Am. J. Trop. Med. Hyg., 78(4), 2008, pp. 666–668
Copyright © 2008 by The American Society of Tropical Medicine and Hygiene

Short Report: First Isolation of West Nile Virus in the Caribbean

Roberto Barrera,* Elizabeth Hunsperger, Jorge L. Muñoz-Jordán, Manuel Amador, Annette Diaz, Joshua Smith, Kovi Bessoff, Manuela Beltran, Edgardo Vergne, Mark Verduin, Amy Lambert, and Wellington Sun

Dengue Branch, DVBID, Centers for Disease Control and Prevention, 1324 Calle Cañada, San Juan, Puerto Rico 00920

Abstract. A sentinel chicken program for West Nile virus (WNV) surveillance was initiated in July 2006 in eastern Puerto Rico, yielding the first seroconversions on June 4, 2007. WNV was isolated from sentinel chicken serum and mosquito pools (*Culex nigripalpus*, *Culex bahamensis*) for the first time in Tropical America. Preliminary sequence analysis of the prM and E genes revealed a 1-amino acid difference (V159A) between the Puerto Rican 2007 and the NY99. This mutation has been observed in the current dominant clade circulating in the United States. Sentinel chicken surveillance was a useful tool for the detection of West Nile virus in the tropics.

WNV has been spreading southward into the Caribbean Basin and Latin America since 2001 when the first human case was reported from the Cayman Islands.[1] Serological evidence of WNV transmission has been accumulating, and cross-reactive WNV antibodies have been detected in humans from Mexico, The Bahamas, and Cuba, in horses from Guadalupe, Mexico, Central America, Cuba, Puerto Rico, Colombia, and Venezuela, and in resident birds from Jamaica, Dominican Republic, Cuba, Puerto Rico, and Venezuela.[2–6] In November 2006, 4 serologically confirmed human WNV encephalitis cases were reported by Argentina.[7] WNV may be following the same patterns of southward dissemination as other arboviruses into the Caribbean and Central and South America via migratory birds.[8]

In spite of growing evidence for the presence of WNV in tropical America, no outbreaks have yet been reported from the region. It is also interesting to note that high bird mortality, as observed in the Nearctic, has not been reported. WNV has been isolated in tropical America from a dead raven in Tabasco, Mexico (18° N lat.),[9] and in subtropical Argentina (23°49′ S lat.) from dead horses,[10] but no isolates have yet been reported from other areas in Central and South America or the Caribbean. One explanation for the paucity in WNV detection in tropical America may be the lack of active WNV surveillance. One would hypothesize that the likelihood of identifying or isolating WNV would be maximized in the Caribbean Basin, given its location in the flight paths of infected migratory birds. Another plausible explanation is that WNV has not been able to establish persistent enzootic foci in the region, so that reported serological evidence may be caused by frequent but unsuccessful virus re-introductions. Here, we report the first WNV isolation in the Caribbean, specifically from sentinel chicken serum and mosquitoes in natural and rural areas of eastern Puerto Rico.

To investigate the presence of WNV in Puerto Rico, a sentinel chicken program was established in July 2006 in Ceiba and Naguabo municipalities, near the place where a resident bird was previously found with WNV antibodies[3] and close to the area where the Puerto Rico passive surveillance system detected 3 asymptomatic horses with WNV antibodies in 2004.[11] The research protocol was approved by CDC's Animal Care and Use Committee (06-012). Sixty sentinel chickens were pretested for flavivirus reactive antibodies and placed in 12 pens, each housing 5 chickens. The 12 pens were placed in 8 natural habitats (2 herbaceous wetlands, 2 mangrove forests, 2 deciduous forests, and 2 evergreen forests) and in 4 nearby human populated areas (2 rural and 2 urban areas). Blood samples were taken from all sentinel chickens every 2 weeks and tested by a chicken-specific MAC-ELISA[12]; 1,247 blood samples were analyzed before initial seroconversion was detected (July–June 2006).

