfection mixture was prepared as follows (per well): 1 µl Lipofectamine was diluted in 24 µl Opti-MEM & incubated at RT/ 5 min while 320 ng DNA was diluted in 25 µl Opti-MEM. DNA mix was combined with the diluted Lipofectamine, and incubated at RT/ 20 min. The reaction was scaled up according to the number of wells to be transfected. The transfection mixture was added drop wise to each well and incubated as per requirements. 2] Suspension cells were seeded into 24 well plates on the same day of transfection in Opti-MEM. Transfection mixture was prepared as follows): 5 µl Lipofectamine was diluted in 50 µl of Opti-MEM while 1µg DNA was diluted in 50 µl Opti-MEM, the mixture was incubated and added to the cells as mentioned for adherent cells. 24 h post transfection, cells were supplemented with CM with antibiotics & incubated as per requirements.

Reporter assays

1] HEK 293 cells were seeded in 96 well flat bottom plate at a density of $2x10^4$ cells per well in 100 µl antibiotic free CM, a day before transfection; 2] 160 ng of *promoter-*

reporter plasmid DNA+160 ng of HHV-6.IE-GFP *effector plasmid* DNA was transfected using Lipofectamine, incubated for 48 h and processed for Luciferase assay as per manufacturer's instructions; 3] For control reactions, effector plasmid was replaced with an equal amount of pEGFP-N2 (empty vector backbone) and processed similarly. GFP luminescence was also measured simultaneously for all reactions for normalization.

Assay was performed in 96 well plates, using Steady-Glo Luciferase assay system and luminescence signal was detected using a microplate reader (Mithras LB-940; Berthold, Germany). 1] An aliquot of required volume of Luciferase reagent was removed from -20ºC (covered in a foil), kept at 4ºC for thawing and subsequently kept at RT to normalize temperature variations between cells and assay reagent; 2] at the time of assay, media volume in all the wells was made 100 µl by replacement with fresh CM to minimize volume errors and the plate was kept at RT till harvesting; 3] 100 µl of assay reagent was added per well and incubated at RT/ 5 min for efficient lysis (avoiding exposure to light). 100 µl of the lysate was transferred to each well of a 96 black microwell plate and luminescence signal was acquired.

Testing functionality of amplicon vector

1] PJH6 cells were seeded in 24 well plates at $0.5x10^6$ cells/ 0.3 ml Opti-MEM/ well; 2] cells were transfected with 1 µg of vector DNA and 5 µl Lipofectamine following manufacturer's instructions; 3] 0.5 ml CM was added/ well after 2 h; 4] 24 h post transfection, cells were diluted 1:10 in CM and transferred to T-25 flask; 5] 7 days post transfection the cell line supernatant was collected, filtered, and used for transduction of target cells. 3-5 days post transduction, cells were observed for GFP expression by flow cytometry and fluorescence microscopy; 6] transgene expressing cells were sorted on the basis of GFP fluorescence in order to enrich the population of transduced cells; 7] for generation of stable cell lines, transduced cells were selected using G418 (600 µg/ ml)

for 3 weeks followed by GFP based sorting in order to purify a pool of high transgene expressing stable clones.

Flow cytometry

It is a laser based technology that facilitates multi-parametric analysis of the physical and chemical characteristics of cells at a very high speed. FACS is a specialized type of flow cytometry that additionally provides physical separation of cells of particular interest form a heterogeneous mixture based on their light scattering and fluorescent characteristics. Transfected/ transduced cells were analyzed/ sorted by FACS (FACS Calibur/Aria, Beckton-Dickinson, USA).

1] Cells were washed and resuspended in PBS at $0.5x10^6$ cells/ ml and all the samples were acquired for GFP expression and analyzed using Cell Quest software; 2] for cell sorting, samples were prepared in CM at a high cell density and collected in media with 50% serum and double antibiotic concentration.

Protein extraction from mammalian cells

1] Target cells were terminated by typsinization, washed in PBS, spun and either processed for protein extraction immediately or stored at -80ºC; 2] cell pellets were resuspended in lysis buffer containing PI mixture by vortexing the tube vigorously and incubated in boiling water for 5 min. In case of frozen pellets, the lysis buffer was added immediately upon removal from -80ºC to avoid any degradation; 3] tubes were allowed to cool to RT, transferred on ice and the cell lysates were sonicated (Branson sonicator, USA) by giving 3 cycles of 10 pulses at 50% output, keeping samples on ice; 4] tubes were centrifuged at 16000xg for 30 min/ 4ºC, supernatant was transferred to fresh tubes, protein was quantified, and the lysates were stored at -20ºC.

Protein estimation

It was carried out by Lowry's method, which involves reaction of proteins with cupric sulfate and tartrate in an alkaline solution resulting in formation of copper-protein complexes which reduce the Folin reagent, producing a water-soluble product whose blue color is measured at 750 nanometers (nm). 1] BSA stock solution 2 mg/ ml in DW was diluted to 100 µg/ ml concentration and used to prepare protein standard containing 2, 5, 10, 15, 20 and 30 µg BSA; 2] 1 ml of Lowry mix was added to all the samples and standards (including a blank sample containing water), tubes mixed by gentle vortexing and incubated at RT for 10 min. 0.5 ml of Folin reagent was added to each tube, mixed again, and incubated at RT for 30 min; 3] The tubes were vortexed gently and 250 µl aliquot from each tube was transferred to a 96 well plate and the absorbance was measured at 750 nm. Relative protein concentrations were calculated with reference to the protein standards.

SDS-PAGE

It is a technique widely used to separate proteins according to their electrophoretic mobility (charge and molecular weight). Binding of SDS promotes linearization of the polypeptide chains and imparts a negative charge to them, thereby favoring fractionation based on size.

1] Resolving gels ranging from 8%-12% (as per requirements) were made by combining the following: 30 % acrylamide (8-12 ml), 9.0 ml of 1.25 M Tris.Cl (pH 8.8), 0.3 ml of 20% SDS, 0.3 ml 10% APS, 0.012 ml TEMED and DW to make volume to 30 ml. The gel was poured into pre-assembled gel casting apparatus and a thin layer of water was added on the top; 2] to prepare a resolving gel with 6-12 % gradient, 15 ml of 6% gel, and 15 ml of 12% gel was prepared, poured into the 2 chambers of the pre-set gradient former apparatus, and then poured in the gel casting apparatus to get a continuous

gradient starting from 12% at the base to 6% at the top of the gel; 3] after polymerization of resolving gel, a stacking gel (3.5%) was prepared by mixing 30 % acrylamide 2.25 ml, 1.25 M Tris Cl (pH 6.8) 1 ml, 20% SDS-0.05 ml, 10% APS 0.1 ml, TEMED 15 µl, and DW to make volume 15 ml. The water layer was drained off from top of the resolving gel, comb was inserted and stacking gel solution was poured and left for polymerization; 4] comb was removed, electrophoresis buffer was added, protein samples along with a protein molecular weight marker were loaded in the wells and electrophoresis was carried out initially at constant 70 V for 1 h, followed by 100 till the dye front reached the base; 5] Resolved gel was removed on one of the glass plates, stacking gel was gently sliced off, while the resolving gel was rinsed once with DW and processed for immunoblotting.

Immunoblotting

It is an analytical technique to identify target protein from separated proteins transferred to a membrane (nitrocellulose or PVDF) by treatment with antibodies specific to the target protein.

1] The resolved gel was rinsed in water as before and immersed into transfer buffer for ~ 15 min. Blotting sheets and PVDF membrane was cut to the size of resolving gel. Membrane was activated by soaking in 100% methanol for 1 min and kept in transfer buffer; 2] transfer assembly was set up by placing the following in the given order inside a transfer cassette: a single fiber sheet, triple layer of filter paper followed by the membrane, on top of which the gel was placed, followed by again triple layer of filter paper and single fiber sheet; 3] the assembly was inserted vertically inside the transfer apparatus tank which was filled with transfer buffer, placing the gel facing towards negative electrode. Transfer was carried out routinely at constant 25 V with maximum current output for O/ N at RT; or at constant current 300 mA for 3 h, RT; 4] the cassette

was disassembled, membrane was quickly transferred to TBS and stained with Ponceu to check the efficiency of protein transfer. The stain was removed by rinsing the membrane with TBS; 5] membrane was blocked by incubating in either 5% milk or 3% BSA for 1 h at RT and incubated with appropriately diluted primary antibody at 4ºC, O/N on a rocker; 6] membrane was washed thrice with TBST, 10 min each at RT, incubated with secondary antibody for 1 h at RT and washed similarly; 7] The signal was detected by incubating the membrane with ECL+ detection reagent for 2 min, followed by exposure to the X-ray films in dark which were developed using a developing machine (Promax, USA).

CHAPTER 4

RESULTS

I. Ultra structure of the isolate and virus infectivity study

EM of the HHV-6 isolate

In order to analyze the ultrastructural features of the isolate, the concentrated virus preparation derived from PJH6 culture supernatant were processed for EM studies. Images of the virus particles exhibited features typically documented for the all human herpesvirus family members (Fig-10). In addition to this, the study also indicated the production of virus particles by the cell line.

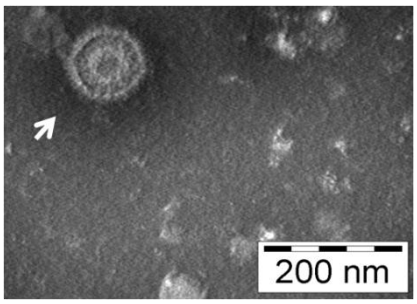


Fig-10. Electron micrograph of a virus particle (arrow) obtained from clarified culture supernatant of PJH6 cell line.

Infectivity of the isolate

Three different lineages of HHV-6 negative cell lines SupTI, U937 & Daudi were treated with PJH6 culture derived supernatant and target cell genomic DNA was used for HHV-6 specific nested PCR. The expected amplifications of 429 bp specific for HHV-6B (23) was observed only in the infected cells, thus, confirming their infection and all the samples were β-globin PCR positive showing the expected 536 bp amplification (Fig-11a). As the producer cell line is EBV transformed and thus EBV and HHV-6 dual positive, the EBV status in the recipient cells was also ascertained by an EBV specific PCR that yields a 239 bp amplicon (104). The infected SupTI cells were EBV PCR negative thus confirming that the PJH6 cell line produced only infectious HHV-6 (Fig-11b).

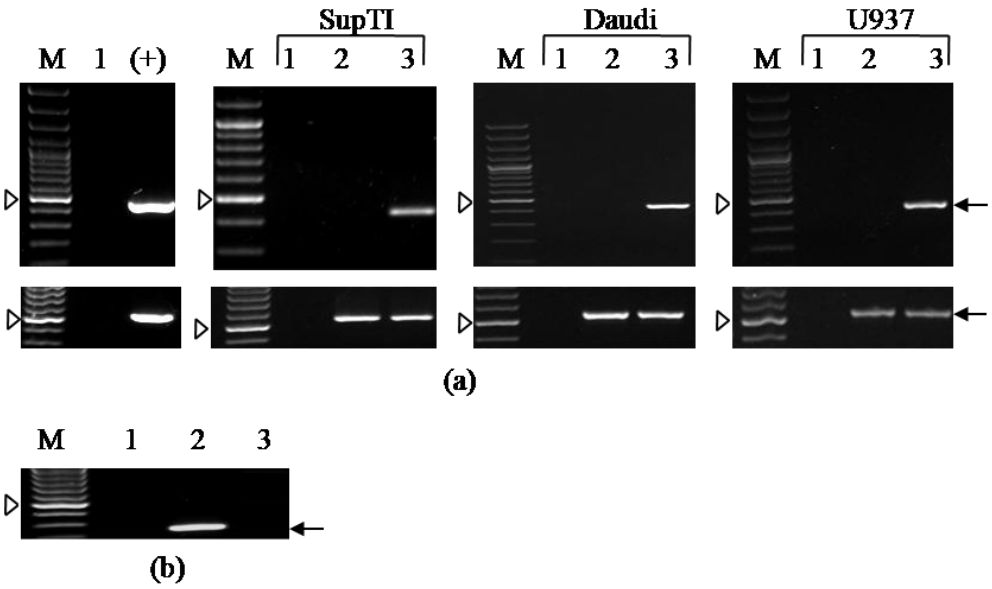
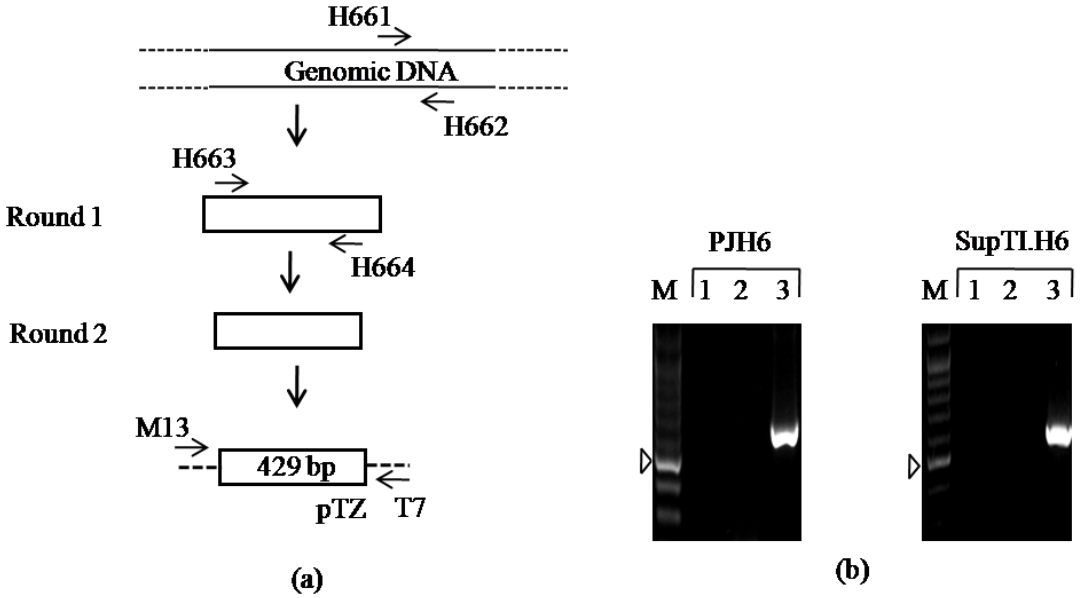


Fig-11. Confirmation of HHV-6 infection of the target cell lines by nested PCR. *(a) HHV-6 PCR (upper panels) lane 1: RC, lane 2: uninfected cells, lane 3: infected cells, (+): PJH6 cells; (lower panel) β-globin PCR product from corresponding samples; (b) EBV PCR, lane 1: RC, lane 2: PJH6 cells, lane 3: infected SupT1 cell line; M: 100 bp marker; arrow heads indicate 500 bp marker bands and arrows indicate respective PCR amplification products.*

Sequencing of PCR products from infected cells

In order to confirm the identity of the infection in the recipient cells, HHV-6B specific nested PCR products from PJH6 cell line (donor) and infected SupTI cell line (recipient) were cloned in pTZ vector (Fig-12a). The cloning was confirmed by performing PCR using M13 forward (M13 F) and T7 reverse (T7 R) primes flanking the MCS of pTZ (Fig-12b). Sequencing of the clones revealed an absolute sequence identity thereby further confirming infection of target cells by the isolate from the producer cell line (Fig-12c).

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Query  7    AGCTCGGTACCTCGCGAATGCATCTAGATTATCGCTTTCACTCTCATAAGGTGCTGAGTG  66
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3    AGCTCGGTACCTCGCGAATGCATCTAGATTATCGCTTTCACTCTCATAAGGTGCTGAGTG  62

Query  67   ATCAGTTTCATAACCAAATGTTATCATTTCTGATATTAAAGGGTTGTCATGGAAACCATC  126
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  63   ATCAGTTTCATAACCAAATGTTATCATTTCTGATATTAAAGGGTTGTCATGGAAACCATC  122

Query  127  ACATAGATTCACTAGACTTTCTACAGCTTCCAATTCTTTGTCTATTTTACTTTCTGGATT  186
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123  ACATAGATTCACTAGACTTTCTACAGCTTCCAATTCTTTGTCTATTTTACTTTCTGGATT  182

