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West Nile Virus: A Reemerging Global Pathogen

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Guest Editor,
Series on West Nile virus

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Dr. Roehrig is chief of the Arbovirus Diseases Branch, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention. His research interests focus on the immunology of vector-borne viral diseases; protein biochemistry; and specific disease interests—equine and human encephalitides, dengue fever, and rubella.

The recognition of West Nile (WN) virus in the Western Hemisphere in the summer of 1999 marked the first introduction in recent history of an Old World flavivirus into the New World (1,2). The United States is not alone, however, in reporting new or heightened activity in humans and other animals, and incursions of flaviviruses into new areas are likely to continue through increasing global commerce and travel. Similar expansion of other flaviviruses has been documented. Dengue viruses, perhaps the most important human flaviviral pathogens, have spread from roots in Asia to all tropical regions (3-5). Japanese encephalitis (JE) virus has recently encroached on the northern shores of Australia and may soon become endemic in that continent (6-9). This issue of Emerging Infectious Diseases focuses on current understanding of the biology, ecology, and epidemiology of WN virus.

WN virus, a member of the family Flaviviridae (genus *Flavivirus*) (10), was first isolated in 1937 in the West Nile district of Uganda (11). Flaviviruses have a 30- to 35-nm icosahedral core composed of multiple copies of a 12-kDa capsid protein. The capsid encloses a single-stranded, positive-sense RNA of approximately 12,000 nucleotides (Figure 1). The capsid is enclosed in a host cell-derived envelope that has been modified by the insertion of two integral membrane glycoproteins, E (53 kDa) and prM (18-20 kDa). The virion is 45 nm to 50 nm in diameter (Figure 2). Late in virus maturation, the prM protein is cleaved to M protein (8 kDa) by a cellular protease, and the M protein is incorporated into the mature virion. The genome also encodes seven nonstructural proteins (NS1, NS2a, NS2a, NS3, NS4a, NS4b, and NS5) that make up the intracellular replication machinery of the virus. E-glycoprotein, the most immunologically important structural protein, is the viral hemagglutinin

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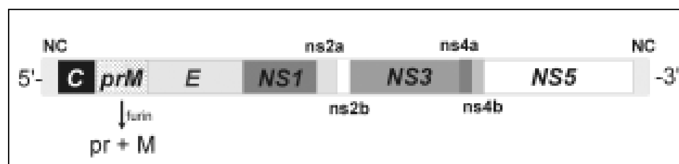

Figure 1. Genomic structure of flaviviruses. The flavivirus genome is 11,000 to 12,000 nucleotides long. Both the 5'- and 3'- ends contain noncoding (NC) regions. The genome encodes 10 proteins, 3 of which are structural proteins (C, M, and E), and 7 of which are nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). The M protein is synthesized as a precursor (prM) protein. The prM protein is processed to pr + M protein late in the virus maturation by a convertase enzyme (furin).

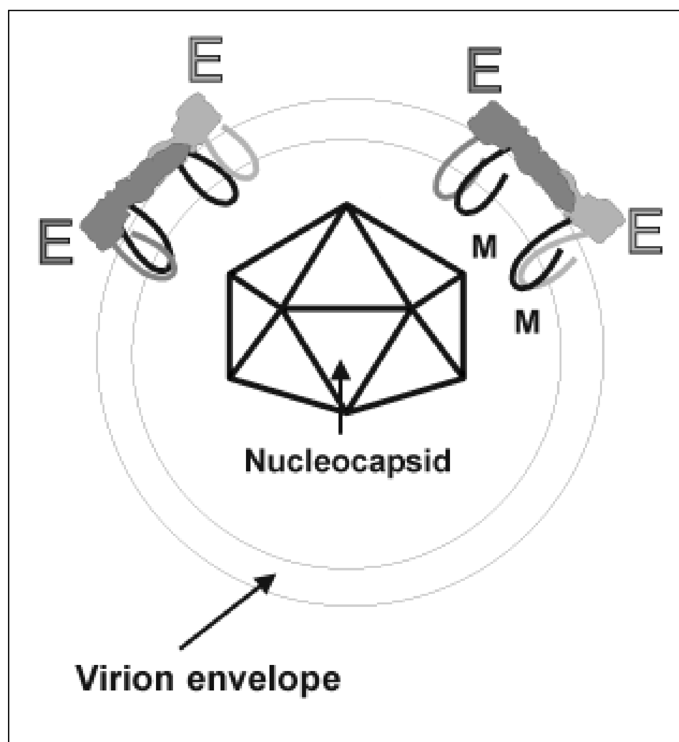

Figure 2. Diagram of the flavivirus virion. An icosahedral nucleocapsid (half shown here) encloses the virion RNA. The virion has an envelope derived from the host cell membranes. E-glycoprotein (E), an integral membrane protein, is arranged as homodimers (head-to-tail) and associates with the other integral membrane proteins prM protein (in immature virions).

and also mediates virus-host cell binding. It elicits most of the virus neutralizing antibodies. WN virus is a member of the JE virus serocomplex (Table) (12), which contains a number of viruses also associated with human encephalitis: JE, St. Louis encephalitis (SLE), Murray Valley encephalitis, and Kunjin (a subtype of WN). All flaviviruses are closely related antigenically, which accounts for the serologic cross-reactions observed in the diagnostic laboratory. Members of the JE complex are even more closely related, often needing specialized tests (e.g., virus neutralization assays) to differentiate the infecting flavivirus (13). Because of the close antigenic relationships between the flaviviruses, acute- and convalescent-phase serum specimens from patients are required to fully assess antibody response. A useful outgrowth of the recent WN virus activity has been the development, standardization, and implementation of rapid techniques for antibody and virus detection (14-16). These rapid, sensitive techniques permitted identification of overwintering mosquitoes in New York City in 2000 and two human WN encephalitis cases in Israel in 1999 (17,18).

Since the original isolation of WN virus, outbreaks have occurred infrequently in humans, those in Israel (1951-1954 and 1957) and South Africa (1974) being most notable. Since the mid-1990s, however, three disturbing epidemiologic trends for WN virus have emerged: 1) increase in frequency of outbreaks in humans and horses (Romania 1996; Morocco 1996; Tunisia 1997; Italy 1998; Russia, the United States, and Israel 1999; and Israel, France, and the United States 2000)(19-23); 2) apparent increase in severe human disease (2,19,20,22,24,25) (confirmed human infections in recent outbreaks: Romania, 393 cases; Russia [Volgograd], 942 cases; United States, 62 cases in 1999 and 21 in 2000; Israel, 2 cases in 1999 and 417 in 2000); and 3) high avian death rates accompanying the human outbreaks, in outbreaks in Israel and the United States.

Recent outbreaks of WN virus have been accompanied by an apparent evolution of a new WN virus variant. WN virus can be divided genetically into two lineages (26-29). Only members of lineage 1 WN viruses have been associated with clinical human encephalitis (the lineage of the WN virus causing the human outbreak in South Africa in 1974 is under contention). Lineage 1 WN viruses have been isolated from Africa, India, Europe, Asia, and North America. In addition, Kunjin virus, an apparent subtype of lineage 1 WN viruses, cocirculates in Australia with a second encephalitis virus member of the JE virus complex, Murray Valley encephalitis virus (26). Lineage 2 WN viruses are maintained in enzootic foci in Africa and have not been associated with clinical human encephalitis. Among lineage 1 WN viruses, the viruses causing the recent human and equine outbreaks throughout Europe and Asia have been most closely related to a WN virus first isolated in Romania in 1996 (ROM96) and subsequently in Kenya in 1998 (25,30,31). The WN virus responsible for the U.S. outbreak (NY99) is genetically distinguishable from the ROM96-like viruses. The closest relative of NY99 virus was a virus circulating in Israel from 1997 to 2000 (Isr98). Only the United States and Israel have reported illness and death in humans and animals caused by this Isr98/NY99 variant of WN virus (18,28). The reason for this is not known. The genotype of NY99 WN virus in the United States has remained stable. Very few genomic changes occurred in the NY99 WN virus between the 1999 and 2000 WN virus outbreaks (32; Lanciotti, pers. comm.).

Table. Distribution of Japanese encephalitis (JE) virus serocomplex viruses

Virus	Abbreviation	Geographic location
Cacipacore	CPC	South America
Koutango	KOU	Africa
Japanese encephalitis	JE	Asia, Oceania, Australia[a]
Murray Valley encephalitis	MVE	Australia
Alfuy	ALF	Australia
St. Louis encephalitis	SLE	North America, South America
West Nile encephalitis	WN	Africa, Asia, Europe, North America
Kunjin	KUN	Australia
Yaounde	YAO	Africa

[a]JE virus has occasionally been introduced into Australia. Classification from (12).

The 2000 WN virus outbreak in humans and birds in Israel was caused by cocirculation of both the ROM96 and the Isr98 variants of WN virus (33; C. Banet, manuscript in preparation). Although these are the first reports of two genetic variants of WN virus causing a single WN encephalitis outbreak in humans and birds, similar mixed human flavivirus outbreaks have been documented for dengue virus (34).

The close genetic relationship between WN virus isolates from Israel and New York suggests that the virus was imported into North America from the Middle East. The means of its introduction (infected bird, mosquito, human, or another vertebrate host) will likely remain unknown. A striking feature of the initial human epidemic in New York City in 1999 was the high number of avian deaths in the accompanying epizootic, particularly in American Crows (*Corvus brachrhynchos*) and other corvids (35,36). Subsequent work demonstrating near 100% death rates among experimentally infected American Crows with NY99 WN virus has confirmed this observation (R. McLean, pers. comm.). Although one early study showed high death rates among Egyptian Hooded Crows (*Corvus corone*) and House Sparrows (*Passer domesticus*) experimentally infected with the prototype Egypt 101 WN virus strain (37), the epizootic in Israel in 1997 to 2000 was the first in the Old World demonstrating high avian death rates (38). Whether high avian death rates in the United States are due to higher virulence of the circulating strains or to higher susceptibility in North American birds requires further evaluation.

High avian death rates during the 1999 epizootic in the New York City area prompted an avian mortality surveillance system to track the spread of WN virus in the eastern and southern United States. Surveillance showed expansion of viral activity to 12 states in 2000, extending from the Canadian border to North Carolina, a distance of 900 km (39). Pronounced northward spread of the virus from New York City was noted in the late spring and early summer and southward spread in the late summer and fall—a pattern consistent with bird migration. Through 2000, avian mortality rate surveillance has documented WN virus infection in 76 North American native and captive bird species. Although American Crows were by far the most commonly identified species, this may reflect the lethality of infection in this species, rather than its importance as a reservoir host.

Despite the substantial geographic expansion of WN virus activity documented by avian mortality surveillance in 2000, human infections were noted only in New York City and surrounding counties in New Jersey and Connecticut (39). Ten of the 21 infected persons identified in 2000 lived on Staten Island, the only part of New York City without documented WN virus infections in humans in 1999. The reason that the 2000 human epidemic remained focal despite a widely geographically expanding epizootic is unknown. Extensive spring and early summer larval mosquito control efforts in urban areas of the Northeast likely contributed to decreased human exposure to mosquitoes.

In addition to high mortality rates of 5% to 14% among persons with neurologic symptoms in the recent U.S., Romanian, Russian, and Israeli outbreaks, other clinical aspects (e.g., profound motor weakness and infrequency of skin rash and lymphadenopathy) differ from those of earlier outbreaks (19,20,22,25,39,40). Serologic surveys accompanying the Romanian (1996) and two U.S. outbreaks (1999 and 2000) indicated that severe neurologic illness developed in <1% of persons infected with WN virus, with systemic febrile illness developing in approximately 20% of those infected (40,41).

In the United States in both 1999 and 2000, infections in humans peaked in August and in horses in September (39,42), suggesting either different mosquito species transmitting the virus to humans and horses or temporal differences in exposure to the same species. In 2000, 14 mosquito species in five states had evidence of WN virus infection (by culture or nucleic acid amplification) (39). Since mosquitoes of the genus *Culex* are the principal maintenance vectors in the Old World, not surprisingly, *Cx. pipiens* and *Cx. restuans*—common, ornithophilic maintenance vectors for SLE in the northeastern United States (43)—were by far the most frequently identified species with WN virus in 2000 (39). However, which species are most important for transmission to humans or horses remains unknown. Extensive mosquito collections from Connecticut and New York State indicated that *Cx. pipiens* was present in high numbers and had high WN virus infection rates in early August, coinciding with a subsequent peak in human disease in the New York City area (44,45). One important observation was the high WN virus infection rates in and abundance of *Cx. salinarius* mosquitoes on Staten Island in 2000, which temporally coincided with the human outbreak (46). This species indiscriminately feeds on both birds and mammals and readily bites humans.

Experience with WN virus in the Old World and SLE virus in the Americas may provide clues to the eventual outcome of WN virus in the Americas. The broad geographic distribution of WN virus in Africa, Europe, the Middle East, and western Asia suggests potential for wide geographic distribution in the Americas. The principal mosquito vectors and avian host species for SLE virus vary regionally; the broad range of mosquito vectors and avian host species for WN virus in the Old World also suggests that a similar pattern can occur in the Americas for WN virus (23). Further study of the ecology and epidemiology of WN virus in areas where the virus has been endemic for a long time (e.g., the Nile Delta in Egypt) will provide additional clues about what can be expected in the Americas.

Outbreaks caused by WN and SLE viruses have been difficult to predict, in part because of our incomplete knowledge of the viruses' complex ecology. Weather data suggest that hot, dry summers may promote human outbreaks caused by these two viruses (25,40,47,48). The mean July temperature in the New York City area in 1999 was among the highest on record, while 2000 was comparatively cool. However, climate and weather influence mosquito populations and arboviral recrudescence in complex ways; simple generalizations about weather have had poor predictive value for SLE forecasting and will likely be equally unpredictive for WN virus forecasting in any given area (48,49).

In the United States, first attempts have been made to predict WN virus human epidemics in a county on the basis of avian mortality data (50); efforts to interpret avian mortality or other surveillance data at a more local level for more focused emergency mosquito control are at an even earlier stage of development (46,51). To prevent WN virus infection in humans, extensive early season larval control has been recommended and undertaken, as have the development and dissemination of public health messages for reducing personal exposure to mosquito bites (52). The efficacy and cost-effectiveness of these prevention measures, along with application of pesticides to control adult mosquitoes, require further evaluation. These evaluations are likely to be hindered by the sporadic nature of human WN epidemics. Given our incomplete and evolving knowledge of the ecology and public health impact of WN virus in the Americas, as well as the efficacy of control efforts, the virus will remain an important public health challenge in the next decade.

References

1. Asnis DS, Conetta R, Teixeira AA, Waldman G, Sampson BA. The West Nile Virus outbreak of 1999 in New York: the Flushing Hospital experience. Clin Infect Dis 2000;30:413-8.
2. Nash D, Mostashari F, Fine A, Miller J, O'Leary D, Murray K, et al. Outbreak of West Nile virus infection, New York City area, 1999. N Engl J Med 2001;344:1807-14.
3. Gubler DJ. Dengue and dengue hemorrhagic fever in the Americas. P R Health Sci J 1987;6:107-11.
4. Gubler DJ, Clark GG. Dengue/dengue hemorrhagic fever: the emergence of a global health problem. Emerg Infect Dis 1995;1:55-7.
5. Gubler DJ. The global pandemic of dengue/dengue haemorrhagic fever: current status and prospects for the future. Ann Acad Med Singapore 1998;27:227-34.
6. Ritchie SA, Phillips D, Broom A, Mackenzie J, Poidinger M, van den Hurk A. Isolation of Japanese encephalitis virus from *Culex annulirostris* in Australia. Am J Trop Med Hyg 1997;56:80-4.
7. Hanna JN, Ritchie SA, Phillips DA, Shield J, Bailey MC, Mackenzie JS, et al. An outbreak of Japanese encephalitis in the Torres Strait, Australia, 1995. Med J Aust 1996;165:256-60.
8. Hanna JN, Ritchie SA, Phillips DA, Lee JM, Hills SL, van den Hurk AF, et al. Japanese encephalitis in north Queensland, Australia, 1998. Med J Aust 1999;170:533-6.
9. Mackenzie JS, Broom AK, Hall RA, Johansen CA, Lindsay MD, Phillips DA, et al. Arboviruses in the Australian region, 1990 to 1998. Commun Dis Intell 1998;22:93-100.
10. Gubler DJ, Roehrig JT. Togaviridae and Flaviviridae. In: Collier L, Balows A, Sussman M, editors. Topley and Wilson's microbiology and microbial infections. London: Arnold Publishing; 1999. p. 579-600.
11. Smithburn KC, Hughes TP, Burke AW, Paul JH. A neurotropic virus isolated from the blood of a native of Uganda. Am J Trop Med 1940;20:471-92.
12. Heinz FX, Collett MS, Purcell RH, Gould EA, Howard CR, Houghton M, et al. Family: *Flaviviridae*. In: Van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, et al., editors. Virus taxonomy: classification and nomenclature of viruses. 7th Report of the International Committee on Taxonomy of Viruses. San Diego: Academic Press; 1999. p. 859-78.

13. Roehrig JT. Arboviruses. In: Specter S, Hodinka RL, Young SA, editors. Clinical virology manual. 3rd ed. Washington: American Society for Microbiology; 1999. p. 356-73.
14. Johnson DJ, Ostlund EN, Pedersen DD, Schmitt BJ. Detection of North American West Nile Virus in animal tissue by a reverse transcription-nested polymerase chain reaction assay. Emerg Infect Dis 2001;7:739-41.
15. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol 2000;38:4066-71.
16. Briese T, Glass WG, Lipkin WI. Detection of West Nile virus sequences in cerebrospinal fluid. Lancet 2000;355:1614-5.
17. Nasci RS, Savage HM, White DF, Miller JR, Cropp BC, Godsey MS, et al. West Nile virus in overwintering *Culex* mosquitoes, New York City, 2000. Emerg Infect Dis 2001;7:742-4.
18. Giladi M, Metzkor-Cotter E, Martin DA, Siegman-Igra Y, Korczyn AD, Rosso R, et al. West Nile encephalitis in Israel, 1999: The New York connection. Emerg Infect Dis 2001;7:659-61.
19. Weinberger M, Pitlik SD, Gandacu D, Lang R, Nassar F, Ben David D, et al. West Nile fever outbreak, Israel, 2000: Epidemiologic aspects. Emerg Infect Dis 2001;7:686-91.
20. Chowers MY, Lang R, Nassar F, Ben-David D, Giladi M, Rubinshtein E, et al. Clinical characteristics of the West Nile Fever outbreak, Israel, 2000. Emerg Infect Dis 2001;7:675-8.
21. Murgue M, Murri S, Zientara S, Durand B, Durand J-P, Zeller H. West Nile outbreak in horses in southern France, 2000: the return after 35 years. Emerg Infect Dis 2001;7:692-6.
22. Weiss D, Carr D, Kellachan J, Tan C, Phillips M, Bresnitz E, et al. Clinical findings of West Nile virus infection in hospitalized patients, New York and New Jersey, 2000. Emerg Infect Dis 2001;7:654-8.
23. Hubálek Z, Halouzka J. West Nile fever—a reemerging mosquito-borne viral disease in Europe. Emerg Infect Dis 1999;5:643-50.
24. Cernescu C, Ruta SM, Tardei G, Grancea C, Moldoveanu L, Spulbar E, et al. A high number of severe neurologic clinical forms during an epidemic of West Nile virus infection. Rom J Virol 1997;48:13-25.
25. Platonov AE, Shipulin GA, Shipulina OY, Tyutyunnik EN, Frolochkina TI, Lanciotti RS, et al. Outbreak of West Nile virus infection, Volgograd Region, Russia, 1999. Emerg Infect Dis 2001;7:128-32.
26. Scherret JH, Poidinger M, Mackenzie JS, Broom AK, Deubel V, Lipkin I, et al. The relationships between West Nile and Kunjin viruses. Emerg Infect Dis 2001;7:697-705.
27. Berthet FX, Zeller HG, Drouet MT, Rauzier J, Digoutte JP, Deubel V. Extensive nucleotide changes and deletions within the envelope glycoprotein gene of Euro-African West Nile viruses. J Gen Virol 1997;78:2293-7.
28. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333-7.
29. Jia XY, Briese T, Jordan I, Rambaut A, Chi HC, Mackenzie JS, et al. Genetic analysis of West Nile New York 1999 encephalitis virus. Lancet 1999;354:1971-2.
30. Savage HM, Ceianu C, Nicolescu G, Karabatsos N, Lanciotti R, Vladimirescu A, et al. Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serologic and molecular characterization of a virus isolate from mosquitoes. Am J Trop Med Hyg 1999;61:600-11.
31. Miller BR, Nasci RS, Godsey MS, Savage HM, Lutwama JJ, Lanciotti RS, et al. First field evidence for natural vertical transmission of West Nile virus in *Culex univittatus* complex mosquitoes from Rift Valley province, Kenya. Am J Trop Med Hyg 2000;62:240-6.
32. Ebel GD, Dupuis AP II, Ngo K, Nicholas D, Kauffman E, Jones SA, et al. Partial genetic characterization of West Nile virus strains, New York State, 2000. Emerg Infect Dis 2001;7:650-3.
33. Hindiyeh M, Shulman LM, Mendelson E, Weiss L, Grossman Z, Bin H. Isolation and characterization of West Nile virus from the blood of viremic patients during the 2000 outbreak in Israel. Emerg Infect Dis 2001;7:748-50.
34. Gubler DJ, Kuno G, Sather GE, Waterman SH. A case of natural concurrent human infection with two dengue viruses. Am J Trop Med Hyg 1985;34:170-3.
35. Eidson M, Komar N, Sorhage F, Nelson R, Talbot T, Mostashari F, et al. Crow deaths as a sentinel surveillance system for West Nile virus in the northeastern United States, 1999. Emerg Infect Dis 2001;7:615-20.
36. Steele KE, Linn MJ, Schoepp RJ, Komar N, Geisbert TW, Manduca RM, et al. Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, New York. Vet Pathol 2000;37:208-24.
37. Work TH, Hurlbut HS, Taylor RM. Indigenous wild birds of the Nile Delta as potential West Nile virus circulating reservoirs. Am J Trop Med Hyg 1955;4:872-88.
38. Swayne DE, Beck JR, Smith CS, Shieh W-J, Zaki SR. Fatal encephalitis and myocarditis in young domestic geese (*Anser anser domesticus*) caused by West Nile virus. Emerg Infect Dis 2001;7:751-3.
39. Marfin AA, Petersen LR, Eidson M, Miller J, Hadler J, Farello C, et al. Widespread West Nile virus activity, eastern United States, 2000. Emerg Infect Dis 2001;7:730-5.
40. Tsai TF, Popovici F, Cernescu C, Campbell GL, Nedelcu NI. West Nile encephalitis epidemic in southeastern Romania. Lancet 1998;352:767-71.
41. Centers for Disease Control and Prevention. Serosurveys for West Nile virus infection—New York and Connecticut Counties, 2000. MMWR Morb Mortal Wkly Rep 2001;50:31-9.
42. Centers for Disease Control and Prevention. Update: West Nile virus activity—Eastern United States, 2000. MMWR Morb Mortal Wkly Rep 2000;49:1044-7.
43. Mitchell CJ, Francy DB, Monath TP. Arthropod vectors. In: Monath TP, editor. St. Louis encephalitis. Washington: American Public Health Association; 1999. p. 313-79.
44. Andreadis TG, Anderson JF, Vossbrinck CR. Mosquito surveillance for West Nile virus in Connecticut, 2000: Isolation from *Culex pipiens, Cx. restuans, Cx. salinarius,* and *Culiseta melanura*. Emerg Infect Dis 2001;7:670-4.
45. White DJ, Kramer LD, Backenson PB, Lukacik G, Johnson G, Oliver J, et al. Mosquito surveillance and polymerase chain reaction detection of West Nile virus, New York State. Emerg Infect Dis 2001;7:643-9.
46. Kulasekera V, Kramer L, Nasci RS, Mostashari F, Cherry B, Trock SC, et al. West Nile virus infection in mosquitoes, birds, horses, and humans, Staten Island, New York, 2000. Emerg Infect Dis 2001;7:722-5.
47. Han LL, Popovici F, Alexander JP, Laurentia V, Tengelsen LA, Cernescu C, et al. Risk factors for West Nile virus infection and meningoencephalitis, Romania, 1996. J Infect Dis 1999;179:230-3.
48. Reiter P. Weather, vector biology and arboviral recrudescence. In: Monath TP, editor. The arboviruses: epidemiology and ecology. Boca Raton: CRC Press; 1999. p. 245-55.
49. Reiter P. Climate change and mosquito-borne disease. Environ Health Perspect 2001;109:141-61.
50. Eidson M, Miller J, Kramer L, Cherry B, Hagiwara Y, West Nile Virus Bird Mortality Analysis Group. Dead crow densities and human cases of West Nile virus, New York State, 2000. Emerg Infect Dis 2001;7:662-4.
51. Hadler J, Nelson R, McCarthy T, Andreadis T, Lis MJ, French R, et al. West Nile virus surveillance in Connecticut in 2000: An intense epizootic without high risk for severe human disease. Emerg Infect Dis 2001;7:636-42.
52. Centers for Disease Control and Prevention. Epidemic/epizootic West Nile virus in the United States: revised guidelines for surveillance, prevention and control. Available at URL: http://www.cdc.gov/ncidod/dvbid/westnile/publications.htm

Crow Deaths as a Sentinel Surveillance System for West Nile Virus in the Northeastern United States, 1999

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In addition to human encephalitis and meningitis cases, the West Nile (WN) virus outbreak in the summer and fall of 1999 in New York State resulted in bird deaths in New York, New Jersey, and Connecticut. From August to December 1999, 295 dead birds were laboratory-confirmed with WN virus infection; 262 (89%) were American Crows (*Corvus brachyrhynchos*). The New York State Department of Health received reports of 17,339 dead birds, including 5,697 (33%) crows; in Connecticut 1,040 dead crows were reported. Bird deaths were critical in identifying WN virus as the cause of the human outbreak and defining its geographic and temporal limits. If established before a WN virus outbreak, a surveillance system based on bird deaths may provide a sensitive method of detecting WN virus.

West Nile (WN) virus (family Flaviviridae) causes inapparent infection, mild febrile illness, meningitis, encephalitis, or death in humans and horses in Europe, Africa, Asia, and Australia (1). Wild birds are considered the principal hosts of WN virus, and mosquitoes, particularly *Culex* species, are the primary vector (1). Bird deaths had not been frequently documented in previous human WN virus outbreaks, although infected carcasses of a variety of bird species were found in Israel in 1998 (1,2), and deaths were observed after experimental infection in crows and sparrows (3).

As early as the end of June 1999, an unusual number of dead and dying crows were noted by residents of northern Queens in New York City (NYC). In July, a local veterinarian noted neurologic illness in some birds with unstable gait. Although not then recognized, the earliest cases of human illness due to West Nile virus occurred in this area, beginning in the first week of August (4). After initial evaluation of dead birds by the New York State Department of Environmental Conservation's Wildlife Pathology Unit and the Wildlife Conservation Society, a virus isolated from specimens by the National Wildlife Health Center and the U.S. Department of Agriculture's National Veterinary Services Laboratory was identified as WN virus by the Centers for Disease Control and Prevention (CDC) on September 23 (5). The virus was also recovered by the Connecticut Agricultural Experiment Station in specimens from a Connecticut bird on September 13 (6). A West Nile virus genomic sequence identical to that derived from the bird isolates was then observed in a brain specimen from a human encephalitis case (7).

In response to the initial indications of WN virus in bird specimens, surveillance systems for bird deaths and laboratory testing were established and used in the assessment and control of the outbreak. We reviewed data from systems in New York State, New Jersey, and Connecticut to describe how surveillance of bird deaths was used in 1999 to guide public health action, as well as the advantages and disadvantages of using dead birds as sentinels for West Nile virus in a given geographic area.

Methods

Sightings of Ill or Dead Birds

Local health departments were requested to collect and report dead birds to the state health departments of New York

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and Connecticut. Sighting reports for ill or dead birds that were not submitted for laboratory testing were not systematically maintained in New Jersey in 1999. Data collected included date of the report, date of death or sighting of the birds, whether the birds were dead or appeared ill, street address where the birds were seen or found, number of birds, and species of birds. Mapping was based on the earliest date provided for the death or sighting. New York State's surveillance data for bird deaths were collected prospectively from September 23, 1999, through November 30, 1999, and retrospectively through May 1, 1999. Connecticut's reporting system was active from September 30, 1999, through November 4, 1999.

In New York State, a geographic information system was used to geocode locations of WN virus-positive birds and to generate maps. Because of incomplete address information, dot-density mapping was used with random placement of the birds within townships for dead crow sightings in New York State and WN virus-positive birds in Connecticut and New Jersey. To assess changes in crow populations, the National Audubon Society's Christmas Bird Count (8), adjusted for party-hours (sum of hours spent counting by each group performing the count), was used.

Specimen Collection

Recently dead birds with no other obvious causes of death were submitted for testing in all three states. Although initially New York State requested submission only of birds found within 1 mile of each other within 72 hours, that requirement was soon dropped. Connecticut prioritized the submission of birds based on towns with multiple reports of dead birds and then in areas near the towns where WN virus was confirmed. WN virus testing was limited to birds collected from September 13 through October 29, 1999. New Jersey initially accepted all dead bird specimens but later reduced the testing of specimens from several counties where numerous positives had been identified. Mapping was based on the date the dead bird was found.

In their respective states, dead birds were necropsied and specimens were processed for virus testing by the New York State Wildlife Pathology Unit, the New Jersey Department of Health and Senior Services Public Health and Environmental Laboratory, and New Jersey Division of Fish and Wildlife Pathology Laboratory, as well as the Department of Pathobiology at the University of Connecticut.

Laboratory Testing

Methods for detecting WN virus in avian tissues at CDC have been described (9). Briefly, tissue samples were prepared by macerating approximately 0.5 cm^3 of brain tissue in 1.8 mL of BA-1 diluent in a glass TenBroeck tissue grinder (Bellco Glass, Inc., Vineland, NJ). These homogenates were clarified by centrifugation. Virus isolation was attempted in duplicate 100-µL aliquots of the supernatant by Vero plaque assay in 6-well plates. A 75-µL aliquot from each sample was tested by either the traditional or TaqMan reverse-transcriptase-polymerase chain reaction (RT-PCR) assays or both.

In Connecticut, brain tissue was assayed for WN virus as described (6), using cytopathic effect in Vero culture to screen for viruses and specific WN virus RT-PCR for identification. A similar strategy was used at the National Wildlife Health Center, but kidney or spleen suspensions were used in place of brain.

Results

Ill or Dead Bird Sightings

New York State received 13,654 reports of 17,339 dead birds from 32 county health departments and from the New York City Department of Health, which represents five boroughs (counties). Dates of death ranged from May 1 to November 30. The predominant species reported was the American Crow (*Corvus brachyrhynchos*) (5,697 sightings, 33%). Before August, there were few retrospective dead crow sightings, and these were confined primarily to the NYC boroughs of Queens and the Bronx and to lower Westchester County. Continued geographic spread of dead crow sightings was noted in August (Figure 1a). Reported sightings peaked in September (Figure 1b), with the largest numbers from NYC and lower Westchester County and wide distribution into Long Island and north along the Hudson River. Although dead crow reports did not dramatically decrease until November, they began to decline in number and density in October (Figure 1c). Later reports were also distributed farther north along the upper Hudson Valley. Most of the dead bird sightings were of single dead birds, rather than clusters of dead birds found together.

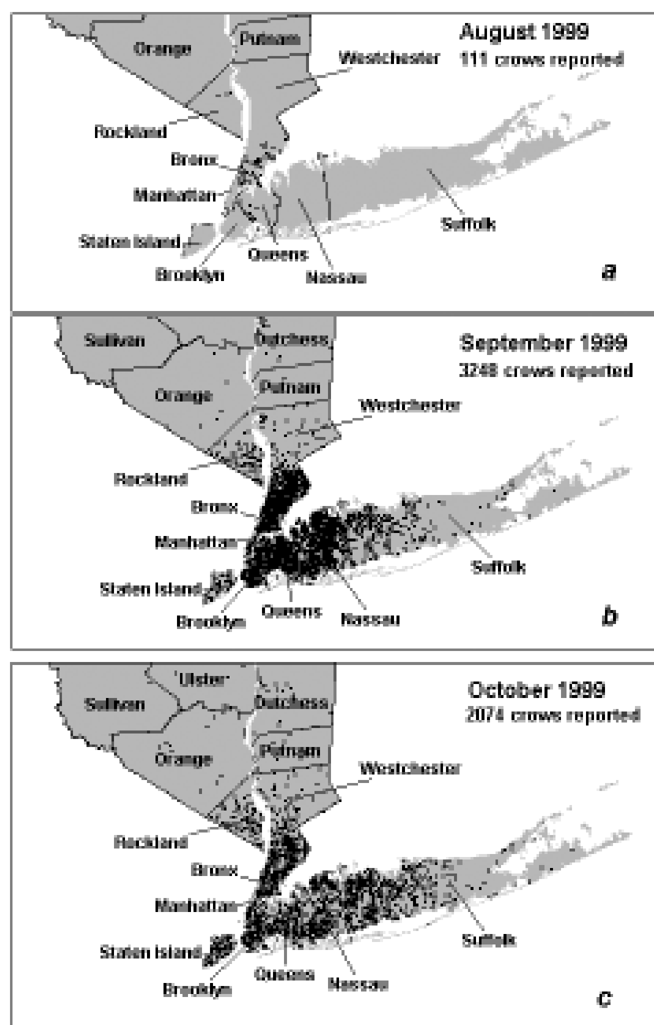

Figure 1. Dead crow sightings, August-October, 1999, New York State.

In Connecticut, the Department of Public Health received reports of dead birds from health departments representing 40 of 169 Connecticut towns. Thirty-five of these 40 towns had reported 1,040 dead crow sightings by the time surveillance ended. The earliest report of a dead crow was in Stratford on September 1, and the latest was in New Fairfield on November 5. The peak number of deaths in a week was 279 during the week of September 26 to October 2, although not all reports included the date of the sighting. Of the 10 towns where more than 10 dead crows were sighted, all were coastal towns, including 8 in Fairfield County and 2 in New Haven County. However, towns in 6 of the 8 Connecticut counties received 1 to 10 reports of dead crows.

Laboratory Testing

Of 671 dead birds tested, 295 had laboratory-confirmed WN virus infection (142 from New York State, 78 from New Jersey, and 75 from Connecticut). The proportions testing positive were 39% for New York State, 37% for New Jersey, and 77% for Connecticut. WN virus-positive dead birds provided evidence of possible viral activity in four New York State counties, all five NYC boroughs, 16 New Jersey counties, and two Connecticut counties. Viral activity, as indicated by WN virus-positive birds, spread from a central cluster in NYC and adjacent New York State counties in August (Figure 2a) to northeastern New Jersey and southwestern Connecticut in September (Figure 2b). In October, a "central clearing" with fewer WN virus-positive birds in the NYC area was evident (Figure 2c), while a wider distribution of infected birds was seen in southern New Jersey. In Connecticut, where testing was primarily in towns near areas with confirmed WN virus-infected birds, fewer WN virus-positive birds were identified in October than in earlier months.

Two hundred sixty-two (89%) of the WN virus-positive dead birds were American Crows. However, WN virus was isolated from dead birds of 19 other species, including the Fish Crow (*C. ossifragus*, 7), Chilean Flamingo (*Phoenicopterus chilensis*, 4), Blue Jay (*Cyanocitta cristata*, 4), Red-tailed Hawk (*Buteo jamaicensis*, 2), Mallard (*Anas platyrhynchos*, 2), and one each of the following species: Rock Dove (*Columba livia*), Belted Kingfisher (*Ceryle alcyon*), Laughing Gull (*Larus atricilla*), Herring Gull (*L. argentatus*), Black-crowned Night Heron (*Nycticorax nycticorax*), Sandhill Crane (*Grus canadensis*), Guanay Cormorant (*Phalacrocorax bougainvillea*), Blyth's Tragopan (*Tragopan blythi*), Bald Eagle (*Haliaeetus leucocephalus*), American Kestrel (*Falco sparverius*), Broad-winged Hawk (*Buteo platypterus*), Cooper's Hawk (*Accipiter cooperii*), Merlin (*Falco columbarius*), and American Robin (*Turdus migratorius*). The noncorvid species were primarily from New York State, except for a Cooper's Hawk and Sandhill Crane reported from Connecticut and a Red-tailed Hawk and Merlin reported from New Jersey.

The earliest collection dates for WN virus-positive birds were August 2-9 in Nassau County, New York (Figure 3), and the latest collection date was November 15, from Rockland

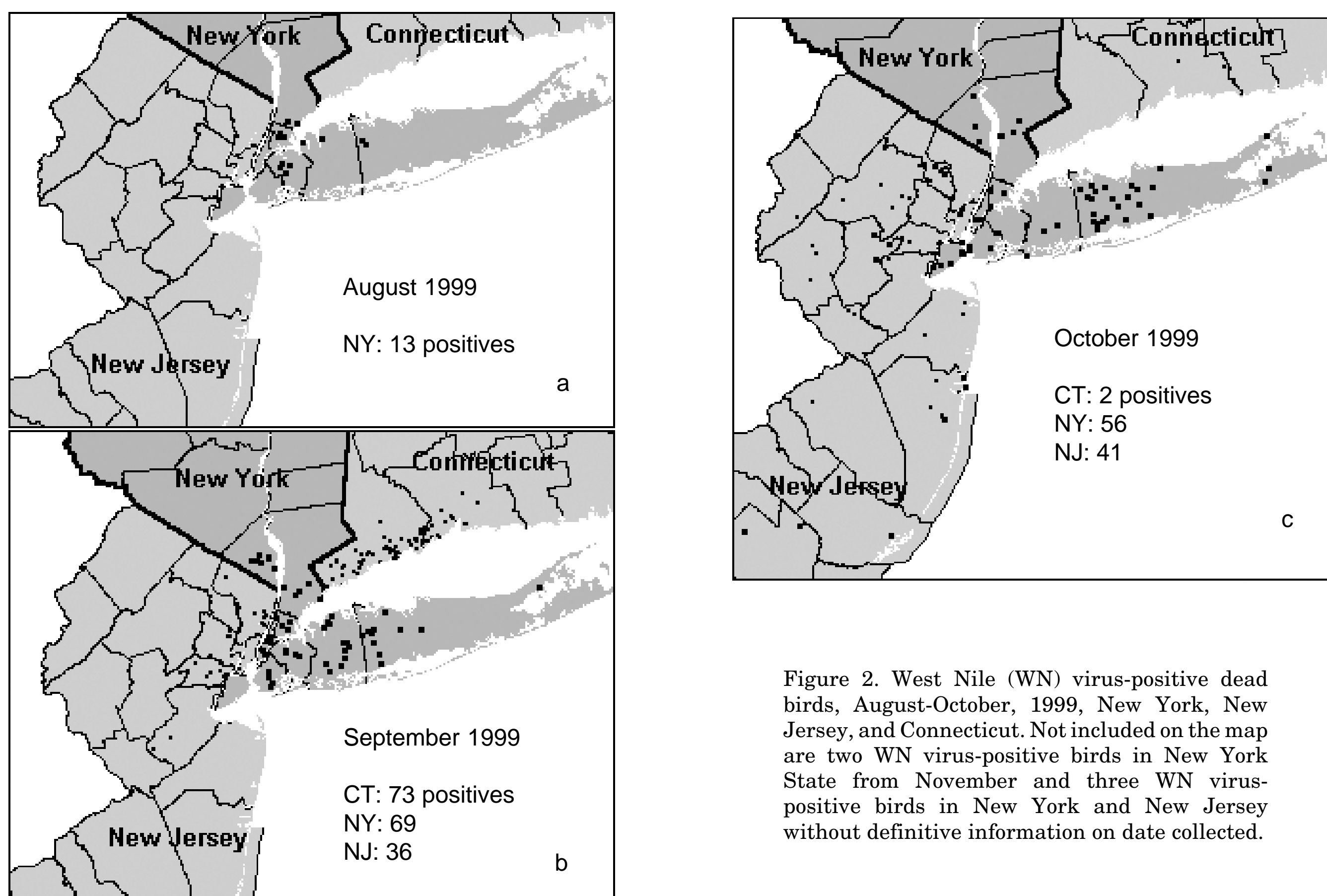

Figure 2. West Nile (WN) virus-positive dead birds, August-October, 1999, New York, New Jersey, and Connecticut. Not included on the map are two WN virus-positive birds in New York State from November and three WN virus-positive birds in New York and New Jersey without definitive information on date collected.

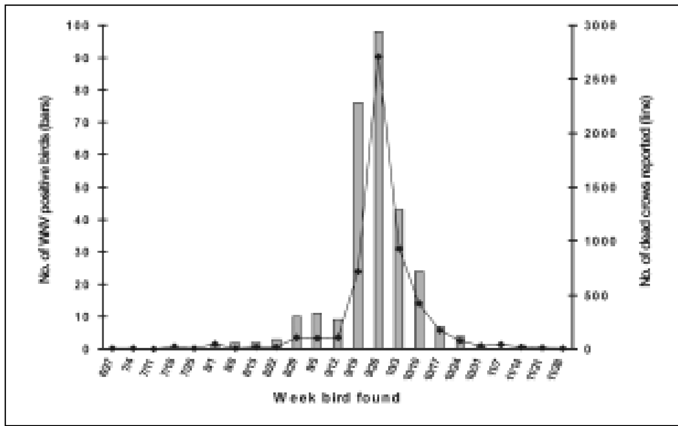

Figure 3. Number of dead crow sightings in New York State and number of West Nile (WN) virus-positive birds in New York State, New Jersey, and Connecticut, by week, June 27-November 30, 1999. Not included are three WN virus-positive birds in New York and New Jersey without definitive information on date collected.

County, New York. The peak in collections of WN virus-positive birds, as well as reports of dead crow sightings in New York State and Connecticut, occurred during the week of September 26, immediately after the first press release announcement that WN virus had been detected in dead birds.

Analysis of the National Audubon Society's Christmas Bird Count data, adjusted for party-hour (8), indicated a decrease in the number of crows sighted in 1999 (after the WN virus outbreak) compared with 1998, with the largest decreases in the NYC WN virus epicenter boroughs of Queens (69%) and the Bronx (65%) (Figure 4). Geographic areas at the periphery of the outbreak in 1999, including Rockland County, Staten Island, and the eastern tip of Suffolk County, had increases in crow sightings in 1999 compared with 1998.

Retrospective testing found no WN virus-positive birds among six archived specimens found dead in the New York City region from May 27 to August 16, 1998 (including two American Crows) or among three specimens collected in April 1999 in the same region.

Conclusion

Although inapparent avian infections were known to occur during WN virus outbreaks, along with occasional avian illnesses and deaths (2,10), the WN virus outbreak in the northeastern United States in 1999 is the first with a recognized substantial avian mortality rate (1).

Interpretation of the results of this surveillance system in 1999 in the Northeast and conclusions about its possible future value as a sentinel for WN virus have several limitations. First, bird death cannot be adequately investigated over wide areas without recognition of its importance by the public and by local and state agencies in those areas. Routine mechanisms were already in place at the local, state, and federal levels to investigate bird die-offs, and wildlife, zoologic, health, and agricultural agencies played a critical role in determining the presence of WN virus in this hemisphere. However, public knowledge of the WN virus outbreak did result in a peak in the number of reported dead birds, occurring immediately after the first press announcement of WN virus. Thus, public awareness of the need to report animal deaths is key to using ill or dead wild animals as sentinels for detection of zoonotic pathogens.

Another limitation is that media coverage was more intense in areas close to NYC and where the first WN

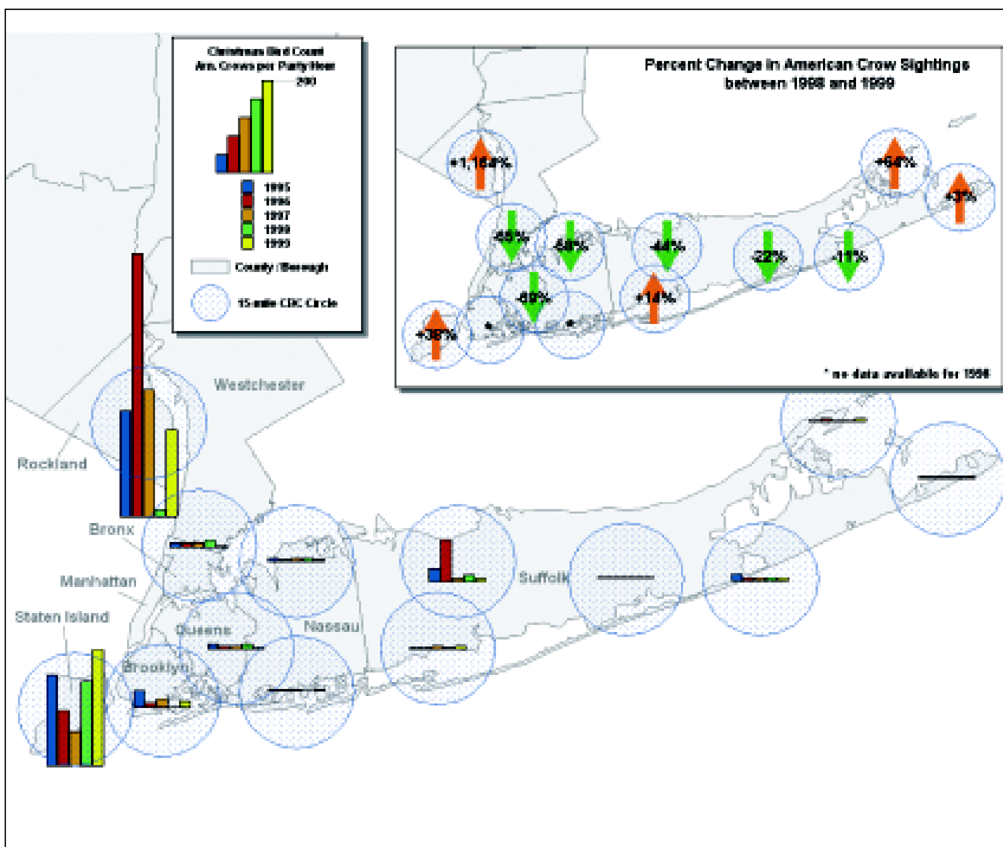

Figure 4. Christmas bird count, number of American Crows reported, adjusted for party-hours, 1995-1999, New York City area (8).

virus-positive birds were found, which may have influenced public awareness of the surveillance system and led to underreporting of dead birds in areas with less media coverage. An active system of surveillance for bird deaths may be necessary to supplement passive reporting systems in areas without strong media coverage and public awareness about the need to report dead birds.

The process of obtaining birds for necropsy and performing laboratory analyses proved to be time-consuming and labor-intensive, so that testing had to be prioritized and limited. Thus, in addition to potential variability in the quality of the reporting of dead bird sightings, additional problems in interpreting data on positive birds may result from differing decision processes and procedures for collection and submission of birds for testing across county and state lines.

Drawing any definitive conclusions about the decreases in 1999 crow counts seen by the National Audubon Society in the epicenter of the outbreak is problematic. The percentage of reductions in the numbers of American Crows seen is based on small numbers of birds per party-hour. In addition, the counts may be influenced by factors such as crow migration in the fall and changes in the number and skill of bird survey participants from year to year.

An additional limitation of the possible usefulness of bird deaths as a sentinel for WN virus is the difference between the outbreaks in humans and birds in the Northeast in 1999. The geographic distribution of positive birds was much greater than that of human cases. No human cases were reported from Connecticut and New Jersey despite positive dead birds in 2 and 16 counties, respectively, and no human cases were reported from one NYC borough and two New York State counties with positive birds (11). Some of the positive birds may not have provided indication of viral activity and risk to humans in the counties where they were found because they could have been infected elsewhere and flown to a different county before their death.

A final limitation is that WN virus was confirmed in humans and birds at the same time, in late September 1999, for humans and birds with onset of illness in early August (11). Therefore, analysis of avian mortality in 1999 cannot definitively determine whether a prospectively established surveillance system could have provided an early warning for detecting human cases in 1999. However, an increase in dead crow sightings in June in 1999 was one indication that such surveillance could have provided an early sign of possible viral activity.

Despite these limitations, the pattern of crow death reports corresponded with the pattern of WN virus-positive birds, and a clear geographic spread for virus detection can be discerned by examining the maps of dead crow sightings and WN virus-positive birds. A laboratory study of Hooded Crows (*Corvus corone sardonius*) in Egypt infected with WN virus by mosquito bites found that the birds died 1-7 days (median 4 days) after being bitten (3). Thus, dead crows may provide a sensitive indicator of continuing WN virus transmission in an area even after WN virus isolations in mosquitoes or cases in humans or other animals are no longer reported, for example, in the autumn.

Although most of the WN virus-positive dead birds in this study were crows, we emphasize that the mortality impact of WN virus on other bird species has not been adequately studied. This report indicates that 20 species of birds were found to be WN virus-positive during 1999, in spite of the fact that surveillance efforts focused on crows. Eight of these 20 positive species represented captive birds from zoological collections. Natural WN virus infection in seven of these species plus an additional three species of captive birds infected in 1999 have been described (12). However, although 11 of the 23 species of birds now known to have been infected with WN virus in the United States in 1999 were captive when infected, 19 are also wild resident bird species. Thus, WN virus clearly represents a threat to both zoo collections and the native avifauna of North America, in addition to people and horses. As such, in 1999 the National Wildlife Health Center and CDC established ongoing dead and live bird surveillance systems along the East Coast of the United States, first on federal and state natural resource lands and then in conjunction with state public health and animal health agencies.

In summary, the WN virus outbreak in the northeastern United States in late summer and early fall 1999 represented the first introduction of WN virus into the Western Hemisphere. This WN virus outbreak was remarkable in the large numbers of observed crow fatalities and the importance of surveillance for monitoring the outbreak and making public health surveillance and disease control decisions. Establishment of surveillance for bird deaths before possible introduction of the virus in an area, along with additional analyses to identify correlates with human cases, will be required to provide more accurate and timely projections of the likelihood of human cases.

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References

1. Komar N. West Nile viral encephalitis. Rev Sci Technol 2000;19:166-76.
2. Malkinson M, Banet C, Weisman J, Pokamonski S, King R. West Nile fever: recent evidence for inter-continental dispersion of the virus by migratory birds. Proceedings of the 11th International Congress of Virology; 1999 August 9-13; Sydney, Australia. Sydney: International Union of Microbiological Societies; 1999. p. 56.
3. Work TH, Hurlbut HS, Taylor RM. Indigenous wild birds of the Nile Delta as potential West Nile virus circulating reservoirs. Am J Trop Med Hyg 1955;4:872-88.
4. Centers for Disease Control and Prevention. Outbreak of West Nile-like viral encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:845-9.
5. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile Virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333-7.

6. Anderson JF, Andreadis TG, Vossbrinck CR, Tirrell S, Wakem EM, French RA, et al. Isolation of West Nile virus from mosquitoes, crows and a Cooper's hawk in Connecticut. Science 1999;286:2331-3.
7. Jia XY, Briese T, Jordan I, Rambaut A, Chi HC, Mackenzie JS, et al. Genetic analysis of West Nile New York 1999 encephalitis virus. Lancet 1999;354:1971-2.
8. National Audubon Society Christmas Bird Count. Available from: URL:http://www.birdsource.org/cbc
9. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol 2000;38:4066-7.
10. Taylor RM, Work TH, Hurlbut HS, Rizk F. A study of the ecology of West Nile virus in Egypt. Am J Trop Med Hyg 1956;5:579-620.
11. Nash D, Mostashari F, Fine A, Miller J, O'Leary D, Murray K, et al. Outbreak of West Nile virus infection, New York City area, 1999. N Engl J Med. In press 2001.
12. Steele KE, Linn MJ, Schoepp RJ, Komar N, Geisbert TW, Manduca RM, et al. Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City. J Vet Pathol 2000;37:208-24.

Serologic Evidence for West Nile Virus Infection in Birds in the New York City Vicinity During an Outbreak in 1999

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As part of an investigation of an encephalitis outbreak in New York City, we sampled 430 birds, representing 18 species in four orders, during September 13-23, 1999, in Queens and surrounding counties. Overall, 33% were positive for West Nile (WN) virus-neutralizing antibodies, and 0.5% were positive for St. Louis encephalitis virus-neutralizing antibodies. By county, Queens had the most seropositive birds for WN virus (50%); species with the greatest seropositivity for WN virus (sample sizes were at least six) were Domestic Goose, Domestic Chicken, House Sparrow, Canada Goose, and Rock Dove. One sampled bird, a captive adult Domestic Goose, showed signs of illness; WN virus infection was confirmed. Our results support the concept that chickens and House Sparrows are good arbovirus sentinels. This study also implicates the House Sparrow as an important vertebrate reservoir host.

West Nile (WN) virus, a mosquito-borne flavivirus native to Europe, Africa, Asia, and Oceania (1), was first detected in North America in the vicinity of New York City in September 1999 (2,3). The virus was associated with an outbreak that included illness and death in humans (4), horses (5), and birds (6,7). In the Old World, birds serve as the vertebrate reservoir hosts in the transmission cycle of WN virus, while humans and other mammals are incidental hosts (1). The North American counterpart to WN virus is St. Louis encephalitis (SLE) virus. SLE virus is a genetically closely related flavivirus with a similar transmission cycle; it is distributed throughout the Americas (8).

Diagnostic tests for SLE and WN virus infections often cross-react. However, SLE virus had never been detected in New York City, and therefore no arboviral surveillance was in place to recognize a flavivirus epizootic in birds or in mosquitoes. Anecdotal evidence suggested that the WN virus epizootic began in late July 1999, when deaths in crows and other birds were observed in the Queens Borough of New York City and later in other boroughs and surrounding counties. In September 1999, the geographic distribution of WN virus in the New York City area and its natural association with potential mosquito vectors and vertebrate reservoir hosts remained unknown.

To generate basic information on the geographic distribution of WN virus and on its vertebrate host associations in the New York City region, a variety of surveys for flavivirus antibodies were conducted in vertebrate populations there. This report describes one such survey, which targeted resident bird populations in the northeastern quadrant of Queens County, where most of the human WN encephalitis cases were clustered, and in the peripheral counties of Kings (borough of Brooklyn), Richmond (borough of Staten Island), Westchester, and Nassau.

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Methods

Site Selection

Northeastern Queens was selected as a central sampling location to coincide with the region of greatest density of human WN encephalitis cases (Figure). Three scattered peripheral locations (Valley Stream, Nassau County; New Rochelle, Westchester County; and Staten Island, Richmond County) were selected in which to investigate potential

Figure. Bird sampling locations in and around northeastern Queens.

spread of WN virus transmission away from the apparent epicenter. Samples were collected from Brooklyn, midway between northeastern Queens and Staten Island, because a human case had been reported there. Specific sites within these locations were chosen by convenience, depending on the availability of resident birds for sampling. When possible, captive birds were sampled because residence histories and ages of these birds could be provided by their owners.

Bird Capture

Wild birds were captured with mist nets (Avinet, Inc.; Dryden, NY), a radio control-operated spring net (Fuhrman Diversified, Inc.; Seabrook, TX), a net gun, or manually when birds were sufficiently tame. Capture of wild birds was authorized by New York State Department of Environmental conservation permit #LCP99-630. Wild birds (but not domestic birds) were marked with uniquely numbered aluminum bands provided by the U.S. Department of Interior Bird Banding Laboratory, as authorized by permit #22866. Use of birds as research subjects for arbovirus seroprevalence studies was registered with the Centers for Disease Control and Prevention (CDC) through Animal Use Protocol #00-26-001-MSA.

Sample Collection

Whole blood was collected by jugular venipuncture or bracheal venipuncture. The volume of blood collected depended on the size of the bird but did not exceed 0.6 mL. Blood was collected in Microtainer serum collection tubes (Becton Dickinson and Co., Paramus, NJ, USA), held at ambient temperature for at least 15 minutes to permit clotting, and placed into coolers. Each night, serum was separated from blood samples collected earlier in the day by centrifugation with a portable microcentrifuge. Serum was transferred into 2-mL cryovials for shipment to the Division of Vector-Borne Infectious Diseases laboratory, CDC, in Fort Collins, Colorado.

Virus Strains

The EG101 strain of WN virus, obtained from the CDC reference collection of arboviruses, has a history of 13 unknown passages and 2 passages in suckling mice. The NY99-4132 strain was obtained from the brain of an American Crow (*Corvus brachyrhynchos*) collected in New York during 1999, provided by W.B. Stone. This strain was passaged once in Vero cells before use. The TBH-28 strain of SLE virus was obtained from the CDC reference collection; it has an unknown passage history that includes at least seven passages in suckling mice.

Plaque Reduction Neutralization Test

Serum samples were screened for flavivirus antibodies in the following manner: Serum samples were heat inactivated for 30 minutes at 56°C to inactivate adventitious microorganisms and nonspecific inhibitors of virus neutralization. Each specimen was diluted 1:5 in a total volume of 75 µL B Buffer (composed of M-199 salts, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 units/mL penicillin, 100 mg/L streptomycin, 1 mg/L Fungizone in 0.05 M Tris, pH 7.6) in sterile 96-well microtiter plates. To these dilutions, we added 75 µL of B buffer that contained approximately 75 Vero PFU of WN virus or SLE virus and 8% normal human serum. The final serum dilution of this mixture was 1:10, and concentration of WN virus was 50 plaque-forming units (PFU)/0.1 mL. The mixture was incubated for 1 hour at 37°C, 5% CO_2. Vero cell monolayers grown in six-well culture plates (Costar, Cambridge, MA, USA) were inoculated with 0.1 mL of the serum-virus mixture and incubated for 1 hour at 37°C, 5% CO_2. Cells were overlaid with 3 mL per well of 0.5% agarose in M-199 medium supplemented with 350 mg/L sodium bicarbonate, 29.2 mg/L L-glutamine, and antibiotics as in B buffer. After 48 hours of additional incubation, a second 3-mL 0.5% agarose overlay containing 0.004% neutral red dye was added for plaque visualization. Plaques were counted on days 3 and 4 after infection of the Vero cells. Controls included B buffer only (cell viability control), bird serum-free virus mixture with B buffer only (to count PFUs in the challenge dose of virus) and flavivirus (WN or SLE) hyperimmune mouse ascitic fluid (diluted 1:200) mixture with virus (to verify challenge virus identity). Serum samples that neutralized $\geq$80% of the challenge virus were selected for further titration against both WN virus and SLE virus.

Flavivirus titers of serum samples that tested positive in preliminary screen tests were determined as follows. With the use of 96-well microtiter plates, six serial twofold dilutions of serum in B buffer were prepared beginning with a dilution of 1:5. Virus mixtures were added as described above, resulting in final serum dilutions of 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320. Endpoint titers were assigned as the greatest dilution in which $\geq$90% neutralization of the challenge virus was achieved. Samples with reciprocal 90% neutralization titers of $\geq$10 were considered positive. Endpoints for samples with reciprocal titers $\geq$320 were not determined unless it was necessary to distinguish between WN and SLE viruses as the cause of infection. A sample that showed a fourfold greater titer for one of the viruses was considered positive for neutralizing antibodies to that virus. If a fourfold difference could not be demonstrated, designation as flavivirus-antibody positive was assigned.

Relative Abundance of Bird Species

To estimate relative abundance of the bird species that we sampled in suburban habitats of northeastern Queens, we relied on subjective estimates of several observers of bird populations in urban and suburban habitats of New York City.

Statistical Analysis

Pearson chi-square statistics were used to compare seroprevalence percentages (SAS 8.0). If 20% of the expected cell frequencies were <5%, p-values were established by the Fisher exact test. Significance was tested at a level of 0.05.

Results

We collected serum samples from 430 birds resident in and around northeastern Queens during September 13-23, 1999, and tested them for flavivirus-neutralizing antibodies. Eighteen species, representing four orders, were sampled. Three species comprised 80% of samples (chicken [38%], Rock Dove or Domestic Pigeon [28%], and House Sparrow [16%]). WN virus-neutralizing antibodies were detected in serum from 9 of the 18 species examined, including representatives of all four orders (Table 1). Overall, approximately one third of

Table 1. Flavivirus-neutralizing antibody in birds during September 1999, by species

Order	Common name	Latin name	Total tested	Percent virus Ab pos. ([95% CI], no.) WN	SLE	FLAV
Anseriformes	Canada Goose	*Branta canadensis*	16	18.8 ([3.9-54.8], 3)	0	6.2 ([0.1-34.8], 1)
	Domestic Goose	*Anser* species	11	63.6 ([30.8-89.1], 7)	0	0
	Mallard/Domestic Duck	*Anas platyrhynchos*	21	4.8 ([0.1-26.5], 1)	0	0
	Muscovy Duck	*Cairina moschata*	1	0	0	0
	Mute Swan	*Cygnus olor*	1	0	0	0
	Ruddy Shelduck	*Tadorna ferruginea*	7	0	0	0
	Wood Duck	*Aix sponsa*	1	0	0	0
Galliformes	Domestic Chicken	*Gallus gallus*	157	56.7 ([48.6-64.6], 89)	0	0
	Common Peafowl	*Pavo cristata*	10	0	0	0
	Turkey	*Meleagris gallopavo*	3	66.7 ([9.4-99.1], 2)	0	33.3 ([0.8-91.0], 1)
Columbiformes	Mourning Dove	*Zenaida macroura*	3	66.7 ([9.4-99.1], 2)	0	0
	Rock Dove	*Columba livia*	120	13.3 ([7.8-20.7], 16)	0.8 ([0.2-4.6], 1)	1.7 ([0.2-5.9], 2)
Passeriformes	American Robin	*Turdus migratorius*	1	0	0	0
	Brown-headed Cowbird	*Molothrus ater*	5	40.0 ([5.3-85.3], 2)	0	0
	Common Grackle	*Quiscalus quiscula*	2	0	0	0
	European Starling	*Sturnus vulgaris*	2	0	0	0
	House Sparrow	*Passer domesticus*	67	26.9 ([16.8-39.1], 18)	1.5 ([0.3-8.0], 1)	3.0 ([0.4-10.4], 2)
	Red-winged Blackbird	*Agelaius phoeniceus*	2	0	0	0
			430	32.6 ([28.1-37.2], 140)	0.5 ([0.05-1.7], 2)	1.2 ([0.3-2.7], 5)

Ab: antibody; CI: confidence interval; WN: West Nile; SLE: St. Louis encephalitis; FLAV: flavivirus.

the birds were positive for WN virus-neutralizing antibodies, whereas 0.5% tested positive for SLE virus-neutralizing antibodies. The six species for which >10 birds were sampled each had at least one WN virus-seropositive bird. Of the eight species represented by at least six individuals, the Domestic Goose was the most frequently exposed to flavivirus infection, followed by Domestic Chicken, House Sparrow, Canada Goose, Rock Dove, and Mallard.

Seroprevalence differences for WN virus in birds sampled in different regions were evaluated for each of five New York counties (Table 2). WN virus-infected birds were detected in all five counties, but seroprevalence was greatest in Queens ($\chi^2_{4df} = 92.0$, $p \leq 0.001$). Differences in seroprevalence in the other four counties were not statistically significant ($\chi^2_{3df} = 3.2$, $p \leq 0.364$). A limitation of this analysis is that bird populations sampled may not be representative within each county.

Table 2. Flavivirus-neutralizing antibody detected in birds during September 1999, by county

County (NY)	Total tested	Percent virus Ab pos. ([95% CI], no.) WN	SLE	FLAV
Queens	253	50.1 ([44.3-56.9], 128)	0	1.2 ([0.2-3.5], 3)
Richmond	43	2.3 ([0.1-12.3], 1)	0	0
Kings	20	5.0 ([0.1-37.5], 1)	0	0
Nassau	61	6.6 ([1.8-15.9], 4)	1.6 ([0.04-8.8], 1)	1.6 ([0.04-8.8], 1)
Westchester	53	11.3 ([4.3-23.0], 6)	1.9 ([0.04-10.0], 1)	3.8 ([0.5-13.0], 2)

Ab: antibody; CI: confidence interval; WN: West Nile; SLE: St. Louis encephalitis; FLAV: flavivirus.

The differences in seroprevalence among species could not be compared across regions where different levels of activity were observed. However, such an analysis was possible within northeastern Queens, where a dozen species were sampled. Again, three species represented approximately 80% of all specimens obtained (Domestic Chicken [56%], Rock Dove [19%], and House Sparrow [8%]). WN virus-neutralizing antibodies were detected in serum from 9 of the 12 species examined; half of these had seroprevalences of ≥50% (Table 3). Sample sizes were adequate to allow comparison of four species. From this analysis, Domestic Chickens and House Sparrows were the most frequently infected with WN virus; Mallards were least frequently infected; and Rock Doves were intermediate.

We evaluated cross-reactivity between WN and SLE viruses by the plaque reduction neutralization test (PRNT). The two specimens that were positive for SLE virus-neutralizing antibodies were negative for WN virus antibodies in the initial screen assay. However, of 140 WN virus antibody-positive specimens tested for SLE antibodies, 9 (6.4%) had 90% neutralization titers of ≥20 for SLE. Typically, WN virus antibody titers were more than eightfold greater than SLE titers, but this finding depended on the strain of WN virus used in the PRNT.

One of the captive birds sampled, an adult male Swan Goose (*Anser chinensis*, a type of Domestic Goose), was recovering from an illness characterized by ataxia at the time

Table 3. Flavivirus-neutralizing antibody in birds in Queens during September 1999, by species

Common Name	Total tested	Percent virus Ab pos. ([95% CI], no.) WN	FLAV
Canada Goose	7	28.6 ([3.6-71.0], 2)	14.2 ([0.3-57.4], 1)
Domestic Goose	7	85.7 ([42.1-99.6], 6)	0
Mallard/Domestic Duck	16	6.3 ([0.2-34.8], 1)	0
Domestic Chicken	141	63.1 ([54.6-71.1], 89)	0
Turkey	3	66.7 ([9.4-99.2], 2)	33.3 ([0.8-90.6], 1)
Mourning Dove	1	100.0 ([2.5-100.0], 1)	0
Rock Dove	49	26.5 ([14.9-41.1], 13)	2.0 ([0.05-11.4], 1)
American Robin	1	0	0
Brown-headed Cowbird	4	50.0 ([6.8-93.2], 2)	0
House Sparrow	20	60.0 ([36.1-80.9], 12)	0
European Starling	2	0	0
Red-winged Blackbird	2	0	0

Ab: antibody; CI: confidence interval; WN: West Nile; FLAV: flavivirus.

of sampling. Its owners were able to provide convalescent-phase serum samples from this bird. WN virus-neutralizing antibody titers for these samples increased from a reciprocal titer of 10 in the acute-phase specimen to 40 in the convalescent-phase specimen, confirming WN virus infection. This is the first confirmed case of WN virus disease in a Domestic Goose in North America.

Relative abundance of bird species, in concert with seroprevalence, is needed to identify candidate avian reservoir hosts for WN virus. We estimated the relative abundances of the six species for which at least seven birds were surveyed in Queens (Table 4). From this analysis, we estimated that House Sparrows contributed 82% to 97% of all WN virus infections in these six species. Rock Doves contributed 3% to 16%, and the other four species contributed negligibly to the total number of infections.

Table 4. Estimated relative abundance of six bird species with West Nile virus seroprevalence and estimated relative number of infections, suburban northeastern Queens

Bird species	Relative abundance	WN virus Ab prevalence [95% CI]	Relative no. of infections (%)[a]	Percentage range[b]
House Sparrow	6,000	0.60 [0.36-0.81]	4186 (92)	82-97
Rock Dove	1,000	0.27 [0.15-0.41]	314 (7)	3-16
Mallard	60	0.06 [0.002-0.35]	4 (<1)	<1-<1
Canada Goose	60	0.29 [0.04-0.71]	20 (<1)	<1-2
Domestic Chicken	3	0.63 [0.55-0.71]	2 (<1)	<1-<1
Domestic Goose	1	0.86 [0.42-1.00]	1 (<1)	<1-<1

Ab: antibody; CI: confidence interval.
[a]Adjusted relative to Domestic Goose.
[b]This range is determined as follows for each species. For lower bound, the lowest bound of the seroprevalence CI is used to estimate the total relative number of infections; the upper bound of this CI is used for all other species. The converse is assumed for the calculation of the upper bound of the percentage.

Conclusion

In our study, we investigated seroprevalence for WN virus in resident birds in New York City during September 1999. Seropositive birds were widely spread throughout the New York City region, and local transmission was documented in all five counties surveyed. However, transmission was significantly greater in certain neighborhoods (e.g., northeastern Queens). Comparing the seroprevalences in bird species at one such focus (northeastern Queens), we identified several species of birds that were frequently exposed and that thus could be useful sentinels or important reservoir hosts in the WN virus transmission cycle. Geese, chickens, House Sparrows, and Rock Doves in Queens all had high-level seroprevalences, consistent with the exposure of these species to WN virus in the Romanian outbreak of 1996 (9). These species should be considered for use as captive or free-ranging sentinels for WN virus activity.

Vertebrate seroprevalence data may provide clues to the identity of important reservoir hosts. An important reservoir for WN virus must be abundant relative to other bird species, frequently exposed to infection, and biologically capable of infecting hematophagous arthropods (10). Although we did not directly evaluate abundance or competence, we estimated relative abundance (Table 4). Other studies have evaluated competence of various birds experimentally infected with the New York strain of WN virus. Chickens were unable to develop sufficient viremia to infect large proportions of *Culex* mosquitoes that feed on them (11-13). Although 3-week-old Domestic Geese (*A. anser*) develop infectious-level viremia (14), adult Canada Geese were incompetent (CDC, unpub. data). Rock Doves were similarly incompetent, but House Sparrows maintained infectious-level viremia for several days (CDC, unpub. data). Thus, of the species we evaluated for seroprevalence, the House Sparrow was an important reservoir host because of its abundance, high seroprevalence, and biological competence.

Although some abundant species such as House Sparrow and Rock Dove were well represented in our survey, others were not, such as several icterid species (blackbirds), European Starling, American Robin, and American Crow. Crows were noticeably absent from our study sites and may have been locally extirpated by WN virus. Further studies are required to generate estimates of seroprevalence in these abundant resident bird species.

Seroprevalence data in birds may be difficult to interpret. To rule out alternative flavivirus infection, the birds sampled in our study were tested for antibodies to both WN and SLE viruses. As a result, we detected evidence of SLE (but not WN) virus infection in two resident birds: an adult House Sparrow from New Rochelle (Westchester County) and a 1-year-old captive pigeon in Valley Stream (Nassau County). We also collected age data on the birds sampled and found that numerous seropositive birds were aged as "hatching year" birds, thus confirming that transmission occurred in the current year. We did not have an adequate sample of birds of

a single species of different known ages to evaluate whether the seroprevalence patterns in age categories fit an epizootic rather than an enzootic pattern.

Our seroprevalence data should be interpreted with caution. The main conclusions are 1) birds were heavily exposed to WN virus in certain locations in New York City (e.g., northeastern Queens); 2) at least some, if not all, WN virus activity in northeastern Queens occurred in 1999; 3) certain species such as geese, chickens, House Sparrows, and Rock Doves were frequently infected and are likely to serve as effective WN virus sentinels in urban transmission foci; and 4) House Sparrows in particular served as hosts for most of the avian WN virus infections in the bird populations we sampled in northeastern Queens and appear to be an important reservoir host there.

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References

1. Komar N. West Nile viral encephalitis. Rev Sci Tech 2000;19:166-76.
2. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999; 286:2333-7.
3. Anderson JF, Andreadis TG, Vossbrinck CR, Tirrell S, Wakem EM, French RA, et al. Isolation of West Nile virus from mosquitoes, crows, and a Cooper's Hawk in Connecticut. Science 1999;286:2331-3.
4. Nash D, Mostashari F, Fine A, Miller J, O'Leary D, et al. The outbreak of West Nile virus infection in the New York City Area in 1999. N Engl J Med 2001;344:1807-14.
5. Office International des Epizooties. West Nile fever in the United States of America in horses. Disease Information 1999;12:150-1.
6. Steele KE, Linn MJ, Schoepp RJ, Komar N, Geisbert TW, Manduca RM, et al. Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, New York. Vet Pathol 2000;37:208-24.
7. Eidson M, Komar N, Sorhage F, Nelson R, Talbot T, Mostashari F, et al. Crow deaths as a sentinel surveillance system for West Nile virus in North America, 1999. Emerg Infect Dis 2001;7:615-620.
8. Monath TP, editor. Saint Louis encephalitis. Washington: American Public Health Association; 1980.
9. Savage HM, Ceianu C, Nicolescu G, Karabatsos N, Lanciotti R, Vladimirescu A, et al. Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serologic and molecular characterization of a virus isolate from mosquitoes. Am J Trop Med Hyg 1999;61:600-11.
10. Scott TW. Vertebrate host ecology. In: Monath TP, editor. The arboviruses: Epidemiology and ecology. Vol I. Boca Raton (FL): CRC Press; 1988. p. 257-80.
11. Turell MJ, O'Guinn M, Oliver J. Potential for New York mosquitoes to transmit West Nile virus. Am J Trop Med Hyg 2000;62:413-4.
12. Senne DA, Pedersen JC, Hutto DL, Taylor WD, Schmitt BJ, Panigrahy B. Pathogenicity of West Nile virus in chickens. Avian Dis 2000;44:642-9.
13. Langevin SA, Bunning M, Davis B, Komar N. Experimental infection of chickens as candidate sentinels for West Nile virus. Emerg Infect Dis 2001;7:726-9.
14. Swayne DE, Beck JR, Smith C, Shieh W, Zaki S. Fatal encephalitis and myocarditis in young domestic geese (*Anser anser domesticus*) caused by West Nile virus. Emerg Infect Dis 2001;7:751-3.

West Nile Virus Isolates from Mosquitoes in New York and New Jersey, 1999

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An outbreak of encephalitis due to West Nile (WN) virus occurred in New York City and the surrounding areas during 1999. Mosquitoes were collected as part of a comprehensive surveillance program implemented to monitor the outbreak. More than 32,000 mosquitoes representing 24 species were tested, and 15 WN virus isolates were obtained. Molecular techniques were used to identify the species represented in the WN virus-positive mosquito pools. Most isolates were from pools containing *Culex pipiens* mosquitoes, but several pools contained two or more *Culex* species.

In late August 1999, an outbreak of human encephalitis was detected in New York City (NYC) (1). The first cases occurred in a small area in northern Queens and were immunoglobulin M seropositive against St. Louis encephalitis (SLE) virus. The etiologic agent was West Nile (WN) virus (2,3), a member of the Japanese encephalitis virus complex (genus *Flavivirus*, family Flaviviridae), which includes other mosquito-transmitted human pathogens such as Japanese encephalitis virus, SLE virus, Murray Valley encephalitis virus, and Kunjin viruses (4). Both SLE virus, which is a native North American arbovirus, and WN viruses are zoonotic agents maintained in a transmission cycle involving bird and mosquito species (4,5).

Outbreak investigations identified human and animal cases, virus-positive dead birds, seropositive live birds, and virus-positive mosquitoes, indicating widespread virus transmission throughout the NYC metropolitan area (6,7). Sixty-two laboratory-confirmed human cases with clinical illness occurred (46 in NYC, 15 in surrounding suburbs in Westchester and Nassau counties, and 1 in a Canadian tourist who visited NYC) (8). The earliest detected onset of human illness occurred during the first week of August and the latest during the third week of September 1999 (2). In this report, we describe the mosquito surveillance program conducted in response to the outbreak and discuss mosquito species associated with WN virus transmission in 1999.

Materials and Methods

Surveillance designed to monitor mosquito populations associated with the outbreak and determine the species and proportion of mosquitoes carrying the virus was initiated in NYC and surrounding counties during the first 2 weeks of September. NYC and most surrounding counties had not maintained systematic mosquito surveillance and control programs before this outbreak. As a result, no information was available about the density or distribution of mosquito species in the area (1). The exceptions were Nassau and Suffolk counties, NY, and all counties in New Jersey (NJ), where comprehensive mosquito control programs, including surveillance for eastern equine encephalitis (EEE) virus activity, had been in effect for many years. As widespread virus transmission became apparent, mosquitoes were collected from a broader geographic area. Existing mosquito control programs participated by expanding mosquito sampling and providing specimens for testing.

Mosquitoes were collected from September 2, 1999, through October 29, 1999. Some *Culex* species mosquitoes collected earlier in the season as part of long-term EEE virus monitoring programs were provided by Suffolk and Nassau counties to assess evidence of infection in mosquitoes before the onset of human cases. Carbon dioxide-baited CDC miniature light traps (9) or traps of similar design were used to collect host-seeking adult female mosquitoes of various species. CDC gravid traps (10) or traps of similar design were used to collect gravid female mosquitoes (i.e., those that had taken a blood meal and were searching for a site to lay eggs) of the genus *Culex.* Although WN virus has been isolated from >40 mosquito species and several species of ticks (11), *Culex* species mosquitoes have been frequently associated with transmission of SLE and WN viruses (4,12,13).

Mosquitoes were placed in labeled tubes, frozen and held at -70°C, and shipped to the Centers for Disease Control and Prevention, Fort Collins, Colorado. The specimens were identified to species if possible, but the condition of certain morphologically similar *Culex* mosquitoes often prevented this. Morphologic characteristics essential for accurate species identification are often damaged during mosquito

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collection and shipping (and as a result of natural aging of mosquitoes). Therefore, many specimens were only identified to the level of genus or to a species group (e.g., *Cx. pipiens/ restuans* group, which includes the morphologically similar *Cx. pipiens* and *Cx. restuans* species). All specimens, including those that appeared to contain blood meals or partially digested blood meals, were tested for virus. Therefore, the virus infection rate in the mosquito population reflects the proportion of mosquitoes that had contacted a viremic host. Specimens were grouped into pools of 50 (by species, date, and location of collection) and were tested for virus. Every mosquito pool was tested by a Vero cell plaque assay (14), which is sensitive to all North American mosquito-transmitted pathogenic viruses and many nonpathogenic mosquito-transmitted viruses. After WN virus was determined to be the etiologic agent, a WN virus-specific reverse transcriptase-polymerase chain reaction (RT-PCR) assay (15) was used in conjunction with the Vero cell plaque assay to detect and identify WN virus in mosquito pools. Other viruses isolated in the plaque assay were identified by virus-specific RT-PCR (R. Lanciotti, unpub. data). The identity of the mosquitoes in virus-positive pools was subsequently determined or verified by species-diagnostic PCR (16). This technique, based on interspecific nucleic acid sequence variation, identifies *Cx. pipiens*, *Cx. restuans*, or *Cx. salinarius* (in combination or alone) in a pool of 50 mosquitoes.

Results

During the surveillance program, 32,814 mosquitoes representing 25 species were collected and tested for WN virus in 1,853 pools (Table 1). More than half of mosquitoes tested (18,016) were in the genus *Culex*; most of these could not be identified to species but were likely *Cx. pipiens* or *Cx. restuans*. In the remaining specimens, the predominant species were the floodwater mosquitoes *Aedes vexans* and *Ae. trivittatus*. The collection period, number of *Culex* mosquitoes, and number of other mosquito species tested for each of the 10 NY and 10 NJ counties providing specimens are listed in Table 2. The number collected and tested was not a good representation of the relative population density of *Culex* and other species mosquitoes because sampling was not consistent across participating counties. The total number collected was higher in areas where sampling was more intense. The numbers of *Culex* and other species within a county were representative of the relative abundance of various mosquito larval habitats where mosquito traps were placed (e.g., permanent water sites appropriate for *Cx. pipiens* and *Cx. restuans* development vs. floodwater habitats appropriate for *Ae. vexans* and *Ae. trivittatus*).

Table 1. Mosquito species identification by morphologic characteristics, New York and New Jersey, 1999

Genus	Species	Total
Aedes	*albopictus*	8
	canadensis	26
	cantator	55
	cinereus	426
	japonicus	64
	sollicitans	178
	sticticus	175
	taeniorhynchus	187
	triseriatus	132
	trivittatus	3,274
	vexans	7,956
	unidentified *Aedes* sp.	901
Anopheles	*bradleyi*	1
	punctipennis	23
	quadrimaculatus	77
	walkeri	32
	unidentified *Anopheles* sp.	12
Coquillettidia	*perturbans*	155
Culiseta	*melanura*	587
Culex	*erraticus*	4
	pipiens	511
	pipiens/restuans	4,686
	restuans	215
	salinarius	1,866
	territans	8
	unidentified *Culex* sp.	10,726
Psorophora	*ferox*	245
	unidentified *Psorophora* sp.	6
Uranotaenia	*sapphirina*	31
Unidentified genus	unidentified mosquito sp.	256
Total		32,814

Table 2. Mosquito species tested for West Nile virus, New York and New Jersey, 1999

	Collection dates		No. tested	
	From	Through	*Culex* sp.	Other sp.
New York counties (borough)				
Bronx[a]	9/2/99	10/26/99	166	4,679
Kings (Brooklyn)[a]	9/11/99	10/26/99	122	24
New York (Manhattan)	9/11/99	10/26/99	1,344	93
Queens[a]	9/10/99	10/26/99	6,245	156
Richmond (Staten Island)	10/2/99	10/26/99	18	38
Nassau[a]	8/19/99	10/22/99	1,301	846
Orange	9/13/99	9/13/99	80	16
Rockland	9/13/99	10/5/99	171	1,877
Suffolk[a]	6/8/99	10/20/99	6,849	1,217
Westchester[a]	9/8/99	10/19/99	334	1,206
New Jersey counties				
Bergen	9/22/99	10/20/99	48	328
Burlington[b]	10/4/99	10/26/99	0	234
Camden[b]	10/4/99	10/25/99	0	53
Cape May[b]	9/15/99	10/30/99	0	90
Essex	9/24/99	10/12/99	18	521
Hudson[a]	9/9/99	10/20/99	1,281	3,255
Middlesex	9/24/99	9/30/99	9	25
Ocean[b]	9/29/99	9/29/99	0	3
Salem[b]	9/29/99	10/28/99	0	142
Warren	10/28/99	10/28/99	7	3

[a]Counties in which West Nile virus-infected *Culex* species mosquitoes were collected.
[b]Other species tested are primarily *Culiseta melanura* collected as part of New Jersey's long-term eastern equine encephalitis surveillance program.

Suffolk County, NY, was an exception. Total collections in Suffolk County were very large, and *Culex* species mosquitoes were selectively submitted for testing. Several NJ counties provided mainly *Culiseta melanura* mosquitoes for testing. This species feeds almost exclusively on birds and is the primary enzootic vector of EEE virus. These specimens were solicited to determine if WN virus-infected birds were being fed upon as they migrated south in late summer and early fall. WN virus-infected mosquitoes were collected in six NY counties and one NJ county.

WN virus was isolated from 15 pools of mosquitoes (Table 3). All isolates were from *Culex* species. Identification of the species composition of these pools by molecular techniques indicated that six pools contained exclusively *Cx. pipiens* and

Table 3. West Nile virus-positive mosquito pools, New York and New Jersey, 1999

County	Collection date	Species[a] (morphologic id.)	Species[b] (molecular id.)
Queens, NY	9/12/99	*Culex pipiens*	*Cx. pipiens*
	9/13/99	*Cx. pipiens*	*Cx. pipiens*
	9/13/99	*Cx.* species	*Cx. pipiens/restuans*
	9/19/99	*Cx.* species	*Cx. pipiens*
	9/20/99	*Cx.* species	*Cx. pipiens*
	10/10/99	*Cx. pipiens/restuans*	insufficient sample
Kings (Brooklyn), NY	9/12/99	Cx. species	*Cx. pipiens*
	9/15/99	*Cx.* species	*Cx. restuans/salinarius*
Bronx, NY	9/12/99	*Cx.* species	*Cx. restuans/salinarius*
Nassau, NY	9/29/99	*Cx. pipiens*	*Cx. pipiens*
	10/3/99	*Cx.* species	*Cx. pipiens/restuans/salinarius*
	10/10/99	*Cx. pipiens/restuans*	*Cx. pipiens/restuans/salinarius*
Suffolk, NY	10/4/99	*Cx. species*	*Cx. restuans/salinarius*
Westchester, NY	10/1/99	*Cx. restuans*	*Cx. restuans/salinarius*
Hudson, NJ	9/28/99	*Cx. pipiens*	insufficient sample

[a]Species identification by morphologic characteristics.
[b]Species identification by species-specific polymerase chain reaction primers.

seven contained two or more *Culex* species (combinations of *Cx. pipiens*, *Cx. restuans*, and *Cx. salinarius*). Two pools contained insufficient material for molecular species identification. The only evidence that another species was involved in WN virus transmission in 1999 was the isolation of WN virus from a pool of *Ae. vexans* mosquitoes collected on September 14, 1999, in southwestern Connecticut (7). The earliest WN virus isolates in NY and NJ came from collections made on September 12, 1999, in Queens, Brooklyn, and the Bronx. The latest WN virus isolate came from collections made on October 10, 1999, in Queens and Nassau County. Most isolates were from Queens, which was the location of most human WN-virus infection cases (6).

Other viruses were isolated from mosquitoes during the surveillance program (Table 4). Flanders virus was isolated from 11 pools of *Culex* species mosquitoes, most of which contained combinations of species. Flanders virus is a widely distributed rhabdovirus frequently found in birds and bird-feeding mosquitoes and apparently nonpathogenic in vertebrates (17). EEE virus was isolated from a pool of *Cs. melanura* collected in Burlington County, NJ. Three isolates of a California serogroup virus were obtained from pools of *Ae. trivitattus* collected in the Bronx and Nassau County, NY. Numerous California serogroup viruses are present in this region of North America (18). Although these California serogroup isolates were not specifically identified for this study, they are likely trivitattus virus, a generally nonpathogenic member of the California serogroup commonly found in *Ae. trivittatus* (19).

The minimum infection rate (MIR) of WN virus in *Culex* mosquitoes, expressed as the number infected per 1,000 specimens tested, was calculated by county for the sampling periods (weeks) during which WN virus was isolated from mosquitoes (Table 5). MIR for a given period and location is an indicator of prevalence of virus in the habitat and of transmission intensity and, in many circumstances, is related to the risk for human disease. All *Culex* mosquitoes collected in a county during a particular week, except *Cx. territans,* which feeds predominantly on amphibians, were combined to determine the denominator for this value because many of the *Culex* specimens could not be identified below genus or species levels. As a result, MIR estimates probably underestimate the infection rate for certain *Culex* species and overestimate the rate for others. MIR for WN virus-infected *Culex* in this outbreak was 0.7/1,000 to 57.1/1,000, although the 95% confidence intervals are very large around MIR estimates calculated from small sample sizes.

Conclusion

Mosquito surveillance, although not implemented until late in the outbreak (well after most transmission to humans that resulted in clinical cases), provided information about transmission dynamics that may prove useful in developing

Table 4. West Nile virus-positive mosquito pools containing viruses other than West Nile virus, collection location, date, species composition, and virus identification

County	Collection date	Species[a] (morphologic id.)	Species[b] (molecular id.)	Virus identification
Bronx, NY	9/9/99	*Aedes trivittatus*	not done	California serogroup
	9/12/99	*Ae. trivittatus*	not done	California serogroup
Nassau, NY	10/15/99	*Culex pipiens/restuans*	*Cx. pipiens/restuans/salinarius*	Flanders
	10/16/99	*Ae. trivittatus*	not done	California serogroup
Suffolk, NY	6/29/99	*Cx. pipiens/restuans*	*Cx. pipiens/restuans*	Flanders
	6/29/99	*Cx. pipiens/restuans*	*Cx. restuans*	Flanders
	7/7/99	*Cx. pipiens/restuans*	*Cx. pipiens/restuans*	Flanders
	7/27/99	*Cx. pipiens/restuans*	*Cx. pipiens/restuans/salinarius*	Flanders
	8/3/99	*Cx. pipiens/restuans*	*Cx. pipiens/restuans/salinarius*	Flanders
	8/10/99	*Cx. pipiens/restuans*	insufficient sample	Flanders
	8/10/99	*Cx. pipiens/restuans*	insufficient sample	Flanders
	8/16/99	*Cx. pipiens/restuans*	insufficient sample	Flanders
	9/28/99	*Cx. restuans*	*Cx. restuans/salinarius*	Flanders
Hudson, NJ	9/22/99	*Cx. pipiens*	*Cx. pipiens*	Flanders
Burlington, NJ	10/11/99	*Culiseta melanura*	not done	Eastern equine encephalitis

[a]Species identification by morphologic characteristics.
[b]Species identification by species-specific polymerase chain reaction primers.

Table 5. West Nile (WN) virus infection rates in *Culex* species mosquitoes, New York and New Jersey, 1999

Sampling period	County	#WN virus-positive pools	Total *Culex* specimens tested[a]	MIR[b]	(95% Confidence Interval)
9/12-9/19	Queens	3	820	3.7	(0.8-10.7)
	Kings (Brooklyn)	2	35	57.1	(7.0-191.6)
	Bronx	1	48	20.8	(0.5-110.7)
9/19-9/25	Queens	2	862	2.3	(0.3-8.3)
9/26-10/2	Nassau	1	198	5.1	(0.1-27.8)
	Hudson, NJ	1	138	7.2	(0.2-39.7)
	Westchester	1	92	10.8	(2.0-54.5)
10/3-10/9	Nassau	1	214	4.7	(0.1-25.8)
	Suffolk	1	810	1.2	(0.03-6.9)
10/10-10/16	Queens	1	1496	0.7	(0.02-3.7)
	Nassau	1	135	7.4	(0.2-40.6)

[a]Excluding *Culex territans*.
[b]Minimum infection rate expressed as number infected per 1,000 specimens tested.

new surveillance systems. *Culex* mosquitoes, particularly *Cx. pipiens*, appear primarily responsible for epizootic transmission. *Cx. pipiens* was quite common in Queens, NY, and other areas where isolates were obtained and transmission activity was documented by avian and human surveillance programs. *Cx. restuans* and *Cx. salinarius* were also implicated in virus transmission. Since these species were found only in combination in WN virus-positive pools, their importance is difficult to assess. *Cx. pipiens* and *Cx. restuans* are ornithophilic, feeding mainly on birds and occasionally on mammals (20). *Cx. salinarius*, which is a pest species common in the region (21), feeds readily on humans and other mammals (20), which suggests that it may be involved in epidemic transmission of WN virus.

Relatively high MIR values in areas where human cases occurred validate use of mosquito-based surveillance to estimate risk for virus transmission to humans. MIRs found in this study are consistent with MIRs calculated for WN virus in mosquitoes reported in other areas. MIR estimates for the primary vector species during WN virus outbreaks range from 0.8/1,000 for *Cx. fatigans* in India (22) to as high as 25.0/1,000 for *Cx. univittatus* in South Africa (23). While it is difficult to associate a quantified risk for human disease to an MIR value, evidence from *Cx. pipiens*-borne SLE outbreaks indicates that widespread transmission to humans is likely when MIR exceeds 3/1,000 but may occur at much lower infection rates (24).

Mosquito-based virus surveillance has its limitations. Adequate estimates of virus distribution and transmission require extensive field and laboratory resources to obtain and process large sample sizes over relatively large geographic areas. In addition, identification of field-collected *Culex* mosquito specimens to species by morphologic characters is difficult, and verification of species composition in pools often requires use of molecular techniques not commonly available to mosquito surveillance programs. The importance of accurate mosquito species identification is underscored by the indication that *Cx. salinarius* may have been involved in WN-virus transmission during 1999. This information was not evident from morphologic identification and was determined only by molecular techniques. Accurate identification of species is essential in estimating risk for transmission to humans and directing mosquito control programs.

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References

1. Centers for Disease Control and Prevention. Outbreak of West Nile-like viral encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:845-9.
2. Centers for Disease Control and Prevention. Update: West Nile Virus encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:944-6,955.
3. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333-7.
4. Hayes CG. West Nile fever. In: Monath TP, editor. The arboviruses: epidemiology and ecology. Vol V. Boca Raton (FL): CRC Press; 1989. p. 59-88.
5. Monath TP. Epidemiology. In: Monath TP, editor. St. Louis encephalitis. Washington: American Public Health Association; 1980. p. 239-312.
6. Centers for Disease Control and Prevention. Update: West Nile-like viral encephalitis-New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:890-2.
7. Anderson JF, Andreadis TG, Vossbrinck CR, Tirrell S, Wakem EM, French RA, et al. Isolation of West Nile virus from mosquitoes, crows, and a Cooper's hawk in Connecticut. Science 1999;286:2331-3.
8. Asnis DS, Conetta R, Teixeira AA, Waldman G, Sampson BA. The West Nile virus outbreak of 1999 in New York: the Flushing Hospital experience. Clin Infect Dis 2000;30:413-8.
9. Newhouse VR, Chamberlain RW, Johnston JF, Sudia WD. Use of dry ice to increase mosquito catches of the CDC miniature light trap. Mosquito News 1966;26:30-5.
10. Reiter P. A portable, battery-powered trap for collecting gravid *Culex* mosquitoes. Mosquito News 1983;43:496-8.
11. Hubálek Z, Halouzka J. West Nile fever, a reemerging mosquito-borne viral disease in Europe. Emerg Infect Dis 1999;5:643-50.
12. Mitchell CJ, Francy DB, Monath TP. Arthropod vectors. In: Monath TP, editor. St. Louis encephalitis. Washington: American Public Health Association; 1980. p. 313-79.
13. Savage HM, Ceianu C, Nicolescu G, Karabatsos N, Lanciotti R, Vladimirescu LL, et al. Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serologic and molecular characterization of a virus isolate from mosquitoes. Am J Trop Med Hyg 1999;61:600-11.
14. Beaty BJ, Calisher CH, Shope RS. Arboviruses. In: Schimdt NJ, Emmons RW, editors. Diagnostic procedures for viral, rickettsial and chlamydial infections. Washington: American Public Health Association; 1989. p. 797-856.
15. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. Rapid detection of West Nile virus from human clinical specimens, field collected mosquitoes, and avian samples by a TaqMan RT-PCR assay. J Clin Microbiol 2000;38:4066-71.

16. Crabtree MB, Savage HM, Miller BR. Development of a species-diagnostic polymerase chain reaction assay for the identification of *Culex* vectors of St. Louis encephalitis virus based on interspecies sequence variation in ribosomal DNA spacers. Am J Trop Med Hyg 1995;53:105-9.
17. Kokernot RH, Hayes J, Will RL, Radivojevic B, Boyd KR, Chan DHM. Arbovirus studies in the Ohio-Mississippi basin, 1964-1967. III Flanders virus. Am J Trop Med Hyg 1969;18:762-7.
18. Calisher CH. Taxonomy, classification, and geographic distribution of California serogroup Bunyaviruses. In: Calisher CH, Thompson WH, editors. California serogroup viruses. New York: Alan R. Liss, Inc.; 1983. p. 1-6.
19. Sudia WD, Newhouse VF, Calisher CH, Chamberlain RW. California group arboviruses: isolations from mosquitoes in North America. Mosquito News 1971;31:576-600.
20. Mitchell CJ, Francy DB, Monath TP. In: Monath TP, editor. St. Louis encephalitis. Washington: American Public Health Association; 1980. p. 313-79.
21. Slaff M, Crans WJ. Impounded water as a major producer of *Culex salinarius* (Diptera: Culicidae) in coastal areas of New Jersey, USA. J Med Entomol 1982;19:185-90.
22. Pavri KM, Singh KRP. Isolations of West Nile virus from *Culex fatigans* mosquitoes from western India. Indian J Med Res 1965;53:501-5.
23. McIntosh BM, Jupp PG, Dos Santos I, Meenehan GM. Epidemics of West Nile and Sindbis viruses in South Africa with *Culex (Culex) univittatus* Theobald as vector. S Afr J Sci 1976;72:295-9.
24. Bowen GS, Francy DB. Surveillance. In: Monath TP, editor. St. Louis encephalitis. Washington: American Public Health Association; 1980. p. 473-99.

Dead Bird Surveillance as an Early Warning System for West Nile Virus

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As part of West Nile (WN) virus surveillance in New York State in 2000, 71,332 ill or dead birds were reported; 17,571 (24.6%) of these were American Crows. Of 3,976 dead birds tested, 1,263 (31.8%) were positive for WN virus. Viral activity was first confirmed in 60 of the state's 62 counties with WN virus-positive dead birds. Pathologic findings compatible with WN virus were seen in 1,576 birds (39.6% of those tested), of which 832 (52.8%) were positive for WN virus. Dead crow reports preceded confirmation of viral activity by several months, and WN virus-positive birds were found >3 months before the onset of human cases. Dead bird surveillance appears to be valuable for early detection of WN virus and for guiding public education and mosquito control efforts.

In the late summer and fall of 1999, New York State (NYS) had the first outbreak of West Nile (WN) virus encephalitis in the Western Hemisphere (1). The nucleotide sequence of the viruses isolated during this outbreak was most similar to that of a 1998 isolate from a goose in Israel (2). By the end of 1999, 62 human cases, 7 fatal, had occurred in New York City (NYC) and two neighboring counties, Nassau and Westchester (3).

