

ISOLATION OF EASTERN EQUINE ENCEPHALITIS VIRUS AND WEST NILE VIRUS FROM CROWS DURING INCREASED ARBOVIRUS SURVEILLANCE IN CONNECTICUT, 2000

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Abstract. The emergence of the West Nile virus (WNV) in the northeastern United States has drawn emphasis to the need for expanded arbovirus surveillance in Connecticut. Although the state of Connecticut began a comprehensive mosquito-screening program in 1997, only since 1999 have there been efforts to determine the prevalence of arboviruses in bird populations in this state. Herein, we report on our results of an arbovirus survey of 1,704 bird brains. Included in this report are the first known isolations of eastern equine encephalitis virus (EEEV) from crows and data on the geographic and temporal distribution of 1,092 WNV isolations from crow species. Moreover, these nine isolations of EEEV identify regions of Connecticut where the virus is rarely found. With the exception of WNV and EEEV, no other arboviruses were isolated or detected. Taken together, these data illustrate the distribution of avian borne EEEV and WNV in 2000 and support the need for ongoing avian arbovirus surveillance in Connecticut.

INTRODUCTION

Eastern equine encephalitis virus (EEEV) (genus: *Alpha-virus*, family: *Togaviridae*) is a single-stranded RNA virus that is transmitted to humans through the bite of an infected mosquito.^{1–3} Like many arthropod-borne viruses (arboviruses), EEEV also infects a number of different types of birds whose infections have resulted in a number of massive bird die-offs throughout many of the eastern United States.^{4–6} In addition to playing important roles in viral dissemination, bird hosts are also necessary for the amplification of many arboviruses.² By providing high viral titer blood meals for mosquitoes that feed on birds, infected birds perpetuate the natural cycle of EEEV and allow for the incidental transmission of EEEV to “dead end” mammalian hosts such as horses and humans.⁴

Despite these integral roles of bird hosts in the transmission cycle of EEEV (and other arboviruses), there has been little surveillance in the wild bird populations of Connecticut compared with mosquitoes.^{7–9} Since its first isolation from mosquitoes in Connecticut in 1938, EEEV has been detected sporadically throughout the state in a variety of hosts, including mosquitoes, horses, donkeys, emus, and pheasants.^{2,7–11} The identification of sporadic cases in domestic and exotic species are typically the outcome of necropsies performed at the Connecticut Veterinary Diagnostic Laboratory. During summer and fall months, EEEV is routinely ruled out in cases of encephalitis in known susceptible species. In 1972, a widespread EEEV epizootic killed several thousand pheasants and nine horses in 22 Connecticut towns combined. Although the majority of these isolates were concentrated in the Connecticut and Thames River valleys of central and southeastern Connecticut, one isolate from a pheasant in Bethany (New Haven County) suggested a more widespread distribution of EEEV in the state.^{2,10}

Another arbovirus that has recently emerged in Connecticut, New York, and other surrounding states is the West Nile virus (WNV). Since its introduction into the greater New York City area in 1999, WNV (genus: *Flavivirus*, family: *Flaviviridae*) has been responsible for deaths in humans, horses and many indigenous bird species of North America including an intense epizootic among crows.^{12–16} In 2000, testing of

dead birds for WNV was established as one component of a statewide surveillance system since positive wild birds were most likely to provide the first indication of the re-emergence of WNV. During our surveillance for WNV in the bird populations of Connecticut, we also discovered EEEV while using techniques of virus isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) in the screening of brain tissues from 1,704 dead birds. In this work, we report on the statewide distribution of WNV in Connecticut and also on the isolation of EEEV from nine dead birds that tested negative for WNV, including the first reported isolation of EEEV from crows and a wild turkey. The results identified discrete foci of avian borne EEEV in Connecticut and areas previously not known to have EEEV activity.

MATERIALS AND METHODS

Specimen processing. Wild bird carcasses from around the state were collected by local health departments and selected for testing by staff of the Department of Environmental Protection Wildlife Division based on species, physical condition, and other surveillance data. Selected birds were necropsied by the Connecticut Veterinary Diagnostic Laboratory, Department of Pathobiology, University of Connecticut (Storrs, CT) and specimens of brain tissue were submitted to the Connecticut Department of Public Health Laboratory (Hartford, CT) for WNV testing under bio-safety level 3 conditions.

