Isolation of West Nile Virus from Mosquitoes, Crows, and a Cooper's Hawk in Connecticut

John F. Anderson,[1*] Theodore G. Andreadis,[2*] Charles R. Vossbrinck,[2*] Shirley Tirrell,[3] Edward M. Wakem,[4] Richard A. French,[4] Antonio E. Garmendia,[4] Herbert J. Van Kruiningen[4]

West Nile (WN) virus, a mosquito-transmitted virus native to Africa, Asia, and Europe, was isolated from two species of mosquitoes, *Culex pipiens* and *Aedes vexans*, and from brain tissues of 28 American crows, *Corvus brachyrhynchos*, and one Cooper's hawk, *Accipiter cooperii*, in Connecticut. A portion of the genome of virus isolates from four different hosts was sequenced and analyzed by comparative phylogenetic analysis. Our isolates from Connecticut were similar to one another and most closely related to two WN isolates from Romania (2.8 and 3.6 percent difference). If established in North America, WN virus will likely have severe effects on human health and on the health of populations of birds.

An outbreak of arboviral encephalitis associated with mosquitoes was recognized in late August 1999 to be occurring in New York City (*1*). St. Louis encephalitis virus (SLE) was identified initially as the causative agent, but a Kunjin/WN-like virus was later reported to be the likely etiologic agent (*2*). We began trapping mosquitoes for the testing of viruses on 5 September 1999 (*3*). Traps were placed first in the field in Greenwich, Connecticut, a town located about 18 miles (29 km) northeast of Bronx county, New York City. They were placed in the adjacent town of Stamford on 9 September 1999, and in the following weeks, mosquito traps were placed in 12 additional towns in Fairfield County, Connecticut.

American crows, *Corvus brachyrhynchos* (*4*), were reported dying in Fairfield County, Connecticut, in the second week of September 1999. One crow was collected from Westport, Connecticut, on 13 September 1999 and was tested for virus. Subsequently, 30 additional dead crows from 18 additional towns in Fairfield and New Haven Counties and a Cooper's hawk, *Accipiter cooperii*, from the town of East Haven in New Haven County, were tested for virus. We report isolations of WN virus in the New World from two species of mosquitoes, American crows, and a Cooper's hawk.

A total of 1361 mosquitoes was collected and tested for virus by 14 October 1999 from Greenwich and Stamford, Connecticut, and 2037 additional mosquitoes were captured and tested from the other 12 towns sampled in Fairfield County (*5*). Virus was isolated from one pool of 12 *Culex pipiens* (Fig. 1) and one pool of six *Aedes vexans* collected the evening of 14 September 1999 at the Innis Arden Country Club located in the southern parts of both Greenwich and Stamford, Connecticut. Cell lysate antigen from both isolates reacted in an enzyme-linked immunosorbent assay (ELISA) with mouse antisera to SLE but not with antisera to species in the Togaviridae or Bunyaviridae (*6*). Titers to SLE mouse antisera were 1:320.

Virus was isolated from brain tissue of the dead crow collected from Westport, Connecticut, on 13 September 1999 (*7*). This bird had histopathologic evidence of encephalitis characterized by perivascular cuffs of mononuclear cells, predominately lymphocytes, and multifocal neuronal satellitosis and neuronophagia, consistent with viral encephalitis. Cell lysate antigen was prepared and found to react in an ELISA at a titer of 1:640 with mouse immune antisera to SLE (*6*).

Virus isolations were made from 27 of 30 additional crows that died in Fairfield and New Haven Counties, Connecticut, in September through 12 October 1999 (*8*), and from the brain of a Cooper's hawk (*9*). Crows died in Connecticut along a 62-mile (100-km) corridor from Greenwich on the New York border eastward to Madison, Connecticut, in towns bordering directly on Long Island Sound or inland by about 15 miles (24 km). The gross lesions in the crows consisted of subdural hemorrhage or coelomic hemorrhage, or both, and, in about one-third of the birds, emaciation and occasional fecal staining of feathers (suggestive of seizure activity). Microscopically, these crows had evidence of multifocal viral encephalitis. Cell lysate antigen from all isolates reacted in an ELISA at titers of ≥1:320 with mouse immune antisera to SLE. None reacted with the reference antisera to the other species of viruses tested (*6*).

