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Development of an algorithm for production of inactivated arbovirus antigens in cell culture

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A B S T R A C T

Arboviruses are medically important pathogens that cause human disease ranging from a mild fever to encephalitis. Laboratory diagnosis is essential to differentiate arbovirus infections from other pathogens with similar clinical manifestations. The Arboviral Diseases Branch (ADB) reference laboratory at the CDC Division of Vector-Borne Diseases (DVBD) produces reference antigens used in serological assays such as the virus-specific immunoglobulin M antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA). Antigen production in cell culture has largely replaced the use of suckling mice; however, the methods are not directly transferable. The development of a cell culture antigen production algorithm for nine arboviruses from the three main arbovirus families, *Flaviviridae*, *Togaviridae*, and *Bunyaviridae*, is described here. Virus cell culture growth and harvest conditions were optimized, inactivation methods were evaluated, and concentration procedures were compared for each virus. Antigen performance was evaluated by the MAC-ELISA at each step of the procedure. The antigen production algorithm is a framework for standardization of methodology and quality control; however, a single antigen production protocol was not applicable to all arboviruses and needed to be optimized for each virus.

1. Introduction

There are over 500 arthropod-borne viruses, or arboviruses, geographically distributed throughout the world, over 150 of which cause disease in human and/or animal populations (Monath and Heinz, 1996; Burke and Monath, 2001; Gubler, 2002; Weaver, 2005; Cleton et al., 2012; Rosenberg et al., 2013; Centers for Disease Control and Prevention, 2014). Some arboviruses, such as dengue, Japanese encephalitis, and most recently chikungunya (CHIK) viruses, have wide geographical distribution and cause large seasonal epidemics (Powers et al., 2000; Staples et al., 2009). Others, such as West Nile virus, Zika virus and again, CHIK virus, are emerging or reemerging, and may cause sporadic outbreaks in regions in which they were not previously detected (Lanciotti et al., 1999; Solomon and Winter, 2004; Lanciotti et al., 2007, 2008). Other arboviruses, such as Powassan (POW), have low or unknown incidence, and may be detected due to emergence or increased surveillance (Ei Khoury et al., 2013).

Arbovirus infections may present with clinical symptoms similar to those of other bacterial or virological infections, such as an influenza-like illness, encephalitis, or polio-like myelitis (Burke and Monath, 2001). In addition, arboviruses within a serocomplex may cause similar disease syndromes and may be clinically indistinguishable from one another. Laboratory diagnosis is necessary to identify arbovirus infections and differentiate between other bacterial or viral pathogens, particularly if there is an effective treatment or vaccine available. The virus-specific immunoglobulin M (IgM) antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) can be used for rapid detection of acute arbovirus infections, as IgM antibody is produced early in infection, rises rapidly to detectable levels, and is less cross-reactive than IgG antibodies (Johnson et al., 2000; Martin et al., 2000; WHO, 2003; Wong et al., 2003, 2004; Johnson et al., 2005). The CDC Division of Vector-Borne Diseases (DVBD) Arboviral Diseases Branch (ADB) reference laboratory produces antigens for the MAC-ELISA for a wide array of arboviruses, most of which are not available commercially.

Viral antigen used in serological assays was previously generated from sucrose-acetone extracted suckling mouse brain (SMB) preparations. In order to reduce the use of animals, viral antigen production has shifted toward cell culture. This has necessitated

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modification and optimization of the methods previously used during SMB antigen production to cell culture, such as virus inactivation and concentration. The development of a cell culture antigen production algorithm for nine arboviruses from the three main arbovirus families, *Flaviviridae*, *Togaviridae*, and *Bunyaviridae*, is reported here. Cell culture conditions and inactivation and concentration procedures were optimized for each virus, using the MAC-ELISA as the performance indicator.

2. Materials and methods

2.1. Viruses

Yellow fever virus (YFV) strain 17D; St. Louis encephalitis virus (SLEV) strain TBH-28; Powassan virus (POWV) strain LB; Chikungunya virus (CHIKV) strains 181/25 and S27; Mayaro virus (MAYV) strain TR15537; Sindbis virus (SINV) strains EgAr 339, 16260, 80-2449, AUS C 263, AUS C 377, AUS MRM 39, INDA 1036, MAL AMM 2215, Michalovce, Reed Warbler, SAAR 86 and UGMP 684; La Crosse virus (LACV) strain Original; Jamestown Canyon virus (JCV) strains 61V-2235 and MN256-260; and Tahyna virus (TAHV) strain Bardos 92 were obtained from the Arbovirus Reference Collection at the Centers for Disease Control and Prevention (CDC), Division of Vector-Borne Diseases (DVBD) in Fort Collins, Colorado.

