POOR REPLICATION OF WEST NILE VIRUS (NEW YORK 1999 STRAIN) IN THREE REPTILIAN AND ONE AMPHIBIAN SPECIES

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Abstract. Because West Nile (WN) virus primarily cycles between mosquitoes and birds, North American reptiles and amphibians have not been evaluated as reservoir hosts of this virus. We infected three species of reptiles and one species of amphibian: Iguana iguana (green iguana), Thamnophis sirtalis sirtalis (Florida garter snake), Trachymes scripta elegans (red-ear slider), and Rana catesbeiana (North American bullfrog). After inoculation with WN virus, some of the green iguanas, bullfrogs, and garter snakes showed low but detectable viral loads in the blood, oral or cloacal swabs, and/or organs.

INTRODUCTION

West Nile (WN) virus primarily cycles between adult ornithophilic mosquitoes and birds.1,2 This virus was first documented in North America in 1999,3 but involvement of neartic amphibia and reptiles in the ecology of WN virus is presently unknown. In Russia, WN virus was isolated from a lake frog (Rana ridibunda), and subsequent experiments demonstrated that these frogs develop high-titered viremia capable of infecting biting mosquitoes, suggesting that frogs could be important reservoir hosts for WN virus in Europe.4 However, WN virus studies with North American herpetofauna are lacking.

North American reptiles and amphibians may be competent amplification hosts for WN virus, and play important roles in WN virus transmission. To test this hypothesis, we infected four species of herpetofauna found in North America: Iguana iguana (green iguana), Thamnophis sirtalis sirtalis (Florida garter snake), Trachymes scripta elegans (red-ear slider), and Rana catesbeiana (North American bullfrog). We report on viremia profiles and tissue tropisms for these four species after infection by subcutaneous inoculation, and for green iguanas after mosquito-borne infection.

MATERIALS AND METHODS

Virus strains. We used the NY99-4132 strain of WN virus, passaged 3–4 times in Vero cells, that was isolated from crow brain provided by W. Stone (New York State Department of Environmental Conservation, Albany, NY).

Sources of animals and animal care. All animals were acquired through commercial sources, were housed as cohorts of two or three in plastic 58-quart containers with screen lids and had benefit of full-spectrum lighting and heat lamps 12 hours per day. Food and water was provided ad libitum. Species-specific diets were determined following suppliers recommendations or published guidelines.5,6

Mosquitoes and mosquito inoculation. Colonized 7–14-day-old Aedes aegypti DQ (DeQuincey, LA) mosquitoes were inoculated intrathoracically with approximately 0.35 μL of WN virus (containing approximately106.6 plaque-forming units [pfu]). Infected mosquitoes were placed in groups of five inside screened half-pint paper cartons and incubated in a 16:8 hour light:dark photoperiod at 28°C and a relative humidity of 80% for 8–11 days before feeding.

West Nile virus infection. We injected study animals with approximately 2,000 pfu of WN virus subcutaneously using a volume of 0.1 mL delivered via a 1 mL syringe attached to a 27-gauge subcutaneous needle. Some iguanas were infected by mosquito bite by holding each iguana against a carton containing infected mosquitoes. Each iguana was considered infected when at least one mosquito became engorged.

Specimen collection. Because the animals were small, blood samples were taken from anesthetized animals via cardiocentesis. Sampling individual animals for blood frequently (e.g., daily) was not practical due to the long anesthetic recovery time of ectothermic vertebrates.7 Instead of daily blood sampling, two or three individuals of each species were humanely killed with Beuthanasia-D (Schering-Plough Animal Health, Kenilworth, NJ) (0.1 mL/0.1 kg given intramuscularly) for collection of blood samples each day post-infection (pi) for seven days. Blood (0.2 mL) was sampled via cardiocentesis using a 27-gauge 0.5-inch needle and added to 0.9 mL of BA-1 diluent (Hanks’ M-199 salts, 1% bovine serum albumin, 350 mg/L of sodium bicarbonate, 100 units/mL of penicillin, 100 mg/L of streptomycin, 1 mg/L of Fungizone in 0.05 M Tris, pH 7.6), producing an approximate 1:10 serum dilution. Blood samples were centrifuged at 3,750 rpm for 10 minutes to separate serum from clotted blood, and stored at −70°C.

To obtain oral and cloacal swabs, cotton-tipped applicators were inserted into either the oral cavity or the cloaca of living animals and then placed in 0.5 mL of BA-1 diluent to release any virus. Samples were stored at −70°C.

Two or three individuals of each species were also killed 12–15 days pi. In addition, a group of five needle-inoculated iguanas and one uninfected cage-mate were killed at 28 days pi. Blood samples were obtained from these animals for detection of neutralizing antibodies and organ samples were removed from the carcasses. Six to 10 organ samples (approximately 0.5 cm3 in size) were harvested from each animal. These included heart, kidney, lung, liver, small intestine, gonad, eye, skin, brain, and spleen. Organ samples were ground in 2 mL of BA-1 diluent containing 20% fetal bovine serum, using TenBroeck glass tissue grinders (Bellco Glass, Inc., Vineland, NJ). Each resulting homogenate was transferred to a 1.7-mL Eppendorf (Brinkman Instruments, Westbury, NY) microcentrifuge tube and clarified by microcentrifugation at 7,500 rpm for 2 minutes. Each supernatant was transferred to a cryovial and stored at −70°C. Whole blood sampled post-mortem was centrifuged at 3,750 rpm for 10 minutes or separation of serum.