Although WN virus infection was confirmed in dead birds shortly before it was confirmed in humans, no WN virus-positive dead birds were identified from time periods before the onset of symptoms in the first human cases, despite subsequent WN virus testing of birds collected earlier (4). Whether dead bird surveillance could provide an early warning for human WN virus cases could not be definitively established by analyses of 1999 data on dead bird surveillance. However, sightings of dead crows preceded laboratory confirmation of viral activity in any species, and testing of dead birds provided valuable information about the temporal and geographic spread of the virus (4).

We evaluate the usefulness of dead bird surveillance in 2000 for detecting geographic spread of WN virus and providing an early warning of the risk for transmission to humans. We also discuss lessons learned for other states that may be instituting a similar system.

Methods

For WN virus surveillance, the New York State Department of Health (NYSDOH) developed and implemented an integrated electronic system based on the department's existing infrastructure for secure web-based electronic health information interchange with local health units, health-care facilities, and providers (5). The functional component of the infrastructure is called the Health Information Network, into which local health units entered data about sightings of ill or dead birds.

Freshly dead birds were submitted by local health units to the New York State Department of Environmental Conservation's Wildlife Pathology Unit for necropsy, which included evaluation of gross pathologic indications of WN virus infection and other possible causes of death. Organs collected for laboratory testing included brain, kidney, heart, liver, and spleen. Necropsy results were entered by the Wildlife Pathology Unit into the Health Information Network.

Local health units were permitted to send any species of birds for possible necropsy and WN virus testing. However, American Crows, Blue Jays, and Fish Crows, members of the Corvid family, which was most affected by the WN virus outbreak in 1999, were a top priority for submission, followed by raptors and house sparrows. As the outbreak progressed, birds from counties without documented WN virus were given higher priority, as well as migrating species of birds.

Most laboratory testing on dead birds was done at the NYSDOH Wadsworth Center, as described (6). WN virus infection was confirmed by at least two positive assays. Additional testing for overflow specimens was done at the National Wildlife Health Center laboratory in Madison, Wisconsin, as described (4).

Data from the Health Information Network were downloaded into Microsoft Excel and Microsoft Access files, and those software programs, along with SAS (Chapel Hill, NC), were used for descriptive statistical analyses. Microsoft PowerPoint was used for graphic representations of data and MapInfo (Troy, NY) for mapping. For data analysis, data were aggregated by report week, as requested by the Centers for Disease Control and Prevention for national surveillance.

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Results

For 2000, 71,332 ill or dead birds, of which 17,571 (24.6%) were American Crows, were reported through the Health Information Network. Of 3,976 dead birds tested by NYSDOH's Wadsworth Center or the National Wildlife Health Center, 1,263 (31.8%) were positive for WN virus. These WN virus-positive birds represented 63 species, 30 families, and 14 orders (7); most were American Crows (846 birds, 67%).

Most of the ill or dead birds (62,339 [87.4%]) were found singly. For sightings of multiple birds, the number of birds reported ranged from 2 to 100 (mean 2.8). Only 675 (0.95%) of the birds were seen alive and ill; the others were reported as dead. Symptom information was provided for 582 of the ill birds, with "neurologic signs" listed for 413 (71%). Four of these tested positive for WN virus after death.

Of the dead birds tested for WN virus, 1,576 (39.6%) had one or more signs compatible with WN virus (8), such as emaciation, splenomegaly, hepatomegaly, cardiac or pericardial lesions, or possible signs of encephalitis (Table). Of these birds, 832 (52.8%) subsequently tested positive for WN virus (overall positive predictive value for pathologic findings). Before the onset date for the first human case in NYS in 2000 (July 20), the sensitivity of gross pathologic findings (the proportion of WN virus-positive birds that had suspicious pathology) was highest in American Crows (51.8%). The overall positive predictive value (PPV) for pathologic findings was 27.9% for this time period. The overall specificity for the necropsy evaluation was high for most species tested, with 90.3% of WN virus-negative birds having no gross pathologic indication of WN virus. The negative predictive value (NPV) for necropsy evaluation was 85.3% before the onset of human cases.

For birds collected on or after the human case onset, the overall sensitivity and PPV increased to 68% and 55.1%, respectively, while the specificity and NPV decreased to 62.1% and 73.9%, respectively. The least sensitive species was the House Sparrow; 18.8% of those testing positive had pathologic signs on necropsy. Before the onset of the first human case, American Crows had significantly higher sensitivity, PPV, specificity, and NPV than other species combined. After the onset of the first human case, crows were significantly higher in sensitivity and PPV but significantly lower in specificity and NPV ($p<0.05$). When values for all species combined before human case onset were compared with values after onset, sensitivity and PPV significantly increased, while specificity and NPV significantly decreased ($p<0.001$).

Signs of trauma were found on necropsy in 1,885 (47%) of the birds tested for WN virus. Of these birds, 480 (25.5%) subsequently tested positive for WN virus (PPV). In comparison, 1,308 (63%) of the 2,091 birds without trauma tested negative for WN virus (NPV). American crows without trauma were significantly more likely to test positive for WN virus (568 [49.1%] of 1,158) than crows with trauma (278 [32.9%] of 845) ($p<0.001$).

The first laboratory confirmations that the virus was still present in the United States were from areas affected in 1999: isolations in February 2000 of virus from a mosquito pool in New York City (9) and a hawk in Westchester County (tested by the University of Connecticut and the Connecticut Agriculture Experiment Station) (Figure 1, bars). However, the first evidence of viral transmission during the 2000 season was two dead crows collected in Rockland County (a county in the lower Hudson Valley affected by the outbreak in 1999) on May 22 and confirmed as positive for WN virus on June 9. One crow from Suffolk County, Long Island (another area affected by the outbreak in 1999), found dead on April 1, 2000, frozen until August, then submitted for laboratory testing, also was confirmed as positive for WN virus, making it the earliest identification of viral activity in the 2000 mosquito season.

Table. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of pathology results[a] for West Nile (WN) virus, New York State, 2000, before and after onset of first human case on July 20

	No. pos. on WN virus testing		No. neg. on WN virus testing	
Species	No. pos. on necropsy (%)[b]	No. neg. on necropsy	No. pos. on necropsy	No. neg. on necropsy (%)[c]
Jan 1 - Jul 19				
American Crow[d]	29 (51.8)	27	34	551 (94.2)
Blue Jay	7 (25)	21	37	101 (73.2)
Fish Crow	0 (--)	2	1	18 (94.7)
American Robin	0 (--)	0	5	18 (78.3)
House Sparrow	0 (--)	1	2	35 (94.6)
Other species	2 (25)	6	19	186 (90.7)
Total[e]	38 (40) (PPV=27.9%)	57	98	909 (90.3) (NPV=85.3%)
Jul 20 - Dec 31				
American Crow[d]	624 (79.0)	166	303	269 (47.0)
Blue Jay	76 (61.3)	48	124	126 (50.4)
Fish Crow	16 (84.2)	3	10	3 (23.1)
American Robin	7 (43.8)	9	32	16 (33.3)
House Sparrow	3 (18.8)	13	11	32 (74.4)
Other species	68 (33.5)	135	166	614 (78.7)
Total[e]	794 (68.0) (PPV=55.1%)	374	646	1,060 (62.1) (NPV=73.9%)
Total (all year)	832 (65.8%)	431	744	1,969 (72.6%)

[a]Gross postmortem signs considered indicative of possible WN virus infection included one or more of the following: emaciation, splenomegaly, hepatomegaly, cardiac or pericardial lesions, and possible signs of encephalitis.
[b]Sensitivity of pathologic findings on gross necropsy for detecting WN virus.
[c]Specificity of pathologic findings on gross necropsy for ruling out WN virus.
[d]Differences between American Crows and other species combined significant at 0.05 level.
[e]Differences between time periods (all species combined) significant at 0.001 level.

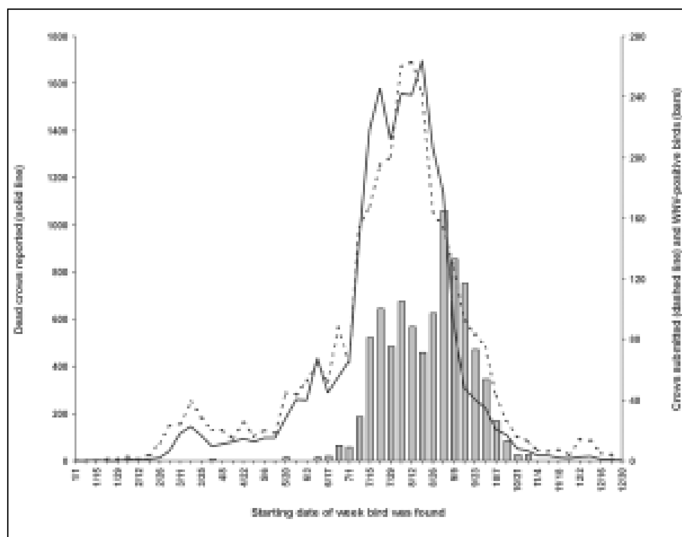

Figure 1. Sightings of ill or dead crows, dead crows submitted for possible West Nile virus testing, and West Nile virus-positive dead birds (all species) by week, New York State, 2000.

The numbers of ill or dead crow reports remained low (<10 per week) early in the year (Figure 1, solid line). Increases in dead crow sightings occurred just before the collection date for the first WN virus-positive crow of the season on April 1 and the same week that the first crows to be identified as positive were found in May, even though the results were not known until 2 weeks later. The steep increase in dead crow sightings in early July predates the onset date for the first human case (July 20) and the increase in WN virus-positive birds by several weeks. Although only a small proportion of the ill or dead crows seen were submitted for possible necropsy and WN-virus testing (Figure 1, dashed line), the number of crows submitted closely parallels the number of crows seen and reported over time.

With regard to geographic spread of the virus, dead crow reports during January-March were concentrated in the areas affected by the outbreak in 1999, as well as into the Hudson River Valley. During the period before the onset of the first human case (Figure 2a), dead crow reports increased to 4,600 in these areas, and sightings began to occur along other bodies of water, including Lake Champlain in the northeastern corner of the state, the Mohawk River and various lakes in central NYS, and Lake Erie and Lake Ontario in western NYS. Many of the state's largest cities (by human population size) are also in some of these same areas. In the period after human WN virus cases began to occur (Figure 2b), 12,530 dead crows were sighted; the highest number were from counties with viral activity in 1999. Increased expansion of reports into other counties of the state clustered around bodies of water and some population centers.

The geographic spread of the virus, as indicated by surveillance with laboratory testing of dead birds, was similar but lagged behind the dead crow reports by several months. Before the first human case, the 91 WN virus-positive dead birds in 2000 were confined primarily to the four counties near NYC with viral activity in 1999 and two of NYC's five boroughs, although WN virus-positive birds were also found in four upstate counties (Figure 2c). Subsequently, 1,171 WN virus-positive birds were reported from all but one NYS county and all five NYC boroughs (Figure 2d). The first WN virus-positive bird found in 2000 outside the 1999 outbreak area was a Rock Dove collected in central NYS on July 6.

For the first laboratory confirmation of viral activity in 2000 in 60 of the 62 NYS counties and NYC boroughs, 30 (50%) had an American Crow, 8 (13.3%) had a Blue Jay, 1 had a Fish Crow, and 21 (35%) had other bird species. One county reported a positive mosquito pool before a positive bird. The first positive "other" species included House Sparrow, Song Sparrow, Ovenbird, Catbird, Robin, Cedar Waxwing, Ruffed Grouse, Rock Dove, Mourning Dove, European Starling, Wood Thrush, Common Grackle, Ring-billed Gull, Greater Black-backed Gull, Mute Swan, Great Horned Owl, Cooper's Hawk, American Kestrel, and Red-tailed Hawk. For the counties without an American Crow or other corvid as their first positive bird species, confirmation of viral activity would have been delayed 1 to 47 days (median 13) or 1 to 41 days (median 11), respectively, if noncorvid species had not been tested. Fifteen counties with viral activity confirmed by dead bird testing (25%) never had a WN virus-positive American Crow, and nine counties never had a WN virus-positive corvid.

Conclusion

At the end of 1999, it was unknown whether a human outbreak of WN virus would recur and whether dead bird surveillance could detect any reappearance of viral activity before human infection. A dead bird surveillance system (established in NYS in 1999 after the bird and human WN virus outbreaks were recognized) was refined for 2000 to include real-time reporting of dead bird sightings by all local health units, using the state's web-based Health Information Network and laboratory testing by the NYSDOH's Wadsworth Center. In 2000, dead bird surveillance (both dead crow sightings and laboratory testing of birds) provided an early warning of WN virus activity before the first human case in NYS, both temporally and geographically. However, test results for many of the WN virus-positive birds were not known soon enough to guide prevention and control activities before the onset of illness in the first human case.

The earliest warning was provided by the dead crow sightings, with the geographic distribution of dead crow reports from earlier time periods overlapping that of WN virus-positive birds from later time periods. Before the first human case, the wider distribution of dead crow sightings compared with the distribution of WN virus-positive birds may reflect the amount of testing done. Although submissions of crows for testing occurred in proportion to the level of dead crow sightings (Figure 1), the number of birds submitted for testing may have been insufficient to confirm low levels of viral activity in some areas.

To provide an early warning of viral activity, dead bird surveillance requires capacity at the local level to let the public know where to report dead birds, as well as a system for answering phone calls, recording data, and collecting birds for testing. Resources for bird necropsies and laboratory testing are also required. The usefulness of this system for monitoring WN virus is influenced by the amount of effort expended by the public and local agencies to notice and report the dead birds. Unlike ill humans, ill or dead birds are dependent on humans to observe and investigate their condition.

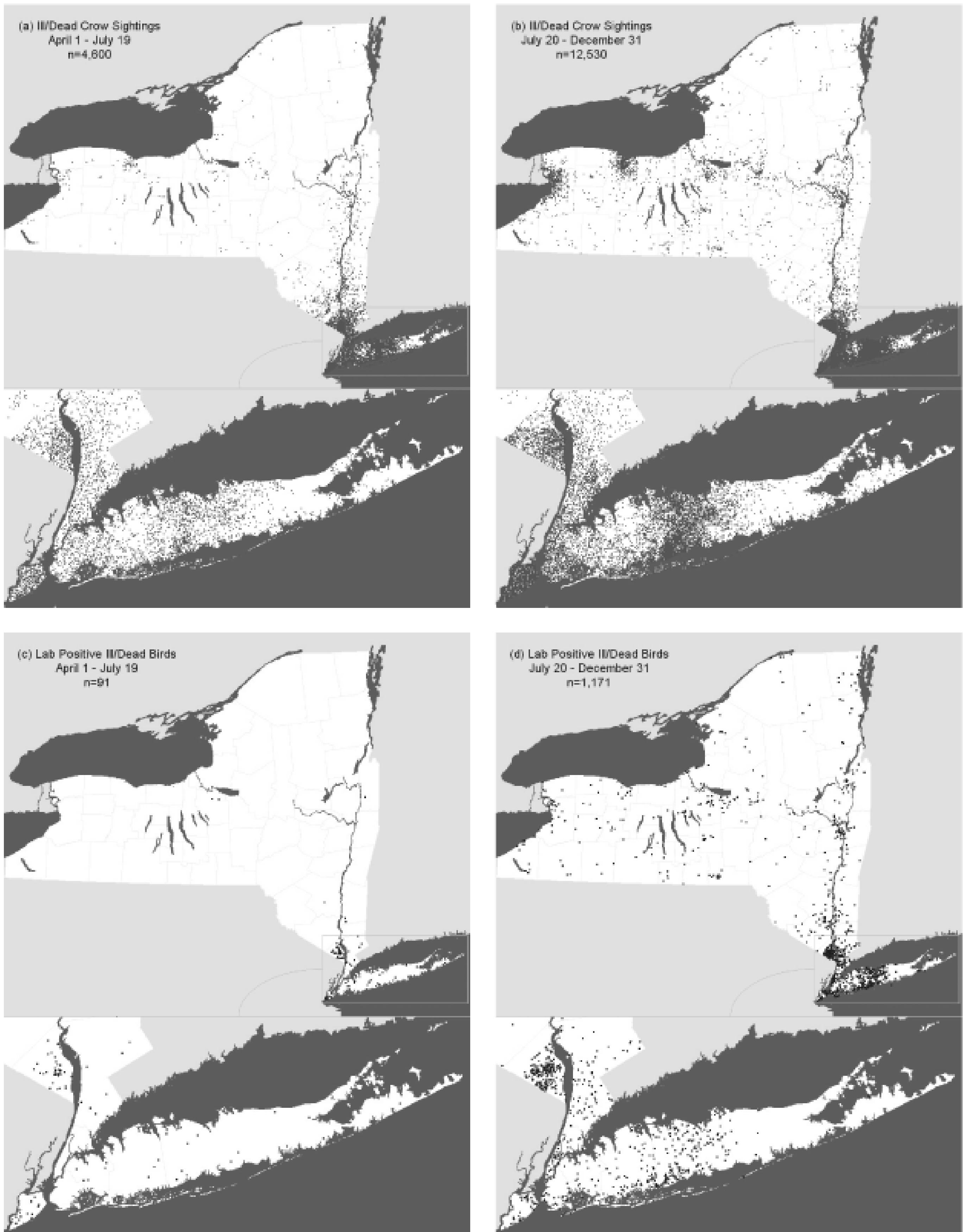

Figure 2. Maps of ill or dead crow sightings (a,b) and West Nile virus-positive dead birds of any species (c,d), New York State, 2000.

Successful dead bird surveillance can be based on a number of factors, including frequency and extent of information provided to the public to encourage reporting of dead birds, the number of people living in an area to see dead birds, and enhanced public interest when new WN virus findings or reports are issued. Potential limitations to dead bird surveillance for WN virus include absence of or scarcity of American Crows in some geographic areas or the possibility that crows will become increasingly immune to WN virus, with a consequent reduction in their case-fatality rate.

Because of the resources required for reporting and testing dead birds, agencies responding to WN virus must make decisions about whether to cast a wider net, with a more sensitive surveillance system capable of detecting the earliest viral activity, or a narrower net, with a more specific surveillance system that eliminates birds less likely to have WN virus. To provide the earliest warning of viral activity to encourage subsequent surveillance, prevention, and control, we recommend unrestricted testing by species, presence of trauma, number of dead birds seen in the area, or pathologic findings before laboratory confirmation of viral activity in an area. Once viral activity has been confirmed, laboratory testing may be conducted primarily to verify continued viral activity, and more specific submission criteria, such as restrictions to American Crows without trauma and with compatible pathologic findings, may be adopted to conserve scarce laboratory resources.

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References

1. Centers for Disease Control and Prevention. Update: West Nile Virus encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:944-6,955.
2. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333-7.
3. Nash D, Mostashari F, Fine A, Miller J, O'Leary D, Murray K, et al. Outbreak of West Nile virus infection, New York City area, 1999. N Engl J Med 2001. In press.
4. Eidson M, Komar N, Sorhage F, Nelson R, Talbot T, Mostashari F, et al. Crow Deaths as a Sentinel Surveillance System for West Nile Virus in the Northeastern United States, 1999. Emerg Infect Dis 2001;7:615-20.
5. Gotham IJ, Eidson M, White DJ, Wallace BJ, Chang HG, Johnson GS, et al. West Nile virus: A case study in how New York State health information infrastructure facilitates preparation and response to disease outbreaks. Journal of Public Health Management and Practice 2001;7(5):79-89.
6. Shi P-Y, Kauffman EB, Ren P, Felton A, Tai JH, Dupuis II AP, et al. High throughput detection of West Nile virus RNA. J Clin Microbiol 2001;39:1264-71.
7. Bernard KA, Maffei JG, Jones SA, Kauffman EB, Ebel GD, Dupuis AP, et al. West Nile virus infection in birds and mosquitoes, New York State, 2000. Emerg Infect Dis 2001;7:679-85.
8. Steele KE, Linn MJ, Schoepp RJ, Komar N, Geisbert TW, Manduca RM, et al. Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, New York. Vet Pathol 2000;37:208-24.
9. Centers for Disease Control and Prevention. Update: Surveillance for West Nile virus in overwintering mosquitoes—New York, 2000. MMWR Morb Mortal Wkly Rep 2000;49:178-9.

West Nile Virus Surveillance in Connecticut in 2000: An Intense Epizootic without High Risk for Severe Human Disease

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In 1999, Connecticut was one of three states in which West Nile (WN) virus actively circulated prior to its recognition. In 2000, prospective surveillance was established, including monitoring bird deaths, testing dead crows, trapping and testing mosquitoes, testing horses and hospitalized humans with neurologic illness, and conducting a human seroprevalence survey. WN virus was first detected in a dead crow found on July 5 in Fairfield County. Ultimately, 1,095 dead crows, 14 mosquito pools, 7 horses, and one mildly symptomatic person were documented with WN virus infection. None of 86 hospitalized persons with neurologic illness (meningitis, encephalitis, Guillain-Barré-like syndrome) and no person in the seroprevalence survey were infected. Spraying in response to positive surveillance findings was minimal. An intense epizootic of WN virus can occur without having an outbreak of severe human disease in the absence of emergency adult mosquito management.

West Nile (WN) virus was first detected in the United States in September 1999 during the investigation of an outbreak of encephalitis in humans in New York City (1). Surveillance initiated in September 1999 showed epizootic activity in all boroughs of New York City and in neighboring counties in New York, New Jersey, and Connecticut (2). Human cases of severe neurologic illness requiring hospitalization occurred in a limited central area of the larger epizootic focus. No human cases were detected in Connecticut or New Jersey (2). The epicenter of human illness was in northern Queens, where an estimated 2.6% of the population was acutely infected with WN virus and the rate of confirmed human illness requiring hospitalization was approximately 18.2 per 100,000 population (3).

The initial response to the 1999 outbreak of human illness and confirmation of WN virus activity in birds and mosquitoes in all three states included extensive spraying for adult mosquitoes to reduce the immediate risk to humans. In planning for the surveillance and public health response to the threat of WN virus reemergence in 2000, several response strategies were developed. While all three of the states initially affected and New York City developed similar surveillance and basic mosquito control strategies (4-6), the threshold for using pesticides to kill adult mosquitoes differed. Many counties in New York and some in New Jersey followed initial guidelines from the Centers for Disease Control and Prevention (7,8), which recommended focal adult mosquito control (adulticide) in a 2-mile area around the finding of either a WN virus-positive dead bird or a positive mosquito pool (7). The objective of focal spraying around WN virus-positive dead birds was to reduce any immediate risk to humans from mosquitoes that may have fed on infected birds and become infected.

In contrast, the Connecticut strategy for implementing adult mosquito control was to wait until surveillance indicators suggested a more substantial risk for an outbreak of severe human illness, rather than depending on the finding of WN virus-positive birds alone. In Connecticut, spraying was prompted by the finding of multiple WN virus-positive mosquito pools or a confirmed case of WN virus infection in a horse or human (4). This threshold was rarely reached during 2000 in Connecticut. Thus, spraying to kill adult mosquitoes was minimal: it was done only three times in local areas 2 miles in radius, once in July and twice in late September.

We describe the year 2000 WN virus surveillance experience in Connecticut to demonstrate, in a setting with minimal adult mosquito control, the magnitude of epizootic activity that can occur in the absence of severe human illness.

Methods

Prospective surveillance to detect the presence and possible amplification of WN virus was established in mid-April 2000. Surveillance included monitoring bird deaths and WN virus infection in dead crows, trapping and testing mosquitoes for WN virus, and testing horses and hospitalized

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humans with neurologic symptoms for WN virus infection. In October 2000, a human seroprevalence survey was done in two towns with intense epizootic activity. A preexisting mosquito trapping and surveillance system established to conduct surveillance for eastern equine encephalitis virus was expanded to include additional trapping sites in areas where WN virus was identified in 1999, as described (4,9). We summarize positive mosquito trapping data (9) and describe the other surveillance systems.

Bird Deaths

For surveillance of bird deaths, each of the 106 local health departments was asked to publicize a telephone number for reporting dead bird sightings and then to collect information in a standard line-list format, including the date a bird was found, species, and address. Once a week, the line list was submitted to the state Department of Public Health (DPH), where the information was entered into a statewide database with town- and county-specific information.

Testing Dead Crows

Each local health department was asked to collect dead crows and submit them for WN virus testing. Because of low submission rates from many towns, a request was made to submit all dead crows beginning August 3. Dead crows were stored in either a refrigerator or freezer (if stored >48 hours) until transport to the University of Connecticut Veterinary Diagnostic Laboratory for necropsy. At necropsy, a portion of brain tissue was excised, frozen at -20°C, and sent within several days to the DPH laboratory, where it was inoculated into Vero cells. Cultures were monitored daily for 7 days for cytopathic effect, and WN virus was identified by indirect immunofluorescence of infected cells with monoclonal antisera provided by the Centers for Disease Control and Prevention (CDC) or by reverse transcription-polymerase chain reaction with WN virus-specific primers specified by CDC (10; Lanciotti R, personal communication).

Neurologic Illness in Horses

Veterinarians statewide were informed of symptoms of WN virus infection in horses and requested to inform the Connecticut State Veterinarian of any suspicious cases. A newsletter published by the Connecticut Veterinary Medical Association in January 2000 contained the first notice, followed by a direct mailing to all licensed veterinarians in August 2000. Specimens of serum, whole blood, cerebrospinal fluid, or brain tissue were collected from rabies-negative animals and submitted to the U.S. Department of Agriculture, National Veterinary Services Laboratories in Ames, Iowa, for testing. Equine specimens were initially evaluated by an immunoglobulin (Ig) M-capture enzyme-linked immunosorbent assay (ELISA), a reverse transcriptase nested polymerase chain reaction, or virus isolation. A plaque-reduction neutralization test was used to confirm suspect serologic IgM-capture ELISA reactions.

Neurologic Illness in Humans

Encephalitis is a reportable condition in Connecticut. A newsletter was sent to all acute-care hospitals and physicians statewide to encourage reporting of encephalitis cases and testing of cerebrospinal fluid and serologic specimens from persons hospitalized with encephalitis or fever and Guillain-Barré syndrome (11). Free testing of these specimens for IgM antibodies to WN virus was offered at the DPH laboratory. Frozen brain tissue, if available, was also requested for virus isolation. Enhanced surveillance was initiated in April through monthly mailings to physicians and hospitals. Beginning in July, infection control practitioners (ICPs) in Fairfield and New Haven counties were contacted weekly and queried regarding any new cases admitted to their hospitals. As part of their hospital surveillance, infection control practitioners were asked to review logs of emergency room and hospital admissions and cerebrospinal fluid results. In early August, criteria for free testing were expanded to include patients ≥18 years old hospitalized with aseptic meningitis. Efforts were made to collect clinical information and acute-phase cerebrospinal fluid and serologic specimens on all reported suspected cases. Convalescent-phase serology was requested for those for whom acute-phase specimens tested negative and were obtained less than 8 days before onset of illness.

Free testing was done at DPH by IgM-capture ELISA on acute-phase specimens and both IgM-capture and IgG ELISA on convalescent-phase specimens. Specimens with an optical density of patient serum dilution with viral antigen (P) compared with mean optical density of normal human serum (N) (P/N ratio) of >1.0 were confirmed by serum dilution-plaque reduction neutralization testing.

Human Seroprevalence Survey

In mid-October 2000, a seroprevalence survey was conducted of residents of an area of southern Stamford and southeastern Greenwich, Connecticut, with a population of approximately 99,000 persons in a 17.9-square mile area, for a population density of 5,543 persons per square mile (3). This population area was chosen because it had one of the highest town-specific crow mortality rates in Connecticut, the largest number of confirmed WN virus-positive dead crows (96; 8.8% of those that tested positive in Connecticut) and 5 of the 14 pools of positive mosquitoes found statewide. A stratified cluster sampling method was used. Serologic samples were screened at the DPH laboratory by IgM-capture ELISA, with confirmation of reactive specimens at CDC as described.

Statistical Analysis

All bird deaths, dead crow testing, and human surveillance data were entered into spreadsheets and analyzed by using Epi-Info (12). Overall and weekly bird and crow mortality rates were calculated by town and by county per square mile and per 100,000 population. Population estimates as of July 1, 1998, and town size in square miles were obtained from the Connecticut Department of Economic and Community Development (13). The statewide rate of dead crow sightings per week per square mile excluded the area of 20 towns that did not participate in the dead bird surveillance system (11% of surface area, 4% of population).

Confidence intervals for the human seroprevalence study were calculated by the exact binomial interval (14).

Results

Surveillance findings of the various surveillance systems are summarized by geographic area (Figure 1). Fairfield and New Haven counties had the highest number of dead birds, all 14 WN virus-positive mosquito pools, and four of the seven

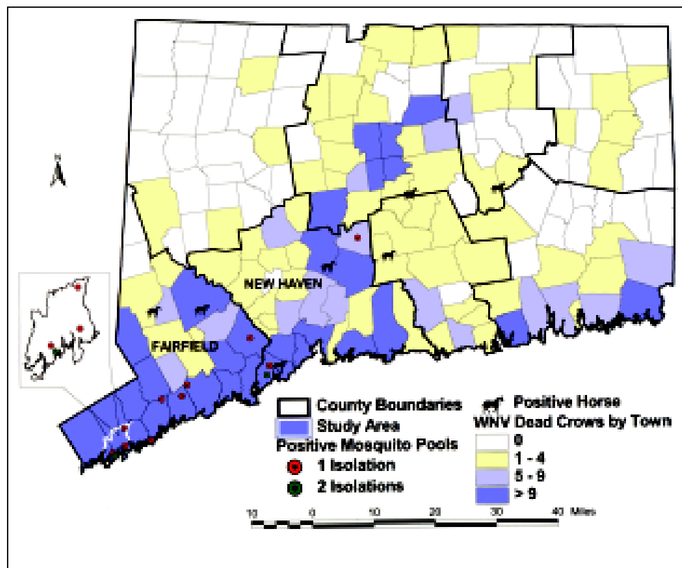

Figure 1. Location of West Nile (WN) virus-positive mosquito pools and horses, of towns by number of WN virus-positive birds, and of the site where the WN virus seroprevalence survey was performed, Connecticut, 2000.

confirmed horse cases. More detailed findings by specific surveillance system follow.

Bird Mortality

A total of 10,735 dead bird sightings were reported from April 17 to November 4, 2000. Of the 8,952 with species identified, 4,335 (48%) were crows. For the last week in June, before the first WN virus-positive crow was found, the percentage of dead bird sightings that were crows was 42%, with no statistically important variation by county (range 41% to 50%, p>0.05 by chi-square). However, beginning in early August, the percentage of crows among dead birds began to increase, first in Fairfield, then in New Haven, followed by the other counties (Figure 2). By early September, approximately two thirds of all dead bird sightings in Fairfield and New Haven counties (77% and 65%, respectively) were crows.

The number of dead crow sightings per square mile per week averaged 0.03 statewide during the surveillance period. However, there were remarkable differences by place and

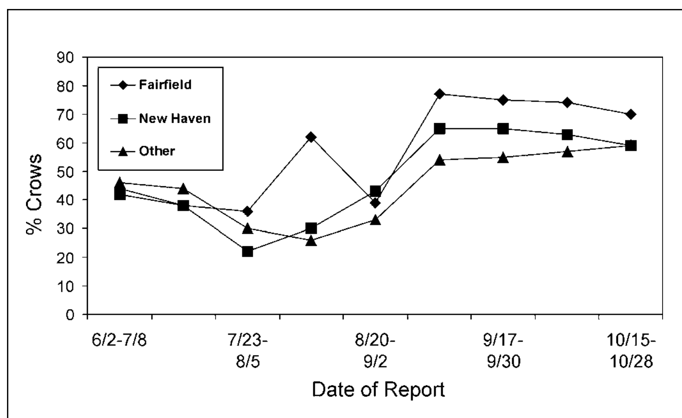

Figure 2. Percentage of dead bird sightings identified as crows, by county and 2-week intervals, June 25-October 28, 2000, Connecticut.

time, especially after WN virus activity was detected in early July. The highest rates consistently occurred in Fairfield County and in coastal Fairfield towns (Darien, Stamford, Fairfield) and in a coastal New Haven County town (Milford) (Table 1). County-specific rates rose to a sustained high of 0.3 dead crows per square mile in Fairfield County beginning in mid-August. No other county had a rate higher than 0.1. The highest town-specific rate was 2.2 dead crows per square mile in mid-August in Darien, the town just east of Stamford. In the area of Stamford and Greenwich where the seroprevalence survey was conducted, the highest rate was 2.3 in mid-August, with a sustained average rate of 2.0 during the next 4 weeks.

Table 1. Dead crow sightings per square mile per week, selected towns and counties, Connecticut, May-October, 2000

County	Area (sq. mi.)[a]	No. of sightings	No. sightings/ sq. mi.	Week
Fairfield[b]	606	189	0.3	8/13 - 8/19
Fairfield	606	187	0.3	9/17 - 9/23
Fairfield	606	183	0.3	9/10 - 9/16
Fairfield	606	164	0.3	9/24 - 9/30
Fairfield	606	155	0.3	8/20 - 8/26
New Haven	606	84	0.1	9/3 - 9/9
Town (County)				
Darien (FF)	12.9	29	2.2	8/13 - 8/19
Milford (NH)	22.6	39	1.7	9/3 - 9/9
Milford	22.6	38	1.7	9/10 - 9/16
Milford	22.6	35	1.5	8/20 - 8/26
Fairfield (FF)	30.0	32	1.3	9/17 - 9/23
Stamford (FF)	37.7	45	1.2	8/13 - 8/19
Stamford	37.7	45	1.2	8/20 - 8/26
Stamford	37.7	44	1.2	9/3 - 9/9
Serosurvey area (FF)	17.9	41	2.3	8/13 - 8/19

[a]Source: Connecticut Department of Economic and Community Development. FF = Fairfield County; NH = New Haven.
[b]Area of Fairfield County = 625.9 square miles. However, one town did not submit logs and its area (19.8 square miles) is not included in the calculations.

Because the number of dead crow sightings might depend partly on the population size of any given county or town, the number of dead crow sightings per 100,000 population per week was also examined. This index also reached a sustained peak beginning in mid-August in Fairfield County and in the same towns in Fairfield County with high rates of dead crows per square mile (Table 2). In the seroprevalence survey area, this index peaked at 41.4 in mid-August.

Testing Dead Crows

A total of 1,574 crows were tested for WN virus between May 1 and November 4, 2000, most (97%) after July 1. Overall, 70% (1,095) tested positive. Once positive crows were found, the percentage increased rapidly in each county, beginning with Fairfield (Figure 3). In Fairfield County, the percentage of dead crows testing positive reached 81% in early August and peaked at 93% in late September.

Mosquito Trapping and Testing

Fourteen mosquito pools tested positive for WN virus: four pools of *Culex restuans*, five pools of *Cx. pipiens*, two pools of *Cx. salinarius*, and three pools of *Culiseta melanura* (9).

Table 2. Number of dead crow sightings per 100,000 population per week, selected counties and towns, Connecticut, May-October, 2000

County	Population[a]	No. Sightings	No. Sightings per 100,000 population	Week
Fairfield	823,698[b]	189	22.9	8/13 - 8/19
Fairfield	823,698	187	22.7	9/17 - 9/23
Fairfield	823,698	183	22.2	9/10 - 9/16
Fairfield	823,698	164	19.9	9/24 - 9/30
Fairfield	823,698	155	18.8	8/20 - 8/26
New Haven	793,504	84	10.5	9/3 - 9/9
Town (County)				
Darien (FF)	18,085	29	160.4	8/13 - 8/19
Darien	18,085	19	105.1	8/6 - 8/12
Darien	18,085	17	94.0	9/24 - 9/30
Darien	18,085	16	88.5	9/10 - 9/16
Milford (NH)	50,027	39	78.0	9/3 - 9/9
Fairfield (FF)	53,740	38	70.7	9/17 - 9/23
Stamford (FF)	110,689	45	40.7	8/20 - 8/26
Serosurvey area (FF)	99,000	41	41.4	8/13 - 19

[a]Population of Fairfield County = 838,362. However, one town did not submit logs and its population (14,664) is not included in the calculations. FF = Fairfield County; NH = New Haven.
[b]Source: Connecticut Department of Economic and Community Development population estimates as of 7/1/98.

The first positive pool, which was *Cx. restuans*, was collected on July 11 in Stamford, 6 days after the first WN virus-positive crow was found in the same town. In response, a single evening ground spraying to kill adult mosquitoes in a 2-mile radius around the trap site was conducted in late July. Three positive pools were collected in August (two *Cx. restuans*, one *Cx. pipiens*), nine in September and one in October (9). Nine WN virus-positive pools were from Fairfield County, and five were from New Haven County (Figure 1). Five WN virus-positive pools (two *Cx. restuans*, three *Cx. pipiens*, one in July, four in mid-September) were collected in the area included in the human seroprevalence survey. The only additional ground spraying done in response to positive mosquito findings was in Milford, New Haven County, in late September, in response to a horse case and the finding of three WN virus-positive mosquito pools. Detailed information on the results of mosquito surveillance, including mosquito species trapped, mosquito infection rates, and mosquito density indices is published in this issue (9).

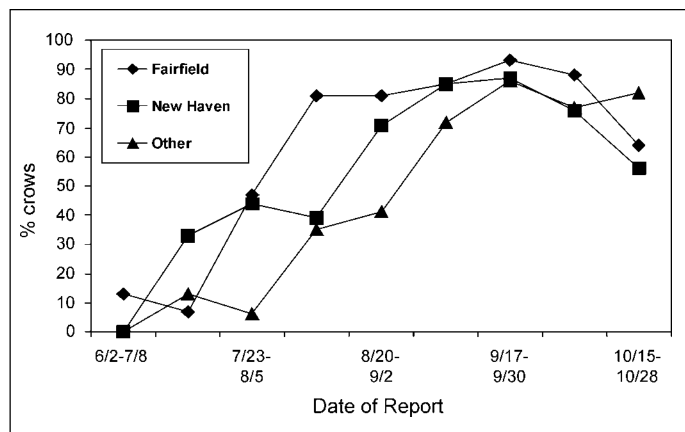

Figure 3. Percentage of dead crows testing positive for West Nile virus, by county and 2-week intervals, June 25-October 28, 2000, Connecticut.

Neurologic Illness in Horses

Seven of 33 horses with neurologic signs tested during the surveillance period had evidence of acute WN virus infection. Onset dates ranged from August 29 to October 10, coincident with the peak number of WN virus-positive mosquito pools (Figure 4). The WN virus-infected horses were scattered over five counties (Figure 1). Only one of the horse cases, onset date September 5, occurred in an area with high levels of bird deaths and positive mosquito pools (Milford, Tables 1,2). In this area, the WN virus-positive mosquito pools were all captured in new traps set up near the stable after the horse was confirmed to have WN virus infection. Although spraying was considered after confirmation of WN virus in each horse with neurologic signs, the only time adulticide spraying was done in response to a horse case without positive mosquito findings was in late September, in a 2-mile radius around a horse case in a suburban area with low-level WN virus activity in birds (peak number of five dead crows reported in a week and a peak density of 0.08 dead crows per square mile in a week).

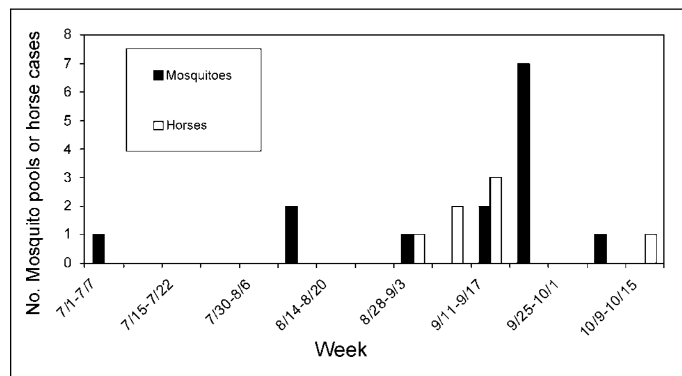

Figure 4. Number of West Nile virus-positive mosquito isolates and horse cases, by week of collection or symptom onset, Connecticut, 2000.

Neurologic Illness in Humans

One hundred fifty-seven serum and cerebrospinal fluid specimens were tested from 86 persons hospitalized with severe neurologic illness with onset of symptoms during May 1 to October 31, 2000. Of these 86 persons, 44 had encephalitis or meningoencephalitis, 41 had aseptic meningitis, and 1 had fever and Guillain-Barré syndrome. Fifty-three (62%) of the hospitalized persons were from Fairfield or New Haven counties, where active surveillance was done.

Although human surveillance was intended to detect severe neurologic illness, at least one serologic specimen was submitted for testing from each of 85 persons who did not meet the surveillance criteria. Of these, one was confirmed positive for WN virus by testing at CDC of acute- and convalescent-phase serum specimens collected 14 days apart. Serum IgM was positive on both specimens, and both IgG and neutralizing antibody titers had greater than fourfold increases. This person, an older woman, sought medical attention for possible WN virus infection after finding a dead

crow and experiencing mild headache without fever in late August. The town of residence and likely exposure to WN virus was Norwalk, a town in Fairfield County with a high level of epizootic activity (peak number of 24 dead crows reported in a week and a density of 1.05 dead crows per square mile in a week).

Human Seroprevalence Survey

Seven hundred thirty-one persons submitted serologic specimens. Three specimens had screening IgM titers that were greater than controls. On further testing, only one specimen was WN virus positive by IgM-capture ELISA (P/N ratio 4.26) and was not found to have neutralizing antibodies indicative of recent WN virus infection. A second specimen from the same person, obtained >2 weeks later, also did not have neutralizing antibodies. Thus, the point seroprevalence of WN virus infection was 0% (95% confidence interval 0-0.52%) (3).

Conclusion

The potential magnitude of the WN virus problem in the United States and the most appropriate short- and long-term public health responses are not yet known. Because severe outbreaks have occurred in temperate northern hemispheric climates with human population infection rates of up to 4% and attack rates of severe illness as high as 12-40 per 100,000 population (3,15,16), the threat must be taken seriously. The major public health challenge is to determine what surveillance indicators suggest that an outbreak of severe human illness is likely and what public health actions are effective to prevent outbreaks or modify outbreak potential. Thus, in these first years that WN virus is circulating in North America, effective arboviral surveillance systems must be established and surveillance data must be thoroughly evaluated.

The Connecticut experience is highly relevant to future public health planning, as spraying for adult mosquitoes was rarely used in 2000, and certainly not at a level that could have affected either the natural course of the WN virus epizootic or human health risk. Ground spraying was done only three times in 2000, all in small areas 2 miles in radius in response to unusual surveillance findings. Spraying was done only once before late September, in late July in an area that included approximately 30% of the seroprevalence survey area.

In 2000, Connecticut had an intense epizootic of WN virus activity, particularly in Fairfield and New Haven counties, with no outbreak of human disease and only very low levels of human infection. The level of epizootic activity in coastal Fairfield county initially paralleled that in Staten Island, New York, the only county with a clear outbreak of human illness in 2000 (17). The first indication of WN virus was on the same date, July 5, with a finding of a WN virus-positive crow. In each area, this first WN virus-positive crow was followed within one week by the finding of WN virus-positive *Culex* mosquitoes and many more WN virus-positive crows.

After that, the experiences in New York and Connecticut diverged. In Staten Island, positive mosquito pools were detected with increasing frequency before the end of July, and the first person ill with a case of WN virus infection was hospitalized in late July (8). In addition, a peak observed crow mortality rate of 5.8 per square mile occurred during the week beginning July 15 (18). In contrast, in Connecticut, no more WN virus-positive mosquito pools were found in July. The peak observed crow mortality rate in a town, 2.2 dead crows per square mile, occurred in mid-August, and no human cases of severe neurologic illness due to WN virus were diagnosed.

There were some important differences in the timing of peak surveillance indicator activity in Connecticut. Most positive mosquito pools occurred in mid to late September and were associated with increased incidence of WN virus neurologic disease in horses. In contrast, the number of dead crows per square mile peaked in mid-August through mid-September, when the single documented human case of WN virus infection in Connecticut and most of the human cases in 1999 and 2000 in New York and New Jersey occurred (2,17). On the other hand, in Staten Island, New York, the one county with a true outbreak of serious human neurologic disease in 2000, many WN virus-positive mosquito pools were identified before and during the outbreak of human illness, which peaked in mid-August (8,17). Thus, continued collection and analysis of data from crow deaths and mosquito surveillance in areas both with and without human cases of severe WN virus-related illness are needed to determine what surveillance indices are most sensitive and specific in predicting the risk for an outbreak of WN virus among humans and to guide future decisions regarding adult mosquito control.

Whether crow mortality rates will remain a good indicator of the amplification of WN virus epizootic activity is not known. The percentage of dead bird sightings that are crows, percentage of dead crows testing positive for WN virus, and number of observed crow deaths per square mile were each quantitative surveillance indicators that reflected the level of epizootic activity in Connecticut in 2000. However, to the extent that WN virus affects the crow population by diminishing it or selecting for relative resistance, quantitative crow mortality indices may become less useful as measures of epizootic activity. Until then, assuming public interest in reporting dead crows can be maintained, they can be used as quantitative surveillance indicators of the level of epizootic activity and may, at higher levels than observed in Connecticut in 2000, prove to be indicators that could be used to predict the potential for a human outbreak of severe illness.

WN virus was first detected in 2000 where activity had occurred the year before, and amplification was greatest there. This suggests that the virus successfully overwintered in Connecticut as well as New York and New Jersey, re-emerged, began to amplify, and then spread to other parts of Connecticut and New England. If this pattern persists, given that nearly all of New England and the mid-Atlantic states had WN virus epizootic activity in 2000, WN virus amplification will likely start earlier over a much larger geographic territory in 2001 than in 2000 and the threat to human health could be much larger.

There are a number of limitations to the Connecticut WN virus surveillance system and to conclusions that can be drawn from these findings. First, surveillance did not occur in the absence of any mosquito control measures. Although there was little spraying, there were intensive efforts in Fairfield and New Haven counties beginning in May 2000 to reduce *Cx. pipiens* breeding habitat and to kill larval mosquitoes. These activities, which intensified and began to be used statewide once the presence of WN virus was known, may

have contributed to reducing human risk at the level of epizootic activity recorded in birds and horses.

Second, bird and mosquito surveillance was not equally intense in each town. Even before July, dead bird reports of all species per 100,000 human population were twice as frequent in Fairfield as in other counties. In addition, reporting and bird submissions depended in part on population density. Thus, less densely populated areas tended to have much lower rates of dead crow sightings per square mile than more densely populated areas. Mosquito surveillance was also more intense in some parts of the state than others. Although trap distribution was statewide, many towns in lower Fairfield County had results from two or three traps per week, while most other towns had no traps at all. However, despite these limitations, the intensity of both bird and mosquito surveillance in densely populated lower Fairfield County was high, making it possible to describe the epizootic there in accurate detail.

A third limitation is crow denominators. Ideally, the intensity of the ornithologic impact of WN virus might be measured by number of crow deaths per unit of crow population. However, the total crow population in any geographic area is unknown, and its variability from year to year can only be crudely measured. Thus, specific crow mortality indicators may not be generalizable from one setting to another and from one year to the next.

A fourth limitation is that the potential for amplification of WN virus and for humans to become infected may differ greatly from one area to another. The natural relative abundance of amplifying and bridge mosquito vectors and of the bird species most important to amplification likely differ from one ecologic niche and geographic area to the next, including within towns or cities. Thus, the ecology of the area of Connecticut where the most intense epizootic activity was documented, the seroprevalence survey area, and Staten Island may differ sufficiently that, although they are similar in population density, the intrinsic potential for a human outbreak of WN virus may be very different. Until the ecology of WN virus in the United States is better understood, comparisons between different areas must be made with this qualification.

Finally, the measurement of human WN virus infection focused on severe illness, not asymptomatic infection or mild outpatient illness. Thus, in the absence of severe cases it is difficult to know exactly how much human infection occurred. However, the seroprevalence study suggests that the number of human infections was very low and that the absence of severe human illness, a roughly 1 in 150 event (3), was not a chance finding.

Clearly, much is still to be learned about the impact of WN virus in the United States and the Americas. Surveillance in all forms needs to be continued and data need to be analyzed to monitor the impact of WN virus on human and animal species over time and to determine and refine thresholds for public health intervention.

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We thank all the local health departments in Connecticut for conducting surveillance for bird deaths and collecting dead crows; Lynn Wilcox, Susan Smolenski, Skip Sirpenski, Tim Brennan, Gloria Arnold, and Craig Morin for their supportive work with bird surveillance, testing, and mapping results; Jenny Dickson, Greg Chasko, Helen Cavaciuti, and Kelly McCurdy for facilitating bird surveillance; Meghan Tucker, Kimberly Holmes, and Tomas Foral for their work in veterinary diagnosis; Susan Smith for help with active surveillance in New Haven County; and Anthony Iton, Caroline Baisley, and Anthony Marfin, whose leadership and support made the seroprevalence survey possible.

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References

1. Centers for Disease Control and Prevention. Outbreak of West Nile-like encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:845-9.
2. Centers for Disease Control and Prevention. Update: West Nile encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:944-6,955.
3. Centers for Disease Control and Prevention. Serosurveys for West Nile virus infection—New York and Connecticut counties-2000. MMWR Morb Mortal Wkly Rep 2001;50:37-9.
4. State of Connecticut, Department of Environmental Protection, Department of Public Health, Connecticut Agricultural Experiment Station and Department of Agriculture. West Nile virus surveillance and response plan, July 2000:1-28. Available at http://www.dep.state.ct.us/mosquito/index.asp. Accessed January 2001.
5. New York State Department of Health. New York West Nile virus response plan, May 2000. Available at http://www.health.state.ny.us/nysdoh/westnile/index.htm. Accessed January 2001.
6. New York City Department of Health. Comprehensive arthropod-borne disease surveillance and control plan, 2000. Available at http://www.nyc.gov/html/doh/html/WN virus/WN virusplan.htm. Accessed January 2001.
7. Centers for Disease Control and Prevention. Update: West Nile virus activity—New York and New Jersey, 2000. MMWR Morb Mortal Wkly Rep 2000;49:640-2.
8. Centers for Disease Control and Prevention. Update: West Nile virus activity—Northeastern United States, January-August 7, 2000. MMWR Morb Mortal Wkly Rep 2000;49:714-7.
9. Andreadis TG, Anderson JF, Vossbrinck CR. Mosquito surveillance for West Nile virus in Connecticut, 2000: Isolation from *Culex pipiens, Cx. restuans, Cx. salinarius,* and *Culiseta melanura*. Emerg Infect Dis 2001;7:670-4.
10. Lanciotti R Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol 2000;38:4066-71.
11. State of Connecticut, Department of Public Health. Update on West Nile virus and encephalitis: reporting and laboratory testing. Connecticut Epidemiologist 2000;20:5-8. Available at URL: http://www.state.ct.us/dph/BCH/Infectious%20Diseases/cenl.htm#2000. Accessed May 2001.
12. Dean AG, Dean JA, Couloumbier D, Brendel KA, Smith DC, Burton AH, et al. Epi Info, Version 6: a word processing, database, and statistics program for epidemiology on microcomputers. Centers for Disease Control and Prevention, Atlanta, Georgia, 1994.
13. Connecticut Department of Economic and Community Development, Research Division. 1998-99 town profiles. Connecticut Department of Economic and Community Development, 2000. Available at http://www.state.ct.us/ecd/research/townprof98/index.html. Accessed January 2001.
14. Korn E, Graubard BI. Confidence intervals for proportions with small expected number of positive count estimated from survey data. Survey Methodology 1998:24;193-201.

15. Tsai TF, Popovici F, Cernescu C, Campbell GC, Nedelcu JI, the Investigative Team. An epidemic of West Nile encephalitis in southeastern Romania. Lancet 1998:352;767-71.
16. Platonov AE, Shipulin GA, Shipulina OY, Tyutyunik EN, Frolochkina TI, Lanciotti RS, et al. Outbreak of West Nile virus infection, Volgograd Region, Russia, 1999. Emerg Infect Dis 2001;7:128-132.
17. Centers for Disease Control and Prevention. Update: West Nile virus activity-Eastern US, 2000. MMWR Morb Mortal Wkly Rep 2000;49:1044-7.
18. Eidson M, Miller J, Kramer L, Cherry B, Hagiwara Y, West Nile Virus Bird Mortality Analysis Group. Dead Crow Densities and Human Cases of West Nile Virus, New York State, 2000. Emerg Infect Dis 2001;7:662-4.

Mosquito Surveillance and Polymerase Chain Reaction Detection of West Nile Virus, New York State

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West Nile (WN) virus was detected in the metropolitan New York City (NYC) area during the summer and fall of 1999. Sixty-two human cases, 7 fatal, were documented. The New York State Department of Health initiated a departmental effort to implement a statewide mosquito and virus surveillance system. During the 2000 arbovirus surveillance season, we collected 317,676 mosquitoes, submitted 9,952 pools for virus testing, and detected 363 WN virus-positive pools by polymerase chain reaction (PCR). Eight species of mosquitoes were found infected. Our mosquito surveillance system complemented other surveillance systems in the state to identify relative risk for human exposure to WN virus. PCR WN virus-positive mosquitoes were detected in NYC and six counties in the lower Hudson River Valley and metropolitan NYC area. Collective surveillance activities suggest that WN virus can disperse throughout the state and may impact local health jurisdictions in the state in future years.

During the summer and fall of 1999, the metropolitan area of New York City witnessed an outbreak of human encephalitis caused by West Nile (WN) virus (1-3). This outbreak was the first evidence of WN virus infection in the Western Hemisphere (4-8) and resulted in 62 laboratory-confirmed human cases, 7 fatal (8). Evidence of WN virus infection was documented in mosquitoes, birds, horses, and humans, primarily in the New York, Connecticut, and New Jersey area of the northeastern United States, with the southernmost distribution identified in a dead WN virus-positive crow in Baltimore, MD (9-11). Laboratory investigation into the likely geographic source of the WN virus strain identified in this outbreak indicated 99% homology with a strain found in Israel in 1998 (5).

Guidelines for WN virus surveillance developed in consultation with national experts were distributed by the Centers for Disease Control and Prevention (CDC) and included recommendations for disease prevention and control (12). Surveillance activities conducted in January and February 2000 for overwintering *Culex* mosquitoes in New York City identified WN virus-infected specimens in underground hibernacula (13), indicating that there was a risk of virus maintenance in vector species and potential for reemergence as a human disease risk during the spring and summer months. Our report describes subsequent state and local agency efforts to establish, implement, and evaluate the mosquito and WN virus surveillance programs undertaken during 2000 to minimize risks for human and animal infection.

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Materials and Methods

Local Health Department (LHD) Response

The New York State Department of Health (NYSDOH) developed a system of mosquito pool allocations that would allow rapid detection of virus should WN virus appear anywhere in the area affected by the 1999 outbreak or in peripheral counties in the general metropolitan area or surrounding lower Hudson Valley area. Based on data from the 1999 surveillance, initial priority was placed on mosquito species considered likely first indicators of virus circulation,

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primarily in the *Culex* genus. LHDs conducted early season larval surveillance and habitat descriptions, initiated habitat management and larval control in habitats where immature mosquitoes were present (focusing on container breeding, storm drain, or catch basin habitats), and developed a local database of these habitats. Adult mosquito surveillance activities could start at any time in the individual counties, and mosquito pools could be submitted to the NYSDOH Arbovirus Research Laboratory beginning the first week of June. The NYSDOH provided CO_2-baited CDC miniature light traps (14) and gravid mosquito traps (15) to LHDs to conduct adult mosquito surveillance. Anticipating a total statewide weekly submission of 400 mosquito pools (up to 50 mosquitoes/pool), we provided LHDs with a weekly pool allocation and scheduled day of submission for all mosquito pools to be tested for arboviruses.

The department also provided other surveillance supplies that LHDs would need to conduct initial mosquito surveillance activities or to enhance existing programs. We divided the surveillance equipment among the counties on the basis of 1) 1999 surveillance data and recognized geographic distribution of WN virus and 2) the anticipated geographic distribution of WN virus in the metropolitan NYC and lower Hudson Valley area during 2000. We also allocated pools for eastern equine encephalitis surveillance conducted annually in Long Island and central New York. Mosquito pool allocations for 2000 were divided among the LHDs in NYC and the rest of the state on the basis of human population density and distance from the 1999 epicenter. Weekly pool allocations ranged from 90 pools for NYC, 45 pools for each county on Long Island, 40 pools for Westchester County, and 10-15 pools for counties further upstate that were conducting adult mosquito surveillance.

Mosquitoes submitted for virus testing arrived at the Arbovirus Research Laboratory on a weekly basis; some counties submitted specimens midweek and others at the end of the week to split the initial 400-pool weekly load. Mosquito pools contained specimens from a single species (or combined species such as *Cx. pipiens* and *Cx. restuans*) collected at a single site during the week. In some cases where numbers were below the target of 50 specimens per pool, collections from 2 sequential weeks or from closely adjacent sites were combined. Although the NYSDOH initially requested separation, it is unlikely that all counties effectively removed all blood-fed or gravid females from weekly collections. Therefore, some pools positive by polymerase chain reaction (PCR) may have contained blood-fed or gravid mosquitoes. Mosquito pools submitted to the Arbovirus Research Laboratory were tested for viral RNA by reverse transcription (RT)-PCR techniques following established protocols and reported as described (16). Briefly, mosquitoes were tested by TaqMan RT-PCR with two primer-probe sets. Virus isolation attempts in Vero cell culture followed the detection of PCR-positive pools, as described (16).

State Analysis of Surveillance Data

Surveillance data related to adult mosquito populations, adult mosquito pool submissions for virus testing, and laboratory test results (as well as bird, animal, and human data) were entered into the NYSDOH Health Information Network, a secure statewide health agency communication network. These surveillance data were maintained on a daily basis and available to NYSDOH and the contributing LHD. Local agency data were held confidential for 24 hours before all other LHDs were provided summary information.

All LHDs were responsible for the entry of mosquito surveillance data in the Health Information Network, as described. All surveillance mechanisms (mosquito, bird, animal, and human) provided data for this secure database. Program and laboratory review of the surveillance database, as well as laboratory entry of virus detection results, provided a current picture of mosquito populations and a virus infection registry of statewide data. When used fully and properly by the LHDs, mosquito population dynamics, rates, and location of arbovirus activity could be derived from regular review of this comprehensive database. All analyses conducted for this report were developed through the summary of the information extracted from that network.

Results

Mosquito Surveillance

Counties conducting adult mosquito surveillance during 2000 along with a summary of their general surveillance efforts are listed in Table 1. Although 26 counties collected and submitted pools of mosquitoes for virus testing (Figure 1), PCR-positive mosquitoes were found only in the five NYC boroughs, both counties on Long Island, and four counties in the lower Hudson River Valley. Figure 2 provides the weekly numbers of mosquitoes collected by genus through the

Table 1. Local health department mosquito surveillance programs

Local health department	No. of trap nights	No. of mosquitoes submitted for virus testing	No. of PCR-positive pools
Albany	203	2,305	0
Broome	81	235	0
Cattaraugus	176	1,320	0
Columbia	369	1,161	1
Cortland	47	88	0
Dutchess	1,439	8,319	0
Erie	45	512	0
Madison	58	2,310	0
Monroe	136	1,156	0
Nassau	1,771	25,543	7
Niagara	104	1,697	0
Oneida	726	2,173	0
Onondaga	566	15,599	0
Orange	1,188	17,091	4
Oswego	1,555	12,417	0
Putnam	1,270	10,200	0
Rockland	2,037	28,623	47
Suffolk	960	77,955	120
Ulster	435	8,234	0
Warren	19	423	0
Westchester	2,505	24,478	13
New York City	2,850[a]	75,837	171
Brooklyn			12
Bronx			6
Manhattan			17
Queens			5
Staten Is.			131
Statewide	18,540	317,676	363

[a]Estimated.
PCR = polymerase chain reaction.

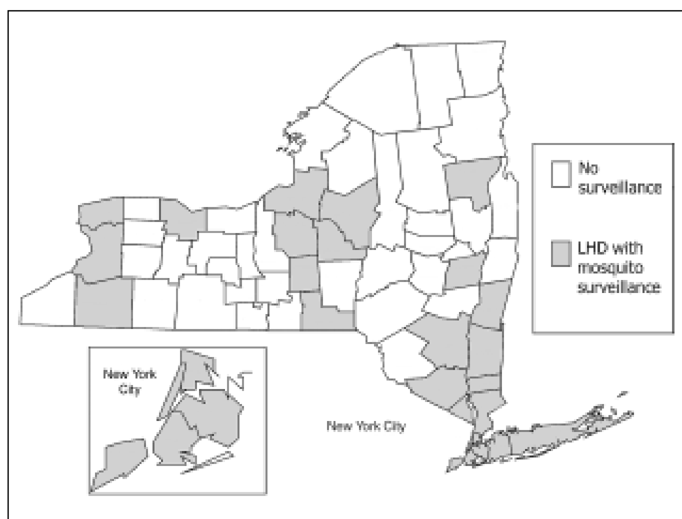

Figure 1. New York counties that conducted adult mosquito surveillance and submitted specimens for West Nile virus testing, 2000.

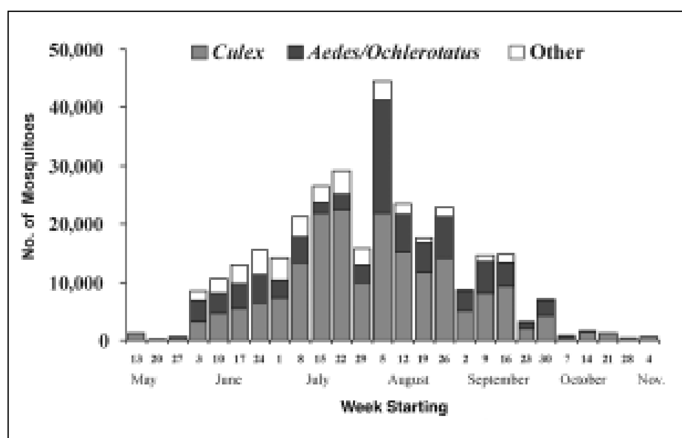

Figure 2. Weekly numbers of *Culex*, *Aedes* (or *Ochlerotatus*), and other genera submitted for testing by local health departments, New York, 2000.

26-week collection season from May 13 to November 4, 2000. A total of 317,676 mosquitoes were collected: 192,538 (60.6%) *Culex* spp., 86,034 (27.1%) *Aedes* (or *Ochlerotatus*) spp., and 39,104 (12.3%) other species. LHDs collected 28 species of mosquitoes during the 2000 adult mosquito surveillance season (3 *Aedes* spp., 4 *Anopheles* spp., 1 *Coquillettidia* sp., 4 *Culex* spp., 2 *Culiseta* spp., 11 *Ochlerotatus* spp., 1 *Orthopodomyia* sp., 1 *Psorophora* sp., and 1 *Uranotaenia* sp.). Of the 9,952 pools submitted for testing, 5,851 (58.8%) were *Culex* spp., 3,072 (30.9%) were *Aedes* or *Ochlerotatus* spp., and 1,029 (10.3%) were other species. Despite the original agreement with LHDs for a maximum weekly submission of 400 pools, submissions exceeded the maximum during 11 weeks of the season, with a peak of 1,200 pools submitted the week of August 5. Laboratory protocols were initially established at a maximum of 100 mosquitoes per pool, but that figure was reduced to 50 mosquitoes per pool in May to enhance the ability to detect virus.

Virus Surveillance in Mosquitoes

Quantified summary data related to virus distribution by county and by mosquito species are provided in Table 2.

Table 2. Local health department reports of West Nile virus specimens that were positive by polymerase chain reaction (PCR)

	PCR-positive pools/pools submitted (% pos)	% of total PCR-positive pools tested statewide
By county		
Brooklyn	12/281 (4.3)	3.3
Staten Island	131/935 (14.0)	36.1
Queens	5/683 (0.7)	1.4
Manhattan	17/262 (6.5)	4.7
Bronx	6/298 (2.0)	1.6
Nassau	7/821 (0.9)	1.9
Suffolk	120/2,044 (5.9)	33.1
Westchester	13/872 (1.5)	3.6
Rockland	47/1,096 (4.3)	12.9
Orange	4/529 (0.8)	1.1
Columbia	1/52 (1.9)	0.3
Total	363/7,873 (4.6)	100.0
By species		
Culex pipiens	79/1,119 (7.1)	21.8
Cx. restuans	0/238 (0)	0
Cx. pip/res	212/3,746 (5.7)	58.4
Cx. salinarius	31/501 (6.2)	8.5
Culex species	19/232 (8.2)	5.2
Ochlerotatus japonicus	5/526 (1.0)	1.4
Oc. triseriatus	3/407 (0.7)	0.8
Aedes vexans	10/1,182 (0.8)	2.7
Oc. cantator	1/87 (1.1)	0.3
Aedes species	1/73 (1.4)	0.3
Anopheles punctipennis	1/54 (1.8)	0.3
Psorophora ferox	1/16 (6.2)	0.3
Total	363/8,181 (4.4)	100.00

NYSDOH surveillance and laboratory testing activities during 2000 led to the identification of 363 PCR WN virus-positive mosquito pools. Results from virus isolation attempts on these PCR-positive pools are included in Table 3. We are also aware of the detection of a single PCR-positive pool of *Culex pipiens/restuans* collected by U.S. Army Center for Health Promotion and Preventive Medicine staff in Brooklyn (B. Pagac, pers. comm.; data not included). Although NYC and Long Island LHDs submitted only 5,324 (53%) of the 9,952 total mosquito pools, 298 (82%) of 363 PCR WN virus-positive pools were collected in these counties. Only 65 (18%) of the 363 PCR WN virus-positive pools were identified in four counties north of NYC and Long Island, despite accounting for 26% (2,549/9,952) of the total pools submitted. Most PCR-positive pools (72%, 47/65) from upstate were from Rockland County. Similarly, 341 (94%) of the 363 PCR-positive pools were *Culex* mosquitoes, primarily in the group of *Cx. pipiens/restuans* mosquitoes that were not separated because of loss of morphologic characteristics during collection or natural aging. Although we found 79 pools of *Cx. pipiens* PCR positive for WN virus, none of 238 pools of *Cx. restuans* were positive. Twenty PCR WN virus-positive pools of *Aedes* or *Ochlerotatus* species mosquitoes were identified, primarily from *Ae. vexans* (10 pools) and *Oc. japonicus* (5 pools).

Temporal analysis of mosquito-based WN virus surveillance is illustrated in Figure 3 for all mosquito species (or groups) during the 2000 season. The first PCR evidence of WN virus infection in mosquitoes occurred in specimens collected the week of July 1, 2000, with one WN virus-positive

Table 3. Comparison of West Nile virus detection in mosquito pools positive by polymerase chain reaction (PCR) tests and isolation in Vero cells

Species	No. pools (indiv) tested	No. pools PCR+/no. mosquitoes tested (MIR)	PCR+ (indiv) and culture +	PCR+ (indiv) and culture -	PCR+ (indiv) and culture not done
Culex pipiens/restuans	3,746 (130,745)	212/130,745 (1.6)	61 (2,555)	83 (4,124)	68 (2,507)
Cx. pipiens	1,119 (30,818)	79/30,818 (2.6)	25 (630)	35 (1,413)	19 (698)
Cx. salinarius	501 (20,236)	31/20,236 (1.5)	13 (486)	13 (666)	5 (190)
Aedes vexans	1,182 (35,010)	10/35,010 (0.3)	1 (11)	8 (252)	1 (21)
Ochlerotatus japonicus	526 (7,209)	5/7,209 (0.7)	0 (0)	5 (36)	0 (0)
Culex spp.	232 (6,466)	19/6,466 (2.9)	9 (296)	4 (187)	6 (300)
Oc. triseriatus	407 (9,278)	3/9,278 (0.3)	1 (16)	2 (51)	0 (0)
Oc. cantator	87 (2,608)	1/2,608 (0.4)	0 (0)	1 (10)	0 (0)
Aedes/Ochlerotatus spp.	73 (1,365)	1/1,365 (0.7)	0 (0)	0 (0)	1 (20)
Anopheles punctipennis	54 (456)	1/456 (2.2)	0 (0)	1 (5)	0 (0)
Psorophora ferox	16 (225)	1/225 (4.4)	1 (10)	0 (0)	0 (0)

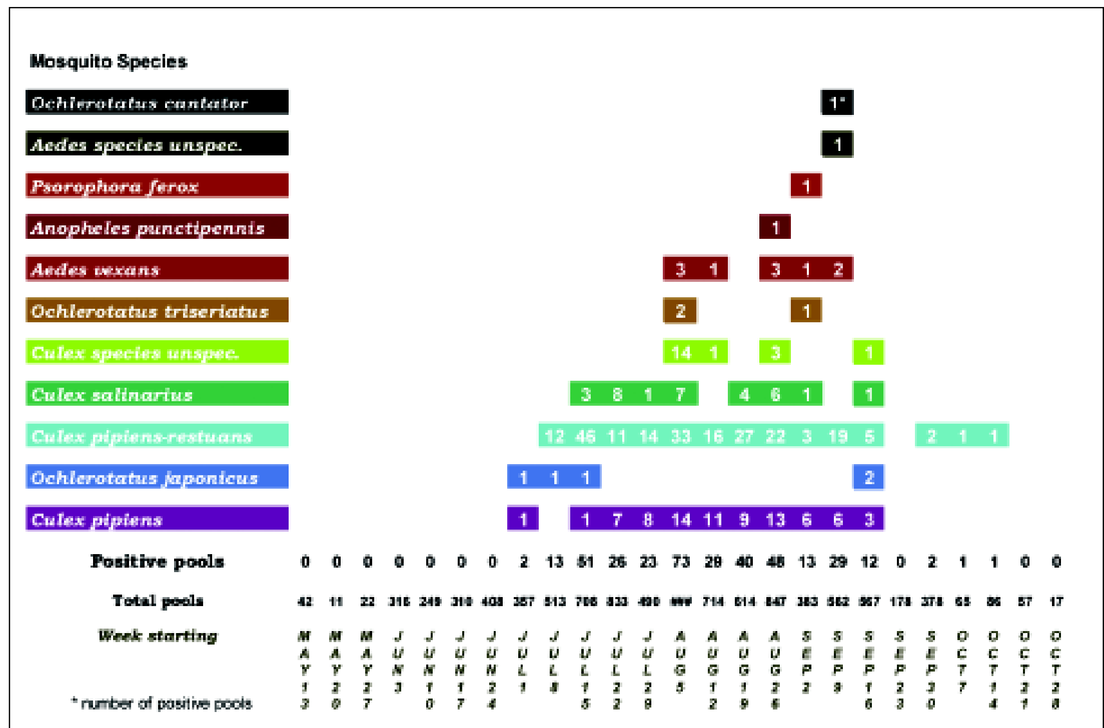

Figure 3. Temporal detection of West Nile virus infection in mosquito species submitted for testing by week of collection, New York, 2000.

pool each in *Cx. pipiens* collected in Richmond County (NYC) and *Oc. japonicus* collected in Westchester County. WN virus activity continued to amplify as the season progressed, with increasing numbers of PCR-positive mosquito pools, increasing geographic distribution of infection, and WN virus infection identified in additional mosquito species. By the end of the season, WNV was identified by PCR in eight mosquito species from a wide geographic area of Long Island and the lower Hudson River Valley, north to Columbia County. The northernmost PCR WN virus-positive mosquito pool was *Oc. japonicus* collected in Columbia County the week of September 16, 2000. The latest PCR WN virus-positive pool was identified in *Cx. pipiens/restuans* collected the week of October 14, 2000, from Suffolk County. (The latest pools of positive mosquitoes collected in 1999 were also collected during mid-October.) A complete list of PCR WN virus-positive mosquito species detected in 2000 appears in Table 2.

We calculated trap-type productivity by species for the six most abundant mosquito species collected statewide during 2000. Dry ice-baited CDC light traps captured 98% of the *Ae. vexans,* 83% of the *Oc. triseriatus,* 77% of the *Cx. salinarius,* 55% of both total *Cx. pipiens* and *Cx. pipiens/restuans* combined, and 21% of the *Oc. japonicus.* The only species collected in predominantly greater numbers by the gravid traps was *Oc. japonicus* (76% of total collections). Although gravid traps collected only 30% of the total number of mosquito pools submitted for virus testing during 2000, 56% of the PCR-positive pools came from gravid trap collections. None of the mosquito pools (n=135) from diurnal resting boxes or aspiration collections (n=11) had evidence of virus infection. However, 205 (6.9%) of 2,957 pools from gravid traps and 158 (2.3%) of 6,828 pools from CDC light traps were PCR positive for WN virus. Since mosquitoes attracted to gravid traps for oviposition would have fed on a host several days previously, a widespread presence of infected hosts could have resulted in these mosquitoes being more likely to acquire virus, thereby explaining the threefold difference in PCR-positive rates in gravid trap collections.

We also investigated the contribution of species minimum infection ratios (MIRs) per 1,000 mosquitoes throughout the season. MIRs (based on PCR-positive tests) were evaluated by individual pool sizes for each species and by analysis of weekly and seasonal aggregated species data within and among LHDs. Figures 4 and 5 illustrate the weekly MIRs by species for each genus (*Aedes, Ochlerotatus*, and *Culex*) through the adult mosquito surveillance season in counties that had evidence of human or equine cases (8). (These figures represent the recapitulation of results after all laboratory data were completed.) WN virus infection (confirmed by PCR) in *Culex* spp. began in early July and continued through the summer, concurrent with human case onsets; weekly MIRs ranged from 1 to 3.5 per 1,000, with a subsequent peak in early October. Although 80 to 100 pools of *Aedes* or *Ochlerotatus* were submitted during June and July, the PCR WN virus-positive *Oc. japonicus* in early July resulted in relatively high genus-specific MIRs. A second peak of elevated *Aedes* and *Ochlerotatus* MIRs occurred in late August and September, concurrent with an observed peak of equine cases. Further review of these data from counties where human or equine cases occurred, using MIR and multifactorial analyses, is required.

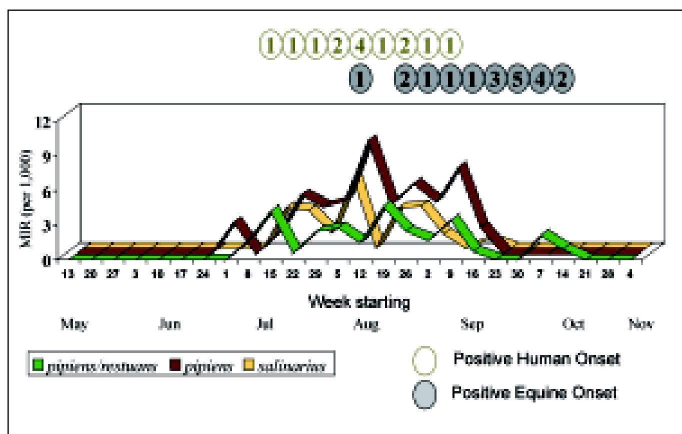

Figure 4. Seasonal fluctuations of minimum infection ratios (MIRs) for dominant *Culex* species (or combined species) and their temporal association with onsets of confirmed human or equine cases, New York, 2000.

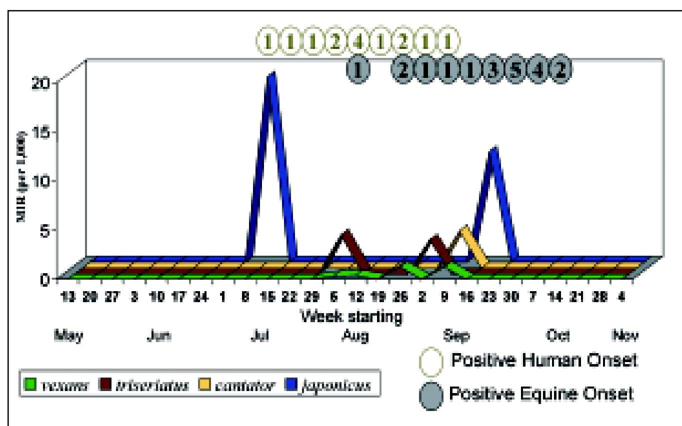

Figure 5. Seasonal fluctuations of minimum infection ratios (MIRs) for dominant *Aedes* or *Ochlerotatus* species and their temporal association with onsets of confirmed human or equine cases, New York, 2000.

By the end of the 2000 surveillance season in New York, PCR WN virus-positive specimens were detected throughout the state (Figure 6). While human disease was restricted to four NYC boroughs (Staten Island, Brooklyn, Manhattan, and Queens), PCR evidence of WN virus infection in mosquitoes was detected in all five NYC boroughs, four adjacent metropolitan counties, and two counties further upstate. Evidence of infection in avian hosts, however, was detected throughout the state (except for Chenango County) (17). Despite the submission of 2,660 pools of primary vector species from 17 upstate counties outside the immediate metropolitan area, we detected PCR evidence of WN virus only in Orange (two pools of *Oc. japonicus* and one each of *Cx. pipiens* and *Cx. pipiens / restuans*) and Columbia (one pool of *Oc. japonicus*) counties. However, MIRs generally reflected the pattern of human cases and dead crow sightings per square mile (17) in three categories: 1) a combination of high number of human cases, dead crow sightings per square mile, and an overall seasonal MIR of 5.27/1,000 tested in Staten Island; 2) a small number of human cases, moderate number of dead crow sightings per square mile, and seasonal MIRs ranging from 0.18 to 2.36/1,000 for counties in and near NYC; and 3) no human cases, low number of dead crow sightings per square mile, and seasonal MIRs ranging from 0 to 0.86/1,000 for upstate counties (Figure 6). Documented WN virus transmission to humans and horses was limited to a much smaller geographic area of New York State, and, over the last two seasons, has included only NYC, Long Island, Westchester County, and Orange County (roughly 50 miles north of NYC).

Discussion

Review of specific local human and mosquito population data will be addressed in separate publications. Our work summarizes statewide surveillance components and identifies some potential flaws due to the geographic aggregation of data. In addition, surveillance may not have been uniformly applied across all municipalities. However, review of these initial surveillance data will lead investigators to design research projects that will better enable the public health

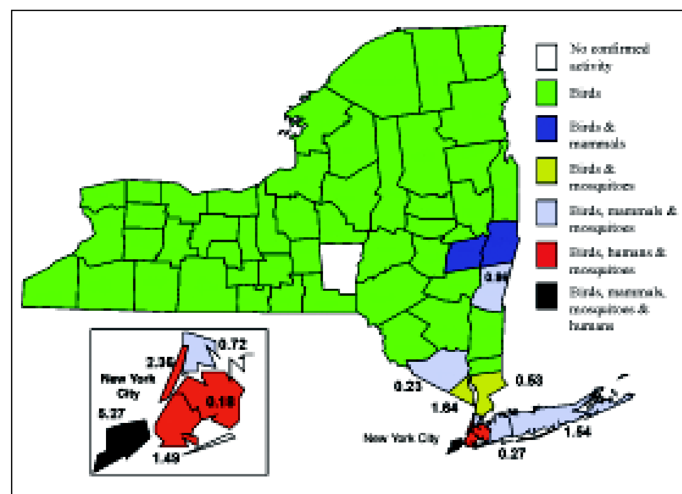

Figure 6. Geographic distribution of West Nile (WN) virus-positive surveillance components in New York State through December 2000, and associated seasonal minimum infection ratios for counties with WN virus-positive mosquito pools.

community to answer questions that continue to arise (e.g., regarding virus transmission, host pathogenicity, immunology, interventions, sociologic aspects, and vaccination or treatment issues).

There may be some risk of faulty interpretation in a retrospective review of statewide field and laboratory data. In addition, we have not fully evaluated the potential effect of mosquito control operations on overall mosquito populations. However, these data provide necessary baseline information related to virus ecology and infection dynamics. The detection of MIRs associated with individual mosquito species may help public health agencies and academic institutions to focus on specific species with high levels of vectorial capacity (18).

The surveillance system in New York State during 2000 led to the submission of >300,000 mosquitoes in almost 10,000 pools for arbovirus screening; most were derived from LHDs that had not previously conducted mosquito or arbovirus surveillance. These efforts required regular internal discussions on laboratory capacity, steps that could be taken to improve it, and proper use of laboratory capacity to answer both routine surveillance and research demands on limited resources.

Although there is an inherent procedural lag between mosquito collection and the availability of laboratory results, this initial analysis supports the natural presence of WN virus PCR-positive *Culex*, *Aedes,* and *Ochlerotatus* mosquitoes 2 weeks before onset of human illness. In addition, peak *Culex* infection during the period of prominent human infection appears to be associated with a peak of PCR WN virus-positive *Aedes* and *Ochlerotatus* immediately before and during the onsets of equine cases, which occurred several weeks after the peak of human onsets. However, laboratory confirmation of positivity for all virus surveillance mechanisms (mosquitoes, birds, humans, and horses) usually lagged behind specimen collection or onset dates by several weeks. In addition, infection dates for human and horse cases are likely to precede onset dates by several days to weeks, depending on the individual incubation periods.

Interpretation of the influence of trap type on yield of PCR-positive pools is complicated by the potential for detecting virus that may have been present in the host on which individual mosquitoes may have fed. Ideally, blood-fed or gravid mosquitoes should be separated from unfed females before laboratory testing. Clearly, however, a blood-fed female mosquito one week will become a host-seeking female a week or two later, and full understanding of the transmission dynamics of WN virus may require this separation, along with specific determination of host meals. Data indicate that agencies with limited budgets may be able to identify the local presence and natural circulation of WN virus more efficiently by focusing on gravid trap-based programs.

Initial analyses of MIRs associated with individual mosquito species indicate that certain species, especially *Cx. salinarius*, *Cx. pipiens, Oc. japonicus* and *Oc. triseriatus*, may play major roles in the natural transmission of WN virus among animal hosts. Several of these species (or species complexes) may be involved with an as-yet-unknown reservoir host capable of maintaining the virus in the absence of overt host pathogenicity. Note, however, that no infectious virus was isolated in approximately half of the mosquito pools tested with detectable RNA. WN virus RNA was detected in five pools of *Oc. japonicus*, for example, but no infectious virus was isolated from these mosquitoes (19). The level of infectious virus may have been too low to be detected and may have increased with further extrinsic incubation, complicating the interpretation of MIRs.

The interrelationships, ecologic niches, and host-meal preferences, for example, of the various *Culex* species may play a major role in the entire ecology of the virus now that it has been introduced to a completely naive hemisphere. In addition, the MIRs of *Culex* and other species associated with the apparently bimodal human and equine case distribution may point to a complex mosquito vector relationship not only among the various *Culex* species, especially *Cx. salinarius* and *Cx. pipiens* and perhaps *Cx. restuans,* but also with other more prominent mammal-feeding species such as *Ae. vexans*, *Oc. triseriatus,* and *Oc. japonicus.* What will happen as this virus continues to spread into other areas of North or Central America where aggressive and more numerous mammal-feeding species exist? How will human disease risks increase if *Oc. sollicitans*, *Cx. tarsalis*, *Ae. aegypti,* or *Anopheles* species become competent vectors under normal field conditions? Are non-mosquito vectors (such as ticks, mites, or black flies) contributing to the total ecologic cycle (e.g., transmission and overwintering) of this virus in nature? Intervention programs must be established that can account for answers to these questions and maintain our original goals of minimizing the risks for human and other animal infections. Given the recognized ability of WN virus to survive in hibernating mosquitoes in the northeastern United States (20) and the current distribution of the virus in birds, mammals, and mosquitoes along the Atlantic seaboard, public health agencies in affected and neighboring states should be prepared to address the emergence of this virus in their jurisdictions in future years.

Another major goal that public health agencies must face is developing a more timely virus surveillance system. This system should allow detection of the virus in mosquito or bird host systems in sufficient time to permit an appropriate intervention to minimize or prevent further mammal infection. Our current surveillance and laboratory testing systems have inherent time lags that may preclude our ability to intervene against infected adult mosquitoes on a timely basis, before humans are exposed.

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References

1. Centers for Disease Control and Prevention. Outbreak of West Nile-like viral encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:845-9.
2. Centers for Disease Control and Prevention. Update: West Nile virus encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:944-6,955.

3. Centers for Disease Control and Prevention. Update: West Nile-like viral encephalitis–New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:890-2.
4. Hubalek Z, Halouzka J. West Nile fever—a reemerging mosquito-borne viral disease in Europe. Emerg Infect Dis 1999;5:643-50.
5. Lanciotti RS, Roehrig JT, Duebel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333-7.
6. Hayes CG. West Nile fever. In: Monath RD, editor. The arboviruses: epidemiology and ecology. Boca Raton (FL): CRC Press, Inc.; 1989.
7. Savage HM, Ceianu C, Nicolescu G, Karabatsos N, Lanciotti R, Vladimirescu A, et al. Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serologic and molecular characterization of a virus isolate from mosquitoes. Am J Trop Med Hyg 1999;61:600-11.
8. Centers for Disease Control and Prevention. Update: West Nile virus activity—eastern United States, 2000. MMWR Morb Mortal Wkly Rep 2000;49:1044-7.
9. Centers for Disease Control and Prevention. West Nile virus activity—New York and New Jersey, 2000. MMWR Morb Mortal Wkly Rep 2000;49:640-2.
10. Anderson JF, Andreadis TG, Vossbrinck CR, Tirrell S, Wakem EM, French RA, et al. Isolation of West Nile virus from mosquitoes, crows and Cooper's hawk in Connecticut. Science 1999;286:2331-3.
11. Komar N. West Nile viral encephalitis. Rev Sci Tech 2000;19:166-76.
12. Centers for Disease Control and Prevention. Guidelines for surveillance, prevention, and control of West Nile virus infection—United States. MMWR Morb Mortal Wkly Rep 2000;49:25-8.
13. Nasci RS, White DJ, Stirling H, Oliver J, Daniels TJ, Falco RS, et al. West Nile virus isolates from mosquitoes in New York and New Jersey, 1999. Emerg Infect Dis 2001;7:626-30.
14. Newhouse VR, Chamberlain RW, Johnston JF, Sudia WD. Use of dry ice to increase mosquito catches of the CDC miniature light trap. Mosquito News 1966;26:30-5.
15. Reiter P. A portable, battery-powered trap for collecting gravid *Culex* mosquitoes. Mosquito News 1983;43:496-8.
16. Shi P-Y, Kauffman EB, Ren P, Felten A, Tai JH, Dupuis AP II, et al. High throughput detection of West Nile virus RNA. J Clin Microbiol 2001;39:1264-71.
17. Eidson M, Miller J, Kramer L, Cherry B, Hagiwara Y, and the WNV Bird Mortality Analysis Group. Dead crow densities and human cases of West Nile virus, New York State, 1999. Emerg Infect Dis 2001;7:662-4.
18. Spielman A, James AA. Transmission of vector-borne disease. In: Warren KS, Mahmood AAF, editors. Tropical and geographical medicine. 2nd ed. New York: McGraw Hill; 1990. p. 146-59.
19. Bernard KA, Maffei JG, Jones SA, Kauffman EB, Ebel GD, Dupuis AP, et al. West Nile virus infection in birds and mosquitoes, New York State, 2000. Emerg Infect Dis 2001;7:679-85.
20. Nasci RS, Savage HM, White DJ, Miller JR, Cropp BC, Godsey MS, et al. West Nile virus in overwintering *Culex* mosquitoes, New York City, 2000. Emerg Infect Dis 2001;7:742-4.

Partial Genetic Characterization of West Nile Virus Strains, New York State, 2000

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We analyzed nucleotide sequences from the envelope gene of 11 West Nile (WN) virus strains collected in New York State during the 2000 transmission season to determine whether they differed genetically from each other and from the initial strain isolated in 1999. The complete envelope genes of these strains were amplified by reverse transcription-polymerase chain reaction. The resulting sequences were aligned, the genetic distances were computed, and a phylogenetic tree was constructed. Ten (0.7%) of 1,503 positions in the envelope gene were polymorphic in one or more sequences. The genetic distances were 0.003 or less. WN virus strains circulating in 2000 were homogeneous with respect to one another and to a strain isolated in 1999.

The first outbreak of West Nile (WN) virus infection in North America (1) was apparently the result of single introduction and subsequent amplification of WN virus among *Culex pipiens* mosquitoes and their avian hosts (2-4). Human disease was accompanied by an epizootic in which high death rates from severe meningoencephalitis and myocarditis were reported in some avian hosts, notably American Crows *(Corvus brachyrhynchos)* (5). RNA virus populations are subject to high mutation rates and may evolve rapidly under certain conditions (6-8). To determine whether WN virus genotypes circulating in New York during the 2000 transmission season differed from those isolated there in 1999, WN virus strains were collected from mosquito pools and dead vertebrates, the complete nucleotide sequences of the envelope genes were determined, and the sequences of these strains were compared with one another and with a strain isolated during 1999.

Materials and Methods

WN virus was isolated from pools of infected mosquitoes collected throughout New York State and from vertebrate tissues submitted by the N.Y. State Wildlife Pathology Unit. Mosquitoes were collected overnight in standard miniature light traps or gravid traps, and they were pooled and sent to the New York State Arbovirus Laboratories. Pools of mosquitoes and vertebrate tissues were homogenized in 2 mL of mosquito diluent (20% heat-inactivated fetal bovine serum [FBS] in Dulbecco's phosphate-buffered saline plus 50 µg/mL penicillin/streptomycin, 50 µg gentamicin, and 2.5 µg/mL fungizone) or 350 µL lysis buffer, respectively, by using an SPEX mixer-mill (Spex CertiPrep, Metuchen, NJ) and glass beads; 500 µL of the resulting suspension was transferred to 1.5-mL microcentrifuge tubes and centrifuged at 16,000 RCF for 10 min; 100 µL of the clarified solution was applied to confluent monolayers of African Green Monkey Kidney (Vero) cells in T-25 flasks, and virus was allowed to adsorb for 1 hr at 37°C, 5% CO_2. After adsorption, 5 mL of minimum essential medium (MEM) containing 2% FBS and antibiotics (as above) was applied to the cells, and they were returned to the incubator. Cultures were checked for signs of cytopathic effect (CPE) daily. When >50% of cells in a culture flask displayed CPE, the culture was harvested, and clarified aliquots of the culture media supernatant were supplemented with FBS (20% of final volume) and stored in 1.5-mL cryovials at -80°C until further use.

Virus stocks were passed by applying 100 µL of the initial culture supernatant to a second confluent monolayer of Vero cells, as above. When CPE was evident in >50% of the cells in the culture, the cells were scraped from the flask and centrifuged with the media in 15-mL conical tubes at 3,000 x *g* for 20 minutes. RNA was extracted from the resulting cell pellet by using RNeasy columns (Qiagen, Valencia, CA) as directed by the manufacturer. The complete envelope sequences were amplified by reverse transcription-polymerase chain reaction (RT-PCR) with primers (Forward [5-CATCGAATTCGTTACCCTCTCTAACTTC-CAAGGGAAGGTG-3] Reverse [5-GTATGGATCCTGATGCTC-CAGTCTGGAAACTGATCGTA-3]) designed to amplify the genomic sequence covering the coding region of the complete prM/M, E, and the N-terminal NS1. These primers also contain engineered restriction sites for use in other experiments that will be described elsewhere. Reaction products were electrophoretically separated on 2% agarose gel, and bands of the predicted size were excised and purified by using the Qiaquick gel extraction kit (Qiagen, Valencia, CA). Purified DNA fragments were sequenced on an ABI PRISM 377XL automated DNA sequencer (Applied Biosystems, CA) with six forward and six reverse primers (Table 1).

Two of the vertebrate strains (3282 and 3356) were processed differently. RT-PCR was conducted directly on RNA isolated from infected tissues. The primers used for the

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Table 1. Primers used in West Nile virus sequencing

Primer	Sequence
WNSE1F	CTC TCT AAC TTC CAA GGG AAG
WNSE2F	CAC TCT AGC GAA CAA GAA GG
WNSE3F	TCT CCA CCA AAG CTG CGT GC
WNSE4F	TAC TAC GTG ATG ACT GTT GGA A
WNSE5F	CCT TGC AAA GTT CCT ATC TC
WNSE6F	TCC TGT TGT GGA TGG GCA TC
WNSE1R	TGT CTT CTG GAT CAT TAC CAG C
WNSE2R	GCC ACC AGG GCA TAT CCA GG
WNSE3R	TTC AAG ATG GTT CTT CCT ATT GC
WNSE4R	GGA ATG GCT CCA GCC AAA GC
WNSE5R	TGT TCT CCT CTG CCC ACC AC
WNSE6R	TCC ATC CAA GCC TCC ACA TC

amplification and sequencing steps will be described elsewhere (Lanciotti et al., manuscript in preparation).

Sequences were aligned with a WN virus strain collected in 1999 (GenBank Accession #AF260967) and a distantly related St. Louis encephalitis virus sequence (AF205490) by using the clustal method on the DNAStar software package. Initial analysis was done by the distance method using MEGA (9). Evolutionary distances were computed by the Kimura 2 parameter method including both transitions and transversions. Distance trees were constructed by the neighbor-joining method, and their robustness was estimated by performing 500 bootstrap replicates.

Results

WN virus strains from diverse locations, times, and host types were assembled for this study (Table 2). The strains were isolated from avian and mosquito hosts collected from midsummer through autumn at the epicenter and at the periphery of the 2000 epizootic. Strains were thus a representative sample of WN virus circulating in New York during 2000.

Nucleotide substitutions occurred at 10 (0.7%) of the 1,503 positions in the envelope gene (Table 3). Of these substitutions, all were transitions, two (0.4%) of which resulted in amino acid changes. The C to U substitution at position 2321 (position numbers refer to Lanciotti et al. [1]) results in a serine to leucine change in envelope amino acid number 452, and the A to G substitution at position 2386 results in an isoleucine to valine change in envelope amino acid position 474. The mean pairwise Kimura 2-parameter distances between the isolates were 0.003 or less. The phylogenetic tree of the nucleotide sequences studied showed similarly minimal distances between the isolates, with low bootstrap confidence values at the nodes separating the branches. WN virus strains circulating in New York State during the 2000 transmission season were relatively homogeneous at both the nucleotide and amino acid levels.

Conclusion

These data represent the first population study of WN virus in North America since its introduction in 1999. The envelope sequences of these virus strains establish a baseline sequence dataset against which strains isolated during future transmission seasons may be compared.

Only the envelope sequences were studied, which were analyzed by using distance matrices and neighbor-joining methods. Although complete genome sequences may have provided additional information, short sequence fragments have often been used in population studies of arboviruses (10-13). Additional criteria (maximum parsimony and maximum likelihood) may have provided corroboration for the close relationships observed; however, the sequences are so similar and the nodes on the neighbor-joining tree so poorly supported that additional analyses seemed unwarranted. Given the close relationship of the strains, it is unlikely that additional nucleotide data or analytic methods would have greatly enhanced our understanding of WN virus population structure in this hemisphere.

Mosquito- and vertebrate-derived sequences appeared to be distributed randomly in the phylogenetic tree. Date of isolation of the strains was similarly unimportant in the clustering of sequences. Additionally, passage history seemed not to affect the gene sequences. RT-PCR amplification directly from infected mosquito pools often failed or produced amplicons that did not conform to size expectations, necessitating Vero cell passage of many strains before amplification. The two sequences obtained from RNA extracts of infected tissue without Vero passage were not different from those passed through these cells twice. The WN virus sequences in this study were homogeneous with respect to passage history, host, and time.

A single nucleotide substitution, a C to U change at position 1974, was present in four of five strains isolated from Staten Island but not from other locations. This mutation caused these strains (3000017, 3100352, 3100365, and 3356) to cluster in the phylogenetic tree, but bootstrap confidence in this clustering pattern, as for all the relationships displayed (Figure), was low. The utility of this particular substitution for molecular epidemiologic studies of WN virus in North

Table 2. Characteristics of strains studied

Strain	Collection date	County/borough	Site/town	Source	Passage history*	GenBank Accession No.
3000017	Jul-2000	Staten Island	Richmond	*Cx. pipiens*	V2	AF346309
3000259	Jul-2000	Suffolk	Calhoun	*Cx. pip/restuans*	V2	AF346316
3000548	Jul-2000	Queens	Country Farm Museum	*Cx. pip/res*	V2	AF346311
3000622	Jul-2000	Westchester	Twin Lake Stable	*Cx. pip/res*	V2	AF346313
3100271	Jul-2000	Rockland	Unknown	*Cx. pip/res*	V2	AF346312
3100352	Jul-2000	Staten Island	Saw Mill Marsh	*Cx. salinarius*	V2	AF346314
3100365	Jul-2000	Staten Island	Fresh Kills Landfill	*Cx. pipiens*	V2	AF346310
842	Jul-2000	Staten Island	Amboy Rd.	American Crow	V2	AF346317
2741	Sep-2000	Albany	SUNY	American Crow	V2	AF346315
3282	Oct-2000	Oswego	New Haven	Ruffed Grouse	P	AF346319
3356	Oct-2000	Staten Island	Mariner's Harbor	American Crow	P	AF346318

*V2=Two vero passages, P=primary RNA tissue extract

Table 3. Nucleotide substitutions in strains studied

Strain	Position									
	1285	1332	1449	1899	1974	2280	2321	2359	2386	2424
NY99-EQHS	C	U	C	U	C	U	C	C	A	C
3000017	-	-	-	-	U	C	-	-	-	U
3000259	-	-	-	-	-	-	-	-	-	-
3000548	-	C	-	-	-	-	-	-	-	-
3000622	-	-	U	-	-	-	-	-	-	-
3100271	-	-	-	-	-	-	-	-	-	-
3100352	-	-	-	C	U	-	-	-	-	-
3100365	-	-	-	-	U	-	-	-	-	-
842	-	-	-	-	-	-	-	U	G	-
2741	-	-	-	-	-	-	-	-	-	-
3282	U	-	-	-	-	-	-	-	-	-
3356	U	-	-	-	U	-	U	-	-	-

Position numbers correspond to Lanciotti et al.(1).

