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A gradient-free method for the purification of infective dengue virus for protein-level investigations

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Abstract

Dengue virus (DENV) is a mosquito-transmitted flavivirus that infects approximately 100 million people annually. Multi-day protocols for purification of DENV reduce the infective titer due to viral sensitivity to both temperature and pH. Herein we describe a 5-h protocol for the purification of all DENV serotypes, utilizing traditional gradient-free ultracentrifugation followed by selective virion precipitation. This protocol allows for the separation of DENV from contaminating proteins – including intact C6/36 densovirus, for the production of infective virus at high concentration for protein-level analysis.

Keywords

Dengue virus; Densovirus; *Aedes albopictus*; Purification; Precipitation; Infectivity

Dengue virus (family, *Flaviviridae*; genus, *Flavivirus*) is a mosquito-transmitted pathogen endemic to tropical and subtropical regions of the globe. Dengue virus (DENV) is the causative agent of Dengue Fever (DF), Dengue Hemorrhagic Fever (DHF), and Dengue Shock Syndrome (DSS) which conservatively affect 100 million people annually (Laughlin et al., 2012). Dengue virus and other flaviviruses are commonly studied after propagation in *Aedes albopictus* C6/36 cells, (Igarashi, 1978; Sakoonwatanyoo et al., 2006) and are purified for protein-level analysis; including the determination of protein–protein interactions (Muñoz et al., 1998), structural studies (Kuhn et al., 2002), and diagnosis (Peeling et al., 2010). Disease-relevant viruses known to infect the *Aedes* mosquitoes include DENV, West Nile virus (Colpitts et al., 2012), Zika virus (Grard et al., 2014), Yellow Fever virus (Hanley et al., 2013), and Chikungunya virus (Li et al., 2012).

Purification of DENV and other viruses propagated in C6/36 cells can be challenging due to a systemic co-infection of these cells by C6/36 densovirus, (family, *Parvoviridae*; sub-family, *Densovirinae*; genus, *Brevidensovirus*). C6/36 densovirus (DNV) constitutively infects C6/36 *Aedes albopictus* cells, causing no cytopathic effect in the host, thus allowing the infection to persist undetected (O'Neill et al., 1995; Chen et al., 2004). DNV can also be found in *Aedes aegypti* and *Aedes albopictus* mosquitoes (Kittayapong et al., 1999). DNV is frequently a contaminant in DENV samples and its prevalence is perpetuated by its stability:

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DNV remains infective after exposure to temperatures up to 65°C and pH 1–11 (Buchatsky, 1989). The presence of DNV in DENV-containing samples makes pertinent the need for purification of viral samples prior to analysis.

Traditionally, the first step of DENV purification is viral concentration. This is commonly performed using either polyethylene glycol (PEG) precipitation (Yamamoto and Alberts, 1970) or ultracentrifugation (Medina et al., 2012). Following concentration, virions are purified through a density, viscosity, or combination gradient, requiring fractional identification of the location of the viral proteins (Ashley and Caul, 1982). This process can take anywhere from 15 to 24 h to complete. However, long purification procedures ultimately reduce the infective titer due to instability of DENV at working temperatures, pH, and the use of multiple freeze-thaw cycles (Manning and Collins, 1979). Furthermore, structural studies of DENV have revealed an irreversible structural change of the viral capsid upon incubation at 37 °C (Fibriansah et al., 2013; Zhang et al., 2013) as well as both reversible (Yu et al., 2008; Zheng et al., 2014) and irreversible (Kuhn et al., 2002; Modis et al., 2004) pH – based structural modifications. Therefore, a simple purification scheme is optimal for preservation of viral structure and infectivity.

Work in our lab on the chemical modification of DENV led us to develop a straightforward purification of the virus. DNV was found to be a frequent contaminant in DENV viral samples in our lab, which necessitated a facile procedure for its separation from DENV (Fig. 1). We were led away from using traditional Tris-buffers, commonly employed for DENV suspension, due to incompatibility with our chemical probes. In doing so, we began to observe DENV precipitation under specific conditions. This precipitation was examined and optimized for successful purification of infective DENV. The following protocol is representative of this optimization.

