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Production of a Sindbis/Eastern Equine Encephalitis chimeric virus inactivated cell culture antigen

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A B S T R A C T

Eastern Equine Encephalitis virus (EEEV) is a medically important pathogen that can cause severe encephalitis in humans, with mortality rates ranging from 30 to 80%. Unfortunately there are no antivirals or licensed vaccines available for human use, and laboratory diagnosis is essential to differentiate EEEV infection 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 EEEV immunoglobulin M antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA). However, EEEV is classified as a HHS select agent and requires biosafety level (BSL) three containment, limiting EEEV antigen production in non-select agent and BSL-2 laboratories. A recombinant Sindbis virus (SINV)/EEEV has been constructed for use under BSL-2 conditions and is not regulated as a select agent. Cell culture production of inactivated EEEV antigen from SINV/EEEV for use in the EEEV MAC-ELISA is reported here. Cell culture conditions and inactivation procedures were analyzed for SINV/EEEV using a recently developed antigen production algorithm, with the MAC-ELISA as the performance indicator.

1. Introduction

Eastern Equine Encephalitis virus (EEEV) is a medically important mosquito-borne human and equine pathogen in North and South America (Griffin, 2001; Wang et al., 2007; Weaver, 2001; Weaver et al., 1999). Primarily transmitted in an enzootic cycle between the mosquito vector *Culiseta melanura* and passerine birds in freshwater, hardwood swamp habitats (Brault et al., 1999; Villari et al., 1995; Wang et al., 2007; Weaver, 2001), transmission of EEEV can occur via bridge vectors to dead-end hosts, such as humans, horses, and other animals (Arrigo et al., 2008; Morris, 1988). There are licensed vaccines for equines; however, no antivirals or licensed vaccines are available for human use (Franklin et al., 2002; Wang et al., 2007). Personal protection from mosquito bites is the only effective prevention strategy during times of active transmission, and treatment options are very limited.

EEEV is a member of the family *Togaviridae*, genus *Alphavirus*, and has been classified into EEEV (formerly North American) and Madariaga virus (formerly South American) (Powers et al., 2012). Madariaga virus is not associated with severe human disease (Aguilar et al., 2007; Arrigo et al., 2008; Tsai et al., 2002; Wang et al., 2007; Weaver, 2001; Weaver et al., 1999); however, EEEV can cause severe encephalitis in humans. The mortality rate of clinical EEEV disease is 30–80% and up to 30% of patients who survive have long-term neurological sequelae (Johnson et al., 2011; Villari et al., 1995; Wang et al., 2007). Clinical signs and symptoms usually begin with high fever, headache, dizziness, and vomiting. Progression to severe encephalitis with coma and paralysis can occur by day 2 of the disease (Wang et al., 2007). Although large EEEV outbreaks have been reported, human infections are generally sporadic, with an average of six cases reported annually in the United States, primarily along the east coast (www.cdc.gov/easternequineencephalitis/Epi.html#map). Due to the sporadic nature of the disease and because clinical symptoms of EEEV infection may be similar to infections by other pathogens, laboratory-based diagnosis is necessary to identify individuals infected with EEEV and to implement prevention and control strategies (Arrigo et al., 2008).

EEEV infection is diagnosed in acute cases by virus isolation, detection of viral RNA in serum or cerebrospinal fluid, or serologically by detection of EEEV-specific immunoglobulin M (IgM)

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antibodies in an enzyme-linked immunosorbent assay (ELISA), with confirmation by the plaque reduction neutralization test (PRNT) (Beaty et al., 1995; Johnson et al., 2011; Lambert et al., 2003). The CDC Division of Vector-Borne Diseases (DVBD) Arboviral Diseases Branch (ADB) reference laboratory produces reagents for arbovirus diagnostics for which there are no commercial assays available, such as the EEEV IgM antibody-capture (MAC)-ELISA. Non-infectious antigens used in the MAC-ELISA are normally derived from live virus that has been inactivated, with the serological reactivity preserved. An algorithm for production of inactivated antigens from arboviruses grown in cell culture was recently developed and evaluated (Goodman et al., 2014). One method could not be used for all of the arboviruses, but rather needed to be optimized for each virus. The method used to inactivate the virus often had a significant effect on antigen reactivity, resulting in either antigen degradation or increased reactivity.