Query  187  TAAAGTATCCTGAATTGCATCATGACTTTTGACAGGAGTTGCTGAGTCGGTAAAGGTCAT  246
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183  TAAAGTATCCTGAATTGCATCATGACTTTTGACAGGAGTTGCTGAGTCGGTAAAGGTCAT  242
```

(c)

Fig-12. Confirmation of target cell infection. *(a) Schematic representation of the steps followed to obtain pTZ clone harboring the HHV-6 nested PCR product; (b) screening of cloned PCR amplified product by PCR using M13 F and T7 R primers, lane 1: RC, lane 2: empty vector, lane 3: vector containing cloned PCR product, M: 100 bp marker-arrowhead indicating the 500 bp band; (c) BLAST alignment data showing 100% sequence identities between PCR amplified product from PJH6 (Query) and HHV-6 infected SupTI cells (SupT1.H6; Subject).*

In-vitro infection to human PBMC

In order to ascertain the infectivity of the isolate for human PBMC, blood samples were collected from previously PCR screened (PCR using PBMC DNA) HHV-6 negative individuals. PBMC cultured both with and without PHA, was incubated with PJH6 culture supernatant. Each PBMC sample became HHV-6 PCR positive, indicating that the isolate could successfully infect PBMC, irrespective of whether mitogen stimulated or not (Fig-13 a & b).

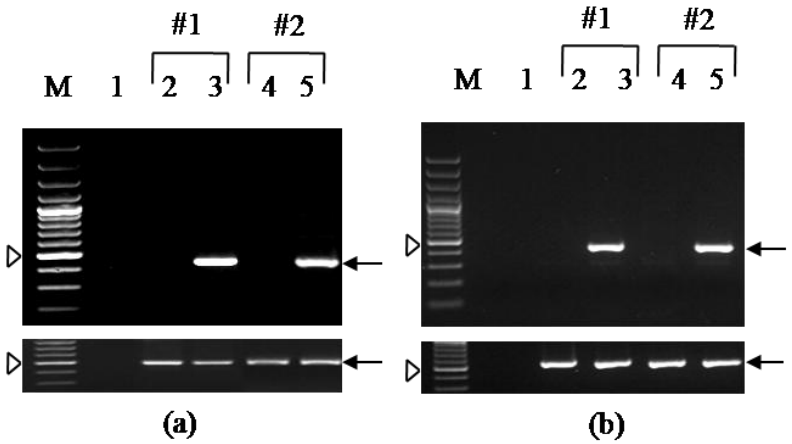


Fig-13. Confirmation of HHV-6 infection of the target PBMC by nested PCR. *HHV-6 PCR (upper panel) from two HHV-6 negative individuals (#1, #2), (a) PHA stimulated PBMC, (b) unstimulated PBMC; lane 1: RC, lanes 2 and 4: uninfected PBMC, lanes 3 and 5: infected PBMC; (lower panel) β-globin PCR product from corresponding samples; M: 100 bp marker; arrow heads indicate 500 bp marker bands and arrows indicate respective PCR amplification products.*

Productive HHV-6 infection of target cell line

SupTI cells which were HHV-6 positive after infection with PJH6 culture supernatant was used as parental cell line in this experiment. The cells were seeded and the cell free culture supernatant was then used to infect a fresh lot of SupTI cells. The status of infection was confirmed by DNA PCR (Fig-14). As evident from the results, the SupTI cells infected using PJH6 culture supernatant cells were also found to produce infectious virus that could infect fresh cells.

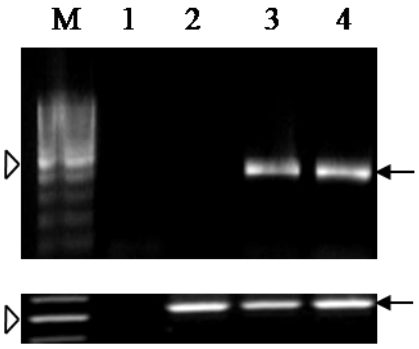
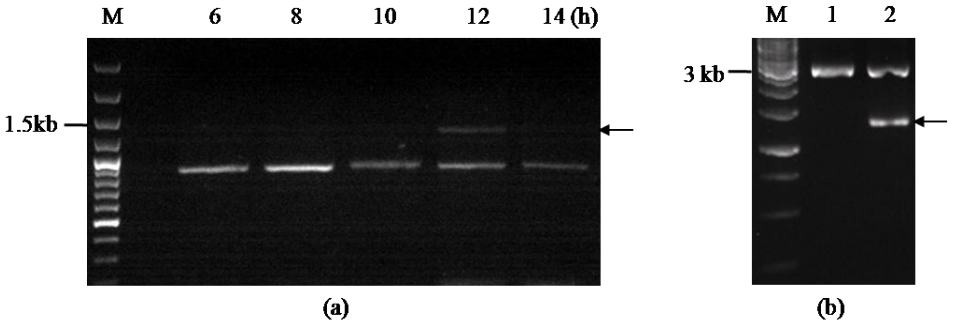


Fig-14. Productive infection of SupT1 cells by PJH6 isolate. *Nested PCR product from SupT1 cells (upper panel) lane 1: RC, lane 2: uninfected cells, lane 3: first lot of infected cells, lane 4: second lot of infected cells; (lower panel) β-globin gene PCR product from corresponding samples; M: 100 bp marker, arrowhead indicates 500 bp marker band, arrows indicate respective PCR amplification products.*

II. Chromosomal integration profile of the isolate

Integration profile of the subject and PJH6 cell line

Previous studies in the lab indicated the presence of unusually high levels of the viral genome in the subject (referred to as donor) as well the PJH6 cell line. We ascertained the status of chromosomal integration of the HHV-6 genome in the donor cells and the cell line. Inverse PCR was performed on the genomic DNA derived from the donor PBMC as well the donor derived cell line and distinct amplified fragments within 1.2-1.5 kb sizes were gel purified and cloned into pTZ (Fig-15a & b). The cloning was confirmed by EcoRI/ HindIII digestions. Sequencing of the cloned fragments followed by BLAST analysis indicated the presence of viral-chromosome junction at chromosome 12q14 in both the cases (Fig-15c & d).



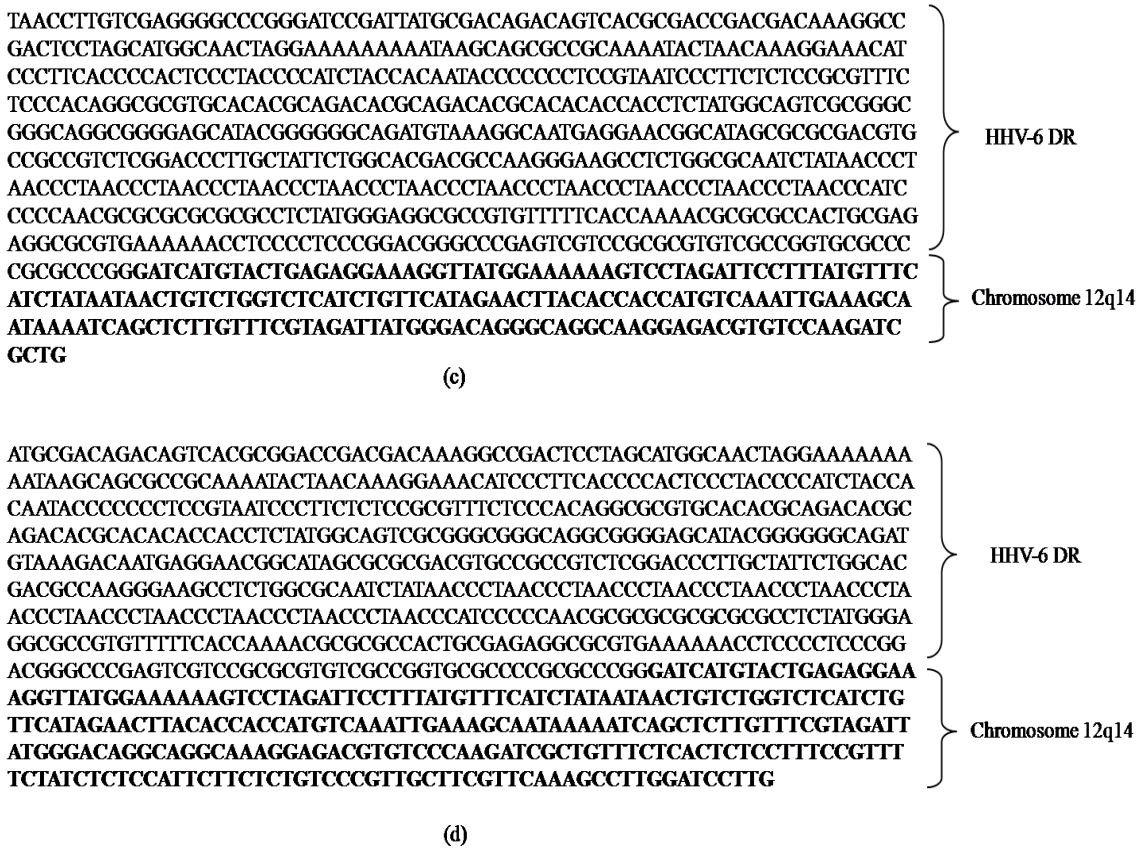


Fig-15. Inverse PCR & HHV-6 integration in the host chromosome. *(a) Representative image of the inverse PCR derived amplifications obtained at indicated time points, M: 100 bp DNA marker; (b) confirmation of the cloning of the PCR product in pTZ vector by EcoRI/ HindIII digestions, lane 1: empty vector, lane 2: vector showing the expected insert release, M: 1kb DNA marker and the arrow indicates the referred genomic fragments; (c & d) HHV-6 integration in the host chromosome, results of BLAST analysis showing the presence of viral-chromosomal junction in the donor PBMC and PJH6 cell line respectively. The chromosomal sequences are shown in bold.*

Vertical transmission of CIHHV-6

In order to ascertain the route of acquisition of CIHHV-6, cloning and sequencing of inverse PCR products from PBMC samples from the donor's parents was also performed and the BLAST analysis indicated the presence of an identical viral-chromosome junction at 12q14 in the father (Fig-16a). Although integration was also observed in genome of mother at chromosome 8q24, the site was different from that found in the subject (Fig-16b). A summary of the results of the integration profiling of the subject and his parents is represented (Fig-16c & d).

ATGCGACAGACAGTCACGCGGACCGACGACAAAGGCCGACTCCTAGCATGGCAACTAGGAAAAAAA
AATAAGCAGCGCCGCAAAATACTAACAAAGGAAACATCCCTTCACCCCACTCCCTACCCCATCTACCA
CAATACCCCCCCTCCGTAATCCCTTCTCTCCGCGTTTCTCCCACAGGCGCGTGCACACGCAGACACGC
AGACACGCACACATCACCTCTATGGCAGTCGCGGGCGGGCAGGCGGGGAGCATACGGGGGGCAGAT
GTAAAGACAATGAGGAACGGCATAGCGCGCGACGTGCCGCCGTCTCGGACCCTTGCTATTCTGGCAC
GACGCCAAGGGAAGCCTCTGGCGCAATCTATAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTA
ACCCTAACCCTAACCCTAACCCTAACCCTAACCCATCCCCCAACGCGCGCGCGCGCCTCTATGGGA
GGCGCCGTGTTTTTCACCAAAACGCGCGCCACTGCGAGAGGCGCGCGAAAAAACCTCCCCTCCCGG
ACGGGCCCGAGTCGTCCGCGCGTGTCGCCGGTGCGCCCCGCGCCCGGGATCATGTACTGAGAGGA
AAGGTTATGGAAAAAAGTCCTAGATTCCTTTATGTTTCATCTATAATAACTGTCTGGTCTCATCT
GTTCATAGAATTTACACCACCATGTCAAATTGAAAGCAATAAAATCAGCTCTTGTTTCGTAGATT
ATGGGACAGGGCAGGCAAGGAGACGTGTCCAAGATCGCTG

HHV-6 DR

Chromosome 12q14

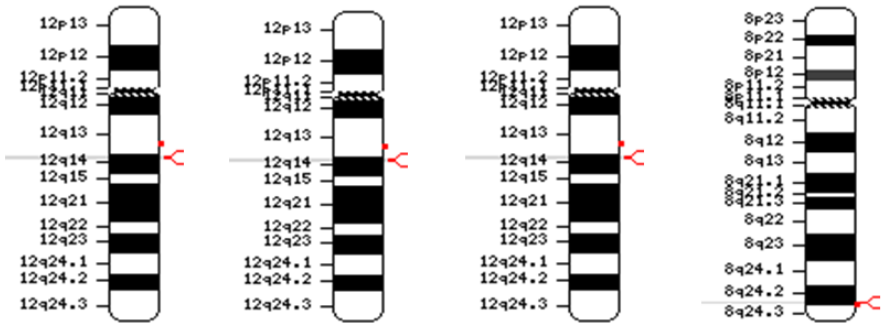
(a)

TGCGACAGACAGTCACGCGGACCGACGACAAAGGCCGACTCCTAGCATGGCAAATAGAAAAAAAAT
AAGCAGCGCTGCAAAATACTAACAAAGGAAACATCCCTTCACCCCACTCCCTACCCCATCTACCACAA
TACCCCCCCTCCGTAATCCCTTCTCTCCGCGTTTCTCCCACAGGCGCGTGCACACGCAGACACGCAGA
CACGCACACACCACCTCTATGGCAGTCGCGGGCGGGCAGGCGGGGAGCATACGGGGGGCAGATGTA
AAGACAATGAGGAACGGCATAGCGCGCGACGTGCCGCCGTCTCGGACCCTTGCTATTCTGGCACGAC
GCCAAGGGAAGCCTCTGGCGCAATCTATAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACC
CTAACCCTAACCCTAACCCTAACCCTAACCCATCCCCCAACGCGCGCGCGCGCGCCTCTATGGGAGGC
GCCGTGTTTTTCACCAAAACGCGCGCCACTGCGAGAGGCGCGTGAAAAAAACCTCCCCTCCCGGACG
GGCCCGAGTCGTCCGCGCGTGTCGCCGGTGCGCCCCGCGCCCGGGATCTCCTGACCTC*ATGATCAGAA*
TAGAAAGCCCAGAAATAGACCCACACAAATAGAGTCAACTGATCTGCCTGCCTATAGGCATTTAATTCCCAGA
*CTCTTGATGAAAGCATCCA*GATCTCATTATTTTTTATTGATGCATAGCATTCCGTGGTGTAGATGTAC
TATAATTTCTTTATCCCATCTGACATTGATGGGCATTTATGTTAATTCCATGTCTTTGCTTTGTGA
ATAGTGCTGCAATGAACGTGTGTGTGCATGTGTCTTTATGATAGACAAATTATATTTCTTTTCGG
TAATACCCGTATGGAGTGCTGGATCTATGTATTTTCTGGTCTTTAGGGTCTTT

HHV-6 DR

Chromosome 8q24

(b)



(c)

Samples analyzed	Integration site
1. Donor PBMC	Chromosome 12q14
2. PJH6 cell line	Chromosome 12q14
3. Father PBMC	Chromosome 12q14
4. Mother PBMC	Chromosome 8q24

(d)

Fig-16. HHV-6 integration in the host chromosome. *Results of BLAST analysis showing the presence of viral-chromosomal junction elucidated in the (a) donor's father and (b) mother PBMC samples. The chromosomal sequences are shown in bold while the gap in mother DNA sequence is shown in italics; (c) integration profile summary with the ideograms of the respective chromosomes, each indicating the site of integration identified for the entire samples analyzed; (d) Tabular representation of the results obtained.*

III. Role of viral IE gene products on activation of select cellular genes

Generation of IE1-GFP construct

Full length HHV-6 IE1 was PCR amplified from PJH6 genomic DNA using primers designed based on sequence of HHV-6B isolate Z29 (GenBank accession no-AF157706; Fig-17a & b). The 3.2 kb PCR amplified fragment was cloned into pTZ and the cloning was confirmed by RE digestion with EcoRI (Fig-17c).The cloned fragment was sequenced from both termini and subjected to BLAST analysis to confirm the identity. Subsequently, it was released from pTZ using XbaI/ SmaI digestions and sub-cloned into NheI/ SmaI sites in MCS of pEGFP-N2, generating IE1with a C-terminus GFP tag. The clones were screened by NotI digestion (Fig-17d).

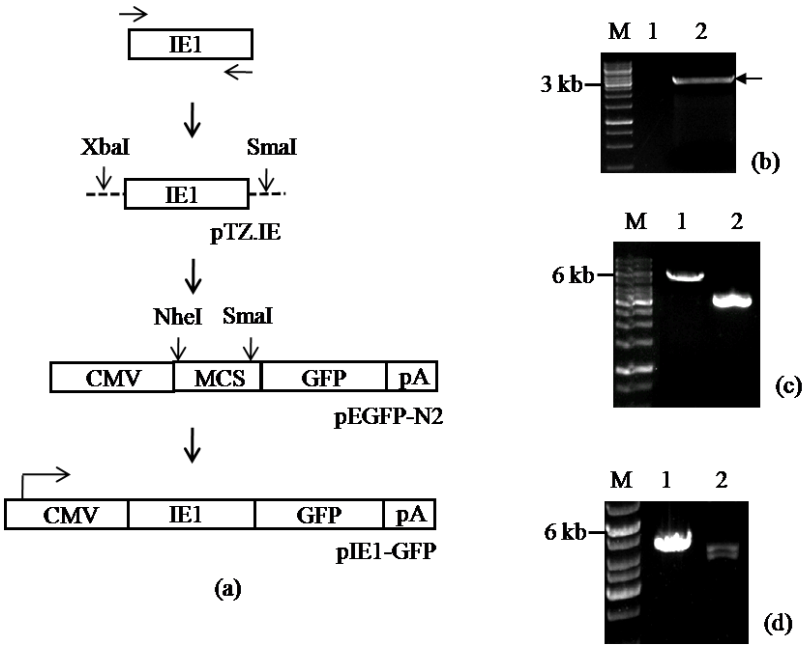


Fig-17. Construction of pIE-GFP. *(a) Cloning steps used to generate pIE1-GFP; (b) PCR amplification of IE1 from PJH6 genomic DNA, lane 1: RC, lane 2: IE1 PCR amplified fragment indicated by the arrow; (c) confirmation of IE1 cloning in pTZ by EcoRI digestion, lane 1: linearized pTZ.IE1-GFP showing an expected ~6 kb band, lane 2: linearized pTZ empty vcetor; (d) confirmation of IE1 sub-cloning in pEGFP-N2 by NotI digestion, lane 1: linearized pEGFP-N2 empty vector, lane 2: pIE1-GFP showing an additional band corresponding to insert and part of vector backbone, M: 1 kb DNA marker.*

IE1 expression in mammalian cells

1 µg of pIE1-GFP or control pEGFP-N2 vectors were transfected into HEK 293 cells and observed for GFP expression by fluorescence microscopy after 48 h (Fig-18a). To confirm the full length expression of IE1 clone, the cells were harvested and 75 µg of the cell lysate per sample was processed for immunoblotting using anti-GFP monoclonal antibody. Apart from the expected band of ~170 kDa (including 29 kDa addition by GFP tag) in pIE1-GFP transfected cells, a higher band at ~ 190 kDa was also obtained. The vector backbone transfected cells showed only 29 kDa band corresponding to GFP expression alone (Fig-18b).

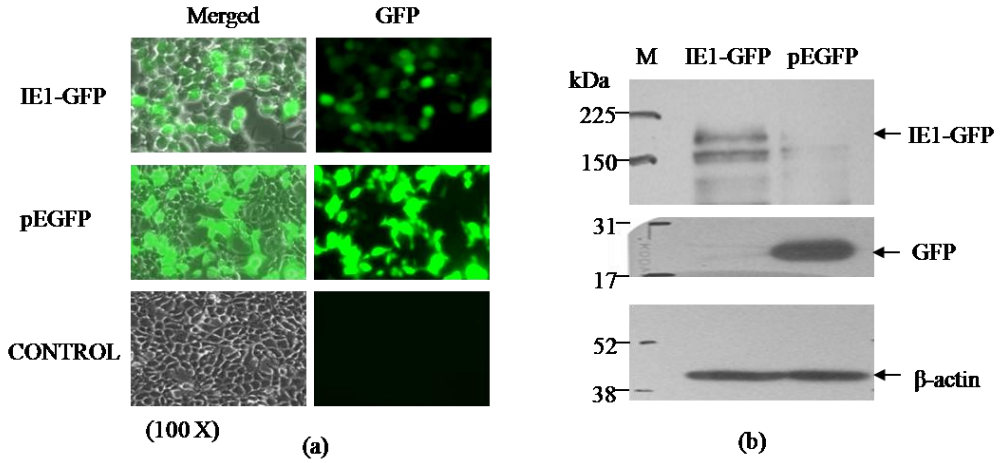


Fig-18. IE1 expression in HEK 293. *(a) Fluorescence microscopy images of transfected cells, upper panel: pIE-GFP, middle panel: pEGFP-N2 and lower panel: untransfected cells, the magnification of the images is indicated; (b) Immunodetection, 75 µg of the cell lysate per sample was resolved and blots were probed with indicated antibodies, upper and middle panels: anti-GFP antibody showing specific bands as indicated by the arrow, lower panel: anti β-actin antibody showing equal loading of the cell lysates.*

Generation of reporter constructs

phsp-70 Luciferase construct

The select hsp-70 promoter region (-1170 to +101 nucleotide (nt); GenBank accession no-NG_011855), was PCR amplified from PBMC genomic DNA obtained from a healthy volunteer (Fig-19a & b). The amplified fragments were cloned into pTZ and the cloning was confirmed by PstI digestion (Fig-19c). The cloned promoter fragment was sequenced to confirm its identity. It was subsequently released from pTZ using KpnI/ BglII digestions and sub-cloned upstream to Luciferase reporter gene at identical sites in the MCS of pGL3-basic vector, thus, generating hsp-70 promoter-Luciferase-pA cassette (phsp-70pr-Luc). The clones were screened by analyzing PstI/HindIII double digestion pattern (Fig- 19d).

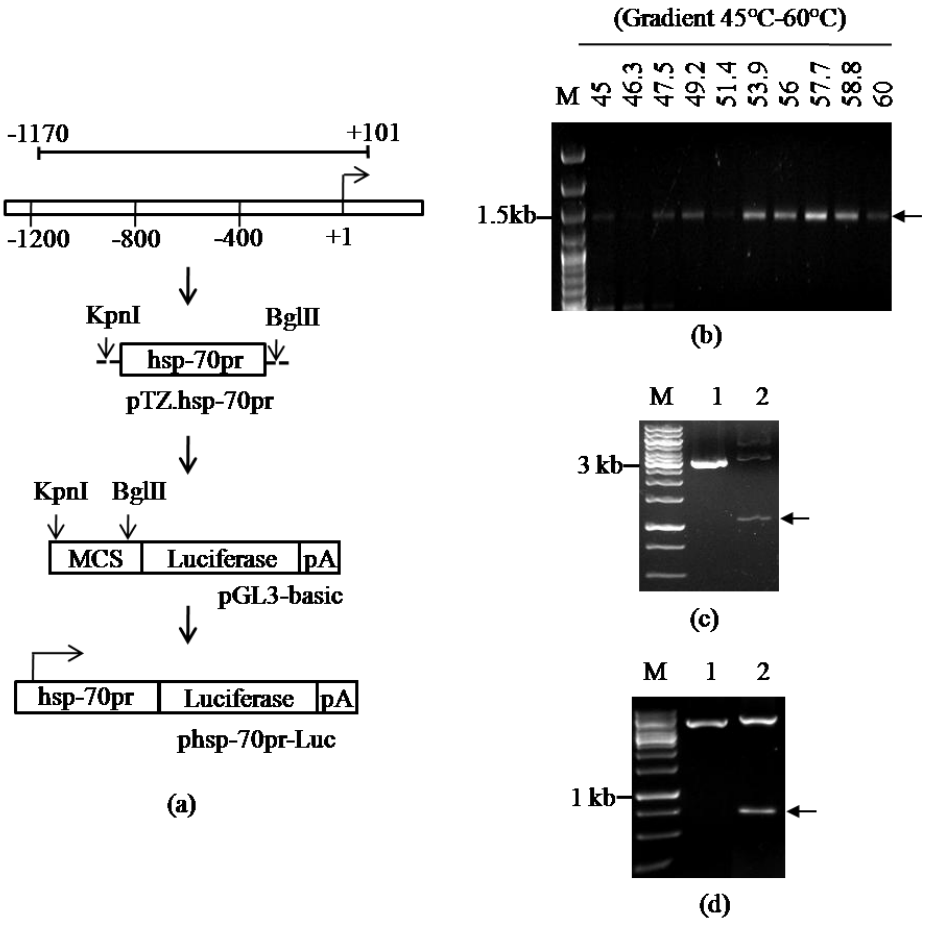


Fig-19. Generation of hsp-70 promoter-Luciferase plasmid. *(a) Cloning steps for generating hsp-70 promoter fragment in pGL3-basic vector; (b) a temperature gradient*

PCR showing amplification of the desired fragment, M: 100 bp DNA marker; (c) PstI digestion pattern of pTZ.hsp-70pr, lane 1: empty vector, lane 2: hsp-70pr insert release from vector backbone is indicated, M: 1 kb DNA marker; (d) PstI/ HindIII double digestion to confirm cloning in pGL3-basic, lane 1: linearized empty vector , lane 2: hsp-70pr fragment release from the vector; M: 1 kb DNA marker, arrows show the referred genomic fragments.

pc-fos Luciferase construct

The select c-fos promoter region (-721 to +5 nt; GenBank accession no-K00650) was PCR amplified using genomic DNA from the T-cell line CEM (Fig-20a & b). The amplified fragments were cloned into pTZ and the cloning was confirmed by PstI digestion (Fig-20c). The cloned promoter fragment was sequenced to confirm its identity. It was subsequently released from pTZ by SacI/BglII digestions and sub-cloned upstream to Luciferase reporter gene at identical sites in the MCS of pGL3-basic vector, thereby generating c-fos promoter-Luciferase-pA cassette (pc-fospr-Luc). The clones were screened by analyzing NheI/ XbaI double digestion pattern (Fig-20d).

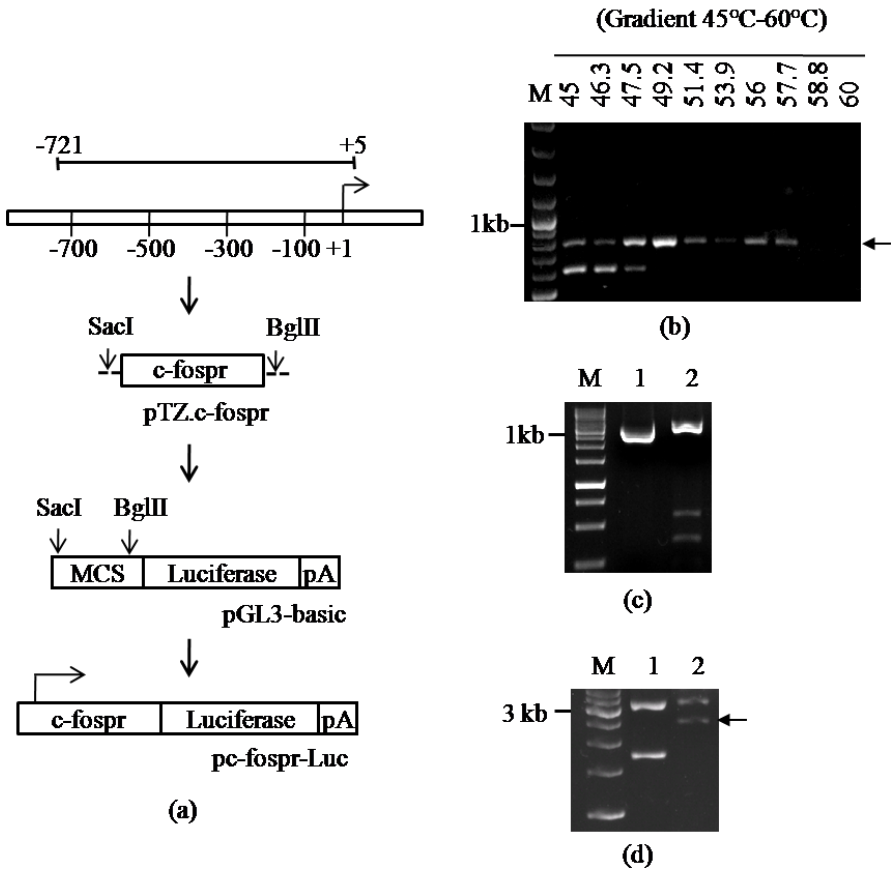


Fig-20. Generation of c-fos promoter-Luciferase plasmid. *(a) Cloning steps for generating c-fos promoter fragment in pGL3-basic vector; (b) a temperature gradient PCR showing amplification of the desired fragment, M: 100 bp DNA marker; (c) PstI digestion pattern of pTZ.c-fospr, lane 1: empty vector, lane 2: c-fospr insert release from vector backbone is indicated, M: 1 kb DNA marker; (d) NheI/ XbaI double digestion to confirm cloning in pGL3-basic, lane 1: empty vector, lane 2: vector showing a shift in the size of the lower band indicating the presence of the cloned c-fospr fragment, M: 1 kb DNA marker, arrows show the referred genomic fragments.*

