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WEST NILE VIRUS SEROSURVEILLANCE IN IOWA WHITE-TAILED DEER (1999–2003)

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Abstract. Sera from white-tailed deer (*Odocoileus virginianus*) were collected in Iowa during the winter months (1999–2003), 2 years before and after West Nile virus (WNV) was first reported in Iowa (2001), and were analyzed for antibodies to WNV. Samples from 1999 to 2001 were antibody negative by blocking enzyme-linked immunosorbent assay (bELISA) and plaque reduction neutralization test ($PRNT_{90}$). Prevalence derived from bELISA (2002, 12.7%; 2003, 11.2%) and WNV $PRNT_{90}$ (2002, 7.9%; 2003, 8.5%) assays were similar. All samples were negative for antibodies against St. Louis encephalitis virus as determined by $PRNT_{90}$. Antibodies to flaviviruses were detected by indirect enzyme-linked immunosorbent assay (iELISA) prior to the first WNV cases reported in Iowa (1999–2001) with prevalence ranging from 2.2% to 3.2%, suggesting the circulation of an additional undescribed flavivirus prior to the introduction of WNV into the area. Flavivirus prevalence as determined by iELISA increased in 2002 and 2003 (23.3% and 31.9%, respectively). The increase in prevalence exceeded estimates of WNV prevalence, suggesting that conditions favored general flavivirus transmission (including WNV) during the 2002–2003 epizootic. These data indicate that serologic analysis of deer sera collected from hunter harvests may prove useful for surveillance and evidence of local transmission of WNV and other pathogens and identify white-tailed deer as a species for further studies for host competency.

INTRODUCTION

West Nile virus (WNV) is a mosquito-borne, zoonotic *Flavivirus* (Flaviviridae) from Africa, the Middle East, Europe, and Asia that emerged as an invasive pathogen in New York City during summer 1999.[1,2] By winter 2004, human and other animal infections had been reported in Canada, all of the contiguous United States, parts of Mexico, and some islands of the Caribbean.[3] The rapidity with which the pathogen disseminated across North America has been remarkable.

WNV is transmitted by many species of mosquitoes and uses a variety of birds as amplifying hosts.[4,5] Although the vector and host competency of North American mosquitoes and birds are known to some extent, there are too few observations on the host competency of mammals to exclude them from being involved in the transmission cycle.[6–8] Experimental infections of horses, dogs, and cats indicate that these species are either dead-end or poorly competent hosts.[9,10] At least some rodents are competent hosts.[11] However, little is known about the host competency of common mammalian wildlife in North America.[12] Because of the considerable investment involved in experimental infection studies, the most economical approach to determine a species role in transmission is to survey wildlife populations for their risks of exposure to the virus. Once high-risk species are identified, their host competency can be tested experimentally. Identification of competent hosts and vectors is critical to understand the ecology of WNV and the factors important in driving disease cycles, and, by implication, forecasting temporal and spatial risks to human populations.[13,14]

Sera collected from white-tailed deer (*Odocoileus virginianus*) during wildlife control operations and hunter-harvests in Iowa from 1999 to 2003 provided an opportunity to determine the deer's exposure to flaviviruses in general, and WNV and Saint Louis encephalitis virus (SLEV) specifically. Herein, we show that WNV is commonly transmitted to white-tailed deer and that this species should be further examined for its role in WNV transmission cycles. The utility of white-tailed deer as a surveillance indicator species also is discussed.

MATERIALS AND METHODS

Sample collection. Whole blood samples (N = 1,079) were collected from professional sharpshooter-killed deer, hunter-killed deer, and road-killed deer during the months of November–January. Most (N = 890) of the samples were collected in the years 1999–2001 and 2003 during the deer herd reduction program in Iowa City, Iowa. All shooting sites, except one, were in the northern part of the city; the other, single site (N = 24) was in the southern part of the city in 2000, 2001, and 2003. Most of the 189 samples in 2002 were collected by the Iowa Department of Natural Resources throughout the state from hunter-killed deer, but some were collected from fresh road-killed deer. The time from death to blood collection varied from 15 minutes to 4 hours. Free-flowing whole blood was obtained from skinned deer hanging from their hind legs when the thoracic cavity contents were removed. Dripping blood was collected in 7-mL serum separator tubes. After clotting, the tubes were centrifuged and the serum removed and stored in cryovials at –80°C until assayed.