EEEV strain NJ-60 is the prototype virus used previously to produce EEEV antigen. EEEV strains require biosafety level (BSL) three containment and are classified as HHS select agents (www.selectagents.gov/Select%20Agents%20and%20Toxins%20List.html). Consequently, only select agent-registered laboratories with BSL-3 capacity are permitted to possess and work with EEEV strains. However, a recombinant Sindbis virus (SINV)/EEEV has been constructed in which genes expressing the EEEV immunogenic structural proteins have been inserted into the SINV backbone (Wang et al., 2007). SINV/EEEV can be used under BSL-2 conditions and is not regulated as a select agent. Previously, SINV/EEEV was shown to be a comparable alternative challenge virus for use in the PRNT by public health laboratories with BSL-2 facilities (Johnson et al., 2011). The use of SINV/EEEV to produce inactivated EEEV antigen needed to be assessed and is reported here. Of particular concern was the effect the inactivation procedure would have on antigen reactivity of the chimeric virus. Cell culture conditions and inactivation procedures were analyzed for SINV/EEEV using the previously developed antigen production algorithm, with the MAC-ELISA as the performance indicator (Goodman et al., 2014).

2. Materials and methods

2.1. Viruses

Sindbis/Eastern Equine Encephalitis (N. American) chimeric virus strain 796 (SINV/EEEV) was obtained from the University of Texas Medical Branch, Galveston, Texas (Wang et al., 2007).

2.2. Tissue culture

Cell lines used in the growth curves were obtained at CDC DVBD. African green monkey kidney (Vero) cells, and baby hamster kidney (BHK-21) clones 13 and 15 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 uM amphotericin B (Sigma–Aldrich, St. Louis, MO).

2.3. Growth curves

Growth curves were performed in T150 cm^2 cell culture flasks (Corning Inc. Life Sciences, Tewksbury, MA), as previously described (Goodman et al., 2014). Briefly, cells were infected at a multiplicity of infection (MOI) of 0.001 PFU/cell. Following adsorption of virus in 10 ml of media at 37 °C for 1 h, cells were maintained in 60 ml of media with 2% FBS (Atlas Biologicals). At 24 h intervals, 1.0 ml of supernatant was removed and stored at −70 °C until tested. Growth curves were carried out for 4 days until cytopathic effect (CPE) reached ~90–100%.

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. Virus titers were recorded as $\log_{10}$ PFU/ml.

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

The CDC MAC-ELISA was used, as previously described (Martin et al., 2000). Positive-to-negative (P/N) ratios were determined, where P was defined as the mean optical density (OD) of the positive control serum reacted on viral antigen and N was defined as the mean OD of the negative control serum reacted on viral antigen. Interpretation of test results were as follows: $P/N < 2$ = negative, P/N 2–3 = equivocal, $P/N > 3$ = positive. Additionally, for a test to be valid, the mean OD of the sample serum reacted on viral antigen had to be at least twice the mean OD of the sample serum reacted on normal cell culture or suckling mouse brain antigen.

2.6. Viral antigen activity

Viral antigen activity was evaluated by the CDC MAC-ELISA, as previously described (Martin et al., 2000; Goodman et al., 2014). EEEV IgM positive and normal control reference sera were obtained from the DVBD diagnostic laboratory. Briefly, untreated live virus or inactivated antigen was serially diluted two-fold and reacted against both constant EEEV IgM positive and normal control sera in the MAC-ELISA. 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.

2.7. Virus production for inactivation and concentration analyses

The optimal virus cell culture type and day of harvest were determined by the growth curves. A second batch was then made under the optimized conditions in four additional T150 cm^2 flask(s). Supernatant was harvested and clarified at 2400 × g for 10 min at 4 °C, and stored at −70 °C with 20% FBS (Atlas Biologicals) until further analysis.

2.8. Virus inactivation methods

2.8.1. Beta-propiolactone (BPL)

Virus cell culture supernatants were thawed in a 44 °C water bath with intermittent shaking. Aliquots of 15 ml were made and BPL (CTC Organics, Atlanta, GA) was added at final concentrations ranging from 0.1% to 0.3%. The BPL-treated aliquots were incubated for 24 h at 4 °C with moderate shaking on a refrigerated shaker plate. Mock-treated control virus 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,