Plaque assay. One hundred microliters of each sample (for samples taken through day 7 pi) were added in duplicate to Vero cell monolayers in six-well plates (Costar, Cambridge,
Samples were allowed to incubate on the cells for one hour at 37°C. The cells were then overlaid with 3 mL per well of 0.5% agarose in M-199 medium supplemented with 350 mg/L of sodium bicarbonate, 29.2 mg/L of L-glutamine and antibiotics as in BA-1 diluent. After 48 hours of additional incubation, a 3-mL 0.5% agarose overlay containing 0.004% neutral red was added for plaque visualization. Plaques were counted on days 3 and 4 after infection of the Vero cells. BA-1 diluent was used as a cell viability control. Plaques from swabs and organ samples were harvested and confirmed as WN virus with a neutralization assay using a known source of antibody to WN virus.

**Neutralization assays.** For virus identification, 75 μL of the virus suspension (containing approximately 200 pfu/0.1 mL) were mixed in a polypropylene 96-well plate with 75 μL of WN hyperimmune mouse ascitic fluid (diluted 1:100 in BA-1 diluent) to produce a final WN virus antibody concentration of 1:200 and a final virus concentration of 100 pfu/0.1 mL. The virus-antibody mixtures were incubated at 37°C for one hour to allow virus neutralization, and then tested by plaque assay. As a negative antibody control, 75 μL of BA-1 diluent was also mixed with 75 μL of the virus suspension. A 90% reduction in the number of plaques in the test well compared with the negative control indicated a positive identification for WN virus.

For detection of neutralizing antibodies, 15 μL of post-mortem serum samples was mixed with 60 μL of BA-1 diluent and 75 μL of a WN virus preparation (200 pfu/0.1 mL) in a polypropylene 96-well plate. The virus-serum mixtures were incubated at 37°C for one hour to allow for virus neutralization. These mixtures were then tested by plaque assay. Controls used only BA-1 diluent (cell viability control), a serum-free virus mixture with BA-1 (to enumerate the number of pfu in the challenge dose of virus), and a WN hyperimmune mouse ascitic fluid (diluted 1:200) mixture with virus (to verify challenge virus identity).

### RESULTS

**Detection of viremia.** Forty-four green iguanas, 24 North American bullfrogs, 19 Florida garter snakes, and 22 red-eared sliders were experimentally infected with WN virus. By sampling three animals each day, we detected viremia in 10 green iguanas and 2 bullfrogs (Figure 1). Four green iguanas inoculated via needle and six inoculated via infected mosquito showed low viremias 1–4 days pi (maximum = 10^3.2 pfu/mL of serum). Only two bullfrogs had detectable viremias: one killed at one day pi (10^3.9 pfu/mL of serum) and one at three days pi (10^2.2 pfu/mL of serum). No garter snakes or red-ear snakes. Each of the three snakes tested had a WN virus-positive spleen. The maximum viral load in organs was 400 pfu/0.5 cm³ of tissue (found in a garter snake spleen).

**Detection of antibody to WN virus.** Two of five needle-inoculated green iguanas tested on day 28 pi had detectable WN virus-neutralizing antibodies (87% and 88% neutralization at a serum dilution of 1:10). One cage-mate iguana failed to seroconvert.

**DISCUSSION**

Ectothermic animals have been hypothesized to play some role in the transmission of arboviruses. Asian field studies have found antibodies to Japanese encephalitis (JE) virus, a close relative of WN virus, in cobra snakes (Naja naja) and in many species of turtles.8–10 Asian laboratory studies have demonstrated JE virus replication in lizards11–13 and snakes.14,15 Laboratory experiments in the United States have confirmed reservoir competence of North American garter snakes for western equine encephalitis virus,16–19 in one New York survey, one painted turtle (Chrysemys picta picta) and one leopard frog (Rana pipiens pipiens) circulated neutralizing antibodies to Saint Louis encephalitis virus.20

Due to the recent emergence of WN virus in North America, we investigated WN virus competence in North American reptilian and amphibian species by experimentally infecting three reptile species and one amphibian species. Although infectious virus particles were detected in the blood of some individuals, the viral titers were quite low. The threshold viremia for infecting Culex pipiens mosquitoes with the NY99 strain of WN virus is approximately 10^7 pfu/mL of serum.21 The maximum viremia that we detected was 10^2–3 pfu/mL of serum, approximately 50-fold lower than necessary for transmission to C. pipiens. These relatively low titers may be infectious to other species. Threshold viremias are unknown for mosquitoes that feed on reptiles and amphibians.

We detected WN virus-neutralizing antibodies in the serum of two of five green iguanas sampled 28 days pi. Cold-blooded vertebrates have relatively slow humoral response times, which may explain the low seroconversion rate. Some individual antibody levels may not peak for several weeks pi and may take months to decrease back to baseline levels.22,23

Although WN virus blood titers were low, infectious virus particles were also isolated from organs and oral or cloacal
swabs of some animals. Thus, some reptilian or amphibian species may contribute to the infection of a predator through oral transmission. Oral transmission of WN virus has been documented in mice and birds. However, the minimum dose required for oral infection of crows or other potential predators is unknown.

In summary, we present experimental infection data for WN virus in green iguanas, North American bullfrogs, Florida garter snakes, and red-eared sliders. Iguanas, bullfrogs, and snakes became infected, but their contribution to the WN virus transmission cycle in nature is yet to be proven.

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