Indirect enzyme-linked immunosorbent assay (iELISA). A WNV iELISA was used to screen for flavivirus-specific immunoglobulin G (IgG) antibodies.[15] This method may detect cross-reactive antibodies raised against other flaviviruses, thus it was viewed as a general screening method for flavivirus antibody detection.[15]

Positive and negative antigens were provided by New York State Department of Health's Wadsworth Center.[16,17] Anti-

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gen was diluted (1:200) in coating buffer (0.015 M $NaCO_3$, 0.035 M $NaHCO_3$, pH 9.6) and applied (50 μL/well) to the inner wells of a 96-well plate (Costar, Corning Incorporated, Corning, NY). Negative antigen was placed in every third column and positive antigen was placed in the remaining columns. Antigen binding to the wells was achieved by placing plates in a humidified chamber (plastic bag with a damp paper towel) and incubating (4°C) them overnight. After incubation, antigen solutions were discarded and wells washed (3×) with PBS-T (0.01 M phosphate buffer, 0.138 M NaCl, 0.0027 M KCl, pH 7.4 with 0.05% Tween 20). Next, blocking buffer (PBS-T with 2% casein) was added (200 μL/well) and plates were incubated (37°C, 1 hour) in a humidified chamber. Blocking buffer was discarded, and test sera diluted in PBS-T with 0.5% bovine albumin (PBS-T-BA) (1:100 dilution) were added (50 μL/well) to duplicate wells. Five known negative control deer sera ($PRNT_{90}$ less than 1:10), one known positive control deer serum ($PRNT_{90}$ greater than 1:10), and one blank (buffer only) were included in each plate. Plates were incubated (37°C, 1 hour) in a humid chamber and after sera were discarded, washed (3×) with PBS-T. After incubation, horseradish peroxidase conjugated rabbit anti-deer immunoglobulin IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), diluted 1:100 in PBS-T-BA, was added (50 μL/well) and incubated (37°C, 1 hour). After incubation, the conjugate solution was discarded and the plates were washed (3×) with PBS-T and developed at room temperature (7 minutes) with tetramethylbenzidine (TMB)-peroxidase substrate (Kirkegaard & Perry Laboratories, Inc.) (50 μL/well). The reaction was stopped by adding (50 μL/well) H_2PO_4 diluted 1:5.

Optical density (OD) was read at a wavelength of 450 nm. Samples were considered positive if OD values were ≥ 3 standard deviations above the mean (OD = 0.055) of negative-control samples from 25 white-tailed deer (PRNT less than 1:10). Positive controls were obtained from 5 deer infected with WNV in Iowa (PRNT greater than 1:10) (National Veterinary Services Laboratories, Ames, IA), 3 deer infected with WNV in New York City (PRNT 1:10) (New York State Diagnostic Laboratory), and from 4 reindeer (> 90% inhibition by blocking ELISA) vaccinated (West Nile-Innovator vaccine, Fort Dodge Animal Health, Wyeth corporation) at the Denver Zoo, Denver, Colorado.

Epitope-blocking enzyme-linked immunosrobent assay (bELISA). A bELISA using the monoclonal antibody (MAb) 3.112G was used to detect immunoglobulin M (IgM) and IgG antibodies against WNV.[18,19] This assay is specifically reactive to WNV, offers a high degree of sensitivity, and enables the assay to be taxon-independent in its ability to detect antibodies against the NS-1 epitope of WNV.[18]

WNV antigen was diluted (1:200) in coating buffer (see above) and applied (100 μL/well) to the inner 60 wells of a 96-well plate. The plate was placed in a humidified chamber and incubated (4°C) overnight. The next morning, the antigen solution was discarded, plates were washed (4×) with PBS-T, and blocking buffer (PBS with 5% skim milk) was added (200 μL/well) to each well. Plates then were incubated (37°C, 40 minutes), after which blocking buffer was discarded and the plates were washed (4×). Fifty microliters of test samples, diluted (1:10) in blocking buffer, were added to each well and incubated (37°C, 2 hours). The test samples were then discarded, plates were washed (4×), and MAb 3.1112G (Chemicon Intern, Temecula, CA) diluted in blocking buffer (1:2,000) was added (50 μL/well) and incubated (37°C, 1 hour). After removal of MAb solution, plates were washed (4×) and horseradish peroxidase–conjugated rabbit anti-mouse IgG (Zymed Laboratories, San Francisco, CA) (1:2,000 dilution) was added (50 μL/well) and incubated (37°C, 1 hour). After removal of conjugate solution, plates were washed (4×). ABTS substrate (2,2' azino-bis [3-ethylbenzthiazoline-6-sulfonic acid]) and peroxidase solutions from the ABTS Microwell peroxidase substrate system (KPL, Gaithersburg, MD) were mixed 1:1, and were added (75 μL/well). Optical density (OD) values were read at 415 nm in an automated plate reader.