Supernatant from C6/36 cells (ATCC, CRL-1660) infected by DENV-1 (virus obtained from BEI, NR-3782) in maintenance media (MEM, 2 mM L-glutamine, 1X NEAA, Pen-Strep, 2% FBS) was collected 6 days post-infection. Virus-containing media was clarified at 3200 rcf on an Eppendorf centrifuge (model 5810R) with a swinging bucket rotor (model A462) for 60 min at 4°C. The clarified supernatant (9 mL) was then layered over 3 mL of a 20% sucrose (w/v) solution prepared in nanopure water in ultracentrifuge tubes on ice (Fig. 2). Ultracentrifugation was carried out at 30,000 rpm, for 3 h at 4°C using a Beckman SW-40 Ti rotor (113,602 rcf). Following ultracentrifugation, the supernatant was quickly and carefully removed. Tubes were inverted to drip-dry for 20 min at room temperature. Pellets were often visible at this stage.

Following this time, pellets were quickly re-suspended in 100 mM HEPES buffer, pH 7.9 with 50 mM NaCl at 4°C. Viral suspensions were immediately centrifuged on a desktop Eppendorf microcentrifuge (model 5145c) at 16,000 rcf with a fixed angle rotor (model F-45-18-11) at room temperature for 10 min. The supernatant, containing pure DNV, was removed and stored, and the remaining visible viral pellet (DENV) was re-suspended in a lower-ionic strength experimental buffer of choice, (for example, 100 mM HEPES pH 7.9, or 10 mM Tris-Cl pH 8, 120 mM NaCl, 1 mM EDTA). The presence of purified DENV in the pellet of this preparation was confirmed by SDS-PAGE with coomassie staining (run

under standard conditions) as well as western blot (Figs. 3 and 4). Purified DENV samples were shown to be infective in C6/36 cells by indirect immunofluorescence assay (IFA), observed three days post infection (Fig. 5). Additionally, purified DENV was shown to be intact by transmission electron microscopy (TEM) (Fig. 6) and did not deviate from previously described structures (Kuhn et al., 2002). Yield of infective DENV from this purification method was analyzed using a TCID₅₀-IFA assay at 4 days post-infection. TCID₅₀ values were calculated by the Reed-Muench Method (Reed and Muench, 1938). The new purification scheme yielded a higher infective viral titer than that which was purified over an inverse density-viscosity gradient, a traditional purification method used by many in the field (Fig. 7) (Kuhn et al., 2002). The new purification method yielded a log(TCID₅₀/mL) value of 6.3 (+/-0.3) while the old method yielded a log(TCID₅₀/mL) value of 2.6 (+/-0.2). Both methods were compared to DENV that was not purified from cellular supernatant, which retained a log(TCID₅₀/mL) value of 8.8 (+/-0.2).

The dengovirus-containing supernatant was also analyzed by SDS-PAGE and coomassie staining (Fig. 3b). Due to the inaccessibility of antibodies for DNV, the identity of DNV was confirmed by tandem mass spectrometry (Fig. 8). Additionally, the presence of purified DNV was confirmed by TEM (Fig. 9), and showed no morphological differences from previously described structures (Chen et al., 2004).

In conclusion, DENV was successfully purified after propagation in *A. albopictus* C6/36 cells and was separated from DNV through a simple precipitation step. All 4 serotypes of DENV were purified by this method (Fig. 10). Because this method was translatable to all four serotypes of DENV, it may prove useful for the other fla-viviruses (such as West Nile, Yellow Fever, Chikungunya, and Zika viruses). Furthermore, the process of “salting in” and “salting out” viral capsids while maintaining infectivity is well documented as a phenomenon of viral crystallography, and was observed from the first viral crystallization of Tobacco Mosaic Virus (Stanley, 1935). That said, to the best of our knowledge, this method of purification has not been previously shown with flaviviruses such as DENV.