America is difficult to ascertain, but in principle, findings of this type may provide useful information in determining the mode or modes of spread of particular WN virus strains in North America.

The envelope sequences studied are highly conserved. RNA viruses are well known to exist as quasispecies, composed of a swarm of competing viral genotypes (14,15). This mode of existence, because of the lack of proofreading and mismatch-repair mechanisms of most viral encoded RNA-dependent RNA polymerases (16), may allow rapid evolution under certain circumstances. Dengue virus, another mosquito-borne flavivirus, is thought to have diversified as the viral population expanded with human and mosquito populations (17). WN virus, having entered a naïve ecosystem and vastly expanded its range, may evolve similarly. Many arboviruses, however, are remarkably conserved across time and space, implying stringent constraints on viral structural proteins and replicative machinery (18). Fitness of vesicular stomatitis virus, an animal RNA virus, has been shown to drop precipitously as the virus passed through a series of population bottlenecks (19). Whether WN virus will follow a pattern of diversification or conservation is unclear. The viruses in this study are likely the result of a single introduction of WN virus, primary expansion during 1999, overwintering, and secondary expansion during the 2000 transmission season. Determining the genetic structure of WN virus populations in subsequent transmission seasons may advance our understanding of WN virus perpetuation, selection, and evolution.

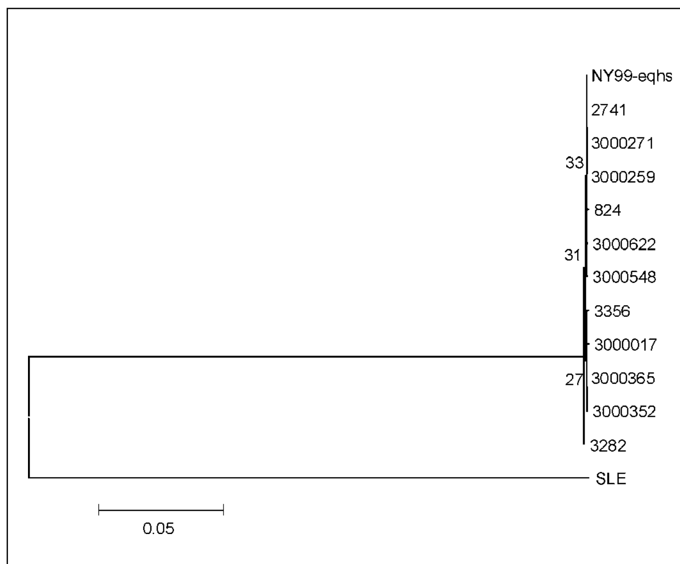

Figure. Phylogenetic relationships among West Nile virus strains collected in 2000. This tree is based on the 1503-bp envelope gene. Distance analysis based on Kimura 2-parameter distance including both transitions and transversions. Numbers at the nodes are bootstrap confidence estimates based on 500 replicates.

Acknowledgments

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References

1. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K. et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333-7.
2. Baqar S, Hayes CG, Murphy JR, Watts DM. Vertical transmission of West Nile virus by *Culex* and *Aedes* species mosquitoes. Am J Trop Med Hyg 1993;48:757-62.
3. Rappole JH, Derrickson SR, Hubalek Z. Migratory birds and spread of West Nile virus in the Western Hemisphere. Emerg Infect Dis 2000;6:319-28.
4. Komar N. West Nile viral encephalitis. Rev Sci Tech 2000;19:166-76.
5. Steele KE, Linn MJ, Schoepp RJ, Komar N, Geisbert TW, Manduca RM, et al. Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, New York. Vet Pathol 2000;37:208-24.
6. Holland JJ, Spindler K, Horodyski F, Grabau E, Nichol S, VandePol S. Rapid evolution of RNA genomes. Science 1982;215:1577-85.
7. Steinhauer DA, Holland JJ. Rapid evolution of RNA viruses. Annu Rev Microbiol 1987;41:409-33.

8. Garmendia AE, Van Kruiningen HJ, French RA, Anderson JF, Andreadis TG, Kumar A, et al. Recovery and identification of West Nile virus from a hawk in winter. J Clin Microbiol 2000;38:3110-1.
9. Kumar S, Tamura K, Nei M. MEGA: molecular evolutionary genetics analysis. University Park (PA): Pennsylvania State University Press; 1993.
10. Kramer LD, Presser SB, Hardy JL, Jackson AO. Genotypic and phenotypic variation of selected Saint Louis encephalitis viral strains isolated in California. Am J Trop Med Hyg 1997;57:222-9.
11. Harris E, Roberts TG, Smith L, Selle J, Kramer LD, Valle S, et al. Typing of dengue viruses in clinical specimens and mosquitoes by single-tube multiplex reverse transcriptase PCR. J Clin Microbiol 1998;36:2634-9.
12. Powers AM, Oberste MS, Brault AC, Rico-Hesse R, Schmura SM, Smith JF, et al. Repeated emergence of epidemic/epizootic Venezuelan equine encephalitis from a single genotype of enzootic subtype ID virus. J Virol 1997;71:6697-705.
13. Ebel GD, Foppa I, Spielman A, Telford SR. A focus of deer tick virus transmission in the northcentral United States. Emerg Infect Dis 1999;5:570-4.
14. Duarte EA, Novella IS, Weaver SC, Domingo E, Wain-Hobson S, Clarke DK, et al. RNA virus quasispecies: significance for viral disease and epidemiology. Infect Agents Dis 1994;3:201-14.
15. Holland JJ, de la Torre JC, Steinhauer DA. RNA virus populations as quasispecies. Curr Top Microbiol Immunol 1992;176:1-20.
16. Steinhauer DA, Domingo E, Holland JJ. Lack of evidence for proofreading mechanisms associated with an RNA virus polymerase. Gene 1992;122:281-8.
17. Zanotto PMDA, Gould EA, Gao GF, Harvey PH, Holmes EC. Population dynamics of flaviviruses revealed by molecular phylogenies. Proc Natl Acad Sci U S A 1996;93:548-53.
18. Pisano MR, Tolou H. The topotype notion and the quasispecies concept: The yellow fever virus as example. Travaux Scientifiques des Chercheurs du Service de Sante des Armees 1996;69-70.
19. Duarte E, Clarke D, Moya A, Domingo E, Holland J. Rapid fitness losses in mammalian RNA virus clones due to Mueller's ratchet. Proc Natl Acad Sci U S A 1992;89:6015-9.

Clinical Findings of West Nile Virus Infection in Hospitalized Patients, New York and New Jersey, 2000

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Outbreaks of West Nile (WN) virus occurred in the New York metropolitan area in 1999 and 2000. Nineteen patients diagnosed with WN infection were hospitalized in New York and New Jersey in 2000 and were included in this review. Eleven patients had encephalitis or meningoencephalitis, and eight had meningitis alone. Ages of patients ranged from 36 to 87 years (median 63 years). Fever and neurologic and gastrointestinal symptoms predominated. Severe muscle weakness on neurologic examination was found in three patients. Age was associated with disease severity. Hospitalized cases and deaths were lower in 2000 than in 1999, although the case-fatality rate was unchanged. Clinicians in the Northeast should maintain a high level of suspicion during the summer when evaluating older patients with febrile illnesses and neurologic symptoms, especially if associated with gastrointestinal complaints or muscle weakness.

Following the emergence of West Nile (WN) virus in New York in 1999, state and local health departments in the eastern United States, in conjunction with the Centers for Disease Control and Prevention (CDC), established surveillance systems for detecting WN virus activity (1). New York City and New Jersey established active and enhanced passive surveillance systems for human disease that encouraged physician, infection control practitioner, and laboratory reporting of suspected cases and provided testing for WN virus. This report details the clinical characteristics of 19 hospitalized human cases that occurred during the summer and fall of 2000; all patients resided in either New York City (NYC) or New Jersey.

Methods

Enhanced surveillance for WN virus human disease in New York and New Jersey during 2000 was instituted to facilitate timely reporting of viral meningoencephalitis and to ensure rapid and accurate laboratory testing for WN virus. In NYC, encephalitis and viral meningitis are reportable conditions. From May to October 2000, the following measures were implemented by the NYC Department of Health to supplement existing passive surveillance: 1) Enhanced passive surveillance—To encourage physician reporting citywide, information on WN virus reporting and testing procedures was widely disseminated to the medical community through invited presentations by departmental medical staff, an agency publication mailed to >65,000 health-care providers, and biweekly broadcast facsimile and e-mail alerts to all NYC hospitals; 2) Hospital-based active physician surveillance—Neurologists, infectious disease consultants, intensive-care physicians, and chief medical residents at 18 sentinel NYC hospitals were called every 2 weeks to ascertain potential cases meeting clinical criteria; 3) Hospital-based active laboratory surveillance—Laboratories at 12 sentinel NYC hospitals submitted all cerebrospinal fluid (CSF) specimens suggestive of a viral cause for WN virus testing at the NYC health department (CSF with negative Gram stain and culture with either a CSF leukocyte count $\geq 5/mm^3$ or protein ≥ 40 mg/dL). A special unit was established within the Communicable Disease Program of the NYC Department of Health to implement these additional surveillance activities. This unit ensured that all suspected cases were prioritized for next-business-day specimen collection and transportation to the city's Public Health Laboratories for WN virus testing.

In New Jersey, bacterial meningitis and encephalitis are reportable to the New Jersey Department of Health and Senior Services (NJDHSS). Active, hospital-based surveillance by infection control practitioners targeted patients admitted with the diagnoses of aseptic meningitis or encephalitis in 42 hospitals in six northern counties. Passive surveillance was enhanced through the distribution of reporting protocols, surveillance criteria, and WN virus educational materials to the medical community. Medical

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providers were reminded to notify the NJDHSS of suspected cases. NJDHSS conducted weekly follow-up with physicians and infection control practitioners to review the status of pending cases.

A patient was considered to have a confirmed WN case if he or she was hospitalized with an illness associated with neurologic manifestations consistent with meningitis or encephalitis, and had laboratory confirmation of WN infection. The four laboratory confirmation criteria used for WN infection, established by CDC (1), are as follows: 1) isolation of WN virus from, or demonstration of WN viral antigen or genomic sequences in tissue, blood, CSF, or other body fluid; 2) demonstration of immunoglobulin M (IgM) antibody to WN virus in CSF by IgM-capture enzyme immunoassay (EIA); 3) ≥4-fold serial change in plaque-reduction neutralizing antibody titer (PRNT) to WN virus in paired, appropriately timed serum or CSF samples; and 4) demonstration of both WN virus-specific IgM (by EIA) and IgG (screened by EIA and confirmed by PRNT) antibody in a single serum specimen.

Patients were classified into three clinical categories: meningitis, if they had fever plus headache, stiff neck, or photophobia; encephalitis, if they had altered mental status or other cortical signs; or meningoencephalitis, if they met both criteria. The categories of encephalitis and meningoencephalitis were combined as "any encephalitis" in some analyses. All syndromes required abnormal CSF findings consistent with a viral cause (CSF with negative Gram stain and culture with either a CSF leukocyte count $\geq 5/mm^3$ or protein ≥40 mg/dL).

IgM-capture EIA was performed at either the NYC Public Health Laboratories or the New Jersey Public Health and Environmental Laboratory; confirmation of positive results by PRNT was performed by CDC or the NY State Department of Health. Viral neutralization testing followed CDC protocol (R. Lanciotti, pers. commun.). Real-time Taqman polymerase chain reaction (PCR) was performed at CDC. Medical chart reviews and patient interviews were completed on all patients with positive tests for WN virus by IgM-capture EIA.

Supplementary medical chart reviews by the physician authors were completed in November-December 2000 after confirmation of initial results. Information abstracted included demographics, symptoms, chronology of illness, admission diagnosis, clinical findings, coexisting illness, laboratory findings, hospital course, diagnostic procedures, complications, level of neurologic involvement, discharge condition, and diagnoses. If a symptom was not specifically mentioned or a physical finding was not noted in the medical record, it was considered to be absent. Patient addresses were geocoded and mapped to compare the geography of the 2000 and 1999 epidemics. Descriptive statistics and Fisher's exact p values were calculated with SPSS (SPSS Chicago, version 10.0) and Epi Info (CDC, Atlanta, version 6.04b). Mapping was done in ArcView (ERSI, Redlands, CA, version 3.2).

Results

Demographics

Nineteen hospitalized WN virus patients were identified in 2000, 14 (74%) from New York and 5 (26%) from New Jersey. The 14 New York cases were from four of the five NYC counties; 10 were from Richmond County (Staten Island), 2 from Kings County (Brooklyn), and one each from New York County (Manhattan) and Queens County. The New Jersey cases occurred in Hudson County (2 cases) and in Bergen, Morris, and Passaic counties (1 case each). Eleven (58%) were male and eight (42%) were female. The median age was 63 years (range 36-87). Eight patients (42%) were ≥65 years of age, and six (32%) of these were ≥75 years of age.

Clinical Illness and Hospital Course

Nine patients were classified as having encephalitis, eight with meningitis and two with meningoencephalitis. All eight patients ≥65 years of age had either encephalitis or meningoencephalitis, accounting for 73% of all cases with encephalitis. The mean age of patients with encephalitis was 71 years (standard deviation [SD]=11.7), compared with 51 years (SD=14.5) for patients with meningitis alone. A history of hypertension (as documented in the past medical history section of the medical record) was present in 8 (73%) of 11 patients with either encephalitis or meningoencephalitis, compared with 3 (38%) of 8 patients with meningitis alone.

The median and mean time periods from symptom onset to hospitalization were 3 and 7.7 days, respectively (range 0-48). One patient became symptomatic 3 days after being hospitalized for an unrelated, noninfectious condition, and another was hospitalized 48 days after the initial onset of symptoms. Patients' onset dates occurred within a 9-week interval from July 19 to September 12, 2000 (Figure 1). The median length of hospital stay was 7 days (range 1-72), and patients ≥65 years of age had a longer median length of stay than those <65 years (11 days vs. 6 days). Five patients were admitted to intensive care units (ICU), and two required mechanical ventilation. The median length of stay in the ICU was 17 days (range 2-47 days).

The patient's temperature on admission ranged from 36.6°C to 40.6°C (median 38.6°C), and 14 patients were febrile upon arrival at the emergency department (fever defined as temperature of ≥38.0°C). Three patients became febrile during their hospital stay, and two did not have documented fever and were determined to have WN infection based on a clinical diagnosis of meningitis or encephalitis and laboratory confirmation. The mean duration of fever was 2.9

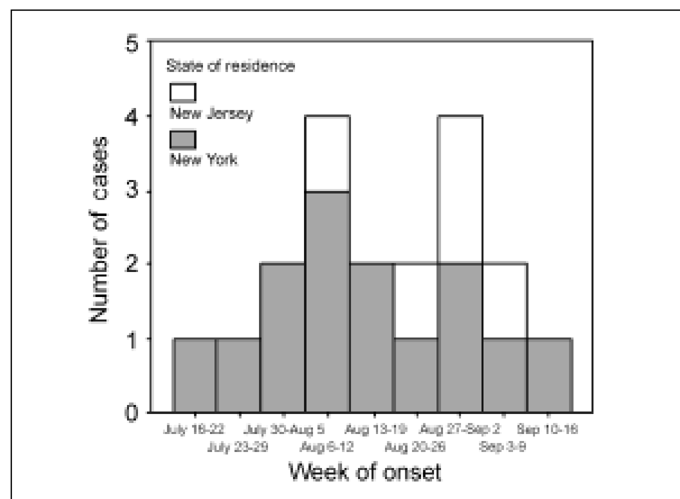

Figure 1. New York City metropolitan area West Nile virus epidemic curve, 2000.

days (range 0-6 days). The two patients without fever were <41 years old. One denied a history of fever, while the other was admitted 7 weeks after onset with an unclear history of fever.

The frequency of symptoms and clinical findings is presented in Table 1. Neurologic and gastrointestinal findings predominated. Of the 19 cases, 16 (84%) presented with at least one neurologic complaint (headache, stiff neck, photophobia, muscle weakness, or change in mental status). Seventeen patients (89%) had one or more abnormalities on neurologic examination. Motor exams in three patients demonstrated muscle weakness (strength <5/5); of the six with abnormal reflexes, four were hyporeflexive, and two had abnormal plantar responses; the two patients with cerebellar abnormalities were ataxic. Both patients with cranial nerve abnormalities died; one had nystagmus and the other had a depressed gag reflex.

Table 1. Frequency of clinical findings in West Nile virus patients, New York and New Jersey, 1999 and 2000

Symptom or physical finding	Frequency No. (%)
Fever	17 (90)
Fatigue	12 (63)
Altered mental status	11 (58)
Headache	11 (58)
Reported weakness	8 (42)
Nausea	8 (42)
Vomiting	8 (42)
Myalgia	6 (32)
Photophobia	6 (32)
Abnormal reflexes	6 (32)
Stiff neck	6 (32)
Abdominal pain	4 (21)
Motor weakness	3 (16)
Cough	3 (16)
Diarrhea	3 (16)
Seizures	3 (16)
Arthralgia	2 (11)
Cerebellar abnormality	2 (11)
Cranial nerve palsy	2 (11)
Shortness of breath	2 (11)

Eleven patients (58%) had at least one gastrointestinal symptom or had abnormal abdominal findings. Three patients had rash. In two of these, the rash was truncal and either macular or papular; the rash in the third patient was not described.

Seventeen patients initially received antibiotics, and eight were treated with acyclovir for presumptive herpes encephalitis. One patient was comatose and was treated with oral ribavirin and alpha-interferon without improvement. This patient died of complications 16 weeks after transfer to a long-term care facility.

Laboratory and Radiology Findings

Eighteen cases were diagnosed based on positive CSF IgM-capture EIA; 9 of these were confirmed by a fourfold rise in PRNT antibodies. An appropriately timed acute- or convalescent-phase serum sample could not be obtained for the remaining patients. Thirteen patients also had real-time Taqman PCR testing of CSF, and one was positive (obtained 8 days after illness onset). One other patient, a 43-year-old man, did not have sufficient CSF for testing; his case was confirmed by positive serum IgM-capture EIA and PRNT results in a single serum specimen. Seventeen patients had an initial CSF pleocytosis, and 15 of these had a differential cell count performed. Nine patients had a predominance of neutrophils ($\geq$50%) in the CSF (Table 2). The presence of neutrophilic pleocytosis was not associated with the more severe presentation of encephalitis (p=0.5).

Three patients had hemoglobin values >2 SD below the gender-specific mean values (73-year-old man, a 73-year-old woman, and an 87-year-old woman). A low platelet count (<150,000/mm^3) occurred in one patient with a previous history of thrombocytopenia, and another patient had a low total leukocyte count (4,400/mm^3). Abnormal serum sodium levels (Na <135 mmol/L) occurred in eight (42%) patients. This finding was noted more frequently in those with encephalitis (hyponatremia in 6/11 with any encephalitis vs. 2/8 with meningitis). In two patients with hyponatremia and encephalitis, the low serum sodium could be explained by

Table 2. West Nile patient laboratory findings, New York and New Jersey, 1999 and 2000

Test	Number tested (%)	Mean value or N with condition (range)	Normal values (2)
CSF			
Leukocyte count, mean	19 (100)	308 mm^3 (0-1782)	0-5 cells/mm^3
Red cell count, mean	16 (84)	115/mm^3 (0-700)	0 cells/mm^3
Protein, mean	19 (100)	111 mg/dL (56-555)	15-50 mg/dL
Glucose, mean	19 (100)	67 mg/dL (48-95)	50-80 mg/dL
Differential,[a] $\geq$50% neutrophils	15 (79)	9 (1-100%)	All mononuclear cells
Complete blood cell count			
Leukocyte count, mean	19 (100)	10,600/mm^3 (4,400-19,700)	4,500-11,000/mm^3
Differential cell count,[a] >77% segs + bands	18 (95)	11 (55-96%)	59% ± 18
Hemoglobin (male), mean	11 (100)	14.5 g/dL (11.8-16.5)	15.5 g/dL ± 1.1
Hemoglobin (female), mean	8 (100)	12.7 g/dL (10.5-14.6)	13.7 g/dL ± 1.0
Other laboratory			
Hyponatremia, serum Na <135 mmol/L	19 (100)	8 (42%)	135-145 mmol/L
Elevated AST, >twice upper limit	17 (90)	4 (24%)	10-35 units/L
Elevated ALT, >twice upper limit	15 (79)	1 (7%)	20-48 units/L
Elevated total bilirubin, >twice upper limit	16 (84)	3 (19%)	0.3-1.0 mg/dL

[a]Values are the number of patients with the laboratory finding; ranges are the values of all patients.
CSF = cerebrospinal fluid; AST = aspartate aminotransferase; ALT = alanine aminotransferase; segs = segmented neutrophils.

other causes. One had spurious hyponatremia caused by hyperglycemia (glucose=598 mg/dL), and the other had elevated blood urea nitrogen (34 mg/dL) and urine specific gravity (1.025) consistent with dehydration.

Radiographic imaging of the brain was conducted in 18 patients (95%). Eleven had computerized tomography, two had magnetic resonance imaging, and five had both. Abnormalities were noted in 10 (56%) patients. Eight had nonacute abnormalities with either evidence of an old infarct, mild communicating hydrocephalus, atrophy, leukomalacia, or ischemia. Two had acute inflammatory changes: one had leptomeningeal enhancement and the other had periventricular hyperintensity of the white matter. Seven of the eight patients with evidence of old brain injury had encephalitis, compared with two of eight with normal brain imaging studies. A patient with Parkinson's disease, diabetes, and hypertension was the only patient in the series to have an electromyogram. Results showed moderate-to-severe peripheral neuropathy, mainly demyelinating, with involvement of sensory and motor neurons consistent with Guillain-Barré syndrome.

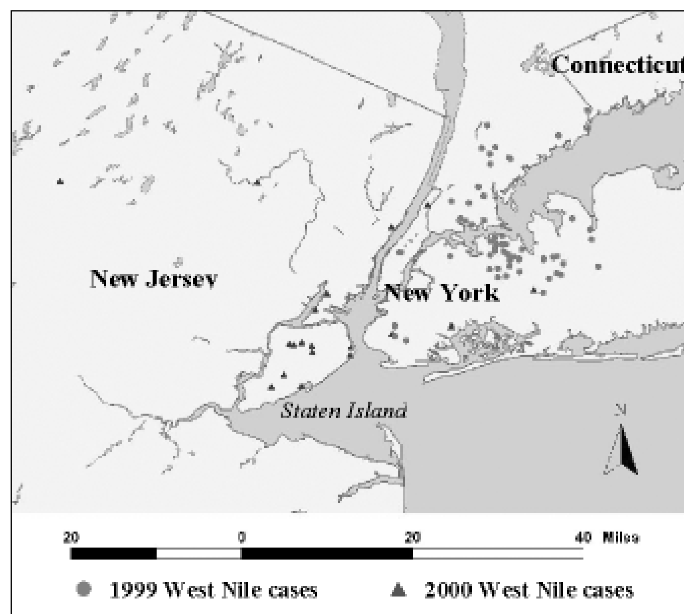

Figure 2. Metropolitan New York area hospitalized West Nile virus patients, 1999-2000.

Outcome

As recorded in discharge summaries, 10 patients (53%) recovered but not to their functional level before illness, 7 (37%) recovered fully, and 2 died (11%). Both deaths occurred in patients >80 years of age, and neither had an autopsy. Thirteen (68%) patients were discharged to home, 4 (21%) were discharged to a long-term care facility, one (5%) died in the hospital, and the location of discharge of one patient (5%) was unknown. When discharged from the acute-care facility, seven (37%) were fully ambulatory, five (26%) were ambulatory with assistance, two (11%) were bedridden, and the condition was unknown for four (21%) patients. Five (26%) patients required in-hospital physical therapy or consultation, three (16%) required speech therapy or consultation, and two (11%) had occupational therapy or consultation.

Temporal and Geographic Trends

WN virus patients had onset dates in the 9-week period from July 19 to September 12, 2000. The temporal distribution of cases was bimodal, with four cases occurring during the weeks of August 6-12 and August 27-September 2 (Figure 1). The epicenter was in Staten Island with the hospitalized human cases encompassing an area of 1,520 square miles (Figure 2). During the first 6 weeks of the epidemic, nine cases occurred on Staten Island, one in Brooklyn, and two in New Jersey. In the final 3 weeks of the epidemic, one case occurred on Staten Island, three cases occurred in other New York City boroughs (one each in Brooklyn, Manhattan, and Queens), and three cases occurred in New Jersey.

Discussion

Nineteen hospitalized adults were diagnosed with WN virus infection in the New York metropolitan area in 2000, a decline of 68% from 1999. The epicenter was located in Staten Island, approximately 20 miles west and south of the 1999 epicenter in northern Queens. Most patients had a febrile illness associated with meningeal signs, altered mental status, or both. The median age of hospitalized patients was lower in 2000 (63 vs. 71 years), and the proportion with encephalitis decreased from 63% to 58% (p=0.1). Gastrointestinal complaints were common, and severe motor weakness was reported less frequently than in 1999 (16% in 2000 vs. 27% in 1999). In 1999, seven deaths were caused by WN virus; in 2000 two were. The case-fatality rates for the 2 years do not differ statistically (11.9% in 1999, 10.5% in 2000, p=0.6).

Routine laboratory findings were nonspecific. CSF findings were consistent with a nonbacterial inflammatory process. Mild hyponatremia was found in eight patients. The syndrome of inappropriate secretion of antidiuretic hormone has been described in viral meningitis, St. Louis encephalitis, and WN virus (3-5). Two of eight patients with hyponatremia had other reasons for this finding (one with suspected dehydration and the other with hyperglycemia), and information on the use of antihypertensive medications, including diuretics, was not collected nor were urine osmolalities measured. A possible association of WN infection with this syndrome cannot be determined from this case series and requires further investigation.

Reasons for the differences seen in the number of human cases over the two epidemic years are speculative. Aggressive mosquito and larval control activities, particularly on Staten Island, may have reduced the infected mosquito population enough to diminish WN virus transmission to humans. Increased immunity in the resident avian population may have also prevented the re-establishment of an enzootic amplification cycle sufficient to cause significant human disease in Queens, the epicenter of the 1999 outbreak. Evidence from an avian serosurvey conducted after the 1999 epidemic supports this theory, since 51% of birds captured in Queens and 2% of those in Staten Island tested positive for WN virus (6).

WN virus infection in hospitalized cases in 2000 occurred over a 9-week period from mid-July to mid-September. A greater proportion of cases occurring outside Staten Island were recognized toward the end of this interval, which may relate to differences in vector ecology or control measures

used. The timing of the 2000 epidemic curve closely resembles that of the recent outbreak in Romania (7) and preceded the 1999 New York epidemic curve by 2 weeks. The earlier onset in 2000 may have resulted from enhanced surveillance efforts that were not in place before the 1999 outbreak was recognized. In the 1996 Romanian outbreak, the predilection for WN virus to cause severe disease with increasing age and the frequency of gastrointestinal complaints were similar to findings in this series. Most encephalitis cases were in persons >50 years of age; vomiting occurred in 63% and diarrhea in 12% of cases (8). The propensity of WN virus to affect the elderly more seriously has been seen with other flaviviruses, most notably St. Louis encephalitis (9). The common contributing factors of age, hypertension, and previous brain insult may relate to a decline in the integrity of the blood brain barrier and facilitated access of WN virus to the central nervous system.

The interpretation of the findings of this case series is limited because of the small number of cases. Only hospitalized patients were included, and most WN virus infections are subclinical. Two additional nonhospitalized WN fever cases, one in Connecticut and one in New Jersey, were detected through surveillance and were not included in this case series (10). Focusing on the most severely ill obscures the true spectrum of WN illness. A 1999 serosurvey in Queens, New York, estimates that for every hospitalized case of WN virus infection there were 24 mild febrile and 110 subclinical illnesses (F. Mostashari, pers. commun.). Surveillance in 2000 focused on adults with aseptic meningitis or encephalitis; patients <18 years old were only included if they had encephalitis. The active laboratory surveillance component, however, included patients <18 years old. Four hundred fifty-three CSF specimens were received through active laboratory surveillance; 13 (3%) were from children <18 years. No positive results in children were found.

Another limitation was that the data described were abstracted from medical records that varied greatly in their completeness and legibility. The frequency of missing, missed, and omitted information was approximately 5%-10%. For some analyses, clinical information not located in the medical record was coded as negative, possibly introducing bias that could have produced spurious or hidden real associations.

WN virus appears to have established an enzootic cycle in the northeast United States with positive avian or mosquito findings extending from New Hampshire to North Carolina (11). Clinicians practicing along the eastern seaboard should consider this diagnosis when evaluating febrile patients during the summer months with neurologic complaints, especially those with a gastrointestinal prodrome or muscle weakness.

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References

1. Centers for Disease Control and Prevention. National West Nile Virus Surveillance System, 2000: Final Plan, May 2000, Atlanta, GA. [document online] Available from url: http://www.cdc.ncidod/dvbid/westnile/resources/WN_Final_Plan_2000_05_26_31.pdf
2. Jacobs DS, DeMott WR, Grady HJ, Horvat RT, Huestis DW, Kasten BL. Laboratory test handbook. Cleveland: Lexi-comp, Inc; 1996.
3. Fajardo JE, Stafford EM, Bass JW, Roscelli JD, Sato AK, Claybaugh. Inappropriate antidiuretic hormone in children with viral meningitis. Pediatr Neurol 1989;5:37-40.
4. White MG, Carter NW, Rector FC, Seldin DW, Drewey SJ, Sanford JP, et al. Pathophysiology of epidemic St. Louis encephalitis. Inappropriate secretion of antidiuretic hormone. Ann Intern Med 1969;71:691-702.
5. Cernescu C, Ruta SM, Tardei G, Grancea C, Moldoveanu L, Spulbar E, et al. A high number of severe neurologic clinical forms during an epidemic of West Nile infection. Rom J Virol 1997;48:13-25.
6. Komar N, Panella NA, Burns J, Disza S, Mascarenhas TM, Talbot TO. Serologic evidence for West Nile virus infection in birds in the New York City vicinity during an outbreak in 1999. Emerg Infect Dis 2001;7:621-5.
7. Tsai TF, Popovici F, Cernescu C, Campbell GI, Nedeclu I, for the Investigative Team. West Nile encephalitis epidemic in southern Romania. Lancet 1998;352:767-71.
8. Ceausu EM, Erscoiu S, Calistru P, Ispar D, Doroboat O, Homos M. Clinical manifestations in West Nile virus outbreak. Rom J Virol 1997;48:3-11.
9. Marfin AA, Bleed DM, Lofgren JP, Olin AC, Savage HM, Smith GC, et al. Epidemiologic aspects of a St. Louis encephalitis epidemic in Jefferson County Arkansas, 1991. Am J Trop Med Hyg 1993;49:30-7.
10. Centers for Disease Control and Prevention. Serosurveys for West Nile virus infection—New York and Connecticut counties, 2000. MMWR Morb Mortal Wkly Rep 2001;50:37-9.
11. Centers for Disease Control and Prevention. Update: West Nile virus activity—Eastern United States, 2000. MMWR Morb Mortal Wkly Rep 2001;49:1044-7.

West Nile Encephalitis in Israel, 1999: The New York Connection

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We describe two cases of West Nile (WN) encephalitis in a married couple in Tel Aviv, Israel, in 1999. Reverse transcription-polymerase chain reaction performed on a brain specimen from the husband detected a WN viral strain nearly identical to avian strains recovered in Israel in 1998 (99.9% genomic sequence homology) and in New York in 1999 (99.8%). This result supports the hypothesis that the 1999 WN virus epidemic in the United States originated from the introduction of a strain that had been circulating in Israel.

West Nile (WN) virus, the causative agent of WN fever and encephalitis, has a wide distribution in Africa, West Asia, and the Middle East, and outbreaks have been reported from Europe, South Africa, and Israel. Wild and domestic birds are the principal amplifying hosts of WN virus, and ornithophilic mosquitoes of the *Culex* species are the major vectors (1).

In late August 1999, the first reported outbreak of WN encephalitis in the Western Hemisphere occurred in New York City and surrounding areas. A high degree of genomic sequence similarity between virus isolates indicated that a single WN viral strain was introduced and circulated during the outbreak (2). A high (≥99.8%) genomic similarity was also found between the U.S. viral isolates and a WN virus strain isolated from the brain of a dead goose in Israel in 1998 (2).

How WN virus was introduced into the United States is not known. The high degree of similarity between the 1999 U.S. isolates and the 1998 Israeli isolate, however, raised the hypothesis that the U.S. epidemic originated from the introduction of a WN virus strain that had been circulating in Israel and surrounding countries (2). We provide more evidence to support this hypothesis.

Case Reports

Case 1

On August 24, 1999, a 75-year-old man was admitted to a Tel Aviv emergency room, with confusion, disorientation, and somnolence of 3 days' duration. Body temperature was 37.5°C. He was conscious but disoriented, with global aphasia. Routine laboratory test results, including cerebrospinal fluid (CSF) examination, were normal. A chest radiograph as well as electroencephalography (EEG) were normal. Computerized tomography (CT) of the brain was noncontributory. Over the next 6 days, the patient's temperature rose to 39.0°C. He became stuporous, and myoclonic jerks, as well as snout and palmo-mental pathologic reflexes, were observed. Repeat lumbar puncture revealed clear CSF with opening pressure of 160 mm H_2O, protein 1.36 g/L, glucose 0.6 g/L, leukocytes 120/mm^3 with 60% polymorphonuclear leukocytes (PMN), and 40% lymphocytes. EEG showed nonspecific, nonfocal, triphasic slow waves. Empirical treatment with acyclovir, ceftriaxone, and erythromycin was begun. During week 2 of hospitalization, the patient became less responsive, with limb spasticity, bilateral ptosis, facial nerve paralysis, and bilateral Babinski response. T2-weighted magnetic resonance imaging showed bilateral nonspecific hyperintense foci in the white matter, with lacunar changes in the striatum. Mechanical ventilation was started. Biopsy of the cerebral cortex and white matter showed reactive gliosis, isolated foci of neuronophagia, and a scanty perivascular lymphocytic infiltrate. Gradual, slow neurologic improvement was noticed starting on week 8 of hospitalization. On week 12, the patient was fully alert, with a tracheostomy but no ventilatory support. He died several months later in a rehabilitation center from bilateral pneumonia.

Case 2

The 75-year-old wife of patient 1 was admitted to the same hospital on August 28, 1999 (4 days after her husband's admission), with fever of 39.0°C, chills, dizziness, and headache. A chest radiograph was consistent with right basilar pneumonia. Routine laboratory test results were notable only for a serum sodium level of 132 mEq/L. Empirical treatment with intravenous cefuroxime and oral roxithromycin was started. On day 4 of hospitalization, the patient became stuporous with severe respiratory acidosis; mechanical ventilation was begun. Brain CT results were normal. Lumbar puncture showed an opening pressure of 200 mm H_2O, protein 2.74 g/L, glucose 1.39 g/L, leukocyte count 25/mm^3, 80% PMN, and 20% lymphocytes. Acyclovir was added, and various antibiotic regimens were given. The patient remained febrile and stuporous and died on day 33 of hospitalization. Postmortem examination revealed mild, diffuse encephalitis involving the brain stem, and isolated

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microinfarction of the basal ganglia. Bilateral pulmonary atelectasis with chronic bronchitis was also noted.

These two patients, a retired engineer and a housewife, shared an apartment in a suburb of Tel Aviv. They did not have any pets and had not left the Tel Aviv area in the preceding 5 years. They had no contact with other patients with similar clinical manifestations, nor had they entertained visitors from other countries, except their son, who had visited Germany 1 month before onset of his father's illness. An inspection by the municipal health authorities did not find mosquito infestation in the local area.

Paired CSF and serum specimens from both patients tested negative for bacteria, mycobacteria, fungi, and a large number of viruses. Results of screening tests of urine and blood for toxic substances, including botulism toxin, were also negative.

Methods

Immunoglobulin (Ig) M-capture enzyme-linked immunosorbent assay (ELISA) and IgG ELISA were performed as described by Martin et al. (3) and Johnson et al. (4), respectively. Antigens were prepared as sucrose-acetone extracts of infected suckling mouse brains or infected C6/36 cell cultures. Positive-to-negative absorbance ratios (P/Ns) were determined by dividing the average optical density (OD) of the unknown sera by the average OD measured for the negative sera, with values >3.0 considered positive. All specimens and controls were tested in triplicate. The serum-dilution plaque reduction neutralization test (PRNT) was performed in Vero cells, as described (5). The following viruses were used: WN virus strain Eg101, dengue-2 (DEN-2) strain New Guinea C, and Japanese encephalitis (JE) virus strain Nakayama. Endpoints were determined at a 90% plaque-reduction level. A titer of 1:20 was considered a positive cutoff for PRNT results.

Fragments of brain cerebral tissue from the two patients were subjected to RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) using two different primer/probe pairs in the TaqMan assay, as described (6). For nucleic acid sequencing, the viral RNA was amplified and copied into five unique DNA fragments using the following WN/Kunjin virus primer pairs: 212/619c, 848/1442c, 1248/1830c, 9661/10,489c, and 10,571/10,815c (numbers denote positions of the primers at the Kunjin virus sequence; GenBank Accession Number D00246). DyeDeoxy Terminator cycle sequencing was performed as described (2).

Results

Table 1 summarizes the serologic test results of both patients. In case 1, IgM antibody to WN virus was detected in serum by day 9 after onset of symptoms (P/N = 13.8). IgM was also detected in CSF on day 14 (P/N = 21.6) but not on day 3. In case 2, IgM antibody to WN virus was detected in both CSF and serum. PRNT results were positive in both cases. Patient 1 had a sixfold increase in antibody titer, 1:10 on day 9 and 1:640 on day 35 after onset of symptoms. In case 1, the positive IgM ELISA result with JE viral antigen is due to known cross-reactive antibody response to closely related flaviviruses.

The TaqMan RT-PCR assay performed on RNA extracted from the patient 1 brain biopsy specimen, obtained 33 days after onset of clinical symptoms, showed WN viral RNA when two different primer/probe sets designed from unique regions of the WN viral genome (Ct-envelope primers = 29.6, Ct-3' non-coding primers = 29.2; where Ct = threshold cycle and Ct values <37.5 are positive) were used. The quantity of viral RNA detected was 8.3 and 9.7 PFU equivalents, based on the standard curve generated in the TaqMan assay. Nucleic acid sequencing of the five RT-PCR-generated DNA fragments yielded 1,861 bp of data, approximately 17% of the total genome. Sequence comparisons demonstrated that the virus strain that infected patient 1 is most closely related to the WN-Israel 1998 strain isolated at the Pasteur Institute from a dead goose in Israel in 1998 (99.9% sequence homology; GenBank Accession Number AF205882) and to the WN-NY99 strain isolated from a dead flamingo in the Bronx Zoo, New York, in 1999 (99.8% sequence homology; GenBank Accession Number AF196835). Alignment of the sequence data revealed three positions of nucleotide differences between these three strains (positions 1,118, 1,285, and 10,851; Table 2). These nucleotide differences confirm that the WN virus strain

Table 1. Antibodies to West Nile virus and clinically related flaviviruses in two encephalitis cases, Tel Aviv, 1999

Case 1	Serum #1			Serum #2			CSF #1	CSF #2
Days[a]		9			35		3	14
Antigen	IgM[b]	IgG[b]	PRNT[c]	IgM	IgG	PRNT	IgM	IgM
WN	13.8	1	10	13.7	3.6	640	0.9	21.6
Den 1-4	2.3	1.2	<10	2.6	1.7	<10	1	1.7
CHIK	0.9	1.2		0.9	1.2		1.2	1
SIN	1	1.2		1.1	1.2		1.1	1.1
POW/TBE	1	1.1		1.2	1.8		0.9	0.8
JE	2.8	0.8	<5	6.2	0.8	20	1.5	1.7
Case 2								
Days[a]		14			NA		7	NA
Antigen	IgM	IgG	PRNT				IgM	
WN	13.5	3.1	80				25.3	
Den 1-4	1.7	1.3					1.4	
CHIK	1	1.4					0.9	
SIN	1.3	1.4					0.9	
POW/TBE	1.1	1.4					1	
JE	2.5	0.8	5				2.1	

CSF = cerebrospinal fluid; CHIK, Chikungunya virus; Den 1-4 = dengue virus (types 1-4); JE = Japanese encephalitis virus; NA = not available; POW/TBE = Powassan virus/tick-borne encephalitis virus; PRNT = plaque-reduction neutralization test; SIN = Sindbis virus; WN = West Nile virus.
[a]Days = days after onset.
[b]Immunoglobulin M (IgM) and IgG antibody levels were determined by enzyme immunoassay. Results are expressed as positive-negative absorbance ratios (P/Ns), determined by dividing the average optical density of the test sera by the average optical density measured for the negative control sera, with values >3.0 considered to be positive.
[c]Results of the PRNT are expressed as reciprocal antibody titers, with values ≥20 considered to be positive.

Table 2. Nucleotide differences detected between West Nile (WN) virus genomic sequence data from patient 1, WN-Israel 1998, and WN-NY99

WN virus nucleotide position	Sequences amplified from brain of patient 1	WN-Israel 1998[a]	WN-NY99[b]
1,118	C	C	T
1,285	C	C	T
10,851	G	A	A

[a]WN-Israel 1998 was isolated at the Pasteur Institute from a dead goose found in Israel in 1998.
[b]WN-NY99 was isolated from a dead flamingo in the Bronx Zoo, New York, 1999.

detected in the brain sample from patient 1 is not a laboratory contaminant. RT-PCR performed on an autopsy cerebral cortex brain specimen from patient 2 was negative.

Discussion

Epidemics of WN viral disease occurred in Israel in the 1950s and in 1980 (7,8). During 1997 and 1998, WN virus was reported, for the first time, as the cause of illness and death among domestic geese in Israel. Approximately 3,000 geese with a high seroprevalence of anti-WN virus antibodies were killed to contain the epizootic (9,10). However, no human cases of WN fever were reported in Israel in 1997 to 1998 and, to the best of our knowledge, the two cases described in this report are the first and only human cases of WN fever reported in Israel in the 1990s. It seems likely that other such cases occurred in 1997 to 1999 but were unrecognized, not reported, or both.

Case 1 meets the criteria for the Centers for Disease Control and Prevention surveillance case definition of a confirmed WN encephalitis case (11). Although paired serum specimens were unavailable for case 2, the presence of WN IgM in the CSF (P/N = 25.3) and serum (P/N = 13.5) specimens obtained on day 7 and day 14, respectively, and the presence of WN virus-specific neutralizing antibodies in serum confirm this as a WN encephalitis case as well. The negative RT-PCR result on the autopsy brain specimen in case 2 is probably due to the fact that the specimen submitted for PCR was from the cerebral cortex which, on histopathologic examination, was not involved in the encephalitic process.

Several lines of evidence connect these 1999 Israeli cases with the 1999 New York WN virus outbreak. First, the Israeli and the initial American cases occurred in August 1999. Second, when genomic sequences of WN virus isolates from the New York outbreak were compared with various non-U.S. WN virus strains, the highest similarity ($\geq$99.8%) was found with a WN virus strain from a goose that died in the 1998 Israeli epizootic (2). Similar findings were reported in another study (12). The WN virus sequences obtained by RT-PCR from a brain biopsy of the Israeli male patient shared a >99% homology with the 1999 New York and 1998 Israeli avian WN virus strains, respectively. Finally, in nature avian death caused by WN virus infection is a new phenomenon observed only in Israel and the United States (9,13).

During the summer of 2000, an epidemic of WN fever was observed in Israel, resulting in 417 serologically confirmed cases and 28 deaths (10). Several WN encephalitis cases were reported from the neighborhood of the two patients in our report. Although the genomic sequences of the isolates from 2000 are not yet available, the WN virus strain circulating in Israel since at least 1998 is likely the causative agent of the 2000 Israel epidemic as well as the 1999 New York outbreak. How this strain was transported from Israel to the United States (by infected humans, birds, mosquitoes, or other animals) remains a matter of conjecture.

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References

1. Tsai TF. Flaviviruses. In: Mandell GL, Bennett JE, Dolin R, editors. Principles and practice of infectious diseases. 5th ed. Philadelphia: Churchill Livingstone; 2000. p. 1714-36.
2. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333-7.
3. Martin DA, Muth DJ, Brown T, Johnson AJ, Karabatsos N, Roehrig JT. Standardization of immunoglobulin M capture enzyme-linked immunosorbent assays for routine diagnosis of arboviral infections. J Clin Microbiol 2000;38:1823-6.
4. Johnson AJ, Martin DA, Karabatsos N, Roehrig JT. Detection of anti-arboviral immunoglobulin G by using a monoclonal antibody-based capture enzyme-linked immunosorbent assay. J Clin Microbiol 2000;38:1827-31.
5. Beaty BJ, Calisher CH, Shope RE. Arboviruses. In: Lennette EH, Lennette DA, Lennette ET, editors. Diagnostic procedures for viral, rickettsial and chlamydial infections. 7th ed. Washington: American Public Health Association; 1995. p. 204-5.
6. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol 2000;38:4066-71.
7. Marberg K, Goldblum N, Sterk V, Jasinska-Klingberg W, Klingberg MA. Natural history of West Nile fever, clinical observations during epidemic in Israel. Am J Hyg 1956;64:259-69.
8. Katz G, Rannon L, Nili E, Danon YL. West Nile fever--occurrence in a new endemic site in the Negev. Isr J Med Sci 1989;25:39-41.
9. Israeli Ministry of Health, Jerusalem. Department of Epidemiology Quarterly Report 2000;2:6-7.
10. Israeli Center for Disease Control web site www.icdc-wnf.co.il
11. Centers for Disease Control and Prevention. Case definitions for infectious conditions under public health surveillance. MMWR Morb Mortal Wkly Rep 1997;46(RR-10):12-3.
12. Jia XY, Briese T, Jordan I, Rambaut A, Chi HC, Mackenzie JS, et al. Genetic analysis of West Nile New York 1999 encephalitis virus. Lancet 1999;354:1971-2.
13. Centers for Disease Control and Prevention. Outbreak of West Nile-like viral encephalitis: New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:845-9.

Dead Crow Densities and Human Cases of West Nile Virus, New York State, 2000

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In 2000, Staten Island, New York, reported 10 human West Nile virus cases and high densities of dead crows. Surrounding counties with ≤2 human cases had moderate dead crow densities, and upstate counties with no human cases had low dead crow densities. Monitoring such densities may be helpful because this factor may be determined without the delays associated with specimen collection and testing.

West Nile (WN) virus was first recognized as a cause of encephalitis in humans and other animals in the United States in 1999, and dead bird surveillance in the northeastern states provided a valuable window into the temporal and geographic distribution of viral activity (1). In 2000, a real-time web-based dead bird surveillance system established for New York State (NYS) (2) identified dead crow sightings and laboratory positive dead birds before the onset date for the first human WN virus cases (3). Viral activity appeared to be widely distributed in 2000, with WN virus-positive birds, mammals, or mosquitoes reported from the District of Columbia and 12 states, from New Hampshire to North Carolina (4). However, the 21 human WN virus cases, with a clinical spectrum from mild illness to fatal encephalitis, were limited to New York City (NYC), New Jersey, and Connecticut (5). We compared the number of human cases with dead bird surveillance factors by county in NYS in 2000 to assess possible temporal correlations.

The Study

Fourteen human WN virus cases were confirmed from NYS in 2000, all from NYC (10 from Staten Island, 2 from Brooklyn, and 1 each from Queens and Manhattan) (4,5). A total of 1,263 WN virus-positive dead birds were reported from 61 of 62 NYS counties, including the five NYC boroughs (3). In NYS, 71,332 dead bird sightings were reported from all 62 counties; 17,571 (24.6%) were American Crows (3).

We examined the variability by county for dead bird surveillance factors for NYS in 2000 and report results for the density of dead crow sightings (calculated as the total number of sightings divided by the square-mile area of the county). Estimates of county land area were obtained from 1990 land area data; estimates of human population were obtained from 1999 estimates of the U.S. Census Bureau (6).

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Comparing the total number of human WN virus cases and the dead crow densities by county for 2000 (Figure 1) allows three groups of NYS counties to be distinguished: Staten Island (10 human cases and 33.3 dead crows per square mile), the other boroughs in NYC and surrounding counties that had WN virus activity both in 1999 and 2000 (≤2 human cases and 3-12 dead crows per square mile for each county), and upstate New York (no human cases and <1.0 dead crow per square mile).

For the four NYC boroughs with human WN virus cases, the weekly densities of dead crows were graphed with the dates of onset of human cases. In Staten Island (Figure 2A), a steep rise in the density of dead crows began 2 weeks before the onset of the first human case on July 20 (7), before laboratory confirmation of viral activity. The peak of 5.9 reported crows per square mile coincided with press announcements of a possible human WN virus case (later

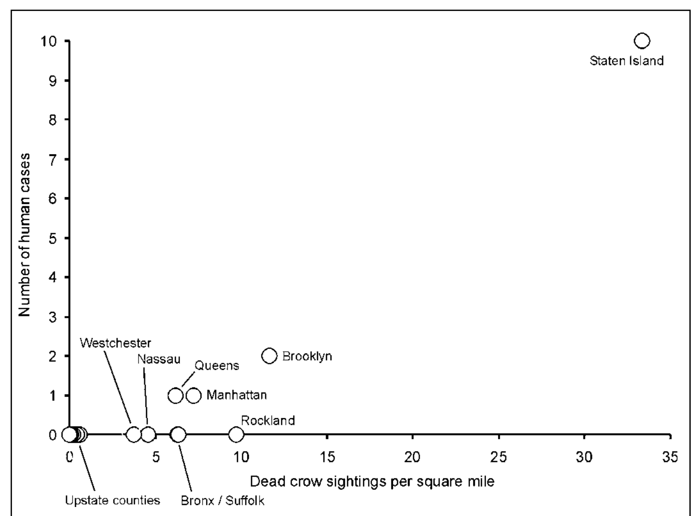

Figure 1. Annual dead crow density (number of dead crow sightings per square mile) compared with number of human cases, New York State, 2000.

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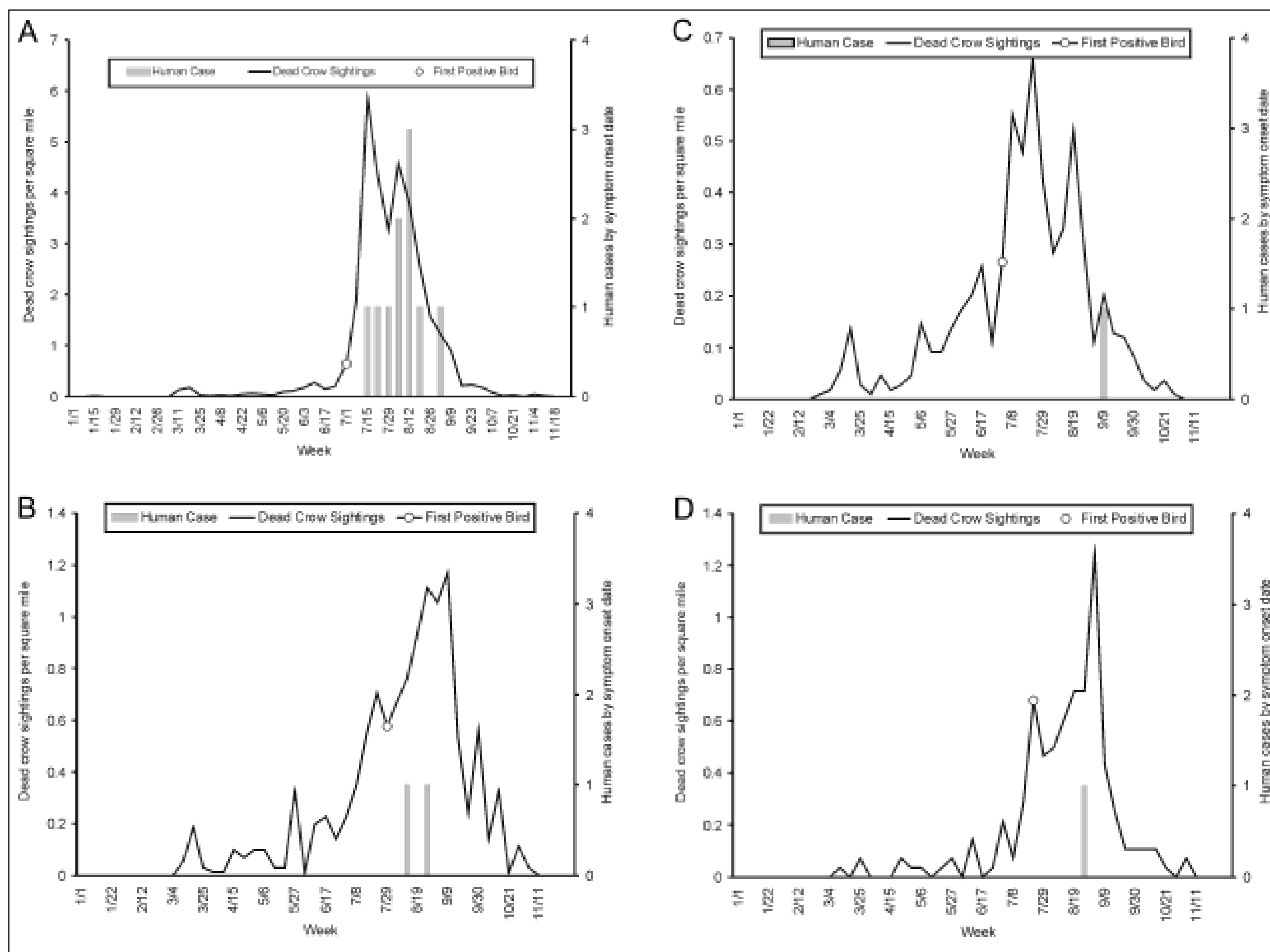

Figure 2. Dead crow density (number of dead crow sightings per square mile) compared with number of human cases, by week. A. Staten Island, axis scale for weekly dead crow density 0 to 7; B. Brooklyn, axis scale for weekly dead crow density 0 to 1.4; C. Queens, axis scale for weekly dead crow density 0 to 0.7; D. Manhattan, axis scale for weekly dead crow density 0 to 1.4.

determined to be negative for WN virus) and the first WN virus-positive crow (collected 2 weeks earlier). In the other three NYC boroughs with one or two human cases, WN virus-positive birds (American Crows in Queens and Manhattan and a Fish Crow in Brooklyn) were found, and dead crow densities increased before the dates of onset of human case (Figures 2B-D), with a maximum weekly dead crow density in Manhattan of 1.25 the week after the date of onset of the human case (Figure 2D).

The rest of the area with WN virus activity both in 1999 and 2000—the Bronx, the two counties immediately north of NYC (Westchester and Rockland), and the two counties to the east (Nassau and Suffolk)—did not have human WN virus cases in 2000, and the weekly dead crow densities never exceeded 1.0. Of the upstate NYS counties with evidence of viral activity only in 2000, none exceeded 0.1 dead crow sightings per square mile per week.

Conclusions

Overall in 2000 and on a weekly basis, three levels of dead crow densities were identified, with high levels in Staten Island, moderate levels in surrounding counties that also had viral activity in 1999, and low levels in upstate counties. Staten Island also had the highest number of human cases, while few human cases were reported from the other surrounding areas with viral activity in 1999 and 2000, and none were reported from upstate counties. This pattern was supported by data from Connecticut showing moderate dead crow densities in a county that had viral activity in both 1999 and 2000 and one WN virus-positive person with a mild illness in 2000 (8). Similarly, Staten Island had a higher proportion of birds that tested positive and higher mosquito infection rates (9,10).

These and other analyses of WN virus in the northeastern United States in 2000 (3,8-10) indicate that dead bird and mosquito surveillance can be useful for monitoring viral activity and the potential for human cases in this geographic area. Tracking dead crow density avoids delays inherent in specimen collection and testing and thus may be more helpful on a weekly basis to permit rapid recognition of trends in viral activity and the potential for occasional human cases or an outbreak.

Whether dead crow densities will be associated with the number of human cases in future years or other geographic areas is unknown. If an area has few crows, crows become immune, or dead crow reporting is inadequate or delayed, an increase in dead crow densities may not be observed before the onset of human cases. Development of spatial statistical procedures to quickly detect geographic clusters of dead crow sightings may be valuable for identification of high-risk areas that cross geopolitical boundaries such as states, counties, or towns.

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References

1. Eidson M, Komar N, Sorhage F, Nelson R, Talbot T, Mostashari F, et al. Crow deaths as a sentinel surveillance system for West Nile virus in the Northeastern United States, 1999. Emerg Infect Dis 2001:7:615-20.
2. Gotham IJ, Eidson M, White DJ, Wallace BJ, Chang HG, Johnson GS, et al. West Nile virus: A case study in how New York State health information infrastructure facilitates preparation and response to disease outbreaks. Journal of Public Health Management and Practice 2001;7(5):79-89.
3. Eidson M, Kramer L, Stone W, Hagiwara Y, Schmit K, and the New York State West Nile Virus Avian Surveillance Team. Dead bird surveillance as an early warning system for West Nile virus. Emerg Infect Dis 2001;7:631-5 .
4. Centers for Disease Control and Prevention. Update: West Nile virus activity—Eastern United States, 2000. MMWR Morb Mortal Wkly Rep 2000;49:1044-7.
5. Centers for Disease Control and Prevention. Serosurveys for West Nile Virus infection—New York and Connecticut counties, 2000. MMWR Morb Mortal Wkly Rep 2001;50:37-9.
6. United States Census Bureau, State and County QuickFacts. Available at: URL: http://quickfacts.census.gov/qfd/.
7. Centers for Disease Control and Prevention. Update: West Nile virus activity—Northeastern United States, January-August 7, 2000. MMWR Morb Mortal Wkly Rep 2000:49;714-7.
8. Hadler J, Nelson R, McCarthy T, Andreadis T, Lis MJ, French R, et al. West Nile virus surveillance in Connecticut in 2000: An intense epizootic without high risk for severe human disease. Emerg Infect Dis 2001;7:636-42.
9. White DJ, Kramer LD, Backenson PB, Lukacik G, Johnson G, Oliver J, et al. Mosquito surveillance and polymerase chain reaction detection of West Nile virus, New York State. Emerg Infect Dis 2001;7:643-9.
10. Bernard KA, Maffei JG, Jones SA, Kauffman EB, Ebel GD, Dupuis AP, et al. West Nile virus infection in birds and mosquitoes, New York State, 2000. Emerg Infect Dis 2001;7:679-85.

Equine West Nile Encephalitis, United States

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After the 1999 outbreak of West Nile (WN) encephalitis in New York horses, a case definition was developed that specified the clinical signs, coupled with laboratory test results, required to classify cases of WN encephalitis in equines as either probable or confirmed. In 2000, 60 horses from seven states met the criteria for a confirmed case. The cumulative experience from clinical observations and diagnostic testing during the 1999 and 2000 outbreaks of WN encephalitis in horses will contribute to further refinement of diagnostic criteria.

In October 1999, West Nile (WN) virus was first confirmed as the cause of illness in a horse in the Western Hemisphere. A Suffolk County, New York, horse was 1 of 25 on Long Island that were eventually diagnosed with WN encephalitis in 1999. Nine (36%) of the infected horses died or were euthanized.

A limited number of veterinary diagnostic tests were available during the 1999 outbreak. Few laboratories were prepared to conduct diagnostic tests, in part because WN virus is categorized as a biosafety level 3 agent (1). The United States Department of Agriculture, Animal and Plant Health Inspection Service (APHIS), Veterinary Services, National Veterinary Services Laboratories (NVSL), Ames, Iowa, which is an international veterinary reference laboratory for diagnosis of eastern, western, and Venezuelan equine encephalomyelitis (EEE, WEE, VEE), provided diagnostic tests for WN virus. Plaque-reduction neutralization tests (PRNT) of equine serum (2) and virus isolation from equine brain or spinal cord tissues were the primary WN virus laboratory methods available in 1999. Most submissions to the NVSL in 1999 were coordinated by APHIS Veterinary Services as exotic disease investigations.

Evidence gathered during the winter of 1999-2000 indicated that WN virus was still present in birds and mosquitoes in the New York City area. In early February 2000, WN virus was isolated from a Red-tailed Hawk that died in Westchester County, New York (3). Adult *Culex* mosquitoes collected from structures in New York City during January and February 2000 were found to be infected with WN virus (4,5). Given these findings, epizootic levels of WN virus activity were thought likely to recur in the summer of 2000.

The laboratory methods used to detect WN virus infection and exposure in horses served well in the initial outbreak in 1999. However, with evidence that the virus had become established in the northeastern United States, the number and range of horses exposed to WN virus were expected to increase. To facilitate detection of new equine WN virus infections, an immunoglobulin (Ig) M-capture enzyme-linked immunosorbent assay (MAC-ELISA) was developed. The assay, modeled after an EEE MAC-ELISA, used an inactivated WN virus antigen from neonatal mouse brain (6). Serum samples collected during the 1999 WN virus outbreak were used to validate the assay. Results of experimental challenge of a small number of horses showed that IgM isotype anti-WN virus antibodies became detectable 8-10 days after infection and persisted <2 months in the challenge model (Ostlund et al., unpub. data). Based on sequential samples collected from a few horses in the New York WN virus outbreak in 1999, the decay of IgM antibodies in naturally infected horses appeared to be similar (7).

Given the possibility of equine cases of WN encephalitis recurring, a case definition was developed by APHIS Veterinary Services in the spring of 2000. Clinical signs used in the definition were based primarily on the 1999 experience in the United States because descriptions of clinical equine cases of WN encephalitis in other parts of the world were limited (8-12). To assure comparability and consistency of results, all diagnostic testing referred to in the case definition was required to be performed or confirmed in the same laboratory. Because specimens might originate from multiple states, the laboratory designated to test all specimens was the NVSL.

We evaluated diagnostic test results in combination with clinical observations to accurately identify cases of WN encephalitis in horses.

Methods

Characterization of Clinical Illness

Dates of onset and signs of clinical illness were obtained from field investigators' interviews with animal owners or care givers or from history forms accompanying specimens submitted to primary or reference diagnostic laboratories. Date of onset of illness was considered to be the first time at which any sign of illness was observed that led to a sign specified in the case definition.

Laboratory Tests

Specimens included serum, whole blood, cerebrospinal fluid (CSF), brain, and spinal cord tissue. Not all submissions included each sample type.

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Virus isolation in rabbit kidney and Vero cell cultures was attempted from brain and spinal cord tissue samples, as well as from whole blood (13). Two cell culture passages were performed with each cell line, and cultures were examined daily for cytopathic effect. WN virus isolates were confirmed by fluorescent antibody testing of infected cell cultures with a monoclonal antibody. In addition, virus isolation attempts for some submissions included intracerebral inoculation of 8 to 16 neonatal mice. When examination for other equine pathogens was indicated (e.g., virus isolates not identified as WN virus), additional virologic tests to identify equine herpesvirus-1 (EHV-1), EEE, WEE, or VEE were performed. EHV-1 isolates were confirmed by fluorescent antibody (14), and alphavirus isolates were identified by complement fixation tests (15).

The 1999 New York avian and equine WN virus isolates were cytopathic in cell culture and formed plaques when cultures were overlaid with agar; a crow isolate was selected as the NY99 prototype for the PRNT. Serum dilutions of 1:10 and 1:100 were examined for WN virus neutralizing antibodies by PRNT in 25-cm^2 flasks (2). One hundred PFU of WN virus were used in the test. Briefly, virus-serum mixtures were incubated at 37°C for 75 minutes and then added to flasks containing confluent monolayers of Vero cells. Following incubation at 37°C for 60 minutes, flasks were overlaid with agar and incubated an additional 72 hours. A second agar overlay containing neutral red was then added, and the flasks were examined the following day. Plaque reduction ≥90% was recorded as positive. A PRNT titer at least 1:10 in equine serum was considered significant (16).

WN virus-specific IgM antibodies in CSF and sera were measured by MAC-ELISA (6,13). All reagents were titrated for optimal performance in the assay. Sera were tested in duplicate at dilutions of 1:100 and 1:1,000; CSF was tested at dilutions of 1:2 and 1:20 in the MAC-ELISA. Briefly, microtiter plates (Immulon 1B, Dynex, Chantilly, VA) were coated with anti-equine IgM (Kierkegaard & Perry Laboratories, Gaithersburg, MD) and blocked with 5% nonfat dry milk. Serum and CSF samples were allowed to bind to the capture antibody overnight at 4°C. After washing, bound equine IgM was reacted with WN virus antigen and control antigen prepared from infected and normal neonatal mouse brain, respectively. After incubation and washing, a flavivirus-specific horseradish peroxidase antibody conjugate (Centers for Disease Control and Prevention, Atlanta, GA) was added. Bound conjugate was detected by reaction with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as the enzyme substrate. The absorbance at 410 nm was measured, and antigen-specific reactions exceeding twice the negative control were considered positive.

Criteria for Confirmed and Probable Equine WN Encephalitis Cases

According to the APHIS Veterinary Services WN encephalitis case definition, a confirmed case was illness in an equine with clinical signs plus one or more of the following: isolation of WN virus from tissue, blood, or CSF; an associated fourfold or greater change in PRNT antibody titer to WN virus in appropriately timed, paired sera; or detection of both IgM antibody to WN virus by MAC-ELISA and an elevated titer (positive at ≥1:10) to WN virus antibody by PRNT in a single serum sample.

A probable case was an equine with clinical signs, located in a county in which WN virus has been confirmed in the current calendar year in any population: mosquito, bird, human, or horse (or within 10 miles of a current-year confirmed equine case), plus one or more of the following: detection of IgM antibody to WN virus by MAC-ELISA but no elevated titer (negative at 1:10) to WN virus antibody by PRNT in a single serum sample taken ≤21 days after onset of illness; positive polymerase chain reaction (PCR) for WN virus genomic sequences in tissue, blood, or CSF; or positive immunohistochemistry for WN virus antigen in tissue. Clinical signs must include one or more of the following: ataxia (including stumbling, staggering, wobbly gait, or incoordination), inability to stand, multiple limb paralysis, or death.

Results

From January 2000 through January 2001, samples from approximately 360 horses for which viral encephalitis was among the differential diagnoses were submitted to the NVSL. Submissions originated from federal, state, university, and private diagnostic laboratories and veterinary practitioners. Eighty-eight submissions contained equine brain or spinal cord tissue; 314 contained serum, CSF, or both, with some submissions including samples for both virologic and serologic tests. The submissions originated from 33 states, with most from the northeastern United States.

In 2000, 60 horses were classified as having a confirmed case of WN encephalitis. Twenty-three (38%) of the 60 cases were fatal, as the horses either died or were euthanized. Clinically, both central nervous system (CNS) and peripheral nervous system (PNS) signs were reported (Table). Most ill horses were reported to be ataxic. Two other common signs included weakness of limbs or going down with difficulty rising. Signs more commonly reported in 2000 than in 1999 included muscle fasciculation, fever, facial paralysis, facial twitching, teeth grinding, and blindness.

Brain tissue samples were submitted from 10 of 60 horses, and CSF was submitted from 6. WN virus was isolated from brain tissue of seven horses that became ill in August or September 2000. Although not included among the diagnostic tests for WN virus case confirmation in 2000, all brain samples were also tested for WN virus RNA by reverse transcription (RT)-nested PCR (RT-nPCR) (17). Brain samples from each of the seven horses yielding a WN virus isolate, plus an additional three confirmed equine WN virus cases, were RT-nPCR positive for WN virus RNA.

No brain samples yielded more than one viral pathogen. However, concurrent with WN virus isolations, EEE virus

Table. Clinical signs in horses with West Nile encephalitis, 2000

Clinical sign	Percentage of horses with sign
Ataxia	85
Weakness of limbs	48
Recumbency, difficulty rising, or both	45
Muscle fasciculation	40
Fever	23
Paralyzed or drooping lip	18
Twitching face or muzzle	13
Teeth grinding	7
Blindness	5

was isolated from 16 equine brain samples submitted in the fall of 2000. The EEE-positive samples were from New Jersey, North Carolina, South Carolina, and Virginia. EHV-1 was isolated in June from the plasma of one horse from Vermont with neurologic illness. WN virus serologic tests were uniformly negative in horses from which EEE or EHV-1 was isolated.

Serum was submitted for all 60 confirmed equine WN encephalitis cases identified in 2000. Fifty-nine of 60 horses had demonstrable WN virus-specific IgM antibodies in acute-phase serum samples. All six CSF samples had detectable IgM antibodies to WN virus when tested at a dilution of 1:2, and three of six CSF samples tested WN virus IgM-positive at the 1:20 dilution.

Neutralizing antibody titers $\geq$1:10 were detected in 55 of 59 serum samples collected at initial visit; insufficient serum was received from one horse to test at the 1:10 dilution by PRNT. Acute-phase serum samples from two of four horses that did not have detectable neutralizing antibody did have IgM antibody to WN virus. Both these horses had fatal cases of encephalitis, and WN virus was isolated from brain tissue.

The illnesses of two horses originally classified as probable cases were later reclassified as confirmed based on additional test results. The first horse was IgM positive ($\geq$1:1,000) but PRNT negative on a serum sample taken 2 days after clinical onset; a second serum sample drawn 14 days later had a PRNT titer of $\geq$1:100. The second horse was IgM positive ($\geq$1:1,000), but PRNT negative at 1:100 with insufficient serum drawn on the first day of clinical illness to test at other dilutions; a second serum sample drawn 22 days later had a PRNT titer of 1:10. One horse met the confirmed case definition by a greater than fourfold change in PRNT titers in paired samples. The PRNT titer in the acute-phase serum was 1:10, and a subsequent sample had a PRNT titer of $\geq$1:100.

Cases of WN encephalitis identified in 2000 had onset of illness from mid-August to the end of October, with 42 (70%) of the 60 cases occurring in a 4-week period from mid-September to mid-October (Figure). Cases were detected in seven northeastern states, six of which had no equine cases of WN encephalitis identified in 1999. Forty-six (77%) of the cases were in New Jersey or New York. Horses ranged in age from 4 months to 38 years (mean 14.0 years). Thirty-six of the horses were male (32 geldings, 3 stallions, 1 colt), and 24 were mares. Cases occurred in at least 11 breeds of horses.

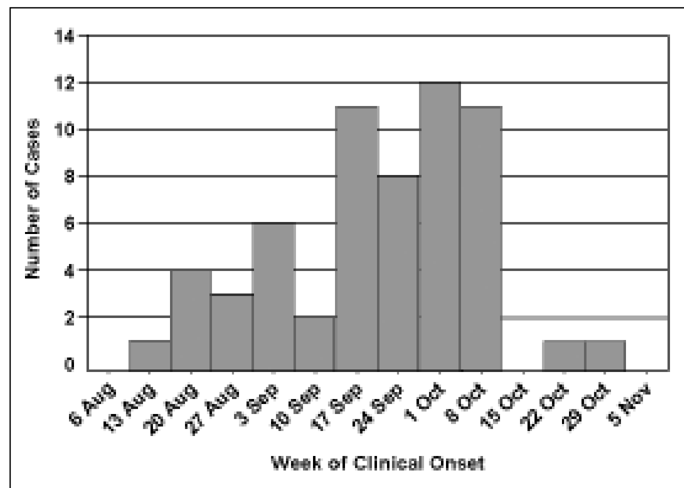

Figure. Equine cases (n=60) of West Nile encephalitis in the United States, by week of clinical onset, 2000.

Conclusion

The emergent nature of WN virus in the United States necessitates reevaluation of the case definition to accommodate clinical observations and additional laboratory methods. Similar to events in human medicine over the past 2 years, valuable diagnostic experience has been acquired to facilitate identification of WN encephalitis in the U.S. equine population. Advances in recognition of clinical signs associated with WN encephalitis in horses and new laboratory diagnostic tests continue to contribute to improved veterinary diagnostic capability.

In 1999, virus isolation and neutralizing antibody detection tests were used to test suspected cases of equine WN encephalitis. In 1999, WN virus was isolated from brain or spinal cord from three horses with WN encephalitis (18). Virus isolates were identified by reverse transcription PCR with RNA extracted from infected cell cultures (19), confirming WN virus as the flavivirus responsible for the 1999 equine epizootic.

In surveillance and diagnostic testing of horses possibly exposed to WN virus in 1999, no serum samples tested from equine submissions from New York counties outside the outbreak area or from 22 other states contained detectable neutralizing WN virus antibodies. To date, there is no evidence that WN virus occurred in the U.S. equine population before 1999.

Neutralizing antibodies to WN virus may persist for $\geq$2 years following infections in humans (8). In limited samples collected in New York from horses that were seropositive in 1999, neutralizing antibody was commonly detected through the following winter (7). More recent testing of the same horses indicates that their WN virus neutralizing antibodies have now persisted for at least 15 months (Ostlund et al., unpub data). Such enduring titers, while perhaps engendering protection from reinfection, have the potential to complicate serologic diagnosis of new infections in a geographic area where WN virus activity had previously occurred. Since all WN virus infections in equines may not give rise to clinical disease, the serologic status of inapparently infected horses is likely to be unknown. Development of subsequent neurologic disease in such an animal could be mistaken for WN virus infection based on persistent neutralizing antibody in the serum. Transfer of WN virus-neutralizing antibodies via colostrum from a seropositive mare in New York to her foal was demonstrated in the spring of 2000 (authors' unpub. observations). Taken together, these data indicate that detection of WN virus-specific neutralizing antibody in a single equine serum sample has limited diagnostic value for new infections in regions where WN virus infections have occurred in previous years.

To assist in identification of recent WN virus infections, the MAC-ELISA method was developed and incorporated into the repertoire of laboratory tests conducted on equine serum and CSF in 2000. Although the kinetics of equine IgM antibody responses to natural WN virus infection were largely unknown, IgM serum antibody responses were expected to wane more rapidly than neutralizing responses to WN virus.

Confidence in confirming equine WN encephalitis cases in the United States was enhanced by the concordance of multiple laboratory test results, including virus isolation. The number of WN virus isolates from horses in North America since 1999 has exceeded all previously published

reports of the disease in horses worldwide. Nearly all infections of WN virus in horses in 2000 were confirmed by at least two laboratory test methods, with the combination of MAC-ELISA and PRNT serologic tests the most dependable in confirming cases in living horses. Fifty-four of 60 confirmed cases had detectable WN virus IgM and neutralizing antibody responses in the acute-phase serum samples. For submissions yielding WN virus from brain, positive WN virus RT-nPCR, or both, IgM was consistently present in serum. Eight of 10 submissions that were WN virus isolation positive, RT-nPCR positive, or both had neutralizing antibody in serum at the time of death. Convalescent-phase serums were needed to support confirmation in three cases. When available, CSF samples from acute-phase cases also yielded positive WN virus IgM results, although testing at a lower dilution than for serum was necessary.

During the 1999 equine outbreak, the primary clinical sign reported (in 18 of 25 cases) was ataxia, either sudden or progressive. Fever associated with clinical illness was documented in only one horse. There were attitudinal changes in many horses, including somnolence, listlessness, apprehension, depression, or periods of hyperexcitability. A greater range of clinical signs were reported for infected horses in 2000 than in the 1999 epizootic.

For a few WN virus suspected cases, the criteria for classification as a confirmed case were only partially met. Illnesses in horses that had only clinical signs not included in the 2000 case definition were not classified as cases by APHIS Veterinary Services. Three clinically ill horses had serum specimens with WN virus IgM titers ≥1:100 and PRNT titers ≥1:10, but none of the signs reported were compatible with the APHIS case definition. Although WN virus infection likely did occur in these horses, they were not included in the WN virus equine encephalitis cases for 2000, since their illness did not reflect encephalitis. In addition, some suspected WN virus cases could not be confirmed in the laboratory because of insufficient samples.

For some horses, the clinical history and geographic location prompted consideration of WN encephalitis but no laboratory tests supported such a diagnosis. Multiple cases of EEE were identified at the National Veterinary Services Laboratory in the fall of 2000, concurrent with the WN virus epizootic. Thus, considering other causes of equine neurologic disease in the differential diagnosis is important.

The surveillance case definition for WN encephalitis in equines used by APHIS Veterinary Services was developed to be as sensitive as possible, yet minimize false-positive case classifications. One of the primary reasons for performing surveillance for equine WN encephalitis is to be able to meet international obligations for disease reporting. Such disease reports can have substantial ramifications for the international movement of horses and other livestock. A high level of specificity in case classification is therefore critical, especially when detecting and reporting the first case of disease in a given geographic area (e.g., a previously unaffected state). Given the specificity of the case definition, failure of a clinically ill equine to meet the criteria for a probable or confirmed case does not completely exclude the possibility that WN virus was the cause of illness.

Based on experience gained in 2000, some modifications will be considered in the diagnostic tests and clinical observations used to identify cases of WN encephalitis in horses in 2001 and future years. Such changes include the addition of a wider range of clinical signs, including PNS signs and additional CNS signs. New laboratory tests will also be incorporated, in particular RT-nPCR, which has been shown to be accurate in detecting WN virus nucleic acid in CNS tissues.

As the range of WN virus activity increases, prevention and control issues for horses becomes even more important. To date, prevention recommendations have been broad and generally targeted at reducing sources of water for mosquito breeding and decreasing equine exposure to biting mosquitoes. Risk factors for equine infection with WN virus are being evaluated through a case-control study conducted by APHIS and animal health officials in states where equine WN encephalitis was detected in 2000. Results of that study may provide information for more specific recommendations on preventing equine infections. Of most use in preventing illness and death of equines may be a vaccine against WN virus. Vaccines for equine use are being developed and could be available for use as early as the summer of 2001.

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References

1. Richmond JY, McKinney RW, editors. Biosafety in microbiological and biomedical laboratories. 4th ed. US Department of Health and Human Services (US). Washington: US Government Printing Office; 1999.
2. Beaty BJ, Calisher CH, Shope RE. Arboviruses. In: Schmidt NH, Emmons RW, editors. Diagnostic procedures for viral, rickettsial and chlamydial infections. 6th ed. Washington: American Public Health Association, Inc.; 1989. p. 797-856.
3. Garmendia AE, Van Kruiningen HJ, French RA, Anderson JF, Andreadis TG, Kumar A, et al. Recovery and identification of West Nile virus from a hawk in winter. J Clin Microbiol 2000;38:3110-1.
4. Centers for Disease Control and Prevention. Update: surveillance for West Nile virus in overwintering mosquitoes—New York, 2000. MMWR Morb Mortal Wkly Rep 2000;49:178-9.
5. Centers for Disease Control and Prevention. Notice to readers: update: West Nile virus isolated from mosquitoes—New York, 2000. MMWR Morb Mortal Wkly Rep 2000;49:211.
6. Sahu SP, Alstad AD, Pedersen DD, Pearson JE. Diagnosis of eastern equine encephalomyelitis virus infection in horses by immunoglobulin M and G capture enzyme-linked immunosorbent assay. J Vet Diagn Invest 1994;6:34-8.
7. Ostlund EN, Andresen JE, Andresen M. West Nile encephalitis. Vet Clin North Am Equine Pract 2000;16:427-41.
8. Hayes CG. West Nile fever. In: Monath T, editor. The arboviruses: epidemiology and ecology. Vol. V. Boca Raton (FL): CRC Press; 1989. p.59-88.
9. Pan American Health Organization. West Nile fever. In: Acha PN, Szyfres B, editors. Zoonoses and communicable diseases common to man and animals. 2nd ed. Washington: Pan American Health Organization; 1987. p. 525-8. Scientific pub. no. 503.
10. Hubálek Z, Halouzka J. West Nile fever—a reemerging mosquito-borne viral disease in Europe. Emerg Infect Dis 1999;5:643-50.

11. Cantile C, Di Guardo G, Eleni C, Arispici M. Clinical and neuropathological features of West Nile virus equine encephalomyelitis in Italy. Equine Vet J 2000;32:31-5.
12. Schmidt JR, El Mansoury HK. Natural and experimental infection of Egyptian equines with West Nile virus. Ann Trop Med Parasitol 1963;57:415-27.
13. Pearson JE. Equine encephalomyelitis (Eastern and Western). In: Office International des Epizooties, Manual of standards for diagnostic tests and vaccines. 3rd ed. Paris: OIE Press; 1996. p. 400-5.
14. Yeargan MR, Allen GP, Bryans JT. Rapid subtyping of equine herpesvirus 1 with monoclonal antibodies. J Clin Microbiol 1985;21:694-7.
15. Department of Health, Education, and Welfare (US) Public Health Service. A guide to the performance of standardized diagnostic complement fixation method and adaption to micro test. Atlanta: Center for Disease Control; 1974.
16. Centers for Disease Control and Prevention. National West Nile Virus Surveillance System, 2000: final plan. May 26, 2000. Available from URL: http://www.cdc.gov/ncidod/dvbid/westnile/publications.htm
17. Johnson DJ, Ostlund EN, Pedersen DD, Schmitt BJ. Detection of North American West Nile virus in animal tissue by a reverse transcription-nested polymerase chain reaction assay. Emerg Infect Dis 2001;7:739-41.
18. Torres A. West Nile fever in the United States of America. Office International des Epizooties Disease Information 2000;13:5-7.
19. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by TaqMan reverse transcriptase-PCR assay. J Clin Microbiol 2000;38:4066-71.

Mosquito Surveillance for West Nile Virus in Connecticut, 2000: Isolation from *Culex pipiens, Cx. restuans, Cx. salinarius,* and *Culiseta melanura*

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Fourteen isolations of West Nile (WN) virus were obtained from four mosquito species (*Culex pipiens* [5], *Cx. restuans* [4], *Cx. salinarius* [2], and *Culiseta melanura* [3]) in statewide surveillance conducted from June through October 2000. Most isolates were obtained from mosquitoes collected in densely populated residential locales in Fairfield and New Haven counties, where the highest rates of dead crow sightings were reported and where WN virus was detected in 1999. Minimum field infection rates per 1,000 mosquitoes ranged from 0.5 to 1.8 (county based) and from 1.3 to 76.9 (site specific). *Cx. restuans* appears to be important in initiating WN virus transmission among birds in early summer; *Cx. pipiens* appears to play a greater role in amplifying virus later in the season. *Cs. melanura* could be important in the circulation of WN virus among birds in sylvan environments; *Cx. salinarius* is a suspected vector of WN virus to humans and horses.

Epizootic West Nile (WN) virus activity was first detected in Connecticut during September and October 1999 (1). Substantial die-offs among American Crows, *Corvus brachyrhynchos,* was observed along a 100-km corridor bordering New York State and Long Island Sound in the southwestern corner of the state (lower Fairfield and New Haven counties). During that period, WN virus was isolated from 72 of 86 crows; a Cooper's Hawk, *Accipiter cooperii;* and a Sandhill Crane, *Grus canadensis,* housed at a local zoo (1,2). Expanded mosquito surveillance in the affected region yielded the first isolates of the virus from two species of mosquitoes, *Aedes vexans* and *Culex pipiens* (one pool each), that were trapped in Greenwich, adjacent to the New York border, in mid-September. Despite substantial crow deaths, no additional virus isolates were obtained from >3,500 mosquitoes collected from several hundred traps placed in urban and suburban locations where WN virus-infected crows were found. Neither was WN virus detected in >45,000 mosquitoes (30 species) trapped from June through October in other areas of the state and tested for arboviruses as part of our annual mosquito surveillance program (3). No human or equine cases of WN virus were reported in the state.

In response to these findings, a comprehensive interagency WN virus surveillance and response plan was developed by the state of Connecticut for 2000. The objectives of this program were to detect WN virus, determine the extent of its geographic distribution, and assess the threat to humans and domestic animals. The plan included surveillance for WN virus in mosquitoes, wild birds, domestic animals, poultry, and humans. Mosquito surveillance was specifically designed to identify potential mosquito vectors, determine their seasonal abundance and spatial distribution in the affected area, and assess viral infection rates relative to virus activity in avian and mammalian hosts. The results of this investigation are reported here.

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Methods

Mosquito Trapping and Identification

Mosquito trapping was conducted from June 1 through October 26, 2000, at 148 (73 permanent and 75 supplemental) locations statewide (Figure 1). The preexisting mosquito

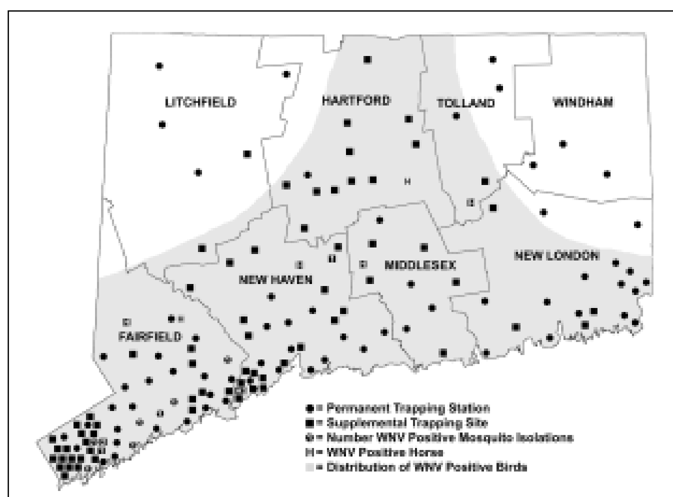

Figure 1. West Nile virus activity in Connecticut, 2000. Locations of mosquito traps, virus isolates from mosquitoes, horse cases, and general distribution of WN virus-positive birds are shown. Source of bird and horse data: Connecticut Departments of Public Health and Agriculture.

surveillance program, consisting of 37 permanent trapping stations principally designed to monitor Eastern equine encephalitis activity (3), was expanded to include 36 new locations, for a total of 73 permanent trap sites. New sites were located in lower Fairfield and New Haven counties, where mosquitoes and dead crows infected with WN virus were found in 1999, and where it was thought that WN virus was most likely to reemerge in 2000. Traps were placed in urban and suburban environs where typical *Culex* spp. habitat was found, including waterways, parks, golf courses, undeveloped wood lots, and temporary wetlands in densely populated residential areas. The 36 preexisting trapping stations in the other six counties (Hartford, Litchfield, Middlesex, New London, Tolland, and Windham) were located mostly in more sparsely populated rural settings that included permanent freshwater swamps (red maple/white cedar), coastal salt marshes, and swamp-forest border locations. Collections were made at 10-day intervals for the entire season (June 1-October 26) at each permanent trap site. The number of trap nights ranged from 12 to 36 (mean 21.7).

Supplemental trapping was conducted at 75 additional locations where dead birds (mostly crows) and horses infected with WN virus were detected during the season and no trapping station was present (Figure 1). These traps were generally placed in the immediate vicinity where the dead birds were recovered in the field or, in the case of the horses, where the animals were stabled. Trapping frequency at the supplemental sites varied; the number of trap nights ranged from 1 to 32 (mean 4.6).

Two trap types were used: 1) a CO_2 (dry ice)-baited Centers for Disease Control and Prevention (CDC) light trap and 2) a sod grass-infused CDC gravid mosquito trap (4,5). Typically, traps were placed in the field during the late afternoon and retrieved the following morning. Adult mosquitoes were transported alive to the laboratory, where they were promptly examined on chill tables with a stereo microscope and identified by using descriptions and keys of Darsie and Ward (6) and Means (7,8). Mosquitoes were pooled by species, collecting site, and date. The number of mosquitoes per pool ranged from 1 to 50. In some instances when both trap types were used at the same site on the same evening, mosquito collections were combined. Mosquitoes were stored at -80°C until tested for virus.

Virus Isolation and Identification

Each frozen mosquito pool was triturated with glass beads and Alundum in 1 mL to 1.5 mL of phosphate-buffered saline containing 0.5% gelatin, 30% rabbit serum, antibiotic, and antimycotic. Following centrifugation for 10 min at 520 x *g*, 100-µL aliquots of each pool of mosquitoes were inoculated onto a monolayer of Vero cells growing in 25-cm^2 flasks at 37°C in 5% CO_2. Cells were examined for cytopathologic effect for up to 7 days after inoculation. Uninoculated flasks were kept as negative controls.

Virus isolates were identified by enzyme immunoassay (ELISA), reverse transcription-polymerase chain reaction (RT-PCR), or both. Reference antibodies for the ELISA were prepared in mice (9) 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. These included seven viruses, in three families, isolated from mosquitoes in North America: Cache Valley, Eastern equine encephalitis, Highlands J, Jamestown Canyon, La Crosse, St. Louis encephalitis, and WN virus. Positive and negative control cell lysates were included in each test.

For molecular identification, Vero cell cultures showing lytic activity were pelleted and processed by using a Qiagen Rneasy mini protocol. The Rneasy column was eluted twice with 40 µL of RNase-free cell culture water. Two microliters of the column eluate was reverse transcriptase amplified by using the Perkin-Elmer GeneAmp EZ rTh RNA PCR kit (Norwalk, CN). Three sets of primers representing five primer sites unique to WN virus were used for redundancy: 1) WN-233F (GACTGAAGAGGGCAATGTTGAGC) and WN-1189R (GCAATAACTGCGGACYTCTGC); 2) WN-200F (TCAATAT-GCTAAAACGCGG) and WN-540R (TTAGAGAGGGTAACT-GCTCC); and 3) WN-451F (GTGCTATCAATCGGCG-GAGCTC) and 540R. Gene amplification was done on an MJ Research PTC-200 DNA Engine (Waltham, MA). The protocol was as follows: 60°C for 30 min, 94°C for 2 min followed by 40 cycles of 94°C for 45 sec, 50°C for 30 sec, and 60°C for 1 min 30 sec. PCR product was run in a 1.5% agarose gel stained with ethidium bromide and electrophoresed at 20 V/CM for approximately 1/2 hr. Band size was checked against the AmpliSize size markers from Bio-Rad Laboratories (Richmond, CA). All WN virus isolates were confirmed by RT-PCR.

Results

Mosquito collection data are summarized in Table 1. A total of 137,199 female mosquitoes representing 32 species in

Table 1. Total number of mosquito species trapped and tested for West Nile virus in Connecticut, June 1–October 26, 2000

Mosquito species	No. locations	No. collected and tested	No. pools
Aedes cinereus	104	9,195	641
Ae. vexans	125	8,310	622
Anopheles barberi	4	5	5
An. crucians	1	6	1
An. punctipennis	126	2,477	516
An. quadrimaculatus	35	98	53
An. walkeri	31	380	82
Coquillettidia perturbans	95	11,516	536
Culex pipiens	125	4,399	473
Cx. restuans	84	4,690	468
Cx. salinarius	100	6,673	466
Cx. territans	26	46	36
Culiseta melanura	108	8,105	625
Cs. morsitans	39	271	79
Ochlerotatus abserratus	57	1,605	136
Oc. atropalpus	1	1	1
Oc. aurifer	56	3,164	187
Oc. canadensis	101	29,172	1,141
Oc. cantator	79	3,514	322
Oc. communis	5	127	8
Oc. excrucians	59	921	146
Oc. grossbecki	1	1	1
Oc. japonicus	82	690	250
Oc. sollicitans	21	1,855	90
Oc. sticticus	63	9,054	327
Oc. stimulans	30	257	51
Oc. taeniorhynchus	13	5,978	153
Oc. triseriatus	113	1,711	418
Oc. trivittatus	119	19,260	761
Orthopodomyia signifera	5	5	5
Psorophora ferox	82	2,361	233
Uranotaenia sapphirina	99	1,352	252
Totals		137,199	9,085

eight genera were collected from the field, identified, and processed for virus isolation. Fifteen species of *Ochlerotatus* (formerly *Aedes*) and two species of *Aedes* were collected, among which *Ochlerotatus canadensis* and *Oc. trivittatus* were the most abundant, followed by *Aedes cinereus, Oc. sticticus, Ae. vexans,* and *Oc. taeniorhychus.* With the exception of *Oc. taeniorhychus* (a salt marsh inhabitant) and to a lesser degree *Oc. sticticus,* each of these species was widely distributed. Of four species of *Culex* collected, *Cx. salinarius* was the most numerous. *Cx. pipiens* and *Culex restuans* were less abundant but were equal in number. Other notably abundant species included *Coquillettidia perturbans, Culiseta melanura, Anopheles punctipennis,* and *Psorophora ferox.*

Virus isolation data are summarized (Table 2, Figure 1). Fourteen isolates of WN virus were obtained from four mosquito species: *Cx. pipiens* (5 isolates), *Cx. restuans* (4 isolates), *Cx. salinarius* (2 isolates), and *Cs. melanura* (3 isolates). Infected mosquitoes were recovered from 11 locations. With the exception of the positive pool from Meriden, a town in northern New Haven County, all isolates were obtained from mosquitoes collected from lower Fairfield and New Haven counties in the southwestern corner of the state, bordering Long Island Sound. The first isolate was obtained from *Cx. restuans* collected on July 11 and the last from *Cs. melanura* collected on October 2. Most (9 of 14) of the isolations were made from mosquitoes collected in mid-September. Minimum field infection rates calculated from season-long collections in each county ranged from 1.8 per 1,000 for *Cx. restuans* to 0.5 for *Cx. salinarius.* Site-specific minimum field infection rates ranged from 1.3 to 76.9. *Culex* spp. infected with WN virus were collected in traps set in densely populated suburban areas (mean population density 2,431 people/sq. mile). *Cs. melanura* infected with WN virus, by contrast, were collected from semipermanent swamp habitats in less populated locales (mean population density 1,407 people/sq. mile). Seven of the 11 locations where infected mosquitoes were found on one occasion only during the season were permanent trapping stations that were monitored from June through October. The number of trap nights at these sites ranged from 26 to 36 (mean 28.6). The trapping effort at the four supplemental sites where isolations were made ranged from 10 to 32 trap nights (mean 15.0).

Isolations from multiple pools of mosquitoes collected at the same site were obtained at Milford and Stamford-2 (Table 2). The Milford site (three isolates) was a stable in a densely populated industrial area adjacent to an isolated wood lot where a horse was diagnosed with WN virus (onset September 4). The first isolate was from a pool of *Cx. salinarius* collected on September 18. Two additional isolates were obtained from *Cx. pipiens* and *Cx. salinarius* collected on September 21. No further isolations were made from mosquitoes collected in traps set at this location on September 27 and October 4. The Stamford-2 site was a small wood lot in a densely populated area. Trapping was conducted on September 13, 20, and 27 and October 3 and 24. Two isolations were obtained from *Cx. pipiens* and *Cx. restuans* collected on September 20.

The weekly collection data for those mosquitoes from which WN virus was isolated (*Cx. restuans, Cx. pipiens, Cx. salinarius* and *Cs. melanura*) are shown (Figure 2). *Cx. restuans* was notably more abundant during early summer (June and July, peak in early July) and was rarely collected in August and September. *Cx. pipiens*, on the other hand, was present in July but was clearly more abundant later in the summer (August and September, peak in late August). With the exception of the early WN virus isolation from *Cx. restuans* in mid-July, all viruses from these two species were isolated when populations of both mosquitoes were on the decline.

Cx. salinarius populations peaked in mid-July and steadily but gradually declined through October. *Cs. melanura* was consistently collected throughout the entire season but there were two discernible peaks of adult abundance, early June and mid-August. WN virus was isolated from both species on the same week in mid-September, when populations were similarly declining.

Conclusion

Our isolations of WN virus from mosquitoes collected in coastal Fairfield and New Haven counties were consistent with epizootic WN virus activity in this region during 2000. Although wild birds (mostly crows) infected with WN virus were recovered throughout south-central Connecticut, the highest rates of dead crow sightings reported (10) were consistently noted in those areas where 13 of 14 mosquito isolations were made. This was also the same general area where WN virus was initially detected in American crows and mosquitoes in 1999 (1). These findings, in concert with the limited flight range of crows during the early summer (11) and

Table 2. West Nile virus isolation data from field-collected mosquitoes trapped in Connecticut, June 1–October 26, 2000

Species	Date collected	Pool size	Location: County	Location: Site	MFIR[a]: County	MFIR[a]: Site	Trap type[b]
Culex restuans	7/11	9	Fairfield	Stamford-1	1.8	6.9	G
	8/7	3		Norwalk-1		32.3	G,L
	8/7	7		Norwalk-2		5.4	G,L
	9/20	18		Stamford-2		55.6	G,L
Cx. pipiens	8/30	1	Fairfield	Greenwich	1.3	29.4	G
	9/11	44		Stamford-3		17.2	G
	9/20	50		Stamford-2		15.9	G,L
	9/12	4	New Haven	Meriden	1.4	41.7	L
	9/21	3		Milford		76.9	G,L
Cx. salinarius	9/18	5	New Haven	Milford	0.5	45.5	L
	9/21	6		Milford		45.5	L
Culiseta melanura	9/19	39	Fairfield	Fairfield	0.8	9.2	L
	9/20	50		Shelton		1.3	L
	10/2	7		Westport		6.8	L

[a]Minimum field infection rate per 1,000 mosquitoes.
[b]G = gravid; L = light; G,L = combined.