The first seroconversion of 7 chickens (12%) was detected in the sentinel chickens in 4 pens from natural areas (1 wetland, 1 mangrove forest, and 2 evergreen forests) in June 4, 2007. Following initial seroconversion, bleeding and sentinel replacement were performed weekly. Seroconversion increased to 40% on June 11, 2007, and was detected in chickens from 11 out of the 12 pens, covering all types of habitats including rural and urban areas. Chickens continued to seroconvert at high rates (max. 45%) throughout the rest of June and July, but the rate sharply declined during August 2007 (2%). Seroconversion has continued through October 2007 indicating a sustained low level of transmission (2–6%).

Plaque reduction neutralization tests (PRNT90)[13] against WNV and Saint Louis encephalitis virus (SLE) were used to confirm that the infecting virus was WNV (Table 1). Serum samples were heat inactivated at 56°C for 30 minutes and serially diluted 2-fold in phosphate-buffered saline (PBS) with 30% heat-inactivated fetal bovine serum. One hundred microliters of 1:20 diluted serum was mixed with an equal volume of diluents containing ChimeraVax WNV or SLE virus and analyzed in duplicate (Acambis, Cambridge, MA). One hundred microliters of the serum–virus suspension was used to inoculate a confluent monolayer of Vero cells in 12-well plates and incubated at room temperature for 1 hour. Two milliliters of medium containing 10% M199 without phenol red, 1% essential amino acids, 1% vitamins, 1% glutamine, 5% inactivated FBS, 0.4% gentamicin, 4% sodium bicarbonate, and 0.6% agarose was added to each well and placed in a 37°C 10% CO_2 incubator for 4 days for WNV and 5 days for SLE virus. Plaques were stained by adding 500 μL of PBS containing 3.2% neutral red to each well at 3 or 4 days post-infection for WNV and SLE virus, respectively. Plaques were counted 24 hours later, and endpoint titers were expressed as the reciprocal of serum dilutions yielding $\geq$ 90% reduction in the number of plaques. Controls included virus only and virus with normal serum.[13]

Chicken serum specimens that were collected a week prior to seroconversion were tested by RT-PCR to attempt virus isolation from these samples.[14] This led to positive identifi-

* Address correspondence to Roberto Barrera, Dengue Branch, Centers for Disease Control and Prevention, 1324 Calle Cañada, San Juan, Puerto Rico 00920. E-mail: rbarrera@cdc.gov

TABLE 1

Plaque reduction and neutralization test (PRNT) results of sentinel chickens from a variety of habitats in eastern Puerto Rico during the first 2 weeks of June 2007

Chicken ID, habitat	PRNT, 90% reduction titers		Interpretation
	WNV	SLE	
31, Evergreen forest	> 320	< 10	WNV
35, Evergreen forest	> 320	80	WNV
71, Mangrove forest	> 320	< 10	WNV
76, Herbaceous wetland	160	10	WNV
78, Herbaceous wetland	> 320	< 10	WNV
80, Deciduous forest	> 320	10	WNV
94, Herbaceous wetland	320	< 10	WNV
36, Rural area	> 320	10	WNV
45, Urban area	160	< 10	WNV
56, Urban area	> 320	< 10	WNV
88, Deciduous forest	> 320	10	WNV
16wh, Mangrove forest	320	< 10	WNV

The chicken serum samples listed were tested with WNV and SLE viruses, and endpoint titers at 90% reduction in plaques are provided. The endpoint titer represents the reciprocal of the dilution of serum that neutralizes the challenge inoculum by 90%. A 4-fold difference between the neutralization titer when comparing one virus to another indicates the infecting virus in the test. All samples were positive for WNV.

cation of WNV RNA in 1 of the sentinel chickens and subsequently WNV isolation from this chicken serum in Vero cells.

