

DETECTION OF WEST NILE VIRUS–INFECTED MOSQUITOES AND SEROPOSITIVE JUVENILE BIRDS IN THE VICINITY OF VIRUS-POSITIVE DEAD BIRDS

ROGER S. NASCI, NICHOLAS KOMAR, ANTHONY A. MARFIN, GEORGE V. LUDWIG, LAURA D. KRAMER, THOMAS J. DANIELS, RICHARD C. FALCO, SCOTT R. CAMPBELL, KELSEY BROOKES, KRISTY L. GOTTFRIED, KRISTEN L. BURKHALTER, STEPHEN E. ASPEN, AMY J. KERST, ROBERT S. LANCIOTTI, AND CHESTER G. MOORE

Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado; Diagnostic Systems Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland; Arbovirus Laboratory, Wadsworth Center for Laboratories and Research, New York State Department of Health, Slingerlands, New York; Vector Ecology Laboratory, Louis Calder Center, Fordham University, Armonk, New York; Entomology Laboratory, Suffolk County Department of Health Services, Yaphank, New York

Abstract. Mosquitoes and wild birds were collected from three sites near locations in the New York City metropolitan area where single, West Nile (WN) virus–positive dead birds were found early in the 2000 transmission season. The mosquitoes were tested for the presence of infectious virus with a Vero cell culture assay and for WN viral RNA by using reverse transcriptase–polymerase chain reaction (RT-PCR) protocols. Serum samples from wild birds were tested for the presence of neutralizing antibodies against WN virus. Infectious WN virus and WN viral RNA were found in *Culex* species adult mosquitoes from each of the three sites, and a seropositive hatch-year house sparrow (*Passer domesticus*) was found in one of the three sites. Molecular techniques used to identify the species in the positive mosquito pools found that most of the pools contained a combination of *Culex pipiens* and *Cx. restuans*. The minimum infection rate in *Culex* species mosquitoes from the sites ranged from 0.2 to 6.0 per 1,000 specimens tested. The results demonstrated that, at least early in the transmission season, detection of a WN virus–positive dead bird indicates a local WN virus transmission cycle. This information is valuable in focusing subsequent surveillance and vector management programs. In addition, the RT-PCR procedure for detecting WN viral RNA in mosquito pools detected more positive pools than did the Vero cell plaque assay.

INTRODUCTION

During late August of 1999, an outbreak of human encephalitis was detected in the New York City area.¹ The etiologic agent was identified as West Nile (WN) virus,^{2,3} a member of the Japanese encephalitis virus complex of the genus *Flavivirus*, family *Flaviviridae*, which includes other mosquito-transmitted human pathogens such as Japanese encephalitis virus, St. Louis encephalitis (SLE) virus, Murray Valley encephalitis virus, and Kunjin viruses.⁴ Prior to 1999, WN virus was known only in the Old World, where disease outbreaks of fever and, occasionally, encephalitis had occurred in southern and northern Africa, the Middle East, southern Europe, and southern Asia. The WN virus is maintained in a zoonotic transmission cycle involving a variety of bird and mosquito species, which vary across the geographic range of the virus, much like the related native Western Hemisphere SLE virus.^{4,5}

Sixty-two laboratory-confirmed human cases occurred during the 1999 outbreak.⁶ Environmental investigations of the outbreak accumulated evidence of virus transmission among local mosquito and bird species.^{2,7,8} Remarkable among these observations were indications that WN virus caused mortality among birds, particularly American crows (*Corvus brachyrhynchos*) and other members of the family *Corvidae* (crows and jays).^{2,9} Widespread avian mortality resulting from natural WN virus infection appears to be a novel occurrence in nature related to the appearance of the virus in North America, and is not reported from the Old World home range of WN virus,¹⁰ though avian mortality can be demonstrated in the laboratory using old world strains of WN virus.¹¹

Demonstration of specific antibody against domestic arboviruses in serum samples from avian populations is commonly used as evidence of local transmission in surveillance programs in the United States.¹² Verification that WN virus was killing crows and other wild birds provided a potentially valuable and sensitive surveillance tool, unique among arbovirus

surveillance programs,⁹ since native North American arboviruses rarely kill native bird species. Surveillance using dead birds was actively promoted and incorporated into state and local WN virus surveillance programs throughout the eastern United States.¹³