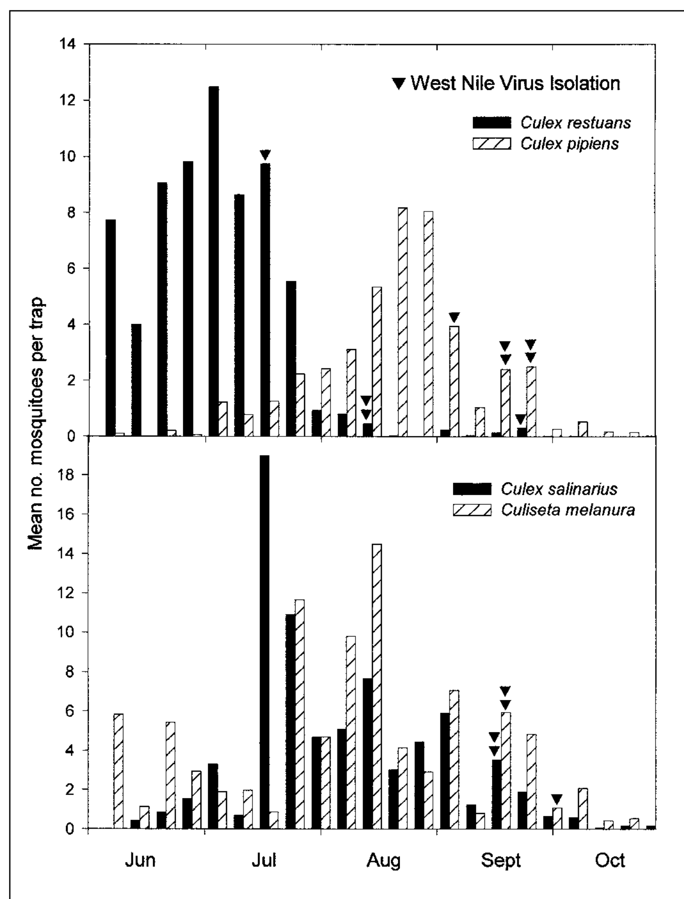

Figure 2. Weekly collection and West Nile virus isolation data for field-collected adult female *Culex restuans, Cx. pipiens, Cx. salinarius,* and *Culiseta melanura* in Connecticut, 2000.

isolation from *Cx. restuans* in mid-July, suggest local reemergence and transmission of the virus in this region, independent of the early seasonal events in New York and New Jersey (12). It is uncertain, however, whether early amplification in this region led to the subsequent spread of the virus to other areas of the state. The mechanism for overwintering of WN virus is also unknown. The detection of WN virus in hibernating *Culex* spp. mosquitoes collected in New York City during January-February (13) and the demonstration of vertical transmission of the virus by mosquitoes in the laboratory (14) and field (15) suggest that vertical transmission could provide a mechanism for persistence of the virus during the winter months.

The relative importance of various mosquitoes as epidemic and epizootic vectors of WN virus in North America is largely unknown. Investigations in Africa, Europe, and Asia (16) have mostly incriminated bird-feeding species, predominantly of the genus *Culex* spp., as the main vectors. Tsai et al. (17) and Savage et al. (18) have suggested that WN virus circulates in Europe in both sylvan and urban transmission cycles involving different species and populations of mosquitoes. In the sylvatic cycle, WN virus is circulated among birds by *Cx. modestus*, *Cx. pipiens*, or both. Because *Cx. modestus* displays a broad host range, it may also transmit the virus to humans. *Cx. pipiens,* on the other hand, is strongly ornithophilic and appears to be more important in amplification of the virus among birds than in transmission to humans in these natural environs. However, in urban areas, *Cx. pipiens* is the only common *Culex* mosquito and is believed to serve both functions.

Our isolates from *Cx. pipiens, Cx. restuans,* and *Cx. salinarius* collected in densely populated communities are consistent with these reports and agree with the preponderance of WN virus-positive pools (406 of 456) obtained from *Culex* species collected from other northeastern states in 2000 (19). The isolations from *Cs. melanura* collected in more rural environs are new host records for WN virus. If proven to be a competent vector, this almost exclusively avian feeder could be important in circulation of the virus among birds in sylvan environments.

The multiple isolates from *Cx. restuans* and *Cx. pipiens* support our hypothesis that these species are important enzootic and epizootic vectors. Both species are strongly ornithophilic (20-25), are widely distributed throughout the region, and occur in both urban and rural environs. Recently completed studies (26,27) have further demonstrated that *Cx. pipiens* is a competent vector for WN virus in the laboratory. The competence of *Cx. restuans* has not been established.

Cx. restuans may be important in initiation of WN virus transmission among wild birds in early summer. It is the most abundant *Culex* species in June and July, and the earliest isolates were from this species in July and August. In contrast, *Cx. pipiens* became abundant in August, with isolations made on August 30 and in September. *Cx. pipiens* may therefore play a greater role in amplification of WN virus later in the season. Reiter (28) has suggested that, in the east-central United States, where *Cx. restuans* populations typically peak in mid-May, this species may play a similar role in recrudescence and early amplification of St. Louis encephalitis virus in the spring. He further speculates that reactivation of previously infected female *Cx. restuans* during periods of unseasonably cold weather in the summer, when it normally estivates, could cause a sudden, synchronous release of virus at a time when it could then be amplified by an increasing *Cx. pipiens* population that peaks in early to mid-July.

The role that *Cx. pipiens* and *Cx. restuans* may play in transmission of WN virus to humans, horses, or other mammals is unclear. Most reports (8,20-25) indicate that both species predominately feed on birds and are reluctant to feed on humans. Blood meal analysis of local populations in Connecticut (25) has further shown that *Cx. pipiens* and *Cx. restuans* acquire blood almost exclusively from passeriform birds. Similar results have been reported for *Cx. pipiens* populations in New York (24) and New Jersey (21). On the other hand, several researchers (8,20,22,29,30) have reported that when *Cx. restuans* is abundant, females will bite wild and domestic animals, and humans. We note that WN virus was isolated from two pools of *Cx. restuans* mosquitoes collected from two locations in Norwalk in Fairfield County on August 7 (Table 2). This was the same community where a mildly symptomatic woman was diagnosed with WN virus with onset in late August (10,19).

Differences in host feeding preferences have also been observed in farm and woodland populations of *Cx. pipiens* in the northeastern United States (22). According to Means (22), *Cx. pipiens* inhabiting commercial bird farms routinely engorge on ducks and pheasants but hardly ever bite humans, but populations in sylvan environments attack humans readily. The human biting behavior of the urban *molestus*

form of *Cx. pipiens (*which breeds in basements, subways, and similar dark, heated places [31]) also cannot be discounted. However, we have no knowledge of the identity, abundance, or distribution of this behavioral form of *Cx. pipiens* in Connecticut. Clearly, more research on the host feeding preferences of these two mosquitoes is needed.

Cx. salinarius, by contrast, is a well-recognized general feeder that feeds indiscriminately on both birds and mammals and will readily bite humans (8,21,30,32,33). In addition to the two isolates reported here, WN virus was detected in 33 pools of this mosquito collected from other areas of the Northeast in 2000 (19). Our two isolates were from females collected at a stable where a horse was diagnosed with WN virus. *Cx. salinarius* should be strongly considered as a possible vector of WN virus to humans, horses, and other animals.

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References

1. Anderson JF, Andreadis TG, Vossbrinck CR, Tirrell S, Wakem EM, French RA, et al. Isolation of West Nile virus from mosquitoes, crows, and a Cooper's Hawk in Connecticut. Science 1999;286:2331-3.
2. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286;2333-7.
3. Andreadis TG, Anderson JF, Vossbrinck CR. Mosquito arbovirus surveillance in Connecticut, 1999: Isolation and identification of West Nile virus. Proceedings Northeastern Mosquito Control Association 1999;45:57-67.
4. Lampman RL, Novak RJ. Oviposition preferences of *Culex pipiens* and *Culex restuans* for infusion-baited traps. J Am Mosq Control Assoc 1996;12:23-32.
5. Reiter P. A portable, battery-powered trap for collecting gravid *Culex* mosquitoes. Mosquito News 1983;43:496-8.
6. Darsie RF Jr, Ward RA. Identification and geographic distribution of mosquitoes of North America, north of Mexico. Mosquito Systematics Supplement, American Mosquito Control Association 1981;1:1-313.
7. Means RG. Mosquitoes of New York. Part I. The genus *Aedes* Meigen with identification keys to genera of Culicidae. New York State Museum Bulletin 1979;430a.
8. Means RG. Mosquitoes of New York. Part II. Genera of Culicidae other than *Aedes* occurring in New York. New York State Museum Bulletin 1987;430b.
9. Ansari MZ, Shope RE, Malik S. Evaluation of Vero cell lysate antigen for ELISA of flaviviruses. J Clin Lab Anal 1993;7:230-7.
10. Hadler J, Nelson R, McCarthy T, Andreadis T, Lis MJ, French R, et al. West Nile virus surveillance in Connecticut in 2000: An intense epizootic without high risk for severe human disease. Emerg Infect Dis 2001;7:636-42.
11. Caceamise DF, Reed LM, Romanowski J, Stauffer PC. Roosting behavior and group territoriality in American crows. The Auk 1997;114:628-37.
12. Centers for Disease Control and Prevention. Update: West Nile virus activity--New York and New Jersey, 2000. MMWR Morb Mortal Wkly Rep 2000;49:640-2.
13. Centers for Disease Control and Prevention. Update: surveillance for West Nile virus in overwintering mosquitoes--New York, 2000. MMWR Morb Mortal Wkly Rep 2000;49:178-9.
14. Baqar S, Hayes CG, Murphy JR, Watts DM. Vertical transmission of West Nile virus by *Culex* and *Aedes* species mosquitoes. Am J Trop Med Hyg 1993;48:757-62.
15. Miller BR, Nasci RS, Godsey MS, Savage HM, Lutwama JJ, Lanciotti RS, et al. First field evidence for natural vertical transmission of West Nile virus in *Culex univittatus* complex mosquitoes from Rift Valley Province, Kenya. Am J Trop Med Hyg 2000;62:240-6.
16. Hubalek Z, Halouzka J. West Nile fever-a reemerging mosquito-borne viral disease in Europe. Emerg Infect Dis 1999;5:643-50.
17. Tsai TF, Popovici F, Cernescu C, Campbell GL, Nedelcu NI. West Nile encephalitis epidemic in southeastern Romania. Lancet 1998;352:767-71.
18. Savage HM, Ceianu C, Nicolescu G, Karabatsos N, Lanciotti R, Vladimirescu A, et al. Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serologic and molecular characterization of a virus isolate from mosquitoes. Am J Trop Med Hyg 1999;61:600-11.
19. Centers for Disease Control and Prevention. Update: West Nile virus activity—eastern United States, 2000. MMWR Morb Mortal Wkly Rep 2000;49:1044-7.
20. Hayes RO. Host preferences of *Culiseta melanura* and allied mosquitoes. Mosquito News 1961;179-87.
21. Crans WJ. Continued host preference studies with New Jersey mosquitoes, 1963. In: Proceedings of the 51st Annual Meeting of the New Jersey Mosquito Extermination Association; 1963. p. 50-8.
22. Means RG. Host preferences of mosquitoes (Diptera: Culicidae) in Suffolk County, New York. Ann Entomol Soc Am 1968;61:116-20.
23. Edman JD. Host-feeding patterns of Florida mosquitoes III. *Culex (Culex)* and *Culex (Neoculex)*. J Med Entomol 11;1975:635-53.
24. Tempalis CA. Host-feeding patterns of mosquitoes, with a review of advances in analysis of blood meals by serology. J Med Entomol 11;1975:635-53.
25. Magnarelli LA. Host feeding patterns of Connecticut mosquitoes. Am J Trop Med Hyg 1977;26:547-52.
26. Turell MJ, O'Guinn ML, Oliver J. Potential for New York mosquitoes to transmit West Nile virus. Am J Trop Med Hyg 2000;62:413-4.
27. Turell MJ, O'Guinn ML, Dohm B, Jones JW. Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. J Med Entomol 2001;38:130-4
28. Reiter P. Weather, vector biology, and arboviral recrudescence. In: Monath TP, editor. Arboviruses: epidemiology and ecology. Vol 1. Boca Raton: CRC Press; 1988. p. 245-55.
29. Barr AR. The mosquitoes of Minnesota. University of Minnesota Agricultural Experiment Station Technical Bulletin 1958;228.
30. Murphey FJ, Burbutis PP, Bray DF. Bionomics of *Culex salinarius* Coquillett. II. Host acceptance and feeding by adult females of *C. salinarius* and other mosquito species. Mosquito News 1967;27:366-74.
31. Burbutis PP, Jobbins DM. Notes on certain characteristics of two populations of *Culex pipiens* Linn. In: Proceedings of the 50th Annual Meeting of the New Jersey Mosquito Extermination Association; 1963. p. 289-97.
32. Edman JD. Host-feeding patterns of Florida mosquitoes III. *Culex (Culex)* and *Culex (Neoculex)*. J Med Entomol 1974;11:95-104.
33. Cupp EW, Stokes GM. Feeding patterns of *Culex salinarius* Coquillett in Jefferson Parish, Louisiana. Mosquito News 1976;36:332-5.

Clinical Characteristics of the West Nile Fever Outbreak, Israel, 2000

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West Nile (WN) virus is endemic in Israel. The last reported outbreak had occurred in 1981. From August to October 2000, a large-scale epidemic of WN fever occurred in Israel; 417 cases were confirmed, with 326 hospitalizations. The main clinical presentations were encephalitis (57.9%), febrile disease (24.4%), and meningitis (15.9%). Within the study group, 33 (14.1%) hospitalized patients died. Mortality was higher among patients ≥70 years (29.3%). On multivariate regressional analysis, independent predictors of death were age ≥70 years (odds ratio [OR] 7.7), change in level of consciousness (OR 9.0), and anemia (OR 2.7). In contrast to prior reports, WN fever appears to be a severe illness with high rate of central nervous system involvement and a particularly grim outcome in the elderly.

West Nile (WN) virus was first isolated and identified from the blood of a febrile woman in Uganda in 1937 (1); fever was her only known symptom. In the early 1950s, several reports from outbreaks in Israel were the first to detail the clinical characteristic of this illness; the clinical picture that emerged was that of a benign febrile disease in young adults (2,3). In a later outbreak from Israel in 1957, a linkage between WN virus infection and severe central nervous system (CNS) disease was first noted, and a correlation between the age of patients and severity of disease was established (4).

In the last decade, two outbreaks gained attention: the first from Romania in 1996, where a high percentage of CNS involvement was noted, and the second from New York in 1999, in which several cases of flaccid paralysis were described (5,6). The outbreak in New York was the first time WN fever was reported in the Western Hemisphere (7).

From August to October 2000, 417 laboratory-confirmed WN fever cases occurred in Israel; 326 were hospitalized cases. We collected clinical data on 233 of the hospitalized population from 12 different hospitals throughout the country. We report the clinical characteristics of these patients.

Methods

Study Population

A case of WN fever was defined as illness in a patient with a clinical picture consistent with WN fever and with anti-WN virus immunoglobulin (Ig) M antibodies detected in either a serum or cerebrospinal fluid (CSF) specimen. The study populat:ion was persons who were hospitalized and diagnosed from August 1, 2000, to October 31, 2000. Patients with headache and abnormal CSF findings, with no confusion or change in level of consciousness (i.e., drowsiness to coma), were diagnosed as having meningitis. Patients with an altered level of consciousness, confusion, or focal neurologic signs were diagnosed as having encephalitis, regardless of CSF findings. Patients with severe weakness of limbs or flaccid paralysis were diagnosed as having myelitis. Patients with fever and no CNS symptoms were diagnosed as having febrile disease.

A total of 325 WN fever patients were hospitalized in 20 hospitals. Data were obtained from 12 (60%) of the hospitals and 233 (71%) of the 326 patients. The geographic location of hospitals that participated in the study was similar to that of nonparticipating hospitals. No exclusion was made based on patient characteristics.

Demographic, epidemiologic, and clinical data were collected by infectious disease specialists in each hospital, using a structured questionnaire. Information was obtained by interviewing the patients and reviewing medical records or by reviewing the charts in cases in which the diagnosis was confirmed after the discharge of the patients.

Serologic Testing

Serologic diagnosis of WN virus infection was based on an IgM-capture enzyme-linked immunosorbent assay (ELISA), performed in serum or CSF samples. The assay, which was

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developed in the Central Virology Laboratory during 1999 and 2000, includes the following steps: coating of ELISA plate with goat anti-human IgM and stepwise incubation with the patient's serum (diluted 1:100 and 1:2,000) or CSF (diluted 1:10 and 1:100 for CSF); WN virus antigen was prepared from Vero cells infected with an Israeli gull isolate from 1999; mouse anti-flavivirus monoclonal antibodies (TropBio, Australia); horseradish peroxidase conjugated goat anti-mouse antibody (Jackson Imunoresearch Laboratories, West Grove, PA, USA), and o-phenylenediamine as substrate. Cross-reaction with other flaviviruses was not thoroughly evaluated; however, no other known endemic cross-reacting *Flavivirus* infects humans in Israel.

Statistical Analysis

The Fisher exact test was applied to compare differences between diagnosis by age: <70 years or ≥70 years. A two-step process was used to determine which of the proposed risk factors were independently associated with death. A bivariate logistic regression analysis was used to determine the association of individual risk factors with death. Subsequently, a multivariate logistic regression analyses was performed, with a forward elimination model. Variables for this analysis were selected if they fulfilled the following criteria: 1) level of significance of p<0.15 in the bivariate analysis; 2) data on variables were available in >92% of patients; and 3) variables on which sufficient information was available and occurred in more than 10% of the cases. Data were analyzed with the SAS program (version 6.12).

Results

The age distribution of the 233 patients for whom data were available is delineated in Figure 1. The mean age of the patients was 59 (±23.5 standard deviations) years old. The median age was 65 years (range 3 to 95 years). A large percentage (79.8%) of patients had one or more coexisting illnesses or conditions, including hypertension, diabetes mellitus, ischemic heart disease, renal failure, obstructive lung disease, and immunodeficiency such as organ transplantation, malignancy, and chemotherapy.

Three main clinical presentations were prominent in hospitalized patients with WN fever: encephalitis (57.9%), febrile illness (24.4%), and meningitis (15.9%). Compared with persons <70 years of age (n=134), older persons (n=99) were more likely to have encephalitis (80% vs. 41%) and were less likely to have febrile illness (18% vs. 30%) or meningitis (1% vs. 27%) (p<0.00001).

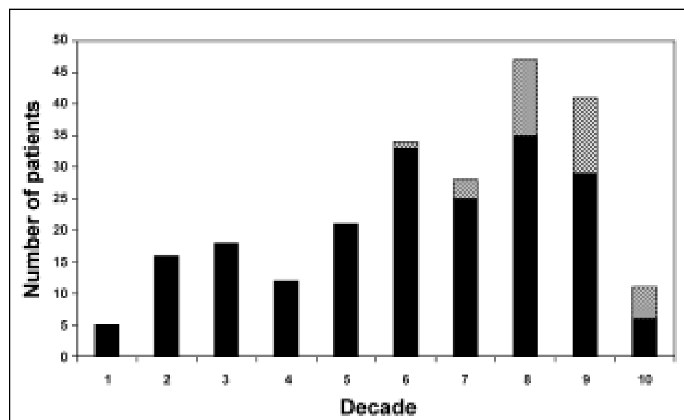

Figure 1. Age distribution of 233 hospitalized patients with West Nile fever. Fatal cases are shown in hatched bars.

Table 1 presents the symptoms and signs during the disease course. Fever (≥38°C) was almost universally present. Sixty percent of the patients had fever above 39°C and 20% had fever above 40°C. Headache, myalgia, chills, and rash were common, as were gastrointestinal complains such as abdominal pain and diarrhea. Lymphadenopathy was infrequent (Table 1).

Table 1. Signs and symptoms of 233 hospitalized patients with West Nile fever

Symptoms and signs	No. (%)
Fever ≥38°C	229 (98.3%)
Headache	135 (57.9%)
Change in level of consciousness	109 (46.8%)
Confused state	92 (39.5%)
Vomiting	73 (31.3%)
Nuchal rigidity	67 (28.7%)
Rash	51 (21.8%)
GI symptoms[a]	43 (18.5%)
Coma	39 (16.7%)
Myalgia	36 (15.4%)
Focal neurologic signs	22 (9.4%)
Lymphadenopathy	10 (4.3%)

[a]Abdominal pain, diarrhea; GI = gastrointestinal.

Symptoms and signs that suggested CNS involvement were common: nuchal rigidity was present in 28.7%, confusion in 39.5%, and change in level of consciousness in 46.8%; 17.7% of the hospitalized patients deteriorated into coma. Thirty-seven patients received enteral ribavirin as an experimental therapy

Lumbar puncture was performed in 153 patients (65.6%). The findings were characteristic of viral infection and demonstrated mild leukocytosis (median 77 leukocytes/mm^3; range 0 to 1,750), high protein (median 85 mg/dL; range 18 to 1,900), and normal glucose (median 67 mg/dL; range 2.8 to 197).

Brain computerized tomography (CT) scan was performed for 105 patients. The findings were interpreted as normal in 62 patients (58%). Abnormal findings included old infarcts (18%), cortical atrophy (13%), meningioma (4%), old hemorrhages (2%), and multiple brain metastasis (1%). None of these findings were attributable to WN virus infection. Electroencephalogram (EEG), performed in 43 patients, was consistent with encephalitis in 34 (79%). No specific pattern for WN virus infection was found.

Laboratory results are depicted in Table 2. Anemia was documented in 41.1% of the patients. Leukocytosis, thrombocytopenia, and leukopenia were documented in 35.9%, 14.9%, and 8.6% of patients, respectively. Liver function tests were mildly impaired in 20% of patients. Mild electrolyte abnormalities and elevated urea were noted.

Anti-WN virus IgM antibodies were detected in serum (143 cases), CSF (27 cases) or both (63 cases). Of note, many of these samples were obtained on the first week of symptoms (83 of serum samples and 50 of CSF samples) and were found to be already positive at that time (Figure 2).

Table 2. Laboratory abnormalities, on admission, in hospitalized patients with West Nile fever

Laboratory results	No. (%)
Hb <12 g/dL in F Hb <13.5 g/dL in M	91/221 (41.1)
WBC <4.8 K/µL	19/220 (8.6)
WBC >10.8 K/µL	79/220 (35.9)
PLT <140 K/µL	33/221 (14.9)
SGOT >37 U/L	36/200 (18)
Alkaline phosphatase >117 U/L	41/197 (20.8)
Na <135 mEq/L	72/216 (33.3)
K <3.5 mEq/L	29/215 (13.5)
Urea >50 mg/dL	39/213 (18.3)

Abbreviations used in this table: n = abnormal results/available results; WBC = leukocyte count; PLT = platelets; SGOT = serum glutamic oxalacetic transaminase.

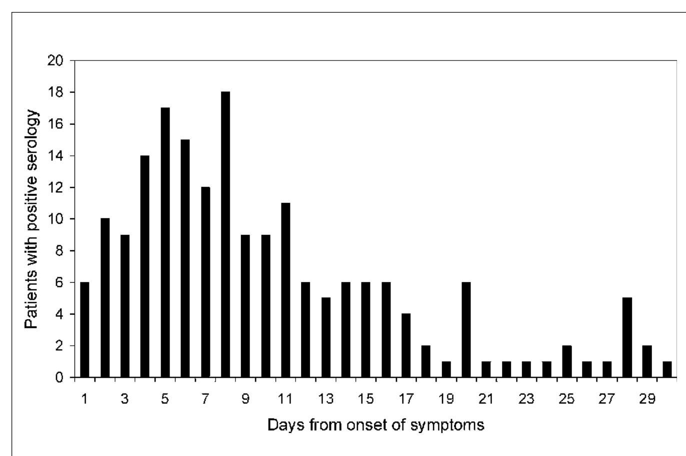

Figure 2. Number of positive blood samples for immunoglobulin (Ig) M serology and time from beginning of symptoms. Blood samples were obtained from the patients upon initial suspicion of the diagnosis of West Nile fever. Bars represent the numbers of persons with positive serology at a given time after the onset of symptoms.

Thirty-three patients (14.1%) in the study group died during hospitalization. Their median age was 80 years (range 54 to 95). The mortality rate among patients ≥70 years increased to 29.3%. All fatal cases but one were ≥68 years. A 54-year-old woman with myasthenia gravis, treated with azathioprine, was the youngest to die from WN virus encephalitis.

We tried to identify clinical and laboratory variables associated with death. By bivariate logistic regression analysis, age ≥70 years, headache, confusion, changes in level of consciousness, anemia on admission, and ribavirin therapy were found to be associated with death. Lack of a coexisting condition was protective (p<0.05). No single coexisting condition was found to be a significant predictor of death.

Sixteen patients were immunocompromised because of heterogeneous conditions, i.e., organ transplantation, malignancy, and chemotherapy. Only four of these patients were ≥70 years. Mortality rate was higher among the immunocompromised (5/16) than among those not immunocompromised (28/217); p=0.052, odds ratio [OR] 3.1, 95% confidence intervals 1.0-9.5).

Multivariate logistic regression analysis was performed with all variables in Table 3. Three variables were independently correlated with death: age ≥70 years (OR 7.7, p= 0.0004), change in level of consciousness (OR 9.0, p=0.0007), and anemia at presentation (OR 2.7, p=0.028).

Discussion

In this report we summarized the clinical characteristics of 233 hospitalized patients during a large-scale outbreak of WN fever in Israel. Our data indicated that WN fever was a severe disease with significant CNS involvement and high mortality (14.1% death rate in the study group). Furthermore, a clear correlation between age and increased severity and death was noted. Eighty percent of hospitalized patients ≥70 years of age had encephalitis, and deaths in this age group rose to 29.3%. Such findings raise the question of whether old age was a risk factor in itself or whether coexisting conditions associated with advanced age account for the observed increased risk. Importantly, in the bivariate regression analysis no single coexisting condition or illness was correlated with death. Moreover, in the multivariate regression analysis, age ≥70 years was an independent risk factor. These findings agree with the results of a case-control study in Romania in which no correlation between an underlying medical condition and meningoencephalitis was found (8).

Immunosuppressive therapy, which nearly reached statistical significance, may be the exception to this finding. Notably, the only fatality in a patient <68 years occurred in a woman treated by immunosuppressive therapy.

The reasons for the high death rates observed in the present epidemic are not clear. Mortality was higher than

Table 3. Variables entered into the multivariate regression analysis

Variables	Alive 200 (%)	Dead 33 (%)	O.R. (95% CI)	p value*
Age ≥70	70 (35.0)	29 (87.9)	13.5 (4.5-39.8)	0.0001
No coexisting condition or illness	53 (26.5)	2 (6)	0.2 (0.1-0.9)	0.03
Diabetes mellitus	31 (15.5)	9 (27.3)	2.0 (0.9-4.8)	0.1
Ischemic heart disease	29 (14.5)	9 (27.3)	2.2 (0.9-5.2)	0.07
Temp (mean +/-SD)	39.1 +/-0.7	39.3 +/-0.64	1.6 (0.9-2.7)	0.08
Headache	123 (61.5)	12 (36.4)	0.4 (0.2-0.9)	0.02
Confusion	68 (34.0)	24 (72.7)	5.2 (2.2-11.7)	0.0001
Change in level of consciousness	80 (40)	29 (87.9)	15.3 (4.5-51.9)	0.0001
Hb<12 g/dL in F Hb<13.5 g/dL in M	72 (36)	19 (57.5)	3.0 (1.4-6.7)	0.006
PLT<140 K/mL	25 (12.5)	8 (24.2)	2.1 (0.8-4.6)	0.1
Ribavirin therapy	22 (11.0)	15 (45.4)	6.7 (3.0-15.2)	0.0001

[a]Only factors included were those that were significant with p<0.15 by bivariate regression analysis, were available in more than 92% of the cases, and occurred in more than 10% of the cases.
SD = standard deviation; OR = odds ratio; CI = confidence intervals; Hb = hemoglobin; PLT = platelets.

that described in the outbreak in Romania in 1996 (14.3% vs. 4.3%, respectively), despite the fact that a high percentage of CNS involvement (89%) was noted in Romania as well (9). High background immunity may account for lower morbidity and deaths. However, the outbreak in Romania was the first reported from that area, and therefore occurred in a population with low background immunity. In fact, the seroprevalence after the outbreak in Romania was reported to be 4.1% (5), compared with seroprevalence of 18.6% in a healthy adult population in Israel in the 1980s (10). Thus, background immunity of the population could not explain the difference in death rates.

Introduction of a new, more virulent strain of WN virus is another possible explanation for the high death rate. Studies in North America support this hypothesis. Mortality during the outbreak in New York (11.4%) was closer to that from Israel. Viral isolates from the outbreak in New York showed a 99.8% genomic similarity to WN virus strain from the brain of a dead goose in Israel in 1998, as well as a fatal human case from Israel in 1999 (11,12). In addition, avian deaths caused by WN viral infection were reported only from North America and Israel, a phenomenon that had not been reported previously. Taken together, these findings suggest that a new strain of WN virus identified in the United States and Israel may be responsible for the higher death rates in these countries.

The signs and symptoms of the disease are not specific, and their percentages seem to differ considerably in the outbreaks reported. Data from confirmed cases of outbreaks in Israel in the early 1950s indicate that fever, headache, and lymphadenopathy were almost universal findings (80% to 100%), with rash in 50% and gastrointestinal complaints in 20% to 30% (2). The study from Romania showed high prevalence of fever and headache but noted only 5% rash, 11% gastrointestinal symptoms, and 2.4% lymphadenopathy (9). In our series, fever, headache, and change in level of consciousness were the most frequent findings; rash was less frequent (28.1%). Differences in study population and case definitions, as well as methods of data collection, may account for such discrepancies. Alternatively, the variation in clinical manifestations may have occurred because of differences between viral strains.

Laboratory results were mostly normal. The mild changes in electrolyte and urea could be attributed to changes in fluid balance in patients with vomiting and diarrhea. Anemia on admission was reported in a high proportion of our patients and was found to be independently associated with fatal outcome. Anemia has not been reported as an important laboratory abnormality in other flaviviral infections (13); with the limited data available, determining whether it was caused by the WN fever or whether it antedated the infection could not be assessed.

A large number of patients underwent brain CT and EEG examinations. CT showed abnormalities in 40% of the patients, but none were specific to the disease and reflected mostly the age of the patients. These results agree with published findings from New York (6).

Generally, the signs, symptoms, laboratory findings, and imaging results in WN fever are nonspecific. Similar to reports from Romania, we found that a high number of blood and CSF specimens obtained during the first week of symptoms were already positive by IgM serology (14). Therefore, this specific test is important for timely diagnosis.

Our study had some limitations. Because the patient population was distributed in multiple hospitals, the physical examination, clinical assessment, and chart review were performed by different physicians. This may have resulted in some skewing of the percentages of the different signs and symptoms attributed to the disease.

Despite a detailed analysis of the clinical manifestations, signs, symptoms, and laboratory results of patients with WN fever, no findings are diagnostic. A febrile illness with neurologic manifestations in elderly patients hospitalized in the summer or fall should raise the possibility of WN fever and prompt a work-up to establish the diagnosis.

Finally, this study identified a specific group with increased risk for death from WN fever. The elderly might be a suitable target group for protective vaccines.

Dr. Chowers is an infectious disease specialist in Meir Medical Center, Kfar Sava, Israel, which is affiliated with Tel Aviv University.

References

1. Smithburn JS, Hughes TP, Burke AW, Paul JH. A neurotropic virus isolated from the blood of a native of Uganda. Am J Trop Med Hyg 1940;20:471-92.
2. Marberg K, Goldblum N, Sterk VV, Jasinska-Klinberg W, Klinberg MA. The natural history of West Nile fever: clinical observations during an epidemic in Israel. Am J Hyg 1956;64:259-69.
3. Goldblum N, Sterk VV, Paderski B. West Nile fever. The clinical features of the desease and the isolation of West Nile virus from the blood of nine human cases. Am J Trop Med Hyg 1954;59:89-103.
4. Spigland W, Jasinska-Klingberg W, Hofshi E, Goldblum N. Clinical and laboratory observations in an outbreak of West Nile fever in Israel in 1957. Harefua 1958;54:275-81.
5. Tsai TF, Popovici F, Cernescu C, Campbell GL, Nedelcu NI. West Nile encephalitis epidemic in southeastern Romania. Lancet 1998;352:767-71.
6. Asnis DS, Conetta R, Teixeira AA, Waldman G, Sampson BA. The West Nile virus outbreak of 1999 in New York: the Flushing Hospital experience [published erratum appears in Clin Infect Dis 2000 May;30:841]. Clin Infect Dis 2000;30:413-8.
7. Centers for Disease Control and Prevention. Update: West Nile-like viral encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:890-2.
8. Han LL, Popovici F, Alexander JJ, Laurentia V, Tengelsen LA, Cernescu C, et al. Risk factors for West Nile virus infection and meningoencephalitis, Romania, 1996. J Infect Dis 1999;179:230-3.
9. Ceausu E, Erscoiu S, Calistru P, Ispas D, Dorobat O, Homos M, et al. Clinical manifestations in the West Nile virus outbreak. Rom J Virol 1997;48:3-11.
10. Cohen D, Zaide Y, Karasenty E, Schwartz M, LeDuc JW, Slepon R, et al. Prevalence of antibodies to West Nile fever, sandfly fever Sicilian, and sandfly fever Naples viruses in healthy adults in Israel. Public Health Rev 1999;27:217-30.
11. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333-7.
12. Giladi M, Metzkor-Cotter E, Martin DA, Siegman-Igra Y, Korczyn A, Rosso R, et al. West Nile encephalitis in Israel, 1999: The New York connection. Emerg Infect Dis 2001;7:659-61.
13. Tsai T. Flaviviruses. In: Mandell G, Bennett J, Dolin R, editors. Principles and practice of infectious diseases. Fifth ed. Philadelphia: Churchill Livingstone; 2001:1714-36.
14. Cernescu C, Ruta SM, Tardei G, Grancea C, Moldoveanu L, Spulbar E, et al. A high number of severe neurologic clinical forms during an epidemic of West Nile virus infection. Rom J Virol 1997;48:13-25.

West Nile Virus Infection in Birds and Mosquitoes, New York State, 2000

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West Nile (WN) virus was found throughout New York State in 2000, with the epicenter in New York City and surrounding counties. We tested 3,403 dead birds and 9,954 mosquito pools for WN virus during the transmission season. Sixty-three avian species, representing 30 families and 14 orders, tested positive for WN virus. The highest proportion of dead birds that tested positive for WN virus was in American Crows in the epicenter (67% positive, n=907). Eight mosquito species, representing four genera, were positive for WN virus. The minimum infection rate per 1,000 mosquitoes (MIR) was highest for *Culex pipiens* in the epicenter: 3.53 for the entire season and 7.49 for the peak week of August 13. Staten Island had the highest MIR (11.42 for *Cx. pipiens*), which was associated with the highest proportion of dead American Crows that tested positive for WN virus (92%, n=48) and the highest number of human cases (n=10).

The emergence of West Nile (WN) virus in 1999 in four U.S. states (1) was followed by its spread to 12 states in 2000 (2). An enzootic cycle was established between birds and mosquitoes, and WN disease was observed in humans, horses, and birds in both years (2,3). Bird deaths due to WN virus are unusual outside North America, with the exception of deaths of geese in Israel (4) and pigeons in Egypt (5). In 1999 in North America, WN disease, characterized by meningoencephalitis and myocarditis, was observed in 14 species of wild and captive birds (6). WN virus has been detected in a number of mosquito genera in North America, including *Culex* and *Aedes* species (2,7). Vector competence has been confirmed experimentally for some North American species, including *Cx. pipiens*, *Ae. vexans*, and *Ae. japonicus* (8,9).

We have summarized surveillance data for WN virus in dead birds and mosquitoes for New York State in the 2000 transmission season. A quantitative and kinetic analysis of data within and outside the epicenter is shown for both the bird and mosquito samples. Vertebrate and invertebrate WN virus infections are compared for counties in the epicenter.

Materials and Methods

Bird and Mosquito Samples

Dead birds were collected and mosquitoes were trapped by local county health units and other agencies as part of the New York State WN virus surveillance effort. Dead birds were necropsied at the Wildlife Pathology Unit at the Department of Environmental Conservation. Kidney, brain, heart, liver, or spleen were harvested and stored at -70°C. Additional avian tissue samples sent to the National Wildlife Health Center were not included in this analysis because selection criteria and testing procedures differed. Mosquitoes were trapped, speciated, grouped into pools of 5 to 50, and stored at -70°C. For some pools, *Cx. pipiens* and *Cx. restuans* were not separated but were pooled together and denoted as *Cx. pipiens-restuans*. The *Aedes* genus is being reclassified into two genera, *Aedes* and *Ochlerotatus* (10), but is classified as *Aedes* in this manuscript. Avian tissue samples and mosquito pools were sent to the Arbovirus Laboratory at the Wadsworth Center for WN virus testing. The transmission season was defined as May 15 to October 31, 2000, with the first and last positive samples collected on May 22 and October 31, 2000, respectively. Two positive birds were found earlier in the year (a hawk on February 6 and a crow on April 1), but they were not followed by other positive specimens and did not therefore appear to represent the beginning of the mosquito-borne WN virus transmission season.

WN Virus Testing

Samples were processed and WN virus assays were performed as described by Shi et al. (11). Briefly, RNA was extracted from bird tissue or pools of ≤50 mosquitoes, and real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed (TaqMan, ABI Prism 7700 Sequence Detector, Applied Biosystems, Foster City, CA). Confirmatory tests included a second TaqMan primer-probe set, standard RT-PCR, virus isolation in cell culture, and

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immunofluorescence assays (for avian tissues). A sample was confirmed as positive if at least two different test results were positive.

Other Arbovirus Testing

Virus isolation was attempted for all *Aedes* species (with the exception of some *Ae. vexans* pools), all *Culiseta* species, and most WN virus-positive mosquito pools (with the exception of some *Cx. pipiens* and *Cx. pipiens-restuans* pools). Samples were inoculated onto monolayers of Vero or C6/36 cells. Viruses from positive cultures were typed by using serogroup-specific polyclonal antisera for bunyaviruses (California and Bunyamwera serogroups), flaviviruses, alphaviruses, and rhabdoviruses (Hart Park serogroup). California serogroup isolates were further characterized by sequence analysis.

Definition of the Epicenter and Radial Regions

The epicenter of the New York State epizootic was defined as follows. The minimum infection rate (MIR) of each mosquito species was calculated for each county or New York City borough by the standard formula: (number of WN virus-positive mosquito pools/total number of mosquitoes tested) x 1000. The MIR was calculated only when at least 1,000 mosquitoes were tested per species per county or borough. When the MIR was at least 1.0 for any mosquito species in a region, the county or borough was included in the epicenter. In addition, any counties that bordered at least two other epicenter counties or boroughs were included in the definition. The epicenter included the five boroughs of New York City (the Bronx, Brooklyn, Manhattan, Queens, and Staten Island) and the four counties immediately east and north of New York City (Nassau, Suffolk, Rockland, and Westchester counties). For both 1999 and 2000, all human cases of WN virus in New York State were in one of these counties (2,7).

Counties outside the epicenter (the "non-epicenter") were divided into four radial regions, R1 to R4, with increasing distance from the epicenter (Figure 1). Radial regions were defined as follows: R1 = Putnam, Orange, Dutchess, Sullivan, and Ulster counties; R2 = Columbia, Delaware, Greene, Rensselaer, Montgomery, Albany, Otsego, Broome, Cortland, Schenectady, Schoharie, and Chenango counties; R3 = Fulton, Essex, Hamilton, Herkimer, Allegany, Lewis, Chemung, Madison, Cayuga, Schuyler, Yates, Washington, Warren, Tompkins, Tioga, Steuben, Onondaga, Seneca, Saratoga, Ontario, Oswego, and Oneida counties; and R4 = Monroe, Wyoming, Cattaraugus, Wayne, Chautauqua, Erie, Clinton, Genesee, Jefferson, Orleans, St. Lawrence, Niagara, Franklin, and Livingston counties.

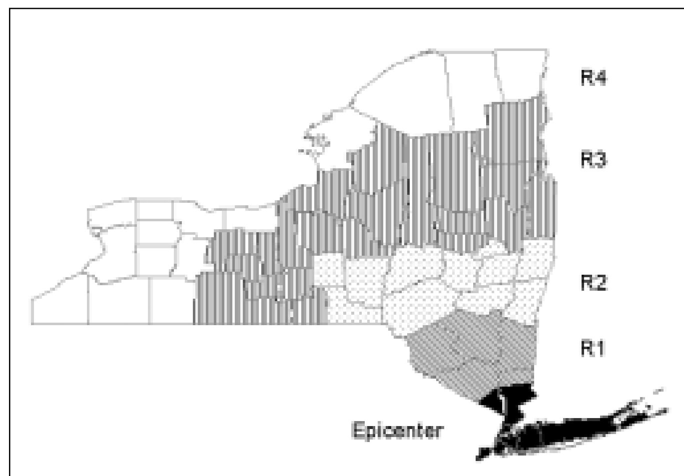

Figure 1. Map of New York State showing the epicenter and radial regions used for analysis. The non-epicenter was defined as R1, R2, R3, and R4. Counties included in the regions are defined in Materials and Methods.

Results

During the 2000 transmission season, WN virus testing was performed on 3,403 dead birds, representing 153 species, 46 families, and 18 orders. The 1,201 WN virus-positive birds represented 63 species, 30 families, and 14 orders (Table 1). The percentage of WN virus-positive birds was 35% for all birds submitted for testing from throughout the state. Avian species that were >35% positive and for which at least 10 birds were tested included American Kestrel (57%, n=14), Cedar Waxwing (60%, n=10), Ovenbird (50%, n=18), American Crow (47%, n=1,687), Fish Crow (47%, n=45), and Red-tailed Hawk (43%, n=14). The discrepancies in number of birds tested make comparisons between species difficult.

The percentage of WN virus-positive birds was analyzed for the epicenter and non-epicenter regions. Data are included for avian species for which at least 10 birds were tested in one of the regions (Table 2). For all submitted birds, 51% and 23% WN virus-positive birds were found in the epicenter and non-epicenter regions, respectively. WN virus infection in dead birds was highest for American Crows (67%) in the epicenter. In the non-epicenter, WN virus infection for crows was lower, similar to infection in all birds in this region. High numbers of crows were tested in both regions, and the percentage positive differed by almost threefold.

WN virus infection in dead birds was examined over time for American Crows and all other birds in the epicenter and four radial regions in New York State (Figure 2). Comparison of American Crows in the five regions (Figure 2A) revealed the highest peak in the epicenter during September. In addition, the peak for American Crows in the epicenter was much broader than for the other four regions. For September, the peaks for American Crows in R1 and R2 were greater than those in the more distant regions, R3 and R4, suggesting a minor extension of the epicenter during this month. Comparison of all birds except American Crows in the five regions (Figure 2B) revealed little difference between the regions, even for the epicenter. These data support the hypothesis that the susceptibility to WN disease was greatest in crows in the epicenter.

We tested 9,954 mosquito pools with 317,668 mosquitoes, representing 28 species and eight genera (Table 3). Of eight positive species representing four genera, most positive pools were *Culex* species (n=341), compared with only 22 positive pools in the other three genera. All but five of the positive pools were collected in the epicenter. The MIR was calculated for each species in the epicenter for which at least 1,000 mosquitoes were tested (Table 3). In the epicenter, the MIR ranged from 0.47 to 3.55. The MIR of *Cx. pipiens* was the highest for an individual species. All the pure *Cx. restuans* pools were negative, and the MIR for *Cx. pipiens-restuans* was almost half that of the pure *Cx. pipiens* pools; therefore, the positive mosquitoes in the mixed pools of *Cx. pipiens-restuans*

Table 1. Birds positive for West Nile virus in New York State during the 2000 season[a]

Order	Family	Common name	No. tested	% positive
Anseriformes	Anatidae	Domestic Goose	2	50
		Canada Goose	15	33
		Mute Swan	3	33
Apodiformes	Trochilidae	Ruby-throated Hummingbird	5	20
Caprimulgiformes	Caprimulgidae	Common Nighthawk	2	50
Charadriiformes	Charadriidae	Killdeer	3	33
	Laridae	Herring Gull	9	33
		Ring-billed Gull	66	32
		Greater Black-backed Gull	7	29
	Rynchopidae	Black Skimmer	1	100
	Scolopacidae	Ruddy Turnstone	1	100
Ciconiiformes	Ardeidae	Least Bittern	1	100
		Green Heron	3	33
		Great Blue Heron	29	10
Columbiformes	Columbidae	Mourning Dove	83	19
		Rock Dove	41	17
Coraciiformes	Alcedinidae	Belted Kingfisher	6	33
Falconiformes	Accipitridae	Red-tailed Hawk	14	43
		Sharp-shinned Hawk	17	35
		Cooper's Hawk	30	30
		Broad-winged Hawk	7	14
	Falconidae	Merlin	5	100
		American Kestrel	14	57
Galliformes	Meleagrididae	Domestic Turkey	1	100
		Eastern Wild Turkey	3	67
	Phasianidae	Peacock	8	25
		Ring-necked Pheasant	16	25
	Tetraonidae	Chicken	14	29
		Ruffed Grouse	131	21
Gruiformes	Rallidae	Virginia Rail	2	50
Passeriformes	Bombycillidae	Cedar Waxwing	10	60
	Corvidae	Fish Crow	45	47
		American Crow	1,687	47
		Blue Jay	500	29
	Fringillidae	Zebra Finch	1	100
		Song Sparrow	5	60
		American Goldfinch	4	50
		House Finch	8	38
		Cardinal	3	33
	Icteridae	Red-winged Blackbird	6	17
		Common Grackle	53	13
	Mimidae	Gray Catbird	22	23
		Northern Mockingbird	10	20
	Parulidae	Black-throated Blue Warbler	1	100
		Canada Warbler	1	100
		Warbler	1	100
		Yellow-rumped Warbler	1	100
		Ovenbird	18	50
	Ploceidae	House Sparrow	127	13
	Sturnidae	European Starling	23	17
	Turdidae	Veery	3	33
		Eastern Bluebird	4	25
		American Robin	74	22
		Wood Thrush	5	20
	Tyrannidae	Eastern Phoebe	2	50
Pelecaniformes	Phalacrocoracidae	Cormorant	2	100
		Double Crested Cormorant	2	50
Psittaciformes	Cacatuidae	Cockatoo	1	100
		Cockatiel	5	60
	Psittacidae	Macaw	1	100
		Parakeet	9	22
Strigiformes	Strigidae	Snowy Owl	2	100
		Great Horned Owl	16	19

[a]Season defined as May 15, 2000, through October 31, 2000.

Table 2. Summary of birds tested for West Nile virus in New York during the 2000 season[a]

Common name	Epicenter No. tested	Epicenter % WN virus positive	Non-epicenter No. tested	Non-epicenter % WN virus positive
American Crow	907	67	780	23
Fish Crow	31	55	14	29
Blue Jay	191	40	309	23
Cooper's Hawk	11	27	19	32
Sharp-shinned Hawk	<10	NA[b]	14	36
American Robin	11	9	59	22
House Sparrow	107	8	20	40
European Starling	15	7	<10	NA
Common Grackle	27	7	26	19
Gray Catbird	<10	NA	16	25
Ovenbird	<10	NA	12	75
Common Yellow Throat	19	0	<10	NA
Mallard	<10	NA	12	0
Ring-billed Gull	<10	NA	66	32
Great Blue Heron	<10	NA	28	7
Rock Dove	16	0	25	28
Mourning Dove	<10	NA	77	19
Ring-necked Pheasant	<10	NA	15	27
Chicken	<10	NA	10	30
Ruffed Grouse	<10	NA	130	21
Great Horned Owl	<10	NA	15	20
Total[c]	1,502	51	1,901	23

[a]Season defined as May 15, 2000, through October 31, 2000. Bird species were included only if at least 10 birds were tested for one of the regions throughout the season.
[b]NA = not applicable because of the low number of birds tested.
[c]All birds tested in each region throughout the season.

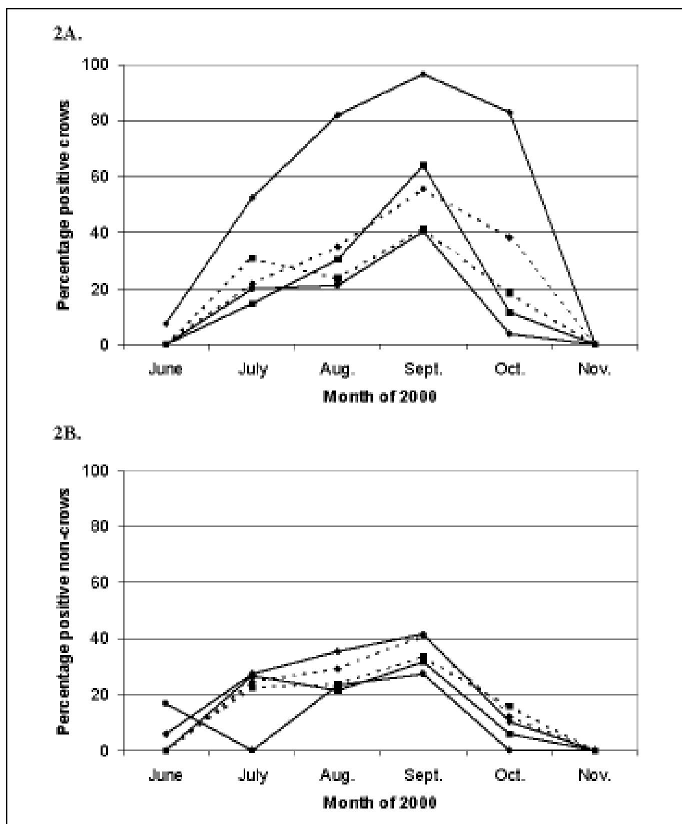

Figure 2. Percentage of West Nile virus-positive birds in different regions of New York State over time. Percentage of positive American Crows (2A) or birds excluding American Crows (2B) in the epicenter and radial regions with increasing distance from the epicenter, R1 to R4 (Figure 1). The epicenter (◆ solid line), R1(◆ dashed line), R2 (■ solid line), R3 (■ dashed line), and R4 (• solid line).

were most likely *Cx. pipiens*. The MIRs for *Cx. pipiens* and *Cx. pipiens-restuans* were compared with the percentage of positive American Crows in the epicenter for each week of the transmission season (Figure 3). The MIRs for *Cx. pipiens* and *Cx. pipiens-restuans* peaked 3 and 2 weeks, respectively, before the peak for positive crows. These data support the hypothesis that *Cx. pipiens* is an important enzootic vector of WN virus in New York.

The epicenter was examined as individual boroughs and counties to compare vertebrate and invertebrate WN virus infections (Table 4). The percentage of positive American Crows was calculated, and human and equine cases were noted for each county over the entire season. The MIRs of mosquitoes from each county were calculated for species with at least 1000 mosquitoes tested. Six mosquito species or groups met this criterion. The highest number of vertebrates infected with WN virus was found in Staten Island and was associated with the highest mosquito MIRs. This borough had measurable MIRs for five mosquito groups: *Cx. pipiens*, *Cx.* species, *Cx. pipiens-restuans*, *Cx. salinarius,* and *Ae. vexans.*

Virus isolation was performed on mosquito samples by cell culture. WN virus was isolated from 110 samples, including *Cx. pipiens-restuans* (n=60), *Cx. pipiens* (n=25), *Cx. salinarius* (n=13), *Culex* species (n=9), *Ae. triseriatus* (n=1), *Ae. vexans* (n=1), and *Psorophora ferox* (n=1). No virus was isolated from any of the WN virus RNA-positive pools of *Ae. japonicus*, *Ae. cantator*, and *An. punctipennis*. Pools that were negative on Vero cell culture were passaged repeatedly in *Aedes albopictus* (C6/36) cells; these further attempts to isolate virus were unsuccessful. Other viruses isolated were trivittatus virus from *Ae. trivittatus* (n=4) and *Ae. triseriatus* (n=1), Cache Valley virus from *Ae. trivittatus* (n=2) and *Ae. triseriatus* (n=2), and Flanders virus from *Cx. pipiens-restuans* (n=7), *Cx. pipiens* (n=2), and *Cs. melanura* (n=11). Eastern equine encephalitis virus and California group viruses other than trivittatus were not isolated from any of the *Culiseta* or *Aedes* pools.

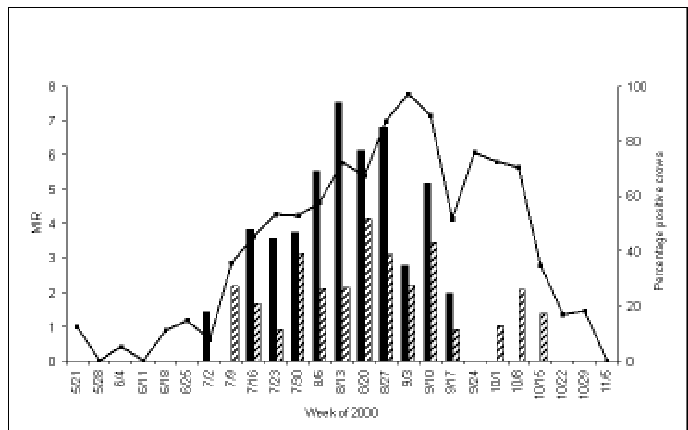

Figure 3. Weekly minimal infection rate per 1,000 mosquitoes (MIR) and percentage of crows positive for West Nile virus in the epicenter. Solid bars designate *Culex pipiens*. Hatched bars designate *Cx. pipiens-restuans*. Solid line designates percentage of positive crows.

Table 3. Summary of mosquitoes tested for West Nile virus in New York State in 2000

Mosquito species	Epicenter				Non-epicenter		
	Total mosquitoes	Total pools	Total positive pools	MIR[a]	Total mosquitoes	Total pools	Total positive pools
Aedes abserratus-punctor	NC[b]	NC	NC	NC	214	9	0
Ae. canadensis	8,018	167	0	0	8,269	145	0
Ae. cantator	1,615	65	1	0.62	993	22	0
Ae. cinereus	340	15	0	NA[c]	247	10	0
Ae. communis	NC	NC	NC	NC	335	12	0
Ae. communis group	NC	NC	NC	NC	235	8	0
Ae. intrudens	21	1	0	NA	NC	NC	NC
Ae. japonicus	3,342	257	2	0.60	3,871	271	3
Ae. sollicitans	5,003	131	0	NA	1	1	0
Ae. stimulans	NC	NC	NC	NC	42	1	0
Ae. stimulans group	153	3	0	NA	540	17	0
Ae. taeniorhynchus	395	11	0	NA	11	1	0
Ae. trichuris	NC	NC	NC	NC	23	2	0
Ae. triseriatus	2,956	203	3	1.01	6,335	206	0
Ae. trivittatus	4,289	184	0	0	2,453	79	0
Ae. vexans	21,486	761	10	0.47	13,490	422	0
Aedes species	1,340	72	1	0.75	NC	NC	NC
Anopheles crucians	13	2	0	NA	NC	NC	NC
An. punctipennis	165	37	1	NA	291	17	0
An. quadrimaculatus	66	18	0	NA	59	3	0
An. walkeri	NC	NC	NC	NC	833	22	0
Anopheles species	16	2	0	NA	NC	NC	NC
Coquillettidia perturbans	8,167	157	0	0	10,874	206	0
Culiseta melanura	8,189	211	0	0	6,281	178	0
Cs. morsitans	NC	NC	NC	NC	1,821	85	0
Culiseta species	98	3	0	NA	NC	NC	NC
Culex pipiens	22,120	831	78	3.53	8,698	288	1
Cx. pipiens-restuans[d]	114,517	3,208	211	1.84	16,228	537	1
Cx. restuans	3,403	190	0	0	794	48	0
Cx. salinarius	19,541	483	31	1.59	704	17	0
Cx. territans	76	15	0	NA	NC	NC	NC
Culex species	5,358	200	19	3.55	1,108	32	0
Orthopodomyia alba	NC	NC	NC	NC	101	3	0
Psorophora ferox	63	10	1	NA	162	6	0
Uranotaenia sapphirina	208	19	0	NA	419	18	0
Unidentified	1,173	26	0	0	105	6	0
TOTAL	232,131	7,282	358		85,537	2,672	5

[a] MIR = minimal infection rate per 1,000 mosquitoes. MIR calculated as (number of WN virus-positive pools by RT-PCR/total mosquitoes tested) x 1,000. MIR was calculated only if a minimum of 1,000 mosquitoes was tested from a defined region throughout the season. None of the counties outside the epicenter met this criterion.
[b]NC = not collected.
[c]NA = not applicable because <1,000 mosquitoes were collected.
[d]*Cx. pipiens* and *Cx. restuans* were not separated and were pooled together.

Table 4. Comparison of infection in vertebrates and minimal infection rate in mosquitoes for WN virus in the epicenter of the New York epizootic of 2000

Borough or county	% positive crows (n)[b]	No. human cases[c]	No. equine cases[d]	MIR[a]					
				Culex pipiens	*Cx. pipiens-restuans*[e]	*Culex* species	*Cx. salinarius*	*Aedes vexans*	*Ae. japonicus*
Staten Island	92% (48)	10	1	11.42	9.9	6.92	2.61	0.79	NC[f]
Brooklyn	73% (48)	2	0	3.12	1.42	NA[g]	0.67	NA	NC
Manhattan	85% (34)	1	0	2.91	3.86	NA	NA	NA	NC
Queens	64% (25)	1	0	0.16	0.24	0	0.2	NA	NA
Suffolk	70% (188)	0	8	NC	2.74	NC	NC	0.40	NA
Bronx	44% (9)	0	2	2.38	NA	NA	0.87	0	NC
Rockland	76% (280)	0	0	NA	1.98	NA	NA	0.44	NA
Westchester	44% (128)	0	0	0.51	0.73	NC	NC	0	0.43
Nassau	56% (147)	0	4	NC	0.28	NC	NC	0.45	NC

[a]MIR = minimal infection rate per 1,000 mosquitoes. Mosquito species were included only if a minimum of 1,000 total mosquitoes was collected throughout the season for the county. MIR was calculated as (number of WN virus-positive pools by RT-PCR/total mosquitoes tested) x 1,000.
[b]Percentage WN virus-positive crows throughout the transmission season with total number of crows tested in parentheses.
[c]Human cases reported by the Centers for Disease Control and Prevention (2).
[d]Equine cases reported by S. Trock (personal communication).
[e]*Cx. pipiens* and *Cx. restuans* were not separated and were pooled together.
[f]NC = not collected.
[g]NA = not applicable because <1,000 mosquitoes were collected.

Conclusion

In the 2000 transmission season in New York State, we found 63 bird species infected with WN virus, compared with 14 species in 1999 (6). The percentage of WN virus-positive birds was higher in the epicenter than outside it. This high percentage almost entirely reflects infected crows in the epicenter; no increase in WN virus infection was noted in birds other than crows. In contrast, high WN virus infection of dead crows was not observed outside the epicenter, where the percentage of WN virus positivity was similar in crows and other birds over the entire season. High numbers of dead crows were also observed in 1999 (3,6). The cause of the increased sensitivity of crows to WN disease or infection is unknown, but may be due to virus-host interactions, mosquito-bird interactions, mosquito feeding preferences, crow population density, or crow behavior. The presence of WN virus in dead birds does not indicate a definitive diagnosis of WN virus as the cause of death. Many of the birds did not show gross pathologic lesions of WN disease (12). In addition, the rate of WN virus-positive birds in our surveillance samples is not equivalent to prevalence of infection, since we are sampling only dead birds.

The analysis of percentage positive birds over time revealed differences between various regions in New York State. The percentage of WN virus-positive crows was highest in the epicenter compared with other regions of New York State throughout the season, supporting the importance of crows as indicators in the epizootic. The percentage of WN virus-positive crows was higher than that for all other birds early in the season only in the epicenter. Outside the epicenter, the percentages of WN virus-positive crows and all other birds were similar until the peak month of September. At this time, the two radial regions closest to the epicenter showed higher infection in crows than in other birds, suggesting that the intense level of viral activity may have spread beyond the epicenter. An explanation for this apparent spread may be increased movement of crows during the fall. The surveillance data on avian deaths have implications for future surveillance activities. The similar percentages of positive crows and other birds outside the epicenter indicate the importance of testing all birds, not only crows, outside the epicenter.

Sampling errors are likely with the avian surveillance samples. The samples were from dead birds submitted to the Wildlife Pathology Unit, which relied on the cooperation of the general public, individual county health departments, and other agencies; therefore, surveillance samples do not represent a random sampling of dead birds throughout the state. The size and degree of urbanization of various bird species may have resulted in differences in submission. For example, large urban-dwelling species, such as crows, were more likely to have been submitted than small rural dwellers. In addition, more birds were likely sampled from areas with human cases, greater media coverage, and higher human population. The similarity in WN virus infection in birds other than crows from different regions suggests that the impact of sampling bias was not significant. Additionally, specimens submitted for testing to the Wadsworth Center from the Wildlife Pathology Unit represent a sample of those submitted by the public. Sampling at this level may introduce further bias into our surveillance sample. The similarity in WN virus infection in birds other than crows outside the epicenter, however, suggests that such bias was minimal.

The highest MIR for mosquitoes in the epicenter was for *Cx. pipiens*. Positive pools of *Cx. pipiens* also were identified in 1999 in New York (13). *Cx. pipiens-pipiens* mosquitoes feed almost exclusively on birds (14); thus, they are likely an important enzootic vector in the bird-mosquito cycle in North America. Other *Cx.* species have been implicated as enzootic vectors worldwide (15). High MIRs of *Cx. pipiens* and *Cx. salinarius* were associated with human and equine WN virus cases and high infection rates of crows in counties in the epicenter. *Cx. salinarius* feeds on both birds and mammals (16); therefore, it is a likely candidate as a "bridge vector," transmitting the virus from bird to mammal. *Cx. salinarius* has been proposed as a bridge vector for Eastern equine encephalitis virus (17). All MIR data were calculated by using RT-PCR-positive pools and therefore cannot be directly compared with MIRs calculated by using infectious virus-positive pools. No virus was isolated from five RNA-positive pools of *Ae. japonicus*, even after six serial passages through C6/36 cells. Much attention has been focused on this species because it has been reported to be a highly competent laboratory vector of WN virus (9), but current field data do not support this experimental observation. Different populations of *Ae. japonicus* have been described in the eastern United States (18), and differences in vector competence between the populations may explain the discrepancy between the field and experimental data.

The possibility of sampling error also exists for the mosquito surveillance samples. Individual counties collected mosquitoes in different numbers and set traps by different criteria. In addition, some counties used mosquito adulticides or larvicides during the season. These sources of bias are unlikely to have been uniformly introduced, and their impact on our analyses is unclear.

The results from the 2000 surveillance season for WN virus leave a number of unanswered questions. Many avian species can become infected with WN virus, but the prevalence of infection for each species is unknown without systematic serosurveys of the wild bird population. It is also unknown which birds have a high enough viremia for efficient transmission to the vector. The apparent mortality rate caused by WN virus is higher for crows than for other birds, but laboratory experiments are required to determine WN virus mortality rates and the pathogenic mechanisms in crows and other avian species. In addition to crows, many other birds, such as raptors and other corvids, also showed significant pathology. Some nonmigrating species (e.g., ruffed grouse) have potential use as an indicator species for WN virus infection. Vector competence, blood meal identification, and transovarial transmission studies for the potential mosquito vectors are also important research areas.

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References

1. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333-7.
2. Centers for Disease Control and Prevention. Update: West Nile Virus activity—Eastern United States, 2000. MMWR Morb Mortal Wkly Rep 2000;49:1044-7.
3. Centers for Disease Control and Prevention. Outbreak of West Nile-like viral encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:845-9.
4. Office of Internationale des Epizooties. West Nile fever in Israel in geese. Disease Information 1999;12:166.
5. Work TH, Hurlbut HS, Taylor RM. Isolation of West Nile virus from hooded crow and rock pigeon in the Nile Delta. Proc Soc Exp Biol Med 1953;84:719-22.
6. Steele KE, Linn MJ, Schoepp RJ, Komar N, Geisbert TW, Manduca RM, et al. Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, New York. Vet Pathol 2000;37:208-24.
7. Centers for Disease Control and Prevention. Update: West Nile virus encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:944-55.
8. Turell MJ, O'Guinn M, Oliver J. Potential for New York mosquitoes to transmit West Nile virus. Am J Trop Med Hyg 2000;62:413-4.
9. O'Guinn ML, Dohm DJ, Jones JW, Turell MJ. Potential mosquito vectors of West Nile virus in the United States. Am J Trop Med Hyg 2001;62:286.
10. Reinert JF. New classification for the composite genus *Aedes* (Diptera: Culicidae: Aedini), elevation of subgenus *Ochlerotatus* to generic rank, reclassification of other subgenera, and notes on certain subgenera and species. J Am Mosq Control Assoc 2000;16:175-88.
11. Shi P-Y, Kauffman EB, Ren P, Felton A, Tai JH, Dupuis II AP, et al. High throughput detection of West Nile virus RNA. J Clin Microbiol 2001;39:1264-71.
12. Eidson M, Kramer LD, Stone W, Hagiwara Y, Schmit K, New York State West Nile Virus Avian Surveillance Team. Dead bird surveillance as an early warning system for West Nile virus. Emerg Infect Dis 2001;7:631-5.
13. Centers for Disease Control and Prevention. Update: West Nile-like viral encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:890-2.
14. Spielman A. Population structure in the *Culex pipiens* complex of mosquitos. Bull World Health Organ 1967;37:271-6.
15. Hubalek Z, Halouzka J. West Nile fever—a reemerging mosquito-borne viral disease in Europe. Emerg Infect Dis 1999;5:643-50.
16. Crans WJ. Continued host preference studies with New Jersey mosquitoes, 1963. In: Proceedings of the Annual Meeting of the New Jersey Mosquito Extermination Association 1964;51:50-8.
17. Vaidyanathan R, Edman JD, Cooper LA, Scott TW. Vector competence of mosquitoes (Diptera: Culicidae) from Massachusetts for a sympatric isolate of eastern equine encephalomyelitis virus. J Med Entomol 1997;34:346-52.
18. Fonseca DM, Campbell SR, Crans WJ, Mogi M, Miyagi I, Toma T, et al. *Aedes* (Finlaya) *japonicus* (Diptera: Culicidae), a newly recognized mosquito in the United States: Analyses of genetic variation in the United States and putative source populations. J Med Entomol 2001;38:135-46.

West Nile Fever Outbreak, Israel, 2000: Epidemiologic Aspects

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From August 1 to October 31, 2000, 417 cases of West Nile (WN) fever were serologically confirmed throughout Israel; 326 (78%) were hospitalized patients. Cases were distributed throughout the country; the highest incidence was in central Israel, the most populated part. Men and women were equally affected, and their mean age was 54±23.8 years (range 6 months to 95 years). Incidence per 1,000 population increased from 0.01 in the 1st decade of life to 0.87 in the 9th decade. There were 35 deaths (case-fatality rate 8.4%), all in patients >50 years of age. Age-specific case-fatality rate increased with age. Central nervous system involvement occurred in 170 (73%) of 233 hospitalized patients. The countrywide spread, number of hospitalizations, severity of the disease, and high death rate contrast with previously reported outbreaks in Israel.

In early August 2000, infectious disease specialists in hospitals in central Israel noted an increasing number of elderly patients admitted for encephalitis. By mid-August, when several blood and cerebrospinal fluid (CSF) samples tested positive for antibodies for West Nile (WN) virus, an outbreak was suspected. Eventually a WN fever epidemic that affected the entire country was recognized.

This article outlines the epidemiologic aspects of this WN fever epidemic; Chowers et al. (1) details the clinical characteristics. These are the first in-depth descriptions of an outbreak caused by WN virus involving an entire country.

Methods

Serologic studies from blood and CSF were performed by one facility, the Central Virology Laboratory, Public Health Services, Israel Ministry of Health, at the Chaim Sheba Medical Center, Tel Hashomer. From August 1 to October 31, 2000, this laboratory reported 417 serologically confirmed cases of WN fever to the Epidemiology Department of the Israel Ministry of Health. These patients are the focus of our study.

Basic demographic data, date of disease onset, and the patients' final outcome (alive or dead) were available from the files of the Department of Epidemiology, Israel Ministry of Health, and health district offices around the country. From early September 2000, infectious disease specialists in hospitals throughout Israel were asked to complete a detailed questionnaire on clinical, epidemiologic, and laboratory data for patients with WN virus at their hospitals. Information on the clinical presentation of the patients at ambulatory settings was obtained from the health district offices.

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Data on the Israeli population by age group, geographic distribution, and type of locality were retrieved from the Israeli Central Bureau of Statistics (2). Israel is divided into six major geographic districts (Jerusalem, Northern, Haifa, Center, Tel-Aviv, Southern), as well as the Judea, Samaria, and Gaza areas. The permanently inhabited places in Israel are divided into urban (≥2,000 population) and rural (<2,000, even if not agricultural) localities.

Diagnosis of WN fever was based on immunoglobulin M (IgM)-capture enzyme-linked immunosorbent assay (MAC-ELISA) in serum or CSF samples of patients. The assay, which was developed in the Central Virology Laboratory during 1999-2000, includes the following steps: a) binding of anti-human IgM to the ELISA plate; b) incubation with the patient's serum or CSF sample (dilutions of 1:100 and 1:2,000 for serum and 1:10 and 1:100 for CSF); c) incubation with WN virus antigen prepared from Vero cells infected with an Israeli gull isolate from 1999 (Banet C, manuscript in prep.); d) incubation with a mouse anti-flavivirus monoclonal antibody (JCU/KUN/2B2, cat. no. 01-031-02, TropBio Pty Queensland, Australia); and e) incubation with peroxidase-conjugated goat anti-mouse antibody (cat. no. 115-035-071, Jackson ImmunoResearch Laboratories, West Grove, PA) and substrate. Cross-reactivity with other flaviviruses was not thoroughly evaluated; however, no other endemic cross-reacting flavivirus is known to infect humans in Israel. A

patient with a positive MAC-ELISA from serum or CSF was considered to have a serologically confirmed case of WN fever.

Serologic studies for WN virus were not routine in Israel before 2000; this changed in early August 2000, when the first cases of WN fever were documented. Blood or CSF samples for serologic studies were submitted by primary physicians or infectious disease consultants caring for hospitalized patients, based on clinical suspicion. Although not encouraged by the Israeli health authorities, blood samples for serologic studies were also submitted by primary physicians caring for ambulatory patients.

Contingency tables were tested for statistical significance by the chi-square test. Continuous variables were tested by the Student *t* test. SAS software (SAS Inc., Cary, NC) was used for data analysis.

Results

Geographic Distribution

From August 1 to October 31, 2000, 417 cases of WN fever were confirmed by the Central Virology Laboratory. Patients were distributed throughout 113 localities in Israel. The most populated localities in the coastal plains of Israel were the most heavily affected, followed by the northern parts. The Negev and Jerusalem areas were the least heavily affected. No cases of WN virus fever were reported from the Judea, Samaria, and Gaza areas. Incidence rates by geographic district are given in Figure 1.

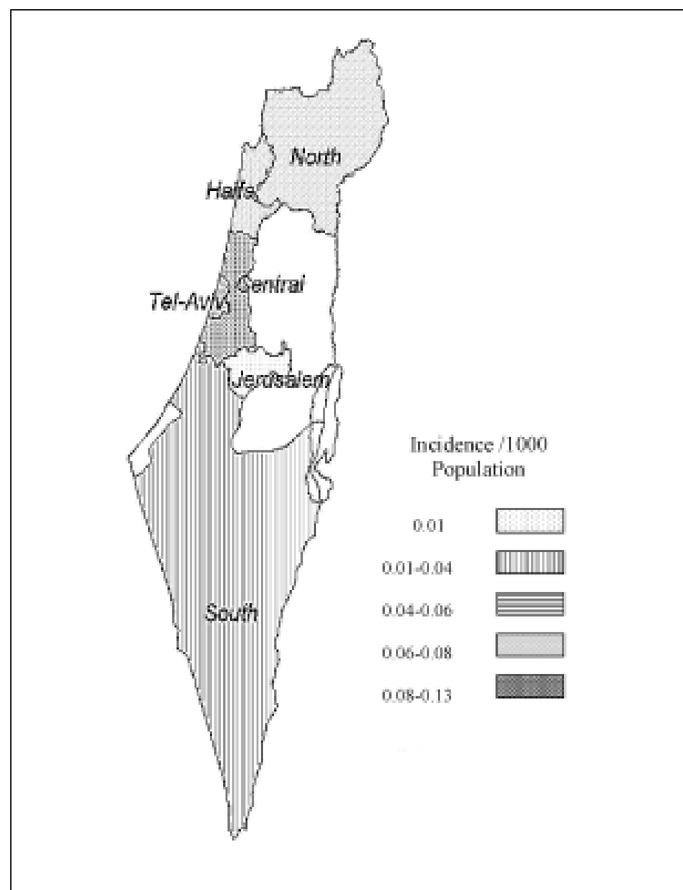

Figure 1. Incidence of West Nile fever infection by district, Israel, 2000.

Three hundred thirty-nine (81.5%) patients resided in urban localities, and 77 (18.5%) in rural localities; however, the incidence of WN virus infection was 2.3-fold higher in rural localities (0.14 cases per 1,000 population) than in urban areas (0.06 cases per 1,000 population, p<0.001).

The Epidemic Curve

The exact date of disease onset was available for 379 (91%) patients with serologically confirmed WN fever. According to these data, the outbreak started at the end of July-early August, 2000, peaked in the second week of September, and abated by the end of October (Figure 2). The main wave of the epidemic started in the central parts of Israel (Center and Tel-Aviv districts), where 41% (2.5 million) of the Israeli population resides. One week later, the epidemic spread to the northern parts of Israel (Haifa and Northern districts), but peaked at the same time as in the central areas. A much smaller wave occurred in the south of Israel (Southern District): it started at the end of August and peaked at the end of September, 2 weeks later than the rest of the country (Figure 3).

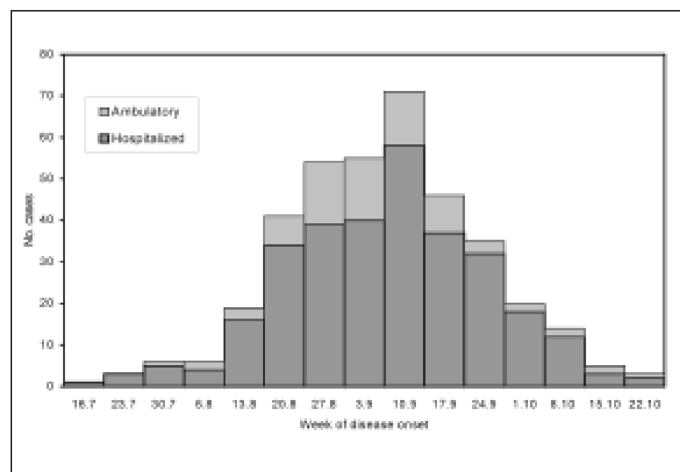

Figure 2. Serologically confirmed West Nile fever cases by week of disease onset, Israel, 2000.

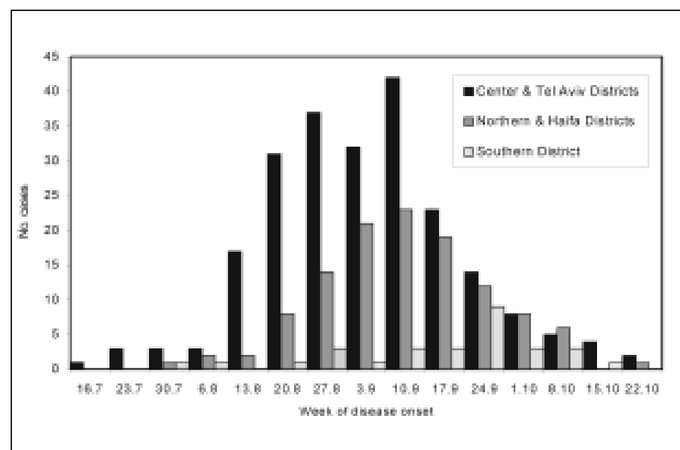

Figure 3. Distribution of West Nile fever cases by week of onset and geographic district, Israel, 2000.

Patient Characteristics and Outcome

Of the 417 patients with serologically confirmed WN virus infection, 209 (50.1%) were female; age ranged from 6 months to 95 years (mean 54.5 ± 23.8 years, median 57). Only 6% (25 patients) were ≤14 years; 46% (192 patients) were ≥60 years. The overall incidence per 1,000 population was 0.07; incidence increased dramatically with age, from 0.01 in the 1st decade of life to 0.48 and 0.87 in the 9th and 10th decades, respectively (Figure 4). Notably, 15 (11.9%) of 126 hospitalized patients who were ≥60 years of age were residents of nursing homes.

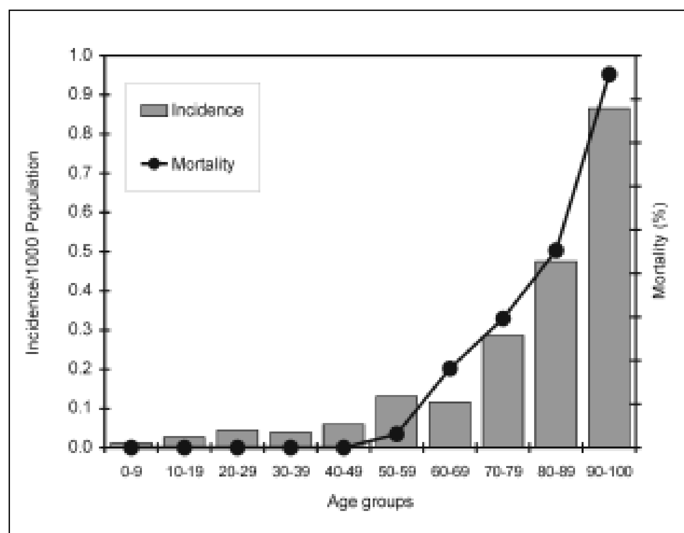

Figure 4. Incidence and deaths from West Nile fever by age group, Israel, 2000.

Three hundred twenty-six patients (78%) were hospitalized in 20 hospitals throughout Israel, and 91 (22%) were diagnosed in ambulatory settings. Ambulatory patients were significantly younger than hospitalized patients (mean age 44 ± 17.8 years vs. 57.4 ± 24.3 years, respectively, $p<0.0001$).

Clinical data were available for 233 (71%) of the 326 hospitalized patients and 37 (52%) of the 71 ambulatory patients. While 170 (73%) of the hospitalized patients had central nervous system (CNS) involvement (encephalitis 133 patients, meningitis 37), only 3 (8%) of the ambulatory patients had mild encephalitis. Further details are presented in a companion article (1).

Thirty-five (10.7%) of the 326 patients hospitalized with WN fever died during hospitalization (33 patients) or within 1 week after discharge (2 patients). None of the 91 ambulatory patients died. The case-fatality rate for all 417 patients was 8.4% (35 of 417 patients); this rate did not differ significantly between females (19 [9.1%] of 209) and males (16 [7.7%] of 208). The mean age of the patients who died was 79.1 ± 9.2 years (median 80), the youngest being 54 and the oldest 95 years old. In comparison, the mean age of the patients who survived was 52.2± 23.3 years ($p <0.0001$).

The case-fatality rate increased dramatically with age (Figure 4). Among all patients ages ≥60 years, the case-fatality rate was 17.7% (34 of 192). Predictors of death in hospitalized patients are discussed in a companion article (1).

Discussion

WN fever is endemic in Israel (3-13); however, the impact of the 2000 outbreak was unprecedented. It involved all age groups, affected all parts of the country, resulted in the hospitalization of 326 patients within a 3-month period, and claimed the lives of 35 persons. The most prominent feature of this outbreak was an exceptionally high rate of CNS illness.

Israeli researchers in the 1950s were the first to characterize the clinical presentation of WN fever, but by the end of the 20th century, WN virus infection was an almost forgotten disease in Israel. Moreover, previous reports did not prepare the infectious disease community and health authorities for the scope, magnitude, and severity of the recent outbreak (14). Infectious disease experts quickly responded to the outbreak by arousing public opinion, enforcing preventive measures, exploring novel therapies (15, Huminer D, et al., unpub. data), and collaborating to form a detailed national clinical database.

Several WN fever outbreaks were reported from Israel in the 1950s and one in 1980 (Table 1) (4-12); most patients were young soldiers in training, who contracted the infection in army camps. Many of them were transferred to military hospitals in accordance with army routines (not necessarily because their illness was severe). The most common manifestation of WN fever in soldiers was an acute febrile illness with headache; a generalized rash occurred in approximately one third. CNS involvement was unusual, and the outcome was excellent.

Information about WN fever in the civilian population is more limited. In 1951, an outbreak occurred in a small agricultural settlement (Maayan Zvi) south of Haifa; 41% of its 303 inhabitants became ill (5,6). All age groups were affected, but only one child (0.8%) had mild aseptic meningitis, and no deaths were reported. Most adults in the settlement were 20 to 35 years old. Another report described a WN fever outbreak among 65 civilians residing in the Hadera area in 1957 (11); 2 patients (a child and an adult) had encephalitis (3.1%), which resolved without sequelae. No deaths were reported.

Standing out among these reports was a description of WN virus infection in 49 elderly patients, ages 66 to 86 years (mean 70 years), who were residents of four nursing homes in the Hadera area, the center of the 1957 epidemic (11). Encephalitis developed in one third of these patients and four died (8.1%). Serologic tests for WN virus were positive in 53% of the 49 patients (and 75% of those with encephalitis), and autopsy was compatible with encephalitis in one of four fatal cases. This was the first time that infection caused by WN virus was associated with severe encephalitis and death.

Some researchers believe (8,11) that a large outbreak of febrile illness accompanied by rash that occurred in 1941 (4), before the state of Israel was established, was also due to WN virus. Obviously, this could not be confirmed at the time, since the WN virus was a newly discovered virus (16) with unknown epidemiology, and diagnostic tests were not yet available. However, the clinical characteristics and time of year support the diagnosis of WN virus infection. An estimated 500 people in the Central and Tel-Aviv districts became ill. If one assumes that the diagnosis was correct, this was probably the largest outbreak in Israel, considering that the total population at that time was only 449,000 (2). In that outbreak no CNS involvement and no deaths were reported.

Table 1. Reported outbreaks caused by West Nile virus in Israel

Year (Ref)	Area	Type of locality	No. studied	Age	CNS involvement	Reported deaths	Documentation
1941 (4)	Center & Tel-Aviv	Urban & rural	500	All ages	None	None	None
1951 (5,6)	Haifa	Agricultural settlement	123	All ages	Meningitis (1/123, 0.8%)	None	Virus isolate (1/123, 0.8%) Serology (14/26, 54%)
1950 (7)	Hadera and coastal plain	Army camps and communal settlement	105	17-40 y	Nuchal rigidity ("a few cases")	None	Virus isolate (9/50, 18%) Serology (18/24, 75%)
1950-53 (8)	Military[a] hospital	Army camps	400	17-23 y	Meningitis (1/400, 0.3%)	None	NA
1953 (9)	Tel Hashomer Hospital[a]	Army camps	70	18-20 y	None	None	Virus isolate (13/70, 18%) Serology (50/70, 68%)
1953-1954 (10)	Center and North Israel	Army camps	300	Young adults	N/A	None	Virus isolate (40/205, 20%) Serology (66/151/ 57%)
1957 (11)	Hadera area	Army camps	300	18-28 y	Encephalitis (1/300, 0.3%)	None	Virus isolate (8/50, 40%) Serology (139/154, 88%)
		Urban & rural	65	All ages	Encephalitis (2/56, 3.1%)	None	Serology (23/50, 46%)
		Nursing homes	49	66-86 y	Encephalitis 16/49 (33%)	4/49, 8.2%	Serology (53%) (in patients with encephalitis: 9/12, 75%)
1980 (12)	Negev Desert	Army camps	32	18 y	Meningitis (1/32, 3.1%) Nuchal rigidity (3/32, 9.3%)	None	Serology (10/11, 91%)
2000 (PR)	Country-wide	Urban and rural	417	0.5-95	Encephalitis (135/256, 51%) (35/265, 13%)	35/417, 8.4%	Serology (417/417, 100%)

[a]Soldiers were transferred to military hospitals according to army routines, not necessarily because of severe illness.
CNS = central nervous system; y = year; PR = present report.

The time of year in which WN fever outbreaks appear in Israel has remained almost constant for the last 5 decades. The previously reported outbreaks (4,6), as well as the recent one, occurred between late July and early October. In comparison, the 1996 epidemic in southeast Romania ended by mid-September (17), and the 1999 epidemic in New York abated by the end of September (18-20).

The severity and scope of the year 2000 outbreak in Israel can be better appreciated when compared with other WN virus outbreaks worldwide in the last decade (Table 2) (17,20-24). The Israeli outbreak most resembles the Romanian outbreak in 1996 (17), although both the incidence (7 vs. 4 per 100,000 population, respectively) and the death rate of hospitalized patients (10.7% vs 4.3%, respectively) were higher in Israel.

The vectors of WN virus in Israel are mosquitoes of the *Culex* species (*Cx. pipiens* and *Cx. perexiguus*) (25,26). The reservoir is wild birds, including pigeons, storks, and crows (25,27,28). In 1997, the virus infected the commercial geese population in Israel (Banet C, manuscript in prep.), a phenomenon that has not been previously described. This resulted in neurologic illness and death in the affected flocks. During 1998 and 1999, 18% to 20% of the 60 flocks in the country were affected, and approximately 5,000 geese died or were killed (O. Nir Markusfeld, pers. comm.). The virus was also isolated from live and dead horses (Banet C, manuscript in prep; 28).

Table 2. Reported deaths in WN virus outbreaks during the last decade

Year	Location	No. cases studied	Deaths (%)	Reference
1994	Algeria	13	13.3[a]	(21)
1996	South Romania	393	4.3	(17)
1999	New York	61	11.5	(20,22)
1999	South Russia	1,000	4.0	(23)
2000	New York, New Jersey	19[b]	10.5	(24)
2000	Israel	417	8.4	Present report

[a]Patients were mainly young children.
[b]Hospitalized patients only.

Human cases were not reported to the Ministry of Health during the 1990s until 1999, when three cases were diagnosed: a young woman with encephalitis who had fully recovered and two elderly patients who died of the disease (29,30). In late 1999 and the first quarter of 2000, the Central Virology Laboratory conducted a serologic survey in Eilot region, a rural area located in the Southern District. This survey revealed two additional cases of acute encephalitis and a 22% rate of IgM seropositivity to WN virus (E. Mendelson, pers. comm.).

A high degree of similarity (>99.8%) was found between the virus isolated from the brain of a dead Israeli goose in 1998 and the U.S. WN virus isolates from the 1999 epidemic (31). In the same study, an earlier Israeli WN virus isolate from 1952 was found to be phylogenetically closer to the 1996 Romanian isolate. More recent studies have also indicated that two phylogenetically distinct WN virus strains are cocirculating among humans (32) as well as avians and equines in Israel (Banet C, manuscript in prep.).

WN virus has reemerged in Israel with renewed virulence and vigor. One possible explanation is the waning of immunity among the Israeli population. Serologic studies from the 1950s in a small number of soldiers residing in epidemic areas revealed a 50% seropositivity during the outbreak season and a 14% seropositivity off-season (10). Other studies revealed 48% to 73% seropositivity in endemic

areas, compared with 7% to 12% in nonendemic areas (13). The only large-scale study was published in 1999 by Cohen et al. (3), who tested stored sera of 1,060 soldiers, 18 to 55 years of age, which were collected between 1982 and 1989. Seroprevalence for WN virus was found to increase with age: from 7% in the 18- to 20-year age group to 10.5% in the 21- to 30-year age group, and as high as 41.9% for people ages 40 to 55. While revealing a high level of background immunity, these results do not support waning of seropositivity to WN virus with age.

The elderly population in Israel has almost doubled since the 1950s. In 1951, the proportion of patients $\geq$60 years was 6.8% (33); it increased to 13.2% in 1998, according to the most recent census (2). Forty-six percent of the WN fever cases and 97% of the fatal cases in the 2000 epidemic occurred in this age group. Unfortunately, no data are available on seroprevalence in the elderly in Israel. Pending results of a recently performed large serologic study in the general population, including nursing homes, may shed more light on this issue.

The changing face of WN virus epidemiology is not unique to Israel, but is a global phenomenon (34,35). Increased CNS invasiveness and a high case-fatality rate were also features of the outbreaks in southeast Romania and New York (17-20,36,37). This apparent increase in virulence had been also noted among birds in New York City (38) and commercial geese in Israel (Banet C, manuscript in prep.), who became sick and died as a result of WN infection. As more data are accumulating, it is tempting to speculate on a possible alteration in the virulence of WN virus. The altered strain may have been introduced into local avian populations by migratory birds, and then into humans and equines (Banet C, manuscript in prep.; 39).

The potential for causing large-scale outbreaks, with substantial illness and death, and the spread of WN virus across the globe call for international cooperation in developing effective vaccines and planning innovative strategies for mosquito control.

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References

1. Chowers MY, Lang R, Nassar F, Ben-David D, Rubinshtein E, Itzhaki A, et al. Clinical characteristics of West Nile fever outbreak, Israel, 2000. Emerg Infect Dis 2001;7:675-8.
2. Statistical abstracts of Israel. Jerusalem, Israel: Central Bureau of Statistics; 2000. Pub. no. 51.
3. Cohen D, Zaide Y, Karasenty E, Schwarz M, LeDuc JW, Slepon R, et al. Prevalence of antibodies to West Nile fever, sandfly fever Sicilian, and sandfly fever Naples viruses in healthy adults in Israel. Public Health Rev 1999;27:217-30.
4. Leffkowitz M. On the frequent appearance of an unclear infectious disease. Harefuah 1942;22:3-4.
5. Bernkopf H. The isolation of the West Nile virus in Israel. Harefuah 1953;45:99-101.
6. Bernkopf H, Levine S, Nerson R. Isolation of West Nile virus in Israel. J Infect Dis 1953;93:207-18.
7. Goldblum N, Sterk VV, Paderski B. West Nile fever. The clinical features of the disease and the isolation of West Nile virus from the blood of nine human cases. Am J Hyg 1954;59:89-103.
8. Radt P. Clinical observations on patients with West Nile fever during outbreaks of the disease in 1950-1953. Harefuah 1955;49:41-4.
9. Marberg K, Goldblum N, Sterk VV, Jasinska-Klingberg W, MA K. The natural history of West Nile fever. I. Clinical observations during an epidemic in Israel. Am J Hyg 1956;64:259-69.
10. Goldblum N, Sterk VV, Jasinska-Klingberg W. The natural history of West Nile fever. II. Virological findings and the development of homologous and heterologous antibodies in the West Nile infection in man. Am J Hyg 1957;66:363-80.
11. Spigland I, Jasinska-Klinberg W, Hofshi E, Goldblum N. Clinical and laboratory observations in an outbreak of West Nile fever in Israel. Harefuah 1958;54:275-81.
12. Katz G, Rannon L, Nili E, Danon YL. West Nile fever-occurrence in a new endemic site in the Negev. Isr J Med Sci 1989;25:39-41.
13. Flatau E, Kohn D, Daher O, Varsano N. West Nile fever encephalitis. Isr J Med Sci 1981;17:1057-9.
14. Siegel-Itzkovich J. Twelve die of West Nile virus in Israel. BMJ 2000;321:724.
15. Shimoni Z, Niven M, Pitlik D, Bulvik M. Treatment of West Nile virus encephalitis with intravenous immunoglobulin. Emerg Infect Dis 2001;7:759.
16. Smithburn KC, Hughes TP, Burke AW, Paul JH. A neurotropic virus isolated from the blood of a native of Uganda. Am J Trop Med Hyg 1940;20:471-92.
17. Tsai TF, Popovici F, Cernescu C, Campbell GL, Nedelcu NI. West Nile encephalitis epidemic in southeastern Romania. Lancet 1998;352:767-71.
18. Centers for Disease Control and Prevention. Outbreak of West Nile-like viral encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:845-9.
19. Centers for Disease Control and Prevention. Update: West Nile-like viral encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:890-2.
20. Centers for Disease Control and Prevention. Update: West Nile virus encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:944-6, 955.
21. Le Guenno B, Bougermouh A, Azzam T, Bouakaz R. West Nile: a deadly virus? Lancet 1996;348:1315.
22. Centers for Disease Control and Prevention. Guidelines for surveillance, prevention, and control of West Nile virus infection—United States. MMWR Morb Mortal Wkly Rep 2000;49:25-8.
23. Lvov DK, Butenko AM, Gromashevsky VL, Larichev VP, Gaidamovich SY, Vyshemirsky OI, et al. Isolation of two strains of West Nile virus during an outbreak in southern Russia, 1999. Emerg Infect Dis 2000;6:373-6.
24. Marfin AA, Petersen LR, Edison M, Miller J, Hadler J, Farrelo C, et al. Widespread West Nile virus activity, eastern United States, 1999-2000. Emerg Infect Dis 2001;7:730-5.
25. Nir Y, Avivi A, Lasovski Y, Margalit J, Goldwasser R. Arbovirus activity in Israel. Isr J Med Sci 1972;8:1695-701.
26. Samina I, Margalit J, Peleg J. Isolation of viruses from mosquitoes of the Negev, Israel. Trans R Soc Trop Med Hyg 1986;80:471-2.
27. Nir Y, Goldwasser R, Lasowski Y, Avivi A. Isolation of arboviruses from wild birds in Israel. Am J Epidemiol 1967;86:372-8.
28. Annual report. Beit-Dagan, Israel: The Israeli Veterinary Services; 2000.
29. Israel Ministry of Health. West Nile fever. Circular #44/2000 (September 19, 2000) 2000;2.
30. Giladi M, Metzkor-Cotter E, Martin DA, Siegman-Igra Y, Korczyn AD, Rosso R, et al. West Nile encephalitis in Israel, 1999: the New York connection. Emerg Infect Dis 2001;7:659-61.
31. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333-7.
32. Hindiyeh M, Shulman LM, Mendelson E, Grossman Z, Weiss L, Bin H. Isolation and characterization of West Nile virus from the blood of viremic patients during the 2000 outbreak in Israel. Emerg Infect Dis 2001;7:748-50.

33. Statistical abstracts of Israel. Jerusalem, Israel: Central Bureau of Statistics; 1958-59. Pub. no. 10.
34. Solomon T, Cardosa MJ. Emerging arboviral encephalitis. Newsworthy in the west but much more common in the east. BMJ 2000;321:1484-5.
35. Marra CM. Encephalitis in the 21st century. Semin Neurol 2000;20:323-7.
36. Sampson BA, Ambrosi C, Charlot A, Reiber K, Veress JF, Armbrustmacher V. The pathology of human West Nile virus infection. Hum Pathol 2000;31:527-31.
37. Shieh WJ, Guarner J, Layton M, Fine A, Miller J, Nash D, et al. The role of pathology in an investigation of an outbreak of West Nile encephalitis in New York, 1999. Emerg Infect Dis 2000;6:370-2.
38. Steele KE, Linn MJ, Schoepp RJ, Komar N, Geisbert TW, Manduca RM, et al. Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, New York. Vet Pathol 2000;37:208-24.
39. Rappole JH, Derrickson SR, Hubalek Z. Migratory birds and spread of West Nile virus in the Western Hemisphere. Emerg Infect Dis 2000;6:319-28.

West Nile Outbreak in Horses in Southern France, 2000: The Return after 35 Years

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On September 6, 2000, two cases of equine encephalitis caused by West Nile (WN) virus were reported in southern France (Hérault Province), near Camargue National Park, where a WN outbreak occurred in 1962. Through November 30, 76 cases were laboratory confirmed among 131 equines with neurologic disorders. The last confirmed case was on November 3, 2000. All but three cases were located in a region nicknamed "la petite Camargue," which has several large marshes, numerous colonies of migratory and resident birds, and large mosquito populations. No human case has been confirmed among clinically suspected patients, nor have abnormal deaths of birds been reported. A serosurvey has been undertaken in horses in the infected area, and other studies are in progress.