Percent inhibition of MAb binding was calculated using the formula[18] $100 - [(TS - B)/(CS - B)] \times 100$, where TS is the OD of test serum, CS is the OD of negative control serum, and B is the background OD. The percent inhibition was calculated once the control serum OD values exceeded 0.3. An inhibition value of ≥ 30% was considered positive for the presence of anti-WNV antibodies.[19]

Plaque reduction neutralization test (PRNT). Differential diagnosis for antibody positive samples was confirmed by plaque reduction neutralization test (PRNT) against WNV and SLEV.[19,20] Heat-inactivated samples (56°C, 30 minutes) were serially diluted twofold in BA-1 (Hanks M-199 salts, 50 mM Tris [pH 7.6], 1% bovine serum albumin, 0.35 g of sodium bicarbonate/L, 100 units of penicillin/mL, 50 μg of gentamicin/mL, 2.5 μg fungizone/mL), starting at a dilution of 1:5. Diluted serum (100 μL) was mixed (1:1) with additional diluent containing approximately 200 PFU (plaque forming units) of virus and incubated (4°C) overnight. Each serum-virus suspension (100 μL) was inoculated onto confluent Vero cell monolayers in six-well plates and incubated (37°C, 60 minutes). Overlay medium (MEM supplemented with 5% fetal bovine serum, 0.5% agarose, and antibiotics as in BA-1) was added (2 mL/well for WNV, and 3 mL/well for SLEV) and the plates were incubated (37°C; 2 days for WNV; 5 days for SLEV), after which a second overlay containing 0.004% neutral red was added. Plaques were counted 24 to 48 hours after the second overlay, and titers expressed as the reciprocal of the highest dilution of the serum that yielded ≥ 90% reduction in the number of plaques ($PRNT_{90}$).

Statistical tests. Data were analyzed by χ^2 and the binomial difference between proportions tests.

RESULTS

Prevalence of antibodies against flaviviruses as determined by indirect ELISA. White-tailed deer were exposed to flaviviruses throughout the observation period. Initially, prevalence of antibodies against flaviviruses was similar and low: 2.2%, 2.9%, and 3.2% for the years 1999, 2000, and 2001, respectively (Table 1; $\chi^2 = 0.4$, $P = 8.17$). The prevalence of antibodies against flaviviruses increased to 23.3% and 31.9% of the sampled population during 2002 and 2003, respectively (Figure 1A). This change in prevalence ($\Delta = +20.1\%$) represented a 628% increase in flavivirus antibody prevalence between the years 2001 and 2002 ($P < 0.001$). Deer populations continued to show an increase (36.9%) in prevalence of antibodies against flaviviruses in the subsequent year (cf. 2002 versus 2003; $\Delta = +8.6\%$, $P = 0.041$).

TABLE 1
Summary of flavivirus and WNV antibody prevalence as determined by screening iELISA, bELISA, and PRNT*

Year	Age	N	iELISA n+ (%)	bELISA n+ (%)	WNV $PRNT_{90}$ n+ (%)	WNV $PRNT_{80}$ n+ (%)
1999	Fawn	117	4 (3.4)	0 (0.0)	0 (0.0)	0 (0.0)
	Yearling	50	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Adult	102	1 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)
	All	269	6 (2.2)	0 (0.0)	0 (0.0)	0 (0.0)
2000	Fawn	118	3 (2.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Yearling	48	3 (6.3)	0 (0.0)	0 (0.0)	0 (0.0)
	Adult	141	3 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)
	All	307	9 (2.9)	0 (0.0)	0 (0.0)	0 (0.0)
2001	Fawn	45	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Yearling	26	1 (3.9)	0 (0.0)	0 (0.0)	0 (0.0)
	Adult	55	3 (5.5)	0 (0.0)	0 (0.0)	0 (0.0)
	All	126	4 (3.2)	0 (0.0)	0 (0.0)	0 (0.0)
2002	Fawn	24	5 (20.8)	3 (12.5)	3 (12.5)	4 (16.7)
	Yearling	33	4 (12.1)	2 (6.1)	1 (3.0)	1 (3.0)
	Adult	123	33 (26.8)	19 (15.4)	10 (8.1)	16 (13.0)
	All†	189	44 (23.3)	24 (12.7)	15 (7.9)	22 (11.6)
2003	Fawn	62	10 (16.1)	3 (4.8)	3 (4.8)	5 (8.1)
	Yearling	53	20 (37.7)	6 (11.3)	4 (7.5)	7 (13.2)
	Adult	73	30 (41.1)	12 (16.4)	9 (12.3)	12 (16.4)
	All	188	60 (31.9)	21 (11.2)	16 (8.5)	24 (12.8)

* SLE $PRNT_{90}$ and SLE $PRNT_{80}$ were all negative and are not shown in the table.
† This category includes additional animals for which no age category could reliably be assigned.