pc-myc Luciferase construct

The select c-myc promoter region (-1986 to +15 nt; GenBank accession no-V00568) was PCR amplified from PBMC genomic DNA obtained from a healthy volunteer (Fig-21a & b). The amplified fragments were cloned into pTZ and the cloning was confirmed by EcoRI/ HindIII digestions (Fig-21c). The cloned promoter fragment was sequenced to confirm its identity. It was subsequently released from pTZ using KpnI/ BglII digestions and sub-cloned upstream to Luciferase reporter gene at identical sites in the MCS of pGL3-basic vector, thus, generating c-myc promoter-Luciferase-pA cassette (pc-mycpr-Luc). The clones were screened by analyzing BglII/ KpnI double digestion pattern (Fig-21d).

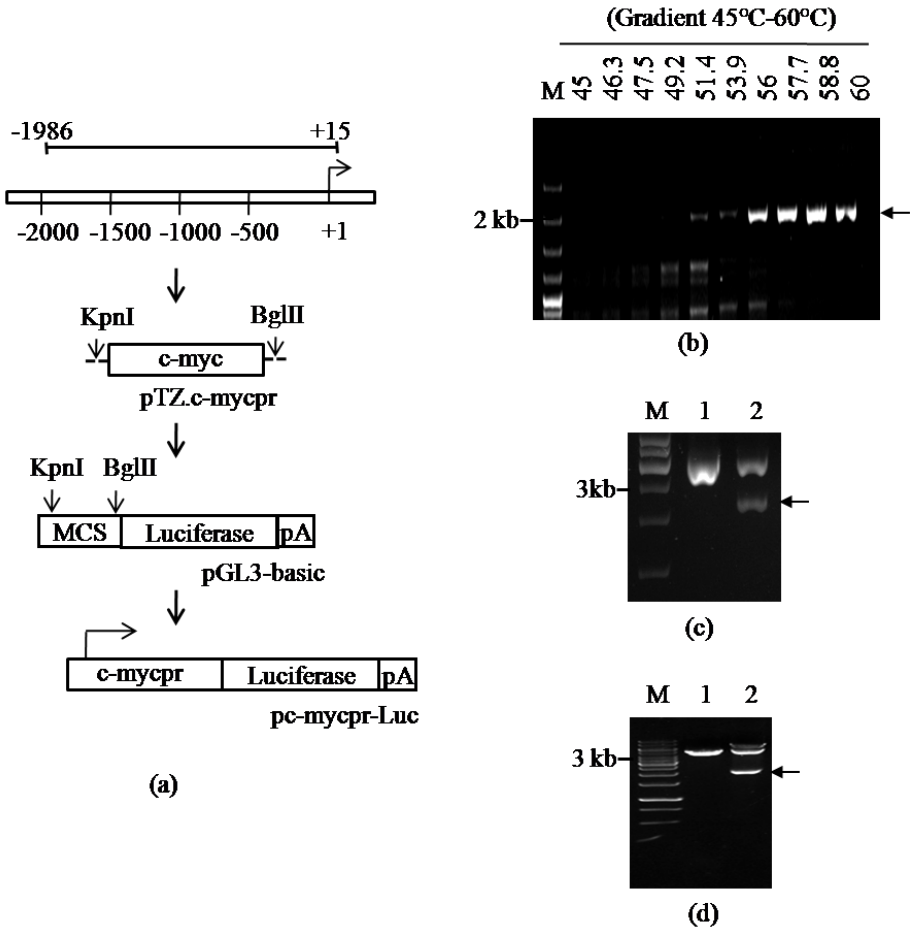


Fig-21. Generation of c-myc promoter-Luciferase plasmid. *(a) Cloning steps for generating c-myc promoter fragment in pGL3-basic vector; (b) a temperature gradient PCR showing amplification of the desired fragment, M: 100 bp DNA marker; (c) EcoRI-HindIII double digestion pattern of pTZ.c-mycpr, lane 1: empty vector, lane 2: c-mycpr insert release from vector backbone is indicated, M: 1 kb DNA marker; (d) BglII/ KpnI double digestion to confirm cloning in pGL3-basic, lane 1: linearized vector backbone, lane 2: c-myc promoter fragment release from the vector is indicated; M: 1 kb DNA marker, arrows show the referred genomic fragments.*

Reporter assays

HEK 293 cells were co-transfected with each of hsp-70-Luc, c-fos-Luc, c-myc-Luc reporter constructs in presence of equal amounts of either IE-GFP (effector plasmid) or pEGFP-N2 (control plasmid). IE mediated transactivation of the said promoters was documented by Luciferase assay 48 h post transfections. An appreciable up-regulation in promoter strength of all the three promoters was observed in the presence of IE, in comparison to the control (Fig-22a). Luciferase values were normalized with GFP, which

was measured in parallel for each sample. The increase in relative luciferase activity obtained was highly significant for all the data sets. The relative fold increase with respect to the basal promoter activities also showed considerable up-regulations for hsp-70, c-myc and c-fos promoters respectively (Fig-22b).

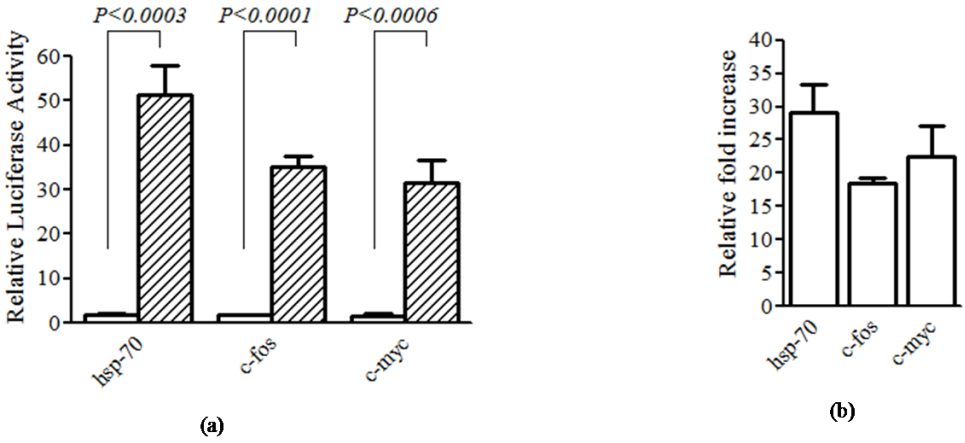


Fig-22. HHV-6 IE1 transactivates cellular promoters. *(a) Cellular promoters showing increased activity in presence of pIE1-GFP as compared to GFP alone. The empty columns represent cells co-transfected with promoter-reporter constructs and pEGFP-N2 empty vector, while the hatched columns represent co-transfection in presence of vector bearing IE1 gene, p-values are indicated for each data set; (b) graph showing the relative fold increase for all the promoters tested with respect to their basal promoter activities, the bars in the figure represent mean ± s.d. of 3 independent experiments.*

HHV-6 induced gene expression in SupTI cells

In order to validate the results obtained in the reporter assays, the effect of viral infection on the steady state levels of hsp-70, c-fos and c-myc genes in the target cells was ascertained. As IE-1 gene is essentially expressed at early time points post infection, cultures were terminated 8 h after infection and processed for RNA extraction, RT-PCR & densitometry. The status of infection in SupTI cells was first confirmed by performing nested PCR (Fig-23a). RT-PCR analysis indicated a significant increase in steady state levels of hsp-70 and c-fos in presence of the input virus preparation (Fig-23b & c). However, no change in the c-myc expression was observed at the indicated time point. Fold increase relative to the basal expression levels was also calculated that showed almost 3.25 and 1.5 fold increase for hsp-70 and c-fos genes respectively (Fig-23d).

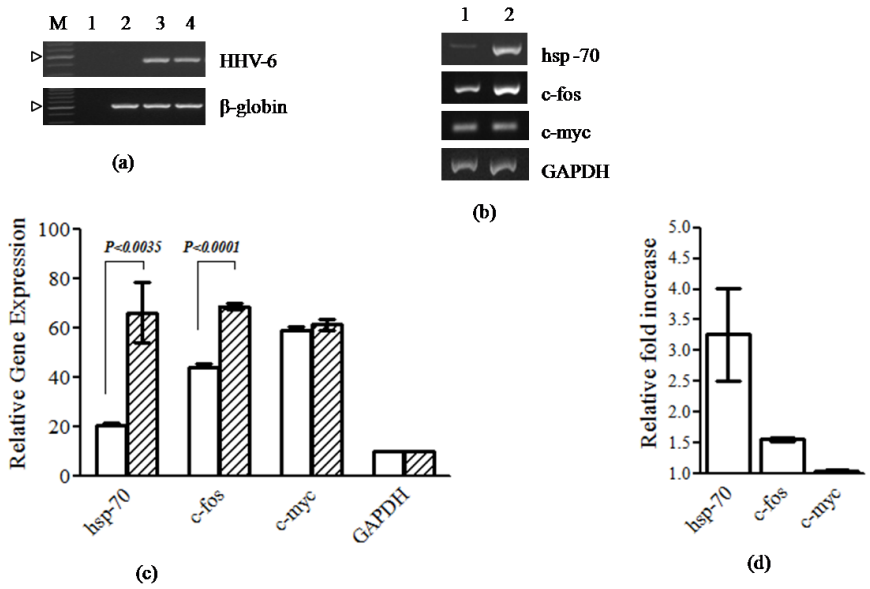


Fig-23. HHV-6 induced gene expression in SupTI cells. *(a) Confirmation of HHV-6 infection of SupTI cells by HHV-6 nested PCR (upper panel) lane 1: RC, lane 2: uninfected cells, lane 3: infected cells; lane 4: PJH6 cells, (lower panel) β-globin PCR product from corresponding samples, M: 100 bp DNA marker, arrowhead indicates 500 bp marker bands; (b) RT-PCR for hsp-70, c-fos, and c-myc expression in (1) uninfected and (2) infected cells. Equal volumes of PCR products corresponding to an equal amount of cDNA template were loaded on agarose gels for comparative analysis and GAPDH was used as an internal control; (c) densitometry analysis of the RT-PCR where the relative gene expression levels denote the ratio of quantity of PCR products from the target gene and the quantity of GAPDH products. The empty columns represents the uninfected cells and the hatched columns represent the infected cells, the p-values in case of significant up-regulations are indicated; (d) graph showing relative fold increase in gene expression with respect to their basal expression levels, the bars in the figure represent mean ± s.d. of 3 independent experiments.*

IV. Harnessing the isolate to develop a basic gene transfer vector

pGL3-basic plasmid was used as a backbone for the assembly and generation of the amplicon vector. The vector components including the Origin of replication (ori) and packaging-cleavage cassette (pac), transgene expression cassette and the selection marker cassette were first assembled individually in different plasmids and then stepwise combined together into the pGL3-basic.

ori and pac cassettes

Region encompassing HHV-6 ori (nt nos 68351-69570; Genbank accession no-NC_000898) was PCR amplified from PJH6 genomic DNA (Fig-24a &b) and cloned into pTZ and the cloning was confirmed by EcoRI digestion (Fig-24c). The cloned fragment was sequenced from both termini to confirm its identity. Using this clone as a template, the cleavage-packaging signal sequences, pac1 and pac2 (referred to as pac) was incorporated in an orientation similar to the replicating viral genome (86), downstream to the ori sequence by consecutive 3 rounds of PCR with primers having overlapping ends (Fig-24a, d). The complete ori-pac amplified cassette was subsequently cloned in pTZ and the cloned fragment was confirmed by BglII/ EcoRI digestions (Fig-24e) and sequence configuration was confirmed by sequencing.

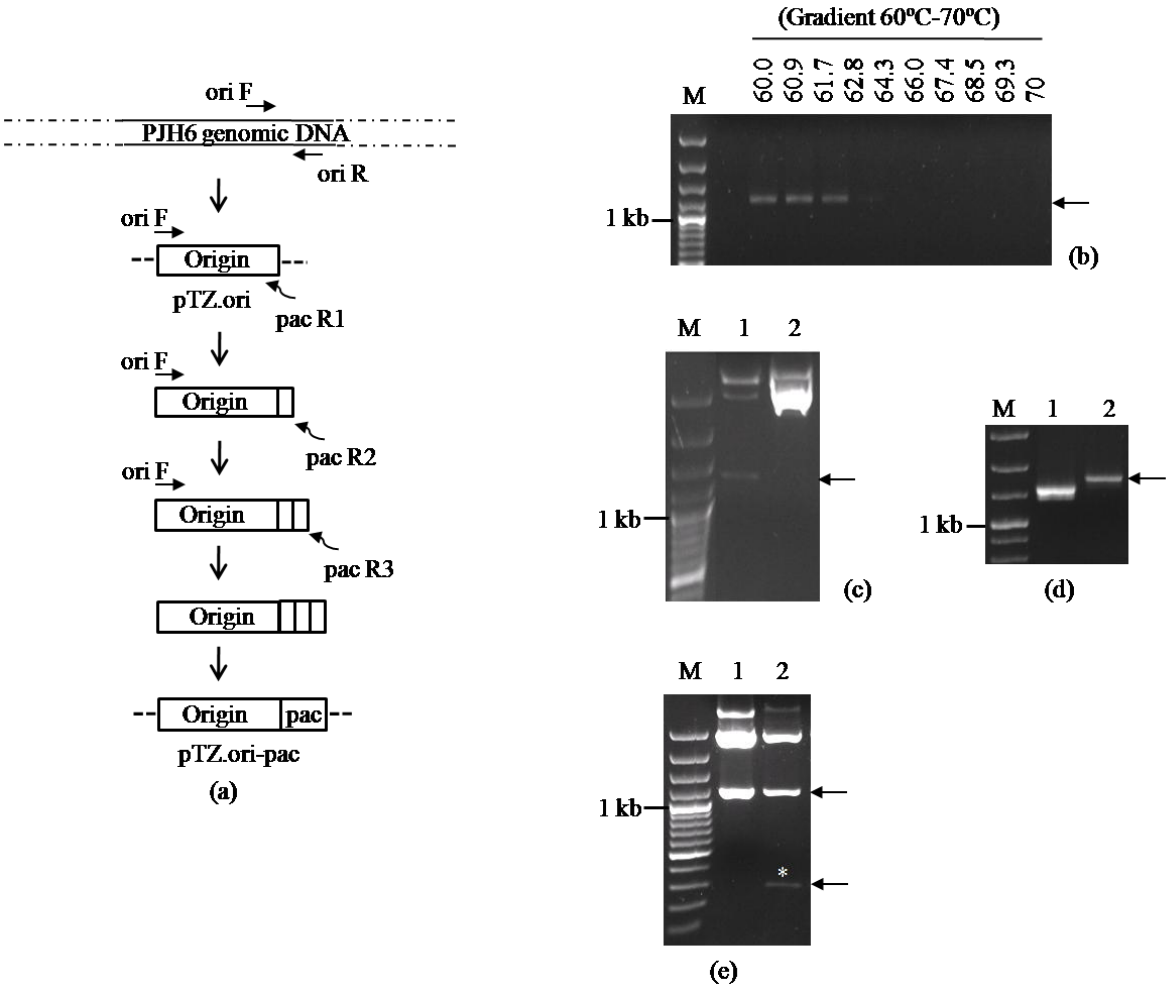


Fig-24. Generation of HHV-6 ori-pac-clevage cassette. *(a) PCR strategy followed to assemble the complete ori-pac-clevage cassette; (b) a temperature gradient PCR showing amplification of ori, M: 100 bp DNA marker; (c) EcoRI digestion pattern of pTZ.ori, lane 1: insert release from vector backbone is indicated, lane 2: empty vector; (d) addition of the pac signal at the rear end of ori by overlapping PCR, lane 1: ori, lane 2: ori with pac1 and pac2; (e) BglII/ EcoRI double digestion to confirm cloning in pTZ, lane 1: pTZ.ori showing ori release from pTZ, lane 2: pTZ.ori-pac showing both ori, and pac fragment (indicated by*) release from pTZ, M: 1 kb DNA marker, arrows show the referred genomic fragments.*

Generation of transgene expression cassette pLTR-GFP-pA

An HIV-1 LTR promoter was used to drive the EGFP transgene expression. The LTR promoter fragment was PCR amplified (Fig-25a & b) using genomic DNA from the SupTI cell line infected with HIV-1, strain GT50 *(lab isolate, unpublished).* The amplified fragment was cloned into pTZ and the cloning was confirmed by digestion with SacI (Fig-25c). The sequence of the cloned fragment was ascertained from both ends. Subsequently, it was released from pTZ by EcoRI/PstI digestions, sub-cloned into NdeI/ PstI sites in pEGFP-N2 by replacing the CMV promoter with LTR promoter, thus generating pLTR-GFP-pA. The clones were screened for the presence of LTR by KpnI digestion (Fig-25d). However, as a large part of MCS from pTZ as well as pEGFP-N2 was retained between LTR and GFP and the same was removed by digestion with BamHI followed by self-ligation. The removal of MCS was confirmed by BamHI digestion (Fig-25e). Further, the complete LTR-GFP (~1.37 kb) fragment was PCR amplified and polished by Klenow fragment. pGL3-basic was digested with NcoI/ XbaI (to release luciferase gene) and the RE generated ends were polished and ligated with LTR-GFP fragment, thus generating pLTR-GFP-pA. The clones were screened for the presence of the LTR-GFP fragment by PCR (Fig-25f).

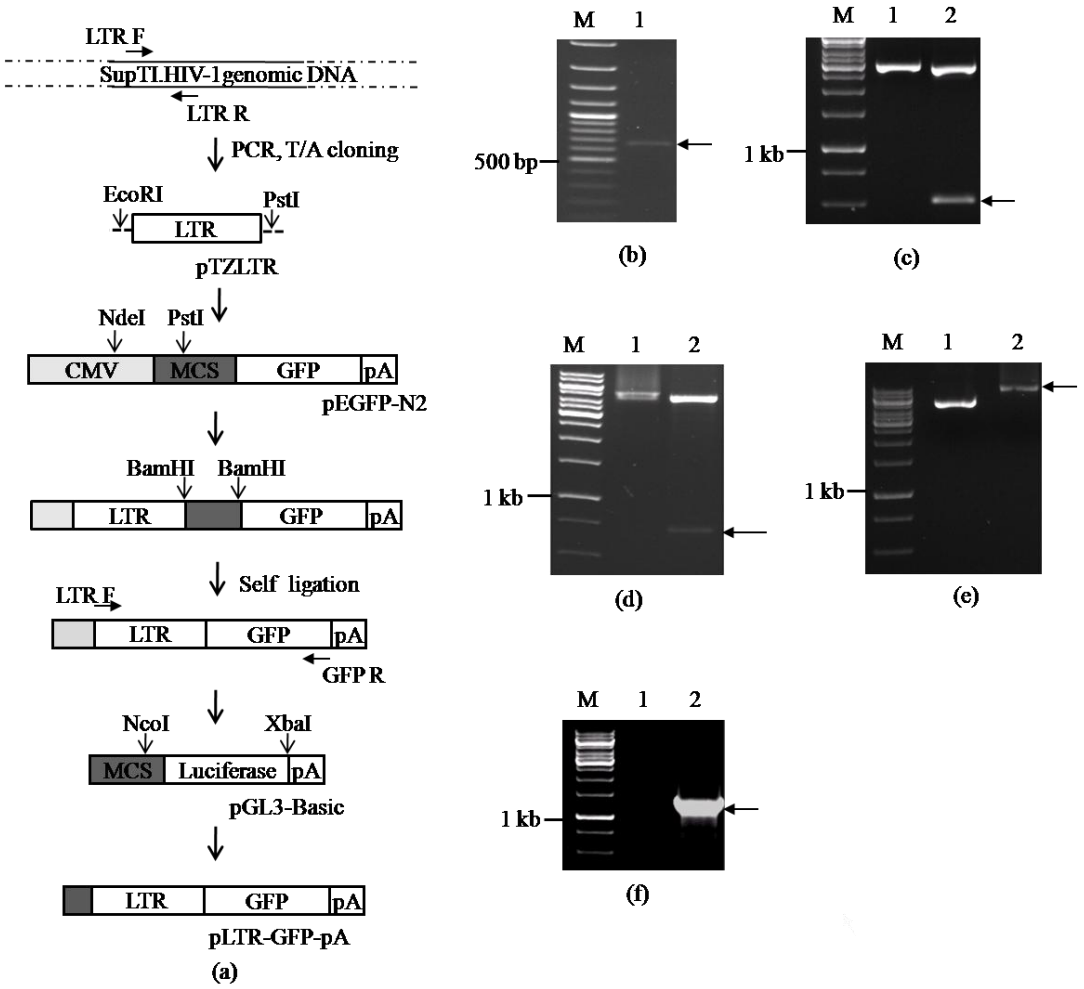


Fig-25. Generation of LTR-GFP-pA construct. *(a) Cloning steps for generating LTR-GFP fragment in pGL3-basic vector; (b) PCR amplification of the LTR promoter desired fragment, M: 100 bp DNA marker; (c) SacI digestion pattern of pTZLTR, lane 1: empty vector, lane 2: partial LTR insert release from vector backbone is indicated, M: 1 kb DNA marker; (d) KpnI digestion to confirm LTR cloning in pEGFP-N2, lane 1: linearized vector backbone, lane 2: LTR fragment release from the vector; (e) BamHI digestion to confirm the removal of the MCS between LTR and GFP, lane 1: linearized vector prior to the removal of MCS, lane 2: undigested vector indicating the loss of MCS; (f) PCR using LTR F and GFP R primers to confirm the LTR-GFP cloning in pGL3-Basic, lane 1: empty vector, lane 2: the PCR amplified LTR-GFP cloned fragment is indicated and the arrows show the referred genomic fragments.*

Generation of selection marker cassette pCMV-Neomycin-pA

Neomycin (Neo) gene was released from pTZ using XbaI/ SmaI digestions, and sub-cloned into NheI/PmeI sites in pCDNA3.1(+) downstream to CMV promoter (Fig-26a). The clones were screened for the presence of Neo fragment by NcoI digestion (Fig-26b). The complete CMV-Neo assembly was then released from pCDNA3.1 by BglII/ NheI

digestions and cloned at BglII/ XbaI sites in pGL3-Basic, replacing luciferase fragment and generating pCMV-Neo-pA. The clones were screened by PstI digestion (Fig-26c).

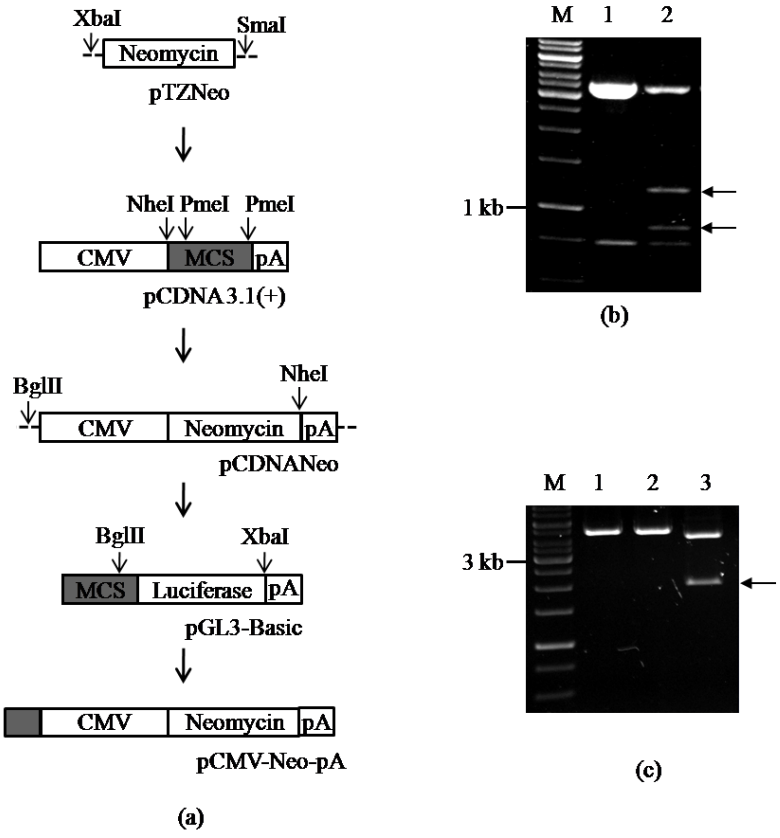


Fig-26. Generation CMV-Neo-pA construct. *(a) Cloning steps for generating CMV-Neo fragment in pGL3-basic vector; (b) NcoI digestion to confirm Neo cloning in pCDNA, lane 1: digestion pattern of the empty vector, lane 2: vector containing Neo gene showing an expected release of two extra fragments as indicated; (c) PstI digestion to confirm cloning of CMV-Neo in pGL3-Basic, lane 1: pCDNA empty vector, lane 2: pGL3 empty vector, lane 3: pCMV-Neo-pA showing an additional band corresponding to the insert and part of the vector backbone, M: 1 kb DNA marker and the arrows show the referred genomic fragments.*

Amplicon vector assembly

The vector was assembled in two steps where first the selection marker and transgene expression cassettes were cloned into a single platform. Subsequently, HHV-6 Ori-Pac cassette was added to the same construct resulting in generation of an amplicon vector.

Step I: Cloning of transgene and selection marker cassettes

The selection marker construct was digested with SalI/ BglII to release the complete CMV-neo-pA cassette (Fig-27a & b). The ~1.7 kb released fragment was subsequently cloned at SalI/ BamHI sites in pLTR-GFP-pA, thereby generating pLTR-GFP-pA.CMV-neo-pA construct. The clones were screened by SacI digestion (Figure-27c).

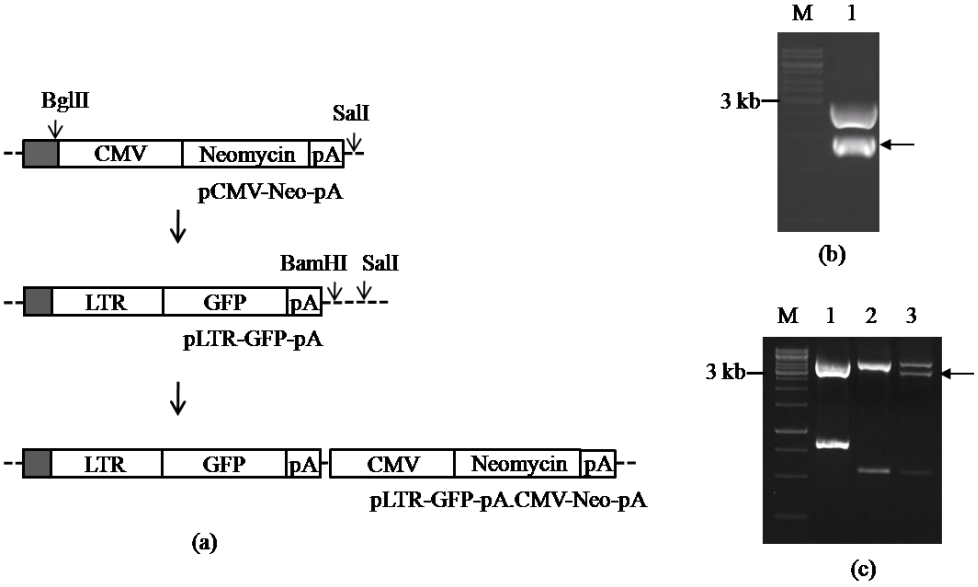


Fig-27. Cloning of transgene and selection marker cassettes. *(a) Cloning steps for generating pLTR-GFP-pA.CMV-Neo-pA; (b) lane 1: pCMV-Neo-pA digested with SalI/ BglII to release the CMV-Neo-pA cassette from the vector; (c) SacI digestion to confirm the sub-cloning of the cassette, lane 1: pCMV-Neo-pA, lane 2: pLTR-GFP-pA, lane 3: pLTR-GFP-pA.CMV-Neo-pA showing an additional band, confirming the incorporation of CMV-Neo-pA cassette into pLTR-GFP-pA, M: 1kb DNA marker and the arrows show the referred genomic fragments.*

Step II: Addition of ori-Pac cassette

pTZ.ori-pac was digested with BamHI to release the ori-pac cassette (Fig-28a & b). This ~1.3 kb released fragment was incorporated in the step I derived clone at BglII site, thus generating pH6ori-pac.LTR-GFP-pA.CMV-neo-pA construct (referred to as amplicon vector) in a pGL3-Basic backbone. The clones were screened by BglII/ EcoRI digestions (Fig-28c). The total size of the amplicon vector constructed is ~7.8 kb.