After the 1999 outbreak, further investigations detected WN virus-infected mosquitoes and dead birds during the cold winter months of 2000.^{14–16} From May 25 through July 28, 2000, 26 WN virus-positive dead crows and blue jays (*Cyanocitta cristata*) were found scattered throughout five counties in New York and New Jersey, primarily in the New York City metropolitan area,¹⁷ prompting questions about the meaning of these early-season surveillance observations. This study was conducted to test the hypothesis that the presence of a single, WN virus-infected dead bird was indicative of local WN virus transmission. To test this hypothesis, mosquitoes and hatch-year resident birds collected in close proximity to the time and place where a WN virus-positive bird was found dead were tested for WN virus infection.

MATERIALS AND METHODS

Study sites and specimen collection. Three study sites were selected from among the locations where single, dead, WN virus-positive birds were collected during late June and early July 2000. Dead birds were reported by residents to the county or city health departments, collected by health department staff, and submitted for necropsy to the Wildlife Pathology Unit of the New York State Department of Environmental Conservation. Multiple tissues from each animal were then forwarded to the Arbovirus Laboratories at the Wadsworth Center of the New York State Department of Health for viral assay using a reverse transcriptase–polymerase chain reaction (RT-PCR) and two sets of primers/probes specific for WN virus.¹⁸ The sites were located in Babylon (Suffolk County), New Rochelle (Westchester County), and Floral Park

(Queens County), which are in the New York City metropolitan area, but not within the College Park area of Queens where human cases were clustered during 1999. Adult mosquitoes and wild birds were collected within 2–4 weeks of the date on which the bird was collected and within 3.2 km of the location where the bird was found. No additional WN virus-positive birds were collected near the sites between the time the original bird was reported and our collections were completed. The exact location (street address) where the dead bird was collected in each study site was obtained from the respective county's surveillance program. A dead, WN virus-positive American crow was found on June 25, 2000 at the Babylon site, and collections were performed from July 15 to July 17, 2000. The virus-positive American crow at the New Rochelle site was found on June 22, 2000 and collections were performed from July 10 to July 21, 2000. At the Floral Park site, a dead blue jay was found on July 5, 2000 and collections were performed from July 22 to July 24, 2000. The duration between collection of the dead bird and initiation of mosquito/bird sampling was due to the time required to test and confirm laboratory results on the dead birds.

Carbon dioxide-baited, Centers for Disease Control and Prevention (CDC), miniature light traps were used to collect host-seeking adult female mosquitoes, and CDC gravid traps were used to collect female mosquitoes that had previously taken a blood meal and were in search of a site to lay eggs.^{19,20} Wild birds were collected using mist nets, and were identified to species, sex, and age, tagged with appropriate leg bands, and a blood sample was collected by jugular venipuncture.⁸ Bird-collecting sites were situated near stables or barns where house sparrows (*Passer domesticus*) were plentiful. House sparrows were specifically targeted because they were implicated as potentially important reservoir hosts in 1999,⁸ and because large numbers of juvenile (hatch-year) individuals are easily collected in these locations and do not disperse far from the nesting area.

Mosquito processing. Mosquitoes were sorted by date and location of collection, and were identified to species when possible. The condition of certain morphologically similar *Culex* mosquitoes often prevented identification to species level. Morphologic characters essential for accurate species identification are often damaged through the collection and shipping process, and as a part of natural aging of the mosquito. As a result, many specimens were initially identified only to the level of genus, or to a species group (e.g., *Culex pipiens/restuans* category, which includes the morphologically similar *Cx. pipiens* and *Cx. restuans*, or *Culex* species category, which may contain *Cx. pipiens*, *Cx. restuans*, and/or *Cx. salinarius*).