West Nile (WN) fever is a mosquito-borne flaviviral infection transmitted in natural cycles between birds and mosquitoes, particularly *Culex* species. In humans, WN infection is usually an asymptomatic or mild febrile illness; however, encephalitis cases are reported with some fatalities in older patients. WN virus is also a cause of animal disease, especially in horses.

WN virus was discovered in 1937 in the blood of a woman in the West Nile Province of Uganda who had a mild febrile illness (1). Since then, both sporadic cases and major outbreaks of WN fever in humans and equines have been reported in Africa, the Middle East, Europe, and Asia (2), and many aspects of WN infection have been well documented elsewhere since the early 1950s (3-7). During the last 5 years, many reports about WN virus have been published (8-17).

In France, the first reported outbreak occurred during the summer of 1962 in the Camargue region (Bouches-du-Rhône Province). At that time, several horses had neurologic disorders. As many of these horses were living wild, the exact number of animals with clinical symptoms was not known. However, among domestic horses for which information was available, 50 cases with neurologic signs, 25% to 30% of them fatal, were reported during the summer of 1962, with a peak between August 15 and September 15. The disease was mainly characterized by ataxia, weakness, and amaurosis (6). Several human cases of encephalitis were also reported during the same period in Camargue and Languedoc (Herault Province). However, no precise data were available for these patients except for one who was hospitalized with fever and meningitis and who had antibodies against group B arboviruses (18). WN virus infection could not be confirmed until 1964, when the virus was isolated in September from *Culex modestus* mosquitoes and the blood of two entomologists working in the field (19). Subsequently, 13 human patients, recorded from September 1962 to September 1964, were confirmed by hemagglutination-inhibition and neutralization tests to have infection compatible with WN virus (5), including one fatal case (September 1962). In 1963 and 1964, a serosurvey was conducted in 47 horses located in Camargue, including 10 animals who had neurologic signs in 1962. Neutralizing antibodies against WN virus were detected in 6 of 37 animals without clinical symptoms and 6 of 10 with previous disease (6). In 1965, WN virus infection was confirmed in three horses with neurologic signs, including one fatal case from which virus was isolated from the spinal cord. The same year, virus also was isolated from *Cx. modestus* mosquitoes (20).

After 1965, there was no evidence of WN virus infections in humans or horses. During a serosurvey (hemagglutination-inhibition assay) conducted in Camargue from 1975 to 1979, a low frequency of antibody response against WN virus was observed in 235 human samples (4.9%) and 99 horse samples (2%) (21). In contrast, a high frequency was observed against Tahyna virus (31% in humans and 9% in horses), a Bunyavirus belonging to the California group that induces febrile illness with central nervous system signs and has been reported in many countries in Europe as well as in Africa and Asia (22).

Materials and Methods

The Outbreak

On September 6, 2000, WN immunoglobulin M (IgM)-capture enzyme-linked immunosorbent assay (MAC-ELISA) and indirect IgG ELISA results were positive for two samples

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from horses. These horses were located in the same village in the south of France (Lansargues, Hérault), approximately 10 km from Montpellier (Figure 1). On August 24 and 28, they had signs of acute neurologic disorders, characterized by high fever and paresis of the hindquarters, then paralysis of the hind legs and inability to get up. The horses were euthanized on August 30 and September 1, respectively. Retrospectively, the same veterinary practitioner reported the case of a horse in the same village, which had clinical symptoms compatible with WN virus infection on August 3 and died 9 days later. WN infection was confirmed on September 8 by detection of WN viral RNA in a brain biopsy of one horse sampled for rabies diagnosis.

An alert was launched by both the ministry of health and the ministry of agriculture. Mosquito larvicide, targeted at *Cx. modestus* mosquitoes, was applied on September 9 to an area of about 200 ha near the confirmed cases. Restricted movement measures imposed by the Commission of the European Communities, were applied to equines within a 25-km radius area around a holding on which WN fever was confirmed in equines during the previous 30 days. These equidae were held for 21 days in isolation quarantine, after which MAC-ELISA was performed by a method derived from Zeller et al. (23). Briefly, IgM antibodies were captured with a goat anti-horse mu-chain antibody (Sigma Chemical Co., St. Louis, MO). WN antigen, prepared on Vero E6 cells and inactivated by beta-propiolactone, was added. Specific binding was demonstrated by using a WN mouse immune ascitic fluid and a peroxydase-labeled anti-mouse antibody. IgG antibodies in sera were detected by a method derived from Tsai et al. (11). Plates were coated with WN antigen, and IgG antibodies were revealed by a peroxydase-labeled anti-horse IgG antibody (Biosis, Compiègne, France). Sera were considered positive if the optical density was >3 standard deviations above the mean of negatives.

Figure 1. Geographic location of horses with laboratory-confirmed West Nile virus infection, France.*
*Open circle indicates location of confirmed cases.

Results

As of November 30, we had received samples from 129 horses and 2 donkeys clinically suspected of having WN virus infection by veterinary practitioners (neurologic signs such as ataxia, paresis, or paralysis, with or without fever >38.5°C). A confirmed case was defined as illness in an equine with clinical suspicion of WN virus infection and a positive WN virus IgM antibody test result; a probable case had a negative WN virus IgM test result and a positive IgG antibody test result. A total of 58 equines were defined as having confirmed cases (57 horses and 1 donkey) and 18 horses as probable cases. Twenty (34%) of the animals with confirmed cases and one (6%) of the probable cases died. Eight of the 58 confirmed cases had a negative IgG antibody test result; 4 of these 8 died. Of the probable cases, two had samples obtained 15 and 23 days after illness onset; the rest had samples obtained during the acute phase of illness. The clinical symptoms of the confirmed and probable cases were similar, as was the age distribution of the animals (mean 12.5 ± 5.3 vs. 12.0 ± 6.6 years for confirmed and probable cases, respectively).

Most positive samples (confirmed and probable) were reported in September (82.9%). The last case was reported on November 3. The clinical symptoms included mainly fever (>38.5°C), ataxia, paresis, and paralysis (Table).

Ages of confirmed and probable cases ranged from 3 to 30 years (mean 12 years, median 10 years). There were 4 stallions, 20 mares, and 49 geldings (no information for 3 horses). Most fatal cases (57.1%) were recorded before September 15 (Figure 2); among fatal cases, 18 (86%) were euthanized, including one donkey that had neurologic signs

Table. Clinical features of disease in 76 horses with confirmed or probable West Nile virus infection

Clinical signs	No. of horses (%)
Fever (>38.5°C)	47 (62%)
Ataxia	55 (72%)
Paresis/paralysis	36 (47%)
Tremor	7 (9%)
Hyperesthesia	6 (8%)
Grinding teeth	3 (4%)
Abnormal behavior	2 (3%)
Hepatitis	1

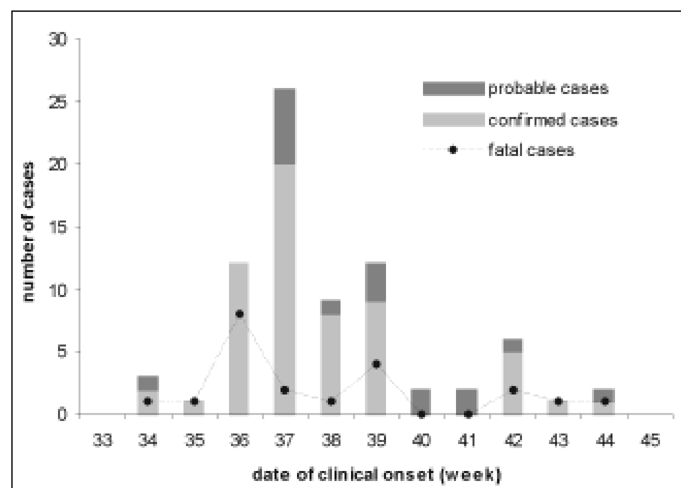

Figure 2. West Nile confirmed, probable, and fatal equine cases, by week of clinical onset, France.

followed by a short period of remission and then severe hepatic failure. Ages were not known for 4 of the 21 fatal cases. Of the 17 horses for which information was available, 41.2% and 29.6% were in the 6- to 10- and 16- to 20-year age categories, respectively (Figure 3).

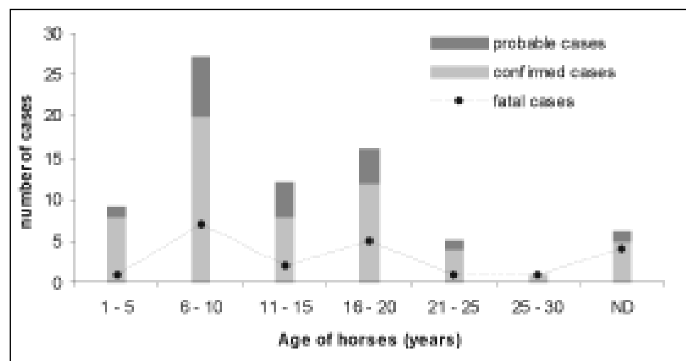

Figure 3. Age of horses with confirmed, probable, and fatal West Nile virus infection, France.*
*ND = not determined.

All but three confirmed and probable cases were located in an area within a radius of 15 km, in a region in Hérault and Gard provinces called "la petite Camargue." Thirty-one (40.8%) of the horses were located within a 5-km radius of the first two reported cases (Lansargues). Three cases, all fatal, were located near this area, in Bouches du Rhone Province approximately 30 km from the first reported cases and 15 km outside the area where confirmed cases were reported. These animals, according to the owners, had not moved from this area during the 3 weeks preceding the onset of symptoms. However, because of the economic consequences of the restricted movement measures imposed by the Commission of European Communities, we assume some owners may not have observed the restrictions.

We also received 33 samples from other animals, some of them with neurologic signs, in the infected area during the outbreak: 16 cows, 8 goats, and 9 others (e.g., camel, dog, zebra). WN ELISA results were negative for all of them.

No human suspected of having WN infection has been laboratory-confirmed among 51 persons tested, including 33 hospitalized with signs of encephalitis or meningoencephalitis and 18 others with fever or living in close contact with horses. All these samples were obtained from persons living or traveling in the infected area during the outbreak. In contrast, WN IgG antibodies were detected in 3 of 33 gamekeepers working in this area. Two had WN neutralizing antibodies: one had no WN IgM antibodies; the other (who had no history of travel during recent years) had low but detectable IgM antibodies.

Virus Isolation and Molecular Characterization of Virus Isolates

WN virus was isolated after one passage into C6/36 and Vero E6 cells from the rachidian bulb of the first confirmed case and from cerebellum, cortex, and lumbar spinal cord of another horse that died on September 6. Viral RNA was extracted from culture supernatants and a reverse transcription-polymerase chain reaction (RT-PCR) was performed with primers located in the envelope gene fragments WN240 and WN132, as described (24). Nucleic acid sequences were obtained on an automated Applied Biosystems sequencer (PPE Biosystems, Foster City, CA). WN virus sequences were aligned by using the multiple sequence alignment software CLUSTAL.

Phylogenetic analysis of an informative region of the E glycoprotein gene (Figure 4), using tree-view, showed that the WN France-2000 isolate belonged to lineage 1 and was closely related to both horse Morocco-1996 and Italy-1998 isolates. It is also closely related to mosquito isolates from Senegal-1993, Kenya-1998, and Romania 1996, as well as to the recent human isolate from Volgograd-1999. It is distinguishable from the group including both the New York-1999 and Israel-1998 isolates, as well as a WN virus recently isolated in our laboratory from the brain of a human fatal case that occurred during an outbreak in the governorates of Mahdia and Sfax on the Tunisian coast in 1997 (H. Triki, unpub. data).

General Survey in Horses

To determine the number of infected horses and thus the number of asymptomatic infections, a serosurvey study has been undertaken, which includes all horses located within a 10-km radius of confirmed cases. A total of 5,133 sera were collected from September to November 2000 from the three provinces where cases were reported (Herault, Gard, and Bouches du Rhone). Preliminary results showed 428 (8.3%) horses with IgG antibodies; 248 had IgM antibodies. Analysis of these data is in progress, especially to determine rates of seropositivity for each commune. (A commune is the smallest French administrative subdivision, which approximately corresponds to an English parish).

The geographic locations of the seropositive horses were compared with those of the clinically confirmed and probable cases (Figure 5).

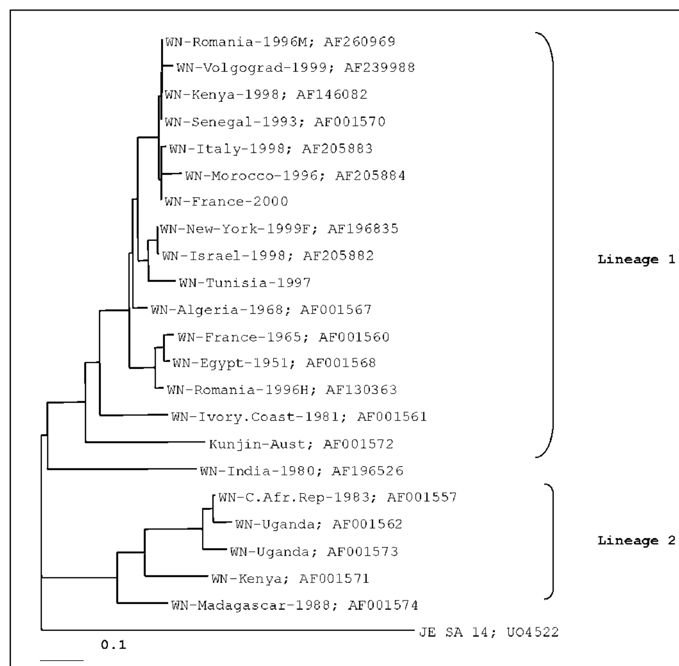

Figure 4. Phylogentic trees based on nucleic sequence data of E-glycoprotein gene fragments of 254 bp.*
*GenBank accession numbers for the sequences included in the tree are indicated.

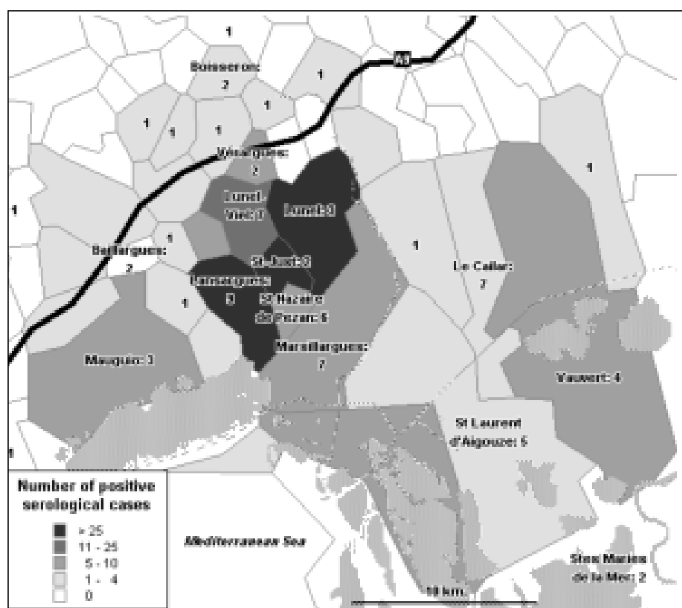

Figure 5. Geographic location of confirmed and probable clinical cases and serologically positive cases (according to serosurvey) of West Nile infection in equines, France. Data are grouped by commune, the boundaries of which are indicated. (The commune is the smallest French administrative subdivision, which approximately corresponds to an English parish). Numbers indicate clinical cases. Of the 76 cases, 73 are shown; the rest occurred more than 10 km outside the area. Names of communes in which more than one clinical case occurred are indicated. For each commune, the color (see key) indicates the number of positive serologic cases. The first cases were reported in Lansargues.

Discussion

In this report we described the 2000 WN outbreak in horses in southern France. Only a few reports of WN virus encephalomyelitis cases in equines have been published. In Egypt, a high prevalence of WN antibodies (54%) was observed during a serologic survey conducted from January to May 1959 in 436 equines (horses, donkeys, and mules). One suspected case was fatal and confirmed by viral isolation from the brain (25). In France during the 1962 WN outbreak in Camargue, several horses were suspected to be infected (6). In Morocco (provinces of Kenitra and Larache), 94 equines were affected from August to mid-October 1996; 42 died. The disease was reported in all age categories (9), and virus was isolated from a brain biopsy sample (10). In Italy, from August to beginning October 1998, 14 horses in Tuscany had laboratory-confirmed WN virus infection, and 6 animals died (12). Virus was isolated from a brain biopsy sample (V. Deubel, unpub. data). In Israel in 1998, 18 serum samples from horses with encephalomyelitis had WN-neutralizing antibodies, and virus was isolated from the brain of a stork (26). In 1999, thousands of geese were destroyed when WN virus was identified in commercial flocks (27). In the northeastern United States, 20 horses were infected by WN virus in 1999; 9 died (28). In 2000, 63 equine cases, approximately 35% fatal, were confirmed. The first case was identified in mid-August 2000 (29).

In France, the outbreak started in August 2000 and ended in November. Most positive samples (80%) were obtained before September 30. The death rate during this outbreak (28%) was lower than observed in Morocco (45%), Italy (43%), and North America (45% and 35%). Most fatal cases (57.1%) occurred at the beginning of the outbreak, before September 15. At that time, most veterinary practitioners thought that the disease could not be cured and did not apply symptomatic treatment.

Horses, as well as humans, are generally considered to be dead-end hosts of WN infection; however, little is known about the duration and magnitude of viremia. Experimental infections in horses and donkeys in Egypt (25) and in France (30,31) showed undetectable or low viremia of short duration. However, these experiments were conducted in different conditions with different WN strains; therefore, it is difficult to extrapolate from these results.

During the present outbreak, we were unable to detect WN virus (by RT-PCR and inoculation into cell culture) in the blood of a few animals tested, including animals with virus identified in the brain. In contrast, using intracerebral inoculation into mice, virus was isolated from blood samples during previous epidemics in Egypt, Israel, South Africa, and France.

During the French outbreak, WN IgM antibodies were not detected in 18 IgG-positive animals with neurologic signs from which blood samples were obtained. In the absence of a second blood sample and in the context of the outbreak, we concluded that these animals had a recent WN virus infection. This conclusion was supported by previous serosurveys conducted in 1999 and 2000 that demonstrated absence of *Flavivirus* antibodies in horses (unpub. data). These data suggest that in some cases IgM response was very low or nondetectable by commonly used techniques. This may have implications for the diagnosis of recent infections.

The outbreak appeared limited to a restricted area within a 15-km radius. This region, near Camargue National Park, where the 1962 outbreak occurred, is characterized by its original flora (wetlands, rice fields, garriguea [a geographical dry area, typical of the mediterranean basin]) and fauna (more than 300 migratory and resident bird species and large populations of mosquitoes). No abnormal deaths of birds were reported. An epidemiologic investigation of WN virus in birds, including five common nesting species, is in progress. The vector(s) involved in the present outbreak is (are) still unknown.

Thousands of horses live in this region. Thus, our large serosurvey should allow us to precisely determine the geographic distribution of the infection and the number of asymptomatic infections in the geographic area where the outbreak occurred.

Two gamekeepers had WN-neutralizing antibodies, and one of them also had IgM antibodies. Thus, human transmission occurred during this outbreak. However, in the absence of a serologic survey, evaluating the level of human infection among persons living in the infected area is not possible.

The main concern for 2001 is the possibility of persistence of virus transmission and thus the risk of human infections. Natural vertical transmission of WN virus in *Culex* mosquitoes (32) or survival in overwintering mosquitoes (3,33) could explain the persistence of the virus. However, human transmission likely depends on several factors, including environmental factors, vectors, and amplifying host conditions. Usually in most countries where WN outbreaks have been documented, a few cases are reported during subsequent years (34).

Several questions about WN virus infection are still unresolved, among them whether WN infection is a major

health problem for humans and horses. The main problem during this outbreak was not the disease itself but the economic consequences from the restricted movement measures imposed by the Commission of the European Communities (cancellation of horse exhibitions, layoffs of employees in equestrian centers). Gaining more knowledge about the role of horses in virus transmission under natural conditions is important.

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References

1. Smithburn KC, Hughes TP, Burke AW, Paul JH. A neurotropic virus isolated from the blood of a native of Uganda. Am J Trop Med 1940;20:471-92.
2. Zeller H. West Nile: une arbovirose migrante d'actualité. Med Trop 1999;59:490-4.
3. Taylor RM, Work TH, Hurlbut HS, Rizk F. A study of the ecology of West Nile virus in Egypt. Am J Trop Med 1956;5:579-620.
4. Bernkopf H, Levine S, Nerson R. Isolation of West Nile virus in Israel. J Infect Dis 1953;93:207-18.
5. Panthier R, Hannoun C, Beytout D, Mouchet J. Epidémiologie du virus West Nile: étude d'un foyer en Camargue. III. Les maladies humaines. Annales de l'Institut Pasteur 1968;115:435-45.
6. Joubert L, Oudar J, Hannoun C, Beytout D, Corniou B, Guillon et al. Epidémiologie du virus West Nile: étude d'un foyer en Camargue. IV. La méningo-encéphalomyélite du cheval. Annales de l'Institut Pasteur 1970;118:239-47.
7. McIntosh BM, Jupp PG, Dos Santos, Meenehan GM. Epidemics of West Nile and Sindbis viruses in South Africa with *Culex (Culex) univittatus* Theobald as vector. S Afr J Med Sci 1976;72:295-300.
8. Le Guenno B, Bougermouth A, Azzam T, Bouakaz R West Nile: a deadly virus? Lancet 1996;348:1315.
9. Tber Abdelhaq A. West Nile fever in horses in Morocco. Bulletin de l'Office International des Epizooties 1996;11:867-9.
10. El Harrack M, Le Guenno B, Gounon P. Isolement du virus West Nile au Maroc. Virologie 1997;1:248-9.
11. Tsai TF, Popovici F, Cernescu C, Campbell GL, Nedelcu NI. West Nile encephalitis in southeastern Romania. Lancet 1998;352:767-71.
12. Cantile C, Di Guardo G, Eleni C, Arispici M. Clinical and neuropathological features of West Nile virus equine encephalomyelitis in Italy. Equine Vet J 2000;32:31-5.
13. Platonov AE, Shipulin GA, Shipulina AY, Tyutyunnik EN, Frolochkina TI, Lanciotti RS, et al. Outbreak of West Nile virus infection, Volgograd region, Russia, 1999. Emerg Infect Dis 2001;7:128-32.
14. Siegel-Itzkovich J. Twelve die of West Nile virus in Israel. BMJ 2000;321:724.
15. Rappole JH, Derrickson SR, Hubalek Z. Migratory birds and spread of West Nile virus in the Western Hemisphere. Emerg Infect Dis 2000;6:319-27.
16. Novello AC. West Nile virus in New York State: the 1999 outbreak and response plan for 2000. Viral Immunol 2000;4:463-7.
17. Hubalek Z, Halouzka J, Juricova Z. West Nile fever--a reemerging mosquito-borne viral disease in Europe. Emerg Infect Dis 1999;5:594-5.
18. Panthier R. Epidémiologie du virus West Nile: étude d'un foyer en Camargue. I. Introduction. Annales de l'Institute Pasteur 1968;114:519-20.
19. Hannoun C, Panthier R, Mouchet J, Eouzan JP. Isolement en France du virus West Nile à partir de malades et du vecteur Culex modestus Ficalbi. C R Acad Sci 1964;259:4170-2.
20. Panthier R, Hannoun C, Oudar J, Beytout D, Corniou B, Joubert L, et al. Isolement du virus West Nile chez un cheval de Camargue atteint d'encéphalomyélite. C R Acad Sci 1966;262:1308-10.
21. Rollin PE, Rollin D, Martin P, Baylet R, Rodhain F, Hannoun C. Résultats d'enquêtes séroépidémiologiques récentes sur les arboviroses en Camargue: populations humaines, equines, bovines et aviaries. Medecine et Maladies Infectieuses 1982;12:77-80.
22. Karabatsos N, editor. International catalogue of arboviruses, including certain other viruses of vertebrates. 3rd ed., and Supplements 1986-98. San Antonio: American Society of Tropical Medicine and Hygiene; 1985. p. 977.
23. Zeller HG, Fontenille D, Traore-Lamizana M, Thiongane Y, Digoutte JP. Enzootic activity of Rift valley Fever virus in Senegal. Am J Trop Med Hyg 1997;56:265-72.
24. Berthet FX, Zeller HG, Drouet MT, Rauzier J, Digoutte JP, Deubel V. Extensive nucleotide changes and deletions within the envelope glycoprotein gene of Euro-African West Nile viruses. J Gen Virol 1997;78:2293-7.
25. Schmidt JR, El Mansoury HK. Natural and experimental infection of Egyptian equine with West Nile virus. Ann Trop Med Parasitol 1963;57:415-27.
26. Malkinson M, Banet C, Weisman Y. Intercontinental spread of West Nile virus by wild birds--recent epidemiological findings in Israeli livestock and birds. In: Proceedings of the 2nd International Conference on Emerging Zoonoses, Strasbourg, France, 1998.
27. Office International des Epizooties. West Nile fever in Israel in geese. Disease Information 99;12:166.
28. Office International des Epizooties. West Nile fever in the United States of America: in horses. Disease Information 1999;12:150-1.
29. Centers for Disease Control and Prevention. Update: West Nile virus activity--eastern United States, 2000. MMWR Morb Mortal Wkly Rep 2000;49:1044-7.
30. Oudar J, Joubert L, Lapras M, Guillon JC. Reproduction expérimentale de la méningo-encéphalomyélite du cheval par l'arbovirus West Nile. II. Etude anatomo-clinique. Bull Acad Vét 1971;Tome XLIV:147-58.
31. Joubert L, Oudar J, Hannoun C, Chippaux M. Reproduction expérimentale de la méningo-encéphalomyélite du cheval par l'arbovirus West Nile. III. Relations entre la virologie, la sérologie et l'évolution anatomo-clinique. Conséquences épidémiologiques et prophylactiques. Bull Acad Vét 1971;Tome XLIV:159-67.
32. Miller BR, Nasci RS, Godsey MS, Savage HM, Lutwama JJ, Lanciotti RS, et al. First field evidence for natural vertical transmission of West Nile virus in *Culex Univittatus* complex mosquitoes from Rift Valley province, Kenya. Am J Trop Med Hyg 2000;62:240-6.
33. Centers for Disease Control and Prevention. Update: surveillance for West Nile virus in overwintering mosquitoes--New York. MMWR Morb Mortal Wkly Rep 2000;49:178-9.
34. Cernescu C, Nedelcu N, Tardei G, Ruta S, Tsai TF. Continued transmission of West Nile virus to humans in southeastern Romania, 1997-1998. J Infect Dis 2000;181:710-2.

The Relationships between West Nile and Kunjin Viruses

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Until recently, West Nile (WN) and Kunjin (KUN) viruses were classified as distinct types in the *Flavivirus* genus. However, genetic and antigenic studies on isolates of these two viruses indicate that the relationship between them is more complex. To better define this relationship, we performed sequence analyses on 32 isolates of KUN virus and 28 isolates of WN virus from different geographic areas, including a WN isolate from the recent outbreak in New York. Sequence comparisons showed that the KUN virus isolates from Australia were tightly grouped but that the WN virus isolates exhibited substantial divergence and could be differentiated into four distinct groups. KUN virus isolates from Australia were antigenically homologous and distinct from the WN isolates and a Malaysian KUN virus. Our results suggest that KUN and WN viruses comprise a group of closely related viruses that can be differentiated into subgroups on the basis of genetic and antigenic analyses.

Kunjin (KUN) and West Nile (WN) viruses belong to the Japanese encephalitis (JE) antigenic complex of the *Flavivirus* genus in the family Flaviviridae (1). The *Flavivirus* genus comprises >70 antigenically related, positive-stranded RNA viruses (2,3). KUN and WN viruses are maintained in a natural transmission cycle involving mosquito vectors and bird reservoir hosts, with humans and horses believed to be incidental hosts (4,5). Clinical symptoms most commonly associated with infection with KUN and WN viruses include febrile illness or mild encephalitis. WN virus has been associated with fatal cases of acute meningoencephalitis and fulminant hepatitis (6).

Early cross-neutralization studies with polyclonal antisera raised to single strains of WN and KUN viruses revealed that these viruses shared a close relationship but were antigenically distinct (7-9). This close relationship was also shown genetically by Coia et al. (10), who compared the sequence of the MRM61C KUN isolate with that of a Ugandan strain of WN (WNFCG) (11-13) and showed that the nucleotide and amino acid sequence identity between the two viruses was 82% and 93%, respectively, in the coding region of the genome. Although genetic studies have shown that KUN virus exists in Australia as a single topotype with <2% nucleotide divergence (14,15), Berthet et al. (16) demonstrated that WN viruses were divided into two lineages. Although these comparisons demonstrated a close relationship between the two viruses, further sequence information is needed from additional isolates of both viruses to fully establish their phylogenetic association within the genus. This report describes the results of sequence analyses of 31 Australian KUN isolates; a KUN isolate from Sarawak, Malaysia; and 28 WN isolates from Africa, India, Europe, and New York (Tables 1 and 2). These virus isolates had all been identified as WN or KUN virus by traditional antigenic means. The Koutango (KOU) isolate was also included in this study, as it belongs to the JE serogroup and is closely related to the KUN/WN group of viruses (9,17).

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Materials and Methods

Virus and Cell Culture

Virus strains sequenced in this study are listed with their sources of isolation in Table 1. African green monkey (Vero) cells were grown at 37°C in M199 (Gibco, New York) with 20 mM HEPES (Gibco) and supplemented with 2% L-glutamine and either 10% fetal bovine serum (FBS) for growth or 2% FBS for maintenance. Viruses were cultured in Vero cells by inoculating cell monolayers with virus at a multiplicity of infection of 1. Infected cell culture supernatants were harvested when ≥70% of the cells exhibited cytopathic effect. Infected supernatant was clarified by centrifugation at 2000 x *g* at 4°C for 15 min, and aliquots were stored at -70°C. A line of *Aedes albopictus* (C6/36) cells was cultured in M199 without HEPES and supplemented with FBS for growth or maintenance, as described. The cells were incubated at 28°C in a humidified atmosphere with 5% CO_2.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Sequencing

A single-step RT-PCR procedure (22) was performed on each virus isolate. The region amplified within the envelope

Table 1. West Nile and Kunjin virus isolates and nucleotide sequences used in this study

Virus ID	Year of isolation	Source of isolation	Place of isolation	GenBank Accession Number
KUN35911	1984	Horse brain	Hunter Valley, NSW,[a] AU	AF196511 (E gene)
KUNP1553[b]	1994	*Culex* sp.	Marble Bar, WA, AU	AF297856 (NS5/3'UTR) AF196495 (E gene)
KUNCH16465C	1974	*Cx. ann.*	CH, Qld, AU	AF297841 (NS5/3'UTR) AF196504 (E gene)
KUNCH16514C	1974	*Cx. ann.*	CH	AF297842 (NS5/3'UTR) AF196501 (E gene)
KUNCH16532C	1974	*Cx. ann.*	CH	AF297843 (NS5/3'UTR) AF196513 (E gene)
KUNCH16549E	1974	*Cx. ann.*	CH	AF297844 (NS5/3'UTR) AF196520 (E gene)
KUNM695	1982	*Cx. ann.*	Victoria, AU	AF297852 (NS5/3'UTR) AF196496 (E gene)
KUNM1465	1983	*Cx. ann.*	Victoria, AU	AF297851 (NS5/3'UTR) AF196522 (E gene)
KUNMRM5373	1966	*Oriolus flavocintus* (bird)	MRM, Qld, AU	AF297859 (NS5/3'UTR) AF196509 (E gene)
KUNMRM16	1960	*Cx. ann.*	MRM	AF196505 (E gene)
KUNMRM61C	1960	*Cx. ann.*	MRM	AF196516 (E gene)
KUNOR130	1973	*Cx. ann.*	OR, East Kimberley, WA, AU	AF297857 (NS5/3'UTR) AF196492 (E gene)
KUNOR134	1973	*Cx. ann.*	OR	AF196506 (E gene)
KUNOR166	1973	*Cx. ann.*	OR	AF196499 (E gene)
KUNOR205	1973	*Aedes tremulus*	OR	AF297858 (NS5/3'UTR) AF196515 (E gene)
KUNOR354	1974	*Cx. ann.*	OR	AF297855 (NS5/3'UTR) AF196518 (E gene)
KUNOR393	1974	*Cx. ann.*	OR	AF196503 (E gene)
KUNOR4	1972	*Cx. ann.*	OR	AF196523 (E gene)
KUNCX255	1982	*Cx. ann.*	Wyndham, East Kimberley	AF297845 (NS5/3'UTR) AF196514 (E gene)
KUNCX238	1982	*Cx. ann.*	Wyndham, East Kimberley	AF196502 (E gene)
KUNBoort	1984	Horse spinal cord	Boort, Victoria, AU	AF297840 (NS5/3'UTR) AF196519 (E gene)
KUNFC15	1986	*Cx. ann.*	West Kimberley, WA, AU	AF297846 (NS5/3'UTR) AF196510 (E gene)
KUNHu6774	1991	Human	Southern NSW, AU	AF297847 (NS5/3'UTR) AF196493 (E gene)
KUNK6547	1991	*Cx. ann.*	SE Kimberley, WA, AU	AF196521 (E gene)
KUNK1738	1989	*Cx. ann.*	OR	AF297848 (NS5/3'UTR) AF196494 (E gene)
KUNK5374	1989	*Cx. ann.*	SE Kimberley, WA, AU	AF297849 (NS5/3'UTR) AF196517 (E gene)
KUNK2499	1984	*Cx. ann.*	OR	AF196498 (E gene)
KUNK6590	1991	*Cx. ann.*	Broome, West Kimberley, WA, AU	AF297850 (NS5/3'UTR) AF196500 (E gene)
KUNSH183	1991	Chicken	Victoria, AU	AF297853 (NS5/3'UTR) AF196491 (E gene)
KUNWK436	1979	*Cx. ann.*	Camballin, West Kimberley, WA, AU	AF297854 (NS5/3'UTR) AF196507 (E gene)
KUNV407	1983	*Cx. ann.*	Jabiru, NT, AU	AF196508 (E gene)
KUNMP502-66	1966	*Cx. pseudovishnui*	Sarawak, Borneo, Malaysia	AF196534 (E gene)
HB6343	1989	Human	CAR	AF196542 (NS5/3'UTR) AF196528 (E gene)
ArTB3573	1982	Tick	CAR	AF196541 (NS5/3'UTR) AF196527 (E gene)
MgAn798	1978	*Coracopsis vasa* (bird)	Madagascar	AF196543 (NS5/3'UTR)
63134Ent 280	<1963	Human	Uganda	AF196539 (NS5/3'UTR) AF196530 (E gene)
ArA1Dj	1968	Mosquito	Algeria	AF196536 (NS5/3'UTR) AF196529 (E gene)
ArNa1047	unknown	Mosquito	Kenya	AF196535 (NS5/3'UTR)
G2266	1955	*Cx. vishnui*	Sathuperi, India	AF196537 (NS5/3'UTR) AF196525 (E gene)
G22886	1958	*Cx. vishnui*	Sathuperi, India	AF196538 (NS5/3'UTR) AF196524 (E gene)
804994	1980	Human brain biopsy	Bangalore Field Station, Karnataka, India	AF196540 (NS5/3'UTR) AF196526 (E gene)
Sarafend	unknown	unknown	unknown	AF196533 (E gene)
KOU DakAad 5443	1968	*Tatera kempi* (rodent)	Senegal, Africa	AF196532 (E gene)

[a]NSW = New South Wales; AU = Australia; WA = Western Australia; *Cx. ann.* = *Culex annulirostris;* CH = Charleville; Qld = Queensland; MRM = Mitchell River Mission; OR = Ord River; NT = Northern Territory; CAR = Central African Republic; UTR = untranslated region.
[b]P1553 was isolated from a culture of C6/36 cells inoculated with culture fluid derived from a mosquito pool from which Edge Hill (EH) virus had also been isolated (Annette Broom, pers. comm.).

Table 2. Additional West Nile and Kunjin virus sequences included in this study

Virus ID	Year of isolation	Source of isolation	Place of isolation	GenBank Accession Number	Region of genome	Reference
KUNMP502-66	1966	*Culex pseudovishnui*	Sarawak	L49311	NS5/3'UTR	17
NY99	1999	*Phoenicopterus chilensis* (Chilean flamingo)	NYC[a]	AF196835	E	18
NY99	1999	Human	NYC	AF202541	NS5/3'UTR	21
ISR98	1998	Goose	Israel	AF205882	E	V. Deubel,unpub. data
Rom96	1996	Human	Romania	AF130363	E	19
Rom97-50	1997	Unknown	Romania	AF130362	E	20
ArB310	1967	*Culex* sp.	CAR	AF001566	E	16
Mor96	1996	Unknown	Morocco	AF205884	E	V. Deubel,unpub. data
Italy98	1998	Unknown	Italy	AF205883	E	V. Deubel,unpub. data
ArD93548	1993	*Cx. neavei*	Senegal	AF001570	E	16
AnD27875	1979	*Galago senegalensis*	Senegal	AF001569	E	16
PaH651	1965	Human	France	AF001560	E	16
AnMg798	1978	*Coracopsis vasa* (bird)	Madagascar	AF001559	E	16
ArMg978	1988	*Cx. univittatus*	Madagascar	AF001574	E	16
MP22	unknown	unknown	Uganda	AF001562	E	16
UGA-B956	unknown	unknown	Uganda	AF208017	NS5	21
ArD78016	1990	*Aedes vexans*	Senegal	AF001556	E	16
HB83P55	1983	Human	CAR	AF001557	E	16
Eg101	1951	Human	Egypt	AF001568	E	16
Eg101	1951	Human	Egypt	AF260968	NS5	Bowen et al., unpub. data
ArA3212	1981	*Cx. guiarti*	Ivory Coast	AF001561	E	16
KUNMRM16	1960	*Cx. ann.*	MRM	L48979	NS5/3'UTR	17
KUNMRM61C	1960	*Cx. ann.*	MRM	L48978	NS5/3'UTR	17
Sarafend	unknown	unknown	unknown	L48977	NS5/3'UTR	17
KOUDakAad 5443	1968	*Tatera kempi* (rodent)	Senegal	L48980	NS5/3'UTR	17
WNFCG	1937	Human	Uganda	M12294	E and NS5/3'UTR	11

[a]NYC = New York City; *Cx. ann.* = *Culex annulirostris;* CAR = Central African Republic; MRM = Mitchell River Mission; UTR = untranslated region.

(E) gene used the primers KUN5276 (GCG TGT GGT TCT TCA AAC TCC A) and WN4752 (TGC GTG TCC AAC CAT GGG TGA AGC) with the isolates Sarafend, MP502-66, and a strain of KOU virus, DakAad 5443. Primer KUN5276 was used with primer KUN4778 (ATA ATG ACA AGC GGG CTG ACC C) for the remaining isolates. The region of the virus genome encompassing the terminus of the nonstructural protein, NS5 and the 5' end of the 3' untranslated region (3'UTR), was amplified by using the previously published universal flavivirus PCR primers EMF1 and VD8 (23).

Both strands of the PCR product were then sequenced on a 377 automated sequencer (Applied Biosystems International [ABI], Foster City, CA, USA) by using the same primer pair. The two sequences derived from each PCR product were initially aligned by using the program SeqEd (ABI) and a consensus sequence determined. The consensus sequences were then aligned by using the program Clustal W (24), and results were further analyzed by using phylogenetic programs in Bionavigator (http://www.bionavigator.com). Percentage nucleotide similarity was calculated by the Old Distance (GCG) program, and bootstrap confidence levels were calculated with 1,000 replicates by using the Consense program (25). Sequences determined in this study have been deposited in GenBank (National Institutes of Health, Bethesda, MD, USA) (Table 1). Additional sequences included in this analysis are listed in Table 2.

Enzyme-Linked Immunosorbent Assay (ELISA)

Antigenic profiles of each isolate were compared by using a panel of anti-KUN monoclonal antibodies (MAbs) (26,27) and anti-WN MAbs (28,29) in ELISA as described (26). All MAbs were produced to the E protein except for 3.1112G, which was specific for the NS1 protein.

Results

Genetic Analysis

In accordance with previous reports (16,18,21), the phylogenetic trees generated from both E gene and NS5/3'UTR sequences grouped most of the isolates into two major lineages (Figures 1 and 2). Australian KUN isolates and WN isolates from North, West, and central Africa; southern and eastern Europe; India; the Middle East; and New York constituted lineage I. Lineage II comprised WN isolates from West, central, and East Africa and Madagascar. Genetic lineage was not significantly associated with date or source of isolation, with most isolates of both lineages coming from human, mosquito, and avian sources between 1950 and 1990. However, as noted, all viruses isolated during outbreaks of human or avian disease in the last decade belonged to lineage I. Lineage I viruses grouped together with an average sequence identity of 80% (E gene) and 77% (NS5/3'UTR), while the viruses of lineage II contained a single cluster with an average identity of 82% and 83%, respectively. The lineage I viruses were further separated into three clusters: the Australian KUN isolates; the Indian WN viruses; and WN isolates from Africa, the Middle East, Europe, and North America. The divergence observed between lineage I and lineage II viruses was in the range of 16.5% to 30.8% and 19% to 36.5% for sequences of the E gene and NS5/3'UTR, respectively. High bootstrap confidence levels (100%) for the sequences of the NS5/3'UTR also support the separation of the

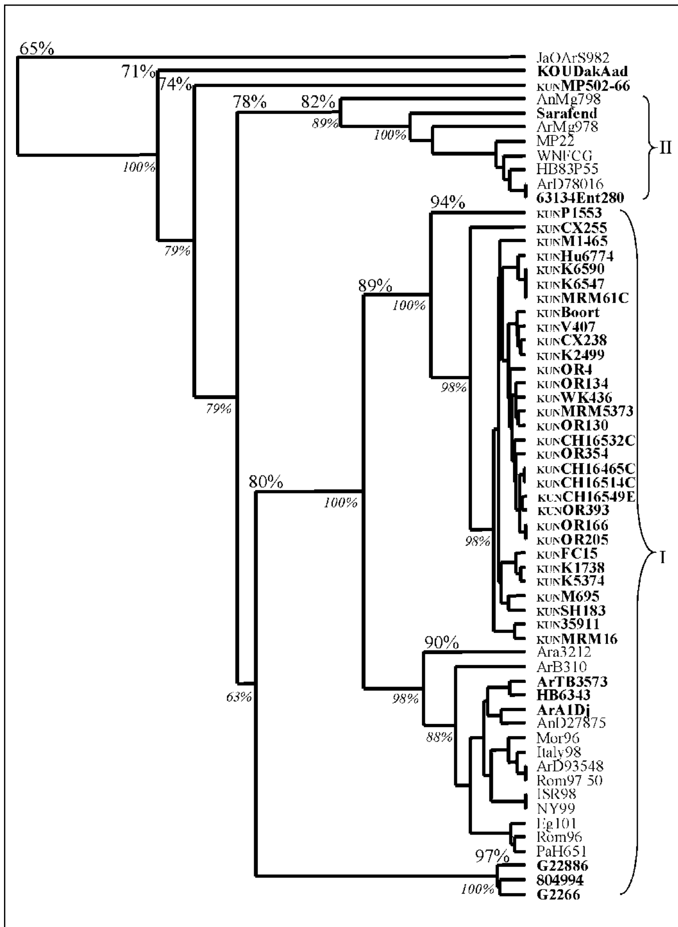

Figure 1. Phylogenetic tree constructed by the neighbor-joining algorithm based on E gene nucleic acid sequence data. Numbers above branches indicate average percentage nucleotide similarity between limbs, while the values in italics indicate the percentage bootstrap confidence levels. Isolates highlighted in bold are sequences obtained in this study. Dendrogram outgrouped with the Japanese encephalitis isolate, JaOArS982 (30; GenBank Accession Number M18370).

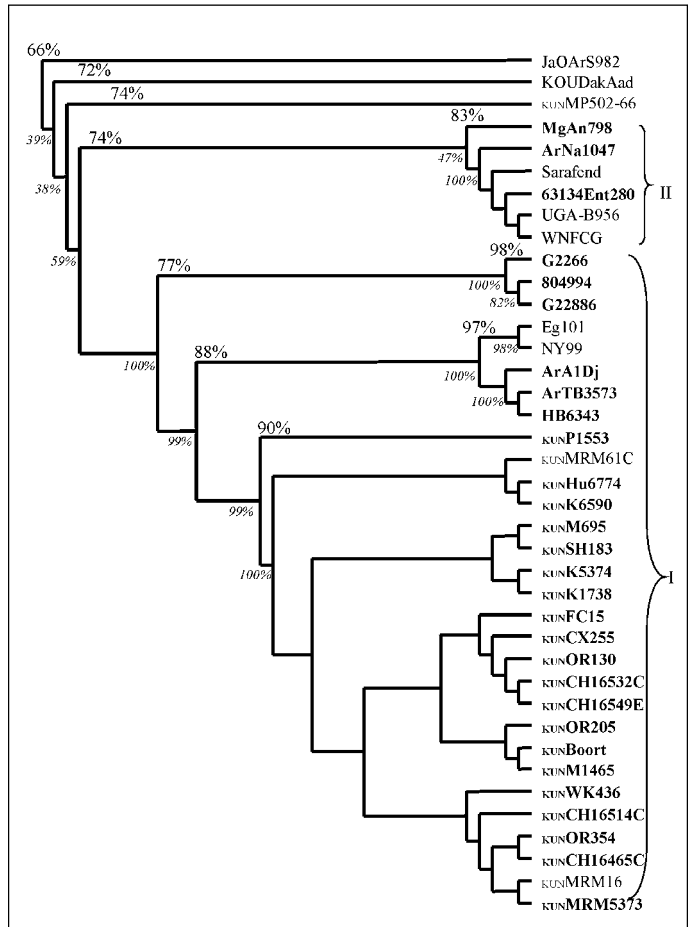

Figure 2. Phylogenetic tree constructed using the neighbor-joining algorithm based on nucleic acid sequence data encompassing the 3' end of the NS5 gene and 5' end of the 3' UTR untranslated region). Numbers above branches indicate average percentage nucleotide similarity between limbs, while the values in italics indicate the percentage bootstrap confidence levels. Isolates highlighted in bold are sequences obtained in this study. Dendrogram outgrouped with the JE isolate, JaOArS982 (30; GenBank Accession Number M18370).

two lineages and the branching of the NY99 cluster of WN viruses with the Australian KUN viruses in lineage I, rather than with the WN group of viruses in lineage II. The clustering of the Indian WN group in lineage I based on sequences in the E gene, however, was at a lower bootstrap confidence level (63%).

The sequence of the virus from Malaysia, KUN MP502-66, grouped outside the two lineages described. Similarly, the KOU virus, which was 72%-73% identical to KUN MP502-66, did not group with either lineage. The range of percentage divergence between KUN MP502-66 and KOU viruses with the lineage I and lineage II viruses (Table 3) shows that these two isolates display similar divergence from all other isolates in this study, supporting their grouping outside the two main lineages.

The viruses of lineage I group together in three tight clusters. The first of these includes the Australian KUN viruses, which were 94% identical when sequences of the E gene were compared and 90% when the sequences of the NS5/3'UTR were compared. High bootstrap confidence levels (100% for sequences from the E gene and 99% for sequences from the NS5/3'UTR) separated the Australian KUN viruses from the other isolates. However, extremely low bootstrap confidence levels were observed for most of the branches between the Australian KUN viruses in both dendrograms, which also suggests that these viruses are closely related and cannot be definitively separated from each other. The Indian viruses also cluster together, with a sequence identity of 97% and 98% for sequences of the E gene and NS5/3'UTR, respectively. The WN isolates in the remaining cluster of lineage I are 90% and 97% identical, respectively, for the regions sequenced. When compared with the Australian KUN isolates, this cluster, which includes the 1999 New York isolate, shared a sequence identity of 89% for the E gene and

Table 3. Range of percentage divergence between the Malaysian and Koutango isolates with lineage I and lineage II viruses

	E gene		NS5/3'UTR	
	Lineage I	Lineage II	Lineage I	Lineage II
MP502-66	20%-30%	20%-30%	21%-35%	21%-25%
KOU	25%-30%	29%-32%	26%-39%	22%-25%

UTR = untranslated region.

88% for the NS5/3'UTR. Similarly, when the sequences of the Australian KUN isolates were compared with those of the WN Indian viruses, they were 80% identical for the E gene and 77% identical for the NS5/3'UTR. In comparison, the two clusters of WN viruses in lineage I and the WN isolates in lineage II shared an average sequence identity of only 78% and 71% for the E gene and NS5/3'UTR, respectively. These results demonstrate that the sequences of some WN isolates are more closely related to the Australian KUN viruses than to other WN isolates.

The high degree of nucleotide sequence homology within clusters is consistent with the observed similarity of the amino acid sequences. The most notable variation in amino acid sequence in this study appears around the potential glycosylation site at amino acid 154 of the E protein (Figure 3). The Australian KUN viruses generally contain either the glycosylation motif NYS at this position or the sequence NYF, which abolishes glycosylation of the E protein. In contrast, the KUN virus SH183 has a 154N→K substitution, which also ablates the potential for glycosylation at this site. In comparison with the KUN prototype, the amino acids 159 (T→I, T→V, or T→Q) and 162 (A→T) of all the WN isolates in this study contain an amino acid substitution. The KUN isolate P1553 also differs from the KUN prototype at amino acid 159 (T→I). Two aberrant isolates, 63134Ent280 and WNFCG, incur a deletion of four amino acids (154 through 157), which also abolishes the glycosylation site.

Our results concur with those of Berthet et al. (16), who suggested the presence of signature motifs within the E gene that support the segregation of WN viruses into two lineages. These signature residues include the amino acid substitutions from lineage I→II as follows: 172A→S, 205T→S, and 210T→S. The amino acid substitution 208T→A holds true in general; however, two of the Indian isolates (lineage I) have K at this position and WNFCG (lineage II) has E. Of particular note is the substitution at amino acid 199. The Australian KUN isolates (199S) share the same amino acid as the lineage II WN viruses, while the lineage I viruses contain an N residue at this position. We have also identified an additional three signature motifs (I→II) at amino acids 128R→W, 129T→I, and 131L→Q. When we attempted to place the Malaysian KUN isolate within either lineage by using these signature motifs, the residues at 128, 129, 131, 172, and 208 were similar to those of lineage I viruses, but the residues at

```
                  128                          154
                  ↓                            ↓
MRM61C        TKATGRTILKENIKYEVAIFVHGPTTVESHGNYSTQTGAAQAGRFS
OR205         .................................F............
SH183         ...............................K..............
P1553         ....................................I.V.......
ArA1Dj        .................................P..I..T......
ArTB3573      .................................P..I..T.....I
HB6343        .................................P..I..T......
NY99          ...I................................V..T......
ISR98         ...I................................V..T......
Rom96         .................................P..I..T......
804994        .................................A..Q..T......
G22886        .................................A..Q..T......
G2266         ...............................S....Q..T......

63134Ent280   .....WI.Q......................----KI..T......
WNFCG         .....WI.Q......................----KI..T......
Sarafend      .....WI.Q...........................I..T......

MP502-66      .R...............................P..V..T......
KOUDakAad     ..PW..S.Q........S........L.....FP..RT.T...TI.
JaOArS982     S..I....QP.......G.....T..S.N.....A.V..S..AK.T
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                 172                        199   205 210
                 ↓                          ↓     ↓   ↓
MRM61C        ITPAAPSYTLKLGEYGEVTVDCEPRSGIDTSAYYVMTVGTKTFL
OR205         ............................................
SH183         ............................................
P1553         ..............................N.............
ArA1Dj        ..............................N.............
ArTB3573      ..............................N.............
HB6343        ..............................N.............
NY99          ..............................N.............
ISR98         ..............................N.............
Rom96         ..............................N.............
804994        ..............................N........K....
G22886        ..............................N........K....
G2266         ..............................N.............

63134Ent280   ...S................................S..A.S..
WNFCG         ...S................................S..E.S..
Sarafend      ...S................................S..A.S..

MP502-66      .........M.........IG.........D....LS....S..
KOUDakAad     VS.S...S.....D.....I.......V...........S.S..
JaOArS982     ...N...I.....D.....L.......LN.E.F......S.S..
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Figure 3. Amino acid alignment of the region surrounding the potential glycosylation site of the E protein (shown in bold). KUN viruses not shown display the identical amino acid sequence as the prototype or the isolate OR205, depending on the glycosylation status of the virus. Alignment was performed with the Clustal W program.

205 and 210 were consistent with those of lineage II viruses. Residue 199 (D) was unlike any of the other viruses. The KOU isolate displayed more similarities with the lineage II WN viruses (residues 131, 172, 199, and 210) when signature motifs were compared. Residues 129 and 208 differed from viruses of both lineages.

We have identified signature motifs within the NS5 protein that correlate with the separation of the two lineages. Substitutions between lineages I→II include 860A→T, 869Q→H, 878I→V (except for the isolate MgAn798, which has 878I→L), and 899L→V (except for the isolate ArNa1047, which has 899L→I) (Figure 4). At amino acid 877, the lineage I WN viruses are separated again from the lineage II WN viruses with an A→S substitution; however, the KUN isolates (including MP502-66 from Malaysia) have the same motif as the lineage II WN viruses (877S). The amino acid substitution at 903 separates the Indian WN viruses (903S) from the WN and KUN viruses of both lineages (903T), instead grouping them with the Malaysian isolate and the KOU virus. Once again, the signature motifs cannot be used to classify the Malaysian isolate and KOU virus into either lineage.

Nucleotide sequences in the 3'UTR of the viruses included in this study had a highly variable region in both length and nucleotide sequence immediately downstream of the open reading frame stop codon (Figure 5). Deletions as well as point mutations were observed in this region, which varied from 38 (MgAn798) to 129 (ArNa1047) nt in length. The Australian KUN viruses displayed only point mutations when compared with the KUN prototype, except for the isolate P1553, which contained a 7-nt insertion, consistent with the WN viruses of lineage I. The long deletion in the nucleotide sequence immediately downstream of the stop codon of the WN prototype virus, WNFCG (53 nt), has been described (31); it is also present in the sequences of another two lineage II WN viruses analyzed in this study, Sarafend (53 nt) and MgAn798 (65 nt). The rest of the 3'UTR for these viruses was found to be highly conserved.