Prevalence of antibodies against West Nile virus as determined by blocking ELISA. There was no evidence of WNV transmission to white-tailed deer between 1999 and 2001 (Table 1; Figure 1B). Prevalence of antibodies against WNV was 12.7% in 2002 and 11.2% in 2003. These rates were similar ($P = 0.661$).

Prevalence of neutralizing antibodies to WNV and SLEV as determined by PRNT. None of the samples positive by iELISA ($N = 19$) prior to 2002 was positive by WNV $PRNT_{90}$ or SLEV $PRNT_{90}$ (Table 1). Nor were any of the 2002 and 2003 iELISA positive samples positive by SLEV $PRNT_{90}$. The prevalence for neutralizing antibodies against WNV ($PRNT_{90}$) was 7.9% ($N = 188$) and 8.5% ($N = 186$) for 2002 and 2003, respectively. The values from the $PRNT_{80}$ (Table 1) assay were were statistically identical to those estimated by bELISA. The difference in prevalence estimates by iELISA and bELISA indicate an unknown flavivirus was circulating in deer populations prior to 2002, and that an increase in unknown flavivirus transmission occurred concurrently with WNV transmission in 2002 and 2003 (Figure 1C).

The sensitivity of the iELISA compared with $PRNT_{90}$ was 98.18% and its specificity was 93.03%. The sensitivity of the bELISA compared with $PRNT_{90}$ was 58.18% and its specificity 98.72%.

There were no differences in antibody prevalence between males and females in 2002 ($Z = 0.016$; $P = 0.98$) and 2003 ($Z = 1.585$; $P = 0.113$) by $PRNT_{90}$ (Table 1). There also was no association between age and positive WNV serology in 2002 ($\chi^2 = 5.74$; $P = 0.057$) and 2003 ($\chi^2 = 3.26$; $P = 0.196$) by $PRNT_{90}$ (Table 1). Prevalence for the naive age class, fawns, indicates that the annual exposure to WNV was around 5%.

DISCUSSION

Through careful wildlife management practices over the past century, population sizes of white-tailed deer in the United States have increased dramatically, leading to a bonanza of hunting opportunities for sportsmen, but causing problems of deer overpopulation in many areas. Because the numbers of deer harvested are large, the opportunity for cost-effective serological surveys exists.[21,22] Serologic surveys of deer may be particularly useful in locating foci of recent arbovirus activity because these animals seldom roam great distances, are relatively long-lived, and are easily accessible to biting or sucking arthropods.[23,24] Hunter-killed deer are also useful in measuring temporal changes in annual antibody prevalences because these deer are only sampled once during a multiyear surveillance period.

The detection of WNV-specific antibodies in white-tailed deer during 2002–2003 was consistent with other reports of WNV activity in the area. The prevalence as determined by WNV-specific bELISA and WNV-$PRNT_{90}$ were concordant. The slightly higher prevalence found by $PRNT_{90}$ over bELISA, while not statistically different, may be attributable to differing efficiencies of the two assays in detecting neutralizing versus non-neutralizing antibodies.[18,19,25] Because the