The sorted mosquitoes were placed in 12 × 75-mm tubes in pools of 50 or fewer and shipped on dry ice to the Arbovirus Laboratory at the Wadsworth Center for Laboratories and Research of the New York State Department of Health for virus and WN viral RNA testing. Each pool was tested with a 5' nuclease RT-PCR assay using three sets of primer/probes targeting different regions of the WN virus RNA genome, and with a cell culture assay.²¹ The cell culture assay was conducted by inoculating a 150- μ L aliquot of clarified supernatant from the mosquito pool onto subconfluent Vero cell monolayers in T-25 flasks and observing daily for evidence of a cytopathic effect (CPE). When a CPE was observed, infected cells were spotted onto slides, fixed with acetone, and

stained with the WN virus monoclonal antibody H5.46 in an indirect immunofluorescence assay using goat anti-mouse IgM as the secondary antibody.

Specimens were considered WN virus positive by RT-PCR if they tested positive by at least two of the primer-probe sets. Specimens were considered WN virus positive by cell culture assay when positive immunofluorescence occurred.

Subsequent to virus testing, an aliquot of each mosquito pool containing evidence of infectious WN virus and/or WN viral RNA was sent to the CDC Division of Vector-Borne Infectious Diseases in Fort Collins, Colorado for further mosquito species identification. The pools were tested with a species-diagnostic PCR assay based on interspecific nucleic acid sequence variation in the first and second internal transcribed spacers (ITS 1 and ITS 2) of the nuclear ribosomal DNA gene array that can identify *Cx. pipiens*, *Cx. restuans*, and *Cx. salinarius* mosquitoes in combination or alone in a pool of 40 mosquitoes.²² The DNA was extracted from each mosquito pool homogenate and three different reaction mixtures were prepared for each DNA sample such that each reaction mixture contained one primer that binds to a conserved sequence at the 5' end of the 28S rDNA region and one of three primers that bind to species-specific sequences in the internal transcribed spacer regions. After amplification, mosquito species identification was determined by the presence of an appropriately sized amplicon from each reaction.

Bird serum processing. Serum samples were tested at the CDC Division of Vector-Borne Infectious Diseases laboratories for antibodies against WN and SLE viruses with a plaque-reduction neutralization test previously used for this purpose.⁸ The procedure used WN virus strain NY99-4132 (obtained from the brain of an American crow collected in New York in 1999) and the TBH-28 strain of SLE virus. Screening neutralization tests used a 1:10 serum dilution and Vero cells grown in six-well plates. Samples that contained evidence of flavivirus neutralization in initial screening tests were further tested in two-fold serial dilution series against both WN and SLE viruses to determine 90% neutralization titers for each of these closely related viruses. A four-fold greater titer to one of these viruses indicated that virus as the etiologic agent.

RESULTS

Mosquitoes. A total of 17,220 mosquitoes belonging to the genus *Culex* was collected from the three sites and tested for evidence of WN virus infection (Table 1). Only 50 individuals of other species, primarily *Aedes vexans*, were collected and tested from the sites. No virus was isolated from the non-*Culex* specimens and they were eliminated from further analysis. Evidence of WN virus was found in mosquito pools from each of the three sites. The number of pools from each site containing evidence of WN viral RNA or infectious virus varied depending on the detection system that was used. The RT-PCR detected more positive pools than did the cell culture assay procedure. The minimum infection rate (MIR) calculated using the number of pools containing evidence of WN viral RNA with the RT-PCR procedure was from two to six-fold greater than the MIR calculated using cell culture assay results.

Molecular identification of the mosquito species contained in the pools that were RT-PCR positive for WN viral RNA indicated that most consisted of a combination of *Cx. pipiens*

TABLE 1

Mosquito pools tested with RT-PCR and cell culture assay, indicating the number of specimens and pools tested and the number of pools producing positive results for infection with West Nile virus with each technique*

Site in New York	No. of specimens	No. of pools	Number of positive pools		Minimum infection rate‡ estimated from	
			RT-PCR	Cell culture assay	RT-PCR	Cell culture assay
Babylon	4,728	102	30	5	6.3	1.0
New Rochelle	6,150	123	4	2	0.6	0.3
Floral Park	6,342	128	2	1	0.3	0.2

* RT-PCR = reverse transcriptase-polymerase chain reaction.

† Includes all specimens belonging to the genus *Culex*.

