Am. J. Trop. Med. Hyg., 80(5), 2009, pp. 862–863

Short Report: Comparative Thermostability of West Nile, St. Louis Encephalitis, and Western Equine Encephalomyelitis Viruses during Heat Inactivation for Serologic Diagnostics

Ying Fang, Aaron C. Brault, and William K. Reisen*

Center for Vectorborne Diseases and Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, California

Abstract. During the monitoring of arbovirus seroprevalence in wild birds collected in California, we inadvertently made two isolates of western equine encephalomyelitis virus (WEEV) from California quail sera being tested by plaque reduction neutralization assay for antibodies against St Louis encephalitis (SLEV) and West Nile (WNV) viruses despite heating the sera at 56°C for 30 minutes. These data prompted us to examine the thermostability of these viruses during heat treatment. The flaviviruses, SLEV and WNV, at titers up to 10^6 plaque-forming units (PFU), were readily inactivated by the standard protocol of heating at 56°C for 30 minutes. In contrast, solutions containing 10^5 and 10^6 PFU of WEEV required 2 hours for complete inactivation. Occasional presence of live virus within sera could lead to false negatives using standard plaque reduction neutralization test protocols.

The plaque reduction neutralization test (PRNT) is the "gold standard" for serologic diagnostics and relies on antibodies within serum samples to specifically neutralize target viruses. However, complement and adventitious viruses within sera can confound PRNTs and lead to false negatives. For this reason, protocols recommend that sera be heated to 56°C for 30 minutes to inactivate complement and remove confounding viruses,[1] especially when more than one virus may be circulating within the target population.

We have been monitoring the prevalence of antibodies against western equine encephalomyelitis virus (WEEV; *Togaviridae, Alphavirus*), St. Louis encephalitis virus (SLEV; *Flaviviridae, Flavivirus*), and West Nile virus (WNV; *Flaviviridae*, *Flavivirus*) in California birds for the past 10 years. In our standard protocol, sera initially are screened against WEEV or flavivirus antigen using an enzyme immunoassay (EIA).[2] If positive by EIA, confirmation is attempted and, in the case of the flavivirus positives, the virus is identified as either SLEV or WNV using an endpoint $PRNT_{90}$. Before serologic testing, sera are inactivated by heating in a water bath at 56°C for 30 minutes per standard procedures.[1] During August 2006, sera from two California quail (*Callipepla californica*) that had high EIA positive/negative (P/N) well ratios of 5.0 and 8.1 against SLEV antigen could not be confirmed as either WNV or SLEV by PRNT; P/N ratios against WEEV antigen were < 2.0. Cell monolayers of wells for the WNV and SLEV PRNT 6-well plates, regardless of dilution, were obscured by large plaques after 3 days at 37°C by a rapidly growing virus. This was especially evident for the SLEV PRNT given the relatively slow plaque formation by SLEV compared with WNV. At retest, 100 µL was cultured separately in Vero cells on 6-well plates and harvested, and an aliquot was tested by multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) for WNV, SLEV, and WEEV RNA. RT-PCR from these extractions resulted in TaqMan Ct scores of 15.7 and 16.6, correlating with the WEEV-specific probe, respectively, and values > 40 Ct for the WNV and SLEV probes, indicating that these quail had antibodies against WNV or SLEV but were acutely infected with WEEV when initially sampled and that these samples were not adequately heat inactivated by our standard protocol. Viremia titers after initial heat inactivation were estimated to be $10^{3.5}$ and $10^{2.3}$ plaque forming units (PFU)/0.1 mL, respectively, by Vero cell plaque assay. These results led us to evaluate the efficacy of the standard heat inactivation protocol for WEEV, SLEV, and WNV.

Stocks of WEEV (BFS1703 strain), SLEV (Kern217), and WNV (NY99) were diluted to ~10^6 PFU/0.1 mL, after which six 10-fold dilutions were prepared in diluent (phosphate buffered saline [PBS], 15% fetal bovine serum, antibiotics) and placed in duplicate in a water bath set at 56°C and in a control bath set at 4°C. These are the standard temperatures used for our heat inactivation protocol and for holding blood samples during viremia assays, respectively. Samples were removed at 30, 45, 60, 90, and 120 minutes, frozen at –80°C, and tested for infectious virus titer on Vero cell cultures.

All three viruses were stable at 4°C, showing minimal change in titer. In general, the flaviviruses SLEV and WNV were rapidly inactivated by the standard protocol of 56°C for 30 minutes (Figures 1A and 1B). A single plaque was detected in the WNV 10^6 PFU/0.1 mL group at 30 minutes, but none were detected at 45 minutes. In a similar preliminary study, 79 PFUs were detected for the SLEV 10^6 PFU/0.1 mL group at 30 minutes, but none were detected at 45 minutes (data not shown). In contrast, WEEV was remarkably stable at 56°C for 30 minutes (Figure 1C). Mean titers decreased ~10^2 PFU/0.1 mL during the first 30 minutes, resulting in failure to detect virus in the 10^1–10^3 PFU/0.1 mL groups. For the 10^6 group, 10^4 PFUs were detected after 30 minutes at 56°C, decreasing to 2,800 PFU after 45 minutes, 1,000 PFU after 60 minutes, 56 PFU after 90 minutes, and 1 PFU/0.1 mL after 120 minutes when the experiment was terminated. Similar slow inactivation was seen for the 10^5 and 10^4 groups (Figure 1C). Therefore, it was possible that the viremia titers of the quail at capture were $10^{5.5}$ and $10^{4.3}$ PFU/0.1 mL, respectively; within the range of peak viremia titers seen on Day 2 postinfection after experimental infection of adult California quail using the Kern5547 strain of WEEV.[3] In addition, after further heat inactivation for