```
              860      869     877                                    899 903
               ↓        ↓       ↓                                      ↓   ↓
MRM61C        RARATWAENIQVAINQVRSIIGDEKYVDYMSSLKRYEDTTLVEDTVL
ArTB3573      ..................A............................
HB6343        ..................A............................
ArA1Dj        ..................A............................
NY99          ..................A............................
G2266         ..................A...E..........R...EV.....S..
G22886        ..................A...E..........R...EV.....S..
804994        ..................A...E..........R...EV.....S..
63134Ent280   .T........H........V..E..........R......V......
WNFCG         .T........H........V..E..........R......V......
Sarafend      .T........H........V..E.......G..R......V......
ArNa1047      .T........H........V..E.R.....G..R.....II......
MgAn798       .T........H........L..EG.........R......V......
MP502-66      ......................E..F.......R....I.....S..
KOUDakAad     .P........HA......A.......I....T.R....ASV...S..
JaOArS982     .S........YA......AV..K.N.....T..R....VLIQ..R.I
```

Figure 4. Amino acid alignment of the distal region of the NS5 protein. The KUN viruses not shown display a similar amino acid sequence to the prototype, except for a few minor point mutations not found within the signature motifs. Alignment was performed with the Clustal W program.

Antigenic Analysis

The MAb 10A1, produced to the KUN isolate OR393 (26), reacted specifically with the Australian KUN isolates in ELISA and did not react with the KUN isolate from Malaysia (MP502-66) nor with KOU virus or any of the lineage I or lineage II WN viruses (Table 4). The MAb 546 (29), produced to the WN strain Eg101, reacted with all the lineage I and lineage II WN isolates except WN-Sarafend; it did not react with the KOU, KUN, or Malaysian viruses. The MAbs 2B2, produced to the KUN isolate MRM 16 (27), and 2B4, produced to the WN isolate H442 (28), reacted with all the isolates in the study, while the MAbs 3.67G and 3.91D, again produced to the KUN isolate OR393 (26), reacted with all the isolates except WN-Sarafend. The MAb 3.1112G, produced to the NS1 protein of KUN isolate OR393 (26), reacted with all isolates except KOU. The Mab binding patterns (Table 4) clearly digress and fail to differentiate KUN and WN isolates into two distinct groups. Instead, they define five distinct antigenic groups: Australian KUN viruses, Malaysian KUN virus, lineage I and lineage II WN viruses, WN-Sarafend, and KOU virus.

Conclusion

The results of the phylogenic analysis in this report clearly illustrate that the KUN, WN, and KOU viruses make up a closely related group of viruses, which can be further subdivided into several subgroups on the basis of genetic and antigenic data. Previous phylogenic studies have also shown that KUN and WN viruses share a close relationship (16-18,21). This report however, further defines this relationship by using a comprehensive panel of both viruses. Also included in this study were several anomalous isolates, including an isolate from Southeast Asia (MP502-66), a laboratory-adapted WN strain of uncertain passage history and origin (Sarafend), and a flavivirus from West Africa (KOU), which has been shown to be closely related to the KUN/WN group of viruses.

The region sequenced in the E gene spans a glycosylation site that, although highly conserved among viruses of the JE antigenic subgroup, is absent from many KUN and WN isolates (16,26; Scherret JH, Khromykh AA, Mackenzie JS, Hall RA, unpub. data). While glycosylation at this site has been associated with neuroinvasiveness of WN isolates in mice (32,33), the biological significance of E protein glycosylation is still unclear. Indeed, sequence analysis of the E gene of WN viruses responsible for fatal outbreaks of encephalitis in Romania (Rom 96) and New York (NY99) showed that only the latter contained a potential glycosylation site, casting doubt on the importance of E protein glycosylation in viral pathogenesis. However, our studies and those of others have shown that limited passage of WN and KUN viruses in some cell types can alter the glycosylation status of the E protein and that analysis of passaged viral isolates should be interpreted with caution

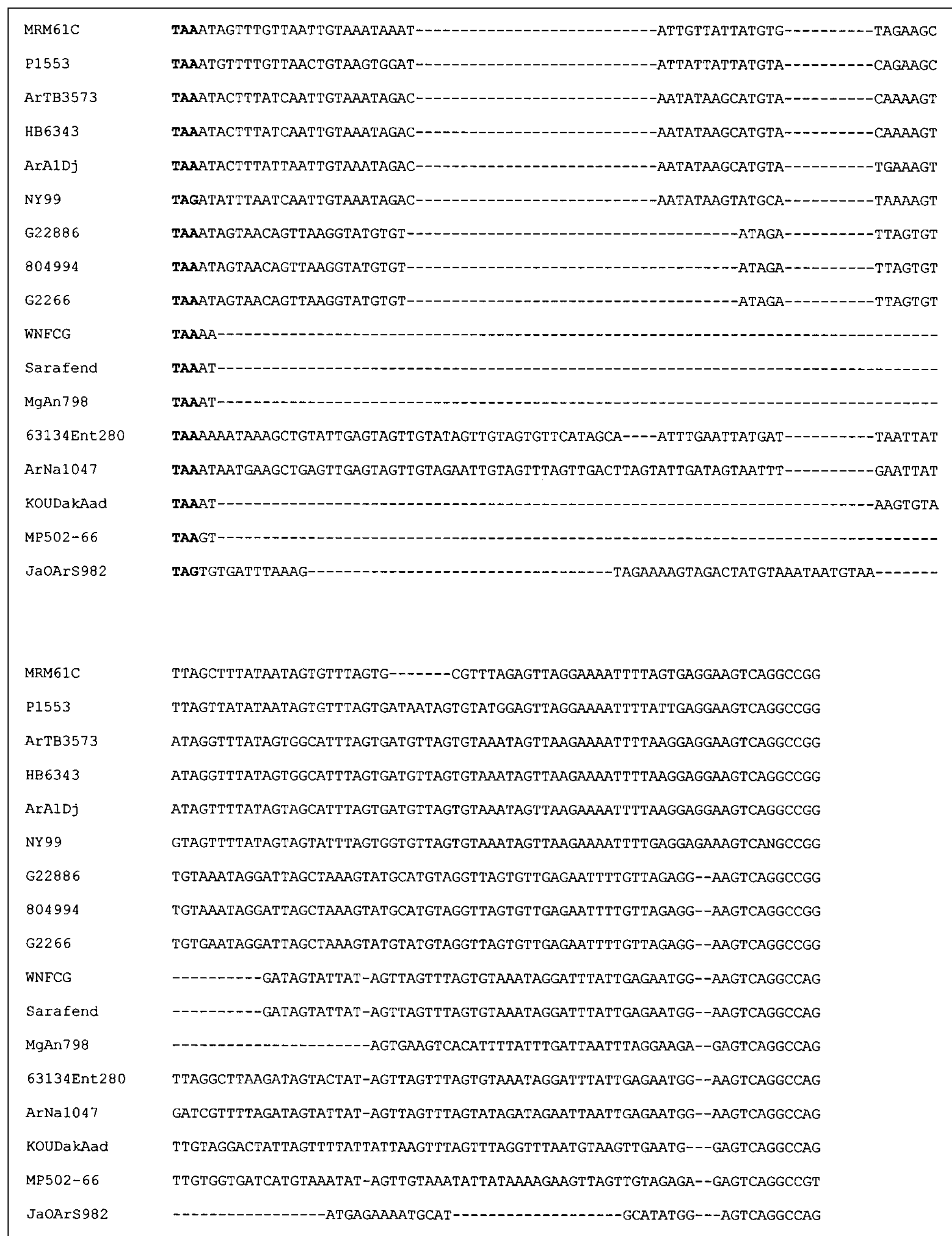

Figure 5. Nucleotide sequence alignment of the 3'UTR (untranslated region) proximal to the open reading frame stop codon (shown in bold) showing distinctive insertions or deletions. Alignment was performed with the Clustal W program.

(33; Scherret JH, Khromykh AA, Mackenzie JS, Hall RA, unpub. data).

The 3'UTR of flaviviruses ranges in length from 400 nt to 600 nt and is thought to play a crucial role in the initiation and regulation of viral translation, replication, and assembly. It includes a potential stable secondary RNA structure at its terminus (2,34-38), and upstream it contains several domains that appear to be conserved among mosquito-borne flaviviruses (2,39, 40). Men et al. (41) have suggested that deletions in the distal 80 nt to 90 nt would most likely lead to disruption of the stem-loop and loss of viability. In contrast, the region sequenced in this study contains highly variable regions suitable for genetic classification and analysis of the relationships among viruses, which had been subjected to deletions or insertions or both during evolution (17).

Phylogenetic trees constructed from sequence data from both regions identified two major lineages, consistent with previous reports (16,18,21). These two lineages did not separate the KUN isolates from the WN isolates; rather, they emphasized the close link between KUN and WN viruses of lineage I. Nevertheless, within lineage I, the Australian KUN isolates formed a tight cluster with an average nucleotide divergence of 6% for the E gene and 10% for the NS5/3'UTR. In contrast, the WN isolates were spread between the two lineages in three clusters, with a divergence of up to 30.6% for sequences of the E gene and 28.3% for sequences of the NS5/3'UTR. Signature motifs in the deduced amino acid sequences of the E and NS5 proteins also support the separation of the viruses into two lineages.

The virus from Malaysia, KUN MP502-66, and the African virus, KOU, pose a conundrum as to their relationship with the WN and KUN group of viruses. Statistical support for clustering with either of the WN lineages was poor, suggesting that they represent two single-isolate lineages. Although our previous findings suggested that the Malaysian KUN isolate may represent an evolutionary link between the KUN and WN viruses (17), the lack of sequence identity between KUN MP502-66 and the KUN/WN group of viruses in our study suggests that these viruses have evolved separately from a common ancestor.

Table 4. Binding patterns of anti-KUN and anti-WN monoclonal antibodies to virus isolates in enzyme-linked immunosorbent assay (ELISA)[a]

	Monoclonal antibodies (MAb)						
Virus	10A1	546	2B2	2B4	3.91D	3.67G	3.1112G
KUN[b]	+[c]	-	+	+	+	+	+
KUN MP502-66	-	-	+	+	+	+	+
WN[d]	-	+	+	+	+	+	+
WN Sarafend	-	-	+	+	-	-	+
KOU	-	-	+	+	+	+	-

[a]Infected C6/36 cell monolayers in 96-well plates were fixed with acetone and used as the antigen in the ELISA.
[b]All Australian KUN isolates exhibited identical MAb binding patterns.
[c]A result was considered positive if consecutive twofold dilutions of MAb produced an OD >0.25 and at least twice that shown on uninfected cells.
[d]All West Nile isolates except Sarafend produced identical MAb binding patterns.

The binding patterns of MAbs to KUN and WN isolates did not differentiate these viruses into the same phylogenetic lineages observed in the dendrograms, although they did support the sequencing results by identifying the Australian KUN viruses, the Malaysian KUN virus, and KOU virus as distinct antigenic groups. The WN-specific MAb used in this study, 546, could not distinguish subgroups within the WN group of viruses; however, Besselaar and Blackburn (28) and Damle et al. (42) have differentiated Indian WN isolates from lineage I South African strains by using MAbs, consistent with the earlier studies of Hammam et al. (43,44). These findings support our sequence data, which show tight clustering of the Indian isolates on a separate branch from other WN isolates in the phylogenetic trees (Figures 1 and 2). Additional MAbs to the E protein of WN viruses may be required to differentiate between lineage I and lineage II viruses.

The unique binding pattern of anti-E MAbs to the Sarafend WN isolate is difficult to explain in light of the E gene sequencing results and amino acid alignments, which show that this virus is similar to other lineage II viruses. However, Sarafend also differs from other WN viruses in the way that it buds from the cell membrane of infected cells (45). Sequencing of the entire prM and E genes of this virus may identify the basis for structural differences in the envelope heterodimer that account for the loss of MAb binding sites and unusual virion maturation.

Phylogenetic analyses enable more precise determination of the relationships among similar viruses and consequently aid in identifying the origin of unknown viruses in subsequent outbreaks. The importance of defining the relationship between the KUN and WN viruses was emphasized during the 1999 outbreak of viral encephalitis in New York City (46,47). Until recently, WN and KUN had been classified as distinct virus types in the *Flavivirus* genus. However, the latest report by the International Committee on Taxonomy of Viruses (25) recognized that KUN and WN should not be classified as two separate species and designated KUN as a subtype of WN. Our results suggest that this definition requires further consideration. The species should perhaps be further subdivided into at least six subtypes on the basis of the clusters of viruses displayed in the phylogenetic trees. Subtypes would then include lineage II WN group, Indian WN group, Australian KUN group, lineage I WN group, Malaysian group, and KOU group.

Indeed, the assessment of viruses from each subgroup for transmissibility by the major mosquito vectors of each geographic region and relative virulence and amplification in primate, equine, and avian species will provide valuable information on the likelihood and possible consequences of the spread of these viruses to new geographic regions. Additional studies of cross-protection between subgroups by natural infection or immunization with vaccines derived from these viruses and the specificity and sensitivity of serologic and molecular assays for each subgroup in monitoring and diagnostic applications will be useful in defining control strategies.

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References

1. Heinz FX, Collett MS, Purcell RH, Gould EA, Howard CR, Houghton M, et al. Family *Flaviviridae*. In: van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, et al., editors. Virus taxonomy: Classification and nomenclature of viruses. 7th Report of the International Committee for the Taxonomy of Viruses. San Diego: Academic Press, 2000; p. 859-78.
2. Chambers TJ, Hahn CS, Galler R, Rice CM. Flavivirus genome organization, expression, and replication. Annu Rev Microbiol 1990;44:649-88.
3. Kuno G, Chang GJJ, Tsuchiya R, Karabatsos N, Cropp CB. Phylogeny of the genus Flavivirus. J Virol 1998;72:73-83.
4. Hayes C. West Nile Fever. In: Monath TP, editor. The arboviruses: epidemiology and ecology. Vol. III. Boca Raton (FL): CRC Press; 1988. p. 59-88.
5. Marshall ID. Murray Valley and Kunjin encephalitis. In: Monath TP, editor. The arboviruses: epidemiology and ecology. Vol. III. Boca Raton (FL): CRC Press, 1988; p. 151-89.
6. Monath TP, Heinz FX. Flaviviruses. In: Fields BN, Knipe DM, Howley PM, editors. Fields virology. 3rd ed. Philadelphia: Lippincott-Raven, 1996; p. 961-1034.
7. Westaway EG. The neutralization of arboviruses. II. Neutralization in heterologous virus-serum mixtures with four group B arboviruses. Virology 1965;26:528-37.
8. De Madrid AT, Porterfield JS. The flaviviruses (group B arboviruses): a cross-neutralization study. J Gen Virol 1974;23:91-6.
9. Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, et al. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. J Gen Virol 1989;70:37-43.
10. Coia G, Parker MD, Speight G, Byrne ME, Westaway EG. Nucleotide and complete amino acid sequences of Kunjin virus: Definitive gene order and characteristics of the virus-specified proteins. J Gen Virol 1988;69:1-21.
11. Castle E, Nowak T, Leidner U, Wengler G, Wengler G. Sequence analysis of the viral core protein and membrane associated proteins V1 and NV2 of the flavivirus West Nile virus and of the genome sequence for these proteins. Virology 1985;145:227-36.
12. Wengler G, Castle E, Leidner U, Nowak T. Sequence analysis of the membrane protein V3 of the flavivirus West Nile virus and of its gene. Virology 1985;147:264-74.
13. Castle E, Leidner U, Nowak T, Wengler G. Primary structure of the West Nile flavivirus genome region coding for all nonstructural proteins. Virology 1986;149:10-26.
14. Lobigs M, Weir RC, Dalgarno L. Genetic analysis of Kunjin virus isolates using HAE III and TAQ I restriction digests of single-stranded cDNA to virion RNA. Aust J Exp Biol Med Sci 1986;64:185-96.
15. Flynn LM, Coelen RJ, Mackenzie JS. Kunjin virus isolates of Australia are genetically homogeneous. J Gen Virol 1989;70:2819-24.
16. Berthet FX, Zeller HG, Drouet MT, Rauzier J, Digoutte JP, Deubel V. Extensive nucleotide changes and deletions within the envelope glycoprotein gene of Euro-African West Nile viruses. J Gen Virol 1997;78:2293-7.

17. Poidinger M, Hall RA, Mackenzie JS. Molecular characterisation of the Japanese encephalitis serocomplex of the Flavivirus genus. Virology 1996;218:417-21.
18. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern U.S. Science 1999;286:2333-7.
19. Tsai TF, Popovici F, Cernescu C, Campbell GL, Nedelcu NI. West Nile encephalitis epidemic in southeastern Romania. Lancet 1998;352:767-71.
20. Savage HM, Ceianu C, Nicolescu G, Karabatsos N, Lanciotti R, Vladimirescu A, et al. Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serological and molecular characterization of a virus isolate from mosquitoes. Am J Trop Med Hyg 1999;61:600-11.
21. Jia XY, Briese T, Jordan I, Rambaut A, Chi HC, Mackenzie JS, et al. Genetic analysis of the West Nile New York 1999 encephalitis virus. Lancet 1999;354:1971-2.
22. Sellner LN, Coelen RJ, Mackenzie JS. A one-tube, one manipulation RT-PCR reaction for detection of Ross River virus. J Virol Methods 1992;40:255-64.
23. Pierre V, Drouet MT, Deubel V. Identification of mosquito-borne flavivirus sequences using universal primers and reverse transcription/polymerase chain reaction. Res Virol 1994;145:93-104.
24. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673-80.
25. Felsenstein J. PHYLIP—Phylogeny Inference Package (Version 3.2). Cladistics 1989;5:164-6.
26. Adams SC, Broom AK, Sammels LM, Hartnett AC, Howard MJ, Coelen RJ, et al. Glycosylation and antigenic variation among Kunjin virus isolates. Virology 1995;206:49-56.
27. Hall RA, Burgess GW, Kay BH, Clancy P. Monoclonal antibodies to Kunjin and Kokobera viruses. Immunol Cell Biol 1990;69:47-9.
28. Besselaar TG, Blackburn NK. Antigenic analysis of West Nile virus strains using monoclonal antibodies. Arch Virol 1988;99:75-88.
29. Gould EA, Buckley A, Higgs S, Gaidamovich S. Antigenicity of flaviviruses. Arch Virol Suppl 1 1990;137-52.
30. Sumiyoshi H, Mori C, Morita K, Kuhara S, Kondou J, Kukushi Y, et al. Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. Virology 1987;161:497-510.
31. Khromykh AA, Westaway EG. Completion of Kunjin virus RNA sequence and recovery of an infectious RNA transcribed from stably cloned full-length cDNA. J Virol 1994;68:4580-8.
32. Halevy M, Akov Y, Ben-Nathan D, Kobiler D, Lachmi B, Lustig S. Loss of active neuroinvasiveness in attenuated strains of West Nile virus: pathogenicity in immunocompetent and SCID mice. Arch Virol 1994;137:355-70.
33. Chambers TJ, Halevy M, Nestorowicz A, Rice CM, Lustig S. West Nile virus envelope proteins: nucleotide sequence analysis of strains differing in mouse neuroinvasiveness. J Gen Virol 1998;79:2375-80.
34. Brinton MA, Fernandez AV, Dispopto JH. The 3'-nucleotides of flavivirus genomic RNA form a conserved secondary structure. Virology 1986;153:113-21.
35. Proutski V, Gaunt MW, Gould EA, Holmes EC. Secondary structure of the 3'-untranslated region of Yellow Fever virus: Implications for virulence, attenuation and vaccine development. J Gen Virol 1997;78:1543-9.
36. Rice CM, Lenches EM, Eddy SR, Shin SJ, Sheets RL, Strauss JH. Nucleotide sequence of yellow fever virus: Implications for Flavivirus gene expression and evolution. Science 1985; 229:726-33.
37. Takegam T, Washizu M, Yasui K. Nucleotide sequence at the 3' end of Japanese encephalitis virus genomic RNA. Virology 1986;152:483-6.
38. Wengler G, Castle E. Analysis of structural properties which possibly are characteristic for the 3' terminal sequence of the genome RNA of flaviviruses. J Gen Virol 1986;67:1183-8.
39. Hahn CS, Hahn YS, Rice CM, Lee E, Dalgarno L, Strauss EG, et al. Conserved elements in the 3' untranslated region of the flavivirus RNAs and potential cyclization sequences. J Mol Biol 1987;198:33-41.
40. Deubel V, Kinney RM, Trent DW. Nucleotide sequence and deduced amino-acid sequence of the non-structural proteins of dengue type 2 virus, Jamaica genotype: comparative analysis of the full-length genome. Virology 1988;165:234-44.
41. Men R, Bray M, Clark D, Chanock RM, Lai CJ. Dengue Type 4 virus mutants containing deletions in the 3' noncoding region of the RNA genome: Analysis of growth restriction in cell culture and altered viremia pattern and immunogenicity in Rhesus monkeys. J Virol 1996;70:3930-7.
42. Damle RG, Yeolekar LR, Rao BL. Strain analysis and epitope mapping of West Nile virus using monoclonal antibodies. Acta Virol 1998;42:389-95.
43. Hammam HM, Clark DH, Price WH. Antigenic variation of West Nile virus in relation to geography. Am J Epidemiol 1965;82:40-55.
44. Hammam HM, Price WH. Further observations on geographic variation in the antigenic character of West Nile and Japanese B viruses. Am J Epidemiol 1966;83:113-22.
45. Ng ML, Howe J, Sreenivasan V, Mulders JJ. Flavivirus West Nile (Sarafend) egress at the plasma membrane. Arch Virol 1994;137:303-13.
46. Briese T, Jia XY, Huang C, Grady LJ, Lipkin WI. Identification of a Kunjin/West Nile-like flavivirus in brains of patients with New York encephalitis. Lancet 1999; 354:1261-2.
47. Fine A, Layton M, Miller J, Cimini D, Vargas MC, Inglesby A, et al. Update: West Nile virus encephalitis, New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:944-6.

Rapid Determination of HLA B*07 Ligands from the West Nile Virus NY99 Genome

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Defined T-cell epitopes for West Nile (WN) virus may be useful for developing subunit vaccines against WN virus infection and diagnostic reagents to detect WN virus-specific immune response. We applied a bioinformatics (EpiMatrix) approach to search the WN virus NY99 genome for HLA B*07 restricted cytotoxic T-cell (CTL) epitopes. Ninety-five of 3,433 WN virus peptides scored above a predetermined cutoff, suggesting that these would be likely to bind to HLA B*07 and would also be likely candidate CTL epitopes. Compared with other methods for genome mapping, derivation of these ligands was rapid and inexpensive. Major histocompatibility complex ligands identified by this method may be used to screen T cells from WN virus-exposed persons for cell-mediated response to WN virus or to develop diagnostic reagents for immunopathogenesis studies and epidemiologic surveillance.

West Nile (WN) virus is the cause of a potentially fatal form of viral encephalitis that suddenly emerged in the New York City area during 1999. The virus is a member of the Flavivirus family, which includes St. Louis encephalitis (SLE), Japanese encephalitis (JE), hepatitis C, and dengue viruses (1,2). WN virus is common in West Asia, Africa, and the Middle East but was not reported in the Americas until the New York outbreak in 1999. The source of virus introduction to New York City is unknown; potential sources include an infected host (human or bird), an infected vector (mosquito), or bioterrorism (1,3). The WN-NY99 virus associated with the New York 1999 outbreak appears to have been circulating in Israel since 1997 (1). Other close relatives to the WN-NY99 virus were isolated in Italy (1998), Morocco (1996), Romania (1996), and Africa (1989, 1993, and 1998).

Surveillance data indicate intensified transmission and geographic expansion of the WN virus-NY99 outbreak in the northeastern United States during 1999 and 2000. Twelve states and the District of Columbia reported WN virus activity in 2000, a substantial increase over the four states reporting activity in 1999. WN-NY99 is expected to continue to spread along the East Coast of the United States in 2001 and thereafter, as a result of overwintering of mosquitoes and avian migratory patterns (4,5).

Virology

WN virus's genome is 11,000 nucleotides long. The following structural proteins have been identified: envelope glycoprotein (env gp E), capsid (C), and premembrane protein (prM). The following nonstructural proteins have also been identified: NS-1, NS-2A/NS-2B, NS3, NS-4A/NS-4B; and NS-5 (RNA-directed polymerase) (1). Isolates that have been completely sequenced include the WN-NY99 virus originally obtained from a Chilean flamingo, a WN-NY99 equine isolate, the Italy 1998 virus, the Romania 1996 virus, and the prototype Eg101 virus. Although the latter viruses are closely related to WN-NY99, they are not identical to each other or to WN-NY99 (6). A virus isolated in Israel, Israel 1998, appears to be identical to WN-NY99; completion of its genome sequence is under way at the Institute Pasteur, France.

Immunology and Immunopathogenesis

An extensive body of research is available on the immunology of flaviviruses in the murine model; however, relatively little research has been done on human immune response to WN virus. Some information on human T-cell responses to related viruses (e.g., JE virus, dengue) has been obtained (7,8). Both CD4 T-helper cells and cytotoxic T-cells that respond to JE virus and dengue proteins have been identified, and their epitopes have been mapped (9). Some of the JE virus CD4 T epitopes are identical or nearly identical to sequences in WN virus (10). Langerhans cells in the epidermis may play a role in the upregulation of immune response to the virus, processing antigen and presenting it to T cells (11,12). Mobilization of dendritic cells and antigen presentation by these cells to T cells in the lymphoid follicles may be involved in the development of immune responses to WN virus (13).

Cytotoxic T-cell responses (restricted by class I major histocompatibility complex [MHC] and MHC class II) and T helper responses (restricted by class II MHC) appear to be critical components of human immune response to members of the flavivirus family (14,15). Cell-mediated immunity to WN virus may prove to be an important barrier to infection of the central nervous system, and vaccines that promote the development of T-effector cells may provide protection from WN virus encephalitis or may be used to treat patients who have WN virus-related illnesses. Further research to test

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these hypotheses will require the development of reagents such as the T-cell epitopes defined in this study.

Applying Bioinformatics to Defining T-Cell Epitopes

New bioinformatics tools developed by the TB/HIV Research Lab and EpiVax (Providence, RI) enable researchers to move rapidly from genome sequence to epitope selection (16). EpiMatrix is a computer-driven pattern-matching algorithm that identifies T-cell epitopes. BlastiMer permits the analysis of protein sequences for homology with other known proteins.

The goal of this project was to demonstrate the utility of a bioinformatics and computational immunology approach for the rapid selection of T-cell epitope reagents. Defining these reagents will permit the evaluation of cell-mediated responses in the immunopathogenesis of WN virus, promote the development of diagnostic reagents such as tetramers (17), and provide components for epitope-based preventive or therapeutic vaccines (18-20). A secondary goal was to determine the time required to select and screen epitope candidates in vitro, since time may be a critical factor in the development of vaccines and diagnostic reagents in response to emerging infectious pathogens.

On the basis of experience with the EpiMatrix HLA B*07 prediction tool, we selected peptides for this pilot study that were expected to be restricted by HLA B*07. In studies of HIV-1 peptides, 60% of peptides selected by EpiMatrix HLA B*07 stimulated T-cell responses in vitro. We therefore expected that approximately 60% of WN virus peptides selected by the same criteria would bind to HLA B*07 and stimulate T-cell responses.

We screened 16 WN virus peptides and identified 12 epitope candidates, 5 of which exhibited strong binding to HLA B*07 at a range of peptide concentrations in vitro. The largest source of delay in the screening process was peptide synthesis (4 weeks from placement of order to receipt of the first set of peptides and 8 weeks until delivery of the final set of peptides). This process could be accelerated if more rapid access to MHC ligands were necessary.

The binding studies we describe are a first step to confirming immunogenicity. In cases such as WN virus, in which access to T cells from infected persons is limited, both the bioinformatics step and the binding assays can be carried out without clinical specimens. Once the epitope candidates selected by this method are confirmed in cytotoxic T-cell (CTL) assays, they may be useful for 1) screening exposed persons for T-cell responses, 2) investigating the immunopathogenesis of WN virus disease in humans, 3) as components of diagnostic kits developed for WN virus surveillance, 4) as reagents for measuring WN virus vaccine-related immune responses, and possibly 5) as components of a subunit vaccine for WN virus. Confirmation of T-cell response to the peptides will depend on availability of peripheral blood cells from WN virus-infected patients during the 2001 transmission season. Additional peptides also need to be identified and screened for binding to other HLA alleles, to broaden the MHC specificity of the diagnostic reagent or immunopathogenesis tools developed by this approach.

Methods

Bioinformatics Analysis

We obtained the NY 1999 WN virus sequence from GenBank (GenBank accession number AF196835) (21). The 3,433 amino acids in the GenBank translation were parsed into 3,424 10-amino acid long frames, each 10 amino acid-long peptide sequence overlapping the previous peptide sequence by nine amino acids. The sequences of these 3,424 decamers were stored in a database.

Each of the peptides in the database was then evaluated by EpiMatrix, a matrix-based algorithm that ranks 9 and 10 amino acid peptides by estimated probability of binding to a selected MHC molecule (22). The estimated binding potential (EBP) is derived by comparing the EpiMatrix score with those of known binders and presumed nonbinders. The EBP describes the proportion of peptides with EpiMatrix scores as high or higher than known binders for a given MHC molecule. Both retrospective and prospective studies of EpiMatrix predictions have confirmed the accuracy of this T-cell epitope selection method (22-24). EpiMatrix is available for use by HIV researchers on the TB/HIV Research Laboratory website (http://tbhiv.biomed.brown.edu/) and under collaborative and commercial arrangements with the TB/HIV Research Laboratory and EpiVax, Inc. (Providence, RI), respectively.

Table 1 illustrates the process of selecting candidate B*07 ligands from the WN virus genome. Of six overlapping peptides in the region of the WN virus sequence shown (Table 1), WN virus B7 0019 scored in the same range as known B*07 ligands and HLA B*07-restricted epitopes (EBP 22.49). Therefore, this peptide would be considered the most likely candidate to show binding to HLA B*07 of the six peptides in this illustration.

Table 1. Scoring overlapping peptides by the EpiMatrix motif HLA B*07

	EpiMatrix analysis of West Nile virus protein NS-1		
AA start	Peptide no. (B*07 rank)	Sequence	EBP[a]
1123	WNB7 3119	GMEIRPQRHD	0.04
1124	WNB7 2818	MEIRPQRHDE	0.08
1125	WNB7 0591	EIRPQRHDEK	1.12
1126	WNB7 2660	IRPQRHDEKT	0.10
1127	WNB7 0019	RPQRHDEKTL	22.49
1128	WNB7 2661	PQRHDEKTLV	0.10

[a]EBP = estimated binding potential, which is the value that EpiMatrix uses to describe the probability that the peptide will bind to B*07 in vitro and in vivo. In this example, six overlapping peptides in the region of the WNV sequence coding for the NS-1 protein (WNV genome AA 1123 to 1128) are shown. WNVB7 0019 received the best EpiMatrix score (22.49) and was therefore selected for in vitro studies.

EBPs for the WN virus peptides ranged from >20% (highly likely to bind) to <1% (very unlikely to bind) (Figure 1). We also scored 10,000 random peptides of natural amino acid composition (25) derived from the ExPASy (Expert Protein Analysis System) proteomics server at the Swiss Institute of Bioinformatics (Randseq, http://www.expasy.ch/tools/ randseq.html). We compared the HLA B*07 EpiMatrix scores of this set of random peptides with those of a set of >300 known binders (compiled and maintained at EpiVax) and with the scores of the set of WN virus peptides selected for this study (Figure 2).

Selection of Peptides

Peptides with EpiMatrix EBP scores in the range of 7 to 50 are more likely to bind to MHC and stimulate T cells in vitro (23). Peptides with an EBP score >50 are less likely to be immunogenic, although they may bind to B7 in vitro (16,23).

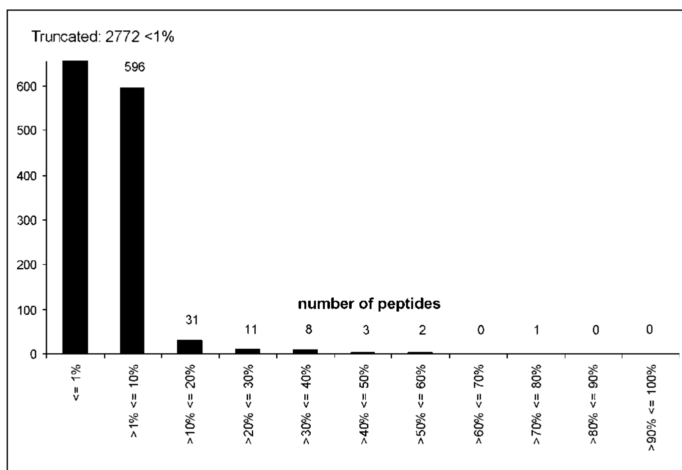

Figure 1. Distribution of scores for the complete set of 3,424 peptides obtained by parsing the West Nile (WN) virus genome into 10 amino-acid long peptides, each overlapping by 9 amino acids, as scored on the EpiMatrix motif for HLA B*07. Peptides with estimated binding potential (EBP) scores >7 and <50 with the HLA B*07 motif are highly likely to bind to HLA B*07 in T2 B7 assays and to stimulate T cells. WN virus peptides with EBP scores between 20 and 50 were considered for study.

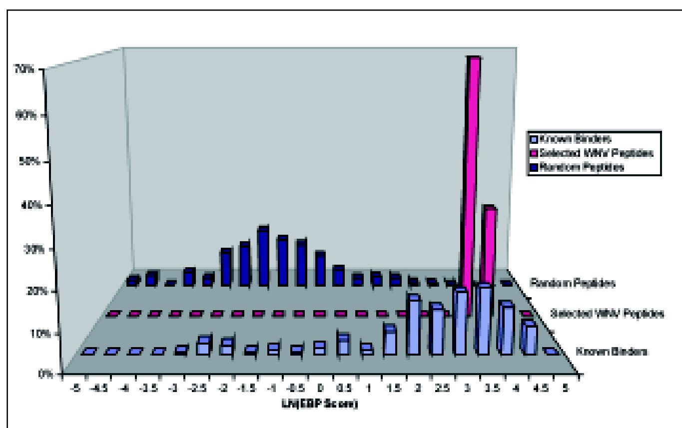

Figure 2. EpiMatrix HLA B*07 score distributions for a random set of 10,000 peptides (dark blue), a set of 20 West Nile (WN) virus peptides selected for screening (magenta), and a set of known HLA B*07 ligands (light blue) are compared. The natural log of estimated binding potential (EBP) for all three sets (random, known binders, and WN virus selections) fell within the range -5 to 5. Scores for the set of WN virus peptides selected for this study are higher than those of most random peptides and are within the same range as scores of published HLA B*07 binders.

Therefore, peptides scoring >50 in the WN virus set were excluded. Based on these criteria, a final set of 22 peptides with EBP scores from 20 to 50 (Table 2a, 2b) was selected for screening in vitro. We excluded 3,329 WN virus peptides with EBP scores <7, 70 potential B*07 binders with EBP scores >7 and <20, and 3 peptides with scores >50 (Table 2b).

Four of the lowest scoring WN virus peptides (EBP 0.00%, Table 2a) were also selected to test the hypothesis that low scoring peptides derived from WN virus would not bind to HLA B*07 in vitro (predicted nonbinders). One well-defined B*07-restricted epitope, GPGHKARVLA (derived from HIV), was also chosen as a positive control for the assays (26).

Tables 2. Selected West Nile virus peptides and their EpiMatrix scores

a. Peptides selected for screening in vitro

Peptide no. (B*07 rank)	Source	Sequence	AA start	EBP
WNB7 0004	NS-1	AVKDELNTLL	861	48
WNB7 0005	mpM	APAYSFNCLG	286	47
WNB7 0006	NS-2A	AAKKKGASLL	1337	45
WNB7 0007	NS-2A	NPMILAAGLI	1357	37
WNB7 0008	NS-3	IPAGFEPEML	1680	36
WNB7 0009	env gp E	TPAAPSYTLK	460	36
WNB7 0010	NS-5	VPCRGQDELV	3259	33
WNB7 0011	NS-5	GPGHEEPQLV	2635	32
WNB7 0013	NS-5	EPPEGVKYVL	2895	31
WNB7 0015	NS-5	KPTGSASSLV	2842	29
WNB7 0017	NS-3	RPRWIDARVY	2098	24
WNB7 0018	NS-4A	VPGTKIAGML	2223	23
WNB7 0019	NS-1	RPQRHDEKTL	1127	22
WNB7 0020	NS-3	SPHRVPNYNL	1777	22
WNB7 0023	NS-5	RPAADGRTVM	3112	21
WNB7 0024	NS-2A	TPGLRCLNLD	1306	21
WNB7 3399	pre-mpM	PEDIDCWCTK	185	0
WNB7 3403	NS-1	PETPQGLAKI	827	0
WNB7 3411	NS-3	PFPESNSPIS	1830	0
WNB7 3415	NS-5	PRTNTILEDN	2073	0

b. Peptides excluded from screening in vitro

Peptide no. (B*07 rank)	Reason for not testing	Sequence	AA start	EBP[a]
WNB7 0001	EBP>50	RPSECCDTLL	2663	72
WNB7 0002	EBP>50	GPIRFVLALL	42	60
WNB7 0003	EBP>50	GPREFCVKVL	2703	55
WNB7 0012	Human-like	AGMLLLSLLL	2229	31
WNB7 0014	Poor quality	MPAILIALLV	1177	30
WNB7 0021	Poor quality	IPMTIAGLMF	1405	22
WNB7 0025	Poor quality	SVNMTSQVLL	2760	20
WNB7 0016	Not expressed	IPTAAGKNLC	148	26
WNB7 0022	Not expressed	MPRVLSLIGL	21	21

[a]EBP = estimated binding potential; env gp E = envelope glycoprotein E; prM = pre-membrane protein; nonstructural proteins NS-1, NS-2A, NS-2B, NS-3, NS-4A, NS-4B, and NS-5.

Cross-Reactive Analysis

After the EpiMatrix analysis, the Conservatrix tool (EpiVax, Providence, RI) was used to align and compare the WN virus sequences with those of other related flaviviruses (21). In an intermediate step designed to avoid selecting epitopes that may have cross-reactivity with "self," each of the highly selected epitopes was passed through the Blast engine at the National Center for Biotechnology Information, using the BlastiMer tool (EpiVax, Providence, RI). Any sequence that was similar to (i.e., >80% identical to the 10 amino acid WN virus NY99 sequence) a peptide component of equivalent length in the human genome (accessible and published to date) was excluded from the study set.

Peptide Synthesis

Peptides corresponding to the epitope selections were prepared by 9-fluoronylmethoxycarbonyl synthesis on an automated Rainen Symphony/Protein Technologies synthesizer (Synpep, Dublin, CA). The peptides were delivered 90% pure as ascertained by high-performance liquid chromatography, mass spectrophotometry, and UV scan. The peptides were shipped as lyophilized powder, which was diluted in a minimal volume of dimethyl sulfoxide and then diluted to stock concentrations in RPMI 1640 medium (Sigma, St Louis,

MO). Peptides that could not be purified to specifications within the study period were not evaluated.

MHC Binding Studies

The T2B7 binding assay method (23,24) relies on the ability of exogenously added peptides to stabilize the class I MHC/beta 2 microglobulin structure on the surface of transporters associated with antigen processing (TAP)-deficient cell lines (27,28). Briefly, the HLA B*07 T2 cell line was prepared for the assay by incubating overnight (16 hours) at 26°C. Before the binding assay, these cells were washed twice in serum-free media. Solutions of the test peptides at three concentrations (final concentrations of 10, 20, and 200 µg/mL in RPMI 1640 (Sigma, St Louis, MO) were plated in triplicate wells of a 96-well, round-bottom assay plate (Becton Dickinson, Lincoln Park, NJ). Sixteen wells containing cells without peptide were included in each plate as background controls.

After 100,000 cells were added to each well, the plates were incubated for 4 hours at 37°C, 5% CO_2, followed by centrifugation at 110 x *g* for 10 minutes at 4°C. The supernatant was discarded, and the remaining cells were resuspended. One hundred µL of anti-HLA-B*07 primary antibody-containing hybridoma supernatant was diluted in staining buffer (1:10 dilution of ME1 supernatant produced by HB-119 cell line [ATCC, Rockville, MD] in staining buffer: phosphate-buffered saline [PBS], 5% fetal bovine serum, 0.1% sodium azide) was added to all the wells. Primary antibody was incubated with the peptide-pulsed cells for 30 minutes at 4°C. After washing three times with staining buffer, the cells were resuspended, and 100 µL of a 1:250 dilution of fluorescein isothiocyanate (FITC)-labeled secondary antibody (FITC-labeled Goat F[ab']2 anti-mouse IgG [H+L] [Caltag, Burlingame, CA]) in staining buffer was added to all the wells. The binding studies are predicated on the assumption that the primary antibody recognizes an epitope on the HLA with a configuration that is unchanged by the stabilizing peptide. The plates were incubated for 30 minutes at 4°C, then washed three times with staining buffer. The contents of each well were then resuspended in 200 µL of fixing buffer (PBS, 1% paraformaldehyde).

The 16 negative control wells in each plate contained no peptide but did contain cells, primary antibody, and secondary antibody. An additional set of wells was plated with peptide at the highest concentration (200 µg/mL), but no primary antibody was added to the wells as a control for nonspecific secondary antibody binding. One positive control peptide (the known B*07 binder) was tested in triplicate at three concentrations (final concentrations of 10, 20, and 200 µg/mL in RPMI 1640) in each assay plate.

Following fixing, the presence of fluorescent secondary antibody on the surface of T2 cells (gated to the appropriate cell size) was measured at 488 nm on a FACScan flow cytometer (Becton Dickinson). The mean linear fluorescence of 10,000 events was measured and compared with the background fluorescence of cells plated in control wells. The entire assay was repeated 4 times, so that each peptide was tested in a total of 36 wells (triplicate wells, three concentrations, four assays).

The B*07 molecule was considered to be stabilized on the surface of the T2B7 cells if the average of the mean linear fluorescence for the triplicate wells at each concentration of peptide was >10% higher than the average of the 16 negative control wells (and $p<0.05$ in two-way comparisons by ANOVA). Binding was rated as strong, moderate, weak, or none, based on the number of significantly positive wells by pair-wise ANOVA (Table 3).

Table 3. West Nile virus T2B7 binding assay results

Peptide no.	AA sequence	EBP[a]	Avg. fold inc. @200[b]	Avg. MFI	Fluorescence ratio (peptide/negative control) 10	20	200	Fluorescence ratio comparisons (p value) control -10	control -20	control -200	Summary
WNB7 0004	AVKDELNTLL	47.77	1.0	842.5	0.91	0.89	0.90	0.021	0.007	0.009	None
WNB7 0005	APAYSFNCLG	47.03	1.2	743.5	0.96	1.09	1.14	0.511	0.127	0.020	Weak
WNB7 0006	AAKKKGASLL	45.3	1.2	708.1	1.01	1.04	1.19	0.717	0.100	0.000	Weak
WNB7 0007	NPMILAAGLI	36.60	1.1	944.6	0.89	0.92	1.00	0.008	0.051	0.949	None
WNB7 0008	IPAGFEPEML	36.02	1.9	1,207.4	1.03	1.18	1.89	0.468	0.000	0.000	Moderate
WNB7 0009	TPAAPSYTLK	35.68	1.5	954.5	1.03	1.13	1.55	0.372	0.001	0.000	Moderate
WNB7 0010	VPCRGQDELV	32.57	1.0	658.6	1.05	1.06	1.04	0.053	0.015	0.118	None
WNB7 0011	GPGHEEPQLV	32.35	1.0	848.9	1.01	1.03	0.98	0.759	0.521	0.689	None
WNB7 0012	AGMLLLSLLL	31.17	--	--	--	--	--	--	--	--	--
WNB7 0013	EPPEGVKYVL	31.07	1.1	681.7	1.08	1.10	1.06	0.004	0.000	0.023	Weak
WNB7 0014	MPAILIALLV	30.23	--	--	--	--	--	--	--	--	--
WNB7 0015	KPTGSASSLV	28.79	1.7	1,070.2	1.04	1.08	1.68	0.243	0.012	0.000	Weak
WNB7 0017	RPRWIDARVY	23.99	1.9	1,714.0	1.18	1.43	1.97	0.003	0.000	0.000	Strong
WNB7 0018	VPGTKIAGML	23.10	1.7	1,088.4	0.98	1.06	1.54	0.533	0.058	0.000	Weak
WNB7 0019	RPQRHDEKTL	22.49	2.8	1,644.6	1.32	1.57	2.77	0.000	0.000	0.000	Strong
WNB7 0020	SPHRVPNYNL	22.40	2.5	1,607.9	1.37	1.59	2.44	0.000	0.000	0.000	Strong
WNB7 0021	IPMTIAGLMF	22.32	--	--	--	--	--	--	--	--	--
WNB7 0023	RPAADGRTVM	20.89	1.5	979.1	1.68	2.01	2.90	0.000	0.000	0.000	Strong
WNB7 0024	TPGLRCLNLD	20.73	1.0	849.6	1.09	1.11	1.01	0.037	0.015	0.821	Weak
WNB7 0025	SVNMTSQVLL	20.09	--	--	--	--	--	--	--	--	--
WNB7 3399	PEDIDCWCTK	0.00	1.1	990.3	0.97	0.96	0.94	0.448	0.396	0.145	None
WNB7 3403	PETPQGLAKI	0.00	1.0	588.9	1.02	1.02	0.99	0.484	0.395	0.716	None
WNB7 3411	PFPESNSPIS	0.00	1.0	626.7	0.90	0.86	0.90	0.020	0.001	0.019	None
WNB7 3415	PRTNTILEDN	0.00	0.9	778.6	0.92	0.91	0.90	0.041	0.020	0.008	None
HIV-1 B7 1291	GPGHKARVLA	28.0	2.2	1,423.8	1.16	1.20	1.72	0.002	0.000	0.000	Strong
			1.5	1,370.9	1.12	1.14	1.78	0.000	0.000	0.000	Strong
			1.6	1,003.9	0.98	1.06	1.66	0.720	0.195	0.000	Weak

[a]EBP = estimated binding potential; MFI = mean fluorescence index and T2B7 binding assay results for each of the peptides.
[b]Average fold increase in fluorescence of cells incubated with peptide at 200 µg/mL.

Results

The 3,424 decamers derived from the WN virus genome were evaluated by EpiMatrix B*07 and evaluated for match to the stored matrix pattern. Most decamers scored for the entire WN virus genome (by the HLA B*07 scoring matrix) had EBP scores <1% (Figure 1). Figure 2 shows the distribution of HLA B*07 scores of a set of 10,000 random peptides (plotted as their natural logs, to allow better distribution of EBP scores <1), compared with scores for the set of >300 known HLA B*07 binders and with the scores of the selected WN virus peptides. The set of peptides selected for study scored well within the EBP range of the comparison set of >300 known HLA B*07 ligands (Figure 1).

Each peptide in the entire WN virus-NY99 dataset of peptides was scored by EpiMatrix. Ninety-five of the 3,424 decamers had EBP scores >7%. Of these 95 peptides, 20 of the 25 with EBP scores between 20% and 50% (Table 2a) were selected for screening. Three peptides with EBP scores >50 (0001, 0002, 0003) were eliminated from the set of peptides tested because scores in this range are less likely to be B*07 ligands and epitopes (TB/HIV Research Lab and EpiVax, unpub. data). The amino acid sequence of peptide 0012 overlapped substantially with the human genome, and for that reason this peptide was also excluded. Three of the original 25 peptides (0014, 0021, 0025) could not be synthesized to sufficient purity within the study timeframe. Two peptides with EBP scores between 50 and 20 (0016 and 0022) were also not tested because they did not fall within a region of the WN virus genome belonging to a mature WN virus protein, based on information in the GenBank database. Sixteen WN virus peptides remained in the final selection.

The final set of 16 WN virus peptides included two from NS-1, four from NS-2A, five from NS-3, one from NS-4A, five from NS-5, one from env gp E, and one from prM (Table 2a). In addition to these peptides, four predicted nonbinder peptides and a known binder (1291) were also synthesized. Twenty-one peptides were tested in vitro in T2B7 binding assays.

Binding Results

Triplicate wells of peptide at 10, 20, and 200 µg/mL were evaluated in each of the T2 B7 binding assays. Table 3 provides information on the mean fluorescence index for the peptide at 200 µg/mL; the average fold increase over background for the peptide at 10, 20 and 200 µg/mL; and the ANOVA analysis for each pairwise comparison (between fluorescence for cells incubated with one of the concentrations of the study peptide and the fluorescence of the cells in control wells).

Twelve of the 16 study peptides demonstrated consistent binding in the four replicate assays. Of these peptides, four (0017, 0019, 0020, and 0023) stabilized HLA B*07 on the surface of T2B7 cells substantially more often than controls in the four replicate assays (strong binders, Table 3). Two WN virus peptides (0008, 0009) stabilized HLA B*07 to a moderate degree. Six WN virus peptides (0005, 0006, 0013, 0015, 0018, and 0024) were weak binders, and four did not bind.

The positive control peptide, 1291, was tested with each set of peptides. The peptide bound significantly over background (based on ANOVA) in all three assays. Four negative control peptides selected for low EBP scores (3399, 3404, 3411, and 3415, all with scores of 0.0%) did not stabilize T2B7 to a significant degree.

Cross Strain Analysis Results

Peptide 0019, a strong binder, was conserved in all strains of WN virus (100% or 10 of 10 amino acids) and Kunjin virus; it was 80% conserved in JE virus strains (8 of 10 amino acids). Peptides 0017 and 0023, two strong binders, were 100% conserved in all strains of WN virus and Kunjin virus, 80% conserved in JE virus and Murray Valley encephalitis (MVE) virus, and 90% conserved in some strains of dengue. Peptide 0020, the fourth strong binder, was conserved in West Nile and Kunjin (100%), JE virus, MVE virus, and dengue (90%).

The two moderate binders 0008 and 0009 were unique. Peptide 0008 was 100% conserved in West Nile virus strains, different by one amino acid from Kunjin virus (closely related to WN virus), and not conserved in any other flaviviruses. Peptide 0009 was conserved only in WN virus and Kunjin virus (100%) and not conserved in any other related flavivirus strains.

Of the weaker binding peptides (0005, 0006, 0013, 0015, 0018, and 0024), 0005 was 100% conserved across WN, Kunjin, SLE, and Sindbis viruses. Peptide 0006 was 90%-100% conserved in WN, Kunjin, JE, and MVE viruses. Peptide 0013, likewise, was conserved in WN and Kunjin but less well in JE virus (80%). WN virus 0015 was conserved in WN virus, Kunjin (100%), JE virus (90%), MVE virus (80%), SLE virus (90%), and dengue (80%). WN virus 0018 was conserved in WN virus, JE virus (90%), MVE virus (80%), and SLE virus (90%). In contrast, peptide 0024 was 100% conserved in all strains of WN virus (100%, or 10 of 10 amino acids) and Kunjin virus but not conserved in any other virus of the flavivirus group.

Estimated Cost

The 3,329 peptides with EBP scores <7% (3,424 to 95) were considered unlikely to bind to HLA B*07. The EpiMatrix approach reduced the number of candidate peptides by 97% (3,329/3,424). Some researchers have adopted a standard overlapping (OL) approach (constructing a set of 10 amino acid-long peptides overlapping by 5 amino acids covering the entire genome [29]). This strategy (10/5 OL set) would have required the synthesis of 685 decamer peptides for the WN virus genome, more than 7 times the number (95) selected by the EpiMatrix approach.

The cost of synthesizing the 16 putative ligands and four controls (at a cost of $250 per peptide) for this project was $5,000. Synthesizing the entire selected set and four controls would have cost $24,750. Had the standard overlapping peptide approach been used, the cost of synthesizing OL peptides would have been approximately $170,000 ($250 for each of 685 peptides). The cost of synthesizing and mapping the complete overlapping set of peptides representing decamer peptides overlapping by 9 amino acids (3,423 peptides) would be $856,000 (Table 4).

If the WN virus B7 peptides behave as observed in previous studies of HLA B*07 peptide datasets (23; De Groot et al., unpub. data), additional HLA-B7 ligands would be identified (approximately 76%; 72 of the set of 95 WN virus peptides with EBP scores >7). If, by performing more overlapping peptide assays, this larger set of 72 (putative) ligands had been found, the cost per ligand with the OL approach would have been approximately $3,600 per ligand, compared with $617 per ligand for 72 ligands with the EpiMatrix approach. If no epitopes were to be missed, the

Table 4. Projected cost of HLA-B7 epitope mapping for the West Nile virus genome

	EpiMatrix	Overlapping (OL)[a] (10 AA long OL by by 5 AA)	Complete OL set (decamers overlapping by 9)
Peptides	20	685	3,424
Peptide synthesis	$5,000	$171,250	$856,000
Time (days)	28	959	4,794
Technician/reagent cost	$2,608	$89,332	$446,527
Cost (synthesis + assay) 12 ligands[b]	$634	$21,715	$108,544
Cost (synthesis + assay) 72 ligands[c]	$617	$3,619	$18,091

[a]The standard overlapping approach, constructing a set of 10 amino-acid long peptides overlapping by 5 amino acids (10/5 OL set) would require the synthesis of 685 decamer peptides, approximately 30 times the number synthesized and tested by the EpiMatrix approach. The "discovery" cost per ligand was calculated by dividing the total cost of synthesis and screening for each of the approaches by the number of ligands expected to be discovered (12 ligands, a low estimate, and 72 ligands, a high estimate).
[b]Based on the assumption that only 12 ligands will be found
[c]Based on the assumption that as many as 72 ligands may be found. In that case, 95 peptides would be synthesized for EpiMatrix, 685 for OL (10 by 5), and 3,434 for the exhaustive approach.

exhaustive approach could be used at an estimated cost of $18,000 per ligand. This approach would have cost approximately five times more than the OL approach and 30 times more than the EpiMatrix approach.

Time Required for Analysis

Analysis of the WN virus genome and selection of the WN virus peptides was performed during one working week. Selected peptides were obtained in batches over a 4-week period. T2B7 binding assays were performed as the peptides arrived. Overall, the T2 B7 binding assays and data analysis took place over 20 working days, and the entire process from peptide selection to completion of data analysis took 8 weeks. Eliminating delays associated with peptide synthesis would have reduced the time required to 4 weeks.

Discussion

Using the EpiMatrix approach, we rapidly identified four excellent B*07-restricted T-cell epitope candidates for WN virus. Overall, 12 (75%) of 16 selected peptides bound in T2B7 binding assays. These binding results compare favorably with those of other T2B7 binding results for HIV-1 (16,23). Xia Jin et al. tested 29 HIV-1 peptides with EBP scores of 7%, of which 10 (35%) bound to T2B7 cells in vitro and 4 (14%) were subsequently demonstrated to be HLA-B7 restricted CTL epitopes in assays performed with CD8+ T-cell lines derived from an HIV-infected patient (23). In a separate study (16) of HLA B*07-restricted peptides, 25 peptides were tested, including a known HLA B*07-restricted epitope (peptide 1291, also used in this study). Nineteen (76%) of 25 peptides were shown to bind to T2B7 cells in vitro, and 60% of the peptides stimulated gamma-interferon release in T-cell assays performed with HIV-1-infected patients' cells.

Based on these experiences with EpiMatrix HLA-B7 selection, additional peptides from the original list of 95 WN virus peptides (EBP scores >7) might be expected to bind to HLA B*07 and stimulate T-cell responses. If the rest of the WN virus B7 peptides behave as observed in the HIV-1 datasets, 21 to 60 additional HLA-B7 ligands might be identified (76%, or 72 of the set of 95 WN virus peptides with EBP scores >7). This observation is also consistent with estimates of the number of epitopes in a given protein (30). Even at this higher number of total ligands, the cost per ligand of the OL approach would still have been more expensive than the EpiMatrix approach. Furthermore, the exhaustive approach would have cost approximately five times more than the OL (10/5) approach and 30 times more than the EpiMatrix approach. The EpiMatrix approach would also be substantially more rapid than OL or exhaustive testing of overlapping peptides.

EpiMatrix is one of several epitope mapping tools available to researchers, including the tool available at the SYFPEITHI (31) website and the HLA binding prediction tool available on the National Institutes of Health (BIMAS) site (32). Neither of these sites returned exactly the same predictions as EpiMatrix for the WN virus genome; however, no direct comparison was made. Either of these web-based epitope-mapping tools could also accelerate the process of epitope mapping the WN virus genome by the approach described here.

The matrix-based approach used by EpiMatrix developers occasionally results in the selection of peptides that do not fit standard anchor-based and extended anchor motifs such as those available on the SYFPEITHI website. As a result, WN virus peptides selected by the EpiMatrix method and included in this study did not always fit the conventional, anchor-based format of proline in position 2 and leucine or phenylalanine in position 9 (17). For example, the sequence of one weak WN virus binder, AAKKKGASLL, has little in common with published HLA B*07 motifs, illustrating how EpiMatrix is able to prospectively identify ligands that do not necessarily match anchor-based motifs.

Although EpiMatrix appears to provide excellent discrimination between most published HLA B*07 ligands and a set of random peptides (Figure 2), there is still overlap between the lower-scoring published HLA B*07 ligands and the scores of some of the random peptides. Since the universe of HLA B*07 ligands is unknown, some of the set of random peptides could be previously unidentified HLA B*07 ligands. Furthermore, EpiMatrix scored several known HLA B*07 ligands very low, reflecting either inaccuracy of the HLA B*07 matrix or inaccurate reporting of these ligands. Further study of these low-scoring HLA B*07 ligands may improve knowledge of the rules determining HLA B*07 binding.

Epitopes that are specific for WN virus could be used to develop diagnostic tests such as tetramer assays for WN virus (17). The tetramer staining assay relies only on the interaction between the tetramer reagent and T-cell receptors on the surface of T cells; it can be performed in <30 minutes on as little as 2 mL of blood. Peptide 0008 was unique to WN virus, with only 8 of 10 amino acids in this sequence conserved in Kunjin virus; the sequence was even less well conserved in other members of the flavivirus family. Peptide 0009 would also be a strong candidate reagent for a diagnostic test, as it was conserved in Kunjin and in many strains of WN virus but not in any other member of the flavivirus family.

The incubation period in humans (i.e., time from infection to onset of disease symptoms) for WN virus encephalitis is

usually 5 to 15 days. Antibodies are detectable within 3 to 7 days; however, to confirm infection, antibody assays must be repeated in the acute and convalescent phases. In contrast, recent tetramer-staining studies (33) indicate that cell responses may be detectable 2 to 3 days after acute infection. The initial CTL response to acute infection with a virus, as measured by tetramer technology, can be dramatic. For example, during the acute immune response to lymphocytic choriomeningitis virus (LCMV) in BALB/c mice, 55% of all CD8+ splenocytes are stained with an LCMV-specific tetramer (34). The method is extremely robust and can detect antigen-specific populations at frequencies as low as 1:5,000 CD8+ T cells, or approximately 1:50,000 peripheral blood mononuclear cells (35). Results of the studies performed here suggest that peptides 0008 and 0009, which are relatively specific for WN virus and which score in the range of EpiMatrix scores shown to be compatible with immunogenicity (24), would be reasonable first candidates for the development of a tetramer-based diagnostic reagent for WN virus.

No specific vaccine or antiviral treatment exists for WN virus infection. CTL response will likely be one critical component of the immune response against WN virus. Development of a preventive or therapeutic vaccine against this public health threat would be greatly expedited if the correlates of immune response were determined and appropriate components rapidly incorporated into a vaccine. Epitopes defined by methods such as the one described here are likely to contribute substantially to the development of new research and diagnostic reagents and vaccines for WN virus and other emerging infectious diseases.

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References

1. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333-7.
2. Wright PJ, Warr HM, Westaway EG. Comparisons by peptide mapping of proteins specified by Kunjin, West Nile and Murray Valley encephalitis viruses. Aust J Exp Biol Med Sci 1983;61:641-53.
3. Stern J. The prospect of domestic terrorism. Emerg Infect Dis 1999;5:517-22.
4. Anderson JF, Andreadis TG, Vossbrinck CR, Tirrell S, Wakem EM, French RA, et al. Isolation of West Nile virus from mosquitoes, crows, and a Cooper's Hawk in Connecticut. Science 1999;286:2331-3.
5. Rappole JH, Derrickson SR, Hubalek Z. Migratory birds and spread of West Nile virus in the Western Hemisphere. Emerg Infect Dis 2000;6:319-28.
6. Jia XY, Briese T, Jordan I, Rambaut A, Chi HC, Mackenzie JS, et al. Genetic analysis of West Nile New York 1999 encephalitis virus. Lancet 1999;354:1971-2.
7. Aihara H, Takasaki T, Matsutani T, Suzuki R, Kurane I. Establishment and characterization of Japanese encephalitis virus-specific, human CD4(+) T-cell clones: flavivirus cross-reactivity, protein recognition, and cytotoxic activity. J Virol 1998;72:8032-6.
8. Okamoto Y, Kurane I, Leporati AM, Ennis FA. Definition of the region on NS3 which contains multiple epitopes recognized by dengue virus serotype-cross-reactive and flavivirus-cross-reactive HLA-DPw2-restricted CD4+ T cell clones. J Gen Virol 1998;79:697-704.
9. Zeng L, Kurane I, Okamoto Y, Ennis FA, Brinton MA. Identification of amino acids involved in recognition by dengue virus NS3-specific, HLA-DR15-restricted cytotoxic CD4+ T-cell clones. J Virol 1996;70:3108-17.
10. Kurane I, Okamoto Y, Dai LC, Zeng LL, Brinton MA, Ennis FA. Flavivirus-cross-reactive HLA-DR15-restricted epitope on NS3 recognized by human CD4+ CD8- cytotoxic T lymphocyte clones. J Gen Virol 1995;76:2243-9.
11. Shen JT, T-To SS, Schrieber L, King NJ. Early E-selectin, VCAM-1, ICAM-1, and late major histocompatibility complex antigen induction on human endothelial cells by flavivirus and comodulation of adhesion molecule expression by immune cytokines. J Virol 1997;71:9323-32.
12. Johnston LJ, Halliday GM, King NJ. Phenotypic changes in Langerhans cells after infection with arboviruses: a role in the immune response to epidermally acquired viral infection? J Virol 1996;70:4761-6.
13. Johnston LJ, Halliday GM, King NJ. Langerhans cells migrate to local lymph nodes following cutaneous infection with an arbovirus. J Invest Dermatol 2000;114:560-8.
14. Lobigs M, Arthur CE, Mullbacher A, Blanden RV. The flavivirus nonstructural protein NS3 is a dominant source of cytotoxic T cell peptide determinants. Virology 1994; 202:195-201.
15. Murali-Krishna K, Ravi V, Manjunath R. Cytotoxic T lymphocytes raised against Japanese encephalitis virus: effector cell phenotype, target specificity and in vitro virus clearance. J Gen Virol 1994;75:799-807.
16. De Groot AS, Bosma A, Chinai N, Frost J, Jesdale BM, Gonzalez MA, et al. From genome to vaccine: in silico predictions, in vivo verification. Vaccine. In press 2001.
17. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, et al. Phenotypic analysis of antigen-specific T lymphocytes. Science 1996;274:94-6.
18. Hanke T, Schneider J, Gilbert SC, Hill AV, McMichael A. DNA multi-CTL epitope vaccines for HIV and *Plasmodium falciparum*: immunogenicity in mice. Vaccine 1998;16:426-35.
19. An LL, Whitton, JL. A Multivalent minigene vaccine, containing B-cell, cytotoxic T-lymphocyte, and Th epitopes from several microbes, induces appropriate responses in vivo and confers protection against more than one pathogen. J Virol 1997;71:2292-302.
20. Nardin EH, Calvo-Calle JM, Oliveira GA, Nussenzweig RS, Schneider M, Tiercy JM, et al. A totally synthetic polyoxime malaria vaccine containing plasmodium falciparum B cell and universal T cell epitopes elicits immune responses in volunteers of diverse HLA types. J Immunol 2001;166:481-9.
21. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389-402.
22. De Groot AS, Jesdale BM, Szu E, Schafer JR, Chicz RM, Deocampo G. An interactive web site providing major histocompatability ligand predictions: application to HIV research. AIDS Res Hum Retroviruses 1997;13:529-31.
23. Jin X, Nixon DF, Roberts CG, Safrit JT, Zhang LQ, Huang YX, et al. Identification of subdominant cytotoxic T lymphocyte epitopes encoded by autologous HIV type 1 sequences, using dendritic cell stimulation and computer-driven algorithm. AIDS Res Hum Retroviruses 2000;16:67-76.
24. Schafer JR, Jesdale BM, George JA, Kouttab NM, De Groot AS. Prediction of well-conserved HIV-1 ligands using a Matrix-based Algorithm, EpiMatrix. Vaccine 1998;16:1880-4.
25. Bairoch A. Amino acid scale: Amino acid composition (%) in the SWISS-PROT Protein Sequence data bank. Release notes for SWISS-PROT release 38 - July 1999. Available at: URL: http://www.expasy.ch/tools/pscale/A.A.SWISS-PROT.html Accessed 01.12.01.

26. Goulder PJ, Phillips RE, Colbert RA, McAdam S, Ogg G, Nowak MA, et al. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nature Medicine 1997;3:212-7.
27. Ljunggren HG, Stam NJ, Ohlen C, Neefjes JJ, Hoglund P, Heemels MT, et al. Empty MHC class I molecules come out in the cold. Nature 1990;346:476-80.
28. Nijman HW, Houbiers JG, van der Burg SH, Vierboom MP, Kenemans P, Kast WM, et al. Characterization of cytotoxic T lymphocyte epitopes of a self-protein, p53, and a non-self-protein, influenza matrix: relationship between major histocompatibility complex peptide binding affinity and immune responsiveness to peptides. J Immunother 1993; 14:121-6.
29. Bond KB, Sriwanthana B, Hodge TW, De Groot AS, Mastro TD, Young NL, et al. An HLA-directed molecular and bioinformatics approach identifies new HLA-A11 HIV-1 subtype E cytotoxic T lymphocyte epitopes in HIV-1 infected Thais. AIDS Res Hum Retroviruses. In press 2001.
30. Woodfolk JA, Sung SS, Benjamin DC, Lee JK, Platts-Mills TA. Distinct human T cell repertoires mediate immediate and delayed-type hypersensitivity to the trichophyton antigen, tri r 2. J Immunol 2000;165:4379-87.
31. Rammensee HG, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 1999;50:213-9. Available at: URL: http://www.uni-tuebingen.de/uni/kxi/
32. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol 1994;152:163-75.
33. Blattman JN, Sourdive DJ, Murali-Krishna K, Ahmed R, Altman JD. Evolution of the T cell repertoire during primary, memory, and recall responses to viral infection. J Immunol 2000;165:6081-90.
34. Murali-Krishna K, Altman JD, Suresh M, Sourdive DJD, Zajac AJ, Miller JD, et al. Counting antigen-specific CD8 T Cells: A reevaluation of bystander activation during viral infection. Immunity 1998;8:177-87.
35. Callan MF, Tan L, Annels N, Ogg GS, Wilson JD, O'Callaghan CA, et al. Direct visualization of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus in vivo. J Exp Med 1998;187:1395-402.

West Nile Virus Infection in the Golden Hamster (*Mesocricetus auratus*): A Model for West Nile Encephalitis

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This report describes a new hamster model for West Nile (WN) virus encephalitis. Following intraperitoneal inoculation of a New York isolate of WN virus, hamsters had moderate viremia of 5 to 6 days in duration, followed by the development of humoral antibodies. Encephalitic symptoms began 6 days after infection; about half the animals died between the seventh and 14th days. The appearance of viral antigen in the brain and neuronal degeneration also began on the sixth day. WN virus was cultured from the brains of convalescent hamsters up to 53 days after initial infection, suggesting that persistent virus infection occurs. Hamsters offer an inexpensive model for studying the pathogenesis and treatment of WN virus encephalitis.

West Nile (WN) virus was first detected in North America in the summer of 1999, during an epidemic and epizootic involving humans, horses, and birds in the New York City metropolitan area (1). The persistence and spread of the virus to several neighboring states during the summer and fall of 2000 suggest that WN virus is now endemic in the United States and that its geographic range probably will continue to expand (2). Although many WN virus infections in humans are asymptomatic or unrecognized, some patients have an acute denguelike illness, and a small percentage have frank meningitis and encephalitis (1-4). The latter complication is most common among the elderly, with recent case-fatality rates ranging from 4% to 11% (3-7).

WN virus is a positive-stranded RNA virus; based on its antigenic and genetic characteristics, it is included in the Japanese encephalitis (JE) serocomplex of the genus *Flavivirus*, family Flaviviridae (8). WN virus was originally isolated from a febrile patient in Uganda in 1937 (9), but it has a worldwide geographic distribution, including most of Africa, southern Europe, central and southern Asia, Oceania, and now North America (3,4,7,10). Despite its wide geographic distribution and frequency, little is known about the pathogenesis of human infection with WN virus, especially encephalitis. One unique pathologic finding in WN virus encephalitis, unlike the encephalitides caused by other closely related flaviviruses, is the targeting of Purkinje cells of the cerebellum (1,7,11-13). We describe preliminary studies of a hamster model for the disease.

Materials and Methods

Hamsters used in our studies were adult (70 to 100 g) females (*Mesocricetus auratus*) obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). They were experimentally infected with a single stock of WN virus strain 385-99. This virus was originally isolated from the liver of a Snowy Owl that died at the Bronx Zoo during the 1999 epizootic in New York City (12); it had been passaged twice in Vero cells.

Virus titrations were done by immunofluorescence (Figure 1) in cultures of C6/36 cells (14) as described (15,16). Blood and 10% brain homogenates were titrated in 24-well tissue culture plates seeded with C6/36 cells. Serial 10-fold dilutions from 10^{-1} to 10^{-6} were made of each sample in phosphate-buffered saline containing 10% fetal bovine serum (PBS); four wells were inoculated with 0.1 mL of each dilution. The cells were subsequently examined for the presence of WN virus antigen by indirect fluorescent antibody (IFA) test with a WN virus-specific mouse immune ascitic fluid and a commercially prepared (Sigma, St. Louis, MO), fluorescein-conjugated, goat antimouse immunoglobulin (15,16). WN virus titers were calculated as the tissue culture infectious

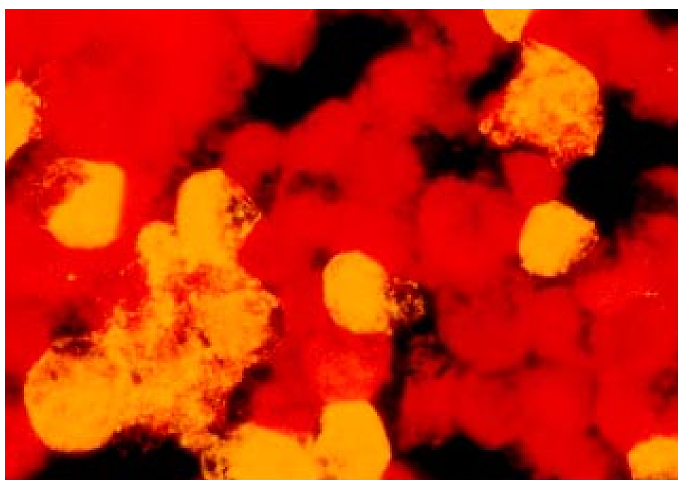

Figure 1. West Nile virus antigen in infected C6/36 cells, as detected by indirect fluorescent antibody testing.

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$dose_{50}$ ($TCID_{50}$) per milliliter of specimen, as described by Reed and Muench (17).

An initial study was done to determine the ID_{50} and the lethal $dose_{50}$ (LD_{50}) of WN virus strain 385-99 for adult hamsters. Serial 10-fold dilutions of the virus stock were prepared from 10^{-1} to 10^{-6} in PBS, and groups of adult hamsters were inoculated intraperitoneally with 0.1 mL of the various dilutions of virus. Hamsters were observed for 28 days, and any deaths were recorded. Brain homogenates from some of the dead animals were inoculated into cultures of Vero or C6/36 cells, which were subsequently examined by IFA to confirm the presence of WN virus. After 4 weeks, serum specimens from surviving hamsters were examined by hemagglutination-inhibition (HI) test for the presence of WN virus antibodies. LD_{50} and ID_{50} values were calculated by the method of Reed and Muench (17). In calculating the ID_{50} of WN virus, both dead and seropositive animals were included. In all subsequent experiments, hamsters were inoculated intraperitoneally with a single virus dose of $10^{4.0}$ $TCID_{50}$.

Antibody Determinations

Antibodies to WN virus in the infected animals were measured by HI and plaque reduction neutralization tests. Antigens for the HI test were prepared from brains of newborn mice infected with the prototype WN virus strain, B956 (9), by the sucrose-acetone extraction method (18). Hamster sera were tested by HI at serial twofold dilutions from 1:20 to 1:5,120 at pH 6.6, with 4 units of antigen and a 1:200 dilution of goose erythrocytes (18).

The plaque reduction neutralization test was done in microplate cultures of Vero cells as described (19), with a constant virus inoculum (~100 PFU of the Egypt 101 WN virus strain) against varying dilutions of hamster serum. Hamster sera were prepared in twofold dilutions from 1:10 to 1:320 in PBS containing 10% fresh guinea pig serum. The serum-virus mixtures were incubated overnight at 5°C before inoculation. Two microplate wells were inoculated with each serum dilution. Plaques were read on the sixth day; samples producing $\geq$90% plaque reduction were considered positive.

Histologic Examination of Tissues

Under Halothane (Halocarbon Laboratories, River Edge, NJ) anesthesia, hamsters were exanguinated by cardiac puncture. The chest cavity was opened quickly, and 20 to 30 mL of 10% buffered formalin was injected directly into the left ventricle to perfuse the body. After refrigeration overnight at 5°C, the body was dissected, and samples of lung, liver, spleen, pancreas, kidney, and spinal cord, as well as the entire brain, were removed and placed in 10% buffered formalin solution for another 24 hours to allow proper fixation. The following day tissue samples were transferred to 70% ethanol for storage. These specimens were subsequently processed, and histologic slides were prepared as described (20). Special stains (Luxol Fast Blue and Nissl's) were also performed on some brain and spinal cord sections. Tissues from six uninfected hamsters were fixed and processed by the same techniques; these tissues were included as controls in all histologic and immunohistochemical examinations.

Immunohistochemical Detection of WN Viral Antigen

After deparaffinization, the formalin-fixed, paraffin-embedded tissue sections (3- to 4-µm thick) were immersed in 3% H_2O_2 for 10 minutes to block endogenous peroxidase activity. This was followed by an antigen retrieval heating step, with a citrate buffer (10% target retrieval solution, DACO, Carpinteria, CA), at 90°C for 30 minutes. A WN virus immune ascitic fluid was used as the primary antibody at a dilution of 1:100. A commercially available mouse-on-mouse immunostain kit (InnoGenex, San Ramon, CA) was used to detect specifically bound primary antibodies and prevent nonspecific binding between species (20).

In Situ TUNEL Assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique was used to assay for apoptosis in stained sections of hamster brain and spinal cord, with the ApopTag peroxidase kit (Intergen Company, Purchase, NY) and 3,3'-diaminobenzidine as the chromogen (20). Slides were counterstained with hematoxylin and mounted with a cover slip for microscopic examination. Different regions of the section were evaluated individually. For semiquantitative assessment of the apoptotic activity, a single 20x-objective microscopic field was selected that contained the highest number of positively stained cells. The activity was scored as follows: 0 = no positive cells; 1 = 5 positive cells; 2 = 6 to 10 positive cells; 3 = 11 to 20 positive cells; and 4 = >20 positive cells.

Results

An initial experiment was carried out to determine the ID_{50} and LD_{50} of the 385-99 WN virus stock in adult female hamsters following intraperitoneal inoculation (Table 1). The hamster ID_{50} of the virus stock was estimated to be $10^{-6.3}$/mL (17), but the LD_{50} was difficult to calculate because the percentage of deaths at various dilutions did not give a clear endpoint. A similar irregular pattern of death or encephalitis, compared with the ID_{50}, was also reported in hamsters experimentally infected with tick-borne encephalitis (TBE) virus (21). Based on these results, $10^{4.0}$ $TCID_{50}$ was selected as the infecting dose of WN virus to be used in subsequent hamster experiments.

The pattern of illness in the hamsters in all experiments was similar. During the first 5 days after infection, the hamsters appeared normal. At day 6 or 7, the animals became lethargic and remained huddled together in the corners of their cages. Food and water consumption by the animals decreased, as did grooming activity. At days 7 to 10, many of the animals had neurologic symptoms, including hind limb paralysis, tremors, difficulty in walking, circling, and loss of

Table 1. Mortality and infection rates among adult hamsters following intraperitoneal inoculation of serial 10-fold dilutions of West Nile virus strain 385-99

Virus titer of inoculum[a]	No inoculated	No. infected (%)	No. dead (%)
$10^{6.0}$	10	10 (100)	5 (50)
$10^{5.0}$	10	10 (100)	7 (70)
$10^{4.0}$	10	10 (100)	6 (60)
$10^{3.0}$	10	9 (90)	6 (60)
$10^{2.0}$	10	8 (80)	2 (20)
$10^{1.0}$	10	8 (80)	2 (20)
$10^{0.0}$	7	1 (14)	0 (0)

[a]Tissue culture infectious $dose_{50}$ ($TCID_{50}$) as determined by titration in mosquito cell (C6/36) cultures.

balance. Many of the severely affected animals died 7 to 14 days after infection. Animals still alive at 14 days usually survived, although some had residual neurologic signs (tremors, muscle weakness, and difficulty in walking).

Viremia and Antibody Response Following WN Virus Infection of Hamsters

Figure 2 shows the pattern of viremia and immune response in 10 adult hamsters following intraperitoneal inoculation of 10^4 $TCID_{50}$ of WN virus strain 385-99. These animals were bled daily for 7 days. Moderate levels of viremia were detected in the hamsters within 24 hours after infection; viremia persisted for 5 or 6 days. HI antibodies were detected in all the animals by day 5, and the titers continued to increase through day 7 (Table 2). No infectious virus was detected in the blood after day 6. The same pattern was observed regardless of outcome (i.e., fatal encephalitis or recovery). Titration of 10% brain suspensions of hamsters that developed clinical encephalitis during the second week of infection yielded virus titers ranging from 10^3 to $10^{6.5}$ $TCID_{50}$, although no infectious virus was detected in peripheral blood at this time and high titers of HI and neutralizing antibodies were present in sera.

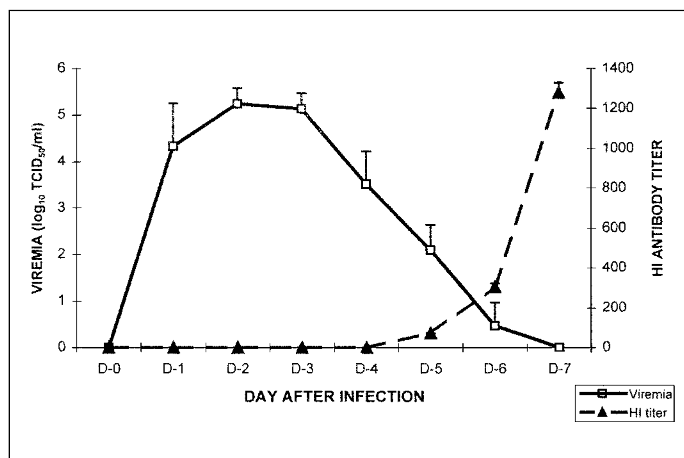

Figure 2. Daily mean (+ standard deviation) virus titers and hemagglutination inhibition antibody levels in 10 hamsters following intraperitoneal inoculation of 10^4 $TCID_{50}$ of West Nile virus strain 385-99.

Table 2. Viremia and hemagglutination inhibition (HI) antibody response in 10 adult hamsters following intraperitoneal inoculation of 10^4 $TCID_{50}$[a] of West Nile virus strain 385-99

Animal no.	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
8001	4.3(N)[b]	5.0(N)	5.0(N)	3.3(N)	1.0(80)	1.0(320)	<0.7(640)
8002	4.7(N)	5.5(N)	5.2(N)	3.5(N)	2.0(80)	<0.7(320)	<0.7(640)
8003	5.3(N)	5.3(N)	5.0(N)	3.5(N)	2.5 (40)	<0.7(320)	<0.7(NT)
8004	2.0(N)	5.0(N)	5.0(N)	4.3(N)	2.5(40)	1.0(160)	<0.7(640)
8005	4.0(N)	5.0(N)	5.5(N)	3.7(N)	1.7(80)	1.0(320)	<0.7(1280)
8006	4.6(N)	5.2(N)	5.7(N)	4.3(N)	2.7(80)	<0.7(320)	<0.7(2560)
8007	4.3(N)	5.7(N)	4.6(N)	4.0(N)	2.0(80)	1.0(320)	<0.7(2560)
8008	4.2(N)	5.8(N)	4.8(N)	1.8(N)	2.0(80)	<0.7(320)	<0.7(640)
8009	5.2(N)	5.2(N)	5.0(N)	3.2(N)	2.8(80)	<0.7(320)	<0.7(1280)
8010	4.7(N)	4.7(N)	5.5(N)	3.5(N)	1.8(80)	0.7(320)	<0.7(1280)

[a]$TCID_{50}$ = tissue culture infectious $dose_{50}$
[b]Level of viremia expressed as log_{10} $TCID_{50}$ of virus/mL of blood. (reciprocal of HI antibody titer) N = < 1:20.

Pathologic Findings in Hamsters with WN Virus Encephalitis

In a third experiment, 60 adult hamsters were inoculated intraperitoneally with 10^4 $TCID_{50}$ of WN virus strain 385-99. Beginning on day 1 postinfection, two hamsters were killed daily for 10 consecutive days; these animals were perfused with 10% formalin to fix tissues for histologic study. Some surviving animals from this experiment were subsequently examined for persistent WN virus infection, as described later.

Histopathologic examination of liver, kidney, lung, myocardium, pancreas, and spleen of the infected hamsters during this initial 10-day period showed no substantial pathologic changes, except for spotty splenic necrosis in a few animals. In contrast, substantial, progressive pathologic changes were observed in the brain and spinal cord.

During the first 4 days, no discernible pathologic changes were observed in the brain or spinal cord. Beginning on day 5, however, neuronal degeneration was seen in many areas, manifested by shrinkage of the perikaryon with intense eosinophilia of the cytoplasm, central chromatolysis, and condensation of the nucleus. Small clusters of large neurons undergoing these changes were located in the cerebral cortex, cerebellar cortex (Purkinje cells), and subcortical gray matter; the hippocampus and basal ganglia were also affected, but less severely. No abnormalities were seen in the spinal cord at this stage. These changes became more extensive on day 6, with involvement of both the superficial and deeper layers of the cerebral cortex, as well as the hippocampus (Figure 3). Large neurons adjacent to the olfactory bulb also showed similar degeneration. Likewise, Purkinje cell degeneration in the cerebellum became more severe and extensive, and scattered degenerating neurons began to appear in the brain stem. At this stage, no inflammatory cell infiltration or perivascular inflammation was seen except for perivascular edema. However, mild perivascular inflammation was observed in the spinal cord. By days 7 and 8 postinfection, neuronal degeneration became more localized, mainly involving the deeper layers of the cerebral cortex, occasional large neurons in the olfactory nucleus, scattered Purkinje cells in the cerebellum (Figure 3), and the brain stem. At this stage, ill-formed microglial nodules and minimal perivascular inflammation began to appear. The microglial nodules consisted of small microglial cells surrounding degenerative neurons (Figure 3). Mild perivascular inflammation with focal neuronal degeneration was observed in the spinal cord, mainly involving the anterior horn. On day 9, more neuronal degeneration, along with psammoma bodies, was seen in the olfactory nucleus; changes in spinal cord were similar to those on day 8. On day 10, most of the abnormalities were localized in the brain stem, which exhibited focal neuronal degeneration surrounded by microglial cell infiltration and "spongiform" neuropil (Figure 3F). Inflammation in the spinal cord was diffuse.

Brains from some surviving hamsters were also examined pathologically on days 12, 14, 19, 28, 35, and 48 after infection. Microscopic changes observed included focal loss of Purkinje cells, occasional microglial clustering, and psammoma bodies.

Immunohistochemical Detection of WN Virus Antigen

WN virus antigen was not detected in the brain during the first 5 days after inoculation. On day 6, clusters or

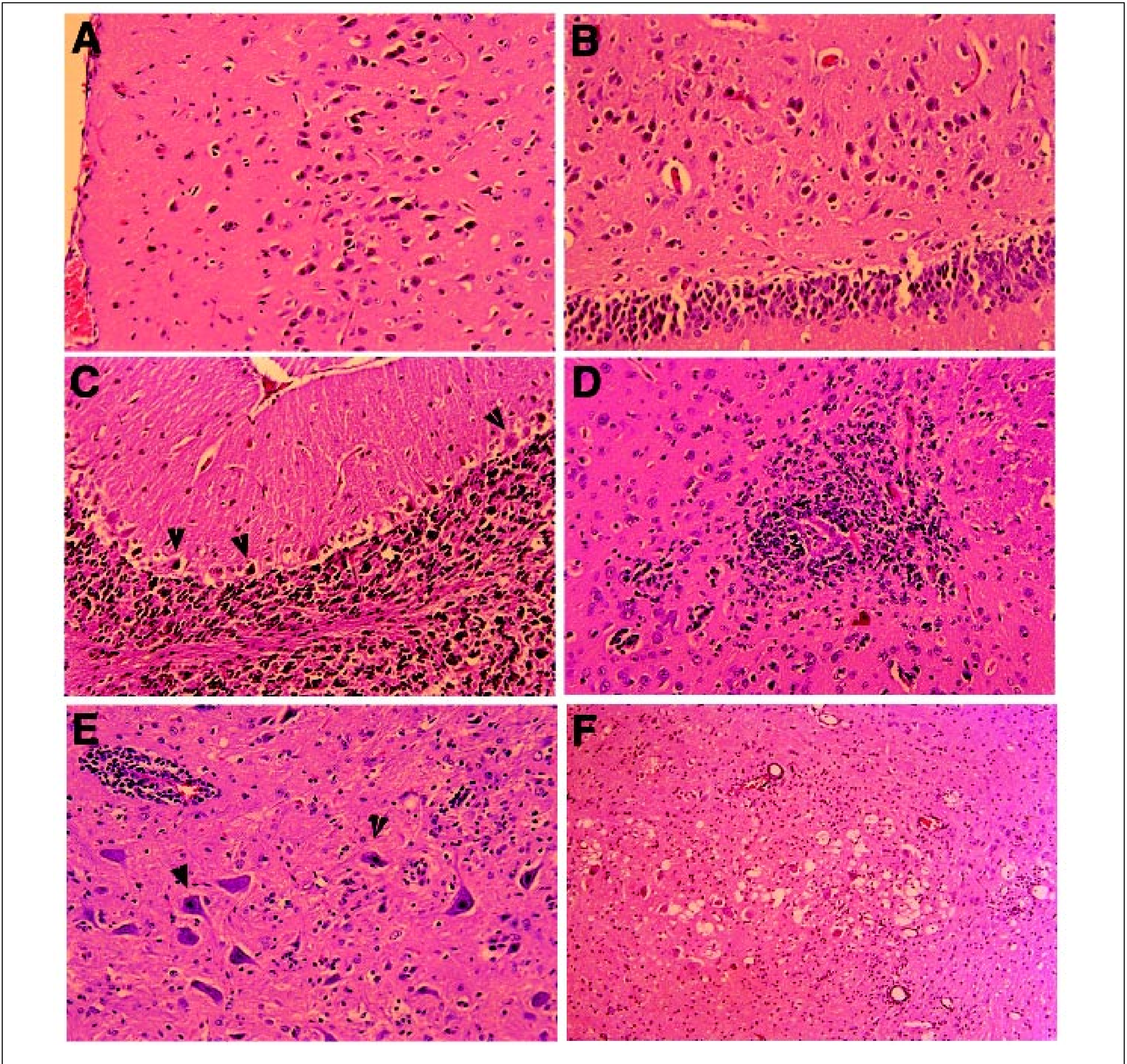

Figure 3. Histologic changes in brains of West Nile virus-infected hamsters. a. Cerebral cortex, with many degenerating neurons, day 6 postinfection. b. Hippocampus, showing large neurons undergoing degeneration, day 6. c. Cerebellar cortex, with frequent Purkinje call degeneration (shrunken cells, arrowheads) and loss, day 8. d. A microglial nodule near blood vessel in basal ganglia, day 9. e. Mild perivascular inflammation (upper left field), neurons with nuclear condensation (arrowhead) and cytoplasmic eosinophilia, in brain stem, day 9. f. Spongiform change in the brain stem, day 10. Magnification 100x.

individual neurons with antigen-positive cytoplasmic staining were observed in the basal ganglia and the brain stem (Figure 4). Other regions of the brain were negative. By day 7, the amount of antigen had increased, and antigen appeared in neurons of the cerebellar cortex, subcortical gray matter, brain stem, basal ganglia, and, to a lesser degree, in the frontal and parietal cortices and the hippocampus. In the cerebrum, foci of positive cells were more prominent immediately adjacent to the ventricles. The amount of viral antigen detected on days 8 and 9 decreased. By day 10 of infection, antigen appeared focally (but strongly) only in the brain stem (Figure 4). None of the hamsters had WN virus antigen in the olfactory bulb by immunohistochemical staining.

Spinal cord sections from two hamsters were stained immunohistochemically on day 9 postinfection. One or two large neurons from each side of the anterior horn were positive for WN virus antigen. The positive neurons were limited in number but were present at most of the spinal levels examined, particularly in the thoracic and lumbar regions.

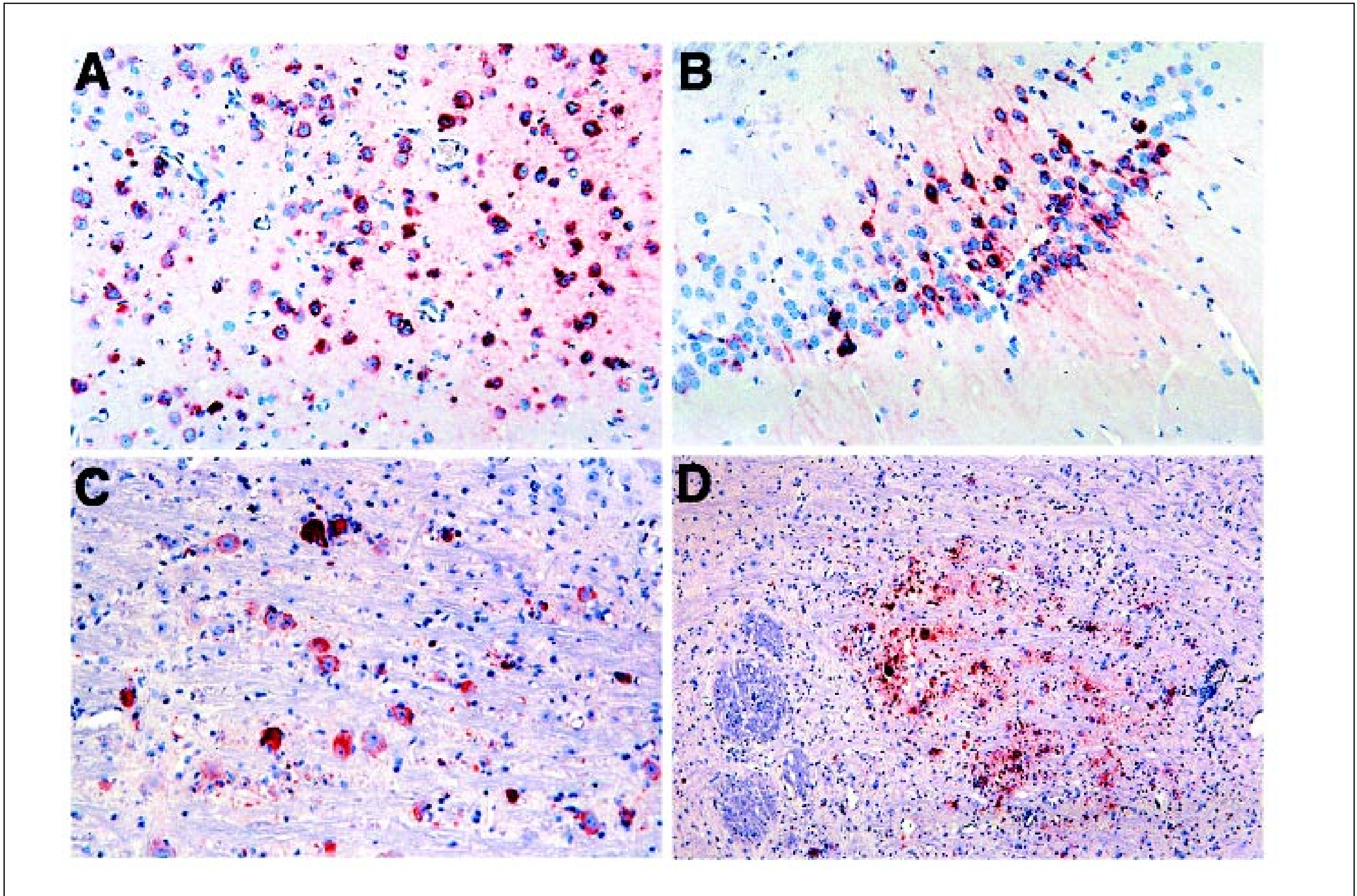

Figure 4. Immunohistochemical detection of West Nile virus antigen in brains of inoculated hamsters. The photomicrographs demonstrate strong cytoplasmic staining (red color) of large and small neurons in different regions. a. Cerebral cortex, day 8 postinfection. b. Hippocampus, day 7. c. Basal ganglia, day 7. d. Brain stem, day 10. Magnification: a-c 100x; d 50x.

In Situ TUNEL Assay

The TUNEL assay, which selectively stains apoptotic cells, was performed on both brain and spinal cord (22). Apoptotic cells were observed in all the areas where neuronal degeneration was seen histologically, but positive-staining cells were most prominent in the hippocampus and basal ganglia (Figure 5).

Brain sections from two or three hamsters from each day postinfection were studied by this method (Figure 6). Rare apoptotic cells began to appear on day 6, and activity gradually increased and peaked on day 9. The positive cells were not limited to neurons but included some endothelial cells of blood vessels in the same microscopic fields. In general, the concentration of apoptotic cells appeared to be most intense in the basal ganglia and brain stem. High activity also appeared transiently in the cerebellar cortex, including both Purkinje cells and scattered medium-sized neurons in the deeper levels (Figure 6).

Persistent WN Virus Infection in Hamsters

Because of an earlier report (23) that WN virus persisted for up to 5½ months in the brains of experimentally infected monkeys, we investigated this possibility in hamsters. Eleven animals that survived intraperitoneal inoculation of WN virus in the third experiment were killed at intervals of 19, 27, 35, 42, and 52 days after infection. A blood sample was taken before death for culture and antibody determinations, and a portion of the cerebellum was also removed at necropsy for culture. WN virus was recovered in Vero cell cultures inoculated with brain homogenates from 5 of 11 convalescent hamsters sampled. WN virus was recovered from one of two hamsters killed on day 19, one of two on day 27, one of two on day 35, one of two on day 42, and one of three on day 52. The positive cultures showed typical WN virus cytopathic effect (CPE) and were confirmed by IFA with a WN virus immune ascitic fluid. IFA of Vero cells from brain cultures without CPE gave negative results. An attempt was made to titrate some of the persistently infected brain samples in C6/36 cells, but the titers were very low ($<10^{0.7}$ to $10^{1.0}$ $TCID_{50}$/mL of 10% brain suspension). Blood cultures of the same hamsters were negative, and the sera had high titers of HI and neutralizing antibodies.

Conclusion

The sequence of events following intraperitoneal inoculation of WN virus into adult hamsters was similar to that described in experimental studies of other flavivirus encephalitides (11,23-27). After a brief viremia of 5 to 6 days' duration, humoral antibodies developed (Figure 2). During this period, the hamsters were asymptomatic. Beginning

Figure 5. In situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining (ApopTaq peroxidase kit, Intergen Company, Purchase, NY) of neurons undergoing apoptotic cell death (brown-colored nuclear staining). a. Cerebellar cortex, showing occasional positively stained Purkinje cells. b. Hippocampus. c. Positively stained neurons within a microglial nodule. d. Positively stained neurons in cerebral cortex. Magnification 200x.

day 6 postinfection, many of the animals had clinical signs of acute central nervous system (CNS) injury (somnolence, muscle weakness, paralysis, tremors, and loss of balance) with a substantial number of deaths occurring on days 7 to 14. Histologically, neuronal degeneration in the brain also was not seen until day 6 after infection. The histopathologic changes began in the cerebral cortex, involving all layers, but gradually only the deeper layers were involved. The observed histopathologic changes eventually spread to the basal ganglia, hippocampus, cerebellar cortex (as Purkinje cell degeneration and loss), and brain stem. At first, neuronal degeneration was not accompanied by microglial cell infiltration or perivascular inflammation. These processes appeared later, with well-formed microglial nodules, sometimes containing a degenerating neuron at the center. The in situ TUNEL analysis confirmed that many of the degenerating neurons underwent apoptosis, leading to the loss of these neurons. This observed sequence suggests that WN virus entered the brain and infected neurons first and that the inflammatory infiltration (perivascular inflammation and microgliosis) was a secondary response to neuronal damage caused by the virus. Supporting this observation is the fact that both histologic abnormalities and appearance of viral antigen (see below) were observed in the brain first and spinal cord second.

Using immunohistochemical staining, WN virus antigen was first detected in the brain on day 6 after infection. This timing correlated well with the onset of encephalitic

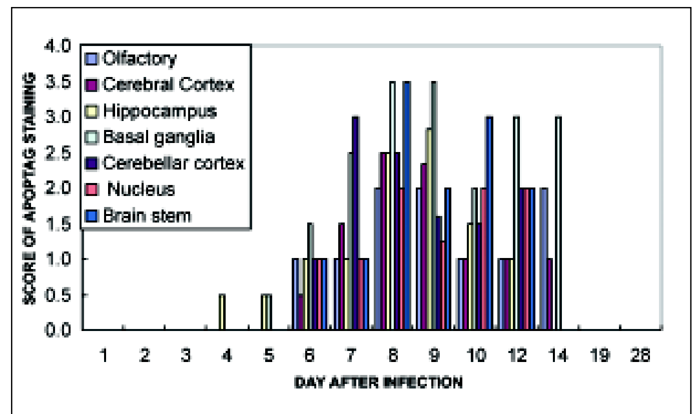

Figure 6. Semiquantitative analysis of ApopTaq-labeled cells in different areas of the brain. Each area was scored individually on a scale of 0 to 4 for positive staining intensity (see Materials and Methods).

symptoms and the observed histopathology in the infected hamsters. Viral antigen was detected in all the areas that showed histologic lesions on routine hematoxylin and eosin (H&E) staining, including cerebral cortex, deep cerebral nuclei, hippocampus, basal ganglia, cerebellar cortex, and the brain stem. Antigen positivity persisted longer in the brain stem. The distribution of the WN virus antigen-positive cells in the brain was focal; these cells usually formed discrete clusters rather than a diffuse pattern. This focal distribution may be caused by regional differences in blood-brain barrier integrity or differential sensitivity of neurons. By day 10, viral antigen was no longer detectable except focally in the brain stem; it completely disappeared afterwards. No viral antigen was detected in the olfactory nucleus in any of the animals examined.

Despite frequent foci of WN virus antigen positivity and neuronal degeneration, inflammatory cell infiltration (i.e., microglial nodules) was not prominent in the hamsters. As shown by the in situ TUNEL analysis, many of the cells undergoing apoptosis were not associated with inflammatory cell attack. This observation suggests that cell death, caused directly by WN virus infection, is the main mechanism of neuronal damage. The exact mechanism by which WN virus initiates the cell death pathway is not clear and will be the subject of future studies. However, experimental studies with Sindbis virus (genus *Alphavirus,* family Togaviridae) (28) and neurovirulent dengue viruses (genus *Flavivirus*) (29) indicate that these mosquito-borne viruses also cause encephalitis by inducing neuronal apoptosis.

Many earlier experimental studies of WN encephalitis were done in monkeys or mice (11,23,24,30-34). Monkeys are no longer a viable option for most investigators because of their cost and the regulatory issues involved in their use. The histopathologic changes reported in the brain and spinal cord of parenterally infected adult mice (24,31,32) are similar to those observed in the WN virus-infected hamsters. However, in preliminary studies with outbred adult Institute for Cancer Research mice, we observed that the New York strain was highly lethal by the intraperitoneal route, but that the viremia following infection was minimal. Other investigators have reported similar results with WN virus strains of Middle Eastern or African origin (24,30,31,33). For this reason, we decided to use hamsters as our animal model, since infection in this rodent species seemed more similar to infection in humans and horses (3,7).

Pogodina et al. (23,34) reported that both WN and tick-borne encephalitis (TBE) viruses induce persistent infection in the CNS of experimentally infected rhesus monkeys, regardless of the route of inoculation or the symptoms (overt or asymptomatic) of the acute infection. These investigators showed that WN virus could be detected for up to 5½ months in the CNS of monkeys after initial infection and that TBE virus could be detected for up to 783 days after infection by cocultivation of trypsinized brain cells on a monolayer of indicator cells (23,34-36). Furthermore, the viruses recovered from the persistently infected monkey brains differed in their phenotypic characteristics (37). The aforementioned monkey experiments were done more than 20 years ago, and the phenotypically altered viruses were not characterized genetically. However, our recovery of WN virus from the brains of persistently infected hamsters supports this earlier Russian work.

The studies of WN virus persistence in the brains of experimentally infected hamsters were not carried beyond 52 days, so the duration and eventual outcome of chronic CNS infection in the animals are unknown. WN virus was recovered from the brains of 5 of 11 convalescent hamsters; but in retrospect, the method of virus assay used (direct culture of a crude brain homogenate) was probably not optimal. Most of the surviving hamsters had WN virus-neutralizing antibody titers $\geq$1:320 when tested 1-2 months after infection. Since a homogenate of brain tissue inevitably contains traces of blood that are present in small vessels, antibodies in the blood may reduce the sensitivity of this culture method (38). Consequently, the cocultivation technique (36) of Pogodina et al. (35) or reverse-transcription polymerase chain reaction would seem preferable, since these assay methods reduce the inhibiting effect of antibodies.

Evidence of persistent infection and chronic progressive neurologic disease following flavivirus encephalitis has been described, especially with Japanese encephalitis and members of the TBE complex (25,39-44). The mechanism and sequelae of persistent CNS infection by flaviviruses are poorly understood but may be of considerable public health importance in the light of the frequency of human infection with some of these agents. Our preliminary results suggest that WN virus infection in hamsters may be a useful experimental model for persistent flavivirus CNS infection. The hamster also provides a reliable and inexpensive animal model for study of the pathogenesis and treatment of WN virus encephalitis.

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References

1. Centers for Disease Control and Prevention. Update: West Nile-like viral encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:890-2.
2. Centers for Disease Control and Prevention. Update: West Nile virus activity—eastern United States, 2000. MMWR Morb Mortal Wkly Rep 2000;49:1044-7.
3. Hayes CG. West Nile fever. In: Monath TP, editor. The arboviruses: epidemiology and ecology. Vol 5. Boca Raton (FL): CRC Press; 1989. p. 60-82.
4. Hubalek Z, Halouzka J. West Nile fever—a reemerging mosquito-borne viral disease in Europe. Emerg Infect Dis 1999;5:643-50.
5. Asnis DS, Conetta R, Teixeira AA, Waldman G, Sampson BA. The West Nile virus outbreak of 1999 in New York: the Flushing Hospital experience. Clin Infect Dis 2000;30:413-8.
6. Tsai TF, Popovici F, Cernescu C, Campbell GL, Nedelcu NI. West Nile encephalitis epidemic in southeastern Romania. Lancet 1998;352:767-71.
7. Komar N. West Nile encephalitis. Rev Sci Tech 2000;19:166-76.
8. Heinz FX, Collett MS, Purcell RH, Gould EA, Howard CR, Houghton M, et al. Family Flaviviridae. In: van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, et al., editors. Virus taxonomy: classification and nomenclature of viruses. San Diego: Academic Press; 2000. p. 859-78.

9. Smithburn KC, Hughes T, Burke A, Paul J. A neurotropic virus isolated from the blood of a native of Uganda. Am J Trop Med Hyg 1940;20:471-8.
10. Taylor RM, Work TH, Hurlbut HS, Rizk F. A study of the ecology of West Nile virus in Egypt. Am J Trop Med Hyg 1956;5:579-620.
11. Manulidis EE. Neuropathology of experimental West Nile virus infection in monkeys. J Neuropath Exp Neurol 1956;15:448-60.
12. Steele KE, Linn MJ, Schoepp RJ, Komar N, Geisbert TW, Manduca RM, et al. Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, New York. Vet Pathol 2000;37:208-24.
13. Sampson BA, Ambrosi C, Charlot A, Reiber K, Veress JF, Armbrustmacher V. The pathology of human West Nile virus infection. Hum Pathol 2000;31:527-31.
14. Igarashi A. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. J Gen Virol 1978;40:531-44.
15. Tesh RB. A method for the isolation of dengue viruses, using mosquito cell cultures. Am J Trop Med Hyg 1979;28:1053-9.
16. Tesh RB, Guzman H, Travassos da Rosa APA, Vasconcelos PFC, Dias LB, Bunnell JE, et al. Experimental yellow fever virus infection in the golden hamster (*Mesocricetus auratus*). 1. Virologic, biochemical and immunologic studies. J Infect Dis 2001;183:1431-36.
17. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. Am J Hyg 1938;27:493-7.
18. Beaty BJ, Calisher CH, Shope RE. Arboviruses. In: Schmidt NJ, Emmons RW, editors. Diagnostic procedures for viral, rickettsial and chlamydial infections, 6th ed. Washington: American Public Health Association; 1989. p. 797-856.
19. Tesh RB, Peters CJ, Meegan JM. Studies on the antigenic relationship among phleboviruses. Am J Trop Med Hyg 1982;31:149-55.
20. Xiao S-Y, Zhang H, Guzman H, Tesh RB. Experimental yellow fever virus infection in the golden hamster (*Mesocricetus auratus*). 2. Pathology. J Infect Dis 2001;183:1437-44.
21. Slonim D, Zavadova H, Simon J. Pathogenicity of tick-borne encephalitis virus. 4. Relation between infective and pathogenic activity for golden hamsters. Acta Virol 1966;10:336-42.
22. Lemasters JJ. Mechanisms of hepatic toxicity. V. Necrapoptosis and mitochondrial permeability transition: shared pathways to necrosis and apoptosis. Am J Physiol 1999;276:G1-G6.
23. Pogodina VV, Frolova MP, Malenko GV, Fokina GI, Koreshkova GV, Kiseleva LL, et al. Study on West Nile virus persistence in monkeys. Arch Virol 1983;75:71-86.
24. Nathanson N. Pathogenesis. In: Monath TP, editor. St. Louis encephalitis. Washington: American Public Health Association; 1980. p. 201-36.
25. Monath TP, Heinz FX. Flaviviruses. In: Fields BN, Knipe DM, Howley PM, editors. Fields virology. 3rd ed. Vol 1. Philadelphia: Lippincott-Raven; 1996. p. 961-1034.
26. Monath TP, Cropp CB, Harrison AK. Mode of entry of a neurotropic arbovirus into the central nervous system. Reinvestigation of an old controversy. Lab Invest 1983;48:399-410.
27. McMinn PC, Dalgarno L, Weir R. A comparison of the spread of Murray Valley encephalitis viruses of high or low neuroinvasiveness in the tissues of Swiss mice after peripheral inoculation. Virology 1996;220:414-23.
28. Lewis J, Wesselingh SL, Griffin DE, Hardwick JM. Alphavirus-induced apoptosis in mouse brains correlates with neurovirulence. J Virol 1996;70:1828-35.
29. Despres P, Frenkiel M-P, Ceccaldi P-E, Duarte dos Santos C, Deubel V. Apoptosis in the mouse central nervous system in response to infection with mouse-neurovirulent dengue viruses. J Virol 1998;72:823-9.
30. Eldadah AH, Nathanson N, Sarsitis R. Pathogenesis of West Nile virus encephalitis in mice and rats. I. Influence of age and species on mortality and infection. Am J Epidemiol 1967;86:765-75.
31. Weiner LP, Cole GA, Nathanson N. Experimental encephalitis following peripheral inoculation of West Nile virus in mice of different ages. J Hyg 1970;68:435-46.
32. Eldadah AH, Nathanson N. Pathogenesis of West Nile encephalitis in mice and rats. II. Virus multiplication, evolution of immunofluorescence, and development of histological lesions in the brain. Am J Epidemiol 1967;86:776-90.
33. Lustig S, Olshevsky U, Ben-Nathan D, Lachmi B-E, Malkinson M, Kobiler D, et al. A live attenuated West Nile virus strain as a potential veterinary vaccine. Viral Immunol 2000;13:401-10.
34. Pogodina VV, Frolova MP, Malenko GV, Fokina GI, Levina LS, Mamonenko LL, et al. Persistence of tick-borne encephalitis virus in monkeys. I. Features of experimental infections. Acta Virol 1981;25:337-43.
35. Pogodina VV, Malenko GV, Fokina GI, Levina LS, Koreshkova GV, Rzhakhova OE, et al. Persistence of tick-borne encephalitis virus. II. Effectiveness of methods used for virus detection. Acta Virol 1981;25:344-51.
36. Shope TC, Klein-Robbenhaar J, Miller G. Fatal encephalitis due to *Herpesvirus hominis*: use of intact brain cells for isolation of virus. J Infect Dis 1972;125:542-4.
37. Pogodina VV, Levina LS, Fokina GI, Koreshkova GV, Malenko GV, Bochkova NG, et al. Persistence of tick-borne encephalitis virus in monkeys. 3. Phenotypes of the persisting virus. Acta Virol 1981;25:352-60.
38. Rajcani J, F. Ciampor SA, Likikova H, Rosenbergova M. Activation of latent *Herpesvirus hominis* in explants of rabbit trigeminal ganglia: the influence of immune serum. Arch Virol 1977;53:55-69.
39. Levy JP, Silvestre D, LeClerc JC, Boiron M. Chronic disease and virus persistence in mice inoculated with Kysanur Forest disease virus. Virology 1966;29:679-83.
40. Zlotnik I, Carter GB, Grant DP. The persistence of louping ill virus in immunosuppressed guinea pigs. Br Exp Pathol 1971;52:395-407.
41. Gresikova M, Calisher CH. Tick-borne encephalitis. In: Monath TP, editor. The arboviruses: epidemiology and ecology. Vol 4. Boca Raton (FL): CRC Press; 1989. p. 177-202.
42. Gaidamovich S Ya. Tick-borne *Flavivirus* infection. In: Porterfield JS, editor. Exotic viral infections. London: Chapman and Hall; 1995. p. 203-21.
43. Ilienko VI, Komandenko NI, Platonov VG, Prozorova IN, Panov AG. Pathogenetic study on chronic forms of tick-borne encephalitis. Acta Virol 1974;18:341-6.
44. Zlotnik I, Grant DP, Carter GB. Experimental infection of monkeys with viruses of the tick-borne encephalitis complex: degenerative cerebellar lesions following inapparent forms of the disease or recovery from clinical encephalitis. Br J Exp Pathol 1976;57:200-10.

West Nile Virus Infection in Mosquitoes, Birds, Horses, and Humans, Staten Island, New York, 2000

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West Nile (WN) virus transmission in the United States during 2000 was most intense on Staten Island, New York, where 10 neurologic illnesses among humans and 2 among horses occurred. WN virus was isolated from *Aedes vexans, Culex pipiens, Cx. salinarius, Ochlerotatus triseriatus,* and *Psorophora ferox,* and WN viral RNA was detected in *Anopheles punctipennis*. An elevated weekly minimum infection rate (MIR) for Cx. *pipiens* and increased dead bird density were present for 2 weeks before the first human illness occurred. Increasing mosquito MIRs and dead bird densities in an area may be indicators of an increasing risk for human infections. A transmission model is proposed involving *Cx. pipiens* and *Cx. restuans* as the primary enzootic and epizootic vectors among birds, *Cx. salinarius* as the primary bridge vector for humans, and *Aedes/Ochlerotatus* spp. as bridge vectors for equine infection.

During the 1999 outbreak of West Nile (WN) virus in the greater New York City (NYC) area, surveillance for virus in mosquito populations did not begin until early September, when the epidemic among humans had already peaked (1). From September through October 1999, WN virus was isolated from nine NYC mosquito pools, including two pools of *Culex pipiens*, six pools of unidentified *Culex* species, and one pool of mixed *Cx. pipiens/restuans*. The mosquitoes were identified by using morphologic characters. Subsequent molecular testing of two of the unidentified *Culex* sp. pools revealed that they were composed of *Cx. restuans* and *Cx. salinarius* (2). Evidence of WN virus infection was found in both dead and live wild birds in NYC in the 1999 outbreak, but there was no systematic monitoring of dead bird sightings in the weeks preceding the first human illness or during the human epidemic.

In anticipation of a possible return of WN virus during 2000, the NYC Department of Health (NYCDOH) established a citywide network of adult mosquito traps and systematically monitored dead bird sightings as part of a comprehensive program for surveillance, prevention, and control of WN virus in the city. An integrated mosquito management program was initiated throughout the city, which included breeding site elimination, larval control, and public education that encouraged residents to remove mosquito sources from their property and to use personal protective behaviors to avoid mosquito bites.

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The first dead bird on Staten Island (SI) in 2000 with laboratory evidence of WN virus was found on July 5, and the first mosquitoes with laboratory evidence of WN virus were collected on SI on July 7. By the end of the mosquito-borne disease transmission season, SI had 71% (10/14) of NYC's human cases, as well as 77% (131/170) of mosquito pools and 33% (61/185) of dead birds with laboratory evidence of WN virus.

SI, one of the five NYC boroughs. has a surface area of 60.2 square miles (156 square kilometers) and a population of 378,977 (3). The population density is 6,295 persons/square mile, less than the density of the other four boroughs (range by borough: 17,409 to 62,765). SI has 115 acres/square mile of park land, compared with the average 80 acres/square mile in the other four boroughs (range by borough: 54 to 155) (3). This island also has 88% (2,942/3,350 acres) of NYC's freshwater wetlands (GIS Unit, Region 2, New York State Department of Environmental Conservation, unpub. data).

During the 1999 WN virus outbreak, SI was the only NYC borough without a human case of WN virus infection. There were no WN virus-infected mosquitoes in the 13 pools collected from SI (a total of 51 adult mosquitoes) October 2-10 (2). The percentage of live wild birds with WN virus antibody in a September 1999 avian serosurvey was 2% (1/43) on SI, compared with 5% (1/20) in Brooklyn and 51% (128/253) in Queens (N. Komar, pers. commun.). Among the eight WN virus-infected dead birds found on SI during 1999, seven were found during October.

We summarize key entomologic, avian, human, and equine surveillance findings from SI from the 2000 outbreak of WN virus, the first year of prospective surveillance for WN virus infection among mosquitoes, birds, horses, and humans in NYC. The results from SI are compared with the other NYC boroughs.

Methods

Adult Mosquito Collection

Adult mosquitoes were collected one night per week from May 2 through November 17 by using CDC miniature light traps baited with dry ice and CDC gravid traps at 21 locations on SI (Figure 1) and 80 locations in the other boroughs. During August 14 to September 2, adult mosquitoes were collected 5-7 nights per week on SI as part of an evaluation of the efficacy of adult mosquito control. All traps were set in the late afternoon and evening and retrieved the following morning. Specimens were immediately frozen with dry ice.

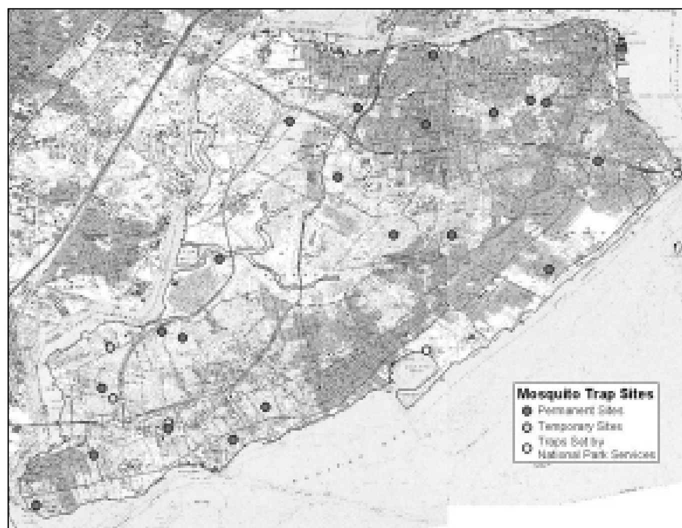

Figure 1. Mosquito trap locations, Staten Island, 2000. NPS = National Park sites.

Adult mosquitoes were identified and sorted to species whenever possible. If the condition of a specimen did not permit species identification, specimens were grouped as *Cx. pipiens*/*restuans* or by genus alone. Up to 50 adult mosquito specimens were pooled (pools of *Cx. salinarius* had up to 100 specimens) by trap site and date. Mosquito pools collected from May 2 to June 2 were submitted to the Centers for Disease Control and Prevention laboratory in Fort Collins, Colorado, and pools from June 3 to November 17 were submitted to the Arbovirus Laboratory of the New York State Department of Health (NYSDOH) for testing.

Minimum infection rate (MIR) was used as the index of virus activity in mosquito populations. MIR is calculated as the number of WN virus-positive pools per number of adult mosquitoes tested and is expressed as the number positive per 1,000 tested. Weekly MIRs were calculated for each species collected on SI and the other boroughs. Mosquito data include only those specimens that were sorted and tested as of December 2000.

Dead Bird Reporting and Collection

Dead bird reports were taken from the public by two systems. An interactive voice-response telephone system allowed callers to leave detailed information about the bird(s) being reported in a recorded message. Reports could also be entered onto a form on the NYCDOH web site. In both systems, the reporter was asked to provide the date the bird was initially found; its exact location, including street address, borough (county), and zip code; species; and cause of death (if known). The density of reported dead birds per square mile of surface area was determined by week for SI and the other boroughs.

Dead bird reports were reviewed before data entry; those meeting predetermined criteria were collected for WN virus testing. Collection was limited to birds recently dead (found in the previous 24 to 48 hours) and in relatively good condition (e.g., little or no decay). The primary criterion for collection was the species of bird. Initially only crows (American or Fish), sparrows, Blue Jays, or any cluster of five or more birds were collected for testing. Collection was expanded to include other species, resources permitting, if they were in good condition. Collection of other species focused on raptors (especially Merlins and American Kestrels) or species not typically reported (e.g., Black Skimmer, Belted Kingfisher). Collected birds were sealed in plastic bags and stored with freezer packs in the field. All specimens were submitted to the Wildlife Pathology Unit of the New York State Department of Environmental Conservation for necropsy; tissue specimens were submitted to the Arbovirus Laboratory of the NYSDOH for WN virus testing.

Laboratory Testing of Mosquitoes and Birds

Adult mosquito pools and avian tissues were tested for WN virus with one set of primers/probes by real-time reverse transcription-polymerase chain reaction (RT-PCR) (TaqMan, ABI Prism 7700 Sequence Detector, Applied Biosystems, Foster City, CA). Confirmatory tests were performed by using a second TaqMan primer/probe set, standard RT-PCR, virus isolation in cell culture, and immunofluorescence assays (avian tissues). A sample was confirmed positive when at least two different tests were positive. Details on virus testing are described elsewhere (4,5).

Human and Equine Case Surveillance

NYCDOH conducted citywide enhanced passive and active hospital-based physician and laboratory surveillance for human WN virus infections, including all three acute-care hospitals on SI. Additional details on human surveillance are described elsewhere in this journal by Weiss et al. (6). Surveillance for equine illnesses was by enhanced passive surveillance. Additional details regarding equine surveillance are described elsewhere in this journal by Trock et al. (7).

Results

Adult Mosquito Collection and Testing