Tissue preparation for culture/RT-PCR. Whole brains were homogenized in buffered Earle’s minimal essential media (EMEM) (10 mM HEPES, 25 mM sodium bicarbonate) supplemented with amphotericin B (10 µg/ml), gentamicin (50 µg/ml), and penicillin (100 units/ml) using either a mortar and pestle or by vortexing briefly with glass beads. The 10% (w/v) suspensions were allowed to settle for an hour at 4°C before proceeding.

Extraction of RNA and RT-PCR. Viral RNA was extracted using the QiAMP Viral RNA purification kit (Qiagen, Inc., Valencia, CA) as per the manufacturer’s procedure. The RT-PCR was performed using the Titan One Tube RT-PCR kit (Roche Diagnostics, Indianapolis, IN). The primers used to detect EEEV were EEE5640, 5'-CGGCAGCGGAATT-TGACGAG-3' and EEE6072C, 5'-ACTTTGACGGCCAC-

TTCTGCTGATGA-3'. The two primer sets used to detect WNV were WN212, 5'-TTGTGTTGGCTCTCTTGGCGT-TCTT-3' with WN619C, 5'-CAGCCGACAGCACTGGA-CATTCATA-3' and WN9794C, 5'-GGAACCTGCTGC-CAATCATAACCATC-3' with WN9483, 5'-CACCTACGC-CCTAAACACTTTCACC-3'.¹⁷ All primer sets, including those used to detect EEEV, were specified by Dr. Robert Lanciotti (Centers for Disease Control and Prevention, Fort Collins, CO). The RT-PCR running conditions were 1 hr at 45°C, 3 min at 94°C, and 40 cycles of amplification (94°C for 30 sec, 61°C for 1 min, and 68°C for 3 min). Amplified products were separated by electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide and viewing under ultraviolet light. The WN212/WN619C primer set yielded a 408-basepair product, the WN9794C/WN9483 primer set yielded a 312-basepair product, and the EEE5640/EEE 6072C primer set yielded a 433-basepair product. The WNV-positive specimens were screened with one primer set and confirmed with the second primer set to reduce the possibility of false positives due to occasional background bands on the gel. The EEEV PCR-positive specimens were confirmed via virus isolation and fluorescent antibody staining test. Due to the specificity of PCR-based diagnostics and the likelihood of detecting other arboviruses, all specimens yielding negative or inconclusive WNV RT-PCR results were inoculated into Vero cell culture.

Cell culture. Ten-fold dilutions of the tissue homogenates were inoculated into 25-cm² flasks of 2–3-day old Vero cell cultures and adsorbed for 1 hr at 37°C. Following adsorption, the inocula were removed and 10 ml of EMEM, supplemented with 10% fetal bovine serum, was added. The cultures were incubated at 37°C, and read daily for 7–10 days.

Fluorescent antibody staining test. Cultures exhibiting a cytopathic effect (CPE) within the 7–10-day incubation period were scraped and the cells were collected by centrifugation at 1,000 revolutions per minute for 10 min at 25°C in an IEC Centra GP8R centrifuge (International Equipment Company, Needham Heights, Mass). Following re-suspension in ~1 ml of sterile saline solution, aliquots of the cell suspension were dropped onto multi-well slides, dried at room temperature, and fixed in acetone for 15 min at 4°C. The fixed slides were probed with a panel of arbovirus mouse monoclonal antibodies and incubated at 37°C for 20 min. The immunopanel included monoclonal antibodies against WNV (MAF+MAB H5-46) (kindly supplied by Dr. Robert Lanciotti, Centers for Disease Control and Prevention, Fort Collins, CO), EEEV (MAB8734), Japanese encephalitis virus (MAB8743), and two strains of St. Louis encephalitis virus (MAB8744 and MAB8749). With the exception of WNV antibodies, the remaining reagents (Chemicon International, Temecula, CA) were diluted 50 fold in phosphate-buffered saline (PBS) with 1.5% bovine serum albumin before use. Following subsequent rinses in PBS and sterile water, the slides were probed with the secondary rabbit anti-mouse monoclonal fluorescein isothiocyanate conjugate (Dako Corp., Carpinteria, CA) at 37°C for another 20 min before visualization via fluorescent microscopy.