‡ Minimum infection rate = number of positive pools per 1,000 specimens tested.

and *Cx. restuans* specimens (Table 2). Only two pools contained a single species.

Birds. One hatch-year house sparrow collected at the Floral Park site contained antibody against WN virus (Table 3) with a WN titer of 1:80. None of the hatch-year house sparrows at the Babylon and New Rochelle sites were WN seropositive. One specimen from Babylon was flavivirus seropositive, but neutralization titers of this sample (WN = 1:40, SLE = 1:20) did not indicate which flavivirus induced the antibody response. The percentage of other birds that contained neutralizing antibodies against WN virus ranged from 0% at the Babylon site to 33% at Floral Park. Of 26 WN-seropositive birds at Floral Park, 25 were adults, and the majority of these were house sparrows (Table 4).

DISCUSSION

All three of the sites investigated yielded WN virus-infected mosquitoes a short distance from sites where single WN virus-positive dead birds were found early in the 2000 transmission season. A seropositive hatch-year house sparrow was found at one of the three sites. These results demonstrate that, at least early in the transmission season, the presence of a single WN virus-positive dead bird is evidence of local virus transmission activity, and is probably not the result of a bird dispersing a great distance from the focus where it was infected. Such indicators of local transmission foci may be extremely valuable in focusing subsequent surveillance efforts²³ to identify sites where the risk of human illness may increase later in the transmission season.

Unfortunately, the presence of single, dead, virus-positive birds and high infection rates in ornithophilic vectors such as *Cx. pipiens* at a transmission focus early in the season are not reliable indicators of the level of virus transmission activity that may occur later in the season. The Babylon site, which

had the highest mosquito MIR of the three sites, experienced intense virus activity in birds, horses, and mosquitoes later in the season, but no clinical human cases were detected there.²⁴ The New Rochelle site, situated in Westchester County, had a mosquito MIR 10-fold lower than that of the Babylon site, but still experienced intense transmission later in the season, as indicated by the numerous virus-positive birds and mosquitoes collected in the area. The Floral Park site had a mosquito MIR similar to that found in New Rochelle and reported numerous WN virus-positive birds and mosquitoes later in the season. In addition, human cases were reported from the Borough of Queens, though not specifically from the Floral Park area.

West Nile virus transmission dynamics developing later in the season depend on numerous local factors such as mosquito abundance and species diversity, host availability, and habitat structure. In addition, transmission activity during the previous year may influence the intensity of transmission during the following season within a site, such as the Floral Park site, which experienced intense virus transmission in 1999.⁸ The extent and type of human exposure may influence epidemic risk. These factors, as well as the intensity of vector control activities that result from early-season detection of transmission, undoubtedly play a major role in the development of WN virus epizootic and epidemic transmission cycles.

These results also emphasize the importance of accurate mosquito species identification. It is likely that, because of differences in blood-feeding patterns,²⁵ *Cx. restuans* is less important than *Cx. pipiens* as an epidemic vector. If this is true, the inability to separate these species prevents the surveillance system from detecting situations that may lead to human cases.

The discrepancy in the number of mosquito pools containing evidence of WN virus between RT-PCR and cell culture assays is consistent with results from other WN virus surveillance programs.^{14,21,24} When initially used for screening mos-

TABLE 2

Identity of mosquito species contained in positive (for West Nile virus RNA) pools determined by molecular identification methods*

Site in New York	No. of positive pools	Mosquito species composition in RT-PCR-positive pools		
		Combined <i>Cx. pipiens/restuans</i>	<i>Cx. pipiens</i>	<i>Cx. restuans</i>
Babylon	30†	27	0	1
New Rochelle	4	4	0	0
Floral Park	2	1	1	0

* RT-PCR = reverse transcriptase-polymerase chain reaction; *Cx.* = *Culex*.

† Two pools had insufficient material for molecular species identification.

TABLE 3

West Nile virus neutralizing antibodies in hatch-year house sparrows and in all other bird species combined, including adult (after-hatch-year) house sparrows

Site in New York	Hatch-year house sparrows No. antibody positive/no. tested (%)	Other birds No. antibody positive/no. tested (%)
Babylon	0/76 (0)	0/47 (0)
New Rochelle	0/82 (0)	1/24 (4.1)*
Floral Park	1/85 (1.2)	25/75 (33.3)†

* The single positive bird was an adult mourning dove (*Zenaidura macroura*).