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This Work		Traditional Method

Clarification/ Ultracentrifugation Concentration Step	4 hours	Clarification/ Ultracentrifugation Concentration Step	4 hours
Resuspend virus in precipitation buffer ↓ Pellet virus for use	1 hour	Gradient - based Ultracentrifugation	>4 hours
		Fraction Analysis (Dot or Western Blot)	6 hours
		Fraction Concentration (eg. dialysis)	>1 hour
Total = 5 hours		Total > 15 hours	

Fig. 1.

The purification route presented in this work reduces the amount of time needed for DENV purification by 10 h.

Fig. 2.

A 20% sucrose cushion prepared for ultracentrifugation, 9 mL of infected media carefully layered over 3 mL of 20% (w/v) sucrose prepared in water.

Fig. 3.

(a) Purified DENV-1 examined by SDS-PAGE and coomassie staining (standard conditions). All three structural proteins (E – envelope, M – membrane, and C – capsid) and a dimer of the envelope protein (E*) were observed. The M and C proteins are of similar molecular weight and were not resolved. Very little of the immature membrane protein (prM – pre-membrane) was present. (b) Examination of the supernatant (sup.) and pellet resulting from “salting-out” DENV-1 followed by centrifugation of the precipitant. DENV-1 is present in the pellet, (lane 2) while DNV remains suspended in the precipitation buffer (lane 1) as observed by SDS-PAGE and coomassie staining.

Fig. 4.

Western blot analysis of the supernatant (sup.) and pellet resulting from DENV-1 purification. (a) Ponceau stain of proteins transferred to nitrocellulose. (b) Fluorescent scan for the observation of DENV-1, envelope protein. The western blot was performed using 1:5000 dilution of monoclonal anti-DENV-1 antibody, specific to domain III of the envelope protein, (mouse, BEI, #NR-4751). A goat anti-mouse IgG secondary antibody (labeled with AlexaFluor 633, Molecular Probes®) at 1:5000 dilution was used for band identification. After incubation, the blot was scanned with a ChemiDoc™ MP scanner (BioRad) using red epifluorescent excitation and a 695/55 nm bandpass filter.

Fig. 5.

Analysis of purified DENV-1 infectivity in *A. albopictus* C6/36 cells prepared by indirect immunofluorescence assay. Viral purification products (both supernatant and pellet) were used to infect C6/36 cells. Equal percentages of purified suspensions were added to C6/36 cells at 80% confluence in T25 flasks in maintenance media (MEM, 2 mM L-glutamine, 1X NEAA, Pen-Strep, 2% FBS). Infected cells were deposited onto a multi-well microscope slides three days post-infection and incubated in a humid chamber at 28°C for 20 min. After incubation, the slides were fixed through submersion in acetone at 4°C for 10 min. Slide were air-dried then re-hydrated in 1X PBS prior to incubation with monoclonal anti-DENV-1 antibodies at 1:200 dilution (Center for Disease Control, Mab D2-1F1-3) at 37°C for 30 min. The slides were washed twice with 1X PBS before secondary incubation with fluorescein isothiocyanate (FITC) – labeled goat anti-mouse IgG antibodies (Sigma) at 1:1000 dilution in 1X PBS (37°C for 30 min). Three final washes with 1X PBS preceded

examination with a Nikon C1si scanning confocal microscope using both bright field and epifluorescence microscopy.

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Fig. 6.

Transmission electron micrographs of purified DENV-1 particles. Viral particle structures vary in size from 40 nm to 55 and are primarily “smooth” in form. For TEM analysis, viral samples were incubated in a 2% formaldehyde solution for 30 min at room temperature, prior to absorption onto a 150 mesh formvar-nickel TEM grid (Electron Microscopy Sciences, catalogue number FF150-Ni). Grids were negatively stained with 2% phosphotungstic acid, then imaged using a FEI Tecnai™ Spirit transmission electron microscope.