A portion of the genome of virus isolates from four different hosts, *Ae. vexans* (isolate 2738, GenBank accession AF206517), *Cx. pipiens* (isolate 2741, GenBank accession AF206518), the crow from Westport (isolate 86814, GenBank accession AF206519), and the Cooper's hawk (isolate 86815, GenBank accession AF206520), was sequenced and analyzed genetically by comparative phylogenetic analysis (maximum likelihood, maximum parsimony, and neighbor joining) with PAUP 4b1 (*10*). The

[1]Department of Entomology, [2]Department of Soil and Water, the Connecticut Agricultural Experiment Station, Post Office Box 1106, New Haven, CT 06504, USA. [3]Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT 06512, USA. [4]Department of Pathobiology, University of Connecticut, 61 North Eagleville Road, Storrs, CT 06269, USA.

*To whom correspondence should be addressed. E-mail: john.f.anderson@po.state.ct.us, theodore.andreadis@po.state.ct.us, charles.vossbrinck@po.state.ct.us

four isolates were nearly identical in the 921–base pair region of the genome sequenced (*11*). The four isolates differed at three nucleotides located at 322, 620, and 662 base positions upstream (3′) from the end of the WN223F primer. While our isolates differed at these three base positions, the crow isolate (86814) differed from the Romanian WN 130362, Romanian WN 130363, Kunjin, Nigerian WN, Japanese encephalitis, and SLE viruses by 26, 33, 101, 176, 297, and 333 base positions, respectively. The Romanian WN isolates differed from each other at 41 base positions (4.5%). Our data suggest that the isolates from Connecticut are closely related to the Romanian WN 130362 isolate (2.8% difference) and less so to the Romanian WN 130363 isolate (3.6% difference).

The earlier study examining sequences from brain tissues of humans who died of encephalitis in New York City (*2*) identified the virus as a Kungin/WN–like virus. Their sequences were compared only with the WN isolate from Nigeria. The isolates from Romania were not included in this previous analysis. We conclude that our isolates from two species of mosquitoes, 28 American crows, and a Cooper's hawk are WN virus and are closely related to the WN virus isolates from Romania (Fig. 2).

A recent epidemic of neurological infections caused by WN virus occurred in Bucharest, Romania, and the lower Danube Valley of southeastern Europe in 1996 (*12*). The mosquito isolate WN 130362 from Romania had similar sequences for the relatively short regions analyzed for isolates from Senegal and Kenya (*12*). It is possible that our isolates, which are closely related to this Romanian isolate and cause neurological disease in birds and in humans (*1*, *2*), may have a similar origin. However, heterogeneity and dispersion by birds of WN virus are so extensive in Africa that strains are unrelated to geographical region (*13*).

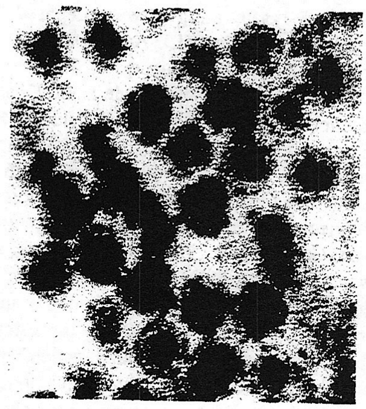

Fig. 1. Electron micrograph of West Nile virus. Vero cell isolation from *Culex pipiens* (isolate 2741). Magnification, ×210,000 (*22*).

WN virus was initially isolated in December 1937 from the blood of a mildly febrile woman living in Omogo, the West Nile district, Northern Province of Uganda (*14*). This virus is now documented to be the most widely distributed flavivirus and occurs in many parts of Africa, Asia, and Europe (*15*, *16*). WN virus was unknown previously in the New World.

