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Short communication

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

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A B S T R A C T

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.

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: 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, com-

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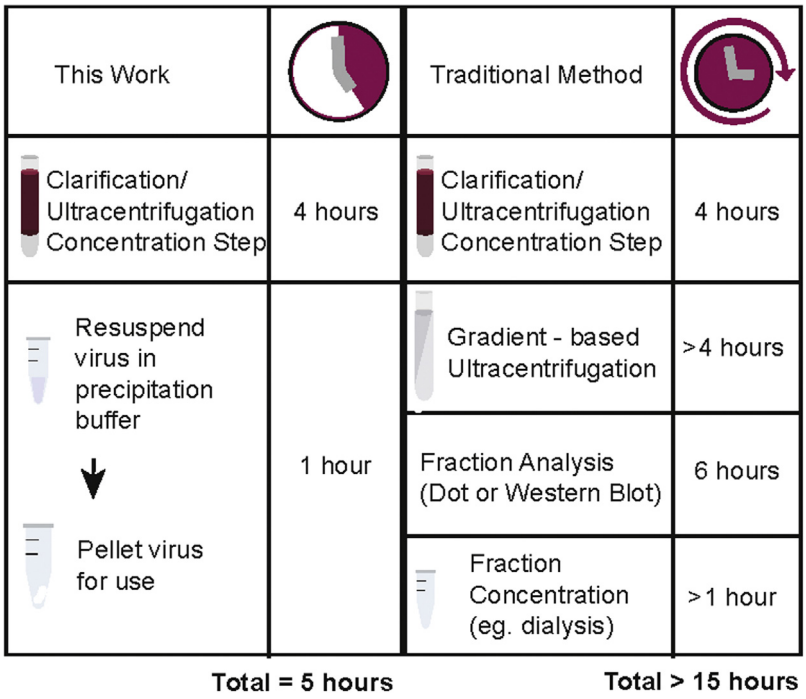

Fig. 1. The purification route presented in this work reduces the amount of time needed for DENV purification by 10 h.

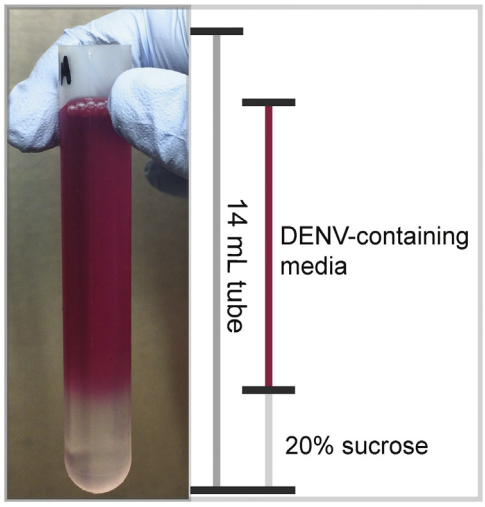

Fig. 2. A 20% sucrose cushion prepared for ultracentrifugation, 9 mL of infected media carefully layered over 3 mL of 20% (w/v) sucrose prepared in water.

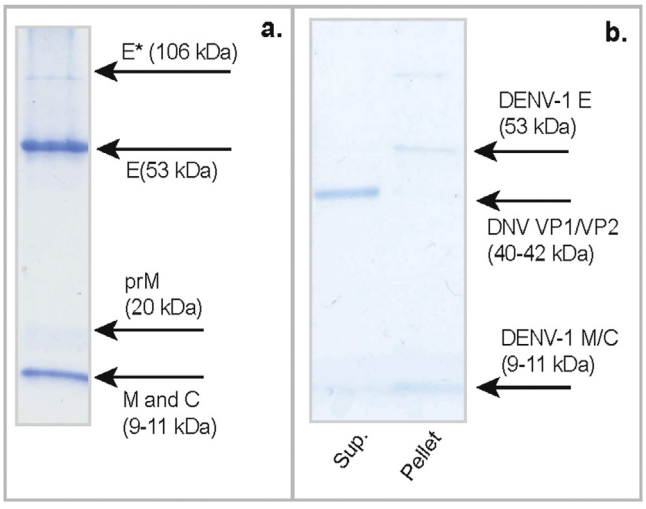

Fig. 3. (a) Purified DENV-1 examined by SDS-PAGE and coomassie staining (standard conditions). All three structural proteins (E – envelope, M – membrane, and C – capsid) and a dimer of the envelope protein (E*) were observed. The M and C proteins are of similar molecular weight and were not resolved. 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.

monly 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