Mosquito captures were performed around the positive pen areas using CDC miniature light/CO_2 traps and CDC gravid traps. Captured mosquitoes were preserved on dry ice, sorted in pools (50 females per pool), and stored at −70°C until they were tested by RT-PCR.[15] The following mosquito species were initially found infected with WNV: *Culex nigripalpus* (51 positive out of 101 pools tested; 50.5%), *Culex bahamensis* (3/8; 37.5%), and *Culex quinquefasciatus* (4/46; 8.7%). WNV isolations were made in both C6/36 and Vero cells[16]: 4 from *Cx. nigripalpus* pools and 1 *Cx. bahamaensis*. Virus isolations were confirmed by RT-PCR and by specific immunostaining using West Nile/Kunjun 393 monoclonal antibody (CDC, catalog no. m28955A) and FITC-conjugated anti-mouse IgG (KPL, Inc., Gaithersburg, MD) in the presence of 4′,6 diamino-2-phenylindole, DAPI (Sigma, St. Louis, MO) according to previously published procedures in both cell lines.[16] Preliminary sequence analysis of the prM and E genes (GenBank library accession number: EU394703) revealed one amino acid difference (V159A) between Puerto Rican 2007 and NY99. This mutation has been observed in the current dominant clade circulating in the United States.[17] No clinical cases of humans or equines had been reported by the end of July. Enhanced surveillance for human WNV infection has been implemented.

These results demonstrate that WNV is actively circulating in Puerto Rico and that sentinel chickens and mosquito surveillance were useful tools for detecting ongoing WNV transmission in areas with pre-existing serologic evidence from a resident bird and horses. In spite of the efforts that were made to capture mosquitoes (June–July 2004) following the recovery of IgG antibodies in a resident bird (February 2004) and 3 horses (May 2004), no mosquito pool was found positive by *Taq*Man RT-PCR that year (CDC, unpublished data). It is likely that detection of WNV IgG antibodies was not an adequate indicator of ongoing or recent WNV transmission. However, WNV surveillance on resident birds and horses using IgG antibodies was useful to identify areas of previous virus transmission, and further surveillance using IgM antibodies in sentinel chickens was useful to detect active transmission at those sites. Although a passive WNV surveillance system on the islands had been in place since 2002 (Puerto Rico Department of Health and CDC's Dengue Branch; unpublished data), only 3 horses out of 4,370 specimens (from dead birds, free-ranging and domestic fowl, domestic porcine, equine, canine, caged primates, and humans) were found infected with WNV-reactive antibodies. Thus, we recommend sustained WNV surveillance with sentinel chickens in areas where the presence of this virus is suspected. Furthermore, human WNV surveillance in Puerto Rico, where dengue is endemic, will require careful interpretation of serologic tests and assiduous efforts to isolate the infecting virus. Our preliminary genetic analysis of the WNV Puerto Rico isolate from a sentinel chicken resulted in a 99.8% homology with NY99 strain, and further studies are being conducted to determine its pathogenesis.

Received October 10, 2007. Accepted for publication January 20, 2008.

Acknowledgments: We appreciate the outstanding field and laboratory work conducted by Yaitza Rosario, Juan Medina Sr., Jesús Flores, Juan Medina Jr., Orlando Gonzalez, Mary Crabtree, and Martin Williams. This work was possible thanks to the support of local residents and institutions where sentinel chickens were located in Ceiba and Naguabo municipalities.

Authors' addresses: Roberto Barrera, Elizabeth Hunsperger, Jorge L. Muñoz-Jordán, Manuel Amador, Annette Diaz, Joshua Smith, Kovi Bessoff, Manuela Beltran, Edgardo Vergne, Mark Verduin, Amy Lambert, and Wellington Sun, Dengue Branch, DVBID, Centers for Disease Control and Prevention, 1324 Calle Cañada, San Juan, Puerto Rico 00920, Telephone: +1 787-706-2399, Fax: +1 787-706-2496, E-mail: rbarrera@cdc.gov.

Reprint requests: Roberto Barrera, Dengue Branch, Centers for Disease Control and Prevention, 1324 Calle Cañada, San Juan, Puerto Rico 00920, Telephone: +1 787-706-2399, Fax: +1 787-706-2496, E-mail: rbarrera@cdc.gov.

