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Serum antibodies to *West Nile virus* in naturally exposed and vaccinated horses

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Abstract

A polyvalent enzyme-linked immunosorbent assay (ELISA) and plaque reduction neutralization tests (PRNT) were used to measure serum antibodies to the *West Nile virus* (WNV) in horses naturally exposed to or vaccinated against this flavivirus in Connecticut and New York State, USA. Relying on a PRNT as a "gold standard", the main objective was to validate a modified ELISA containing a recombinant WNV envelope protein antigen. It was also important to assess specificity by testing sera from horses that had other, undiagnosed illnesses. Sera for the latter study were obtained from 43 privately owned horses during 1995–1996. Analyses by an ELISA and PRNT confirmed the presence of WNV antibodies in 21 (91%) of 23 sera from naturally exposed horses and in 85% of the 20 vaccinated subjects; overall results for both study groups were highly concordant (91% agreement). Humoral responses of naturally exposed and immunized horses were similar. Both serologic tests were useful in confirming past infections of the WNV, but there was no evidence that horses with undiagnosed illnesses were exposed to the WNV prior to the 1999 outbreak.

INTRODUCTION

West Nile virus (WNV), widely distributed in Africa, the middle East, and Europe, was first reported in North America in 1999, following the diagnosis of human infections and reports of fatalities in New York City, USA (Anderson et al., 1999; Lanciotti et al., 1999). Like other flaviviruses (*Flaviviridae*), WNV is transmitted by mosquitoes and can infect a wide range of vertebrate hosts, including horses (Ward et al., 2004). During the period 1999–2003, more than 200 isolations of WNV were made from 17 mosquito species in six genera in Connecticut, USA (Andreadis et al., 2004). The broad feeding habits of key mosquito species include mammals as well as birds (Magnarelli, 1977; Molaei & Andreadis, 2006) and, along with host competence, allow for rapid amplification and spread of select mosquito-borne viruses in nature. Bird migration, dispersal of non-migrating birds (e.g., the House Sparrow, *Passer domesticus*), and the movement of mosquitoes contributed to the rapid spread of the virus from northeastern United States to western and southern sections of the country, Canada, and Mexico

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(Rappole & Hubalek, 2003; Rappole et al., 2006). Thousands of human and horse infections have been reported (Centers for Disease Control and Prevention, 2002).

Horses can be fed upon by infected *Culex* species and other key vectors of WNV in sheltered areas as well as in pastures, and if not vaccinated, may develop severe WNV meningoencephalomyelitis (Davidson et al., 2005). Laboratory diagnosis and ecological studies rely on detecting serum antibodies by an ELISA or neutralizing antibodies by a plaque reduction neutralization test (PRNT).

The WNV is endemic in numerous regions of Europe and is known to reemerge in some areas (Hubalek & Halouzka, 1999). However, in the Tuscany region of Italy, there was no evidence of virus activity prior to a 1998 outbreak (Autorino et al., 2002). Moreover, little is known about humoral responses in horses challenged by an experimental recombinant WNV envelope (E) protein vaccine (Ledizet et al., 2005), compared to those induced by a killed virus vaccine or by natural exposure to the WNV.

The main objectives of the present study were to validate a modified polyvalent ELISA with a recombinant WNV E protein incorporated, to measure total serum antibodies in naturally exposed and vaccinated horses, and to compare ELISA results with those of two PRNT methods being used in different laboratories. It was also important to assess the specificity of serologic tests.

METHODS

Sources of horse sera

There were three main study groups: subjects naturally exposed to WNV (group A) and found to be carrying antibodies to this pathogen; horses vaccinated against WNV (group B); and a panel of sera obtained from ill horses in Connecticut and New York State, USA during the period 1995−1996, prior to the USA WNV outbreak (group C). Twenty three samples in group A were selected from archived collections in the Department of Pathobiology and Veterinary Science at the University of Connecticut (UCONN). Of these, 19 were available for independent, comparative virus neutralization tests in different laboratories. The blood samples were collected between 2000 and 2003 from Connecticut and New York horses with clinical WNV disease; sera were known to contain antibodies to WNV, as determined by serologic testing performed at The Connecticut Veterinary Medical Diagnostic Laboratory (CVMDL) at UCONN. These sera were included in analyses to compare immunoassay methods.

Group B sera, also taken from UCONN collections, represented 20 horses immunized against WNV. These sera were included in analyses to compare antibody titers in naturally exposed and vaccinated horses. There were two subgroups of 10 horses each where subjects were immunized following manufacturers' directions with a licensed killed WNV vaccine (Innovator™) produced by Fort Dodge Animal Health (Fort Dodge, Iowa, USA) and boosted the following year with either the Fort Dodge vaccine or an experimental recombinant WNV E protein subunit vaccine (Ledizet et al., 2005) produced by L^2 Diagnostics (New Haven, Connecticut). As reported by these authors, the horses selected for immunization had no prior histories of WNV infections or vaccinations. Sera obtained before immunization were negative for antibodies to WNV, as determined by an ELISA or microsphere immunoassay. Each horse received two identical injections of the Fort Dodge vaccine 20 to 28 days apart in 2003. Serum samples tested in the present study were obtained approximately two months after a single booster injection in 2004. Antibodies elicited by the vaccines were detectable in the sera by an ELISA and PRNT (Ledizet et al., 2005).