† All seropositive other birds at Floral Park were adults. See Table 4.

TABLE 4

Species of adult birds with West Nile virus neutralizing antibody at the Floral Park, New York site

Species	No. antibody positive/no. tested
House sparrow (<i>Passer domesticus</i>)	18/30
Northern cardinal (<i>Cardinalis cardinalis</i>)	3/3
Rock dove (<i>Columba livia</i>)	1/2
American robin (<i>Turdus migratorius</i>)	1/1
Gray catbird (<i>Dumetella carolinensis</i>)	1/1
Northern mockingbird (<i>Mimus polyglottus</i>)	1/1
Other adult birds	0/5

quito pools for WN virus in 1999, RT-PCR and Vero cell culture assays appeared to be of similar sensitivity.²⁶ Subsequent use on larger sample sizes and on overwintering mosquitoes suggests that RT-PCR is considerably more sensitive than standard cell culture assay in detecting evidence of WN virus in mosquito pools.^{14,21,27} Although we have not fully investigated the meaning of finding WN viral RNA in mosquito pools in the absence of detectable infectious virus, the ability to detect more positive pools suggests that RT-PCR is more sensitive than cell culture assays in detecting evidence of WN virus presence in the field. The important implication of this difference is that RT-PCR may increase our ability to detect WN virus activity early in the season when infection rates are very low.

The finding of numerous seropositive adult birds, in particular at the Floral Park site, was expected. The seroprevalence in adult birds at both the Floral Park and the New Rochelle sites closely matched the WN virus seroprevalence rates found in birds at or near these sites in 1999⁸ and may have been the result of 1999 infections. This observation emphasizes the importance of accurately determining the age of wild birds that are sampled for arbovirus surveillance. Only hatch-year birds should be considered when interpreting current infection rates of enzootic arboviruses, though the presence of acquired maternal antibodies in hatch-year birds may confound interpretation of data. However, house sparrow maternal antibodies against a related flavivirus, SLE virus, are undetectable in nestlings by 16 days of age,²⁸ and maternal antibody against WN virus is probably not detectable in fledged hatch-year house sparrows. A guide for accurately determining the age of most wild birds in North America is available.²⁹

Using infection rates in birds and mosquitoes provides quantitative measures of WN virus transmission that yields valuable information for surveillance programs.^{30,31} Estimation of human risk will depend upon adequate, sustained surveillance in areas where early season indicators such as those investigated in this paper show foci of virus transmission activity.

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Authors' addresses: Roger S. Nasci, Telephone: 970-221-6432, E-mail: rsn0@cdc.gov. Nicholas Komar, Anthony A. Marfin, Kelsey Brookes, Kristy L. Gottfried, Kristen L. Burkhalter, Stephen E. Aspen, Amy J. Kerst, Robert S. Lanciotti, and Chester G. Moore, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, P.O. Box 2087, Fort Collins, CO 80522, Telephone: 970-221-6400, Fax: 970-221-6476. George V. Ludwig, Diagnostic Systems Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, 1425 Porter Street, Frederick, MD 21702-5011, Telephone: 301-619-4941, Fax: 301-619-2492. Laura D. Kramer, Arbovirus Laboratory, Wadsworth Center for Laboratories and Research, New York State Department of Health, 5668 State Farm Road, Slingerlands, NY 12159, Telephone: 518-869-4524. Thomas J. Daniels and Richard C. Falco, Vector Ecology Laboratory, Louis Calder Center, Fordham University, 53 Whipoorwill Road, P.O. Box K, Armonk, NY 10504, Telephone: 914-273-3078, extension 33, Fax: 914-273-6346. Scott R. Campbell, Entomology Department, Suffolk County Department of Health, 335 Yaphank Avenue, Yaphank, NY 11980, Telephone: 631-852-4274.

Reprint requests: Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, P.O. Box 2087, Foothills Campus, Rampart Road, Fort Collins, CO 80522.

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