Fig. 7.

Log(TCID50/mL) values determined from a TCID50-IFA. C6/36 cells were infected with equivalent dilutions of the following: clarified supernatant from infected cells (none), DENV-1 purified by the method reported in this work (new), and DENV-1 prepared through purification over an inverse density/viscosity ultracentrifugation gradient using 60-0% potassium tartrate and 0-30% glycerol (old). Infected cells were fixed and infection was confirmed using immunofluorescence at 4 days post infection (see Fig. 5 for details). TCID50 values were calculated using the Reed-Muench method.

gi|32265026|gb|AAP78909.1| (100%), 40,778.9 Da
 capsid protein [Aedes albopictus C6/36 cell densovirus]
 13 unique peptides, 21 unique spectra, 178 total spectra, 183/356 amino acids (51% coverage)

MADSTTMDHD	GRGTRKR	KREA	DGGSGQG	VGK	GNSNAVKEGY	GPNITEM	IPR
NIFNK	GNHTI	YHVVK	TQKYL	DFNYVTN	QNP	YIIPYQT	AGF
ESNQS	INIMK	ALNAVAL	LGVT	WIKGEIT	FEV	YSVTRQR	LLT
ETSON	MFIAD	ADREPEN	FNL	ATVAATG	PLA	QTTQTLL	LFN
LPQRN	QYTRE	INFQQLT	NNY	MWRPLD	ISAV	HNFRCL	IPMS
LRQTE	FTDET	TAYATSG	ATT	KQTLFR	NRTS	YPRMHL	AQPO
RYQVR	MSTKL	HLNFHMY	PDY	ATNNS	LEYMQ	RQTIQL	PQVA
YEINTQ						ATNGIVA	CMP

Fig. 8.

A map of protein coverage resulting from the Identification of the 40–42 kDa band as C6/36 densovirus, by tandem mass spectrometry. The putative densovirus sample was examined by SDS-PAGE and coomassie staining. A 40–42 kDa band was cut from the SDS-PAGE gel, and digested with trypsin for MS/MS. The spectra were collected using a LTQ Orbitrap LC–MS/MS, followed by analysis with Sequest (Thermo Fisher Scientific, version 1.3.0.339). Scaffold (version Scaffold 4.4.8, Proteome Software Inc.) was used to validate peptide and protein identifications. Peptide identifications were accepted at 98.0% probability with a false discovery rate less than 0.1%. Protein identifications were accepted with 100% probability if they contained 3 identified peptides. This figure was created using Scaffold software.

Fig. 9.

Densovirus particles examined by TEM, showing 20 nm particles, with and without genetic packaging. For TEM analysis, a suspension DNV in 100 mM HEPES pH 7.9, 50 mM NaCl was concentrated using a Nanosep[®] centrifugation device (Pall Life Sciences) with a 100 kDa cutoff, then re-suspended in 75 μ M ammonium acetate buffer. This suspension was then drop deposited on a 150 mesh formvar-nickel TEM grid (Electron Microscopy Sciences, catalogue number FF150-Ni) followed by negative staining with 2% phosphotungstic acid, and imaged using a FEI Tecnai[™] Spirit transmission electron microscope.

Fig. 10.

All four DENV serotypes were purified by the method described in this paper. Viral preparations were analyzed by SDS-PAGE and Western Blot. (a) Ponceau staining of nitrocellulose transfer indicates the presence of envelope proteins with slight variations in migration distance, as well as capsid, membrane, and pre-membrane proteins for each serotype. (b) Western blot against the envelope protein of DENV was performed using a 1:2000 dilution of a 1:1 mixture of BEI #NR-15515 and #NR-4756 (mouse). Followed by incubation with goat anti-mouse IgG secondary antibody (labeled with AlexaFluor 633, Molecular Probes®) at a 1:5000 dilution. The blot was scanned with a ChemiDoc™ MP scanner (BioRad) using red epifluorescent excitation and a 695/55 nm bandpass filter.