Histologic examination. Harvested tissues were fixed in 10% neutral-buffered formalin for 24 hr. After fixation, tissues were trimmed, embedded in paraffin for sectioning (5 µM), and stained with hematoxylin and eosin.

TABLE 1

Number and species diversity of avian specimens tested in Connecticut, 2000

Common name	Number tested	Common name	Number tested
Crow*†	1,593	Northern warbler	2
Grackle	23	Turkey	2
Starling	13	Cardinal	1
Blackbird	12	Chickadee	1
Catbird	10	Cooper's hawk	1
Blue jay	8	Eastern phoebe	1
American robin	4	Goose	1
Bluebird	4	Hawk	1
Pigeon	4	Mockingbird	1
Sparrow	4	Oriole	1
Oven bird	3	Red-winged blackbird	1
Woodpecker	3	Swallow	1
Woodthrush	3	Waterthrush	1
Dove	2	Wren	1
Flicker	2	Wild turkey	1

* Includes American crows and fish crows.

† All isolations of West Nile virus were from crows.

RESULTS

To determine the prevalence of arboviruses in the bird populations of Connecticut, we screened 1,704 bird brain specimens for the presence of WNV. Using predominantly the indigenous crow population as indicators of virus activity, we used both virus isolation and molecular techniques (i.e., RT-PCR) to identify arboviral infections in dead birds. Although other bird species were tested prior to their exclusion in early August 2000, the majority of our surveillance (93%), and all WNV positive specimens were crow species, including the American crow (*Corvus brachyrhynchos*) and the fish crow (*Corvus ossifragus*). Table 1 lists the distribution of different bird species tested.

We used both RT-PCR and cell culture to screen bird brain homogenates for evidence of WNV or other viral infections. Given the relative speed of RT-PCR diagnostics, specimens received early in the week were tested via RT-PCR and specimens received later in the week were screened via cell culture. Our combined approach yielded similar rates of isolation/detection for RT-PCR (76%) and cell culture (81%) screening (data not shown). More importantly, our surveillance effort yielded evidence of a statewide distribution of WNV, with 1,095 infected crows being collected from all eight Connecticut counties (Table 2). Although the surveillance was intense in the two densely populated, coastal counties closest to New York City (Fairfield and New Haven) due to public concern, significant percentages of infected crows were also detected in the landlocked Litchfield (25%), Tolland (54%), and Windham (19%) counties. In addition to these landlocked counties that comprise Connecticut's northern border with Massachusetts, the remaining counties (Middlesex (57%), Hartford (60%), and New London (71%)) exhibited WNV isolation rates comparable to the rates of Fairfield and New Haven counties. Given the locations of these counties along the Connecticut River (i.e., Hartford and Middlesex counties) and on the banks of Long Island Sound (i.e., New London county), our data indicate a statewide distribution of WNV with the highest rates of isolation from wild birds collected in the population centers along Connecticut's waterways.

TABLE 2
Temporal distribution of West Nile virus (WNV)-positive birds by county in Connecticut, 2000

Month of collection	No. positive/total tested Connecticut County*								Totals
	F	H	L	M	NH	NL	T	W	
May	0/20	0/5	0/2	0/5	0/7	0/3	0	0	0/42
June	0/34	0/7	0/1	0/1	0/13	0/5	0	0	0/61
July	19/121	0/13	0/1	0/4	3/13	0/11	1/2	0/1	23/166
August	191/250	38/82	1/5	3/10	66/109	3/14	2/10	0/11	304/491
September	220/247	118/153	3/4	17/24	99/120	47/55	7/9	2/3	513/615
October	77/92	32/50	0/3	15/17	68/94	46/47	4/5	2/6	244/314
November	2/2	0	0	0	0	0	0	0	2/2
Totals	509/766	188/310	4/16	35/61	236/356	96/135	14/26	4/21	1,095/1,704†

* F = Fairfield; H = Hartford; L = Litchfield; M = Middlesex; NH = New Haven; NL = New London; T = Tolland; W = Windham.

† These values include nine of 13 crows that were WNV positive, but were submitted without the information required for inclusion in the table.