2.2. Tissue culture

Cell lines used in the growth curves were obtained at CDC DVBD. African green monkey kidney (Vero) cells, baby hamster kidney (BHK-21) clones 13 and 15 cells, rhesus monkey kidney (LLC-MK2) cells, and Vero clone E6 cells were maintained at 37 °C in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Grand Island, NY) with 8% fetal bovine serum (FBS, Atlas Biologicals, Fort Collins, CO), 1 mM sodium pyruvate (Life Technologies), 27 mM sodium bicarbonate (Life Technologies), 0.1 mM gentamicin (Lonza, Walkersville, MD), and 1 μM amphotericin B (Sigma–Aldrich, St. Louis, MO). *Aedes albopictus* mosquito C6/36 cells were maintained at 28 °C in DMEM (Life Technologies) with 10% FBS (Atlas Biologicals), 0.1 mM non-essential amino acids (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 9 mM sodium bicarbonate (Life Technologies), and 0.1 mM gentamicin (Lonza).

2.3. Growth curves

Growth curves were performed in T150-cm^2 cell culture flasks (Corning Inc. Life Sciences, Tewksbury, MA) at a multiplicity of infection (MOI) ranging from 0.0005 to 0.1 plaque-forming units (PFU)/cell. Following adsorption of virus in 10 ml of media at 37 °C for 1 h, cells were maintained in 60 ml of their respective media as described above, albeit with 2% FBS (Atlas Biologicals). At 24 h intervals, 0.5–1.0 ml of supernatant was removed and frozen at −70 °C until tested. Growth curves were carried out for 3–16 days until cytopathic effect (CPE) reached ~90–100%, or until the cells became overgrown in the negative control flask.

2.4. Virus titration

Virus titers were determined by 1% agarose double-overlay plaque titration assay in Vero cells, as previously described (Beaty et al., 1995). Plaques were visualized with second overlays applied with 0.005% neutral red (Sigma–Aldrich) following incubation for 2 days for CHIKV, MAYV, SINV, LACV, and TAHV; 3 days for JCV; 4 days for YFV; and 6 days for SLEV and POWV. Virus titers were recorded as $\log_{10}$ PFU/ml.

2.5. IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA)

Viral antigen activity was evaluated by the CDC MAC-ELISA, as previously described (Martin et al., 2000). Live virus or inactivated antigen was serially diluted twofold and reacted against both constant IgM positive and normal control sera, obtained from the DVBD diagnostic laboratory, except the SINV IgM positive control, for which no human sera was available. An alphavirus-group reactive mouse/human chimeric monoclonal antibody (cMAb) served as the SINV IgM positive control (Thibodeaux et al., 2011). Virus-specific antigen activity (VSAA) was defined as the optical density (OD) of viral antigen reacted against a constant positive control serum; acceptable VSAA had an OD of >0.8. Nonspecific background reactivity (NBR) was defined as the OD of viral antigen reacted against a constant normal control serum; acceptable NBR had an OD of <0.2. A satisfactory antigen was defined as that which had acceptable MAC-ELISA results, in which both the VSAA and NBR were within acceptable OD ranges; the highest antigen dilution with acceptable VSAA and NBR OD ranges was considered the working antigen dilution, and was a measure of functional antigen concentration.

2.6. Virus production for inactivation and concentration analyses

The optimal virus strain, cell type, and day of harvest were determined by the growth curves from one T150-cm^2 flask, after which a second batch was made in additional T150-cm^2 flask(s) under the optimized conditions. Supernatant was harvested, with volumes ranging from 60 to 500 ml, clarified at 2400 × *g* for 10 min at 4 °C, and stored at −70 °C with 20% FBS (Atlas Biologicals) until further analysis. Flaviviruses grow relatively slowly and it was possible to collect and replenish supernatant on multiple days from one flask. The harvests were then combined to make one batch.

2.7. Virus inactivation methods

2.7.1. Beta-propiolactone (BPL)

Cell culture supernatants were thawed in a 44 °C water bath with intermittent shaking, treated with BPL (CTC Organics, Atlanta, GA) at final concentrations ranging from 0.01% to 0.3%, and incubated for 24 h at 4 °C with moderate shaking on a refrigerated shaker plate. Mock-treated control supernatants (no addition of BPL) were incubated under the same conditions as the BPL-treated samples. Due to acidic BPL by-products, 7.5% sodium bicarbonate (Life Technologies) was added intermittently to adjust the pH (French and McKinney, 1964). Following BPL treatment the samples were stored at −70 °C until further analysis. For hydrolysis analysis, samples were treated with 0.05 or 0.3% BPL and incubated for 48 h at 4 °C with moderate shaking. Following BPL treatment, samples that underwent hydrolysis were incubated at 37 °C for 2 h, and then placed at −70 °C until further analysis.

2.7.2. Gamma-irradiation

Gamma-irradiation using a cobalt-60 source was carried out at the CDC irradiation facility in Atlanta, GA. Small volume aliquots of virus were irradiated with doses ranging from 1 to 6 Mrad in a "kill curve." All material was maintained frozen on dry ice throughout the treatment process. Untreated control supernatants remained frozen without any exposure to gamma-irradiation.

2.7.3. Gamma-irradiation + BPL

Virus supernatant was treated with 5.5–6 Mrad, thawed, concentrated 5× to 6× with Centricon Plus-70,100 kDa Centrifugal Filter Devices (Millipore), and then treated with BPL at final concentrations ranging from 0.01% to 0.1%.