Mosquitoes are the primary vectors of this virus. Experimental transmission of WN virus was first shown with *Aedes albopictus* (*17*). Subsequently, it was isolated from field-caught *Culex* mosquitoes in the Nile Delta of Egypt (*18*). At least 43 species have been shown to be naturally infected (*15*, *16*). *Cx. pipiens*, which is a competent vector (*19*, *20*) and from which we isolated WN virus, is considered to be one of the principal vectors in Europe (*16*) and possibly to be a reservoir for the virus during winter in Egypt (*18*). The other species of mosquito from which we isolated this virus, *Ae. vexans*, has been identified harboring WN virus in Senegal and in Russia (*16*). Our isolations of WN virus from *Cx. pipiens* and from birds confirm the presence of competent vectors in the United States.

Birds were initially documented to harbor WN virus in the Nile Delta (*21*). The virus was isolated from two rock pigeons, *Columba livia*, and a carrion crow, *Corvus corone*. Several bird species are now recognized to be likely reservoirs (*15*, *16*). Carrion crows and house sparrows, *Passer domesticus*, were shown to circulate virus at relatively high titers for at least 4 days (*20*). Our isolation of WN virus from birds and the presence of house sparrows and species of *Corvus*, which are likely capable of circulating this virus at relatively high titers, suggest to us that WN virus is likely to become established in the avian fauna of the United States.

WN virus has been reported to cause disease and death in Egyptian birds. This virus was isolated from the brain, spleen, and serum of a naturally infected young sick rock pigeon (*21*), and in laboratory experiments, all carrion crows and many house sparrows died after exposure to infected mosquitoes (*20*). Many carrion crows, however, survived infection in nature as shown by the relatively high percentage of immune birds after the peak of the transmission season (*20*). Our finding of WN virus in the brain tissue of dead American crows and a Cooper's hawk in Connecticut suggests that this virus also causes the death of North American birds.

Our isolation of WN virus from mosquitoes and birds conclusively documented the presence of this virus in Connecticut during September and October 1999. If established in North America, WN virus likely will continue to have severe effects on human health and on avian populations, such as American crows and raptors, which heretofore have never been exposed to this virus.

Note added in proof: We became aware of the manuscript of Lanciotti *et al.* (*23*) after we had submitted our paper. We believe the two manuscripts complement one another. The report of Lanciotti *et al.* is consistent with our data that virus isolates from mosquitoes and from dead birds in the northeastern United States are WN virus and responsible for the outbreak of encephalitis in humans in New York. While both groups reported similar relatedness of North American isolates to the mosquito isolate from Romania (AF130362), Lanciotti *et al.* documented a previously unreported isolate of WN virus (AF205882) from Israel to be nearly identical to their isolates from New York, Connecticut, and New Jersey. We did not have access to the sequence of this isolate because it was not listed with GenBank.

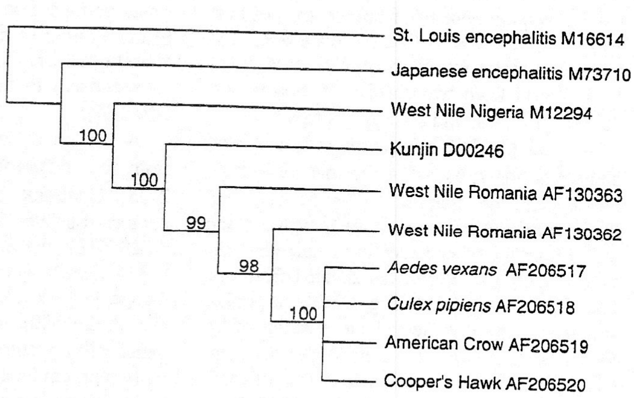

Fig. 2. Bootstrap analysis majority rule (70%) consensus tree (500 replicates) calculated by maximum parsimony analysis of four isolates from Connecticut with other members of the Japanese encephalitis group. Maximum likelihood and neighbor-joining analyses yielded identical tree topologies, suggesting a high degree of support for these relationships.