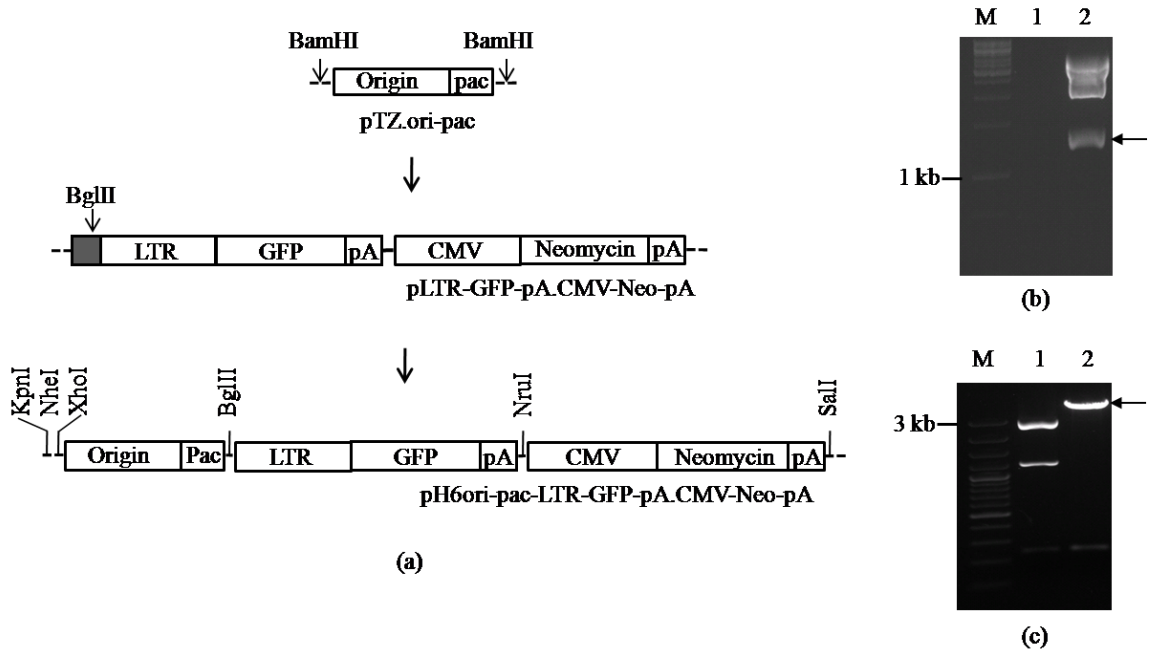
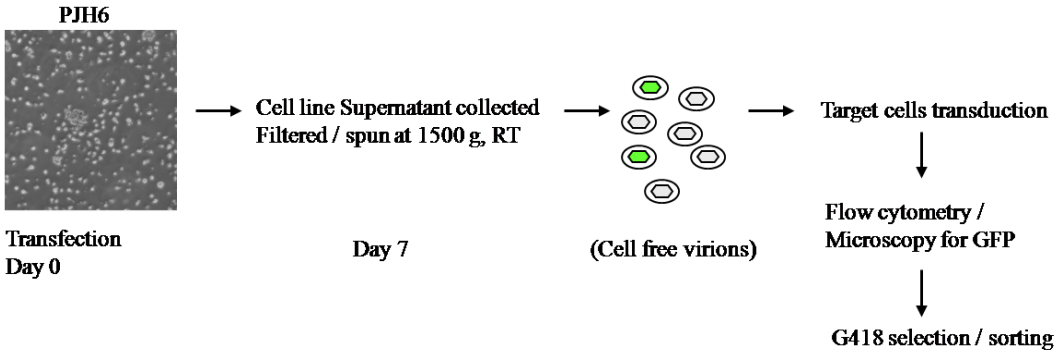


Fig-28. Generation of vector with HHV-6 ori-pac cassette. *(a) Cloning steps for generating pH6ori-pac.LTR-GFP-pA.CMV-Neo-pA construct; (b) lane 1: blank well, lane 2: pTZ.ori-pac digested with BamHI indicating the release the ori-pac cassette from pTZ; (c) BglII/EcoRI digestions to confirm the sub-cloning of the cassette, lane 1: pTZ.ori-pac, lane 2: pH6ori-pac.LTR-GFP-pA.CMV-Neo-pA showing the presence of ori-pac along with an expected up-shift in the size of the vector, M: 1kb DNA marker and the arrows show the referred genomic fragments.*

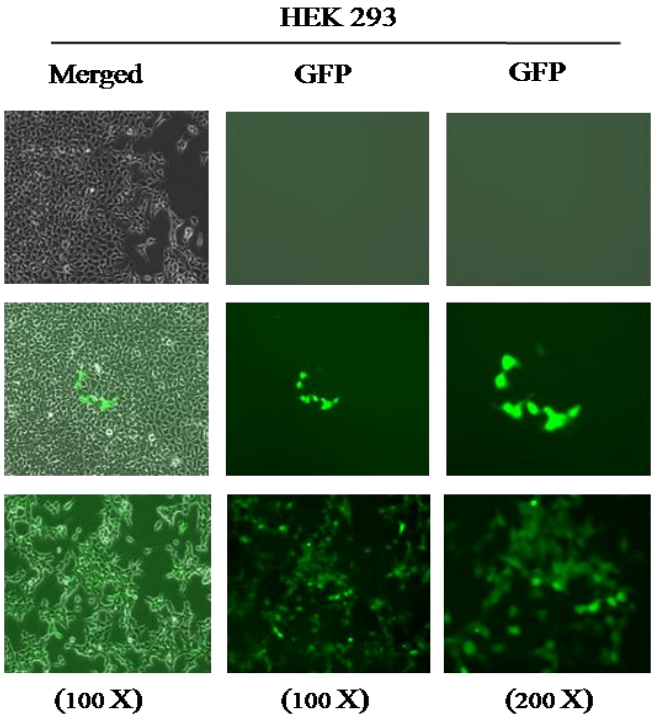
Functionality of the amplicon vector and generation of stable cell lines

PJH6 cells were used as the packaging cell line for generation of amplicon vector derived pseudovirions and this was accomplished by transfection of the vector into PJH6 cells, which resulted in generation of a mixture of wild type and pseudovirions that were used to transduce target two cell lines of different lineages (Fig-29a). GFP transgene expression was monitored after 5 days. The cell lines used were HEK293 and Daoy (a medulloblastoma cell line). Both the cell lines showed transduction evident by GFP transgene expression, indicating that the amplicon vector successfully replicated and packaged into pseudovirions in the presence of wild type virions. Cells transduced with the culture supernatant harvested from PJH6 cells transfected with the control vector (without HHV-6 ori-Pac) were found to be GFP negative. Cells were further selected

with G418. Additionally, the selected cells were also sorted on the basis of their GFP expression which resulted in further enrichment of the pseudovirions transduced cell populations. The selected and sorted cells exhibited high levels of transgene expression which was retained even after several passages over a month (Fig-29b and c).



(a)



(b)

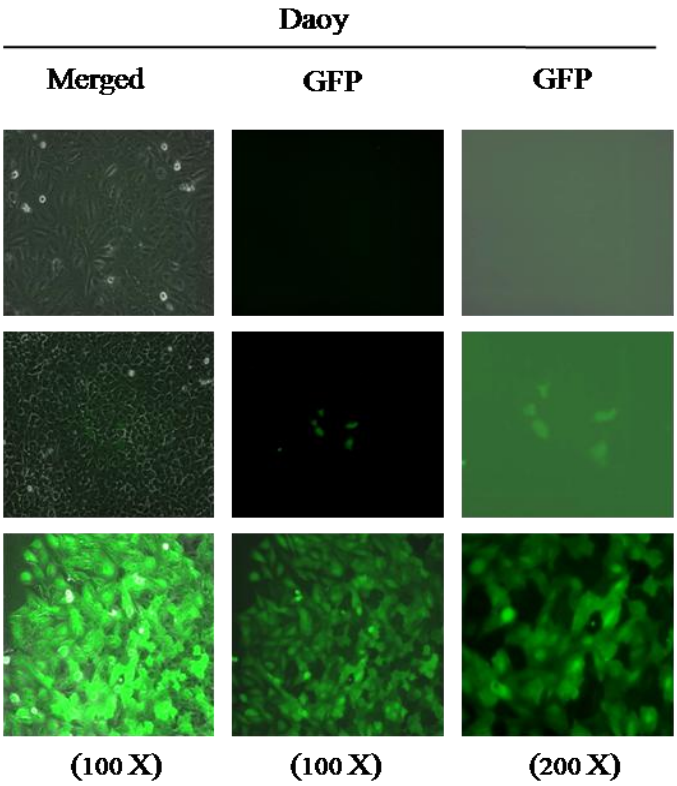


Fig-29. Functionality of the amplicon vector and generation of stable cell lines. *(a) Outline of the steps followed for generation of pseudovirions; (b) HEK 293 cell line and (c) Daoy cell line (middle panels): fluorescence microscopy images at 5 days post transduction showing GFP expression from amplicon vector derived pseudovirions; (upper panels): amplicon vector control transduced cells (without HHV-ori-Pac); (lower panels): G418 selected and GFP sorted cell populations exhibiting a high level of transgene expression, the magnification of the images is indicated.*

V. Development of a single step relative viral titer assay

Generation of a LV construct with HHV-6 inducible promoter-reporter cassette

The complete pLTRC-Luc-EGFP plasmid, derived from pIRES2-EGFP plasmid (105) was linearized using HpaI and was sub-cloned in the lentiviral vector MCS at the SmaI site (Fig-30a & b). Clones were screened by digestion with SalI to obtain a clone with the linearized plasmid insert in a reverse orientation with respect to the LV 5'LTR (Fig-30c).

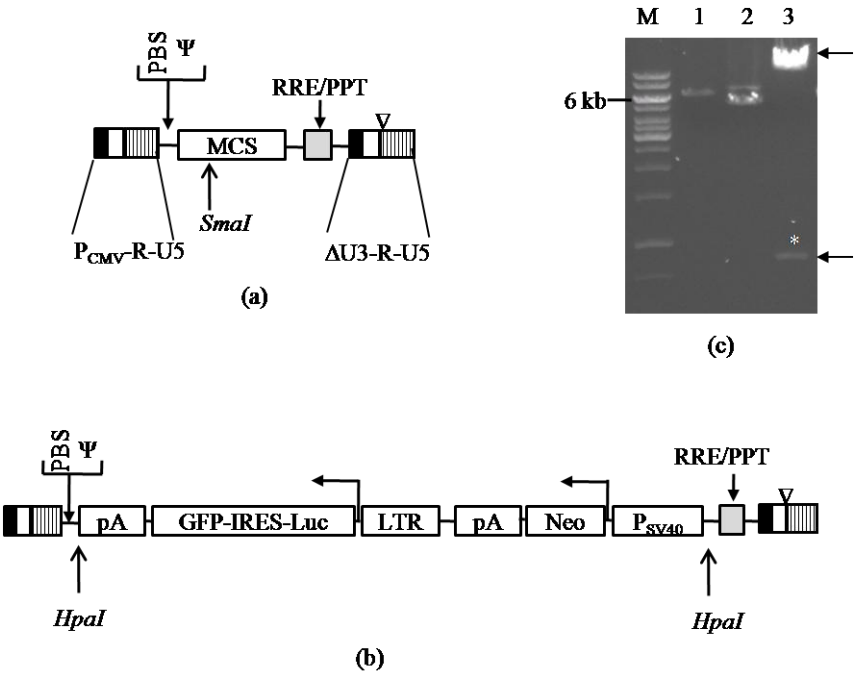


Fig-30. Vector genomic configuration. *(a) Parental lentiviral transducing vector; (b) LV with the incorporated reporter plasmid; (c) confirmation of the clones by SalI digestion, lane 1: linearized LV empty vector, lane 2: linearized transgene expression plasmid, 3: target LV clone showing an up-shift in the size of backbone in addition to a small fragment of the insert (indicated by*) released upon RE digestion, M: 1 kb DNA marker and the arrow indicates the referred genomic fragments.*

Generation of a stable indicator cell line

The lentiviral construct generated was used as a transfer vector and virus was prepared by multi plasmid transfection followed by transduction of HEK 293 cells, which were selected G418 to obtain a stable reporter cell line. Fluorescence imaging of the selected cell line showed GFP expression (Fig-31a). Presence of transgene expression cassette was confirmed by both GFP and Luciferase PCR from the selected cell line DNA (Fig-31b, c & d).

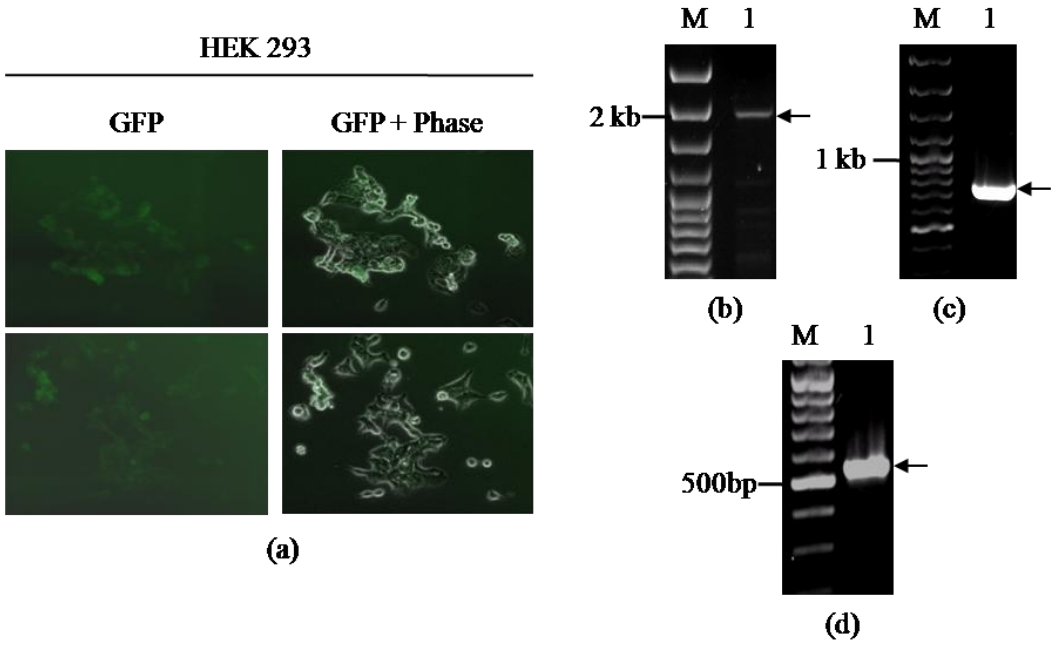


Fig-31. Indicator cell line. *(a) Fluorescence microscopy images of indicator cells post G418 selection; (b) PCR for Luciferase gene; (c) PCR for GFP; (d) β-globin PCR as a quality control, M: 100 bp DNA marker, arrow indicates specific PCR amplifications.*

LTR transactivation in the indicator cell line by HHV-6

Virus stock enriched with both culture supernatant derived virions as well as the intracellular virions (derived from cellular lysate) was concentrated to 50 X by ultracentrifugation and used for titration. Indicator cells were incubated with 100 µl of this stock per well and processed for Luciferase assay at 5 different time points from 8 h to 48 h post infection. A significant up-regulation of the transgene expression levels was obtained in the presence of virus suspension, over the basal expression level (Fig-32). Increasing the incubation period of the cell line with virus preparation from 16 to 24, 36 or 48 h did not appreciably increase the relative level of reporter expression (fold increase).

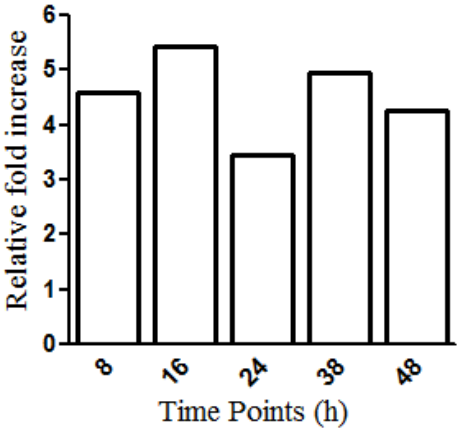


Fig-32. Transgene transactivation in indicator cells. *The columns represent the fold increases in luciferase activity observed in presence of viral preparation relative the basal values (in presence of CM) at the indicated time points; single experiment.*

Titration of virus obtained by ultracentrifugation

In order to ascertain the sensitivity and linearity of the assay, the 50 X concentrated virus stock (described above) was serially diluted and titrated 16 h after virus addition. A dose dependent increase in LTR activation was observed as indicated by the reporter gene expression corresponding to increasing concentrations of the viral suspension tested (Fig-33a). A significant fold increase relative to the control value (reporter activity in presence of CM) was observed for all the viral concentrations tested (Fig-33b).

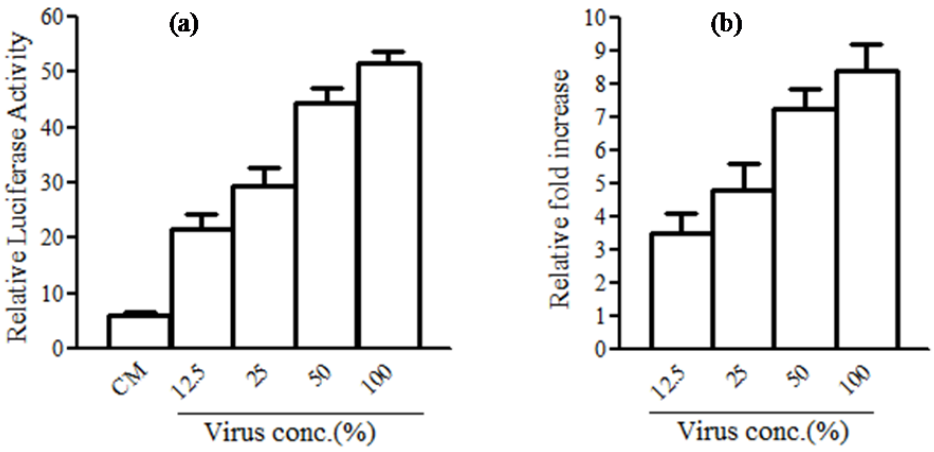
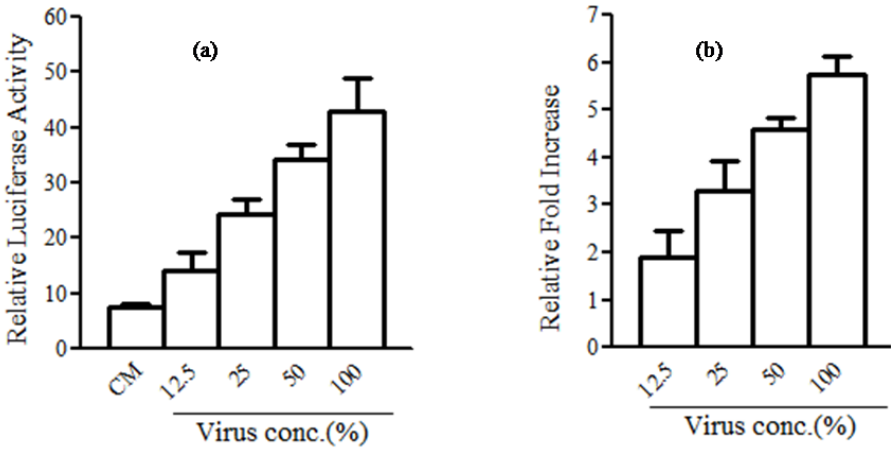


Fig-33. Luciferase reporter transactivation in the presence of virus obtained by ultracentrifugation. *(a) CM: 100 µl culture media, virus conc.: 100 µl of the indicated*

concentrations of viral suspension. The columns represent the observed relative luciferase activity; (b) columns represent the relative fold increases with respect to the basal values obtained using CM; the error bars represent mean ± s.d. of 3 independent experiments.

Titration of virus obtained by PEG precipitation

PJH6 cell culture supernatants were concentrated to 50 X by PEG precipitation and serially diluted (as above) for estimating the viral titer. Increasing concentration of viral suspension showed luciferase reporter activity in a dose dependent manner as before (Fig-34a). The three concentrations of viral suspension (25%, 50% and 100%) showed more than 3, 4 and 5 fold increases in reporter activities, respectively, over the control value (CM, Fig-34b). PEG precipitate from cell free culture media of Daudi cells was also used as inoculum. No appreciable increase in reporter activity was observed as compared to the media control (Fig-34c). The transactivation potential of the total viral pool (cellular lysate + culture supernatant) was also ascertained. A significant enhancement in the reporter transactivation levels upon addition of intracellular virus pool derived from the cellular lysate was evident in comparison to that exerted by the culture supernatants alone (Fig-34d).



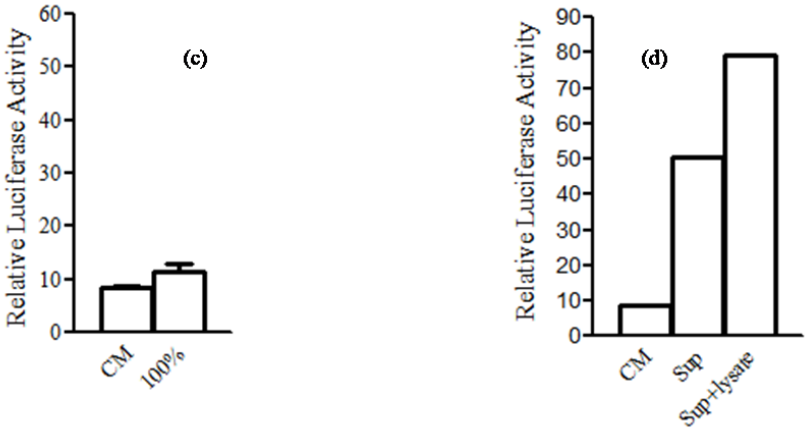


Fig-34. Luciferase reporter transactivation in presence of virus obtained by PEG precipitation. *(a) CM: 5 ml culture media derived precipitate resuspended in 100 µl media, virus conc.: indicated concentrations of PJH6 viral culture precipitates, 100% represents undiluted suspension; (b) relative fold increase with respect to the basal values obtained using CM; (c) 100% represents 5 ml Daudi cell culture derived precipitate resuspended in 100 µl media, columns and error bars are mean ± sd of 3 independent experiments; (d) Sup: PJH6 culture supernatant derived precipitate, lysate: PJH6 cellular lysate derived precipitate.*

CHAPTER 5

DISCUSSION

HHV-6 is essentially a latent virus that ubiquitously infects the human population in early years of life presenting with benign febrile childhood disease like roseola infantum in a few cases, while in rest of the population it silently infects and persists thereafter in the host for a lifetime. Much interest has been generated in the study of HHV-6 basically due to its reactivations or infections in immunocompromised conditions where the virus presents itself as a part of clinically relevant outcomes, which in certain cases have lead to fatal consequences (15). Though not proven conclusively, HHV-6 has also been implicated with the pathology of an increasing number of some diseases like MS, CFS etc., a lot of which have conflicting data in terms of reports by different groups (14, 106, 107). A major reformation in the understanding of the virus biology as well as its disease associations was initiated recently when the 2 variants, HHV-6A & B, were finally recognized as different species by ICTV. At the same time, it generated a lot of gaps in the current understanding of the clinical associations pertaining to the two viral species. Detail understanding can only be accomplished by analyzing more number of viral isolates for each of the species. In this study, we performed a basic characterization for a lab isolate of HHV-6B species, sourced from a healthy adult and propagated by establishing a transformed B-cell line from the PBMC of the said individual; the isolate/cell line was designated as PJH6.

We first performed a morphological analysis of the isolate in order to ascertain its identity as a member of human herpesvirus family. The EM pictures of the cell line derived virus particles exhibited the presence of characteristic features that are typically observed for human herpesviruses. As we had used the concentrated culture supernatant from the PJH6 cell line for our EM analysis, this preliminary data also indicated that the cell line is a spontaneous producer of virus particles, since the parent B-cells that were used to generate the cell line were not stimulated with any chemicals that are generally

used to trigger the herpesvirus replication cycle (59). In the preparations analyzed by EM, the virus particles imaged were without fully formed envelope. However, to our current knowledge, this is indeed the first report describing the spontaneous production of HHV-6B virus particles from a cell line derived from PBMC of a healthy adult.

We next wanted to evaluate the infectivity of the virus particles. The EM studies did not provide any evidence of the expected virion envelope that is typically observed in case of a matured virus particle (108). It was therefore assessed if the cell line supports the completion of lytic virus replication cycle thereby yielding mature virions in the culture supernatant, in addition to those documented by EM. We therefore used only the PJH6 cell culture supernatant for our infectivity studies as opposed to harvesting the virus particles derived from the cellular lysates. When three different lineages of HHV-6 negative mononuclear cell lines were treated with PJH6 culture derived supernatant, the cell lines became HHV-6 PCR positive. Cloning and sequencing of the HHV-6B specific nested PCR products from the donor cell line and infected SupT1 cells showed absolute sequence match, which further confirmed infection of the target cells by the isolate from the producer (PJH6) cell line. We also tested infectivity of the isolate using HHV-6 PCR negative PBMC as target cells, obtained from healthy volunteers. Usually PBMC are mitogen stimulated for infection with HHV-6 (109). However, we found that unstimulated (naïve) PBMC were infected as well as PHA stimulated ones, a feature that can be possibly be attributed to the higher virulence of this isolate.

As HHV-6 has been documented to establish a productive infection in T-cells, we assessed the same for our isolate by in-vitro passaging of the infected SupTI cells. The infected SupTI cells were also found to produce infectious virus that could infect fresh cells, indicating that this isolate also imparts a productive infection to the target cells. As the parent PJH6 cell line was generated by EBV transformation of the donor derived B-