A total of 24,068 adult mosquitoes from 23 species were tested in 967 pools from SI; 131 pools from 6 species had laboratory evidence of WN virus. NYSDOH reported WN virus isolation from *Aedes vexans, Cx. pipiens, Cx. salinarius, Ochlerotatus triseriatus,* and *Psorophora ferox*, and WN viral RNA detection in *Anopheles punctipennis* (8). During the same period, a total of 51,044 adult mosquitoes from 26 species were tested in 1,958 pools collected from the other boroughs; 39 of these pools had laboratory evidence of WN virus (Table). These are the first reports of *Ae. albopictus* and *Cx. erraticus* from New York State.

Laboratory evidence of WN virus was first detected from mosquitoes in a *Cx. pipiens* pool collected July 7 and in a

Table. Adult mosquitoes collected and tested from New York City (NYC), as of December 2000[a]

Genus	Sp.	No. of mosquitoes tested			No. of positive pools		
		Staten Island	Other boroughs	All NYC	Staten Island	Other boroughs	All NYC
Aedes & Ochlerotatus	species	351	1,284	1,635	1		1
Aedes	*albopictus*		90	90			
	vexans	2,497	3,215	5,712	2	2	4
Anopheles	*crucians*	11	2	13			
	punctipennis	47	3	50	1		1
	quadrimaculatus	44		44			
	species	16	37	53			
Coquillettidia	*perturbans*	35	3,010	3,045			
	species	6	6				
Culex	*erraticus*	4		4			
	pipiens	4,820	15,231	20,051	55	19	74
	pipiens/restuans	2,554	11,190	13,744	24	9	33
	restuans	439	2,480	2,919			
	salinarius	10,057	7,687	17,744	28	4	32
	territans	28	45	73			
	species	2,242	3,502	5,744	16	4	20
Ochlerotatus	*canadensis*	21	277	298			
	cantator	14	97	111		1	1
	excrucians	2		2			
	intrudens		21	21			
	japonicus		2	2			
	sollicitans	33	1,699	1,732			
	taeniorhynchus	1	118	119			
	triseriatus	592	180	772	3		3
	trivittatus	193	784	977			
Psorophora	*ferox*	39	19	58	1		1
	columbiae						
	species		1	1			
Uranotaenia	*sapphirina*	28		28			
Unidentified, damaged			64	64			
Total		24,074	51,044	75,106	131	39	170

[a]Pools were collected by the New York City Department of Health, the Centers for Disease Control and Prevention, the National Park Service, and the U.S. Army.

Cx. salinarius pool collected July 17, both from SI. The first human patient on SI became ill on July 20. Additional human cases on SI had onset of disease between July 28 and September 2. Human cases occurred when the MIR among *Cx. pipiens* and *Cx. pipiens/restuans* ranged from approximately 5 to 16/1,000 (July 7 to September 22) and the MIR among *Cx. salinarius* was at least 4/1,000 (6 of 7 weeks from July 15 to September 1). The MIR among *Cx. salinarius* peaked at 9/1,000 in the week ending August 11 (Figure 2).

Dead Bird Reports and Testing

Overall, 14,849 dead birds were reported in NYC during 2000, including 4,910 (33%) from SI. Of the SI birds, 235 (4.8%) were collected and submitted for testing; 60 (26%) had laboratory evidence of WN virus. These included 45 American Crows, 5 Blue Jays, 2 Snowy Owls, and 1 each of the following species: American Kestrel, Canada Goose, Common Grackle, Fish Crow, Greater Black-backed Gull, Mourning Dove, Northern Mockingbird, and sparrow.

Density of total dead birds per square mile on SI began to increase the week ending July 7 and peaked at >14 dead birds/square mile per week during the week ending July 21. The density of dead birds remained >5/square mile until the week ending September 1. This period of increased weekly den-sity of dead birds coincided with the period of elevated MIRs among *Cx. pipiens*, *Cx. pipiens/restuans*, and *Cx. salinarius* (Figure 2).

Human and Equine Cases

Ten human cases of neurologic WN virus infection were reported from SI, and four cases were reported from three other boroughs. Onset of illness for the SI cases ranged from July 20 to September 2 and from August 15 to September 13

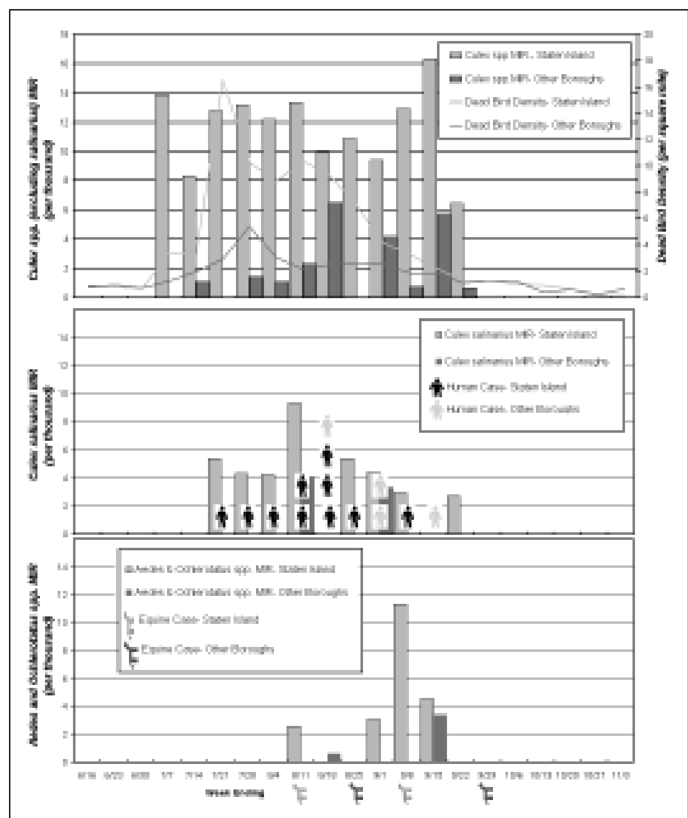

Figure 2. Minimum infection rate (MIR) of *Culex pipiens/restuans*, *Cx. salinarius*, *Aedes* sp., and *Ochlerotatus* sp., dead bird densities, West Nile-infected human and equine cases by week, Staten Island and other boroughs, New York City, 2000

for the other boroughs. Four confirmed equine cases were reported, two on SI (onset August 17 and September 8) and two from another borough (onset August 27 and October 1).

Conclusion

All 10 human infections with WN virus on SI in 2000 occurred when the weekly density of dead birds and mosquito MIRs was elevated. Dead bird density increased to three dead birds/square mile and the MIR for *Cx. pipiens* and *Cx. pipiens/restuans* increased to 8-14/1,000 mosquitoes before the first human case was reported. Monitoring dead bird density and mosquito MIRs may detect increased WN viral activity and predict when the risk of human infection with WN virus is increased.

The first human patient on SI had onset of symptoms on July 20; if one assumes a 3- to 15-day incubation period for WN virus (9), exposure to a WN virus-infected mosquito would have occurred between July 5 and July 17. WN virus was first detected in a pool of *Cx. pipiens* collected on July 7 and in a *Cx. salinarius* pool collected on July 17 from different sites approximately 2 miles from the residence of the first human case. Additional human cases on SI had onset of disease between July 28 and September 2, with exposure to a WN virus-infected mosquito estimated to be between July 13 and August 29. MIRs remained elevated during this period. Based on the observations on SI, we propose a model of transmission that may be applicable to similar habitats elsewhere in the northeastern United States. *Cx. pipiens* and *Cx. restuans* appear to serve as the primary enzootic and epizootic vectors among birds. Blood-meal analyses of mosquitoes collected in NYC during 2000 show that *Cx. pipiens* feeds predominantly on birds (Charles Apperson, pers. commun.), consistent with an earlier observation by Spielman (10). This interaction appears to amplify the amount of virus circulating in spring and early summer. *Cx. salinarius* appears to serve as the bridge vector for human transmission. This mosquito has also been hypothesized as a bridge vector for human transmission of eastern equine encephalomyelitis virus (11). *Aedes/Ochlerotatus* spp. appear to be infected later in the season and serve as bridge vectors for horses.

There are several important limitations to the observations reported in this paper. The data presented reflect a single year of data, and additional surveillance data over time will be needed to determine if there is a consistent correlation between increased dead bird density, elevated mosquito MIRs, and human case onset.

The advance warning provided by dead bird and mosquito data appears to be limited to no more than 10 days. While dead bird reports can be monitored daily and a weekly dead bird density can be quickly determined, MIR data require the labor-intensive and time-consuming steps of pooling by species and laboratory testing. The usefulness of MIR data depends on timely completion of these tasks.

Polymerase chain reaction (PCR) was the primary method of laboratory testing of birds and mosquitoes from SI, and viral culture was not performed to confirm the presence of live WN virus in all specimens. The interpretation of PCR results without viral confirmation requires caution. However, vector competence for WN virus transmission has been demonstrated for *Ae. vexans, Cx. pipiens* (12), *Cx. salinarius* (Michael Turell, unpub. data), and *Oc. triseriatus* (Michael Turell, pers. commun.). These four species made up 98% (112/114) of the positive mosquito pools sorted to species from SI reported in this paper. Vector competence has not been determined for *Ps. ferox* or *An. punctipennis.*

Dead bird data are limited in that avian mortality is not an established surveillance method for which baseline rates are known. We are unable to compare the number of dead birds reported on SI in 2000 with a prior year's data. Furthermore, public interest in reporting dead birds could wane over time, limiting the usefulness of this surveillance technique.

The dead bird densities shown in this paper were calculated at the borough (county) level, and analysis at smaller geographic units may detect more focal areas of transmission that are at greater risk for human infection than surrounding locations. Additional surveillance methods, e.g., live bird surveillance or use of sentinel animals, may provide a more timely warning of increasing risk of human WN viral infection.

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Dr. Kulasekera is a research scientist in charge of the mosquito surveillance program in New York City. Her research interests include ecologic and evolutionary interactions among mosquitoes and the pathogens they transmit.

References

1. Centers for Disease Control and Prevention. Outbreak of West Nile-like viral encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:846.
2. Nasci RN, White DJ, Stirling H, Oliver JA, Daniels TJ, Falco RC, et al. West Nile virus isolates from mosquitoes in New York and New Jersey, 1999. Emerg Infect Dis 2001;7:626-30.
3. City of New York. The 1998-99 green book, official directory of the City of New York. 73rd ed. New York: City of New York; 1998:607-8.
4. Kramer LD, Bernard KA. West Nile virus tissue tropisms in birds and mammals. Ann NY Acad Sci 2001; in press.
5. Shi P-Y, Kauffman EB, Ren P, Felton A, Tai JH, Dupuis II AP, et al. High throughput detection of West Nile virus RNA. J Clin Microbiol 2001;39:1264-71.
6. Weiss D, Carr D, Kellachan J, Tan C, Phillips M, Bresnitz E, et al. Clinical findings of West Nile virus infection in hospitalized patients, New York and New Jersey, 2000. Emerg Infect Dis 2001;7:654-8.
7. Trock SC, Meade BJ, Glaser AL, Ostlund EN, Lanciotti RS, Cropp BC, et al. West Nile virus outbreak among horses in New York State, 1999 and 2000. Emerg Infect Dis 2001;7:745-7.
8. Bernard KA, Maffei JG, Jones SA, Kauffman EB, Ebel GD, Dupuis AP, et al. West Nile virus infection in birds and mosquitoes in New York State, 2000. Emerg Infect Dis 2001;7:679-85.
9. Olejnik E. Infectious adenitis transmitted by *Cx. molestus*. Bulletin of the Research Council of Israel 1952; 2:210-11.
10. Spielman A. Population structure in the *Cx. pipiens* complex of mosquitoes. Bull World Health Organ 1967;37:271-6.
11. Vaidyanathan R, Edman JD, Cooper LA, Scott TW. Vector competence of mosquitoes (Diptera: Culicidae) from Massachusetts for a sympatric isolate of eastern equine encephalomyelitis virus. J Med Entomol 1997;34:346-52.
12. Turell MJ, O'Guinn ML, Dohm DJ, Jones JW. Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. J Med Entomol 2001;38:130-4.

Experimental Infection of Chickens as Candidate Sentinels for West Nile Virus

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We evaluated the susceptibility, duration and intensity of viremia, and serologic responses of chickens to West Nile (WN) virus (WNV-NY99) infection by needle, mosquito, or oral inoculation. None of 21 infected chickens developed clinical disease, and all these developed neutralizing antibodies. Although viremias were detectable in all but one chicken, the magnitude (mean peak viremia $<10^4$ PFU/mL) was deemed insufficient to infect vector mosquitoes. WNV-NY99 was detected in cloacal and/or throat swabs from 13 of these chickens, and direct transmission of WNV-NY99 between chickens occurred once (in 16 trials), from a needle-inoculated bird. Nine chickens that ingested WNV-NY99 failed to become infected. The domestic chickens in this study were susceptible to WN virus infection, developed detectable antibodies, survived infection, and with one exception failed to infect cage mates. These are all considered positive attributes of a sentinel species for WN virus surveillance programs.

West Nile (WN) virus is a mosquito-borne zoonosis maintained by birds in Africa, Eurasia, Oceania, and since 1999, North America (1). Since its emergence in recent years, it has become an important public, veterinary, and wildlife health threat. Monitoring the enzootic transmission of WN virus is critical to obtaining an accurate distribution of virus activity and an assessment of risk for human, livestock, and wildlife populations.

Captive sentinel animals, compared to all other arbovirus surveillance systems, provide more precise data on the location and time in which virus transmission has occurred. Chickens are frequently used as sentinels for surveillance of the bird-transmitted arboviral encephalitides. Chickens were equally or more sensitive than other sentinel birds for detecting St. Louis encephalitis virus transmission in Florida and California (2,3). In California, chickens have provided a more sensitive and cost-effective means to early detection of arbovirus activity in comparison to mosquito- and wild bird-based surveillance systems (4). However, chickens have not been evaluated against criteria for a successful sentinel species for WN virus in North America.

A candidate sentinel bird species for the strain of WN virus circulating in North America (WNV-NY99) would be highly susceptible to mosquito-borne infection yet resistant to disease. It must survive infection in order to develop detectable antibodies. Once infected, it should not develop sufficient viremia to infect biting mosquitoes and should not infect either its flock mates (which may skew surveillance results) or its human handlers. In this study, we evaluated domestic chickens against these criteria for a sentinel species for WNV-NY99. In particular, we inoculated chickens by needle, by mosquito, and orally; we measured susceptibility to infection, development of specific antibody, transmission to cage mates, magnitude and duration of viremia, and potential for viral shedding.

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Materials and Methods

Infection of Chickens

Dekalb Delta hens (Hudson Pullet Farm, Fort Lupton, CO) of various ages (17-60 weeks old) were inoculated with WNV-NY99 (source: *Corvus brachyrhynchos* brain 99-41-32, New York State Wildlife Pathology Unit, 1 Vero passage) by needle, mosquito, or oral inoculation. The needle-inoculated birds (n=5) were injected subcutaneously on the breast with 10,000 Vero PFU per 0.05 mL using a 1-cc syringe and a 26-gauge needle. The mosquito-inoculated birds (n=16) were exposed to three to five infected mosquitoes through the mesh-top of a pint-size ice cream container positioned on an exposed region of the hen's breast. The mosquitoes were removed after at least one mosquito had become engorged. For 16 birds, a noninfected cage mate was provided to evaluate contagiousness in the absence of mosquitoes. Oral inoculation was attempted in three groups of three birds by placing 0.2 mL of sterile water containing either 280 PFU WN virus (group 1), 2800 PFU WN virus (group 2), or one infected dead mosquito (group 3) into the gullet, which stimulated the swallow reflex.

All inoculated chickens and their cage mates were bled daily for 7 days postinoculation (dpi). Each day, 0.2 mL of whole blood was withdrawn by jugular or bracheal venipuncture using a 26-gauge, ½-inch subcutaneous needle and added to 0.9 mL of BA-1 diluent (Hanks M-199 salts, 0.05M Tris ph 7.6, 1% bovine serum albumin, 0.35 g/L sodium bicarbonate, 100 units/mL penicillin, 100 mg/mL streptomycin, 1 mg/mL Fungizone). Samples were permitted to coagulate at room temperature for 30 min, centrifuged at 7,000 rpm for 8 min, and frozen at -70°C. Cloacal and throat samples were also taken during the first 7 dpi by using cotton

swabs and dipping the infected swabs in 0.5 mL of BA-1 before freezing at -70°C. All inoculated hens were observed twice daily during the first 7 dpi of infection for signs of clinical illness. A final serum sample (0.6 mL of whole blood) was taken at 14 dpi to test for seroconversion by plaque-reduction neutralization test (PRNT) (5). A sample that neutralized the challenge dose of WNV-NY99 by at least 90% was considered positive. Three hens were maintained until 28 dpi to monitor the development of neutralizing antibodies during this period.

Infection of Mosquitoes

Colonized *Culex tritaeniorhynchus* mosquitoes were infected by intrathoracic inoculation of 700 nL of a suspension containing $10^{8.2}$ per mL WNV-NY99 (source *Cx. pipiens* pool #NY99-6480 collected in New York, 1999, 1 Vero passage, CDC accession no. B82123), and incubated for 7 to 10 days at 16:8 hours light:dark, 28°C, 80% relative humidity, before feeding on chickens. Successful infection of mosquitoes was confirmed by plaque assay of homogenates of whole mosquitoes (after incubation) or saliva extracted from mosquitoes after feeding (6).

Virus Titration and Identification

The concentration of WN virus infectious particles in fluids (including cloacal swabs, throat swabs, and blood samples) was evaluated by Vero plaque assay (5) of 10-fold serial dilutions. Plaques were counted after 3-5 days of incubation at 37°C, 5% CO_2. Plaques from swabs were harvested and identified by neutralization using a standard antiserum available from the Centers for Disease Control and Prevention reference collection in Fort Collins, CO.

Results

All of the 21 WNV-NY99 parenterally inoculated hens developed neutralizing antibodies and 20 of these had detectable viremia (Table 1). One of 16 in-contact hens had a transient WNV-NY99 viremia of magnitude $10^{2.4}$ PFU/mL on the third day after its cage mate had been injected, and seroconverted. None of nine orally inoculated hens developed WNV-NY99 viremia or antibodies. None of the 46 hens exposed to WNV-NY99 demonstrated overt clinical illness attributable to WN virus.

Three mosquito-infected hens were sampled more frequently (approximately twice per week) after the first week of infection to monitor the pattern of antibody response within 28 dpi (Figure). Neutralizing antibody was detected in one of the three birds as early as 7 dpi (reciprocal 90% neutralization titer = 10), and in all three at 10 dpi (titers = 40, 40, and 80). The titers increased steadily throughout this period, reaching 320, 80, and 160, respectively, by 28 dpi.

We determined the duration and magnitude of WNV-NY99 viremia in the 21 parenterally inoculated hens

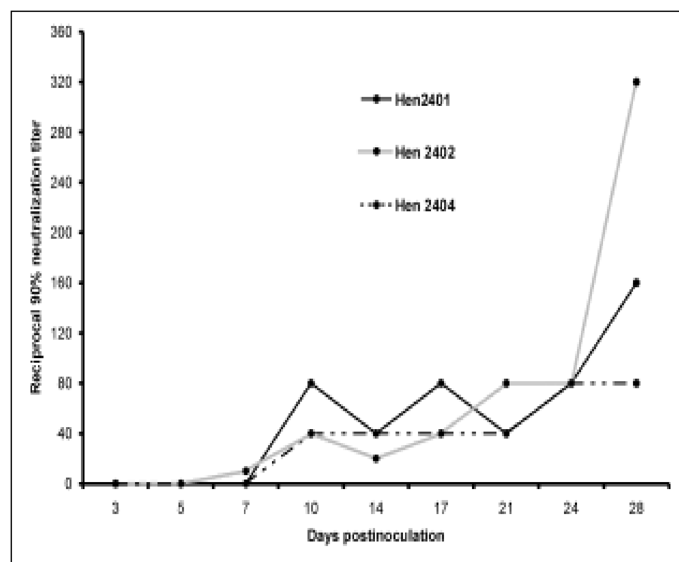

Figure. West Nile virus (WNV)-NY99 neutralizing antibody response in chickens.

Table 1. Viremia in West Nile virus (WNV)-NY99-infected chickens

Chicken ID#	Chicken Age(wk)	Infection mode	No. mosq. fed	Day postinoculation 1	2	3	4	5	Cage mate infection
1103	20	N	NA	--[a]	3.7	2.7	--	--	-
1108	20	N	NA	--	3.8	3.1	--	--	+
1110	20	N	NA	2.0[b]	5.0	3.3	--	--	-
2019	20	N	NA	--	2.3	3.4	2.1	--	-
2027	20	N	NA	1.7	3.4	2.3	--	--	-
1112	17	M	4-5	3.5	3.6	--	--	--	-
1114	17	M	4-5	3.0	3.4	--	--	--	-
1116	17	M	4-5	3.6	3.7	2.9	--	--	-
1118	17	M	4-5	3.6	3.4	--	--	--	-
1120	17	M	4-5	--	--	--	--	--	-
1122	17	M	4-5	--	--	3.0	2.8	--	-
2401	17	M	1	--	1.7	3.4	2.3	--	NT
2402	17	M	2	--	2.7	3.1	--	--	NT
2404	17	M	1	--	2.4	2.2	--	--	NT
2595	17	M	1	--	2.6	3.5	2.2	--	NT
2596	17	M	1	2.4	3.5	--	--	--	NT
1124	60	M	1	2.9	3.6	2.0	--	--	-
1126	60	M	1	--	4.0	3.4	--	--	-
1128	60	M	1	--	3.6	2.9	--	--	-
1132	60	M	1	--	3.9	2.8	--	--	-
1134	60	M	1	4.1	3.9	--	--	--	-

[a]Threshold of detection is 50 PFU/mL serum.
[b]$\log_{10}$ Vero PFU/mL serum.
N = needle; NA = not applicable; M = mosquito; NT = not tested.

(Table 1). All five hens inoculated by needle had detectable viremias that endured 2 to 3 days with mean peak viremia of $10^{3.9}$ PFU/mL (range $10^{3.4}$-$10^{5.0}$). Of the 16 hens inoculated by mosquito, 15 had detectable viremias that endured 2 to 3 days with mean peak viremia of $10^{3.4}$ (max $10^{4.1}$). No virus was detected in blood samples collected 6 and 7 dpi (data not shown).

Cloacal shedding of WNV-NY99 was observed in 12 of 21 (57%) parenterally inoculated hens (Table 2). All 5 of the needle-inoculated birds and all 5 of the 60-week-old mosquito-inoculated birds shed, whereas only 2 of 11 (18%) 17-week-old mosquito-inoculated birds shed. Positive cloacal swabs were observed 2-6 dpi. Peak cloacal swab positivity was 3-5 dpi. Shedding in oral exudates was observed in two of six 17-week-old hens. In these six birds, the number of plaques detected from throat swabs was generally less than that from cloacal swabs (Table 2). Viruses detected in swabs were identified as WN virus by PRNT and were reisolated from a subset of the positive swabs for confirmation of results. To evaluate the viability and stability of WNV-NY99 in fecal material outside the host, fecal urates of chickens were mixed with 100 PFU WNV-NY99. No negative effect of the fecal material was observed when compared with BA-1 diluent. However, viability was reduced by 99% after 24 hours at ambient temperature (data not shown).

Discussion

This study evaluated WNV-NY99 sentinel criteria for chickens by monitoring their response to experimental infection in captivity. We report for the first time quantitative data about WNV-NY99 viremias in chickens inoculated by mosquito bite. Turell (7,8) reported that chicks were infected with WN virus by mosquito bite, but data from these evaluations were not presented. The response of several bird species (including chickens, turkeys, and geese) to needle inoculation of this North American strain of WN virus has been documented (9-11). However, mosquito inoculation has been shown to elicit a different response to infection compared with needle inoculation in several vertebrate-virus systems (12,13).

Three central criteria for an arbovirus sentinel bird are susceptibility to infection, development of detectable antibodies, and survival. Birds that do not survive infection may be lost to surveillance programs designed to detect antibodies as a marker for infection. We found that all the chickens inoculated parenterally in our study, as in other WN virus infection studies in chickens (14,15), became infected, and survived to develop detectable neutralizing antibodies. Evaluation of alternative serodiagnostic assays for immunoglobulin M and hemagglutination-inhibiting antibodies are under way.

Birds used as sentinels for arbovirus surveillance should not contribute to the local arbovirus transmission cycle if they become infected. Detectable viremia in mature chickens (>3 weeks) is unusual for WN virus strains that have been studied previously (14,15), although young chicks do develop viremia $>10^5$ PF/mL (14, 7). Senne et al. (9) reported that WNV-NY99 needle-inoculated 7-week-old hens had viremia sufficient to infect mosquitoes, based on data from an experimental infection study of an African mosquito, *Cx. univittatus*, using an African strain of WN virus (cited in 16). However, new data do not support this statement. A study of vector competence of *Cx. pipiens* collected in New York and infected with WNV-NY99 suggests that the maximum viremia that we observed in the needle-inoculated hens (10^5 PFU/mL) is sufficient to infect about 17% of these mosquitoes; 2% will be able to transmit the virus in a subsequent bloodmeal (7). The maximum viremia detected in mosquito-inoculated hens reached $10^{4.1}$ and is probably well below the level required to maintain the *Cx. pipiens* transmission cycle. Although other species of mosquitoes may have lower thresholds of infection, *Cx. pipiens* is recognized as the important vector in the avian transmission cycle in the northeastern United States (17). Thus, our data imply that chickens are incompetent to retransmit WNV-NY99 to *Cx. pipiens* in New York. However,

Table 2. West Nile virus (WNV)-NY99 PFU in 0.5 mL cloacal or throat swabs of chickens

Chicken		Infection	No. mosq.	Day postinoculation						
ID#	Age (wk)	mode	fed	1	2	3	4	5	6	7
1103	20	N	NA	NT	NT	23[a]	0	NT	5	0
1108	20	N	NA	NT	NT	200	0	NT	0	0
1110	20	N	NA	NT	NT	28	95	NT	3	0
2019	20	N	NA	NT	NT	3	8	NT	0	0
2027	20	N	NA	NT	NT	10	5	NT	0	0
1112	17	M	4-5	0/3[b]	4/0	6/3	23/3	8/0	0/0	0/0
1114	17	M	4-5	0/0	0/0	0/0	0/0	0/0	0/0	0/0
1116	17	M	4-5	0/0	0/0	0/0	0/3	0/0	0/0	0/0
1118	17	M	4-5	0/0	0/0	4/0	1/0	0/0	3/0	0/0
1120	17	M	4-5	0/0	0/0	0/0	0/0	0/0	0/0	0/0
1122	17	M	4-5	0/0	0/0	0/0	0/0	0/0	0/0	0/0
2401	17	M	1	0	0	0	0	0	0	0
2402	17	M	2	0	0	0	0	0	0	0
2404	17	M	1	0	0	0	0	0	0	0
2595	17	M	1	0	0	0	0	0	0	0
2596	17	M	1	0	0	0	0	0	0	0
1124	60	M	1	0	0	0	0	5	NT	NT
1126	60	M	1	0	0	6	5	0	NT	NT
1128	60	M	1	0	0	0	0	1	NT	NT
1132	60	M	1	0	0	3	11	18	NT	NT
1134	60	M	1	0	3	9	24	3	NT	NT

[a]Data presented are from cloacal swabs unless otherwise indicated.
[b]Cloacal swab/nasopharyngeal swab.
N = needle; NA = not applicable; NT = not tested; M = mosquito.

we recognize that conditions may exist in which vector mosquitoes, including strains of *Cx. pipiens*, could theoretically have lower transmission thresholds that permit them to acquire WNV-NY99 infection from mature chickens.

Birds used as sentinels for arbovirus surveillance should not spread arbovirus infections directly to flock mates, because a finding of birds that are seropositive as a result of direct transmission (in the absence of mosquito vectors) would lead to misinterpretation of the true risk for mosquito-borne transmission. In our study, we observed one such transmission event (out of 16 trials). This transmission originated from a needle-inoculated hen. Experimental direct transmission (from needle-inoculated birds) has been observed with other WNV-NY99-infected species, including domestic goslings (11) and American Crows (R.G. McLean, pers. comm.), but not chicken pullets and turkey poults (9,10). The importance of direct transmission of WNV-NY99 among birds in nature remains unknown.

The means by which WNV-NY99 direct transmission among birds occurs may include inhalation of infectious aerosols due to viral shedding in bodily fluids such as fecal material and saliva, ingestion of contaminated food, or contact with viremic blood. The possibility of oral ingestion of WNV-NY99 was tested in nine chickens with negative results. We did, however, document the presence of infectious WNV-NY99 in oral exudates and feces. WNV-NY99 has been reported previously in cloacal swabs of needle-inoculated chicken pullets and turkey poults (9,10) but not goslings (11), and in oropharyngeal swabs of turkeys and goslings (10,11). We observed that the quantity of virus collected in swabs was relatively low (not exceeding 200 infectious virus particles in our preliminary evaluation) and that stability of WNV-NY99 in avian fecal material outside the host was reduced dramatically after 24 hours, suggesting that risk of transmission from infected feces decreases as the time outside the host increases. Because WN virus infection in humans exposed to viral shedding in birds has not been documented, the actual risk is unknown and can be reduced through proper recommended animal-handling techniques, such as the use of disposable gloves and HEPA-filtered masks.

Chickens should be evaluated as sentinels for detecting and monitoring enzootic WN virus transmission. Chickens have been used extensively for surveillance of Kunjin virus (a subtype of WN virus) in Australia (R. Russell, pers. comm.). Pre-existing flocks of domestic chickens were naturally exposed to WN virus in Bucharest in 1996 (37% seropositive) (18), New York City in 1999 (63%) (19), and eastern Suffolk County, NY, in 1999 (30%, S. Campbell, pers. comm.). Thus, based on these data, chickens would seem to be strong candidates for use as sentinels for WN virus.

In summary, we present the first experimental infection study of WNV-NY99 in chickens in which mosquito and oral transmission routes are evaluated. We found that WNV-NY99 viremia in chickens is probably insufficient to infect the primary epiornitic vector, *Cx. pipiens*. The observation of transmission to a hen in contact with a needle-inoculated WNV-NY99-infected hen requires further study on the risk of direct transmission among chickens and to their handlers by contaminated bodily fluids. This experimental infection study provides data that, in part, justify chickens as candidates for WN virus sentinels in North America.

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References

1. Komar N. West Nile viral encephalitis. Rev Sci Tech 2000;19:166-76.
2. Reisen WK, Hardy J, Presser S. Evaluation of domestic pigeons as sentinels for detecting arbovirus activity in southern California. Am J Trop Med Hyg 1992;46:69-79.
3. Morris CD, Baker WG, Stark L, Burgess J, Lewis AL. Comparison of chickens and pheasants as sentinels for eastern equine encephalitis and St. Louis encephalitis viruses in Florida. J Am Mosq Control Assoc 1994;10:545-8.
4. Reisen WK, Presser SB, Lin J, Enge B, Hardy JL, Emmons RW. Viremia and serological responses in adult chickens infected with western equine encephalomyelitis and St. Louis encephalitis viruses. J Am Mosq Control Assoc 1994;10:549-55.
5. Beaty BJ, Calisher CH, Shope RE. Arboviruses. In: Schmidt NJ, Emmons RW, editors. Diagnostic procedures for viral, rickettsial and chlamydial infections. 6th ed. Washington: American Public Health Association; 1989. p. 797-855.
6. Hurlbut HS. Mosquito salivation and virus transmission. Am J Trop Med Hyg 1966;15:989-93.
7. Turell MJ, O'Guinn M, Oliver J. Potential for New York mosquitoes to transmit West Nile virus. Am J Trop Med Hyg 2000;62:413-4.
8. Turell MJ, O'Guinn ML, Dohm DJ, Jones JW. Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. J Med Entomol 2001;38:130-4.
9. Senne DA, Pedersen JC, Hutto DL, Taylor WD, Schmitt BJ, Panigrahy B. Pathogenicity of West Nile virus in chickens. Avian Dis 2000;44:642-9.
10. Swayne DE, Beck JR, Zaki S. Pathogenicity of West Nile virus for turkeys. Avian Dis 2000;44:932-7.
11. Swayne DE, Beck JR, Smith C, Shieh W, Zaki S. Fatal encephalitis and myocarditis in young domestic geese (*Anser anser domesticus*) caused by West Nile virus. Emerg Infect Dis 2001;7:751-3.
12. Osorio JE, Godsey MS, Defoliart GR, Yuill TM. La Crosse viremias in white-tailed deer and chipmunks exposed by injection or mosquito bite. Am J Trop Med Hyg 1996;54:338-42.
13. Edwards JF, Higgs S, Beaty BJ. Mosquito feeding-induced enhancement of Cache Valley virus (Bunyaviridae) infection in mice. J Med Entomol 1998;35:261-5.
14. Taylor RM, Work TH, Hurlbut HS, Rizk F. A study of the ecology of West Nile virus in Egypt. Am J Trop Med Hyg 1956;5:579-620.
15. Parks JJ, Ganaway JR, Price WH. Studies on immunologic overlap among certain arthropod-borne viruses III. A laboratory analysis of three strains of West Nile virus which have been studied in human cancer patients. Am J Hyg 1958;68:106-19.
16. McIntosh BM, Jupp PG. Infections in sentinel pigeons by Sindbis and West Nile viruses in South Africa, with observations on *Culex (Culex) univittatus* (Diptera: Culicidae) attracted to these birds. J Med Entomol 1979;16:234-9.
17. Nasci RS, White DJ, Stirling H, Oliver J, Daniels TJ, Falco RC, et al. West Nile virus isolates from mosquitoes in New York and New Jersey, 1999. Emerg Infect Dis 2001;7:626-30.
18. Savage HM, Ceianu C, Nicolescu G, Karabatsos N, Lanciotti R, Vladimirescu A, et al. Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serologic and molecular characterization of a virus isolate from mosquitoes. Am J Trop Med Hyg 1999;61:600-11.
19. Komar N, Panella NA, Burns JE, Dusza SW, Mascarenhas TM, Talbot T. Serologic evidence for West Nile virus infection in birds in the New York City vicinity during an outbreak in 1999. Emerg Infect Dis 2001;7:621-5.

Widespread West Nile Virus Activity, Eastern United States, 2000

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In 1999, the U.S. West Nile (WN) virus epidemic was preceded by widespread reports of avian deaths. In 2000, ArboNET, a cooperative WN virus surveillance system, was implemented to monitor the sentinel epizootic that precedes human infection. This report summarizes 2000 surveillance data, documents widespread virus activity in 2000, and demonstrates the utility of monitoring virus activity in animals to identify human risk for infection.

In August and September 1999, an epidemic of encephalitis and aseptic meningitis caused by West Nile (WN) virus occurred in New York City (1-3). This epidemic was preceded by anecdotal reports of an extensive die-off among American Crows (*Corvus brachyrhynchos*) and several other bird species in the most affected boroughs of New York City (1-3). The WN virus epidemic in the northeastern United States in 1999 underscores the ease with which an emerging arthropod-borne flavivirus and human pathogen can become established in a new geographic area. In addition, the occurrence of a widespread epizootic as a sentinel event that precedes human infection emphasizes the importance of establishing ecologic surveillance to identify conditions that might result in human infections.

In 1999, establishment of enhanced human and animal infection surveillance was recommended in states either affected in 1999 or at higher risk for becoming affected because of bird migration patterns (4). New York City, the District of Columbia, 20 states along the Atlantic and Gulf coasts, and the Centers for Disease Control and Prevention (CDC) developed and implemented ArboNET, a cooperative WN virus surveillance system designed to provide data to monitor the geographic and temporal spread of WN virus in the United States; to identify areas at increased risk for human infections with WN virus; to develop strategies to prevent WN virus infections in humans or animals or to minimize the number of these infections once an outbreak occurs; and to determine the distribution and incidence of the other domestic arboviruses.

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To accomplish these goals, cooperating jurisdictions performed the following surveillance activities: bird surveillance monitoring, including deaths and seroprevalence among wild birds and seroconversion among sentinel chicken flocks; mosquito surveillance; enhanced equine and nonhuman mammal surveillance; and enhanced passive or active human surveillance (5). The same system collected data regarding confirmed and probable WN virus-infected humans, nonhuman vertebrates, and mosquitoes, in addition to the number of specimens from each species that were collected and tested.

This report summarizes the findings of surveillance data collected in 2000, which document widespread WN virus activity throughout the eastern United States and the utility

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of monitoring WN virus activity in birds and mosquitoes to identify areas at increased risk for human infection.

Methods

This summary includes surveillance data for 2000 that were collected from 20 states (Alabama, Connecticut, Delaware, Florida, Georgia, Louisiana, Maine, Maryland, Massachusetts, Mississippi, New Hampshire, New Jersey, New York, North Carolina, Pennsylvania, Rhode Island, South Carolina, Texas, Vermont, and Virginia), New York City, and the District of Columbia. All states began to submit surveillance data for May 2000 except New York (started with January 2000 data), Vermont (started with June 2000 data), and New Hampshire (started with July 2000 data). Except for Louisiana, New Hampshire, and Maine, which stopped submitting data in October 2000, all other states collected data at least through mid-November 2000.

Data about surveillance activities were gathered by counties in these 20 states and forwarded to a state WN virus surveillance coordinator. At the state level, data aggregated by county and by week of bird report, specimen collection, or illness onset were entered into a standardized database and electronically reported to CDC weekly. Types of data included the numbers of dead crows and dead birds of other species reported by county residents; crows and birds of other species that were tested for evidence of WN virus infection; mosquitoes of a specific species that had been collected; wild birds that were trapped and bled to determine the prevalence of recently developed antibody against WN virus; sentinel chickens that had been bled to identify seroconversion following recent WN virus infection; and ill or dead humans, horses, and other mammals from which a tissue or serum sample had been submitted to determine if illness or death was attributable to WN virus infection.

In addition, humans, nonhuman vertebrates, and mosquitoes with documented WN virus infections were reported continuously to CDC by telephone, facsimile, or e-mail from the 20 states, New York City, and the District of Columbia. Reports were submitted either directly from the state public health laboratory or the WN virus surveillance group. The methods used to document infection differed by state, species, and the type of tissue tested (5). In mosquitoes and nonhuman vertebrates, testing included combinations of reverse-transcription polymerase chain reaction or real-time (TaqMan) polymerase chain reaction to identify WN virus genome in tissue or cerebrospinal fluid (CSF); immunofluorescent or immunohistochemistry studies to demonstrate WN virus antigen in tissue; virus culture from tissue or serum; or serology testing using immunoglobulin (Ig) M-capture enzyme-linked immunosorbent assay (MAC-ELISA) or plaque-reduction neutralization test (PRNT) to identify WN virus-specific antibodies that demonstrate recent infection. In ill humans, WN virus infections were confirmed by isolating WN virus from or demonstrating WN viral antigen or genomic sequences in tissue, blood, CSF, or other body fluid; demonstrating IgM antibody to WN virus in CSF by MAC-ELISA; demonstrating a fourfold serial change in PRNT antibody titer to WN virus in paired, appropriately timed serum samples; or demonstrating both WN virus-specific IgM by MAC-ELISA and IgG antibody in a single serum specimen by various methods. The county, state, specific species, and the week of bird report, specimen collection, or illness onset that corresponded to each reported WN virus-infected human, nonhuman vertebrates, or mosquito were also collected.

Results

Humans

In 2000, 21 persons in the northeastern United States were reported with acute illness attributed to WN virus infection; 19 were hospitalized with severe neurologic illness (12 with encephalitis, 4 with meningitis, and 3 with meningoencephalitis). Of the 19 hospitalized patients, 2 (11%) died. Of the 21 patients, 10 lived in the Staten Island Borough (Richmond County) of New York City (Figure 1). Other patients lived in nine other counties: Kings (Brooklyn), New York (Manhattan), and Queens counties in New York; Hudson, Passaic, Monmouth, Morris, and Bergen counties in New Jersey; and Fairfield County in Connecticut. Patients were 36 to 87 years of age (median 62 years); 13 (62%) were men. Dates of illness onset were from July 20 to September 27 (Figure 2). The peak incidence occurred the week starting August 26, during which five WN virus-infected persons had onset of illness.

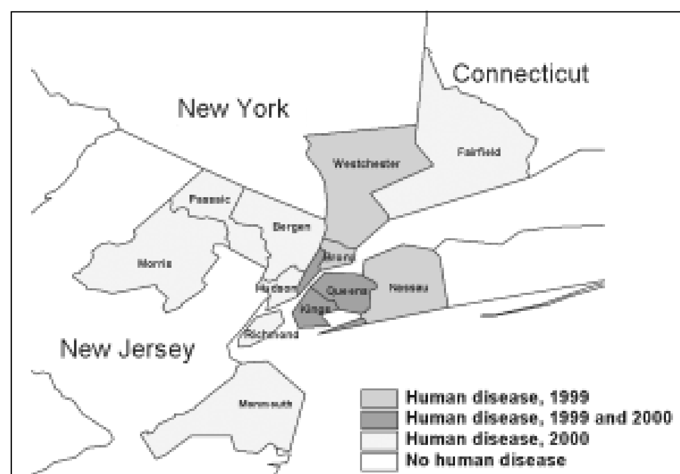

Figure 1. New York and New Jersey counties reporting human illness caused by West Nile virus infection in 1999 (62 cases in 6 counties) and 2000 (21 cases in 10 counties).

Ecologic Surveillance and Human Illness

In all 10 counties subsequently reporting human cases in 2000, a WN virus-infected bird was found an average of 44 days (range 15 to 92 days) before the illness onset date of the first human case (Table 1). In 8 of the 10 counties, infected mosquito pools were collected an average of 32 days (4 to 54 days) before the illness onset date. In the other two counties, no infected mosquito pools were found in 2000 despite intensive collection efforts. Similarly, in the 10 counties that reported human illnesses caused by WN virus infection, the number of dead and ill birds reported by residents increased many weeks before the first human cases (Figure 2).

Crows and Other Birds

In 2000, residents in 321 counties in 16 states reported at least one dead bird to their local or state health department, for a total of 104,816 dead birds (30,601 crows and 74,215

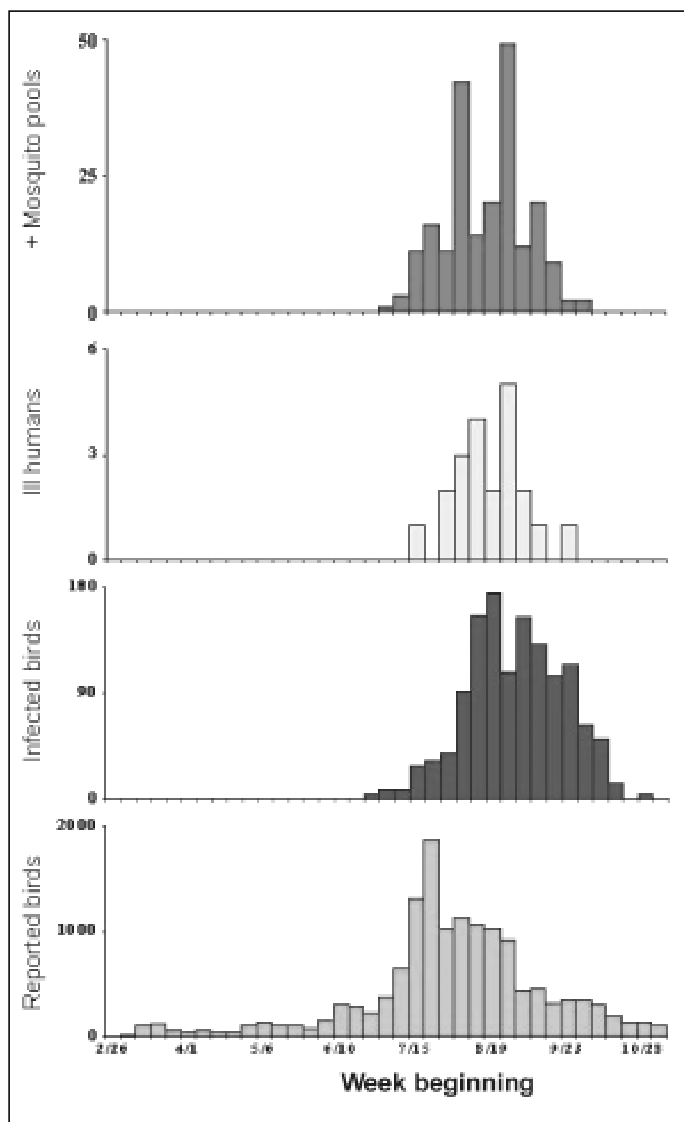

Figure 2. Number of reported dead or ill birds, West Nile (WN) virus-infected birds, human illnesses caused by WN virus infection, and WN virus-infected mosquito pools reported from 10 counties with human cases, United States, 2000.

Table 1. Onset of human illness in 10 counties, in relation to collection of the first West Nile (WN) virus-infected bird and the first WN virus-infected mosquito pool

		No. of days before onset of human illness	
County (no. of human cases	Illness onset first human case	First infected bird	First infected mosquito pool
Bergen, NJ (1)	August 31	92	44
Fairfield, CT (1)	August 25	51	45
Hudson, NJ (2)	August 6	24	*
Kings, NY (2)	August 15	15	4
Monmouth, NJ (1)	September 27	67	37
Morris, NJ (1)	August 26	20	*
New York, NY (1)	August 31	39	50
Passaic, NJ (1)	September 3	41	6
Queens, NY (1)	September 13	72	54
Richmond, NY (10)	July 20	15	13

[a]No infected mosquito pools identified in 2000.

other birds). Of these 104,816 reported birds, 12,961 (12.4%) were submitted for WN virus testing; 4,305 (33.2%) were WN virus infected. Of the 7,580 crows tested, 3,824 (50.4%) were infected, compared with 481 (8.9%) of 5,381 birds of other species tested.

Epizootic activity in birds was widespread (Figure 3). WN virus-infected dead birds were reported from 136 counties in 12 states and the District of Columbia (New York reported 1,263 birds; New Jersey 1,280; Connecticut 1,118; Massachusetts 449; Rhode Island 87; Maryland 50; Pennsylvania 36; New Hampshire 7; Virginia 7; Delaware 1; North Carolina 1; Vermont 1; and the District of Columbia, 5). Crows and related corvid species were the most frequently reported WN virus-infected species. Of the 4,305 reported WN virus-infected birds, 3,824 (88.8%) were *Corvus* species (American Crow, Fish Crow [*C. ossifragus*], Common Raven [*C. corax*]), and 196 (4.6%) Blue Jays (*Cyanocitta cristata*) (Table 2). The remaining 285 (6.6%) reported, WN-virus-infected birds included 59 other bird species. Dead WN virus-infected birds were found over a 9-month period (from a Red-tailed Hawk [*Buteo jamaicensis*] found in Westchester County, New York, on February 6 to an American Crow found

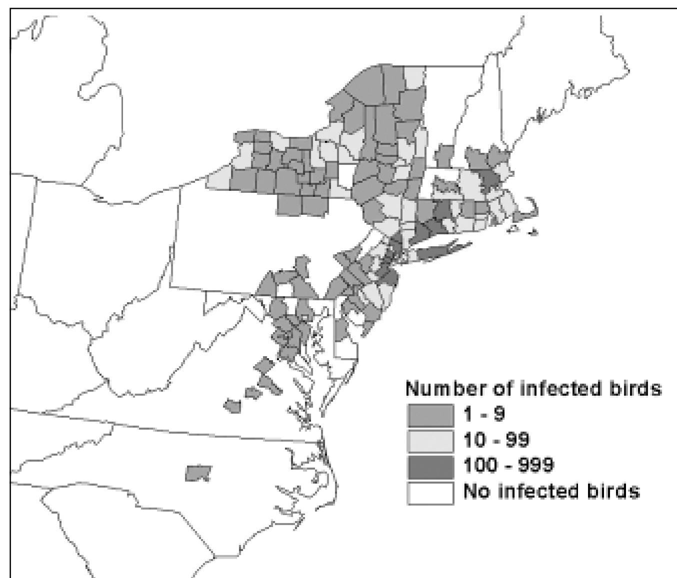

Figure 3. U.S. counties reporting West Nile virus-infected birds, 2000.

Table 2. Species and genera of West Nile virus-infected birds reported to ArboNET in 2000[a]

Species/genus	Common name	No. reported	% of all infected birds
Corvus spp.	Crows	3,824	88.8
Cyanocitta cristata	Blue Jays	196	4.6
Accipiter and *Buteo* spp.	Hawks	30	0.7
Bonasa umbellus	Ruffed Grouse	27	0.6
Larus spp.	Gulls	26	0.6
Passer domesticus	House Sparrows	20	0.5
Turdus migratorius	American Robins	20	0.5
Zenaida macroura	Mourning Doves	17	0.4
Falco spp.	Falcons	14	0.3
46 other species	Mixed	131	3.0

[a]New Jersey collected and tested only *Corvus* species during 2000.

on November 17 in Barnstable County, Massachusetts). However, of the 4,305 ill or dead birds confirmed to have WN virus infection, 3,637 (84.5%) were found from July 1 through September 30.

Mosquitoes

WN virus was isolated from or WN virus gene sequences were detected in 515 mosquito pools in 38 counties in five states: 393 pools in New York, 58 in New Jersey, 46 in Pennsylvania, 14 in Connecticut, and 4 in Massachusetts (Figure 4). Of the infected pools, *Culex* species accounted for 428 (89.2%), including 228 pools of *Cx. pipiens/restuans*, 146 of *Cx. pipiens*, 50 of *Cx. salinarus*, 12 of *Cx. restuans*, and 26 unspecified *Culex* pools (Table 3). *Ochlerotatus* species (formerly in *Aedes* genus) accounted for 29 WN virus-infected pools (including 9 of *Oc. japonicus*, 9 of *Oc. triseriatus*, and 8 of *Oc. trivittatus*), and *Aedes* species accounted for 19 WN virus-positive pools (including 17 pools of *Ae. vexans*). In 2000, by nucleic acid amplification techniques, WN virus genome was identified in at least one pool of all 14 species. Despite attempts to isolate virus from at least one pool of all 14 species, no viral isolate was obtained from three species (*Ae. albopictus*, *Oc. atropalpus*, and *Anopheles punctipennis*).

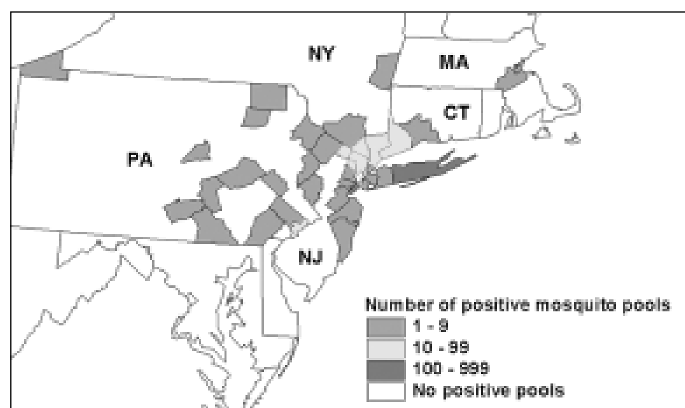

Figure 4. U.S. counties reporting West Nile virus-infected pools of mosquitoes, 2000.

Table 3. Number of West Nile (WN) virus-infected mosquito pools reported to ArboNET in 2000, by species

Species	No. of pools	Method to identify WN virus in ≥1 pool: RT-PCR[a]/ TaqMan	Method to identify WN virus in ≥1 pool: Virus culture
Culex pipiens/restuans	226	--	--
Cx. pipiens	146	Yes	Yes
Cx. salinarius	50	Yes	Yes
Cx. restuans	12	Yes	Yes
Unspecified *Culex* spp.	26	--	--
Aedes vexans	17	Yes	Yes
Ae. albopictus	1	Yes	No
Unspecified *Aedes* spp.	1	--	--
Ochleratatus japonicus	9	Yes	Yes
Oc. triseriatus	9	Yes	Yes
Oc. trivittatus	8	Yes	Yes
Oc. atropalpus	1	Yes	No
Oc. canadensis	1	Yes	Yes
Oc. cantator	1	Yes	Yes
Anopheles punctipennis	1	Yes	No
Culiseta melanura	3	Yes	Yes
Psorophora ferox	1	Yes	Yes

[a]RT-PCR= reverse transcription-polymerase chain reaction.

For the most commonly identified infected mosquito species, collections during the week beginning August 26 yielded the peak number of WN virus-infected mosquito pools (Figure 5). Of 386 positive pools of *Cx. pipiens* or *Cx. restuans* collected during the 2000 transmission season (July 7 to November 4), 63 (16.3%) were collected in this week. Of 50 positive pools of *Cx. salinarius* collected in 2000, 8 (16%) were collected this week, and of 48 positive pools of *Aedes* or *Ochlerotatus*, 11 (23%) were collected this week.

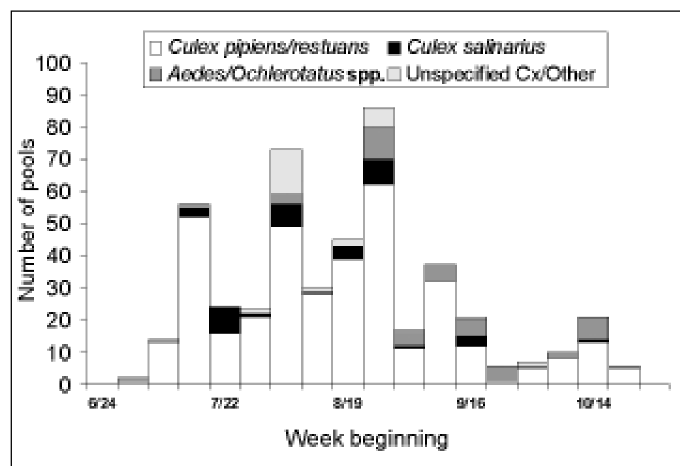

Figure 5. West Nile virus-infected mosquito pools from five northeastern states, by collection week and species group, 2000.

Other Surveillance Components

Veterinary surveillance identified WN-virus infections in 63 horses with neurologic disease from 26 counties in 7 states (28 horses in New Jersey; 21 in New York; 7 in Connecticut; 4 in Delaware; and 1 each in Massachusetts, Pennsylvania, and Rhode Island). Illness onsets were from August 17 to November 1, with a peak of 15 horses with onsets during the week of October 7.

In addition, WN infection was confirmed in six other mammals. Of these, five mammals (big brown bat, *Eptesicus fuscus*; little brown bat, *Myotis lucifugas*; eastern chipmunk, *Tamias striatus*; eastern gray squirrel, *Sciurus carolinenesis*; and domestic rabbit, *Oryctologus cuniculus*) were from four counties (Albany, Columbia, Bronx, and Rensselaer) in New York State and one (eastern striped skunk, *Mephitis mephitis*) was from Fairfield County, Connecticut. All were ill; they were collected from August 31 to September 30.

Seroconversion consistent with recent WN virus infection was documented in 13 sentinel chickens in six counties. In Essex, Sussex, Middlesex, and Morris counties, New Jersey, serum samples were drawn from September 27 to 29; in Westchester and Kings (Brooklyn) counties, New York, samples were collected from August 23 to November 3.

Conclusion

Although WN virus was first indentified in metropolitan New York City in 1999, surveillance data submitted to the ArboNET WN virus surveillance system have shown a

widespread geographic range of virus activity in 2000. Epizootic activity in birds was reported from nine jurisdictions without recognized WN virus activity in 1999 (District of Columbia, Delaware, Massachusetts, New Hampshire, North Carolina, Pennsylvania, Rhode Island, Vermont, and Virginia), as well as the four states that reported activity in 1999 (Connecticut, Maryland, New Jersey, and New York). Similarly, human illnesses attributable to WN virus infection in 2000 were reported from seven counties without identified human illnesses in 1999, as well as three of the six counties that reported human illnesses in 1999.

Despite the widespread virus activity and regional intensification of surveillance activities, 21 acute human illnesses attributable to WN virus infection were identified in 2000, compared with 62 in 1999. Although some decrease in severe human illness may be attributable to vector control and other prevention activities, experience in Europe shows that incidence of human illness can be variable and outbreaks sporadic. Because widespread WN virus epizootic activity probably will persist and expand in the United States, large outbreaks of illness attributable to WN virus infection are possible if adequate surveillance, prevention activities, and mosquito control are not established and maintained.

The large number of avian deaths, particularly among highly recognizable and common birds such as the American Crow, has provided a unique view of a widespread and possibly expanding epizootic from a newly introduced flavivirus. However, a more important question is to what extent avian deaths and mosquito surveillance can serve as early warning sentinels of epizootic activity, so that increased prevention and intervention activities can be implemented before human infections occur. In 2000, all 21 patients had illness onsets at least 15 days after WN virus-infected birds were first collected in the county of residence, suggesting that avian data may be a sensitive indicator of the level of activity associated with subsequent human disease. However, the occurrence of an infected bird in a county was a relatively poor predictor of human illness. Of 136 counties reporting WN virus-infected birds in 2000, 10 (7%) reported humans with illness due to WN virus infection. Further research to identify threshold levels with greater positive predictive value should be undertaken.

The presence of WN virus-infected mosquito pools may be a less sensitive indicator of epizootic activity associated with subsequent human disease. In 2000, 14 of the 21 patients had illness onsets at least 15 days after WN virus-infected mosquito pools were first collected in their county of residence. However, 8 (21%) of the 38 counties with positive mosquito pools reported at least one ill person. Further analysis of 2000 surveillance data, including an assessment of the timing, number, and geographic location of WN virus-infected birds, and an assessment of mosquito-trapping activities, infection rates, and species identified are required to further interpret these data and refine their use.

The avian deaths and mosquito-based surveillance data from the northeastern United States in 2000 indicate that these surveillance modalities may have greater utility as an early warning system for human infections than surveillance among horses and other nonhuman mammal species. Although documented infections among crows occurred as early as April, most reported WN virus illnesses in horses and small mammals occurred relatively late compared with human illnesses. The horse epizootic peaked 6 weeks later and persisted 5 weeks longer than the human epidemic. Similarly, although few infected small mammals were reported, these also occurred relatively later than human illnesses. More data are needed to determine the reasons for this relative delay in horses and small mammals, and, as the epizootic expands, further evaluation of these surveillance modalities in other regions of North America will be required.

The persistence of widespread WN virus activity in 2000 indicates the need for expanded surveillance and prevention activities. In 2001, enhanced ecologic surveillance should be a high priority for states that have been affected or at high risk for being affected by WN virus (6). States with potential for WN virus activity should establish the following: 1) surveillance systems to receive reports of dead and ill crows and other corvids and to collect and test these reported specimens; 2) rapid mosquito surveillance in response to reports of dead WN virus-infected birds to identify potential mosquito vectors, especially those with a propensity to feed on mammals, and to monitor the population densities of those vectors; and 3) enhanced passive surveillance for neurologic disease in horses and other animals to monitor the degree of WN virus transmission outside the bird-mosquito cycle.

Depending on the geographic location of the state, this surveillance should be implemented in the spring and continued until late fall (for states where mosquito activity will cease because of cold weather) or through the winter (for southern states where mosquito activity may be continuous throughout the year).

Even before the recognition of WN virus activity, prevention activities in these states should include programs to 1) eliminate mosquito-breeding habitats in public areas; 2) control mosquito larvae where these habitats cannot be eliminated; 3) promote the increased use of personal protection and reduce peridomestic conditions that support mosquito breeding; and 4) implement adult mosquito control when indicated by increasing WN virus activity or the occurrence of human disease. In addition, because arbovirus infections are endemic in the United States, jurisdictions should have a comprehensive plan and a functional arbovirus surveillance and response capacity that includes trained personnel with suitable laboratory support for identifying arbovirus activity, including WN virus.

In summary, WN virus activity was widespread and possibly expanding in 2000. Although the coordinated, multistate surveillance effort may have led to a wider recognition of epizootic activity in 2000, reports of equine cases from counties that were not affected in 1999 and the large number of reported and WN virus-infected birds strongly suggest that a true expansion occurred. Because of the success of this system in accomplishing its goals, this coordinated, multistate surveillance effort will be expanded in 2001 to include all the continental United States.

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References

1. Centers for Disease Control and Prevention. Outbreak of West Nile-like viral encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:845-9.
2. Centers for Disease Control and Prevention. Update: West Nile viral encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:944-6, 955.
3. Asnis DS, Conetta R, Teixeira AA, Waldmon G, Sampspon BA. The West Nile virus outbreak of 1999 in New York: the Flushing Hospital experience. Clin Infect Dis 2000;30:413-8.
4. Centers for Disease Control and Prevention. Guidelines for surveillance, prevention, and control of West Nile virus infection. MMWR Morb Mortal Wkly Rep 2000;49:25-8.
5. Centers for Disease Control and Prevention. Human West Nile virus surveillance—Connecticut, New Jersey, and New York. MMWR Morb Mortal Wkly Rep 2001;50;265-8.
6. Centers for Disease Control and Prevention. Epidemic/epizootic West Nile virus in the United States: Revised Guidelines for Surveillance, Prevention, and Control (2001). Available from: URL: http://www.cdc.gov/ncidod/dvbid/westnile/resources/WN virus-guidelines-apr-2001.pdf

Exposure of Domestic Mammals to West Nile Virus during an Outbreak of Human Encephalitis, New York City, 1999

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We evaluated West Nile (WN) virus seroprevalence in healthy horses, dogs, and cats in New York City after an outbreak of human WN virus encephalitis in 1999. Two (3%) of 73 horses, 10 (5%) of 189 dogs, and none of 12 cats tested positive for WN virus-neutralizing antibodies. Domestic mammals should be evaluated as sentinels for local WN virus activity and predictors of the infection in humans.

An outbreak of West Nile (WN) viral encephalitis in New York City during the summer of 1999 resulted in numerous human cases and several deaths of elderly patients (1). WN virus, a mosquito-borne flavivirus, had not previously been recognized in New York City; therefore, the city's public health system had no surveillance guidelines in place before the outbreak. Such guidelines require basic epidemiologic and ecologic data. Entomologic studies (2), avian seroprevalence studies (3), and a human serosurvey (4) have been completed. This report presents seroprevalence data in New York City horses, dogs, and cats and discusses the potential of these animals as sentinels for human infection.

The Study

Serum samples were collected, by standard procedures, from healthy stabled horses and from stray dogs at animal shelters in each of the five New York City boroughs (counties) from September 15 to November 1, 1999. Additionally, samples from healthy, privately owned dogs and cats were obtained from veterinary practices in Queens and neighboring communities to the east in Nassau County during routine clinic visits. Serum samples were processed and tested for neutralizing antibodies to WN virus and St. Louis encephalitis (SLE) virus (a closely related flavivirus) as in a previous study (3), except that only samples positive for WN virus-neutralizing antibodies were also tested for SLE virus-neutralizing antibodies. Samples with reciprocal 90% neutralization titers of $\geq$10 were considered positive for flavivirus infection. However, a fourfold difference in titer for one of the two flaviviruses was required for a specific flavivirus to be considered an etiologic agent of the infection. Approximate ages were recorded for each animal sampled.

Neutralizing antibodies to WN virus were detected in horses and dogs in several boroughs (Tables 1, 2). Overall, 2 (3%) of 73 horses and 10 (5%) of 189 dogs, tested positive for WN virus-neutralizing antibodies. All 12 cats tested negative. Reciprocal titers were 80 to $\geq$ 320 for dogs and $\geq$ 320 for both positive horses. In all cases, WN antibody titers were at least fourfold higher than SLE titers (data not shown). Thus, all these infections were attributed to WN virus, and none were attributed to SLE virus. All but one of the 12 infections were in animals from the Queens and Bronx boroughs. The seroprevalence in dogs from these two counties was determined within several age categories (Table 3) to examine whether the pattern of seropositivity in relation to age resembled enzootic or epizootic transmission. More dogs were exposed in the youngest age category than in older categories (p = 0.026, Fisher exact test).

Dogs from Queens were further analyzed for the effect of stray status versus pet status (data not shown). Strays had

Table 1. West Nile virus-neutralizing antibodies in horses, by county

County	Total tested	No. pos. (% [95% CI])
Queens	18	1 (5.6 [0.1-27.3])
Bronx	19	1 (5.3 [0.1-26.0])
Richmond	6	0
Kings	10	0
New York	20	0
Total	73	2 (2.7 [0.3-9.5])

CI: confidence interval.

Table 2. West Nile virus-neutralizing antibodies in dogs, by county

County	Total tested	No. pos. (% [95% CI])
Queens	55	6 (10.9 [4.1-22.2])
Bronx	25	3 (12.0 [2.5-31.2])
Richmond	20	1 (5.0 [0.1-24.9])
Kings	22	0
New York	21	0
Nassau	46	0
Total	189	10 (5.3 [2.6-9.5])

CI: confidence interval.

Table 3. West Nile virus-neutralizing antibodies in dogs from Queens and the Bronx, by age

Age	Total tested	No. pos. (% [95% CI])
<2 yrs	43	7 (16.3 [6.8-30.7])
2-4	20	1 (5.0 [0.1-24.9])
>4 yrs	16	1 (6.3 [0.1-30.2])
Unknown	1	0
Total	80	9 (11.2 [5.3-20.3])

CI: confidence interval.

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WN virus seroprevalence of 15% (3 of 20), whereas pets had 8.6% (3 of 35). Although strays had higher seroprevalence than pets, the difference was not significant (p = 0.657, Fisher exact test).

Conclusions

We present serologic evidence of WN virus infection in New York City horses and dogs in 1999. In epidemiologic studies in the Middle East and Africa, WN virus-infected horses and dogs have been frequently detected in serologic surveys (5,6). Severe disease caused by WN virus in dogs is unknown, but epizootics of WN encephalomyelitis in horses have been described in several countries (7-9). Such an epizootic in New York horses (10) was observed concurrently with our study, with cases clustered in eastern Suffolk County. At least one equine case of WN encephalitis occurred close to New York City in Belmont, Nassau County.

Cats were not adequately sampled to determine valid seroprevalence figures. We were unable to find reference to any other serologic surveys in cats for WN virus antibodies. Cats appear to be "refractory" to infection with SLE virus (11), a common North American flavivirus, because they do not generate a humoral immune response after experimental infection and have not been found to develop antibodies in field serosurveys. Cats do develop antibodies to Powassan virus, another North American flavivirus (12). WN virus was isolated from brain tissue of a cat with neurologic symptoms in New Jersey in 1999 (13). Detectable levels of neutralizing antibodies did not develop in this cat before euthanasia (Centers for Disease Control and Prevention, unpub. data). The role of cats in the epidemiology of WN virus in the New York City region has yet to be determined.

The outbreak of WN virus in New York City and vicinity in 1999 was the first recorded instance of natural WN virus activity in the New World. The definitive date of WN virus introduction to the United States has not yet been established. We evaluated WN seroprevalence in dogs of different ages to determine if prevalence of infection increased with age, which would suggest that WN virus transmission in New York City dogs may have occurred before 1999. However, seroprevalence appeared weighted toward younger animals. This may reflect a skewing of the younger age groups by stray dogs, which probably are more likely to be exposed to biting mosquitoes. Our results support the hypothesis that WN virus was introduced in 1999, although they do not prove it.

Both dogs and horses are presumably dead-end hosts in the WN virus transmission cycle, which involves mosquito vectors and avian reservoir hosts (13). Although infection studies in horses with the New York strain of WN virus have not yet been published, such studies are expected to reaffirm the findings of previous studies (14,15) that these animals develop very low, ephemeral viremia insufficient for infecting mosquitoes (J. Lubroth, R. Bowen, pers. comm.). An infection study in dogs using an African strain of WN virus yielded similar findings (6). Thus, the presence of horses and dogs seropositive for WN virus does not necessarily indicate a human risk for WN infection. In fact, these horses and dogs may offer protection (zooprophylaxis) because they may divert potentially infectious bites of mammalophilic mosquitoes away from human hosts.

Further evidence of a zooprophylactic effect from pet dogs and horses is that seroprevalence in humans was approximately 2.5% in the epicenter of the outbreak in northeastern Queens (4), substantially lower than seroprevalence in dogs or horses in Queens (Table 1). The higher seroprevalence in these species indicates greater exposure to infectious mosquito bites. Whether this exposure in dogs and horses is due simply to increased time spent outdoors at night (when *Culex* species mosquitoes are feeding), greater attractiveness to blood-seeking mosquitoes, or other factors is unknown. Nonetheless, the finding that WN virus antibodies are readily detected in dogs and horses suggests a possible role for these animals as sentinels for human risk due to WN virus transmission.

Sentinels for human infection are frequently used in public health programs for monitoring arbovirus. Typically chickens and wild birds (e.g., house sparrows) have been used as sentinels for mosquito-borne monitoring programs in the United States (16-18) because of the role of birds as primary reservoir hosts for many of these viruses, including WN virus. However, whereas infection in birds may signal enzootic virus activity in birds, it may provide a less effective warning for risk in mammals. Such risk occurs when certain species of mosquitoes that act as "bridge" vectors to mammals become abundant. Thus, using nonhuman mammals as sentinels for human infection may have merit for effective risk management programs. Indeed, horses have been used as public health sentinels for both eastern and western equine encephalitis viruses, mosquito-borne agents of human encephalitis in the United States (19,20).

In summary, the finding that approximately 5% of horses and 10% of dogs were infected with WN virus in certain boroughs within New York City (The Bronx and Queens) in 1999 suggests a possible role for these domestic animals as sentinels for WN virus infection in humans. Seroconversions in these animals may signal increased risk for WN virus transmission to other mammals, including humans. An age-stratified analysis of seroprevalence data in dogs from boroughs of Queens and the Bronx found no evidence of a long-term pattern of infection in dogs, suggesting that all infections probably occurred as a result of the 1999 outbreak.

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References

1. Centers for Disease Control and Prevention. Update: West Nile virus encephalitis—New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:944-6, 955.
2. Nasci RS, White DJ, Stirling H, Oliver J, Daniels TJ, Falco RC, et al. West Nile virus isolates from mosquitoes in New York and New Jersey, 1999. Emerg Infect Dis 2001;7:626-30.

3. Komar N, Panella NA, Burns J, Dusza S, Mascarenhas TM, Talbot T. Serologic evidence for West Nile virus infection in birds in the New York City vicinity during an outbreak in 1999. Emerg Infect Dis 2001;7:621-5.
4. Centers for Disease Control and Prevention. Serosurveys for West Nile virus infection—New York and Connecticut counties, 2000. MMWR Morb Mortal Wkly Rep 2001;50:37-9.
5. Hayes CG. West Nile fever. In: Monath TP, editor. The arboviruses: epidemiology and ecology. Vol V. Boca Raton (FL): CRC Press; 1998. p. 59-88.
6. Blackburn NK, Reyers F, Berry WL, Shepherd AJ. Susceptibility of dogs to West Nile virus: a survey and pathogenicity trial. J Comp Pathol 1989;100:59-66.
7. Joubert L, Oudar J, Hannoun C, Beytout D, Corniou B, Guillon JC, et al. Epidemiology of the West Nile virus: study of a focus in Camargue. IV. Meningo-encephalomyelitis of the horse. Annals of the Institute Pasteur (Paris) 1970;118:239-47.
8. Tber AA. West Nile fever in horses in Morocco. Bulletin of the Office International des Epizooties;1996;108:867-9.
9. Cantile C, Di Guardo G, Eleni C, Arispici M. Clinical and neuropathological features of West Nile virus equine encephalomyelitis in Italy. Equine Vet J 2000;32:31-5.
10. Office International des Epizooties. West Nile fever in the United States of America in horses. Disease Information 1999;12:150-1.
11. McLean RG, Bowen GS. Vertebrate hosts. In: Monath TP, editor. St. Louis encephalitis. Washington: American Public Health Association; 1980. p. 381-450.
12. Keane DP, Parent J, Little PB. California serogroup and Powassan virus infection of cats. Can J Microbiol 1987;33:693-7.
13. Komar N. West Nile viral encephalitis. Rev Sci Tech 2000;19:166-76.
14. Taylor RM, Work TH, Hurlbut HS, Rizk F. A study of the ecology of West Nile virus in Egypt. Am J Trop Med Hyg 1956;5:579-620.
15. Schmidt JR, El Mansoury HK. Natural and experimental infection of Egyptian equines with West Nile virus. Ann Trop Med Parasitol 1963;57:415-27.
16. McLean RG, Mullenix J, Kerschner J, Hamm J. The house sparrow (*Passer domesticus*) as a sentinel for St. Louis encephalitis virus. Am J Trop Med Hyg 1983;32:1120-9.
17. Day JF, Stark LM. Avian serology in a St. Louis encephalitis epicenter before, during, and after a widespread epidemic in south Florida, USA. J Med Entomol 1999;36:614-24.
18. Reisen WK, Lundstrom JO, Scott TW, Eldridge BF, Chiles RE, Cusack R, et al. Patterns of avian seroprevalence to western equine encephalomyelitis and Saint Louis encephalitis viruses in California, USA. J Med Entomol 2000;37:507-27.
19. Goldfield M, Sussman O, Black HC, Horman JT, Taylor BF, Carter WC, et al. Arbovirus infection of animals in New Jersey. J Am Vet Med Assoc 1968;153:1780-7.
20. Potter ME, Currier RW, Pearson JE, Harris JC, Parker RL. Western equine encephalomyelitis in horses in the Northern Red River Valley, 1975. J Am Vet Med Assoc 1977;170:1396-9.

Detection of North American West Nile Virus in Animal Tissue by a Reverse Transcription-Nested Polymerase Chain Reaction Assay

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A traditional single-stage reverse transcription-polymerase chain reaction (RT-PCR) procedure is effective in determining West Nile (WN) virus in avian tissue and infected cell cultures. However, the procedure lacks the sensitivity to detect WN virus in equine tissue. We describe an RT-nested PCR (RT-nPCR) procedure that identifies the North American strain of WN virus directly in equine and avian tissues.

West Nile (WN) encephalitis is an infectious, noncontagious, arthropod-borne viral disease (1). WN virus belongs to the family *Flaviviridae*, genus *Flavivirus*. Mosquito vectors transmit the virus among bird populations and, incidentally, to susceptible mammalian species, including humans and horses. While infected horses may not exhibit clinical symptoms, fatal neurologic disease sometimes develops. The emergence of WN virus in the northeast United States in 1999 and 2000 caused concern among horse owners and veterinary practitioners (2). Animal exposure to WN virus can be confirmed serologically by using immunoglobulin M (IgM)-capture enzyme-linked immunosorbent assay (MAC-ELISA) and plaque reduction virus neutralization (PRNT) assays (3,4). Detecting WN virus in animal tissues, by isolation in cell culture or polymerase chain reaction (PCR), confirms infection. Cell culture isolation of WN virus from equine brain tissue can be difficult. It is speculated that the virus does not replicate to high titer in equine brains (Johnson, unpub. observation). PCR procedures have been developed to detect the New York strain (NY99) of WN virus (3,5). A traditional single-stage reverse transcription PCR (RT-PCR) can identify WN virus in avian tissue and mosquitoes (3) However, the single-stage RT-PCR often lacks the sensitivity to identify WN virus in equine brain (Johnson, unpub. observation). We developed a RT-nested PCR (RT-nPCR) to rapidly and accurately detect WN virus in equine brain.

The Study

Selected primers amplified a portion of the E region of the genome of WN virus NY99, which encodes the envelope protein (GenBank Accession Number AF196835). This region was highly conserved among several WN virus isolates obtained from the United States in 1999, as well as other WN virus strains of the same lineage (5). The Primer Designer 4 computer software program (Scientific and Educational Software, Durham, NC) was used to select primers. First-stage primer sequences, amplifying a 445-bp region, were 1401: 5'-ACCAACTACTGTGGAGTC-3', and 1845: 5'-TTCCATCTTCACTCTACACT-3'. Nested primers amplifying a 248-bp region were 1485: 5'-GCCTTCATACACACTAAAG-3' and 1732: 5'-CCAATGCTATCACAGACT-3'.

Total RNA was extracted from 50-100 mg of tissue by using Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. All samples were extracted and tested in duplicate. A WN virus control was prepared by extracting RNA from a 100-µL volume containing 10 50% tissue culture infective dose WN stock virus. Extracted RNA samples were resuspended in 12 µL RNase-free water and denatured at 70°C for 10 min. Two microliters of each denatured RNA sample was added to 48 µL of RT-PCR mixture with the final composition of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.8 mM deoxynucleoside triphospate (dNTP) pool, 25 units M-MLV RT, 1.25 units RNase inhibitor, 1.25 units AmpliTaq Gold (Applied Biosystems, Foster City, CA), and 37.5 pmol each of the two first-stage primers. Similarly, 2.0 µL of RNase-free water was added to "no-template" controls that were placed between diagnostic samples. Reaction tubes were incubated at 45°C for 45 min and 95°C for 11 min, followed by 35 cycles of 30-sec denaturation at 95°C, 45-sec primer annealing at 55°C, and 60-sec primer extension at 72°C. The final cycle had similar conditions except for a 5-min primer extension period. For the nested reaction, 1.5 µL of the first-stage amplification product was added to a tube containing 48.5 µL of a PCR mixture with a final composition of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.8 mM dNTP pool, 1.25 units AmpliTaq Gold, and 37.5 pmol each of the nested primers. Reaction tubes were incubated for 11 min at 95°C, followed by 35 cycles of the cycling conditions described for the first stage. All incubation and amplification procedures were performed by using a Perkin-Elmer 9600 PCR system (Applied Biosystems, Foster City, CA). RT-nPCR product was analyzed by agar gel electrophoresis followed by ethidium bromide staining and UV visualization. A 248-bp product indicated WN virus RNA was present in the original sample (Figure 1). Duplicate samples with discrepant results were retested. No-template controls were used to detect possible cross-contamination. PCR products of selected reactions were

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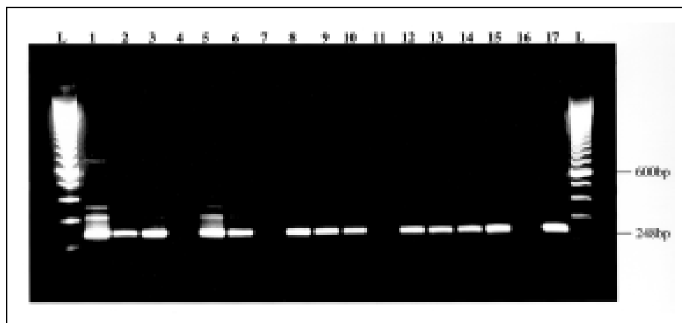

Figure 1. Visualization of reverse transcription-nested polymerase chain reaction product. West Nile virus-positive samples are indicated by a 248-bp band. Lane 1: positive crow brain, NY, 1999. Lane 2: positive crow kidney, NY, 1999. Lane 3: positive Sandhill Crane brain, CT, 1999. Lane 4: negative crow brain. Lane 5-6: positive crow brains, NY, 2000. Lane 7: normal control. Lane 8-10: positive horse brains, NY, 1999. Lane 11: negative horse brain. Lane 12: positive horse brain, RI, 2000. Lane 13: positive horse brain, NJ, 2000. Lane 14: positive horse brain, MA, 2000. Lane 15: positive horse brain, CT, 2000. Lane 16: normal control. Lane 17: NY99 West Nile virus control. L: 100-bp DNA ladder. Band artifacts in lanes 1, 3, 5, and 6 are due to large amount of virus present in those samples.

sequenced and compared with the published sequence of the NY99 strain of WN virus (5, GenBank Accession Number AF196835).

Sensitivity of the RT-nPCR was determined by comparing the endpoint dilution of NY99 WN stock virus detected in Vero cell culture with the endpoint dilution detected by RT-PCR. Tenfold dilutions of virus were prepared. Each dilution was tested in duplicate by cell culture and RT-nPCR. The endpoint dilution in cell culture was $10^{-4.5}$/100 µL. Endpoint dilutions for detection by RT-PCR were $10^{-2.5}$/100 µL after first-stage amplification, and $10^{-8.0}$/100 µL after nested amplification (Figure 2).

Specificity of the RT-nPCR was examined by testing viral RNA extracted from St. Louis encephalitis virus, a closely related flavivirus, as well as bovine viral diarrhea virus and classic swine fever virus, two other *Flaviviridae* family members. Eastern equine encephalitis virus and western equine encephalitis virus, two unrelated North American arboviruses affecting horses, were also tested. The RT-nPCR procedure performed on these samples did not result in observable amplification. Other reference strains of WN virus were not available for testing.

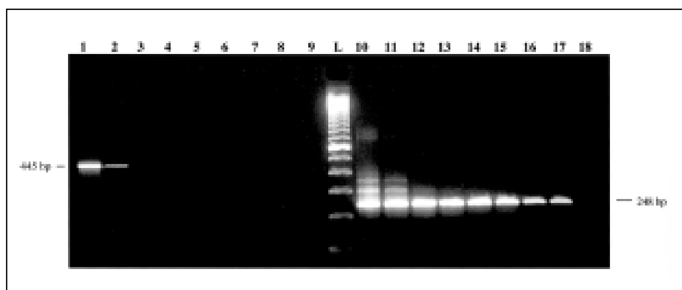

Figure 2. Visual comparison of first stage reverse transcription-polymerase chain reaction (RT-PCR) amplification products with RT-nested (n) PCR amplification products. Lanes 1-9 represent first-stage products of 10-fold dilutions 10^{-1} through 10^{-9}. Lanes 10-18 represent nested amplification products of same dilutions. L: 100-bp DNA ladder.

A total of 128 equine samples were used. Equine brain, blood, and cerebrospinal fluid (CSF) samples collected from suspect WN virus-infected horses were submitted to the National Veterinary Services Laboratories for testing during the 1999 and 2000 outbreak seasons. Diagnostic samples from 31 birds were also tested. All equine and avian brain tissues were tested by RT-nPCR and virus isolation using Vero and rabbit kidney cell cultures. Specimens derived from equine blood samples were also tested serologically, by virus isolation, or both. Five CSF samples from serologically positive horses were tested by RT-nPCR.

Seventy-three equine brains were tested for WN virus by RT-nPCR with 13 yielding positive results (Table). Retrospectively, the first-stage RT-PCR amplification products from the positive samples were examined by agar gel electrophoresis and ethidium bromide staining; two produced a faint band corresponding to the first-stage product of 445 bp (data not shown). The 13 RT-nPCR-positive horses had exhibited typical neurologic signs before death, and sera submitted from all 13 animals were positive for WN virus antibodies by PRNT or MAC-ELISA. WN virus was isolated in cell culture from 10 RT-nPCR-positive horses. Attempts to isolate virus from the remaining three were unsuccessful. The remaining 60 equine brains were negative for WN virus by RT-nPCR and isolation. Sera were available from 15 of the 60 RT-nPCR-negative horses; all 15 were negative for WN virus antibodies. Additional equine samples tested by RT-nPCR were plasma (35 samples), serum (10 samples), buffy coat (5 samples), and CSF (5 samples). Several of these samples were from serologically positive animals; however, all RT-nPCR tests on these samples were negative. Plasma samples were obtained from two ponies experimentally challenged with WN virus; RT-nPCR detected WN virus in plasma from 3 to 7 days postinoculation (dpi) in one pony and 3-6 dpi in the second pony (Table). Virus was isolated from the plasma of one pony at 6 dpi. The ponies were not exhibiting clinical symptoms.

Thirty-one avian brain samples were also tested. Seven of these samples were positive for WN virus by RT-nPCR and virus isolation (Table). The remaining 24 samples were negative by both procedures. Positive RT-nPCR results were also obtained from tissues (e.g., kidney, spleen, and liver) from some positive birds (Figure 1, lane 2).

RT-nPCR amplification products from 6 positive samples collected in 1999 (3 equine, 3 avian) and 12 positive samples

Table. Virus isolation compared with plaque reduction virus neutralization, immunoglobulin M-capture enzyme-linked immunosorbent assay serologic results, or both for West Nile virus samples positive by reverse transcription-nested polymerase chain reaction

RT-nPCR positive samples	Results				
	No. tested	VI positive	VI negative	Sero-positive	Sero-negative
Equine brain	13	10	3	13	0
Equine plasma	8[a]	1	7	0	8[b]
Avian brain	7	7	0	NA[c]	NA

RT-nPCR = reverse transcription-nested polymerase chain reaction; VI = virus isolation; NA = not applicable.

[a]Multiple plasma samples collected from two experimentally challenged ponies were RT-nPCR-positive for West Nile virus between 3 and 7 days after inoculation.

[b]West Nile virus antibodies were not detected in either pony until beyond the time the virus was detected by RT-nPCR.

[c]No avian serum was available for testing.

from 2000 (10 equine and 2 avian) were sequenced and compared with the published NY99 WN virus sequence (5). Within the 248-bp amplified region, two horse samples, one each from 1999 and 2000, had one base change; the remaining 16 samples were identical to NY99 WN virus.

Conclusions

The RT-nPCR proved to be a rapid and reliable method for detecting WN virus in equine as well as avian tissues. While isolation of WN virus from the 10 equine isolates required up to two passages in cell culture and 7-14 days to complete, WN virus was confirmed in tissue by RT-nPCR in <24 hours.

End-point dilution tests determined the RT-nPCR procedure to be 1,000-fold more sensitive than cell culture for detecting WN virus. Additionally, nested amplification increased the sensitivity of the RT-PCR at least 100,000-fold over first-stage amplifiction (Figure 2). Comparison of virus isolation and RT-nPCR results further demonstrated the increased sensitivity of RT-nPCR. All RT-nPCR-positive brain samples were confirmed by virus isolation except three equine brain tissues; WN virus infection in those animals was confirmed serologically. Had the procedure consisted of only first-stage amplification, only two of the positive horses would have been correctly identified.

Specificity of the RT-nPCR test was confirmed by sequence analysis of the amplified products of 18 positive samples and by absence of amplification of St. Louis encephalitis, bovine viral diarrhea, classic swine fever, eastern equine encephalitis, and western equine encephalitis viruses. Complete agreement between negative RT-nPCR tests on brain tissues and all other WN virus diagnostic tests performed further demonstrates the specificity of the assay in identifying animals not infected with WN virus.

Equine blood-derived samples and CSF samples from clinically ill animals were not reliable for determining the presence of WN virus by either RT-nPCR or isolation. Limited information from two experimentally challenged ponies indicated a several-day viremic phase early after infection, before WN virus antibody was detectable. Taken together, our data suggest that the viremic phase of infection occurs before clinical symptoms develop. Therefore, the RT-nPCR would not be expected to detect WN virus in blood of horses exhibiting clinical signs of WN virus encephalitis. Additionally, while WN virus-specific IgM antibodies may be detected in equine CSF during clinical illness, CSF samples do not appear to have diagnostic value for detecting WN virus RNA in horses.

As with all nested PCR procedures, additional manipulation of reaction tubes can lead to cross-contamination, corrupting the test (6). Confidence in the procedure may be increased by testing duplicate samples and including multiple controls.

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References

1. Monath TP, Heinz FX. Flaviviruses. In: Fields BN, Knipe DM, Howley PM, editors. Fields virology. 3rd ed. Philadelphia: Lippincott-Raven Publishers; 1996. p. 961-1034.
2. Anderson JF, Andreadis TG, Vossbrinck CR, Tirrell S, Wakem EM, French RA, et al. Isolation of West Nile virus from mosquitoes, crows, and a Cooper's Hawk in Connecticut. Science 1999;286:2331-3.
3. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol 2000;38:4066-71.
4. Tardei G, Ruta S, Chitu V, Rossi C, Tsai TF, Cernescu C. Evaluation of immunoglobulin M (IgM) and IgG enzyme immunoassays in serologic diagnosis of West Nile virus infection. J Clin Microbiol 2000;38:2232-9.
5. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333-7.
6. Roux KH. Optimization and troubleshooting in PCR. In: Dieffenbach CW, Dveksler GS, editors. PCR primer, a laboratory manual. Plainview (NY): Cold Spring Harbor Laboratory Press; 1995. p. 53-62.

West Nile Virus in Overwintering *Culex* Mosquitoes, New York City, 2000

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After the 1999 West Nile (WN) encephalitis outbreak in New York, 2,300 overwintering adult mosquitoes were tested for WN virus by cell culture and reverse transcriptase-polymerase chain reaction. WN viral RNA and live virus were found in pools of *Culex* mosquitoes. Persistence in overwintering *Cx. pipiens* may be important in the maintenance of WN virus in the northeastern United States.

The 1999 outbreak of human encephalitis in New York City (1) due to infection with West Nile (WN) virus (2) represented the first documented introduction of this virus into the Western Hemisphere. After the outbreak, the Centers for Disease Control and Prevention (CDC) and the U.S. Department of Agriculture recommended that surveillance efforts be enhanced in areas from Massachusetts to Texas along the Atlantic and Gulf coasts (3,4). Of primary concern was the lack of information about the ability of WN virus to persist over the winter in the northeastern United States and reinitiate enzootic or epidemic transmission in spring 2000. Evidence of persistent WN virus transmission in Romania for at least 2 years following the 1996 epidemic (5) increased concern that WN virus would persist and become established in the United States. Since the New York outbreak occurred in an area where mosquito biting activity ceases during winter months, survival of virus-infected female mosquitoes was considered the most likely mechanism for the virus to survive through the winter. Vertical transmission of WN virus by mosquitoes (i.e., passage of virus from infected female to her offspring) has been demonstrated in the laboratory (6) and apparently occurs by entry of virus into mosquito eggs during oviposition (7). Vertical transmission of WN virus has been documented only once in a population of mosquitoes outside the laboratory (8). We describe collection of overwintering mosquitoes during January and February 2000 in New York City and detection of WN viral RNA and live WN virus in the specimens.

The Study

Numerous sites characteristic of harborage for overwintering adult *Culex* mosquitoes in New York City were visited during January 11-13, February 15-16, and February 25, 2000. These sites were concentrated in northern Queens and southern Bronx, where WN virus activity was detected in mosquitoes during 1999 (9). We suspected that the vast sanitary and storm sewer systems in New York City would harbor large populations of overwintering adult mosquitoes. We sampled pipe chases, pump buildings, and dewatering facilities at the Tallman Island sewage treatment facility in Queens and the Hunts Point sewage treatment facility in the Bronx. In addition, we searched for mosquitoes in 15 manholes leading to sanitary and combined sewers, 31 storm sewer catch basins, and 4 large-diameter (1.2 to 2.5 m) storm water outflow pipes in Queens and the Bronx. Other sites included unheated structures associated with utility equipment rooms under the south end of the Whitestone Bridge; pump service buildings and pipe chases associated with municipal swimming pools in Astoria Park, Crotona Park, and Van Cortlandt Park; abandoned buildings at Flushing Airport; the basement of a historical house in Van Cortlandt Park; and historical structures associated with "The Battery" at Fort Totten in Queens.

Adult mosquitoes were located with a flashlight and collected from walls and ceilings of the resting sites by a large, battery-powered backpack aspirator or small hand-held mechanical aspirator. The specimens were held for 24 to 72 h at 21 to 22°C with access to 5% sucrose solution. Dead specimens were removed from the holding cages, frozen as soon as possible after death, and placed in labeled tubes at -70°C. Surviving specimens were frozen, placed in labeled tubes, held at -70°C, and, along with dead specimens, were shipped to the laboratory of CDC's Division of Vector-Borne Infectious Diseases in Fort Collins, CO. The mosquitoes were identified to species if possible, but the condition of certain morphologically similar *Culex* mosquitoes often prevented identification to species level. As a result, many specimens were only identified to genus or species group (e.g., the *Culex* species category may include the morphologically similar species *Cx. pipiens* and *Cx. restuans*). Specimens were grouped into pools of up to 50 mosquitoes by species, date, and location of collection.

A total of 2,383 adult mosquitoes were collected, 2,380 of which were in the genus *Culex;* the pools also included one

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adult *Cx. territans* and one *Cx. erraticus* (Table). The other specimens were *Anopheles punctipennis* or unidentified *Anopheles* species. Structures associated with the sanitary and storm sewer systems produced very few specimens. This discovery was unexpected because hibernating *Cx. pipiens* in peridomestic habitats use storm sewers, basements, unheated outbuildings, and similar protected sites (10). Approximately 88% of the *Culex* mosquitoes came from structures built into hillsides and the battery structures constructed of heavy granite block or concrete at Fort Totten.

The 2,383 mosquitoes were separated into 91 pools for testing, and every pool was screened for viable virus by a Vero cell plaque assay (11). They were also tested for WN viral RNA by WN virus-specific reverse transcriptase-polymerase chain reaction (RT-PCR) and a TaqMan RT-PCR assay (12). No evidence of live virus was observed in any of the pools in the initial Vero cell plaque assay, nor was WN viral RNA detected with the traditional RT-PCR assay. Three of the pools containing mosquitoes morphologically identified as *Culex* species tested positive by the TaqMan RT-PCR assay, indicating the presence of WN virus RNA. The TaqMan RT-PCR WN virus detection procedure has been shown to be more sensitive than traditional PCR and at least as sensitive as the Vero cell plaque assay (12).

The three WN viral RNA-positive pools were subsequently tested for viable WN virus by various techniques. Supernatants from the mosquito pool homogenates were inoculated into cultures of Vero cells, C6/36 *Aedes albopictus* cells, AP/61 *Ae. pseudoscutellaris* cells, and baby hamster kidney (BHK) cells. Flasks (25 mm^2) containing confluent monolayers of cells were inoculated with 0.1 mL of the mosquito pool supernatant and incubated for 1 h at 37°C and 28°C for mammal and mosquito cells, respectively. The appropriate maintenance medium was then added to the flasks. The cells were incubated and observed daily for cytopathic effects (CPE) for 7 days. Cells were harvested on day 7 regardless of CPE, pelleted by centrifugation, resuspended in phosphate-buffered saline (pH 7.2), and used to prepare spot slides (15 µL of cell suspension per spot). The slides were dried, fixed in cold acetone (-20°C for 20 min), and examined with indirect fluorescent antibody (IFA) staining using Broad Group B and WN virus specific antiserum (2,13). Negative cells of each type were included as controls. In addition, adult *Cx. pipiens* mosquitoes were inoculated with 0.34 µL of mosquito pool supernatant and incubated for 7 days at 27°C (14). Ten mosquitoes were inoculated with each of the three pool supernatants. The mosquitoes were then tested for virus by triturating them in BA-1 diluent as described for the Vero cell plaque assay, and injecting supernatants from the triturates into Vero cell cultures. The cells were observed for evidence of cytopathic effect for 7 days then harvested and examined with IFA stain as described. Live WN virus was isolated from the supernatant of one of the three RNA-positive pools inoculated into Vero cell culture. None of the other attempts to isolate virus from these pools were successful. The difficulty in isolating live virus from the RNA-positive pools, despite extensive efforts, may be due to virus death during the collection and shipping process or to a naturally low virus titer in vertically infected, hibernating mosquitoes.

The identity of mosquitoes in two of the WN viral RNA-positive pools was subsequently determined by a species-diagnostic PCR assay that can differentiate between *Cx. pipiens*, *Cx. restuans*, and *Cx. salinarius* in the pool (15). Results indicated that the two pools contained only *Cx. pipiens*. Insufficient material was available from the third RNA-positive pool for species identification by PCR.

Table. Adult mosquitoes collected in overwintering sites, Queens and the Bronx, January and February 2000

Borough	Site	Species	No. mosquitoes
Queens	Tallman Island Sewage Plant	*Culex pipiens*	1
		Cx. restuans	4
		Cx. species	74
	Fort Totten	*Cx. pipiens*[a]	1,034
		Cx. restuans	11
		Cx. erraticus	1
		Cx. territans	1
		Cx. species[b]	1,045
		Anopheles punctipennis	2
		An. species	1
	Other sites combined	*Cx. pipiens*	24
		Cx. restuans	4
The Bronx	Hunts Point Sewage Plant	*Cx.* species	33
	Other sites combined	*Cx. pipiens*	145
		Cx. species	3
	Total		2,383

[a]West Nile (WN) viral RNA detected in two pools of specimens initially morphologically identified as *Culex* species and subsequently identified as *Cx. pipiens* by species-specific polymerase chain reaction (PCR). Live WN virus was isolated from one of these pools.
[b]WN viral RNA detected in one pool of specimens morphologically identified as *Cx.* species. Insufficient material was available to permit species identification by PCR.

Conclusions

Detection of WN viral RNA in three pools and isolation of live WN virus from one pool of overwintering *Cx. pipiens* mosquitoes in New York City indicated that WN virus persisted in vector mosquitoes at least through midwinter, suggesting that the virus would persist until spring and emerge with mosquitoes to reestablish an enzootic transmission cycle in the area. Transovarial transmission of WN virus and preservation of the virus in hibernating mosquitoes are not thought to play an important role in the maintenance of the virus in nature (16,17). However, our observations indicate that approximately 0.04% of the overwintering *Culex* mosquitoes collected at Fort Totten carried viable WN virus, and 0.1% contained WN viral RNA. This finding suggests that WN virus infected, hibernating *Cx. pipiens* were relatively common where virus activity was intense the previous season and likely play an important role in persistence of the virus in an area. This infection rate is similar to rates observed for another flavivirus, St. Louis encephalitis virus, in overwintering *Cx. pipiens* collected in Maryland, where 0.3% were infected (1 isolate from 312 tested), and Pennsylvania, where 0.2% were infected (1 isolate from 406 tested) (18).

What is unclear is the mechanism that produced these infected overwintering mosquitoes. Transovarial transmission of the virus from an infected female to her offspring, which then enter diapause (hibernation physiology and behavior) as adults and survive the winter without taking a

blood meal, is supported by evidence from the field and laboratory (6,8). Alternatively, *Cx. pipiens* infected by feeding on a viremic vertebrate host may have survived the winter. Though blood-fed adult *Cx. pipiens* survive winter conditions (19), they are not considered an efficient mechanism for virus persistence (10). Regardless of the underlying mechanism, WN virus persistence in *Cx. pipiens* clearly contributes to the maintenance of WN virus through the winter season. Future research should address the mechanisms of WN virus maintenance and potential involvement of other mosquito species that may be important vectors in other regions of North America.

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References

1. Centers for Disease Control and Prevention. Update: West Nile Virus encephalitis—New York 1999. MMWR Morb Mortal Wkly Rep 1999;48:944-6,955.
2. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333-7.
3. Centers for Disease Control and Prevention. Guidelines for surveillance, prevention, and control of West Nile virus infection—United States. MMWR Morb Mortal Wkly Rep 2000;49:25-8.
4. Gubler DJ, Campbell GL, Nasci R, Komar N, Petersen L, Roehrig JT. West Nile Virus in the United States: Guidelines for detection, prevention, and control. Viral Immunol 2000;13:469-75.
5. Cernescu C, Nedelcu NI, Tardei G, Ruta S, Tsai TF. Continued transmission of West Nile virus to humans in southeastern Romania, 1997-98. J Infect Dis 2000;181:710-2.
6. Baqar S, Hayes CG, Murphy JR, Watts DM. Vertical transmission of West Nile virus by *Culex* and *Aedes* species mosquitoes. Am J Trop Med Hyg 1993;48:757-62.
7. Rosen L. Further observations on the mechanism of vertical transmission by *Aedes* mosquitoes. Am J Trop Med Hyg 1988;39:123-6.
8. Miller BR, Nasci RS, Godsey MS, Savage HM, Lutwama JJ, et al. First field evidence for natural vertical transmission of West Nile virus in *Culex univitattus* complex mosquitoes from Rift Valley Province, Kenya. Am J Trop Med Hyg 2000;62:240-6.
9. Nasci RS, White DJ, Stirling H, Oliver JA, Daniels TJ, Falco RC, et al. West Nile virus isolates from mosquitoes in New York and New Jersey, 1999. Emerg Infect Dis 2001;7:626-30.
10. Mitchell CJ, Francy DB, Monath TP. Arthropod Vectors. In: Monath TP, ed. St. Louis Encephalitis. Washington DC: American Public Health Assn; 1980. p. 313-79.
11. Beaty BJ, Calisher CH, Shope RS. Arboviruses. Schmidt NJ, Emmons RW, eds. Diagnostic procedures for viral, rickettsial and chlamydial infections. Washington DC: American Public Health Assn; 1989. p. 797-856.
12. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, et al. Rapid detection of West Nile virus from human clinical specimens, field collected mosquitoes, and avian samples by a TaqMan RT-PCR assay. J. Clinical Microbiol 2000;38:4066-71.
13. Lorono-Pina MA, Cropp CB, Ferfan JA, Vorndam VA, Rodrigiez-Angulo EM, et al. Common occurrence of concurrent infections by multiple dengue virus serotypes. Am J Trop Med Hyg 1999;61:725-30.
14. Rosen L, Gubler, DJ. The use of mosquitoes to detect and propagate dengue viruses. Am J Trop Med Hyg 1974;23:1153-60.
15. Crabtree MB, Savage HM, Miller BR. Development of a species-diagnostic polymerase chain reaction assay for the identification of *Culex* vectors of St. Louis encephalitis virus based on interspecies sequence variation in ribosomal DNA spacers. Am J Trop Med Hyg 1995;53:105-9.
16. Taylor FM, Work TH, Hurlbut HS, Rizk F. A study of the ecology of West Nile virus in Egypt. Am J Trop Med Hyg 1956;5:579-620.
17. Malik Peris JSM, Amerasinghe FP. West Nile Fever. In: Beran GW, Steele JH, editors. Handbook of Zoonoses, Second Edition, Section B: Viral. Ann Arbor, MI: CRC Press; 1994. p. 139-48.
18. Bailey CL, Eldridge BF, Hayes DE, Watts DM, Tammariello RF, Dalrymple JM. Isolation of St. Louis encephalitis virus from overwintering *Culex pipiens* mosquitoes. Science 1978;199:1346-9.
19. Bailey CL, Faran ME, Gargan TP, Hayes DE. Winter survival of Blood-fed and nonblood-fed *Culex pipiens* L. Am J Trop Med Hyg 1982;31:1054-61.

West Nile Virus Outbreak Among Horses in New York State, 1999 and 2000

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West Nile (WN) virus was identified in the Western Hemisphere in 1999. Along with human encephalitis cases, 20 equine cases of WN virus were detected in 1999 and 23 equine cases in 2000 in New York. During both years, the equine cases occurred after human cases in New York had been identified.

An outbreak of arboviral encephalitis attributable to West Nile (WN) virus was first recognized in the United States in 1999, when dead crows were reported in and near New York City, and a zoological park noted that some of their exhibition birds had died. These events coincided with initial public health reports of human encephalitis cases diagnosed as St. Louis encephalitis virus in New York City (1). The successful isolation of virus from dead birds allowed the subsequent identification of WN virus as the etiologic agent of both human and animal disease (2).

WN virus is primarily transmitted between mosquitoes and birds, but transmission to mammals can occur when infection occurs in mosquito species that feed on birds and mammals. Encephalitis has been confirmed only in humans and horses (1,3-10). During 1999, 20 equine cases of WN virus encephalitis were confirmed in the United States, all in New York. In 2000, 23 equine cases were identified in New York, with more cases identified in New Jersey, Delaware, Rhode Island, Massachusetts, Connecticut, and Pennsylvania. We summarize these findings.

The Study

1999 Investigations

During fall and early winter 1999, veterinarians with the U.S. Department of Agriculture and the New York Department of Agriculture and Markets (NYSDAM) investigated reports of an unusual cluster of neurologic illness in horses on Long Island. Investigations were initiated by reports from practicing veterinarians. Other veterinarians were contacted to determine whether other similar cases were occurring in the area. Tissue and blood samples were collected and submitted to the National Veterinary Services Laboratories (NVSL) for testing and forwarded to the Centers for Disease Control and Prevention (CDC) for confirmation, as necessary.

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In 1999, a case was defined as an equine with neurologic signs and either a positive plaque reduction neutralization test (PRNT) titer to WN virus or isolation of virus confirmed by primer sequence. Testing was conducted at either CDC or the NVSL (11). Testing for immunoglobulin M (IgM) antibody was not done.

Twenty cases of equine neurologic illness (1 pony and 19 horses, all from Long Island) were laboratory confirmed as WN virus infections. Five additional horses with clinical onset between August 28 and September 24, before the cluster was reported, were considered probable cases. The illness, characterized by acute onset of rear limb ataxia, included muscle tremors, knuckling over at the fetlocks, and in some instances inability to rise. The first horse became ill on August 26; the onset of the last case was October 23 (Figure 1).

Four of the 20 animals died or were euthanized. All survived for 3 or 4 days before euthanasia. Necropsy samples from three of these horses yielded WN virus from brain or spinal cord tissue. The fourth horse had a WN virus titer (>1:320) from a sample collected 3 days after clinical onset. The four dead horses ranged in age from 4 to 21 years old. Sixteen of the horses recovered fully and had neutralizing antibody titers to WN virus from ≥1:100 (NVSL) to ≥1:1,280

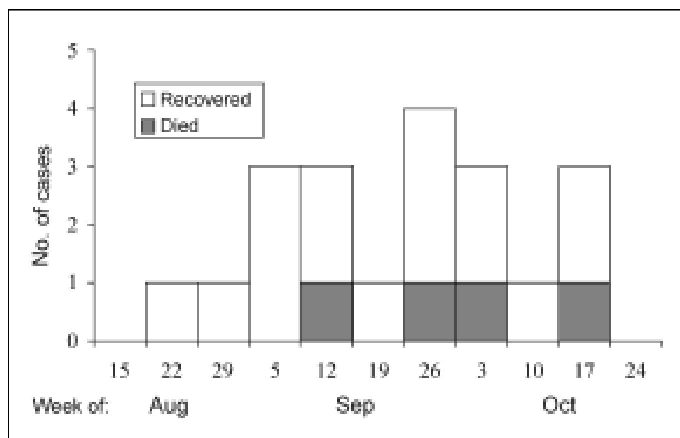

Figure 1. Onset Date of West Nile Case-Horses, New York, 1999.

(CDC). The 20 animals ranged in age from 2 to 28 years old. There were 13 mares, 3 geldings, and 4 stallions.

The 20 cases and 5 probable cases lived on 18 different premises in Nassau or Suffolk counties. Stable mates were identified on 15 of these premises. Samples were collected from 69 asymptomatic stable mates. Of these, 20 (29%) had titers to WN virus ranging from 1:160 to ≥1:1,280. The age of these horses (1 to 37 years old) did not differ significantly from case-horse ages. There were 27 mares, 32 geldings, and 8 stallions.

2000 Investigations

On May 16, 2000, an informational letter, signed by the NYSDAM and the New York State Department of Public Health (NYSDOH), was sent to approximately 1,600 New York veterinarians. The letter summarized the 1999 findings and requested case reporting of suspected equine cases to NYSDAM and suspected cases in companion animals to NYSDOH.

In 2000, 23 WN virus encephalitis cases in horses were confirmed in New York State. Diagnostic samples were submitted to the New York State Animal Disease Diagnostic Laboratory (NYSADDL) in Ithaca. All positive diagnoses were based on IgM antibody and positive WN virus neutralization, a demonstrated fourfold rise in virus neutralization titer from paired serum samples, or detection of viral sequence by reverse transcription-polymerase chain reaction (RT-PCR) performed at the NVSL. One horse was diagnosed by real-time RT-PCR testing at the Arbovirus Laboratory of the Wadsworth Center, NYSDOH. Although no infectious virus was grown in Vero cell culture, the brain sample was also positive by two primer-probe sets (1160 and 3111).

The first equine case had clinical onset on August 18 and lived on Staten Island. The last had onset on November 1 (Figure 2); onset date could not be determined for one horse.

The index horse had positive titers (IgM 1:1,000 and PRNT ≥1:100) for WN virus; six other horses had the same titers. Nine cases had IgM titers of 1:10 and PRNT titers of ≥1:1,000. Six horses had various combinations of IgM (1:10 to 1:100) and PRNT (≥1:100) titers. One horse, with negative serology, was diagnosed by RT-PCR by the NYSDOH Arbovirus Laboratory. This brain sample was also positive by two primer-probe sets.

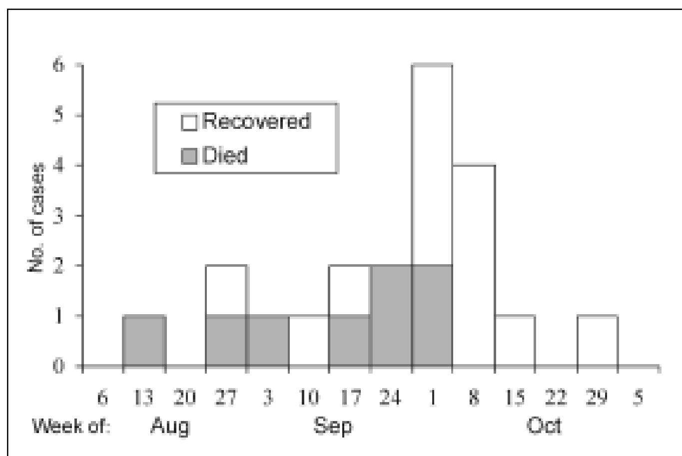

Figure 2. Onset Date of West Nile Case-Horses—New York, 2000.

Horses had ataxia (95.7%), primarily rear limb (90.5%,) and muscle fasciculations or trembling (55%). Many had acute onset (90.5%). Other case-horses were down; some were able to rise with assistance, while others could not stand. Only 32% of the horses had elevated temperatures (Table). Eight horses died or were euthanized; seven died within 3 to 4 days of clinical onset. The average age of horses with fatal cases was 14.4 years, similar to the age of surviving horses. No significant gender differences or breed predispositions for disease were detected. Cases occurred in Suffolk, Orange, Nassau, Bronx, and Richmond counties.

Attempts were made to contact veterinarians who submitted samples to the NYSADDL for WN virus testing on equine sera. When reached, veterinarians were interviewed to determine if the submission was for diagnostic purposes and asked to provide the clinical picture of the ill horse. Only horses with primarily a neurologic component were included in the study (e.g., lameness was excluded). Clinical findings of the 23 case-horses were compared with those of 19 non-case horses with similar clinical signs but with no laboratory evidence of WN virus infection (Table). No statistically significant difference was found. The average age of the non-case horses was 14.4 years (range 2-28). Results indicated that WN virus cannot be diagnosed on the basis of clinical signs alone.

A serosurvey of horses on Staten Island was conducted during September 2000. Ninety-one clinically normal horses from seven stables located within 3 miles of the index case-horse were sampled. Seven seropositive horses were identified at three stables, including the stable mate of the index horse. At one stable, five of six horses sampled had titers to WN virus; one of the five had a positive IgM-capture enzyme-linked immunosorbent assay (MAC-ELISA)(1:100) and PRNT positive at 1:10. Sera from the other four horses were negative by MAC-ELISA but positive at 1:100 with the PRNT at NVSL. Sera from the two other positive horses at the other two stables were negative by MAC-ELISA but positive at 1:10 by PRNT.

Mosquito surveillance conducted by the New York City Department of Health (NYCDOH) within a 2-mile radius of

Table. Horses with neurologic illness—New York, 2000

Sign	Number of case-horses[a]	Number of non-case horses[b]
Ataxia	22/23 (95.7%)	16/19 (84.2%)
Rear	19/21 (90.5%)	15/19 (78.9%)
All four limbs	15/20 (75%)	9/19 (47.4%)
Acute onset	19/21 (90.5%)	12/18 (66.7%)
Fever (>101°F)	7/22 (31.8%)	8/15 (60%)
Mean	102.4°F	104°F
Range	101.4-103	102-106
Muscle fasciculation	11/20 (55%)	5/19 (26.3%)
Almost fall[c]	8/17 (47.1%)	6/18 (33.3%)
Down	9/22 (40.9%)	4/17 (23.5%)
Rise with assist	6/21 (28.6%)	5/18 (27.8%)
Died	8/23 (34.8%)	8/19 (42.1%)
Dull, lethargic	5/19 (26.3%)	10/19 (52.6%)
Hypermetric	4/16 (25%)	4/19 (21.1%)
Agitated	3/19 (15.8%)	2/18 (11.1%)

[a]West Nile (WN) virus confirmed by laboratory testing. Denominator varies depending upon completeness of information provided during interview.
[b]WN virus negative by laboratory testing. Denominator varies depending upon completeness of information provided during interview.
[c]Private practitioners reporting that circling the horse would cause it to fall.

the three positive premises from July to November resulted in 44 WN virus-positive pools. These included *Culex pipiens*, which feed only on birds; *Cx. salinarius*, which feed on both birds and mammals; and *Aedes vexans*, *Ae. triseriatus*, and *Psorophora ferox*, which feed mainly on mammals. In July, *Cx. salinarius* was the only positive mosquito pool in this radius. One week after the index horse became ill, the NYCDOH began trapping mosquitoes at its stable; WN virus was identified in a pool of *Ae. vexans* 10 days after the horse became ill. This was the first identification of this mosquito as positive for WN virus in New York.

Conclusions

In 2000 (unlike in 1999, when resources for laboratory diagnosis of flavivirus infections were limited), MAC-ELISA and real-time RT-PCR were available to detect WN-specific IgM antibodies and WN virus RNA, respectively.

WN infection in horses may cause acute, fatal neurologic disease, but clinical disease often does not occur. Moderate to severe ataxia, weakness, and rear limb incoordination were the most consistent signs; fever was not. Treatment was primarily directed toward relieving clinical signs. In some instances in which the horses were only mildly affected, no treatment was given, and clinical signs resolved in 2 to 7 days. Horses that survived eventually recovered fully.