References and Notes

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3. T. G. Andreadis, J. F. Anderson, S. J. Tirrell-Peck, *J. Med. Entomol.* **35**, 296 (1998). Mosquitoes were captured in dry ice–baited Centers for Disease Control miniature light traps. One mosquito trap was placed at each location per night; the numbers of traps per site ranged from 1 to 6. Mosquitoes were transported alive to the laboratory where they were identified and grouped (pooled) according to species, collecting site, and date. Numbers of mosquitoes per pool ranged from 1 to 50. Mosquitoes were stored at −80°C until tested for virus. Upon thawing, mosquitoes were triturated in tissue grinders or mortars with pestles in 1 to 1.5 ml of phosphate-buffered saline (PBS) containing 0.5% gelatin, 30% rabbit serum, antibiotic, and antimycotic. After centrifugation for 10 min at 520*g*, 100-μl samples of each pool of mosquitoes were inoculated onto a monolayer of Vero cells growing in a 25-cm² flask at 37°C in

5% CO_2. Cells were examined for cytopathologic effect for up to 7 days after inoculation.
4. C. G. Sibley and B. L. Monroe Jr., *Distribution and Taxonomy of Birds of the World* (Yale Univ. Press, New Haven, CT, 1990). Common and scientific names of birds are used in accordance with those listed in this book.
5. Total numbers of mosquitoes by species that were collected in 14 towns in Fairfield County, CT, and tested for virus from 6 September through 14 October 1999: *Ae. vexans*, 1688; *Ae. cinereus*, 172; *Ae. trivittatus*, 131; *Ae. taeniorhynchus*, 123; *Ae. sollicitans*, 109; *Ae. cantator*, 63; *Ae. triseriatus*, 28; *Ae. japonicus*, 19; *Ae. canadensis*, 1; *Anopheles punctipennis*, 82; *An. quadrimaculatus*, 4; *An. walkeri*, 2; *Coquillettidia perturbans*, 15; *Culex pipiens*, 744; *Cx. restuans*, 27; *Cx. erraticus*, 4; *Cx. territans*, 1; *Culiseta melanura*, 76; *Cs. morsitans*, 1; *Psorophora ferox*, 4; and *Uranotaenia sapphirina*, 104.
6. M. Z. Ansari, R. E. Shope, S. Malik, *J. Clin. Lab. Anal.* 7, 230 (1993). Isolates were tested initially in an ELISA against reference antibodies to six viruses, in three families, isolated from mosquitoes in North America. The antibodies were prepared in mice and provided by the World Health Organization Center for Arbovirus Research and Reference, Yale Arbovirus Research Unit, Department of Epidemiology and Public Health, Yale University School of Medicine. The antibodies were to Eastern Equine Encephalomyelitis, Highlands J, Cache Valley, LaCrosse, Jamestown Canyon, and St. Louis Encephalitis viruses.
7. Most dead birds were collected by state or town personnel in Connecticut and sent to the Pathobiology Department at the University of Connecticut, Storrs, where they were examined for postmortem and nutritional condition, gross lesions, and microscopic evidence indicative of encephalitis. Brain tissue from birds with presumed encephalitis were frozen at −70°C and then sent to the Connecticut Agricultural Experiment Station, New Haven, for virus testing. Corresponding brain sections were processed for histologic examination. A 10% suspension of each sampled brain tissue was prepared in 1.5 ml of phosphate-buffered saline by triturating with a mortar and pestle (3). Two to seven tissue samples from each brain were tested for virus. Alundum was added to facilitate homogenization of tissue. Suspensions were centrifuged at 520*g* for 10 min. The supernatant of each sample was then passed through a 0.22-μm filter before inoculation of a 100-μl sample onto a monolayer of Vero cells. Cells were grown and examined for cytopathologic effect (3). Isolates were initially tested against reference antibodies (6).
8. Connecticut towns from which dead crows were collected and virus isolated from brain tissues (number of isolates in parentheses): Bridgeport ($n = 1$), Darien ($n = 1$), Fairfield ($n = 4$), Greenwich ($n = 3$), Hamden ($n = 1$), Madison ($n = 1$), Milford ($n = 1$), New Canaan ($n = 1$), New Haven ($n = 3$), North Haven ($n = 1$), Norwalk ($n = 1$), Redding ($n = 1$), Stamford ($n = 5$), Stratford ($n = 1$), Weston ($n = 1$), Westport ($n = 1$), and Woodbridge ($n = 1$).