cells, status of EBV in the recipient SupT1 cell line was also evaluated. The PJH6 cell line was EBV PCR positive but the SupTI cells infected with virus particles from PJH6 cells were found EBV PCR negative thereby ruling out the possibility of EBV production in PJH6 cell line.

Overall our infectivity data establishes the parent PJH6 cell line as a useful in-vitro model system for continuous generation of infectious virions that can be further used for studying the biological features of HHV-6B. This system particularly demonstrates its usefulness in the present scenario where most of the studies on HHV-6 are essentially carried out on in-vitro infection to the virus permissive cells due to the lack of animal model systems. The in vitro studies are further complicated by the need to infect fresh cells each time for generating virus stocks. This especially becomes a limiting factor, when Cord Blood derived cells are used for the experiments as these are difficult to obtain or even PBMC because of the fact that HHV-6 is ubiquitous in the human population. Thus testing for HHV-6 seronegativity in the blood donors becomes an essential as well as an additional step before performing the actual experiment.

PJH6 was originally derived from a healthy individual exhibiting relatively high level of the viral genome in the PBMC as ascertained by preliminary experiments from our lab. CIHHV-6 has been consistently reported to occur in ~ 1% of the human population worldwide (48). We therefore investigated the status of the virus integration in the PBMC in the donor. The viral-chromosomal junction was mapped by sequencing & BLAST analysis to the chromosome 12q14 confirming the presence of CIHHV-6 (GenBank accession no JX523610) following established method (54). We further attempted to trace the route of CIHHV-6 acquisition in the donor by analyzing the status of donor's parental PBMC samples. Our case study reports an event of vertical transmission of the viral genome from father to son. In this, regard, our case study is

identical to those reported by several other groups showing the transmission of the CIHHV-6 in a typical Mendelian manner (54, 55). All reports of CIHHV-6 to date document viral integrations in the telomeric region, possibly occurring due to the sequence homologies between the viral terminal direct repeat regions and the telomeric region (48). However, integration of this new HHV-6B isolate clearly occurred in a non telomeric region thereby exhibiting an uncommon integration site, the mechanism of which still remains far from speculation. In case of Marek's disease virus integration, it has been described that the presence of mutated TMRs in the viral genome can lead to the non-telomeric intrachromosomal integration of the viral genome (61). In our case, we did not venture into details of the organization of the viral TMRs. Interestingly the donor's mother showed the presence of viral-chromosome junction in the telomeric region of chromosome 8, but the site was different as that observed in the donor sample. A gap of ~100 bp was also observed in between the viral and chromosomal junction sequences, the origin of which is not clearly understood.

As our studies indicated the spontaneous generation of infectious virus particles from the cell line, we wanted to confirm the status of CIHHV-6 in the same. We could successfully map the presence of identical viral-chromosomal junction in the cell line (GenBank accession no JX523611). Currently there is no consensus about the fate of CIHHV-6 genome to be a dead end phenomenon in the viral life cycle or a mode of viral persistence. So far, only one group has demonstrated the production of infectious viral particles from activated primary T-cells sourced from CIHHV-6 positive donors (54). A few reports have also suggested the possibility of CIHHV-6 replication thereby accounting for congenital HHV-6 infections (62). Thus, we report here the production of infectious virus particles from transformed B-cells of a healthy individual with CIHHV-6 infected cells.

Herpesvirus IE gene are associated in the early viral gene expression and very often transactivate transcription from a variety of host promoters. Being extremely diverse in the orientation of their ORFs as well as their nucleotide sequences, they form an interesting group of genes in herpesviruses in general (110). In this report, we made limited characterization of the role of IE1 gene with respect to its transactivation potential for select heterologous promoters and their corresponding genes. We started with the cloning and expression analysis of the isolate derived IE1 gene, for which the entire IE1 ORF was placed under a CMV promoter along with a C-terminal EGFP tag. Noticeably we always observed presence of two closely placed bands at ~170 kDa and ~190 kDa instead of a single band for the IE1-GFP protein. This could possibly be attributed to the SUMOlyation status of the IE1 protein as has been reported previously elsewhere (63). The IE1 protein from HHV-6B species contains two sites for SUMO modifications. Thus, the ~170 kDa band may represent the SUMO un-conjugated form while the protein migrating at ~190 kDa possibly represents the SUMO-modified form of IE1.

Previous reports have described that IE1 is capable of transactivating several heterologous promoters containing distinct transcription factor binding sites by basically interacting with regulatory proteins common to many transcriptional units (29). This feature of IE1 makes it a candidate gene for promoting changes in cellular gene expression thereby enabling the establishment of a favorable cellular environment for the viral infection. We therefore analyzed the in-vitro transactivation potential of IE1 encoded protein upon the host genes, which are primarily involved in growth regulation and cell cycle control. Our results clearly indicated a significant up-regulation in the basal activities of all the three target promoters, hsp-70, c-fos & c-myc, in the presence of IE1. Our results are similar to those described for CMV IE1 transactivation of the

same target promoters (73). Though CMV IE1 and HHV-6 IE1 do not share homologies at the level of protein sequence (29), yet it is evident from this data that much of their functional properties have remained conserved. In comparison to the reported CMV IE1 mediated transactivation (73), our isolate derived IE1 gene products exhibited a higher level of transactivation for all the three promoters in the plasmid co-transfection experiments. The up-regulations observed were as high as ~30 folds for hsp-70 promoter in comparison to only 4 folds in case of HCMV. This could either reflect higher transactivation capabilities of HHV-6 IE1 or might be simply attributed to the difference in the basic properties of the CMV & HHV-6 IE1 gene. Alternatively, the observed differences may also be partially influenced by the higher sensitivity of the luciferase assay over the chloramphenicol acetyl transferase (CAT) reporter system used for CMV study.

Since infection based assay design is always closer to natural scenario in comparison to plasmid based transactivation, we next analyzed transactivation of the corresponding genes at RNA transcript level in case of infection of target SupT1 cells by the wild type PJH6 derived virus. Interestingly, our results showed a similar pattern of up-regulation for hsp-70, a moderate increase for c-fos, while c-myc expression levels remained unchanged post infection. However, the levels of hsp-70 up-regulation observed in case of infection were considerably lower in comparison to those obtained by transfections. We harvested the cells for RNA isolation at 8 h post infection, at the time when the levels of IE1 expression are considerably high as reported earlier (63). The reason for not observing significant levels of up-regulation could possibly be due to the transient elevations in the expression of the target genes post HHV-6 infection. Possibly a time kinetics study to check for the changes in the expression of the selected cellular genes by infection may throw a better picture of the events. Similar experiments have been

performed in case of CMV where the RNA was isolated 12 h post infection and significant induction in expression of endogenous c-myc and c-fos was observed (73). Taking account of both transfection and infection experiments, our results indicate that the HHV-6 IE1 gene products can potentially regulate the expression of hsp-70 although further experiments are needed to elaborate the same. hsp-70 is one of the well characterized heat shock proteins, molecular chaperons that contribute to the cytoprotective effects following cellular stress, however, it has been suggested that elevated hsp-70 expression may assist to the development of neoplastic phenotypes (111). Also, heat shock factors, the family of transcription factors that control expression of heat shock proteins, have also been implicated in the process of malignancies (112). Our earlier study as well as published literature has implied a loosely defined link between HHV-6 and tumorigenessis, though very robust supportive data is lacking (22-26). With this background and implications of hsp-70 in the process of malignancy as cited above, elevated hsp-70 in host cells on HHV-6 infection therefore assumes important significance and merits further enquiries.

In this era of gene therapy using virally derived vectors, the herpesvirus based amplicon vectors are looked upon as very efficient systems for gene delivery for relatively large pieces of DNA. Amplicon based vectors have been previously reported for HSV, EBV, CMV, HHV-6 & HHV-7 (78-80, 82, 85). These vectors have successfully been adapted for numerous applications in the field of gene therapy especially in the case of HSV. A recent study reports successful use of HSV derived vector in bladder sensitive disorder (113). In this study, we tried to harness our isolate for development of a basic amplicon vector. Based on the reported similarities in the basic mechanism of replication of HSV & HHV-6 along with the literature on the basic components required for generation of amplicon vectors, the vector was designed to contain only the viral origin of replication

and the cleavage and packaging motifs from the viral genome. Apart from the viral derived sequences, we also incorporated a GFP marker as a transgene and a selection marker in the basic amplicon to facilitate its downstream processing.

Once all the components were assembled, we tested the functionality of the vector based on the hypothesis that an amplicon vector containing viral replication origin, the cleavage-packaging motifs & the transgene (s), will get replicated in the presence of wild type virion encoded factors and get packaged as pseudovirions that can further infect fresh cells thus delivering the transgene to the target cells. We used PJH6 as the packaging cell line for the amplicon vector replication and generation of pseudovirions. Our results clearly demonstrate the functionality of the amplicon system as indicated by the transgene delivery to both the target cell lines HEK 293 and Daoy, although our efficiency of transduction was low. However, we could overcome this limitation for our experiments as we had incorporated a selection in the basic vector. In addition to this, we further selected out for high transgene expressing cells by cell sorting on the basis of GFP transgene expression. Post sorting and selection, we were able to generate stable clones that exhibited appreciably high levels of transgene expression. Moreover, we observed consistently high levels of transgene expression even after several passages of the transduced cells. The expression was visibly higher in case of Daoy in comparison to HEK 293.

Thus we could generate a basic amplicon vector from our isolate that got packaged as pseudovirions with the help of wild type virions in the PJH6 cell line and the pseudovirions loaded transgene could be delivered to the target cells. Similar HHV-6 amplicon vector has earlier been reported for transgene delivery to target cells (86). Our vector has some additional features for instance the presence of a selection marker cassette as well as presence of additional cloning sites that can be used for incorporation