*Address correspondence to William K. Reisen, Center for Vectorborne Diseases, University of California, Old Davis Road, Davis, CA 95616. E-mail: arbo123@pacbell.net

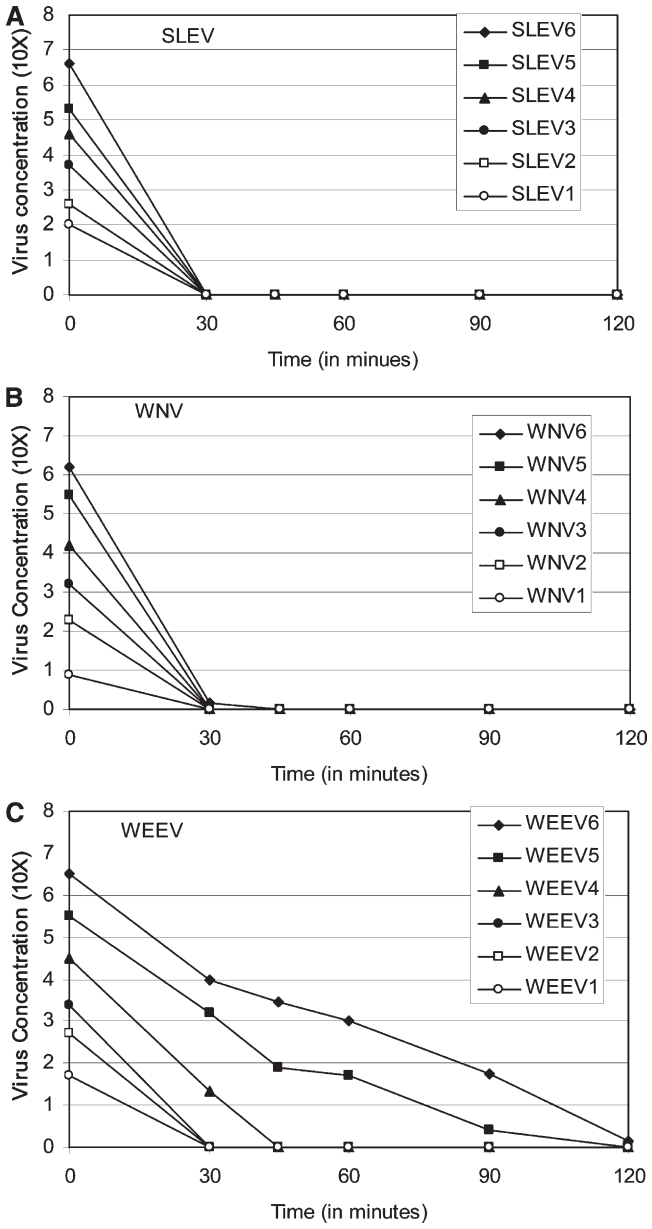

FIGURE 1. Mean virus concentration in PFUs of virus per 0.1 mL for 10-fold dilution series of (**A**) SLEV, (**B**) WNV, and (**C**) WEEV viruses plotted as a function of time immersed in a water bath maintained at 56°C.

1 hour, both sera presumptively positive for antibodies against SLEV by EIA were confirmed by PRNT to be caused by previous WNV infection, with endpoint titers of > 1:80 against WNV and < 1:20 against SLEV. In agreement, we recently reported that the presence of antibody caused by prior WNV infection does not alter the viremia response of birds after WEEV infection.[4]

Results of our experiment indicated that standard World Health Organization protocols were suitable for inactivating flaviviruses, but the current temperature experiment and the isolation of WEEV during PRNT assays performed with field-collected avian sera indicated that these inactivation parameters were not suitable for WEEV and perhaps other alphaviruses. For complete inactivation, samples possibly containing WEEV or other alphaviruses should be heated for > 90 minutes for safety and to preclude false-negative PRNT results. These unexpected WEEV isolations also emphasized the importance of running serum-only controls when doing PRNTs on field-collected sera.

Received November 18, 2008. Accepted for publication January 16, 2009.

Acknowledgment: Sandra Garcia provided technical assistance.

Financial support: This research was funded by Grant RO1-AI55607 from the National Institutes of Allergy and Infectious Diseases, NIH.

Authors' addresses: Ying Fang, Aaron C. Brault, and William K. Reisen, Center for Vectorborne Diseases, University of California, Old Davis Road, Davis, CA 95616.

REFERENCES

1. World Health Organization, 2007. *Guidelines for Plaque-Reduction Neutralization Testing of Human Antibodies to Dengue Viruses.* Geneva: World Health Organization.
2. Chiles RE, Reisen WK, 1998. A new enzyme immunoassay to detect antibodies to arboviruses in the blood of wild birds. *J Vector Ecol 23:* 123–135.
3. Reisen WK, Chiles RE, Martinez VM, Fang Y, Green EN, 2003. Experimental infection of California birds with western equine encephalomyelitis and St. Louis encephalitis viruses. *J Med Entomol 40:* 968–982.
4. Reisen WK, Fang Y, Brault AC. 2008. Limited interdecadal variation in mosquito (Diptera: Culicidae) and avian host competence for Western equine encephalomyelitis virus (Togaviridae: Alphavirus). *Am J Trop Med Hyg 78:* 681–686.