9. The Cooper's hawk was observed alive on the ground on 25 September 1999 and was described as having difficulty standing, spinning in circles, and having seizures. It died 11 hours after being found. Gross pathology of the brain showed extensive hemorrhage.
10. D. L. Swofford, *PAUP: Phylogenetic Analysis Using Parsimony Users Manual* (Illinois Natural History Survey, Champaign, 1993). Data were analyzed by PAUP 4b.1 with maximum parsimony, maximum likelihood, and neighbor-joining analysis. The data set was identical for all analyses. A total of 933 characters was used, including insertions created during (Clustal X) alignment. All characters were unordered and had equal weight; all sites were assumed to evolve at the same rate. Four hundred and forty-six characters were constant, 281 characters were parsimony-uninformative, and 206 characters were parsimony-informative. Gaps were treated as missing. For maximum parsimony analysis, the best tree found = 754; number of trees retained = 1. The branch and bound method of search was used to guarantee finding the shortest tree (or trees). For the bootstrap analysis, 500 replicates were run with the maximum parsimony method. Maximum likelihood analysis settings corresponded to the Felsenstein model. Transition/transversion ratio = 2 (κ = 3.88125); molecular clock was not enforced; trees with approximate likelihoods of 5% or further from the target score were rejected without additional iteration; "MulTrees" option was in effect; topological constraints were not enforced. Score for best tree found by maximum likelihood analysis = 4278.24084; number of trees retained = 1. Trees were run as unrooted. Passage 2 of each virus isolate was grown in Vero cells (3) at 37°C. Infected cells were scraped from the bottom of the flask, centrifuged at 4500*g* for 10 min, and the supernatant was discarded. RNA was extracted from the pellet using the Rneasy mini protocol (Qiagen), eluting the column twice with 40 μl of ribonuclease-free water. Two microliters of each eluate were used in a 50-μl reverse transcription–polymerase chain reaction (RT-PCR) with the GeneAmp EZ rTth RNA PCR kit (Perkin-Elmer). Primers WN-233F-GACTGAAGAGGGCAATGTTGAGC and WN-1189R-GCAATAACTGCGGACYTCTGC used in the reaction were designed to specifically amplify WN and Kunjin viruses based on an alignment of six flavivirus isolates listed in GenBank [SLE virus capsid, membrane, envelope: accession M16614; Japanese encephalitis virus polyprotein: accession M73710; Kunjin virus gene for polyprotein: accession D00246; Nigerian WN virus complete genome: accession M12294; Romania WN virus strain R097-50 polyprotein gene, partial, accession AF130362; Romania WN virus strain 96-1030 polyprotein gene, accession AF130363]. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and submitted to the Keck Biotechnology Center at Yale University, New Haven, CT, for sequencing. Sequences were aligned with Clustalx 1.64B [J. D. Thompson, D. G. Higgins, T. J. Gibson, *Nucleic Acids Res.* 22, 4673 (1994)].
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22. Cells were fixed at 4°C in a 2.5% (v/v) glutaraldehyde–2% paraformaldehyde solution containing 0.1% (w/v) $CaCl_2$ and 1% (w/v) sucrose in 100 mM Na cacodylate buffer (pH 7.4), postfixed in 1% (w/v) OsO_4, dehydrated through an ethanol and acetone series, and embedded in an LX-112–Araldite mixture. Thin sections were poststained with 5% (w/v) uranyl acetate in 50% (v/v) methanol followed by Reynold's lead citrate and examined in a Zeiss EM 10C electron microscope at an accelerating voltage of 80 kV. Virus particles measured 35 to 40 nm.
23. R. S. Lanciotti *et al.*, *Science* 286, 2333 (1999).
24. We thank J. Correia, J. Shepard, M. Vasil, B. Hamid, C. Scott, and T. Blevins for technical assistance. P. Mazik, P. Mazik, R. Wagner, P. Lucas, T. Capanella, R. Nieves, and S. Nieves helped with the collection of mosquitoes in Greenwich and Stamford. K. Hannon and R. Schaper provided the brain from the Cooper's hawk. Supported in part by Hatch Grant 763 and NIH grant P01-AI-30548.

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