of small interfering RNA expression cassette or any other modifications as per requirements. Currently, such vector has a disadvantage of being contaminated by the presence of helper virus, wild type viruses can be eliminated by select anti-viral drug that inhibits HHV-6 growth; the drug will have no effect on the pseudovirions, which lack cellular machinery.

Determination of viral load in a biological sample is essential in evaluating its association with a particular disease especially in a situation like that of HHV-6 where the virus is ubiquitous in distribution and shows instances of chromosomal integration with reactivation potential in immunodeficient conditions, which may arise due to several factors. For in vitro studies also, the estimation of viral titer in an innoculum is an essential step in order to appropriately correlate the experimental dose-response outcome with the infectious agent being analyzed.

For performing most of our infection experiments, we routinely used 1 ml of the PJH6 culture supernatant obtained after 72 h of culture for infecting $1x10^6$ each time, however, the actual estimation of the viral titers was lacking in our experiments. Usually for HHV-6, the standard $TCID_{50}$ method is most frequently used for assessment of the viral titer but the assay has several limitations for instance, apart from being labor intensive & also requires testing of HHV-6 seronegativity if the experiment is performed on PBMC. This increases the experimental time & moreover for reproducibility, sample collection followed by data obtained from at least three different HHV-6 seronegative individuals is generally required.

With the advent of understanding of virus infectivity in vitro, some initial steps including the sample collection, screening, as well as the subsequent PHA stimulation step have been eliminated from the entire procedure by performing the $TCID_{50}$ assays on the cell lines that support viral lytic cycle, for instance Molt-4, SupTI cell lines (114). However,

an initial screening of the available virus permissive cell lines for their susceptibility to the viral particular isolate being examined is required in order to obtain the most suitable cell line for performing the assay due to the differences in the infectivity of the two species HHV-6A & B as well as some inherent differences in the infectivity of different isolates (36).

Reporter based assays are sensitive and quick in comparison to the $TCID_{50}$ determination or IFA based methods (100). Reporter based indicator cell lines have previously been described for other herpesviruses including HHV-8, VZV & CMV but not for HHV-6 (94-96). We attempted to generate a single step relative titer assay based upon the viral property of transactivation of heterologous promoters. We selected HIV1-LTR as the target promoter as the same has been documented to get transactivated by a number of virally encoded factors (14, 30). For the same purpose, we cloned an entire plasmid having the LTR driven reporter gene cassette as well as the selection marker cassette in a LV platform (115) and thereby generated a stably transduced HEK 293 indicator cell line. We hypothesized that upon HHV-6 infection of the indicator cells, an increase in the reporter gene expression would be observed in comparison to the uninfected cells, and the same could be further co-related to the viral titer based upon the amount of inoculums and the respective reporter expression read-outs. We first tested functionality of the cell line by using a single dose of viral preparation obtained by harvesting the PJH6 supernatant as well as the cellular lysates in order to avoid any underestimation of the sensitivity of the assay system in case of low titers. Our results clearly indicated a significant up-regulation of the LTR driven Luciferase activity, indicating appropriateness of the indicator cell line. As we carried out a time kinetics analysis in the same experiments, it was evident that the cells could be harvested for the assay as early

as 16 h post infection as increasing the incubation beyond this time point did not further increase the reporter gene expression appreciably.

We next assayed a panel of serially diluted viral preparation in order to evaluate the sensitivity and linearity of the assay. The results showed a dose dependent increase in the reporter gene expression in response to increasing concentrations of the viral inoculums. We next tried to improve upon our assay system by making it less labor intensive as well as cost effective. For this purpose, we made two modifications in the assay which included removal of ultracentrifugation step for concentrating the virus preparations and harvesting only the supernatant from PJH6 as opposed to an additional preparation of cellular lysates. We replaced the ultracentrifugation step with the relatively simpler PEG precipitation technique and precipitated only supernatant for the assay. Similar panel of virus dilutions were prepared and assayed. We could again observe a similar pattern of the reporter gene activation as observed previously although the levels of up-regulation were comparatively lower. However, this made our assay system simpler and also indicated that the PJH6 supernatant derived virus particles also were capable of infecting the target cells and driving the reporter gene expression considerably. At the same time, supernatant derived from another EBV positive cell line Daudi did not show any appreciable increase in the reporter gene activity in comparison to the controls thereby establishing reliability and specificity of the assay.

Total time required from indicator cell seeding to assay completion is only 30 h. Since we performed the assay in a 96 well plate, the assay required very little starting material and thus can find an application into high-throughput screening as per requirements. The indicator cell line, with reporter construct integrated through lentiviral vector, does not need any antibiotic selection and use of SteadyGlo substrate allows long term signal stability. This is the first single step relative virus titration assay described for HHV-6

and this rapid method can also be adapted to anti HHV-6 compound screening or serum neutralization antibody titer estimation. Additionally, we believe this assay will also be applicable for HHV-6A titer estimation.

Highlights of the present thesis work includes first comprehensive characterization of a HHV-6B isolate from the Indian sub-continent, first evidence of a case of HHV-6B integration in non-telomeric host chromosome, demonstration of HHV-6B IE-1 gene influence on hsp-70 up-regulation and finally establishment of the first indicator based single step assay for HHV-6B titer estimation. Availability of a spontaneously virus producing isolate and the quick viral titer estimation assay will be important tools for further understanding the biology of HHV-6B.

CHAPTER 5

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APPENDIX

LIST OF PCR PRIMERS

TARGET & expected size	PRIMER F: Forward R: Reverse	PRIMER SEQUENCE 5'-3'
Sequencing (T/A cloning)	M13F	GTAAAACGACGGCCAGT
	M13R	CAGGAAACAGCTATGAC
	T7F	TAATACGACTCACTATAGGG
HHV-6 nested PCR (round 1)	H661	CAAGCCCTAACTGTGTATGT
	H662	TCTGCAATGTAATCAGTTTC
HHV-6 nested PCR (round 2) 429 bp	H663	CTGGGCGGCCCTAATAACTT
	H664	ATCGCTTTCACTCTCATAAG
House keeping, gDNA (β-globin) 536 bp	RS42	GCTCACTCAGTGTGGCAAAG
	KM29	GGTTGGCCAATCTACTCCCAGG
EBV 239 bp	EBNA F	GGCTGGTGTCACCTGTGTTA
	EBNA R	CCTTAGGAGGAACAAGTCCC
Integration, inverse PCR	H6int-3F	ATGCGACAGACAGTCACGCGGA CCGAC
	H6int-3R	GCTGCCACACGTGTATTCAATGG CATG
IE1 ORF 3.2 kb	H6 U90 F	AGTTGCGGCCGCACCATGGAGTC AGCAAAAGATA
	H6 U90 R	TGATGCCCGGGATAAATTTGAGC ATTTTCTTCGAA
c-fos promoter(-721 to +5) 726 bp	cFos pr F	AGTTGAGCTCAGCCTCAGAACTG TCTTCAGTT
	cFos pr R	ATGTAGATCTAGAAGGAGTCTGC GGGTGAGT
c-myc promoter (-1986 to +15) 2 kb	cmyc pr F	AGTTGGTACCGAAGAGCCGGGCG AGCAGAGCT
	cmyc pr R	TGTTAGATCTAACGTTGAGGGGCA TCGTCGCGGGA
hsp-70 promoter (-1170 to +101) 1.27 kb	hsp70 pr F	ATGTAGATCTCCCTGGTCGTTGGCG ATGATCT
	hsp70 pr R	AGTTGGTACCTTCCTAGGCCGCACT CCCTTT
c-fos cDNA 291 bp	cfos F	CCTCACCCTTTCGGAGTCCC
	cfos R	CTCCTTCAGCAGGTTGGCAATCT
c-myc cDNA 117 bp	cmyc F	TCAAGAGGTGCCACGTCTCC
	cmyc R	TCTTGGCAGCAGGATAGTCCTT
hsp-70 cDNA 647 bp	hsp70 F	CACCACCTACTCCGACAACAA
	hsp70 R	GCCCCTAATCTACCTCCTCAATG
House keeping, cDNA, 983 bp	GAPDH F	TGAAGGTCGGAGTCAACGGATTTGGT

	GAPDH R	CATGTGGGCCATGAGGTCCACCAC
HIV-1 LTR (GT50) 634 bp	LTR F	TGGATGGGTTAATTTACTCCAAGAA AAG
	LTR R	CCCTGTTCGGGCGCCACTGCTAGAG
HHV-6 ori 1.2 kb	H6.orilyt F	AGGATCCATCGATTACTTTGCGTGCT GTAGGC
	H6.orolyt R	AATTTAACAGCATGCTTTACCATTTAC
HHV-6 packaging signal1	H6 pac R1	GAAAAACACGGCGCCTCCATAGAGGC GCGCGCGCGCGCGAATTTAACAGCAT GCTTT
HHV-6 packaging signal 2	H6 pac R2	CGCGTCTCTCGCAGCGGCGTGTTGGT GAAAAACAC
HHV-6 packaging signal 3	H6 pac R3	ATAGATCTATACCCCCCTTTTTTTAA CCCCCCCGGG GGAGTTTAAAGTAATTTTTG
GFP 719 bp	GFP F	ATGGTGAGCAAGGGCGAGGAG
	GFP R	TTACTTGTACAGCTCGTCCATGC
Luciferase 1.5 kb	LUC F	GAATTCACCATGGAAGACGCCAAAA ACATAAA
	LUC R	TTACACGGCGATCTTTCCGCCCT

INDIVIDUAL PCR CONDITIONS

AMPLICON	Denaturation (ºC)/ time (min)	Annealing (ºC)/ time (min)	Extension (ºC)/ time (min)	Number of cycles
HHV-6 nested Round 1	94/ 1	50/ 1	72/ 1	30
HHV-6 nested Round 2	94/ 1	60/ 1	72/ 1	30
β- globin	94/ 1	55/ 1	72/ 1	30
EBV	94/ 1	52/ 2	72/ 1.2	30
c-fos pr.	94/ 1	56/ 1	72/ 1	30
c-myc pr.	94/ 1	56/ 1	72/ 1	30
hsp-70 pr.	94/ 1	56/ 1	72/ 1	30
c-fos cDNA	94/ 30 sec	55/ 30 sec	72/ 30 sec	30
c-myc cDNA	94/ 30 sec	55/ 30 sec	72/ 30 sec	30
hsp-70 cDNA	94/ 30 sec	55/ 30 sec	72/ 1	30
GAPDH	94/ 1	55/ 1	72/ 1	30
HIV-1 LTR	94/ 1	55/ 1	72/ 1	30
HHV-6 ori	94/ 1	60.9/ 1	72/ 1	30
GFP	94/ 1	55/ 1	72/ 1	30
Luciferase	94/ 1	55/ 1	72/ 1	30

Long PCR conditions

IE1 ORF: initial denaturation : 94ºC/ 2 min
10 cycles: 94ºC/ 15 sec
55ºC/ 30 sec
68ºC/ 2 min
25 cycles: 94ºC/ 15 sec
55ºC/ 30 sec
68ºC/ 2 min 2 sec increase per cycle
Final extension: 68ºC/ 10 min

HHV-6 integration: initial denaturation : 94ºC/ 4 min
9 cycles: 94ºC/ 30 sec
60ºC/ 30 sec
68ºC/ 2 min
25 cycles: 94ºC/ 30 sec
60ºC/ 30 sec
68ºC/ 2 min 2 sec increase per cycle
Final extension: 68ºC/ 1 h

PUBLICATION

Virus Research 173 (2013) 280–285

Contents lists available at SciVerse ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

An infectious HHV-6B isolate from a healthy adult with chromosomally integrated virus and a reporter based relative viral titer assay

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ARTICLE INFO

Article history:
Received 21 November 2012
Received in revised form 3 February 2013
Accepted 4 February 2013
Available online 20 February 2013

Keywords:
CIHHV-6
HHV-6B isolate
HHV-6 titer assay
Indicator cell line
Non-telomeric integration
Productive infection

ABSTRACT

Human herpesvirus 6B (HHV-6B) primary infections occur in early childhood and establish a life-long latency in the most healthy adults. HHV-6B was detectable in the peripheral blood mononuclear cells (PBMC) and granulocytes by serial genomic DNA dilution PCR till 10 pg of template DNA, in a healthy adult. Epstein Barr virus (EBV) mediated transformation of the PBMC resulted in establishment of a B-cell line. Southern hybridization with the PBMC as well as the cell line DNA showed distinct signals for high copy viral genomes and Gardella gel analysis indicated chromosomal integration of the HHV-6B. Integration site analysis in the PBMC and the cell line indicated an atypical viral integration in non-telomeric region of chromosome 12. Cell free culture medium of the cell line could infect different mononuclear cell lines, naïve or mitogen stimulated PBMC and was found to impart productive infection in a recipient T cell line. An HIV-1 LTR driven luciferase based reporter cell line was made and a single step assay was developed for estimating HHV-6B relative concentration in the culture supernatants. This study thus reports a new infectious HHV-6B isolate with uncommon integration site, spontaneous production from a cell line and also development of a simple relative HHV-6B titer assay.

1. Introduction