Our avian borne arbovirus surveillance also isolated EEEV from nine birds that were RT-PCR negative for WNV infection (Table 3). Originally detected via virus isolation in Vero cell cultures exhibiting CPE, all nine EEEV isolates were subsequently confirmed using a fluorescent antibody test and by RT-PCR using primers that do not cross-react with any other alphaviruses, including the Highlands J virus. Whereas the CPE caused by WNV typically developed between 72 and 120 hr, the CPE attributed to EEEV developed within 48 hr.

With the exception of an EEEV isolate from a wild turkey (*Meleagris gallopavo*) in Southbury (New Haven County), the remaining isolates were all recovered from crows, including two isolates from American crows (*Corvus brachyrhynchos*) and seven from crows not identified to species. Histologic examination of the brains from the wild turkey and seven of eight crows revealed pathology characteristic of viral encephalitis (histologic data not shown). The only crow that did not exhibit histologic signs of encephalitis was the crow from Milford, Connecticut. The lesions were characterized by perivascular mononuclear cell accumulations and rare foci of gliosis and could not be differentiated from those caused by WNV. The wild turkey testing positive for EEEV had histopathology indicative of a viral encephalitis characterized by perivascular accumulations of lymphocytes, fewer macrophages, and randomly scattered foci of gliosis and necrosis. In addition, there was petechia of the heart and pericardial effusion with lymphocytic and necrotizing myocarditis, interstitial lymphocytic orchitis, and evidence of intraluminal enteric

hemorrhage. Immunohistochemical staining of tissues and lesions for WNV antigen was negative.

Unlike the WNV isolates, all nine EEEV isolates were collected exclusively from the southwestern and central parts of the state, specifically in New Haven, Fairfield, and Hartford counties. Interestingly, eight of the nine EEEV-positive birds were collected within 40 km of the mouth of the Housatonic River, which forms the border between New Haven and Fairfield counties (Figure 1). The remaining EEEV isolate was obtained in Avon of Hartford County, approximately 58 km northeast of the New Haven/Fairfield epicenter. Our findings suggest the presence of multiple foci of avian borne EEEV in Connecticut and a further town-by-town analysis revealed that EEEV was as prevalent as WNV in the dead crows collected from some Connecticut communities (Table 4).

DISCUSSION

Our surveillance for WNV in the bird population of Connecticut demonstrated a statewide distribution of the virus in all eight counties (Table 2). In fact, six of the eight counties (New London, Fairfield, New Haven, Hartford, Middlesex, and Tolland) yielded overall positivity rates of crows tested greater than 50%, with the exceptions being the inland and landlocked Windham and Litchfield counties. Although aggressive surveillance in the more populated counties (Fairfield, New Haven, and Hartford) skewed the data to suggest a higher prevalence in urban areas of Connecticut, the isolation of WNV from more rural counties (Litchfield and Windham) confirms the dissemination of the virus from its 1999 epicenter in the greater New York City metropolitan area. In agreement with WNV isolation rates from crows during the 1999 epizootic,^{12,13} our results indicate the highest prevalence of WNV during peak arbovirus season (i.e., mid August to early October) (Table 2). Taken together, our data define a statewide distribution of WNV in the crow population of Connecticut.

Unlike WNV, EEEV has a long and well-documented history in many of the states along the eastern seaboard of North America.^{1,3,4-7,11,18} Although EEEV has been isolated from a number of different bird species, to the best of our knowledge, our findings represent the first isolations of EEEV from American crows.^{2,6,19} Since the majority of our EEEV isolates (7 of 9) were collected in the vicinity of the Housatonic River in southwestern Connecticut, our arbovirus surveillance has identified an avian cluster of EEEV in a part of the

TABLE 3

Distribution of avian eastern equine encephalitis virus (EEEV) isolates in Connecticut, 2000

Date of collection	Common name	Town, County	Isolation*/RT-PCR†
9/12/00	Crow	Redding, Fairfield	+/+
9/14/00	Crow	Woodbridge, New Haven	+/+
9/19/00	Crow	Shelton, Fairfield	+/+
9/21/00	Crow	Avon, Hartford	+/+
9/21/00	Wild turkey	Southbury, New Haven	+/+
9/25/00	Crow	Milford, New Haven	+/+
9/28/00	Crow	Trumbull, Fairfield	+/+
10/5/00	Crow	Guilford, New Haven	+/+
10/5/00	Crow	Southbury, New Haven	+/+

* EEEV-infected cultures were tested using monoclonal antibodies (see Materials and Methods).