REFERENCES

1. ProMED-mail, 2001. West Nile virus, humans; Cayman Islands. Archive no. 20011016.2538. Available at: http://www.promedmail.org.
2. Dupuis AP, Marra PP, Kramer LD, 2003. Serologic evidence of West Nile virus transmission, Jamaica, West Indies. *Emerg Infect Dis 9:* 860–863.
3. Dupuis AP, Marra PP, Reitsma R, Jones MJ, Louie KL, Kramer LD, 2005. Serologic evidence for West Nile Virus transmission in Puerto Rico and Cuba. *Am J Trop Med 73:* 474–476.
4. ProMED-mail, 2005. West Nile virus, humans, equines; Cuba. Archive no. 20050202.0355. Available at: http://www.promedmail.org.
5. Komar N, Clark GG, 2006. West Nile virus activity in Latin America and the Caribbean. *Pan Am J Public Health 19:* 112–117.
6. Bosch I, Herrera F, Navarro JC, Lentino M, Dupuis A, Maffei J, Jones M, Fernández E, Pérez N, Pérez-Emán J, Guimarães AÉ, Barrera R, Valero N, Ruiz J, Velásquez G, Martinez J, Comach G, Komar N, Spielman A, Kramer L, 2007. West Nile Virus, Venezuela. *Emerg Infect Dis 13:* 651–653.
7. Ministerio de Salud de la Nación, 2006. Buenos Aires, Argentina: Dirección de Epidemiologia, Ministerio de Salud, Republica Argentina. Accessed August 31, 2007. Available at: http://epi.minsal.cl/epi/html/Actualidad/internacional/VNO_casos_en_humanos.pdf.
8. Lord RD, Calisher CH, 1970. Further evidence of southward transport of arboviruses by migratory birds. *Am J Epidemiol 92:* 73–78.

9. Beasley DWC, Davis CT, Estrada-Franco J, Navarro-Lopez R, Campomanes-Cortes A, Tesh RB, Weaver SC, Barrett ADT, 2004. Genome sequence and attenuating mutations in West Nile Virus isolate from Mexico. *Emerg Infect Dis 10:* 2221–2224.
10. Morales MA, Barrandeguy M, Fabbri C, Garcia JB, Vissani A, Trono K, Gutierrez G, Pigretti S, Menchaca H, Garrido N, Taylor N, Fernandez F, Levis S, Enría D, 2006. West Nile virus isolation from equines in Argentina, 2006. *Emerg Infect Dis 12:* 1559–1561.
11. ProMED-mail, 2004. West Nile virus, equine; Puerto Rico (Fajardo). Archive no. 20040620.1644. Available at: http://www.promedmail.org.
12. Johnson AJ, Langevin S, Wolff KL, Komar N, 2003. Detection of anti-West Nile virus immunoglobulin M in chicken serum by an enzyme-linked immunosorbent assay. *J Clin Microbiol 41:* 2002–2007.
13. Russell PK, Nisalak A, Sukhavachana P, Vivona S, 1967. A plaque reduction test for dengue virus neutralizing antibodies. *J Immunol 99:* 291–296.
14. Langevin SA, Bunning M, Davis B, Komar N, 2001. Experimental infection of chickens as candidate sentinels for West Nile virus. *Emerg Infect Dis 7:* 726–729.
15. Lanciotti RS, Kerst AJ, 2001. Nucleic acid sequence-based amplification assays for rapid detection of West Nile and St. Louis encephalitis viruses. *J Clin Microbiol 39:* 4506–4513.
16. Gubler DJ, Kuno G, Sather GE, Velez M, Oliver A, 1984. Use of mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. *Am J Trop Med Hyg 33:* 158–165.
17. Davis CT, Ebel GD, Lanciotti RS, Brault AC, Guzman H, Siirin M, Lambert A, Parsons RE, Beasley DW, Novak RJ, Elizondo-Quiroga D, Green EN, Young DS, Stark LM, Drebot MA, Artsob H, Tesh RB, Kramer LD, Barrett ADT, 2005. Phylogenetic analysis of North American West Nile virus isolates, 2001–2004: evidence for the emergence of a dominant genotype. *Virology 342:* 252–265.