HHV-6, a double-stranded DNA virus, exists as two species, with characteristic DNA sequences, distribution, pathogenicity and designated HHV-6A and HHV-6B, the later being ubiquitous and latent throughout the adult life of most healthy individuals (De Bolle et al., 2005; Di Luca et al., 1994; Dockrell, 2003). Primary infection with HHV-6B is usually asymptomatic and associated with common, self-limiting childhood illness roseola infantum (Yamanishi et al., 1988). In recent times mesial temporal lobe epilepsy has been linked to HHV-6B infection (Fotheringham et al., 2007). Like some other herpesviruses, latent HHV-6 can be reactivated in clinical conditions associated with immunosuppression including certain malignancies (Clark and Griffiths, 2003; Joshi et al., 2000; Lusso and Gallo, 1995; Ogata, 2009; Tailor et al., 2004). Interestingly, inheritances of chromosomally integrated HHV-6 (CIHHV-6), not only in clinical conditions but infrequently also in healthy individuals, are well documented (Arbuckle et al., 2010; Daibata et al., 1998; Leong et al., 2007; Morissette and Flamand, 2010; Nacheva et al., 2008; Pellett et al., 2012; Tanaka-Taya et al., 2004). Few isolates of HHV-6B are well characterized with complete genome sequenced and establishment of latent HHV-6B containing cell line from patient material was also reported (Bandobashi et al., 1997; Dominguez et al., 1999; Isegawa et al., 1999). Here we report a new infectious HHV-6B isolate, spontaneously produced from a B-cell line derived from the PBMC of a healthy individual with CIHHV-6B. Further, we developed an indicator cell line by lentiviral vector mediated stable integration of a plasmid with HIV-1 long terminal repeat (LTR) driven reporter. The reporter expression levels correlated with the relative concentrations of input HHV-6B isolate viral suspension.

2. Materials and methods

2.1. Materials

Peripheral blood was collected, with due consent, in EDTA containing tube from a 26 year old healthy Indian male (referred herein after as 'donor') with history of sustained periodic allergy like conditions with severe sneezing, runny nose, sore throat and headaches, itchy eyes with constant dryness and swelling of the tear glands, and generalized body itch like prickly heat. Blood was also collected from consenting healthy adult volunteers. PBMC were obtained by Ficoll-Hypaque centrifugation of

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http://dx.doi.org/10.1016/j.virusres.2013.02.006

phosphate buffered saline (PBS) diluted blood. Granulocytes were isolated by hypotonic lysis of red blood cells (RBC) from RBC-granulocyte pellet at the bottom of the centrifugation tube and purity of granulocyte preparations was checked by Giemsa staining. Genomic DNA from cells was isolated by standard detergent lysis and phenol–chloroform extraction method. DNA fragments were analyzed on agarose gel and ethidium bromide (EtBr) stained DNA bands were visualized and documented in a Gel documentation system (Alpha Innotech, USA). Cells were cultured in a humidified incubator at 37 °C with 5% CO_2 and cell lines were procured from National Center for Cell Sciences, Pune, if not indicated otherwise.

2.2. PCR and Southern hybridization

A nested PCR that amplifies a 496 bp amplified fragment in the first round and a 429 bp fragment in the nested round, specific for HHV-6B was used for detection of the viral genome in cellular DNA with all precautions to avoid PCR related contamination (Tailor et al., 2004; Wang et al., 1996). Nested PCR reactions, obtained using reduced template dilution of donor PBMC and granulocyte DNA, were resolved on 1.2% agarose gel and transferred to a nylon membrane (Hybond-N^+; Amersham, UK). Blotted membrane was hybridized overnight at 42 °C with a fluorescene-11-dUTP labeled oligo-probe mapping internal to the PCR product and hybridization signals were captured on X-ray film (X-OMAT; Kodak, USA). Probe labeling and hybridization were carried out using ECL 3′-oligo-labeling and detection system following manufacturer's instructions (Amersham, UK). A target sequence in the EBV capsid protein gp 220 genome was used for EBV PCR that amplifies a 239 bp fragment (Telenti et al., 1990). All oligo primers and probe used are shown in Supplementary Table S1A.Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2013.02.006.

2.3. Establishment of a cell line

A cell line was developed by infection of donor PBMC with the immortalizing strain of EBV harvested from cultures of the marmoset cell line B95-8 (Neitzel, 1986). Briefly, 3×10^6 PBMC were suspended in culture supernatant of B95-8 cell line (from Dr. N. Joshi) diluted 1:1 in RPMI 1640 medium (GIBCO-BRL, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, USA) and 50 μg/ml gentamycin (Nicholas-Piramal, India). Cells were washed in PBS and cultured at 1×10^6 cells/ml for 48 h followed by replacement of half culture medium with fresh medium every third day till distinct clusters of cells were seen. Growing cell populations were maintained for more than 4 months with intermittent freeze downs and phenotypically characterized by flow cytometry (FACS Caliber; BD, USA) and the cell line obtained was designated as PJH6.

2.4. Genomic DNA Southern hybridization and Gardella gel analysis

Aliquots of 10 μg genomic DNA from Jurkat (T-cell line), PJH6 cell line, B95-8 (B-cell line) and donor PBMC were digested with BamHI (New England Biolabs, USA), resolved in a 0.75% agarose gel and blotted on membrane. The blot was hybridized with random-primed ^{32}P-labeled ([α-^{32}P]-dCTP; BRIT, Mumbai) 6.9 kb BamHI fragment of HHV-6B obtained from pH6Z-101 plasmid (from Dr. Philip E. Pellet). Status of the viral genome in host cell was analyzed by resolving cellular DNA in Gardella gel followed by Southern hybridization using the same HHV-6B probe and the ~9 Kb of EBV sequence insert from the BamHI NJ het plasmid (from Dr. D. Saranath) as probe for EBV genome detection (Raab-Traub and Flynn, 1986).

2.5. Virus production and infection of cell lines and PBMC

PJH6 cells were seeded at 0.3×10^6/ml of FBS and antibiotic supplemented RPMI 1640 and cultured for 72 h. Cells were centrifuged at 400xg for 10 min, cell free supernatant was sterile filtered (0.45 μm; Millipore, India) and used for infection of target cells. 1.0×10^6 cells each from two lymphocytic cell lines SupTI (T cell line), Daudi (B cell line), and the monocytic cell line U937, were incubated with 1 ml of the cell free supernatant for 16 h. Cells were washed in PBS and further cultured for 48 h followed by DNA extraction. Additionally, cell free culture supernatant obtained from the infected SupT1 cells was also used to treat fresh SupT1 cells identically. PBMC were obtained from two healthy volunteers who were HHV-6B PCR negative and one lot of cells from each was stimulated with 10 μg/ml phytohemagglutinin (PHA-P; DIFCO, USA) for 48 h. Unstimulated cell fractions were washed in PBS and DNA was extracted. PHA stimulated cells were washed and further cultured for 72 h in medium supplemented with 20 units/ml recombinant IL-2 (Roche, USA) followed by DNA extraction.

2.6. Fluorescence in situ hybridization (FISH)

PHA-P stimulated PBMC from the donor and a healthy control were arrested in metaphase stage using Colchicine. Cells were given a hypotonic treatment, fixed and slides were made according to standard procedures. RNase and protease treated cell preparations on slide were denatured in 70% Formamide (prepared in 2× SSC) at 72 °C for 3 min, dehydrated and air-dried. Earlier mentioned, 6.9 kb HHV-6 specific probe was labeled by Fluorescein11-dUTP incorporation using random primed DNA labeling kit (Fluorescein Gene Images labeling kit, Amersham International, UK). 20 μl of hybridization solution (2× SSC, 50% Formamide, 10% Dextran sulphate, 100 μg/ml of Salmon sperm DNA) containing 20 ng of labeled probe was denatured at 75 °C for 5 min and snap cooled on ice. Denatured probe mixture was applied to slide having treated cell preparation, overlaid with coverslip and incubated overnight at 37 °C in a humidified chamber. The slides were washed twice for 5 min in a 50% Formamide in 2× SSC at 37 °C. Cells were counter stained with propidium iodide. Slides were observed under a Zeiss Axiolab microscope (Carl Zeiss A.G., Germany) and photomicrographs were taken on Kodak max 400 ASA film.

2.7. Inverse PCR (IPCR)

IPCR was carried out following established method with minor modifications (Arbuckle et al., 2010). Aliquots of PBMC genomic DNA from the donor and PJH6 cell line were digested with MboI at 1U/μg for five time points with 2 h intervals between 6 and 14 h. Reactions were heat inactivated and diluted in sterile water to 1 ng/μl. 100 ng of digested DNA from each sample was split into five aliquots each of 20 ng and self ligated with 1U of T4 DNA ligase for 14 h at 15 °C. Reactions were pooled and the DNA was alcohol precipitated. These DNA samples were used as templates for IPCR and distinct amplified fragments within 1.2–1.5 kb sizes were gel purified and cloned into a T/A vector (pTZ57R; Fermentas, Lithuania). The clones were sequenced by cycle sequencing method with fluorescent dye terminators using DNA sequencer (ABI PRISM 377-18; Applied Biosystems, USA) and subjected to BLAST analysis.

2.8. Electron microscopy

Cell free culture supernatant from PJH6 cell line was concentrated by ultracentrifugation at 16000 × *g* at 4 °C for 3 h followed

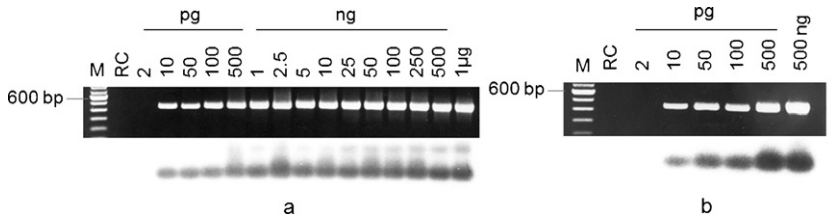

Fig. 1. Genomic DNA template dilution PCR for HHV-6. Nested PCR amplifications from the indicated amount of template DNA, EtBr stained gel (upper panel) and corresponding hybridization signals (lower panel). DNA from donor (a) PBMC and (b) granulocyte; M, Marker; RC, reagent control (no template DNA).

by re-suspension of the pellet in PBS to obtain a 400× virus stock. 50 μl of the stock was mixed with equal volume of gluteraldehyde (Sigma, USA), adsorbed on carbon coated grids, negatively stained with 1% phospo-tungstic acid and imaged under 100 KV operating voltage of a transmission electron microscope (Tecnai 12 Biotwin, FEI, The Netherlands).

2.9. Reporter construct and indicator cell line development

A 5.3 kb plasmid containing HIV-1 LTR-Luciferase-IRES-GFP expression cassette (from Dr. Debashis Mitra) was digested with HpaI (Ravi and Mitra, 2007). The linearized plasmid was cloned at the SmaI site of the multiple cloning site of a HIV-2 derived lentiviral vector (Santhosh et al., 2008). A clone obtained in reverse orientation with respect to the vector 5′LTR was used as the transfer vector (Suppl Fig. S4). Virus was prepared by multi plasmid transfection as described earlier followed by transduction of HEK 293 (Human embryonic kidney) cells (Santhosh et al., 2008). 48 h post transduction, cells were washed and then selected over a period of three weeks with 600 μg/ml G418 (Sigma) to obtain a stable reporter cell line.Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2013.02.006.

2.10. Virus precipitation and reporter assay

50 ml of cell free culture supernatant from PJH6 cell line was mixed with half volume of chilled 30% PEG 8000 (Sigma) solution containing 0.4 M sodium chloride and incubated overnight on ice. The suspension was centrifuged at 14,000 × *g* for 45 min, the supernatant carefully aspirated off and the pellet was thoroughly re-suspended in 1 ml of medium to make the virus stock (50×), which was used to make different dilutions of viral suspension in culture medium. Cells from the reporter cell line were suspended at 5×10^4 cells/ml in DMEM (GIBCO-BRL, USA) supplemented with FBS and antibiotic, dispensed at 100 μl/well of a 96 well flat bottom plate (Nunc, Denmark) and cultured for 14 h. Media were removed from the wells, 100 μl of virus suspensions of indicated concentrations were added and cultures incubated further for 16 h. Media from the wells were removed, 100 μl fresh medium was added in each well and cells were processed for Steady-Glo Luciferase assay following manufacturer's instructions (Promega, USA). Luminescence signal was detected using a microplate reader (Mithras LB-940; Berthold, Germany).

3. Results and discussion

3.1. HHV-6B producing cell line and non telomeric CIHHV-6B