† The isolates and original specimens were confirmed for EEEV using a reverse transcriptase-polymerase chain reaction (RT-PCR) primer set defined by Lanciotti (see Materials and Methods).



FIGURE 1. Distribution of eastern equine encephalitis virus (EEEV) in Connecticut, 2000. The dotted line circle (40-km radius) encompasses eight of the nine EEEV isolates described herein (●). The 40-km circle is bisected by the Housatonic River that divides New Haven and Fairfield counties in southwestern Connecticut. The dark lines in central and eastern Connecticut represent the Connecticut and Thames Rivers, respectively. Also depicted are the locations of previously documented isolates of EEEV, with their corresponding sources and years of isolation. Town borders are depicted as thin black lines. (Δ) = mosquito pool, 1996¹¹; (⊙) = ornithophilic (bird biting) mosquito pools, 1997⁸; (□) = donkey, 1991¹⁰; (○) = horse, 1991⁷; (◇) = emus, 1997⁹; (⊞) = horse, 1953–1957⁷; (☆) = mosquito pools, 1970–1973.²

state where this virus is not frequently isolated from mosquitoes and rarely reported in other animals (Figure 1). Taken together with previous isolations of EEEV from a horse in nearby New Milford (Litchfield County) in 1955,¹⁰ from mosquitoes in Ridgefield and Newtown (both of Fairfield County) in 1998,⁹ and from mosquitoes in Farmington (Hartford County),² our findings confirm the recurring nature of EEEV in the sylvan reservoirs of southwestern Connecticut.

Based on a limited number of specimens tested, these findings also support the tendency of EEEV to occur in discrete

geographic locations.^{5,6,9,11} For example, of the eight dead crows tested from Southbury (New Haven County), two tested positive for WNV and two for EEEV. Similar trends were also evident in other towns where both WNV and EEEV were isolated (Table 4). Although mosquito-borne EEEV was not detected in Connecticut throughout the course of this study,²⁰ the focal nature of these EEEV isolations may be indicative of focal breeding populations of the mosquito species responsible for the EEEV transmission cycle in birds (i.e., *Culiseta melanura*). Whereas the range of *C. melanura* is largely restricted to cedar swamp habitats, the primary mosquito vector thought to be responsible for the bird-to-bird transmission of WNV (i.e., *Culex pipiens*) is found in both urban and sylvan environments.²¹ Taken together, our data are suggestive of a model whereby the distributions of EEEV and WNV in Connecticut are largely determined by the ecologic preferences of their mosquito vectors.

This study attests to the value of avian surveillance in monitoring for the presence of arboviruses in a natural setting. Using molecular biological techniques (i.e., RT-PCR), we were able to screen larger sample sizes for the presence of the virus, thereby providing a more complete picture of viral dissemination throughout Connecticut. Furthermore, as this information was used in the state's effort to access the potential

TABLE 4

Co-occurrence of avian eastern equine encephalitis virus (EEEV) and West Nile virus (WNV) by towns in Connecticut, CT 2000

Town, County	No. of Isolates Total		Tested
	EEEV	WNV	
Avon, Hartford	1	1	5
Guilford, New Haven	1	19	25
Milford, New Haven	1	75	91
Redding, Fairfield	1	4	7
Shelton, New Haven	1	28	37
Southbury, New Haven	2*	2	8
Trumbull, Fairfield	1	15	22
Woodbridge, New Haven	1	0	2

* Includes an isolate from a wild turkey.

risk of WNV transmission to humans, the decreased turn around time afforded by RT-PCR screening enabled for much more rapid diagnostics and proved useful for surveillance purposes.²² However, aside from its time- and cost-saving benefits, this study also illustrates one of the inherent disadvantages of PCR-based diagnostics, namely its specificity. Our isolations of EEEV exemplifies the need for additional confirmatory diagnostics for all RT-PCR-negative specimens when more than one pathogen is a strong possibility. Future applications of real time and multiplex RT-PCR will allow for an even more comprehensive and thorough surveillance effort.

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