The epidemic curves of the 1999 and 2000 equine outbreaks are similar, with equine cases occurring after human and wild bird cases. Horses are unlikely to be the first warning that virus is circulating in an area. In each instance where equine cases occurred, virus activity in wild birds, mosquitoes, or both had already been identified.

There were two case-horses on Staten Island, where 10 human cases occurred (12). No other NY counties with equine cases reported human cases in 1999 or 2000.

Unlike with human cases, veterinarians cannot rely on fever to aid in diagnosing WN virus in horses. In addition, clinical presentation of WN virus in horses is nonspecific. For Staten Island in 2000, an area of intense WN virus activity, equine cases did not serve as an early warning to public health officials that virus was circulating. In other NY counties, equine cases did not precede WN findings in mosquitoes and wild birds, nor did they predict human cases.

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References

1. Centers for Disease Control and Prevention. Outbreak of West Nile-like viral encephalitis--New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:845-9.
2. Centers for Disease Control and Prevention. Update: West Nile-like viral encephalitis--New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:890-2.
3. Cantile C, Di Guardo G, Eleni C, Arispici M. Clinical and neuropathological features of West Nile virus equine encephalomyelitis in Italy. Equine Vet J 2000;32:31.
4. Ceausu E, Erscoiu S, Calistru P, Ispas D, Dorobat O, Homos M, et al. Clinical manifestations in the West Nile virus outbreak. Rom J Virol 1997;48:3-11.
5. Cernescu C, Ruta SM, Tardei G, Grancea C, Moldoveanu L, Spulbar E, et al. A high number of severe neurologic clinical forms during an epidemic of West Nile virus infection. Rom J Virol 1997;48:13-25.
6. Lvov DK, Butenko AM, Gromashevsky VL, Larichev VP, Gaidamovich SY, Vyshemirsky OI, et al. Isolation of two strains of West Nile virus during an outbreak in southern Russia, 1999. Emerg Infect Dis 2000;6:373-6.
7. Nur YA, Groen J, Heuvelmans H, Tuynman W, Copra C, Osterhaus AD. An outbreak of West Nile fever among migrants in Kisangani, Democratic Republic of Congo. Am J Trop Med Hyg 1999;61:885-8.
8. Pantheir R, Hannoun C, Oudar J, Beytout D, Corniou B, Joubert L, et al. Isolation of West Nile virus in a Camarge horse with encephalomyelitis. Comptes Rendus Hebdomadaires Des Seances de L'Academie des Sciences D: Sciences Naturelle (Paris) 1966;262:1308-10.
9. Panthier R. Epidemiology of the West Nile virus: study of an outbreak in Camargue. I. Introduction. Ann Inst Pasteur (Paris) 1968;114:518-20.
10. Tsai TF, Popovici F, Cernescu C, Campbell GL, Nedelcu NI. West Nile encephalitis epidemic in southeastern Romania. Lancet 1998;352:76771.
11. Beaty BJ, Calisher CH, Shope RE. Arbovirus. In: Schmidt NH, Emmons RW, editors. Diagnostic procedures for viral, rickettsial and chlamydial infections. 6th ed. Washington: American Public Health Association 1989;797-856.
12. Kulasekera V, Kramer L, Nasci R, Mostashari F, Cherry B, Trock SC, et al. West Nile virus infection in mosquitoes, birds, horses, and humans, Staten Island, New York, 2000. Emerg Infect Dis 2001;7:722-5.

Isolation and Characterization of West Nile Virus from the Blood of Viremic Patients During the 2000 Outbreak in Israel

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We report the isolation of West Nile (WN) virus from four patient serum samples submitted for diagnosis during an outbreak of WN fever in Israel in 2000. Sequencing and phylogenetic analysis revealed two lineages, one closely related to a 1999 New York isolate and the other to a 1999 Russian isolate.

West Nile (WN) virus (1) outbreaks were recorded in Israel during the 1950s and 1970s (2-4); however, in the last decade, diagnosis ceased and no clinical cases were reported, although seroepidemiologic surveys indicated that the virus continued to circulate (5). Following reports of illness in birds in 1998, the Central Virology Laboratory (CVL) of the Public Health Services reestablished the capability to diagnose WN virus based on serologic assays, including virus neutralization, immunofluorescence, and enzyme-linked immunosorbent assays (ELISA) with immunoglobulin (Ig) G and IgM.

This led to the identification of acute human cases beginning in the fall of 1999 (6). High seroprevalence was found in the southern region of Israel (Eilot region) in the fall and winter of 1999-2000, with IgG levels ranging from 21% to 82% and IgM levels ranging from 0% to 73% in seven communities. Some IgM-positive cases were associated with clinical symptoms compatible with WN fever. Additional acute cases were diagnosed in the central region of Israel during the spring and summer of 2000 (H. Bin, unpub. data).

During the late summer and fall of 2000, an outbreak occurred in the central and northern parts of Israel. Between August 1 and November 30, 439 patients with clinical symptoms were positive by ELISA-IgM testing; 29 of these patients died. The clinical details in records of the patients diagnosed at the CVL indicated that central nervous system (CNS) involvement was a major clinical manifestation in most hospitalized patients. Rates of illness and death increased with age: 69% of the patients were >45 years and 96% of those who died were >65 (Quarterly Report No. 3 of the Department of Epidemiology, Ministry of Health, Jerusalem). Epidemiologic and clinical aspects of the outbreak were also described by Weinberger et al. (7) and Chowers et al. (8), respectively. We describe the isolation and characterization of four viral strains from human serum obtained during this outbreak.

The Study

During the outbreak, patients' samples (serum, cerebrospinal fluid [CSF], or both) submitted to the CVL were immediately divided into two aliquots. One aliquot was immediately used to test for IgM antibodies, and the other was stored at -70°C until further processing. Virologic studies were performed on the frozen acute-phase samples from patients who seroconverted from IgM negative to IgM positive, as well as on CSF from fatal cases. Reverse transcription-polymerase chain reaction (RT-PCR) and virus isolation were attempted simultaneously on 32 patients' samples (17 serum and 15 CSF). RT-PCR and an indirect immunofluorescence assay (IFA) were used to confirm the presence of WN virus in cell culture. Direct sequencing of RT-PCR amplified fragments was performed to characterize the genome of isolated viruses.

Patient samples were analyzed for WN virus by RT-PCR using primer sequences for the envelope gene, as described by Lanciotti et al. and Shi et al. (9,10). Viral RNA was extracted by using the QIAamp Viral RNA Mini Kits (QIAgen Gmbh, Hilden, Germany), and the RT-PCR was performed with Ready to Go Beads (Amersham Pharmacia, Buckinghamshire, England) according to manufacturer's instructions. The primers Kun 108, Kun 848, Kun 998c, and Kun 1830c were used in RT-PCR for sequence analysis (11). Sequence analysis was performed on a 1648-bp fragment of the WN virus genome encoding 309 nt upstream from the pre-membrane protein (preM), the entire preM and membrane protein (M) genes, and 811 nt of the 5' portion of the envelope glycoprotein (E) gene. Purification of the RT-PCR product and sequence and phylogenetic analyses were performed as described (12). Both strands of the amplified PCR products were sequenced. RT-PCR conditions used were 42°C for 45 min, 95°C for 5 min, 60°C for 2 min, 72°C for 2 min, 34 cycles at 93°C for 45 sec, 55°C for 45 sec, and 72°C for 90 sec, followed by 72°C for 7 min; samples were then left at 4°C. PCR products were visualized by staining with ethidium bromide after electrophoresis on 2% agarose gels. With the RT-PCR assay, we can detect as low as 0.01 PFU based on serially diluted titered WN virus isolated from a White-eyed Gull.

Virus isolation was performed on Vero cell monolayers (ATCC CCL-81) by using the tube method. Vero cell monolayers (80%-90% confluent) were washed twice with phosphate-buffered saline, then infected with 100- to 200-µL patient samples. Patient samples were allowed to adsorb for 1 hour at 37°C with gentle swirling every 15 min. Eagle's

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minimal essential medium with 2% fetal calf serum, 100 U/mL penicillin, 200 µg/mL streptomycin, and 12.5 U/mL nystatin was then added to the infected cells. Cells were monitored daily for 7 days for cytopathic effect (CPE). Cells showing CPE demonstrated rounding-up in the early stage of infection and many floating single cells later in the infection. Infected cells that showed CPE were evaluated by RT-PCR, and a sample (100 µL) of the supernatant was passaged on another Vero cell monolayer to confirm WN virus. Infected cells that showed CPE were also evaluated by IFA using the monoclonal antibody JCU/KUN/2B2 (TropBio Pty Ltd., Townsville Queensland, Australia) (13). Cells from monolayers that did not show CPE were passaged onto fresh Vero cell monolayers and monitored for another 7 days. Cells that did not show CPE after 14 days were also tested with the IFA reagent. Only cell cultures that did not stain with IFA were reported negative.

Virus was isolated from serum from four nonfatal WN virus IgM-negative Israeli patients who seroconverted 1 to 2 weeks later. Patient 1 (WN-0043), a 51-year-old woman from the northcentral region, was hospitalized; CNS disease did not develop. Patient 2 (WN-0233), a 20-year-old man from the north, was hospitalized for fever of unknown origin and neutropenia; CNS disease did not develop. Patient 3 (WN-0247), a 5-year-old boy residing in the central region, had meningoencephalitis and was hospitalized. Patient 4 (WN-0304), a 55-year-old woman from the north, had high fever and myalgia and no CNS symptoms; she was not hospitalized.

Two viral isolates were detected from patients 3 and 4 on day 4 after inoculation on Vero cells; the other two isolates were detected from patients 1 and 2 on day 7 after inoculation. All four virus isolates were confirmed as WN virus by IFA. Only two original acute-phase serum samples (patients 3 and 4) were positive by RT-PCR. Negative RT-PCR results and lengthy time until appearance of CPE are apparently consistent with low viral load in patients' serum (Table).

Sequence analysis showed that isolates WN-0233 (GenBank Accession Number AF375043) and WN-0304 (GenBank Accession Number AF375045) had identical sequences over 1,648 nt and isolates WN-0043 (GenBank Accession Number AF375042) and WN-0247 (GenBank Accession Number AF375044) differed by only 1 nt. Such high homology is similar to results reported by Lanciotti et al., who also described identical WN virus sequences from brain samples from two patients (14). WN-0247 differed from WN-0304 by 50 (3%) of 1,648 nts or 25 (2.9%) of 855 nt for the partial E gene sequence. Most differences in the E gene were in the third position of the codon (21 of 25); all of these were synonymous. All four differences in the first and second codon positions encoded different amino acids when isolate WN-0247 was compared with isolate WN-0304.

A 255-nt fragment of the WN virus E gene has previously been used for phylogenetic studies (15-17). A search of the EMBL/GenBank database using the equivalent fragment of the Israeli outbreak isolates indicated that isolate WN-0043 and WN-0247 were identical to WN-flamingo-NY99, while isolates WN-0233 and WN-0304 were most closely related to the WN-Romania-97 isolate AF130362 (3-nt difference, 1.2%) and less closely related to the WN-flamingo-Y99 (9-nt difference, 3.5%).

A similar search, using a 1,648-nt fragment encoding the preM, M, and part of the 5' E gene, allowed the construction of a more detailed phylogenetic comparison (Figure). As with the 255-nt fragment, WN-0043 and WN-0247 were closest to WN-flamingo-NY99 (AF196835, 99.7% homology), while WN-0233 and WN-0304 most closely resembled a 1997 isolate from Romania (AF130362, 98.4% homology). Phylogenetic analysis showed that two lineages of WN virus circulate in Israel. The first is similar to the WN virus isolates from mosquito, horse, and flamingo during the 1999 NY outbreak. The other lineage is similar to the virus isolated from a mosquito pool during the 1997 Romanian outbreak and to the nucleotide sequences reported from the Russian outbreak in

Table. Analysis of West Nile patients' isolates, Israel, 2000 outbreak

Patients	Virus isolate	RT-PCR acute-phase serum	Tissue culture acute-phase serum (CPE+IFA)	RT-PCR infected cell culture	RT-PCR & tissue culture convalescent-phase serum
1	WN-0043	(-)	(+)	(+)	ND
2	WN-0233	(-)	(+)	(+)	ND
3	WN-0247	(+)	(+)	(+)	(-)
4	WN-0304	(+)	(+)	(+)	(-)

+, positive result; -, negative result; ND, not done; RT-PCR, reverse transcription-polymerase chain reaction; CPE, cytopathic effect; IFA, indirect immunofluorescence assay.

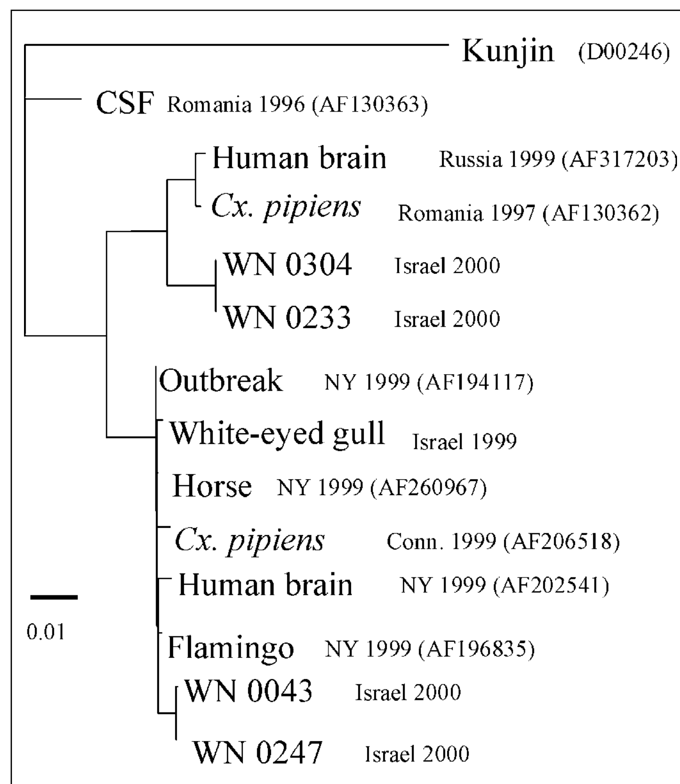

Figure. Phylogenetic comparison of human West Nile virus isolates from the Israel 2000 outbreak with sequences from the EMBL/GenBank database. The PHYLIP DNA Maximum Likelihood program (bootstrap = 100) was used to compare a 1,648-nt sequence encoding the PreM, M gene, and part of the E gene from the four human outbreak isolates with nine sequences from the EMBL/GenBank database (accession numbers in parentheses) and one from a 1999 isolate from an Israeli White-eyed Gull. CSF = cerebrospinal fluid sample.

1999. These two lineages isolated in Israel in the 2000 outbreak differ from the WN sequences obtained in 1996 from Romania (11).

Conclusions

Our sequence analysis shows that at least two lineages of WN virus infected the human population in Israel in 2000. Virus lineage and severity of symptoms were not clearly correlated, although more human isolates would be necessary to confirm this finding. More than one lineage can be found in areas where a virus is endemic and has been circulating for extended periods. More studies, using archived materials, are necessary to determine if there were more than two co-circulating lineages. Yet to be determined is whether changes in the virus genome resulted in a more virulent strain, which caused the high rates of illness and death during the 2000 Israeli outbreak.

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References

1. Rice CM. Flaviviridae: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, et al., editors. Virology. Vol 1. New York: Lippincott-Raven; 1996. p.931-59.
2. Bernkopf H, Levine S, Nerson R. Isolation of West Nile virus in Israel. J Infect Dis 1953;93:207-18.
3. Flatau E, Kohn D, Daher O, Varsano N. West Nile fever encephalitis. Isr J Med Sci 1981;17:1057-9.
4. Lustig S, Halevey M, Fuchs P, Pen-Nathan D, Lachmi BE, Kobiler D, et al. Can West Nile Virus outbreaks be controlled. IMAJ Math Appl Med Bio 2000;2:733-7.
5. Cohen D, Zaide Y, Karasenty E, Schwarz M, LeDuc JW, Slepon R, et al. Prevalence of antibodies to West Nile fever, sandfly fever Sicilian, and sandfly fever Naples viruses in healthy adults in Israel. Public Health Rev 1999;27:217-30.
6. Bin H, Pokamunsky S, Malkinson M, Banet C, Mendelson E. Emerging viral diseases, are we prepared for a new plague? [Abstract H1]. Annual meeting 2000 of the Israel Society for Microbiology, Haifa, Israel, 2000. ISM News 2000;30:24.
7. Weinberger M, Pitlik SD, Gandacu D, Lang R, Nassar F, Ben David D, et al. West Nile fever outbreak, Israel 2000: Epidemiologic aspects. Emerg Infect Dis 2001;7:686-9.
8. Chowers MY, Lang R, Nassar F, Ben David D, Giladi M, Rubinshtein E, et al. Clinical characteristics of the West Nile fever outbreak, Israel, 2000. Emerg Infect Dis 2001;7:675-8.
9. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol 2000;38:4066-71.
10. Shi P-Y, Kauffman EB, Ren P, Felton A, Tai JH, Dupuis AP II, et al. High-throughput detection of West Nile Virus RNA. J Clin Microbiol 2001;39:1264-71.
11. Savage HM, Ceianu C, Nicolescu G, Karabatsos N, Lanciotti R, Vladimirescue A, et al. Entomologic and avian investigations of an epidemic of West Nile fever in Romania, 1996, with serologic and molecular characterization of a virus from mosquitoes. Am J Trop Med Hyg 1999;61:600-11.
12. Shulman LM, Handsher R, Yang CF, Yang SUJU, Manor J, Vonsover A, et al. Resolution of the pathways of poliovirus Type 1 transmission during an outbreak. J Clin Microbiol 2000;38:945-52.
13. Hall RA, Burgess GW, Kay BH, Clancy P. Monoclonal antibodies to Kunjin and Kokobera viruses. Immunol Cell Biol 1991;69:47-9.
14. Lanciotti RS, Rohring JT, Deubel V, Smith J, Parker M, Steele K, Crise B, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the Northeastern United States. Science 1999;286:2333-7.
15. Berthet FX, Zeller HG, Drouet MT, Rauzier J, Digoutte JP, Deubel V. Extensive nucleotide changes and deletions within the envelope glycoprotein gene of Euro-African West Nile viruses. J Gen Virol 1997;78:2293-7.
16. Coia GP, Speight G, Byrne ME, Westway EG. Nucleotide and complete amino acid sequences of Kunjin virus: definitive gene order and characteristics of the virus specific proteins. J Gen Virol 1988;69:1-21.
17. Gould EA, Zanotto P, Holmes EC. The genetic evolution of flaviviruses. In: Saluzzo JF, Dodet B, editors. Factors in the emergence of arbovirus diseases. Paris: Elsevier Press; 1997. p. 51-63.

Fatal Encephalitis and Myocarditis in Young Domestic Geese (*Anser anser domesticus*) Caused by West Nile Virus

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During 1999 and 2000, a disease outbreak of West Nile (WN) virus occurred in humans, horses, and wild and zoological birds in the northeastern USA. In our experiments, WN virus infection of young domestic geese (*Anser anser domesticus*) caused depression, weight loss, torticollis, opisthotonus, and death with accompanying encephalitis and myocarditis. Based on this experimental study and a field outbreak in Israel, WN virus is a disease threat to young goslings and viremia levels are potentially sufficient to infect mosquitoes and transmit WN virus to other animal species.

West Nile (WN) virus belongs to the family *Flaviviridae* in the Japanese encephalitis (JE) serocomplex group and is transmitted through various species of adult *Culex* mosquitos to a variety of mammals and birds (1). WN virus was not recognized in North America until the fall of 1999, when an epizootic began with the death of a wild American Crow (*Corvus brachyrhynchos*) in New York (2); an additional 194 deaths in wild birds were confirmed with WN virus infections that fall (2). Most birds that died were from the order Passeriformes; corvids (crows and jays) accounted for >80% of deaths (1). Simultaneously, WN virus emerged as a cause of 62 cases of encephalitis with seven fatalities in humans (3) and 25 cases of neurologic disease in horses in New York City or on Long Island (1).

WN virus has not affected commercial chickens (*Gallus gallus domesticus*) or turkeys (*Meleagridis gallopavo*), which are predominantly raised indoors with low potential for exposure to mosquito vectors (2). Furthermore, experimental studies in chickens and turkeys inoculated subcutaneously with a New York WN virus isolate had low viremia titers and no clinical disease (4,5). However, natural WN virus infections were associated with severe neurologic signs and death in 160 of 400 8- to 10-week-old domestic geese from a flock in Israel (6). The role of domestic geese as a WN virus reservoir in the Israel outbreak is unknown, but goose infection rates in the Sindbis District of the northern Nile Valley were 27%, similar to rates in buffed-back herons (*Bubulcus ibis ibis*), doves (*Streptopelia senegalensis senegalensis*), and domesticated pigeons (*Columbia livia*) and twice the rate in domesticated chickens and ducks (*Anas platyrhynchos*), suggesting that geese may have a role in local WN virus ecology (7). The U.S. WN virus is closely related to WN virus isolates obtained from humans with encephalitis in Romania (1996) and geese in Israel (1998) (8,9). We report the experimental reproduction of neurologic disease and death in young domestic geese with a WN virus isolated from an American Crow on Long Island.

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The Study

Four 2-week-old Embden geese were needle-inoculated subcutaneously with $10^{3.3}$ mean tissue culture infective doses ($TCID_{50}$) of Vero cell culture propagated WN virus strain 9/99. A similar dosage was used in previous pathogenesis experiments to infect chickens and turkeys (4,5). The goslings were housed in a Biosafety Level 3 agriculture facility (10). Two uninoculated goslings were maintained in contact with the WN virus-inoculated group and two sham-inoculated controls were maintained in isolation.

During the 21-day study, the four WN virus-inoculated geese lost weight, had decreased activity, and were depressed. WN virus-inoculated gosling #80 had intermittent torticollis and opisthotonus and rhythmic side-to-side movement of the head (Figure) and was euthanized 10 days postinoculation (PI) for persistent neurologic signs. Goslings #6 and #86 died 5 and 6 days PI, respectively (Table). On postmortem examination, gosling #86 had subcutaneous hemorrhage around the joints, pale lungs, and petechial hemorrhages in the splenic capsule. Gosling #80 was dehydrated, lacked abdominal body fat, and had a pale beak, enlarged gall bladder, severe thymic and cloacal bursa atrophy, and excess cerebrospinal fluid. All remaining goslings were euthanized 21 days PI and lacked lesions. Goslings #6 and #86 had the most severe histologic lesions, including moderate nonsuppurative meningoencephalitis (Figure) with occasional apoptotic astrocytes, severe diffuse heterophilic (purulent) to lymphocytic myocarditis (Figure) with edema and myocyte necrosis, and vacuolation and apoptosis of pancreatic acinar cells. Gosling #80 had severe meningoencephalitis with necrosis of astrocytes and microglial cells, edema, and microgliosis; lymphohistiocytic choroiditis, uveitis, and from

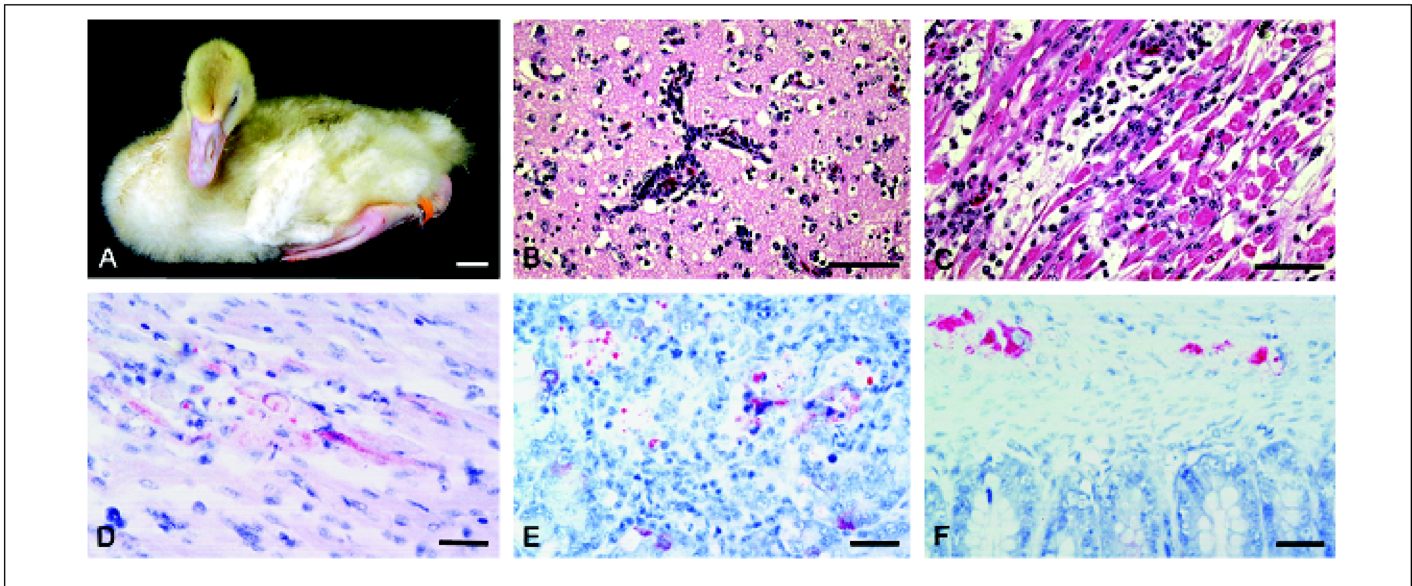

Figure. Two-week-old goslings that were subcutaneously inoculated with 9/99 New York strain of WN virus. (A) Abnormal posture of the head 10 days postinoculation (PI) (#80). The gosling had rhythmic side-to-side movement of the head. Bar = 2.5 cm. (B) Severe nonsuppurative encephalitis at 10 days PI (#80). HE stain. Bar = 50 µm. (C) Severe diffuse myocarditis with edema and myocyte necrosis at 6 days PI (#86). HE stain. Bar = 50 µm. (D) Flaviviral antigens in degenerating myocytes and macrophages within a focus of inflamed myocardium. Immunohistochemical (IHC) strain. Bar = 25 µm. (E) Flaviviral antigens in degenerating parenchymal epithelial cells and foamy macrophages within a focus of pancreatitis. IHC. Bar = 25 µm. (F) Flaviviral antigens in ganglion cells within muscularis mucosae. IHC. Bar = 25 µm.

inflammation of pecten oculi, the protruding vascular structure in the posterior vitreous; and heterophilic (purulent) scleritis and keratitis. WN virus was isolated from brain, heart, kidney, and intestine of goslings in the WN virus-inoculated group (Table). The virus titers were highest in #6, which died 5 days PI; intermediate in #86, which died 6 days PI; and lowest in #80, which was euthanized 10 days PI. WN virus was not isolated from any goslings sampled at the end of the experiment.

Immunohistochemical (IHC) assay, based on a two-step indirect immunoalkaline phosphatase technique described previously (11), was performed on multiple organs of goslings. The primary antibody used in the assay was a mouse polyclonal antibody that cross-reacts with several members of JE serocomplex group flavivirus, including JE virus, St. Louis encephalitis virus, and WN virus. Brain tissues from human JE- and WN virus-confirmed cases (positive controls) and from an unrelated human patient (negative control) were run in parallel. Immunostaining of flaviviral antigens was demonstrated in heart, brain, pancreas, kidney, and autonomic ganglion cells of the intestine (Figure) in WN virus-inoculated goslings, but the distribution and intensity varied with individual WN virus-inoculated goslings.

WN virus was isolated from plasma samples of WN virus-inoculated goslings collected between 1 and 5 days PI, with highest titers 2 and 3 days PI (Table). WN virus was isolated

Table. Virus isolation and titration data from 2-week-old domestic geese inoculated with WN virus and samples taken between 0 and 21 days postinoculation (PI)

Group-bird identification	Plasma virus titers (log_{10} $TCID_{50}$/0.1 mL)[a] by days PI									
	0	1	2	3	4	5	7	10	14	21
Sham controls:										
58	-	NS	-	NS	-	NS	-	-	-	-
73	-	NS	-	NS	-	NS	-	-	-	-
WN virus-inoculates:										
6	-	1.5	5.5	4.5[b]	2.5	2.5[c]	Dead	Dead	Dead	Dead
78	-	3.5	4.5	3.5	1.5[b]	1.5	-	-	-	-
80	-	2.5	5.5	2.5[b]	1.5	-	-	-[c]	Dead	Dead
86	-	2.5	6.5	5.5	2.5	1.5[c]	Dead	Dead	Dead	Dead
In contacts:										
8	-	NS	-	NS	-	NS	-	-	-	-
13	-	NS	-	NS	-	NS	-	2.5	-	-

[a]Endpoints in Vero cell cultures based on CPE and confirmation as WN virus by indirect fluorescent antibody test: NS = No sample, - = No cytopathic effect.
[b]WN virus isolated from oropharyngeal swabs; #6, $10^{1.5}$/0.1 mL, #80, $10^{0.5}$/0.1 mL, #78, $10^{0.5}$/0.1 mL (reported as tissue culture infective doses [$TCID_{50}$] titers in oropharyngeal swab media; originally each swab placed in 1.5 mL of phosphate-buffered saline supplemented with 0.75% bovine serum albumin).
[c]WN virus isolated from tissues at necropsy ($TCID_{50}$/0.01 g): 1) Gosling #6 (5 days PI) - $10^{3.5}$ in brain, $10^{4.5}$ in heart, $10^{1.5}$ in kidney and $10^{3.5}$ in intestine; 2) gosling #80 (10 days PI) - $10^{1.5}$ in brain and $10^{1.5}$ in intestine; 3) gosling #86 (6 days PI) - $10^{3.5}$ in brain, $10^{2.5}$ in heart, $10^{1.5}$ in kidney and $10^{1.5}$ in intestine.

from oropharyngeal swabs on single sampling days in three of four WN virus-inoculated goslings but in low titers ($\leq 10^{1.5}$/0.1 mL). One in-contact gosling (an uninoculated gosling in contact with a WN virus-inoculated gosling) developed transient low-level viremia on 10 days PI (Table). No WN virus was isolated from cloacal swabs of WN virus-inoculated and in-contact goslings. Sham-control goslings were WN virus-free and negative on plaque reduction neutralization assay for anti-WN virus antibodies. WN virus-inoculated goslings had anti-WN virus antibodies 5,7,10, 14, and 21 days PI, and anti-WN virus antibodies were present in one in-contact gosling 14 and 21 days PI.

In the field, the incidence and outcome of WN virus infection vary with the host species. Wild birds are the primary reservoir hosts and, in WN virus-endemic areas, prevalence rates vary from 10% to 53% (12). Seroprevalence studies have identified natural infection in poultry species including broiler chickens and breeders, turkeys, breeder geese, and various species of pigeons in Israel, but veterinary medical investigations identified no associated disease or deaths except in pigeons (Y. Weisman, pers. comm.). However, WN virus infection has been associated with meningoencephalitis, clinical neurologic disease, and myocarditis in young domestic geese (6). The clinical and pathologic features of natural WN virus infections were reproduced in the current study. In humans, infections have usually been asymptomatic, but occasionally illness and death have resulted, accompanied by meningoencephalitis, anterior myelitis, acute pancreatitis, and myocarditis (12). During the 1999 New York outbreak of WN virus, histopathologic examination of central nervous system tissues from four fatal human cases showed varying degrees of neuronal necrosis, with infiltrates of microglia and polymorphonuclear leukocytes, perivascular cuffing, neuronal degeneration, and neuronophagia. IHC assay demonstrated immunostaining of flaviviral antigens in neurons, neuronal processes, and areas of necrosis. No immunostaining was seen in other major organs, including lung, liver, spleen, and kidney. The histopathologic lesions and immunostaining were more prominent in the brain stem and spinal cord (13). Similarly, various animal models, such as mice, hamsters, and rhesus monkeys, have developed fatal encephalitis upon intracerebral inoculation of WN virus (12). In natural cases of WN virus in zoologic birds representing 14 different species and eight orders, deaths were associated with severe myocarditis and encephalitis, and flaviviral antigen was demonstrated in cardiac myocytes and neurons (2,14). Findings from natural cases and experimental studies in various birds and mammals suggest common pathogenic effects of WN virus infections are caused by apoptotic or necrotic cell death in parenchymal cells of the brain, heart, and pancreas.

Conclusions

In our study, subcutaneous inoculation with a New York WN virus isolate produced clinical signs of depression, weight loss, torticollis, opisthotonus, and death in 2-week-old domestic geese, similar to disease in reported field cases. Transient viremia developed 1 to 5 days PI with peak viremia titers (10^{4-6}) 2 days PI. Experimental studies in 1- to 11-day-old chicks demonstrated acquisition of infection from mosquitos, and the resulting viremia titers ($10^{4-6.3}$) efficiently infected naive mosquitoes (7). However, after 3 weeks of age, chickens were refractory to infection by mosquitos, and the resulting low viremia titers were inefficient in infecting mosquitoes (7). In contrast, the peak viremia titers (10^{4-6}) in goslings of the current study were of sufficient magnitude to efficiently infect mosquitos and serve as a reservoir and amplifying host. Furthermore, serologic and virologic data indicated that transmission of WN virus between goslings is possible without a mosquito vector, i.e., by direct contact. In contrast to geese, experimental WN virus infections did not produce clinical disease in chickens and turkeys, and the viremia levels were lower than occurred in the goslings (4,5). WN virus was not directly transmitted to in-contact chickens or turkeys. In addition to various wild birds species of the order Passiformes, young domestic geese can be a reservoir and amplifying host for WN virus and could contribute to the emerging ecology of WN virus in North America.

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References

1. Komar N. West Nile viral encephalitis. Rev Sci Tech 2000;19:166-76.
2. Office International des Epizooties. West Nile fever in the United States of America: in horses. Disease Information 2000;13:150-1.
3. Rappole JH, Derrickson SR, Hubalek Z. Migratory birds and spread of West Nile virus in the Western hemisphere. Emerg Infect Dis 2000;6:319-28.
4. Swayne DE, Beck JR, Zaki SR. Pathogenicity of West Nile virus for turkeys. Avian Dis 2000;44:932-7.
5. Senne DA, Pedersen JC, Taylor WD, Hutto DL, Panigrahy B. Pathogenicity of West Nile virus for chickens. Avian Dis 2000;44:642-9.
6. Office International des Epizooties. West Nile fever in Israel in geese. Disease Information 1999;12:166.
7. Taylor RM, Work TH, Hurlbut HS, Rizk F. A study of ecology of the West Nile virus in Egypt. Am J Trop Med Hyg 1956;5:579-620.
8. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333-7.
9. Anderson JF, Andreadis TG, Vossbrinck CR, Tirrell S, Wakem EM, French RA, et al. Isolation of West Nile virus from mosquitoes, crows, and a Cooper's hawk in Connecticut. Science 1999;286:2331-3.
10. Barbeito MS, Abraham G, Best M, Cairns P, Langevin P, Sterritt WG, et al. Recommended biocontainment features for research and diagnostic facilities where animal pathogens are used. Rev Sci Tech 1995;14:873-7.
11. Zaki SR, Greer PW, Coffield LM, Goldsmith CS, Nolte KB, Foucar K, et al. Hantavirus pulmonary syndrome. Pathogenesis of an emerging infectious disease. Am J Pathol 1995;146:552-79.
12. Malik-Peiris JS, Amerasinghe FP. West Nile fever. In: Beran GW, Steele JH, editors. Handbook of Zoonoses Section B: Viral. Boca Raton: CRC Press; 1994. p. 139-48.
13. Shieh WJ, Guarner J, Layton M, Fine A, Miller J, Nash D, et al. The role of pathology in an investigation of an outbreak of West Nile encephalitis in New York, 1999. Emerg Infect Dis 2000;6:370-2.
14. Steele KE, Linn MJ, Schoepp RJ, Komar N, Geisbert TW, Manduca RM, et al. Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City. Vet Pathol 2000;37:208-24.

Comparative West Nile Virus Detection in Organs of Naturally Infected American Crows (*Corvus brachyrhynchos*)

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Widespread deaths of American Crows (*Corvus brachyrhynchos*) were associated with the 1999 outbreak of West Nile (WN) virus in the New York City region. We compared six organs from 20 crow carcasses as targets for WN virus detection. Half the carcasses had at least one positive test result for WN virus infection. The brain was the most sensitive target organ; it was the only positive organ for three of the positive crows. The sensitivity of crow organs as targets for WN virus detection makes crow death useful for WN virus surveillance.

The 1999 outbreak of West Nile (WN) virus in the New York City area (1) was associated with the deaths of thousands of American Crows (*Corvus brachyrhynchos*), which appeared to be highly susceptible to the virus. Local health authorities selected some of these dead birds for laboratory testing. Generally, brain tissue was targeted for virus isolation as a method of surveillance (2). Although WN virus has frequently been isolated from brain tissue, a rigorous comparison of the brain to other organs of the American Crow has not been undertaken. Accordingly, we compared the sensitivity of the brain with that of other crow organs as targets for WN virus detection by both virus isolation and RNA detection.

The Study

From 20 crow carcasses collected in New Jersey during September and October 1999, we removed sections of brain, liver, spleen, kidney, heart, and lung for WN virus detection by plaque assay and TaqMan reverse transcription-polymerase chain reaction (RT-PCR) (3). The samples were prepared by macerating approximately 0.5 cm^3 of tissue in 1.8 mL of BA-1 (composed of M-199 Hanks salts, 29.2 mg/mL L-glutamine, 0.05 M Tris-HCl, pH 7.6, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 units/mL penicillin, 100 mg/L streptomycin, and 100 µg/mL Fungizone) diluent in a glass Ten Broeck tissue grinder (Bellco Glass, Inc., Vineland, NJ). Virus isolation was attempted in duplicate 100-µL aliquots by Vero cell plaque assay. A 5-µL aliquot from each sample was tested by TaqMan RT-PCR assay, which quantitates WN virus RNA. Sensitivity of each assay for detecting WN virus or RNA in each organ was determined by using only the WN virus-infected carcasses as denominator in the calculations.

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One hundred nineteen tissue samples from 20 crows were assayed for WN virus (Table). Positive test results for WN virus infection were obtained for 10 of the 20 carcasses. WN virus was most often isolated from brain (8 [80%] of 10) and heart (6 [67%] of 9), while WN virus RNA was most frequently detected in brain (10 [100%] of 10) and liver and kidney (each 8 [80%] of 10). The TaqMan assay identified WN virus RNA in seven tissue samples that tested negative by plaque assay, including two brain tissue samples of crows from which all other organ tissues had tested negative. Tissues from the three crows for which only brain provided positive RNA detection were confirmed positive by repeat-testing in triplicate with three different TaqMan RT-PCR primer pairs. WN virus was then isolated by plaque assay from approximately 1 g of brain tissue from one of these specimens (NJN-37, data not shown).

Conclusions

The findings suggest that the brain is the most sensitive target organ (of those tested) from crow carcasses for detecting WN virus with both detection assays ($p = 0.0816$). However, heart, lung, liver, kidney, and spleen were all good sources of WN virus with both assays. (The liver was not a good source of detection with the plaque assay.) Using the TaqMan assay, we were able to identify WN virus RNA in several tissue specimens that were negative by Vero plaque assay. The Taqman assay may be especially useful when organs from necropsied crows no longer contain live virus.

If WN virus continues to spread, rapid detection will be an important public health issue. Since WN virus attacks various internal organs in birds (4), viscera from dead crows can be used to detect the virus in a surveillance program. Our findings, consistent with those of earlier studies, indicate that the brain is the most frequently affected organ among WN virus-infected birds (4) and support the continued use of the brain as the organ of choice from dead crows for surveillance and as a target for WN virus detection in diagnostic assays.

Table. Amount of virus detected by Vero plaque assay and TaqMan reverse transcriptase polymerase chain reaction in American Crow organs

Crow number	Heart	Kidney	Liver	Lung	Spleen	Brain
NJN 5	+++[a]/3.40E+03[b]	+++/5.90E+04	++ /8.48E+04	+++/2.42E+04	+++/4.09E+03	+++/5.41E+03
NJN 6	++/2.36E+03	++/1.12E+04	-[c]/1.10E+02	-/4.61E+02	+/2.02E+02	++/3.12E+02
NJN 7	++/9.57E+03	++/5.52E+03	-/7.61E+01	+/3.46E+03	+/1.26E+02	++/1.76E+03
NJN 8	+++/2.31E+05	+++/5.41E+04	+++/5.61E+05	+++/3.20E+04	+++/4.08E+04	+++/6.18E+04
NJN 9	+++/2.94E+04	+++/1.96E+05	+/2.15E+05	+++/4.36E+05	+++/1.17E+05	+++/1.78E+05
NJN 11	+++/3.62E+04	+++/1.06E+04	+++/1.06E+03	+++/1.31E+04	+++/1.07E+03	+++/2.15E+04
NJN 13	NT/NT	+/2.54E+02	-/1.50E+00	-/1.68E+02	-/-	+/9.28E+01
NJN 29	-/-	-/-	-/-	-/-	-/-	++/6.67E+00
NJN 30	-/-	-/-	-/-	-/-	-/-	-/-
NJN 33	-/-	-/-	-/-	-/-	-/-	-/ 3.24E+00
NJN 37	-/-	-/-	-/-	-/-	-/-	-/ 2.10E-01
NJN 40	-/-	-/-	-/-	-/-	-/-	-/-
NJN 41	-/-	-/-	-/-	-/-	-/-	-/-
NJN 43	-/-	-/-	-/-	-/-	-/-	-/-
NJN 44	-/-	-/-	-/-	-/-	-/-	-/-
NJN 51	-/-	-/-	-/-	-/-	-/-	-/-
NJN 57	-/-	-/-	-/-	-/-	-/-	-/-
NJN 62	-/-	-/-	-/-	-/-	-/-	-/-
NJN 75	-/-	-/-	-/-	-/-	-/-	-/-
NJN 95	-/-	-/-	-/-	-/-	-/-	-/-

[a]Vero cell plaque assay: +++ = ≥100 PFU/200 µL, ++ = 10–100 PFU/200 µL, + = ≤10 PFU/200 µL.
[b]TaqMan RT-PCR assay: PFU equivalents/5 µL.
[c]Negative.
NT = Not tested.

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References

1. Komar N. West Nile viral encephalitis. Rev Sci Tech 2000;19:166-76.
2. Eidson M, Komar N, Sorhage F, Nelson R, Talbot T, Mostashari F, et al. Crow deaths as a sentinel surveillance system for West Nile virus in the northeastern United States, 1999. Emerg Infect Dis 2001;7:615-20.
3. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a Taqman reverse transcriptase PCR assay. J Clin Microbiol 2000;38:4066-71.
4. Steele KE, Linn MJ, Schoepp RJ, Komar N, Geisbert TW, Manduca RM, et al. Pathology of a fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City. J Vet Pathol 2000;37:208-24.

Identification of Arboviruses and Certain Rodent-Borne Viruses: Reevaluation of the Paradigm

Diagnostic and epidemiologic virology laboratories have in large part traded conventional techniques of virus detection and identification for more rapid, novel, and sensitive molecular methods. By doing so, useful phenotypic characteristics are not being determined. We feel that the impact of this shift in emphasis has impaired studies of the biology of viruses. This position paper is a plea to the scientific and administrative communities to reconsider the importance of such information. We also suggest a revised paradigm for virus isolation and characterization and provide a rationale for accumulating biologic (phenotypic) information.

Historical Background

Until about 10 years ago, arthropod-borne viruses (arboviruses) were isolated and then identified by methods now referred to as "classical." That is, clinical or field-collected samples were processed by methods originally established by yellow fever researchers at the Rockefeller Foundation (1-3). As these procedures were shared and adopted by essentially all laboratories conducting arbovirus surveillance and research, they became the standard for arbovirus laboratories worldwide. These techniques were developed to facilitate specific identification of viruses isolated from hematophagous arthropods, vertebrate animals, and human clinical samples. The general scheme included a) isolation of the virus; b) production of a virus seed or stock; c) production of a sucrose-acetone extracted antigen (often inactivated so that it could be used safely for serodiagnostic procedures); d) preparation of an antibody (usually hyperimmune mouse ascitic fluids); e) registration of the virus; and f) deposition of the virus as a voucher specimen in a reference collection (3,4). The accumulation of such reagents by arbovirus laboratories allowed the establishment of reference centers that, with the support and encouragement of the World Health Organization (5,6) and various national governments, distribute useful reagents to regional and local laboratories. Local laboratories, in turn, were then able to conduct serodiagnostic tests for antibody to newly recognized arboviruses, using standardized reagents for virus identification procedures. As an intentional by-product, a network of collaborating centers was established and an international spirit of cooperation and camaraderie evolved, as exemplified by the American Committee on Arthropod-Borne Viruses (ACAV) and its various subcommittees that take responsibility for collating a catalog of the recognized arthropod-borne and rodent-associated viruses (7), evaluating their safety, storing voucher specimens, and determining their antigenic relationships. The resulting catalog, entitled The International Catalogue of Arboviruses and Certain Other Viruses of Vertebrates, has long been the "bible" of arbovirologists. However, with the availability of newer molecular techniques and the current emphasis on genomics, many viruses now are detected by molecular means only. Consequently, few newly discovered viruses are now being registered in the arbovirus catalog, although hundreds of genomic sequences of arboviruses, hantaviruses, arenaviruses, and filoviruses are entered annually in GenBank or other sequence databases. The latter data provide little or no phenotypic information, and, although the ACAV is attempting to provide accessible online biological information regarding arboviruses and other viruses, progress has been slow, in part because of lack of funding and perception of needs. The ultimate goal is to merge genotypic information, such as that deposited in sequence databases, with phenotypic and epidemiologic information, such as that published in the arbovirus catalog, and thereby provide a more accurate and complete picture of the biological characteristics of each virus.

In the heyday of arbovirology (ca. 1960-1975), arbovirus laboratories were fully functional in many parts of the world and both government and institutional support was high. The levels of training, reagent availability, virus discovery, epidemiologic assessments, and research activities were likewise high. As new techniques were developed, the name of the group studying arboviral antigenic relationships was changed from the Subcommittee on *Immunological Relationships* Among Catalogued Arboviruses to the Subcommittee on *InterRelationships* Among Catalogued Arboviruses (SIRACA), to reflect the introduction of molecular techniques as adjunct tools for virus identification and characterization. Few could have predicted the rapid advances to be made or the detail to which the arboviruses would be characterized.

The Apparent Paradigm Shift

As newer techniques (monoclonal antibodies for specific virus identification, immunohistochemistry, RNA fingerprinting, nucleic acid hybridization, and, in particular, polymerase chain reaction and nucleic acid sequencing) were introduced, the earlier techniques were replaced as front-line diagnostic tests, although they remained adequate for most purposes. One reason for this trend was that nucleic acid sequencing and monoclonal antibody mapping of proteins could be used for remarkably rapid and detailed analyses of virus identities and structures by using reagents that had better production consistency and were easier to standardize between laboratories. However, the reliance on genomic sequencing for virus identification has resulted in an apparent quandary: whether to use molecular or other methods for virus identification. Molecular techniques provide information regarding genotypic characteristics. Serologic techniques (hemagglutination inhibition, complement fixation, immunofluorescence using polyclonal or monoclonal antibodies, enzyme-linked immunosorbent assay, neutralization, and vaccination challenge) provide information regarding phenotype. These serologic techniques provide insight into protection and cross-protection against virus infection, information that is of essential epidemiologic and public health significance.

Information Gained, Opportunities Lost

In reality, there is no quandary. Genotypic and phenotypic data are complementary; the phenotype is simply the outward observable characteristic of a virus as determined by its genotype. The genomic sequence provides the foundation for phenotypic expression, but it is not yet possible to deduce completely the phenotype of a virus solely from its genomic sequence. Although some antigenic properties can

be determined by using recombinant antigens, most phenotypic characteristics of a virus (its host range, pathogenicity, cell and tissue tropisms, replication characteristics, and elicitation of protective immunity) still must be determined directly. Because of this, SIRACA continues to support the use of phenotypic assays to identify and classify newly discovered viruses. It is not always necessary to derive genome sequence information for appropriate classification. In fact, for some arboviruses, e.g., those of the families Bunyaviridae and Rhabdoviridae, so little genomic sequence information exists that virus identification must rely on serologic techniques.

Reasons To Accumulate Phenotypic Information

To accurately phenotype a newly discovered virus, infectious virus must be available. Only with an actual isolate is it possible to obtain normal antigenic and other biologic information for comparison with the classical virus databases that have been accrued over many decades. Without a virus isolate, direct cross-protective assays cannot be conducted, and therefore the interrelationships by neutralization of newly recognized arboviruses, hantaviruses, arenaviruses, and filoviruses cannot be determined. Cross-neutralization relationships have been the basis by which most of these viruses have been classified and differentiated (8-15).

Recently, sequencing of virus genomes has opened the fields of viral phylogenetics and molecular epidemiology, allowing comparisons not possible by the older, classical methods. It is now possible to determine rapidly and with some certainty the sources of viruses causing dengue fever (16), West Nile fever (17,18), Venezuelan equine encephalitis (19), hantavirus pulmonary syndrome (20), Ebola fever (21), and outbreaks caused by many other viruses (22). Still, procedures appear to have outpaced process in the study of emerging and reemerging virus diseases.

New Technology Creates New Problems

Detection of viral nucleic acid is not equivalent to isolating a virus. Some hantaviruses have been detected, sequenced and placed in a taxon, and the proteins of some have been expressed without the viruses having been isolated (23). Newly recognized hantaviruses have been described solely on the basis of genomic sequencing, without the agent ever being isolated or the appropriate phenotyping reagents being produced (24-26). Without an isolate, the pathogenic potential, association with human infections and illnesses, and cross-protectivity are difficult to assess. One of the reasons for this development is that agencies funding virus research have opted to support mainly molecular and genetic studies. This funding decision has had a direct effect on the type of virus research carried out at universities, as well as direct and indirect effects on faculty recruitment and graduate education. Research involving the new genetic technologies is promoted as "cutting edge" and "mechanistic," while more classical phenotypic studies are referred to somewhat disparagingly as "descriptive." In truth, both types of research are largely descriptive; genome sequencing and phylogenetic studies of viruses are the molecular equivalents of classical (phenotypic) studies of antigenic properties and antigenic interrelationships. However, both types of research are essential to our understanding of the mechanisms of viral pathogenesis, disease expression, and protective immunity.

Another reason for the lack of phenotypic information about most newly discovered viral pathogens is the increased number of restraints and regulations on the importation, use, and exchange of infectious viruses. The result has been to severely restrict their study to a relatively few high-security laboratories. Inactivated RNA or DNA samples of such agents can be obtained without the need for permits, which favors the use of molecular or genetic methods for studying new viruses. The filoviruses are a case in point. These viruses are extremely hazardous and must be handled under strict Biosafety Level 4 containment. Little is known about their antigenic interrelationships, cross-protectivities, and biological characteristics. Because of the hazards posed by working with these viruses, this is likely to remain the case for the foreseeable future. In contrast, nucleotide sequence analyses of filoviruses provide information adequate for epidemiologic and diagnostic purposes, as well as for phylogenetic studies. Such analyses cannot provide antigenic information for group placement (classification) by neutralization tests or tell us much about pathogenesis or protection. However, in view of their hazardous nature, it would seem prudent for most laboratories to continue assaying filoviruses by molecular techniques, rather than to attempt direct virus isolation.

Despite the remarkable advances in sequencing and phylogenetic analysis, there still is little agreement on the standardization of sequencing approaches, which portions of the genomes of these agents are "best" for designing primers for amplification and diagnostic purposes, and which genome regions will provide the most useful sequence information for taxonomic purposes (for example, the gene coding for the expression of an immunodominant epitope). Uniformity is the sine qua non of such comparisons.

Other issues also impact biological characterization of viruses. For example, little funding is available for the study of animal viruses that are not known or suspected to be pathogens of humans, livestock, or wildlife. The current system of research support in the United States does not encourage the study of orphan viruses until they emerge as proven pathogens, a significant departure from the previous longstanding and productive policy. Likewise, funding agencies have little interest in supporting field studies designed to isolate and identify new viruses. Much lip service is given to the need for biological inventories of species diversity (genetic resources), but in the case of viruses, little funding is available for such studies. As noted, restrictions on the shipment and exchange of some infectious agents, because of biosafety and bioterrorism concerns, have inhibited biological studies with many viruses. Further discussions of these issues are beyond the scope of this paper.

A Solution?

SIRACA continues to emphasize the need for new virus isolates for reference and antigenic studies and for reagent production, even when such isolates are difficult to retrieve. We emphasize that the sources of new arboviruses, hantaviruses, arenaviruses, and filoviruses are field materials, not laboratories. Without support for continued field studies and continued virus isolation, including long-term storage

of representative virus isolates, our knowledge of viral ecology, evolution, and disease emergence will continue to suffer.

In summary, remarkable advances in molecular genetics have allowed rapid and precise identifications of viruses and of their genomes; however, such characterizations thus far can provide only limited information about the phenotype and disease potential of a virus. In addition to more support for studies of viral ecology, pathogenesis, and disease potential, there is a need for serologic reagents with which classical studies can be done. We suggest that infectious materials, in the form of seed virus, be submitted to reference repositories, such as those at the University of Texas Medical Branch, Galveston, Texas; the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado; and the Institut Pasteur, Paris, France. These and other reference centers are supported by home institutions, government agencies, and other funding sources and serve as repositories, rather like museums without the dust.

We suggest that viruses, not simply their genomes, be registered with ACAV, the specialty group on which the International Committee for Taxonomy of Viruses mainly depends for classification of the arboviruses, hantaviruses, arenaviruses, and filoviruses. Financial and enthusiastic and knowledgeable administrative support is needed to continue the task of updating the arbovirus catalog and making it available electronically. As with disease diagnosis, it is the process, not the procedure, that is critical to success.

American Committee on Arthropod-borne Viruses, Subcommittee on InterRelationships Among Catalogued Arboviruses[1]

References

1. Clarke DH, Casals J. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am J Trop Med Hyg 1958;7:561-73.
2. Theiler M, Downs WG. The arthropod-borne viruses of vertebrates. An account of the Rockefeller Foundation virus program, 1951-1970. New Haven: Yale University Press; 1973.
3. Work TH. Isolation and identification of arthropod-borne viruses. In: Lennette EH, Schmidt NJ, editors. Diagnostic procedures for viral and rickettsial diseases, 3rd ed. New York: American Public Health Organization, 1964. p. 312-55.
4. Beaty BJ, Calisher CH, Shope RE. Arboviruses. In: Schmidt NJ and Emmons RW, eds. Diagnostic procedures for viral, rickettsial and chlamydial infections, 6th ed. Washington: American Public Health Association; 1989. p. 797-855.
5. Casals J. Procedures for identification of arthropod-borne viruses. Bull World Health Org 1961;24:723-34.
6. Report of a WHO Scientific Group. Arthropod-borne and rodent-borne viral diseases. Tech Report Series 179. Geneva: World Health Organization; 1985.
7. Karabatsos N. International catalogue of arboviruses including certain other viruses of vertebrates, 3rd ed. San Antonio: American Society of Tropical Medicine and Hygiene; 1985.
8. Calisher CH, Shope RE, Brandt W, Casals J, Karabatsos N, Murphy FA, et al. Recommended antigenic classification of registered arboviruses. I. Togaviridae, *Alphavirus*. Intervirology 1980;14:229-32.
9. Tesh RB, Travassos APA, Travassos JS. Antigenic relationship among rhabdoviruses infecting terrestrial vertebrates. J Gen Virol 1983;64:169-76.
10. Travassos APA, Tesh R, Pinheiro FP, Travassos da Rosa JFS, Peterson NE. Characterization of eight new phlebotomus fever serogroup arboviruses (Bunyaviridae: *Phlebovirus*) from the Amazon Region of Brazil. Am J Trop Med Hyg 1983;32:1164-71.
11. Calisher CH. Antigenic classification and taxonomy of flaviviruses (family Flaviviridae) emphasizing a universal system for the taxonomy of viruses causing tick-borne encephalitis. Acta Virol 1988;32:469-78.
12. Calisher CH, Karabatsos N. Arbovirus serogroups: definition and geographic distribution. In: Monath TP, editor. The arboviruses: epidemiology and ecology, vol. 1. Boca Raton, FL: CRC Press; 1988. p. 19-58.
13. Calisher CH. History, classification, and taxonomy of viruses in the family Bunyaviridae. In: Elliott RM, editor. The Bunyaviridae. New York: Plenum Press; 1996. p. 1-17.
14. Howard CR. Antigenic diversity among the arenaviruses. In: Salvato MS, editor. The arenaviridae. New York: Plenum Press; 1993. p. 37-49.
15. Chu Y-K, Rossi C, LeDuc JW, Lee HW, Schmaljohn CS, Dalrymple J. Serological relationships among viruses in the *Hantavirus* genus, family Bunyaviridae. Virology 1994;198:196-204.
16. Wang E, Ni H, Xu R, Barrett AD, Watowhich SJ, Gubler DJ, et al. Evolutionary relationships of endemic/epidemic and sylvatic dengue viruses. J Virol 2000;74:3227-34.
17. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333-7.
18. Jia X-Y, Briese T, Jordan I, Rambaut A, Chi HC, Mackenzie JS, et al. Genetic analysis of West Nile New York 1999 encephalitis virus. Lancet 1999;354:1971-2.
19. Powers AM, Oberste MS, Brault AC, Rico-Hesse R, Schmura SM, Smith JF, et al. Repeated emergence of epidemic/epizootic Venezuelan equine encephalitis from a single genotype of enzootic subtype ID virus. J Virol 1997;71:6697-705.
20. Nichol ST, Ksiazek TG, Rollin PE, Peters CJ. Genetic identification of a hantavirus associated with an outbreak of acute respiratory illness. Science 1993;262:914-7.
21. Sanchez A, Ksiazek TG, Rollin PE, Miranda MEG, Trappier SG, Khan AS, et al. Detection and molecular characterization of Ebola viruses causing disease in human and nonhuman primates. J Infect Dis 1999;179(suppl 1):S164-S169.
22. Powers AM, Brault AC, Tesh RB, Weaver SC. Reemergence of chikungunya and o'nyong nyong viruses: evidence for distinct geographic lineages and distant evolutionary relationships. J Gen Virol 2000;8:471-9.
23. Schmaljohn C, Hjelle B. Hantaviruses: a global problem. Emerg Infect Dis 1997;3:95-104.
24. Lewis S, Morzunov SP, Rowe JE, Enria D, Pini N, Calderon G, et al. Genetic diversity and epidemiology of hantaviruses in Argentina. J Infect Dis 1998;177:529-38.
25. Hjelle B, Chavez-Giles F, Torrez-Martinez N, Yates T, Sarisky J, Webb J, et al. Genetic identification of a novel hantavirus of the harvest mouse, *Reithrodontomys megalotis*. J Virol 1994;68:6751-4.
26. Vincent MJ, Quiroz E, Gracia F, Sanchez AJ, Ksiazek TG, Kitsutani PT, et al. Hantavirus pulmonary syndrome in Panama: identification of novel hantaviruses and their likely reservoirs. Virology 2000;277:14-9.

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Treatment of West Nile Virus Encephalitis with Intravenous Immunoglobulin

To the Editor: West Nile virus is endemic in Israel. The overwhelming majority of infections are mild and asymptomatic, but there have been periodic symptomatic outbreaks (1). In August 2000, an epidemic of West Nile virus broke out in Israel, with >260 confirmed cases and 20 deaths by the end of September 2000. Hitherto, the only treatment for this condition has been supportive with no proven in vivo specific therapy, although ribavirin has shown promise in in vitro studies (2). We report an apparent dramatic response to intravenous immunoglobulin in an immunosuppressed patient and suggest that this was the result of specific antibodies in the Israeli immunoglobulin used.

A 70-year-old woman was admitted to the hospital because of fever and vomiting of 24 hours' duration. She had a 12-year history of chronic lymphatic leukemia (Rai stage II) but was not on treatment. A routine outpatient assessment 1 week earlier had shown no unexpected findings.

On physical examination the patient appeared generally well, with temperature 39.0°C, regular pulse 100/minute, and blood pressure 130/70. Apart from splenomegaly 2-3 cm below the costal margin, there were no abnormal physical signs, including lymphadenopathy. Chest X ray results were normal. Hb was 12 g/dL, HCt 32%, mean corpuscular volume 84, leukocyte count 280x10^9/L (90% lymphocytes, 13% neutrophils, and 10% monocytes), platelets 280x10^9/L, Coombs negative. Her biochemical profile was entirely within the normal range. Blood and urine cultures were negative. Immunoglobulin G (IgG) was 14.5 g/L, IgM 2.6 g/L, and IgA 2.6 g/L.

Forty-eight hours after admission, dysarthria with episodes of impaired consciousness developed. After a further 24 hours, she was in deep coma (Glasgow Coma Scale, 6). Empiric treatment for presumed central nervous system infection was begun with ceftriaxone, ampicillin, acyclovir, and amphotericin B. Results of cranial computerized tomography were normal. A lumbar puncture was performed and showed clear cerebrospinal fluid (CSF) at normal pressure. CSF protein was 1.04 g/L, glucose 2.4 mmol/L, and leukocyte count 162/mm^3 (90% mononuclear cells). Gram stain was negative, as were bacterial culture, cryptococcal antigen, and results of a polymerase chain reaction test for herpes viruses. IgM antibodies against West Nile virus were positive in both serum and CSF.

With the definite diagnosis of West Nile encephalitis, all antimicrobial treatment was stopped. Because of the chronic lymphatic leukemia and presumed immunosuppression, we decided to give intravenous immunoglobulin (Omr-IgG-am, Omrix Biopharmaceutical Ltd, Tel Hashomer, Israel), 0.4 g/kg, as has been recommended for this condition (3). The patient's neurologic condition remained unchanged (Glasgow coma scale, 5-6) for the next 2 days but then began to improve. Over the subsequent 5 days, her level of consciousness returned to normal.

In light of this apparently dramatic response to treatment with intravenous immunloglobulin, we examined several batches of pooled immunoglobulin from different sources for antibodies to West Nile virus. Intravenous immunoglobulin preparations from donors in Israel, such as our patient received, contained high titers (1:1,600) of such antibodies, while those from the USA had no detectable antibody. We suggest that the use of such antibody-containing immunoglobulin may provide a specific and effective treatment for serious cases of West Nile virus infections, and therefore that formal trials of its use should be carried out.

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References

1. Cohen D, Zaide Y, Karasenty E, Schwarz M, LeDuc JM, Slepon R, et al. Prevalence of antibodies to West Nile fever, sandfly fever Sicilian, and sandfly fever Naples viruses in healthy adults in Israel. Public Health Rev 1999;27:217-30.
2. Jordan I, Briese T, Fischer N, Lau JY, Lipkin WI. Ribavirin inhibits West Nile virus replication and cytopathic effect in neural cells. J Infect Dis 2000;182:1214-17.
3. Cooperative Group for the Study of Immunoglobulin in Chronic Lymphocytic Leukemia. Intravenous immunoglobulin for the prevention of infection in chronic lymphocytic leukemia: a randomized, controlled clinical trial. N Engl J Med 1988;l319:902-7.

Nipah Virus Infection Among Military Personnel Involved in Pig Culling during an Outbreak of Encephalitis in Malaysia, 1998–1999

To the Editor: An outbreak of severe encephalitis affecting 265 patients, 104 (40%) of whom died, occurred during 1998-1999 in Malaysia. It was linked to a new paramyxovirus, Nipah virus, which infects pigs, humans, dogs, and cats (1-4). Nipah virus is most closely related to Hendra virus (4), which was discovered in Australia in 1994 during an outbreak of severe respiratory disease among horses and humans (5-9). Most patients in Malaysia were pig farmers, and human infection was linked to exposure to pigs (10). An operation to cull the approximately 1 million pigs on farms in the outbreak-affected areas was carried out, primarily by 1,638 military personnel. After two soldiers involved in culling became ill with Nipah encephalitis, we conducted a cross-sectional survey of military personnel participating in culling activities in the outbreak-affected states of Malaysia (Negeri Sembilan and Selangor) to assess the prevalence of Nipah infection.

The survey was conducted approximately 2-4 weeks after the end of all culling activities to control the outbreak. All military personnel, enlisted and officers, who had been assigned to culling duty in the states of Negeri Sembilan and Selangor were invited to participate, regardless of specific job assignment. Study teams visited the military bases in each state and administered a survey asking about illness, specific exposures and activities during culling, use

of protective equipment such as gloves, and other pig exposures not associated with culling. A single serum specimen obtained at the time of the interview was tested for the presence of immunoglobulin (Ig) M and IgG antibodies against Nipah virus by enzyme immunoassays (EIA). IgM antibodies were detected by an IgM-capture EIA and IgG antibodies by an indirect EIA. Hendra virus antigens, which cross-react with antibodies against Nipah virus, were used in the serologic assays. In limited laboratory comparisons with Hendra virus and Nipah virus antigens, a good correlation was observed between the IgG and IgM EIA results.

Of 1,474 military personnel listed in the records of the military bases where the survey was performed, 1,412 (96%) responded to the survey and provided serum specimens. The mean age of the participants was 28.3 years (range 19 to 50 years). On average, the soldiers participated in culling for 8.2 days (range 1 to 60 days), for 7.4 hours per day (range 0.5 to 18 hours per day), and at 86 farms (range 1 to 696 farms). During culling, 63% reported physical contact with live pigs and 30.9% with dead pigs. The most common activities reported by the soldiers included shooting pigs (63.2%); herding, hitting, or carrying live pigs (60.5%); changing rifle magazines (39.4%); carrying dead pigs (25.8%); and spraying lime over pig burial sites (14.6%). More than 80% reported wearing gloves, masks, and boots, and 31% reported wearing goggles at all times during culling.

Six (0.4%) of the 1,412 personnel studied had detectable antibody against Nipah virus. All six had IgM antibody, and one also had IgG antibodies. Two of the six antibody-positive persons had been hospitalized with encephalitis during the culling operation. All the antibody-positive personnel were involved in culling in Negeri Sembilan state and reported direct physical contact with live pigs; none reported obvious contact with secretions or body fluids (e.g., blood and urine) of infected pigs. Four of the six antibody-positive persons also reported direct physical contact with dead pigs. All six reported wearing gloves, masks, and boots at all times while working, and three reported wearing goggles.

Comparison of exposures and activities among personnel involved in culling in Negeri Sembilan state (N = 960) and Selangor state (N = 497) showed that the former were more likely to report direct physical contact with both live pigs (69.4% vs. 57.0%, respectively, $p < 0.001$) and dead pigs (41.7% vs. 11.2%, respectively, $p < 0.001$). However, the reported prevalence of sick pigs on farms where pigs were culled did not differ between the two groups (68.6% versus 72.9%, respectively, $p = 0.50$). No significant differences were observed in use of personal protective equipment (gloves, boots, and mask) for those in Negeri Sembilan compared with Selangor, except for wearing goggles (34.8% versus 76.4%, respectively, $p < 0.001$).

Our findings indicate that transmissibility of Nipah virus to military personnel involved in pig culling was low. Four of the six infected persons were apparently well; follow-up of these and the other infected soldiers will be important to determine if any symptoms of disease or long-term sequelae develop. The observation that all the infected persons reported direct contact with live pigs is consistent with the hypothesis that transmission of Nipah virus to humans most likely occurs from close contact with infectious secretions or body fluids of pigs. Respiratory secretions and urine of infected pigs have been shown to contain Nipah virus and may be vehicles of transmission (1,2,4).

We could not document the route of infection for the antibody-positive personnel. Although all six antibody-positive persons reported wearing gloves, masks, and boots while culling, three did not report wearing goggles. Exposure may have occurred through inoculation of the conjunctiva with infectious secretions of pigs; however, bias in reporting use of other protective equipment should also be considered. Given the great severity of Nipah encephalitis and the possible, although small, risk of transmission of virus to military personnel involved in culling, we recommend great care in handling potentially Nipah-infected pigs during such operations.

Acknowledgments

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References

1. Centers for Disease Control and Prevention. Outbreak of Hendra-like virus–Malaysia and Singapore, 1998-1999. MMWR Morbid Mortal Wkly Rep 1999;48:265-9.
2. Centers for Disease Control and Prevention. Update: Outbreak of Nipah virus–Malaysia and Singapore, 1999. MMWR Morbid Mortal Wkly Rep 1999;48:335-7.
3. Chua KB, Goh KJ, Wong KT, Kamarulzaman A, Tan PS, Ksiazek TG, et al. Fatal encephalitis due to Nipah virus among pig-farmers in Malaysia. Lancet 1999;354:1257-9.
4. Chua KB, Bellini WJ, Rota PA, Harcourt BH, Tamin A, Lam SK, et al. Nipah virus: a recently emergent deadly paramyxovirus. Science 2000;288:1432-5.
5. Murray K, Selleck P, Hooper P. A morbillivirus that caused fatal disease in horses and humans. Science 1995;268:947.
6. Murray K, Rogers R, Selvey L, Selleck P, Hyatt A, Gould A, et al. A novel morbillivirus pneumonia of horses and its transmission to humans. Emerg Infect Dis 1995;1:313.
7. Selvey LA, Wells RM, McCormack JG, Ansford AJ, Murray K, Rogers RJ, et al. Infection of humans and horses by a newly described morbillivirus. Med J Aust 1995;162:642-5.
8. Hooper PT, Gould AR, Russell GM, Kattenbelt JA, Mitchell G. The retrospective diagnosis of a second outbreak of equine morbillivirus infection. Aust Vet J 1996;74:2445.

9. Rogers RJ, Douglas IC, Baldock FC, Glanville RJ, Seppanen KT, Gleeson LJ, et al. Investigation of a second focus of equine morbillivirus infection in coastal Queensland. Aust Vet J 1996;74:2434.
10. Parashar UD, Lye MS, Ong F, Mounts AW, Arif MT, Ksiazek TG, et al. Case-control study of risk factors for human infection with a new zoonotic paramyxovirus, Nipah virus, during a 1998-1999 outbreak of severe encephalitis in Malaysia. J Infect Dis 2000;181:1755-9.

Integrated Mosquito Management: No New Thing

To the Editor: Rose displays a fundamental misunderstanding of the history of mosquito control when he states, "Mosquito control in the United States has evolved from reliance on insecticide applications for control of adult mosquitoes (adulticide) to integrated pest management programs that include surveillance, source reduction, larvicides, and biological control, as well as public relations and education" (1).

More than 100 years ago, General William C. Gorgas used a multifaceted approach to control mosquitoes when he and his staff brought yellow fever under control in Havana after the Spanish-American War. He was to repeat this approach in Panama, where the French had lost 20,000 lives to mosquito-borne disease in their failed attempt to construct an isthmusian canal.

In New Jersey at the turn of the century, state entomologist John B. Smith was convinced that the state could be made mosquito free. The laws of 1902 provided for funding to study mosquitoes and resulted in Smith's comprehensive study of the subject (2). Smith's work led to water management as a primary means of controlling mosquitoes on New Jersey's extensive salt marshes. He addressed the issue of biological control by native fish, primarily saltmarsh killifish. Thus, Rose's claim is inaccurate: Integrated mosquito management (IMM) was alive and well at the turn of the century.

When the New Jersey Mosquito Extermination Association was formed in 1913, state mosquito control workers began what has been a long involvement with education and public relations. These critical components of IMM have long been an essential part of mosquito control activities throughout the United States. Reports by various county control agencies in New Jersey reveal an ongoing concern with water management. Indeed, these early reports speak of water management, particularly in the upland environment, as a means of making lands formerly considered useless productive and thus generators of tax revenues.

Regarding surveillance, the laws of 1905 charged the director of the New Jersey Agricultural Experiment Station with conducting surveys of mosquito breeding in the various political entities of the states. The standard tool for surveillance, the New Jersey light trap, was developed in the 1930s and has been in regular use since then. Thus, another key component of IMM was in place at the turn of the last century.

IMM has long been the standard operating procedure in New Jersey and many other states. In the early 20th century, mosquito fighters did not have the array of weapons now available. They had to use the tools available to them: sanitation, habitat management, larvicides, fumigation for adults, screens for exclusion, education, and legal action (i.e., fines for maintaining mosquito-breeding sites on private property).

The association between mosquitoes and disease was very real in the early days of mosquito control. As recently as 1880, 20,000 lives were lost to malaria in the Mississippi River Valley, and malaria was endemic in the Tennessee Valley. Mosquito control in the Tennessee Valley Authority area was not brought about by mosquitocides but by clear-cutting the margins of bodies of water to reduce or eliminate mosquito habitat. The wide-scale use of mosquitocides did not occur until after World War II. Before then, IMM was the only response they had. To ignore these facts does a grave disservice to those who fought in the mosquito wars in the early part of the 20th century.

I also disagree with Rose's discussion of some biological control agents. One has only to look at the number of mosquitoes coming off a flooded high tidal marsh to realize that biological control is useful primarily in areas where mosquito populations do not result in thousands of mosquitoes per trap night. Similarly, some of the limitations listed for various mosquitocides are givens. Mosquito control workers know full well there is no panacea; that is the reason for IMM. It is erroneous, for instance, to list subsurface larvae as a limitation for monomolecular films; where a monomolecular film is present, subsurface larvae cannot emerge because the reduced surface tension does not allow the newly emerged adult to stand on the water's surface. An insect landing on treated water passes through the surface and drowns. Indeed, the greatest drawback of monomolecular films is their effect on insects that require a certain amount of surface tension, such as water striders.

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References

1. Rose RI. Pesticides and public health: integrated metehods of mosquito management. Emerg Infect Dis 2001;7:17-23.
2. Smith JB. Report of the New Jersey Agricultural Experiment Station upon the mosquitoes occurring within the state, their habits, life history, etc. New Brunswick (NJ); 1904. Cited in Headlee TJ. The mosquitoes of New Jersey and their control. New Brunswick (NJ): Rutgers University Press; 1945.

Integrated Mosquito Management—Reply to Dr. Rupp

To the Editor: My article (1) was not intended to delve into the history of mosquito control nor cast aspersions on the great work that was done to fight malaria and yellow fever a century ago. Rather, the article is a short review of contemporary integrated methods of mosquito management and a discussion of how public health pesticides may be affected by the Food Quality Protection Act's amendments to the Federal Insecticide, Fungicide, and Rodenticide Act.

Mr. Rupp contends that the article misinterprets the history of mosquito control and does a disservice to those who fought in the mosquito wars in the early 20th century. Mr. Rupp valiantly defends this early history in his letter,

with reference to programs a hundred years ago, when contemporary pesticides and biological and cultural controls did not exist and the tools of mosquito control were limited to such measures as deep-ditch draining of wetlands in New Jersey, clear-cutting, and use of arsenic compounds and crude petroleum for larval control. Deep-ditch draining was also practiced long ago in other states, such as Florida.

It was but half a century ago, after World War II, that chlorinated hydrocarbons such as DDT came into widespread use for mosquito control until they were banned, and organophosphates such as malathion and naled took their place. For cost and performance reasons, DDT continues to be used in several developing countries for mosquito control. Mr. Rupp refers to old reports of water management as a means of making land formerly considered useless into productive land capable of generating tax revenues. Today, this practice would be considered wetlands conversion and wildlife habitat destruction.

Robert Ward's article in the latest Florida Mosquito Control Association's Wing Beats reminisces about the venerable thermal fog machine, "those hot smelly 'smokers' belching up to eighty gallons of fog material per hour...fireballs, greasy streets and cars, or blinded drivers" (2). Back in those days, many children chased them on bicycles, ignorant of pesticide risks that are now known. Even in recent history, broad-spectrum organophosphates such as parathion and chlorpyrifos, which have potent nontarget effects, were used in aquatic habitats to control mosquito larvae.

Mr. Rupp's comments focus mainly on the mosquito control of a century ago, when the stakes were high because of malaria and yellow fever. The pioneers in mosquito control did marvelous work with the limited tools available to them and their limited knowledge of environmental consequences, but the history of mosquito control has had its time of pesticide reliance and has truly evolved to today's fully integrated mosquito management as briefly described in the article.

Robert I. Rose

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References

1. Rose RI. Pesticides and public health: integrated methods of mosquito management. Emerg Infect Dis 2001;7:17-23.
2. Ward R. Are we just power mad? Wing Beats 2000;11:25-6.

Enteric Fever Treatment Failures: A Global Concern

To the Editor: We read with great interest the report by Threlfall et al. (1) of decreased susceptibility to ciprofloxacin in *Salmonella enterica* serotype Typhi from the United Kingdom. The authors indicate that nalidixic acid-resistant *S.* Typhi with decreased susceptibility to ciprofloxacin is now endemic in India and neighboring countries, constituting a threat to global health. The data are consistent with previous studies from India (2-4). Despite the wide applications and broad-spectrum efficacy of fluoroquinolones, resistance is increasingly observed in many species of gram-negative organisms, including *Salmonella*. Detection in any part of the world of even a few resistant strains with higher MICs to ciprofloxacin is of concern to clinicians and microbiologists. We report our recent observations in cases of treatment failure of enteric fever caused by *S. enterica* serotypes Typhi and Paratyphi A.

Fluoroquinolones have been in use for >15 years and have remained an extremely important weapon against infections. Ciprofloxacin is used widely in India to treat many human infections, even without prescription, although recommendations limit its use to enteric cases caused by multidrug-resistant (MDR) strains. However, concern is increasing that widespread use of these and newer drugs will result in development of resistance against them. Recently, reports have increased worldwide concerning reduced activity of ciprofloxacin and allied drugs against many infectious agents, including *Salmonella* (2-4).

In an ongoing study of drug susceptibility following E-test, >12% of recent isolates of *S.* typhi in our institution have shown increased MICs to ciprofloxacin (range 1.0 to 2.0 µg/mL), with 3% as high as 2.5 µg/mL (3-4). Of >100 strains screened recently, 4 of 18 MDR strains had increased resistance to ciprofloxacin. Of the rest, 9 of 82 had higher MICs to ciprofloxacin alone but were not MDR, and 2 were cases of double infection with *S.* Typhi and *S.* Paratyphi A, common serotypes causing enteric fever in our region. Because resistance to the quinolone group of drugs (caused by gene mutations) develops independent of that in other drugs, which are plasmid encoded, it also may develop in otherwise sensitive strains.

However, our recent observations differ from those of Dr. Threlfall, as well as from past data from India. We have observed that treatment failures did not always correlate with higher MICs to nalidixic acid and ciprofloxacin alone. We have also noted a declining rate of MDR in *S.* Typhi, reflecting increased sensitivity to chloramphenicol, amoxicillin, and trimethoprim. However, *S.* Paratyphi A showed relatively increased resistance to these drugs. The increasing resistance to ciprofloxacin, to which enteric fever treatment failures are often attributed, is now mainly caused by strains susceptible to other common drugs.

Drs. Threlfall and Ward stated that >50% of strains with decreased susceptibility to ciprofloxacin were MDR (1). In contrast, our findings suggest that, despite prolonged doses of ciprofloxacin, treatment failures are still common with isolates sensitive to ciprofloxacin and nalidixic acid. Drs. Threlfall and Ward also emphasized that MDR cases with reduced sensitivity to ciprofloxacin are mainly transmitted by travelers returning from India and Pakistan. This conclusion would be justified as long as phage type E1, comprising MDR strains with higher MICs to ciprofloxacin, is endemic in India. However, problems of reduced action by ciprofloxacin are now thought to be independent of MDR, to result from many other factors, and thus to be of global origin and incidence. Overall, we observe a much higher rate than in the past of reduced susceptibility in *S.* Typhi and *S.* Paratyphi A in our region, causing delayed response in enteric patients. The increasing enteric fever treatment failures noted by our clinicians indicate the need for careful screening of recent isolates.

Fluoroquinolone resistance usually results from mutations in genes for drug targets (gyrA and parC) or potential of the drug being marked as a substrate as a result of overexpression of drug-efflux pumps (5). Drug

resistance attributable to efflux has been reported in a number of gram-negative species, including *Salmonella* and *Pseudomonas*. Strains expressing efflux mechanisms leading to fluoroquinolone resistance are cross-resistant to a number of structurally unrelated antimicrobial agents, permitting multidrug resistance to develop (6). Therefore, inhibition of efflux systems as targets of therapeutic intervention would help prevent emergence of resistance to fluoroquinolones and associated drugs and would further potentiate drug activity. Bacteria exposed to concentrations near their MIC values readily undergo selection for resistance to ciprofloxacin (7). Hence, dosing regimens accounting for both treatment efficacy and susceptibility of clinical pathogens should help control drug resistance that causes frequent treatment failures (8).

Emerging resistance to antimicrobial agents by interacting pathogens is not solely responsible for treatment failures, since many other factors may be involved, e.g., inappropriate antibiotic regimen and dose selection, poor patient compliance, and drug-drug and drug-host interactions. One clinically important drug interaction involving fluoroquinolones is not only by coadministration with other drugs but also results from chelation to divalent and trivalent cations, such as in antacids, iron compounds, or dairy products; such chelation prevents most of the drugs from being absorbed (9).

Efforts should be aimed at shortening treatment duration by adopting efficacious drugs, since rapid, complete eradication of an infecting organism may limit the development of drug resistance. In addition, the rapid and sensitive detection by molecular methods of invasive disease due to *Salmonella* may help avoid overtreatment for fever of unknown origin (10). Finally, development of newer drugs offering similar activity against both enzyme targets (DNA gyrase and topoisomerase-IV), as well as an improved therapeutic index, will definitely strengthen clinical practice.

The challenge ahead is to further our understanding of newer antimicrobial resistance mechanism possibilities stemming from the recent development of structurally modified fluoroquinolones. Additional studies should assess the relevance of pharmacodynamic modeling in determining dosing or predicting efficacy and clinical management for various indications in different patient populations.

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References

1. Threlfall EJ, Ward LR. Decreased susceptibility to ciprofloxacin in *Salmonella enterica* serotype Typhi, United Kingdom. Emerg Infect Dis 2001;7:448-50.
2. Chandel DS, Chaudhry R, Dhawan B, Pandey A, Dey AB. Drug resistant *Salmonella enterica* serotype Paratyphi A in India. Emerg Infect Dis 2000;6:420-1.
3. Rodrigues C, Mehta A, Joshi VR. Nalidixic acid resistant *Salmonella typhi* in Mumbai. Nat Med J India 1999;12:188.
4. Chaudhry R, Chandel DS, Mehta G, Kapoor H, Pang T. Molecular characterisation of drug sensitive and drug resistant strains of *Salmonella typhi*. Med J Indonesia 1998; 7(S1):188.
5. Hooper DC. Emerging mechanisms of fluoroquinolone resistance. Emerg Infect Dis 2000;7:337-41.
6. Poole K. Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria. Antimicrob Agents Chemother 2000; 44:2233-41.
7. Cullmann W, Steiglitz M, Baars B, Opferkuch W. Comparative evaluation of newly developed quinolone compounds, with a note on the frequency of resistant mutatants. Chemotherapy 1985;31:19-28.
8. Schentag JJ. Clinical pharmacology of the fluoroquinolones: studies in human dynamics/kinetic models. Clin Infect Dis 2000;31:S40-S44.
9. Borcherding SM, Stevens R, Nicholas RA, Corley CR, Self T. Quinolones: a practical review of clinical uses, dosing considerations and drug interactions. J Fam Pract 1996;42:69-78.
10. Chaudhry R, Laxmi BV, Nisar N, Ray K, Chandel DS. Standardisation of polymerase chain reaction for the detection of *Salmonella typhi* in typhoid fever. J Clin Pathol 1997;50:437-9.

Enteric Fever Treatment Failures—Reply to Drs. Chandel and Chaudhry

To the Editor: We are pleased that Drs. Chandel and Chaudhry support our concern that the development of low-level resistance to fluoroquinolone antimicrobial agents in *Salmonella enterica* serotype Typhi is a threat to health in both developing and developed countries. They cite their article (1) reporting the recent emergence in India of strains of *S.* Paratyphi A resistant to nalidixic acid and with low-level resistance to ciprofloxacin. This finding has also been observed in the United Kingdom, with >30% of *S.* Paratyphi A infections in 2000 being caused by strains with decreased susceptibility to ciprofloxacin. Of these strains, only one was also resistant to other antimicrobial agents.

Our findings and those of Chandel and Chaudhry clearly demonstrate the inadvisability of the use of ciprofloxacin in the Indian Subcontinent to treat many human infections, regardless of prescription. To maintain the efficacy of fluoroquinolones in both developing and developed countries, this class of antimicrobial agents must be reserved for treatment of invasive disease and not for prophylaxis. For travelers visiting developing countries, ciprofloxacin must be used only when absolutely necessary and not for treatment of uncomplicated gastroenteritis or for travelers' diarrhea syndromes.

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Reference

1. Chandel DS, Chaudhry R, Dhawan B, Pandey A, Dey AB. Drug-resistant *Salmonella enterica* serotype Paratyphi A in India. Emerg Infect Dis 2000;6:420-1.

Mycobacterium tuberculosis Beijing Genotype, Thailand—Reply to Dr. Prodinger

To the Editor: We read with interest the report on the occurrence of *Mycobacterium tuberculosis* strains of the Beijing genotype in Thailand (1). In contrast to our findings in Vietnam (2), Prodinger et al. found no significant association between the Beijing genotype and either young age or drug resistance (1). However, we have some caveats regarding the comparison of these two studies. First, we restricted our analysis to newly diagnosed patients to avoid confounding by possible differences in relapse rates between

M. tuberculosis genotypes. Second, we excluded confounding by geographic collection site. Although this was not a problem in our study (with 58% of isolates in Hanoi and 53% in Ho Chi Minh City representing the Beijing genotype), it might be in Thailand in view of the reported difference between Thailand and Malaysia. Third, the statistical power of the study in Thailand was limited: a difference of 56% in the group <25 years versus 43% in the category >25 years is potentially important, even if not statistically significant with the given sample size. The power of the Thailand study to demonstrate an association with drug resistance is similarly limited.

Despite these caveats, we agree with Prodinger et al. that the epidemiology of the Beijing genotype strains may vary among Southeast Asian countries. For instance, in Hong Kong we found no association between the Beijing genotype and younger age and a weak association with isoniazid (INH) resistance (3).

Various explanations may account for these differences. For instance, if our hypothesis that the selective advantage of the Beijing genotype in Vietnam is due to its association with drug resistance is accurate, then no association with young age and recent transmission would be expected in situations where the Beijing genotype has not (yet) acquired these high levels of drug resistance. Moreover, if a strong program is in place to deal with drug-resistant tuberculosis, this selective advantage may disappear (4).

On the basis of the observation of Prodinger et al., we see no reason to dilute our previous message regarding the emergence of Beijing genotype strains. Ongoing research suggests that the Beijing genotype strains elicit a different immune response than other *M. tuberculosis* genotypes in particular human populations. For instance, in Jakarta, Indonesia, tuberculosis patients infected with Beijing genotype strains were significantly more likely to have febrile responses during the first 2 weeks of treatment (5). In this region we again also found a significant association with INH and streptomycin resistance.

Within the framework of a Concerted Action Project of the European Union, involving 32 institutes within and outside Europe, the worldwide spread of Beijing genotype strains will be examined. We strongly favor study of the genetic makeup of the Beijing genotype to gain insight into the success of this highly conserved family of strains, which appears to be responsible for a substantial part of the worldwide recurrence of tuberculosis, and in particular, of multidrug-resistant tuberculosis.

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References

1. Prodinger WM, Bunyaratvej P, Prachaktam R, Pavlic M. *Mycobacterium tuberculosis* isolates of Beijing geontype in Thailand (Letter). Emerg Infect Dis 2001;7:483-4.
2. Anh DD, Borgdorff MW, Van LN, Lan NTN, van Gorkom T, Kremer K, et al. *Mycobacterium tuberculosis* Beijing genotype emerging in Vietnam. Emerg Infect Dis 2000;6:302-5.
3. Chan MY, Borgdorff MW, Yip CW, de Haas PEW, Wong WW, Kam KM, et al. Seventy percent of the *Mycobacterium tuberculosis* isolates in Hong Kong represent the Beijing genotype. J Clin Microbiol 2001. In press.
4. Dye C, Williams BG. Criteria for the control of drug-resistant tuberculosis. Proc Natl Acad Sci U S A 2000;97:8180-5.
5. Van Crevel R, Nelwan RHH, de Lenne W, Veeraragu Y, van der Zanden AG, Amin Z, et al. *Mycobacterium tuberculosis* Beijing genotype strains associated with febrile response to treatment. Emerg Infect Dis 2001. In press.

In the next issue of
Emerging Infectious Diseases,
September-October 2001

Emerging Infectious Diseases in an Island Ecosystem: The New Zealand Perspective

Potential Infectious Etiologies of Atherosclerosis: A Multifactorial Perspective

The First Isolation of La Crosse Virus from Naturally Infected *Aedes albopictus*

***Ehrlichia* Infected Ticks on Migrating Birds**

Factors Contributing to the Emergence of *Escherichia coli* O157 in Africa

For a more complete list of articles included in the September–October issue, and for articles published online ahead of print publication, see http://www.cdc.gov/ncidod/eid/upcoming-5.htm

Upcoming Events

Fifth International Conference of the Hospital Infection Society
Edinburgh International Conference Centre
Edinburgh, Scotland
September 15-18, 2002

Themes for the conference include recent advances in control and prevention of hospital-acquired infection, antibiotic resistance, information technology and the early detection and control of outbreaks, hospital-wide strategies to enhance patient care, the health of health-care professionals, and clinical governance and infection control standards.

For more information, contact the Conference Secretariat: Concorde Services, HIS2002, Unit 4b, 50 Speirs Wharf, Port Dundas, Glasgow G4 9TB, Scotland, UK; telephone: +44 (0) 141 331 0123; fax: +44 (0) 141 331 0234; e-mail: his@concorde-uk.com; or URL: www.his2002.co.uk

Inaugural EIDIOR Workshop
Perth, Western Australia
September 26-29, 2001

This first international workshop on emerging infectious diseases of the Indian Ocean rim region (EIDIOR) aims to develop a regional EID agenda. Themes will include clinical infectious diseases, laboratory technology, scientific developments and advances, public and environmental health, biocontainment, and biodefense. Preliminary program details will be announced on our website and to those expressing interest in attending. The workshop is scheduled concurrently with the World Melioidosis Congress.

For more information, contact the conference secretariat: Congress West, P.O. Box 1248, West Perth, WA 6872, Australia; telephone: +618 9322 6662; fax: +618 9322 1734; URL: http://www.e-tiology.com/eidior/index.htm

4th European Health Forum—Gastein 2001 Integrating Health across Policies
Bad Gastein, Salzburg, Austria
September 26-29, 2001

Topics of plenary sessions include global influences on health and health services, macroeconomic policy and health, promoting and protecting health across the European Union, health impact assessment in Europe, and the WHO Investment for Health project. Topics of parallel forum sessions include health across policies and sectors, world trade and globalization, health in the single European market, health recommendations for building a new common agricultural policy, and health in the information age.

For further information, contact International Forum Gastein, Tauernplatz 1, A-5630 Bad Hofgastein, Salzburg, Austria; telephone: +43 6432 7110-70; fax: +43 6432 7110-71; e-mail: info@ehfg.org; URL: http://www.ehfg.org/

Health Information for International Travel

The Division of Global Migration and Quarantine, Centers for Disease Control and Prevention, announces the release of the 2001-2002 edition of Health Information for International Travel (the Yellow Book). The new edition contains updated information on vaccinations and malaria risk and prophylaxis, revised disease-specific text and tables, new sections on altitude sickness and international adoption, and improved maps and indexing. For more information contact the Public Health Foundation by telephone at 1-877-252-1200 or online at http://bookstore.phf.org

International Conference on Emerging Infectious Diseases, 2002

The National Center for Infectious Diseases, Centers for Disease Control and Prevention, has scheduled the third International Conference on Emerging Infectious Diseases (ICEID2002) for March 24 - 27, 2002, at the Hyatt Regency Hotel, Atlanta, Georgia, USA. More than 2,500 participants are expected, representing many nations and disciplines. They will discuss the latest information on many aspects of new and reemerging pathogens, such as West Nile virus and issues concerning bioterrorism.

More information about the conference will be posted soon at http://www.cdc.gov/ICEID/index.htm

Contact person is Charles Schable, cas1@cdc.gov

Erratum
Vol.7, No. 2

In the article "Economic Impact of Antimicrobial Resistance," by John E. McGowan, Jr., an error occurred in the estimate in the first paragraph. The second sentence should read, "The Institute of Medicine estimates the annual cost of infections caused by antibiotic-resistant bacteria to be U.S. $4 to $5 billion" (1). We regret any confusion this error may have caused.

Vulimiri Ramalingaswami (1921-2001)

Professor Vulimiri Ramalingaswami, international editor of Emerging Infectious Diseases since 1998, was born on August 8, 1921, at Srikakulam, Andhra Pradesh, India. He passed away on May 28, 2001, after a brief illness at the All India Institute of Medical Sciences Hospital, New Delhi, India.

Professor Ramalingaswami obtained his MBBS degree in 1944 from Andhra University, his MD degree in internal medicine in 1946 from the same university, and D.Phil. and D.Sc. degrees in 1951 and 1967, respectively, from Oxford University, United Kingdom.

Professor Ramalingaswami's research career started at Nutrition Research Laboratories, Coonoor in the Nilgiris (now the National Institute of Nutrition, Hyderabad) in 1947. Since then, he had been very active in various aspects of medical research. He believed in pursuing basic knowledge for a better understanding of causes and mechanisms of human diseases prevalent in developing countries and in the application of that knowledge for human betterment. He believed in promoting a meaningful synthesis of laboratory, clinical, and community-based research. His areas of research were protein energy malnutrition, iodine deficiency disorders, nutritional anemia, and liver diseases in the tropics. He was interested in primary health care, infectious diseases, and health research for development.

The most recent and ongoing activities of Professor Ramalingaswami were in the area of new and reemerging infectious diseases, particularly in the developing world. In 1994, India was struck suddenly by an outbreak of plague–bubonic and pneumonic. A technical advisory committee on plague, established by the Indian government under the chairmanship of Professor Ramalingaswami, reported on the factors responsible for the outbreak and recommended steps for prevention of such outbreaks in the future. The committee's report, "The Plague Epidemic of 1994," was submitted to the government in 1995 and was published in 1996 in a special section of Current Science (71:781-806).

Professor Ramalingaswami was a fellow of the Royal Society; a foreign associate of the National Academy of Sciences, USA; foreign member, Academy of Medical Sciences, USSR; and past president of the Indian National Science Academy. He received Doctor of Medicine degrees from several universities, including the Karolinska Institute, Stockholm, Sweden.

While presenting Dr. Ramalingaswami the Leon Bernard Foundation Award in 1976, Sir Harold Walter, president of the World Assembly, described him as "Physician, research scientist, teacher, and humanist," a very apt description of Professor Ramalingaswami.

The Cover

The Mosquito Net (circa 1912)
John Singer Sargent (1856-1925)

The White House. Gift of Whitney Warren, in memory of President John F. Kennedy, 1964

Neither signed nor dated, The Mosquito Net was retained by Sargent until the end of his life and has long been ranked among his best "private" works–small paintings done for his own delectation rather than for a patron. The woman who posed for the painting was Marion Alice (Polly) Barnard, whose father, Frederick, was a painter and friend of Sargent's. Sargent scholar David McKibbin, who knew the Barnard sisters, specified that it was painted in 1912 at Abries, in the French Alps, a few kilometers from the Italian border.

In 1905, after the death of his mother, Sargent began to take annual trips in the autumn, to Italy and Switzerland, usually in the company of his sister Emily and her friends, including the Barnard sisters. On these trips, the women often posed for Sargent's water colors and oil paintings, and in preparation he brought with him elaborate costumes and accessories. One such accessory was the remarkable mosquito net, designed by Emily and called by Sargent "garde-mangers," or "protection from the eaters."

In this amusing picture, the model is resting on a bed under such a garde-manger. Enveloped in voluminous satin skirts, cushioned by white pillows, the woman has abandoned her reading. The book lies neglected in her hand. Her pensive features are glimpsed through the black enclosure of the mosquito net, whose wire ribs describe a series of strong arcs.

The curtain or wall covering behind her, with its suggestion of a floral pattern, is brushed in quick, unfocused touches in sandalwood and brown, blue, green, and red. It owes much to Édouard Manet's portrait of Stéphane Mallarmé (1876; Musée d' Orsay, Paris), a work that had demonstrable impact on Sargent. The contrast between the flashing brushwork in the satin skirt and the motionless brushwork in the silent face is one measure of his imagination and skill. Reclining attitudes are common in Sargent's art, both in portraits and in small genre pictures, and they are evocative of pervasive fin de siècle indolence.

Courtesy of the White House Historical Association, Washington, D.C., USA

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal has an international scope and is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the Editor at 404-371-5329 (tel), 404-371-5449 (fax), or eideditor@cdc.gov (e-mail).

Emerging Infectious Diseases is published in English and features the following types of articles: Perspectives, Synopses, Research Studies, Policy Reviews, and Dispatches. The purpose and requirements of each type of article are described in detail below. To expedite publication of information, we post journal articles on the Internet as soon as they are cleared and edited.

Chinese, French, and Spanish translations of some articles can be accessed through the journal's homepage at www.cdc.gov/eid. Articles by authors from non-English-speaking countries can be made simultaneously available in English and in the author's native language (electronic version of the journal only).

Instructions to Authors

Manuscript Preparation

Follow "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" (Ann Intern Med 1997:126[1]36-47) (http://www.acponline.org/journals/annals/01jan97/unifreqr.htm).

Begin each of the following sections on a new page and in this order: title page, abstract, text, acknowledgments, references, tables, figure legends, and figures.

Title page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Also provide address for correspondence (include fax number and e-mail address).

Abstract and key words. Avoid citing references in the abstract. Include up to 10 key words; use terms listed in the Medical Subject Headings from Index Medicus (http://www.nlm.nih.gov/mesh/meshhome.html).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Type only on one side of the paper and number all pages, beginning with the title page. Indent paragraphs 5 spaces; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use Courier font size 10 and ragged right margins. Italicize (rather than underline) scientific names when needed.

Electronic formats. For word processing, use WordPerfect or MS Word. Send graphics in native format or convert to .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) formats. The preferred font for graphics files is Helvetica. Convert Macintosh files into one of the suggested formats. Submit slides or photographs in glossy, camera-ready photographic prints.

References. Follow the Uniform Requirements style. Place reference numbers in parentheses, not in superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title in full. List the first six authors followed by "et al."

Tables and figures. Create tables within the word processing program's table feature (not columns and tabs within the word processing program). For figures, use color as needed; send files, slides, photographs, or prints. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced. Place figure keys within the figure.

Access the journal's style guide at http://www.cdc.gov/ncidod/EID/style_guide.htm

Manuscript Submission

Include a cover letter verifying that the final manuscript has been seen and approved by all authors.

Submit three copies of the original manuscript with three sets of original figures and an electronic copy (on diskette or by e-mail) to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS D 61, Atlanta, GA 30333, USA; e-mail eideditor@cdc.gov

Types of Articles

Perspectives, Synopses, Research Studies, and Policy Reviews: Articles should be approximately 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch.

Perspectives: Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases or related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change; human demographics and behavior; technology and industry; economic development and land use; international travel and commerce; and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses: This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. Use of subheadings in the main body of the text is recommended. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text. Photographs and illustrations are encouraged.

Research Studies: These articles report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (e.g., "Here is what we found, and here is what the findings mean").

Policy Reviews: Articles in this section report public health policies that are based on research and analysis of emerging disease issues.

Dispatches: These brief articles are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome. Dispatches (1,000 to 1,500 words) need not be divided into sections. Provide a short abstract (50 words); references, not to exceed 10; figures or illustrations, not to exceed two; and a brief biographical sketch.

Another Dimension: Thoughtful essays on philosophical issues related to science and human health.

Book Reviews: Short reviews (250 to 500 words) of recently published books on emerging disease issues are welcome.

Letters: This section includes letters that give preliminary data or comment on published articles. Letters (500 to 1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

News and Notes: We welcome brief announcements (50 to 150 words) of timely events of interest to our readers. (Announcements can be posted on the journal web page only, depending on the event date.) In this section, we also include summaries (500 to 1,500 words) of conferences focusing on emerging infectious diseases. Summaries may provide references to a full report of conference activities and should focus on the meeting's content.