During our earlier studies that involved HHV-6B detection by PCR, use of PBMC DNA template from a healthy laboratory volunteer, the 'donor', showed an unusually strong amplification (Joshi et al., 2000; Tailor et al., 2004). Subsequently using PBMC DNA from several healthy volunteers along with the donor DNA sample, we could detect the presence of the 496 bp first round PCR product only from the donor DNA by PCR hybridization (Suppl Fig. S1a). These preliminary observations indicated possible presence of high viral copies in the donor PBMC, either due to viral sequence presence in every cell or high viral load in some cells. We therefore probed further to understand the status of HHV-6 in the donor PBMC. The situation was addressed first by nested PCR on progressively reducing template DNA from donor PBMC that resulted in HHV-6B specific amplification from even 10 pg of template DNA (Fig. 1a). Considering a diploid cell contains ~7 pg of DNA, this result therefore implied that HHV-6 was possibly present in each of the donor PBMC. Granulocyte fraction from donor peripheral blood was purified (Suppl Fig. S1b) and similar PCR profile was also obtained from granulocyte derived DNA (Fig. 1b). Specificity of the nested PCR amplifications was confirmed by hybridization with labeled internal probe and the results suggested constitutive presence of the virus in this individual. EBV transformation of PBMC from the individual resulted in the single cell suspension cell line PJH6 that shows infrequent clumping in few hours after seeding and a doubling time of 30 h (data not shown). Absolute expression of cell surface pan B cell marker CD19 confirmed its B cell lineage with activated state shown by HLA-DR expression (Suppl Fig. S2). Distinct signals of comparable intensity in both the donor PBMC and the cell line DNA by genomic Southern hybridization further confirmed presence of very high level of viral DNA in both the cell fractions (Fig. 2a). The signal profiles were similar to that reported for the latent HHV-6B containing Katata cell line using the same probe (Bandobashi et al., 1997). No signal was obtained from the DNA of the T-cell line Jurkat and the B-cell line B95-8 confirming specificity of the hybridization. Gardella in situ gel analysis allows detection of linear and episomal forms of viral DNA on the basis

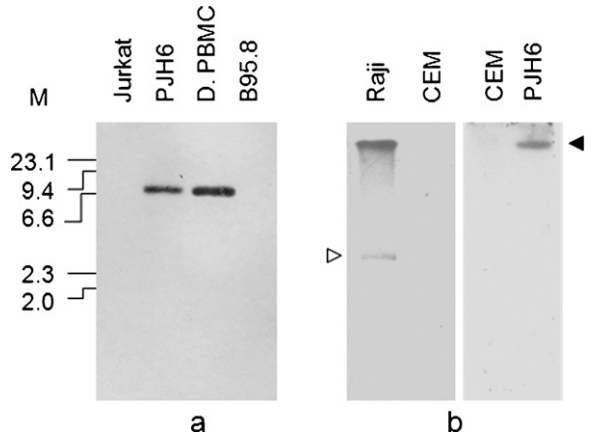

Fig. 2. Detection of HHV-6 DNA by Southern hybridization and Gardella gel analysis. (a) Southern hybridization signals in cellular DNA from indicated cell lines/PBMC, M: λ HindIII DNA size marker; D.PBMC: donor PBMC; (b) Gardella gel analysis. Membrane blotted with electrophoresed total genomic DNA from indicated cell lines was cut to two and hybridized with labeled EBV probe (left panel) and HHV-6 probe (right panel). (◄) indicates signal for integrated genome and (▷) indicates signal for episomal genome.

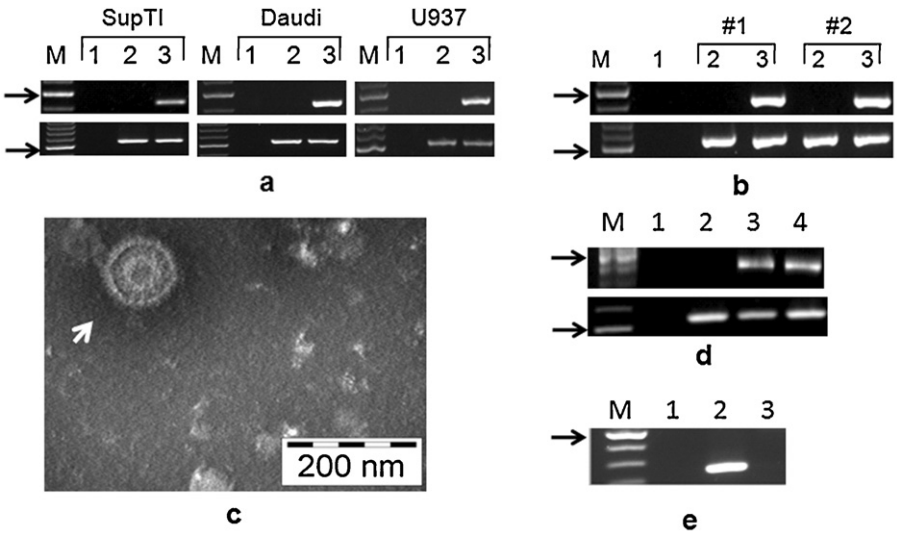

Fig. 3. Infectivity of the HHV-6 isolate and the virion ultra-structure. Nested PCR amplifications from cell line/PBMC derived DNA (upper panels); lane 1: RC, lane 2: uninfected cells, lane 3: infected cells, (a) infection of SupT1, Daudi and U937 cell lines; (b) infection of PBMC from two HHV-6 negative individuals (#1, #2); (c) electron micrograph of a virus particle (arrow) from clarified culture supernatant of PJH6 cell line; (d) productive infection of SupT1 cells by PJH6 isolate, lane 1: RC, lane 2: uninfected cells, lane 3: infected cells, lane 4: second lot of infected cells; (e) EBV PCR using cell line genomic DNA, lane 1: RC, lane 2: PJH6 cell line, lane 3: infected SupT1 cell line; M: 100 bp marker. Beta-globin gene PCR product (536 bp) from all corresponding samples with arrow indicating the 500 bp marker band (lower panels).

of their migration profiles (Decker et al., 1996). HHV-6B probe hybridization signal corresponding to the high molecular weight PJH6 genomic DNA detected only chromosomally integrated linear virus genome. Since the cell line produces infectious virus particles it should contain template concatemers and processed unit length HHV-6B viral genomes (Deng and Dewhurst, 1998). However, these genomic forms were not detectable in the Southern hybridization of the HHV-6B gardella gel though both linear and episomal forms of EBV were detectable in the Raji cell line. DNA from CEM, a T-cell line that harbors neither HHV-6 nor EBV did not show any hybridization (Fig. 2b). Sensitivity of the Southern hybridization was reported to be 1 pg of DNA using another human virus, HPV, (Tham et al., 1991). Lack of detection of the concatemers/unit length HHV-6B genomes in our hybridization experiment therefore could be due to the presence of low copy number of both the forms amounting to below the detection limit (Tham et al., 1991). An early FISH experiment using PBMC from the donor and another HHV-6 positive healthy individual showed detection of single fluorescent signal in the donor PBMC (Suppl Fig. S1c); PBMC from the other individual did not show any signal (data not shown). This preliminary observation led us to a complete characterization of the viral integration in the donor PBMC using the IPCR based approach (Arbuckle et al., 2010). The viral-chromosomal junction was mapped by sequencing the genomic DNA derived IPCR products from both the donor PBMC and PJH6 cell line. BLAST analysis of both the sequences showed the junction of the HHV-6 integration mapping to chromosome 12q14 confirming integration site of the PJH6 isolate (GenBank accession nos JX523610 and JX523611; Supp Fig. S3). All reports of CIHHV-6 to date document viral integrations in the telomeric region, possibly occurring due to the sequence homologies between the viral terminal direct repeat regions and the telomeric region (Arbuckle et al., 2010; Morissette and Flamand, 2010; Nacheva et al., 2008; Pellett et al., 2012). However, integration of this new HHV-6B isolate clearly occurred in a non telomeric region. Cloning and integration site mapping was repeated by two individuals with same results ascertaining this uncommon integration of HHV-6B genome in the host chromosome.Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2013.02.006.

3.2. Infectivity of the HHV-6B isolate

Three different lineages of HHV-6 negative mononuclear cell lines were treated with PJH6 culture derived supernatant and each cell line became HHV-6 PCR positive (Fig. 3a). Cloning and sequencing of the HHV-6B specific nested PCR products from the donor cell line and infected SupT1 cells showed absolute sequence match, which further confirmed infection of the target cells by the isolate from the producer cell line (data not shown). Usually PBMC are mitogen stimulated for infection with HHV-6 but we found that unstimulated (naïve) PBMC were infected as well as PHA stimulated ones from the two originally HHV-6 negative individuals (Fig. 3b). This might be an attribute to the higher virulence of this isolate. The PJH6 cell line therefore is a spontaneous producer of infectious HHV-6B. Electron microscopy also showed typical herpesvirus particle in the clarified culture supernatant of the cell line (Fig. 3c). Further, infected SupTI cells were also found to produce infectious virus that could infect fresh cells, indicating that this isolate imparts a productive infection to the target cells (Fig. 3d). The infected SupTI cells were EBV PCR negative confirming that the EBV transformed PJH6 cell line produced only HHV-6 (Fig. 3e). The cell line could be cultured to a cell density of 1×10^6 cells/ml without any obvious cell death (data not shown). To our knowledge this is the only infectious HHV-6B producing cell line derived by transformation of PBMC from a healthy individual with CIHHV-6.

3.3. A single step assay for relative HHV-6 titer estimation

For all the experiments reported involving infection of target cells, we routinely used 1 ml of PJH6 culture supernatant obtained after 72 h of culture with a fixed initial seeding since it showed HHV-6 PCR positivity in the target cells. However, an appreciation of viral inoculums per se was lacking. Virus titer estimation by target cell infection profiling based assays requires considerable time. Reporter gene based assays are sensitive and quick in comparison to $TCID_{50}$ determination or IFA based methods (Asada et al., 1989). Reporter based indicator cell lines have been described for some herpesviruses, but not for HHV-6 (Gilbert and Boivin, 2005; Inoue et al., 2003; Wang et al., 2006). We recently developed a single

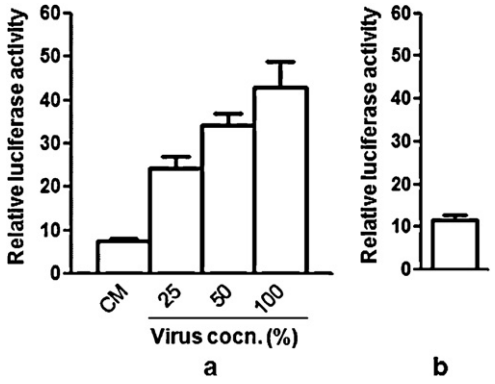

Fig. 4. Reporter bioassay. Luciferase reporter transactivation in presence of PEG precipitate derived suspensions. (a) CM, culture medium (RPMI 1640 with FBS/antibiotic supplementation) derived precipitate resuspended in 100 μl medium (50×), virus conc.: indicated concentrations of PJH6 viral culture precipitates, 100% represents undiluted suspension; (b) Daudi cell culture derived precipitate was resuspended in 100 μl medium (50×). Columns and error bars are mean ± sd ($n = 3$).

step assay for other purpose using the HIV-1 LTR transactivation driven luciferase reporter expression (Chande et al., 2012). Since HHV-6 is known to transcriptionally activate HIV-1 LTR (De Bolle et al., 2005; Dockrell, 2003), we developed a stable cell line with HIV-1 LTR driven luciferase reporter. The complete linearized plasmid harboring LTR driven reporter cassette was sub-cloned in the lentiviral vector multiple cloning sites (Suppl Fig. S4). Since the parental lentiviral transfer vector did not contain any antibiotic selection marker, cloning the whole plasmid with its neo selection cassette allowed G418 selection to obtain the indicator cell line. Increasing concentrations of viral suspensions showed luciferase reporter activity in a dose dependent manner (Fig. 4a). We also used identically obtained PEG precipitate derived neat suspension (50×) from cell free culture supernatant of Daudi cells, which are very low producer of EBV (Luka et al., 1979) and used the same as inoculums. Absence of appreciable reporter expression suggested that the dose dependent reporter activities in presence of PJH6 viral preparations were due to HHV-6 infection of indicator cells and not caused by any cellular metabolites/other agents present in the supernatant (Fig. 4b). Increasing the incubation time of viral suspension with indicator cells beyond 16 h did not increase the reporter expression appreciably (data not shown). Virus concentration by PEG precipitation is a simple procedure and addition of defined dilutions of virus preparation to indicator cells is the only experimental manipulation involved in this assay. Total time required from indicator cell seeding to assay completion is only 30 h. The indicator cell line, with reporter construct integrated through lentiviral vector, does not need any antibiotic selection and use of SteadyGlo substrate allows long term signal stability. This rapid method can be adapted to anti HHV-6 compound screening or serum neutralization antibody titer estimation apart from determining relative titer of HHV-6.

This study reports a new infectious HHV-6B isolate that is constitutively produced from a cell line with chromosomally integrated viral genome and also describes first time a single step reporter based HHV-6 titer assay, these will be valuable tools for further studies on this ubiquitous herpesvirus. The work also documents the first instance of HHV-6 integration in non-telomeric site of host chromosome and further investigations are needed to explain the uncommon situation. Investigations in otherwise healthy individuals with CIHHV-6, presenting episodes like chronic severe allergic bouts, as reported for the concerned donor, might be of clinical interest to further delineate HHV-6 pathobiology.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

R.M. conceived the study with inputs from P.T. and designed the experiments. P.G., P.T., A.G.C. performed experiments and A.B. performed electron microscopy. P.G., P.T., A.G.C., A.B., R.M. analyzed the data and P.G., P.T., A.G.C., R.M. wrote the paper.

Acknowledgements

The authors thank the following investigators for providing important research materials (as indicated). Dr. Phillip E. Pellet, Wayne State University, Michigan, USA (pH6Z-101 plasmid), Dr. Debashis Mitra, National Cell Science Centre, Pune (pLTR.Luc-IRES.EGFP plasmid), Dr. N. Joshi, ACTREC (B95-8 cell line) and Dr. D. Saranath of the former Cancer Research Institute, Mumbai (BamHI NJ het plasmid). The study was supported by Institutional Core Grant. PG and AGC were supported by ACTREC senior research fellowship.

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