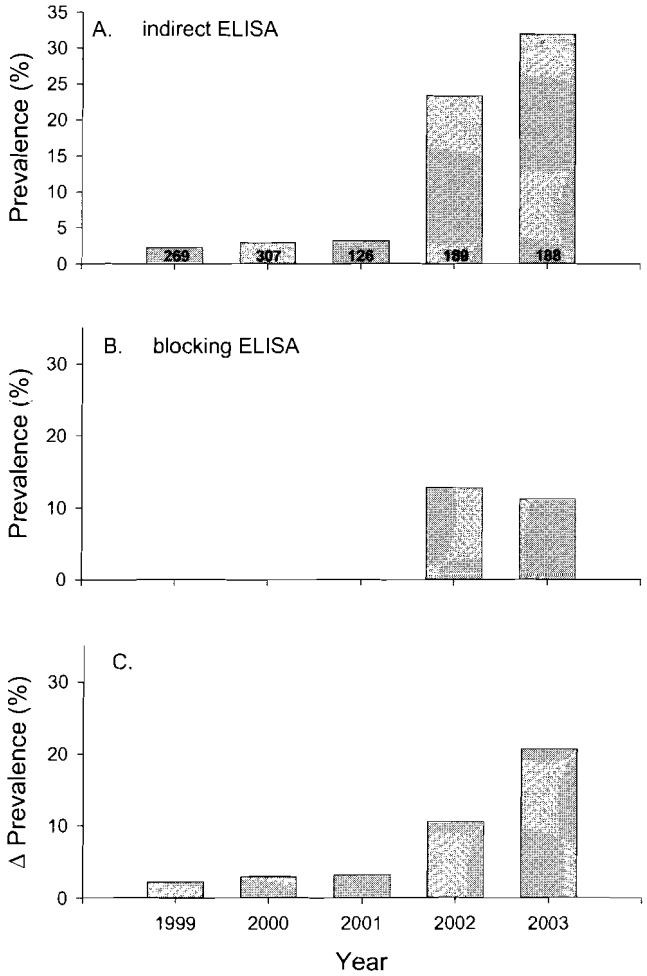

FIGURE 1. Prevalence of (**A**) antibodies against flaviviruses, as determined by indirect ELISA, (**B**) antibodies against West Nile virus, as determined by epitope blocking ELISA, and (**C**) the difference between prevalence as determined by iELISA and bELISA (Δ Prevalence) in Iowa white-tailed deer. Inset numbers indicate sample sizes. The difference in prevalence is taken as an indication of the transmission of some other non-WNV/SLE flavivirus during the course of the study.

samples were obtained in the winter months, 3 to 4 months after the mosquito season was over, it is possible that a higher concentration of neutralizing antibodies was present in the sera. It may also be possible that because the bELISA uses a MAb targeted against an epitope in the NS1 protein, whereas antibody measured by PRNT is primarily directed against E protein epitopes, that the difference in prevalence may reflect a bias in response against these two proteins.

The antibody negative results found by bELISA and WNV $PRNT_{90}$ for 1999–2001 were expected because the first WNV case in Iowa (a dead crow) was reported in September 2001 in eastern Iowa, when the mosquito season was already ending.[3] The prevalence of antibodies by these WNV-specific assays during 2002–2003 was consistent with other estimates of viral activity in the area (Table 2). More interesting was evidence of transmission of a non-WNV, non-SLEV flavivirus prior to the appearance of WNV in Iowa, and the dramatic increase in transmission of the(se) flavivirus(es) during 2002 and 2003. The increase in prevalence of the undescribed flavivirus in 2002 and 2003 suggests that environmental conditions favoring overall flavivirus transmission occurred coincidentally with the WNV epizootic. What these factors might have been remains to be determined. There is evidence for the circulation of other flaviviruses in the United States[5,26] (e.g., the newly discovered tick-borne encephalitis-like virus provisionally named "deer tick virus," similar to Powassan virus[27,28]). Moreover, serological cross-reactions between flaviviruses and pestiviruses also have been demonstrated.[29]

There was no association found between sex or age and positive WNV serology in this study, consistent with previous results.[30] Because the number of samples from fawns and yearlings from 2002 was less than 35 animals per age group, the prevalence found in this study might not be representative of what is happening in the field; further investigations with larger sample sizes for the different age groups is recommended to have a better understanding about the association between age and positive WNV serology. We found a higher prevalence, although statistically not significant, of antibodies to WNV in yearlings than in fawns and also a higher prevalence in adults than in yearlings in 2003. This could be caused by an increased opportunity for older animals to become infected as a result of living in an enzootic area for a longer period of time than younger animals, or by persistence of antibody over multiple years.

Although the antibody prevalences to WNV found in this study confirm WNV exposure in deer, there are no indications what effects WNV might have on the health of deer populations. However, transmission of WNV to white-tailed deer does occur, and it now remains to be determined through experimental infection studies their susceptibility, mortality, and reservoir potential to WNV. This information will lead to a better understanding of the role this species has in the ecological cycle of WNV.

In summary, we have provided serologic evidence for WNV infection in white-tailed deer from Iowa. These results are consistent with human, veterinary, and bird cases reported in Iowa. Even though human case reports are good sources of information to detect activity of a virus in a region, sometimes these reports can be inaccurate because of difficulty in establishing a precise geographic area of exposure and because asymptomatic people do not get tested. Serologic surveillance studies in domestic animals are also useful but have problems because of the introduction of vaccines for WNV. In contrast, surveillance studies of white-tailed deer can provide an accurate, rapid, cost-effective, and easy way to monitor the activity of WNV in a region.

TABLE 2

Number of West Nile virus positive cases reported in Iowa (CDC 2004)

	Year	
	2002	2003
Human	54	147
Equine	1159	96*
Avian	122	87*

* The decline of reported equine and avian WNV cases in 2003 may be due to vaccination of horses against WNV and restriction of dead bird testing by state health laboratories.

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