The third group (C) consisted of serum samples representing 43 privately owned, ill horses from southwestern Connecticut (27 serum samples) and the lower Hudson River region of New York State (16 serum samples). Sera were randomly selected from a larger panel of archived specimens for the present study, tested before for antibodies to *Borrelia burgdorferi* (Magnarelli & Fikrig, 2005) and *Anaplasma* (formerly *Ehrlichia equi*) *phagocytophilum* (Magnarelli et al., 2000), and were stored −60°C at The Connecticut Agricultural Experiment Station (CAES). All horses in group C lived within 30 kilometers of a site in Stamford, Connecticut, where the WNV was first cultured from mosquitoes in North America during 1999 (Anderson et al., 1999). These sera were included in specificity analyses to determine if antibodies to tick-borne pathogens cross-react in an ELISA with the WNV antigen. Details on clinical manifestations and passive surveillance programs for tick-borne infections in horses have been published (Magnarelli et al., 1997, 2000; Magnarelli & Fikrig, 2005).

An additional 37 sera, from healthy horses not exposed to or vaccinated against WNV, served as negative controls. Of these, 28 sera were obtained from equids during 1985 in Kent, Connecticut, a rural area of Litchfield County in the northwestern part of the state. Vaccines for WNV immunization of equids were unavailable prior to 2001. The nine other negative control sera were received from the CVMDL. The positive control serum was from a horse immunized against WNV with the L^2 vaccine at UCONN and contained high concentrations of antibodies (> 1:40960) to the vaccine antigen, as determined by an ELISA.

Enzyme-linked immunosorbent assay

A noncompetitive, polyvalent ELISA described in previous studies (Magnarelli et al., 1984, 2000; Magnarelli & Fikrig, 2005) and conducted at CAES was modified, as described here, to detect total antibodies to the WNV. A recombinant truncated WNV E protein antigen (Ledizet et al., 2005; Wong et al., 2004) was mixed in a carbonate coating buffer solution (pH 9.5) and affixed to flat-bottomed, polystyrene plates (Nunc A/S, Roskilde, Denmark) at a concentration of 1 μg/ml and volume of 100 μL per well. The plates were incubated overnight at 4°C. Unlike previous ELISA methods used at the CAES, preparations were not allowed to dry. The next day, plates were washed four times in phosphate buffered saline solution (PBSS; pH 7.2) containing 0.05% Tween 20 (Magnarelli et al., 1984). A preliminary blocking procedure with mammalian sera was not used as in previous studies conducted at the CAES because early trials with goat or bovine sera showed high background reactivity in test wells for negative controls. Horse sera were diluted in PBSS containing 0.05% Tween 20 to 1 in 160, 1 in 320 and 1 in 640, and samples were added at a 100 μL volume to all test and control wells. Following incubation for one hour at 37°C, the plates were washed. Commercially produced (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA), affinity-purified horseradish peroxidase-labeled goat anti-horse (heavy and light chains specific) immunoglobulins (Ig), which replaced alkaline phosphatase conjugated antibodies used earlier (Ledizet et al., 2005), were diluted to 1 in 8000 in PBSS and were subsequently added at a 100 μL volume to each well. Conjugates that are heavy and light chains specific detect IgM and IgG antibodies (Magnarelli & Anderson, 1989). Following incubation for one hour at 37°C, the plates were washed four times with PBSS. The substrate, also purchased from Kirkegaard and Perry Laboratories, consisted of 2,2′-azinodi (3-ethyl-benzthiazoline sulfonate) and was delivered at a 100 μL volume to each plate well for color production. A microplate spectrophotometer measured absorbance values (optical densities [OD] of preparations, including negative PBSS controls without antigen) at 414 nm after 30 minutes of incubation. Each plate contained controls for positive and negative sera, PBSS, conjugate, and other reagents to either verify antigen reactivity or to check for false-positive results. Likewise, procedures were included to ensure assay standardization. Positive sera, with antibody titers of 1 in 640 in preliminary analyses, were re-tested to determine titration endpoints and to check reproducibility of results.

The general procedures used to determine cut-off values for positive results follow published protocols (Magnarelli et al., 1997, 2000). Twenty eight sera, obtained in 1985 and representing clinically normal horses from Kent, Connecticut, were used to calculate cut-off values for positive ELISA results. The sera were diluted in PBSS to 1 in 160, 1 in 320, and 1 in 640 and tested with the recombinant WNV E protein or without antigen (i.e., controls to assess non-specific, background reactivity). Net absorbance values, defined as the difference in OD between reactions with and without antigen, were determined for each serum dilution. Statistical analyses (mean plus 3 SD) of net OD values were applied to define cutoff values. It was important to minimize or exclude normal background reactions caused by low concentrations of nonspecific antibodies. Net OD positive cut-off values of 0.29, 0.23, and 0.17 were established for serum dilutions of 1 in 160, 1 in 320, and 1 in 640, respectively. The 0.17 OD value was also used to identify positive reactions for serum dilutions of 1 in > 640.

In vitro plaque reduction neutralization tests

Plaque reduction neutralization tests were relied on as primary diagnostic methods ("gold standards") to determine if horse serum antibodies inhibit WNV replication in cultured Vero cells. The materials and methods, described by Wang et al. (2001), were used in the present study at the CAES. Briefly, 75 μL of virus (100 plaque forming units per well) were mixed with 75 μL of heat-inactivated, diluted horse serum in a 96-well microtiterplate and incubated at 37°C for 1 hour. Serum-virus mixtures were then inoculated onto 3-day old confluent monolayers of Vero cells in a 6-well tissue culture plate and incubated at 37°C for one hour. The plates were mechanically shaken every 15 minutes and an agarose overlay was added. After the cells were incubated at 37°C for four days, a second overlay containing 4% neutral red was added on day 5. Virus plaques were counted 18 hours later. Sera were diluted to 1 in 10, 1 in 20, and 1 in 40 initially and, if positive at 1 in 40, were subsequently titrated to 1 in 320 in replicated tests to assess reproducibility of results. Reactions that resulted in an 80% or 95% reduction in viral plaque numbers were noted. Therefore, the recorded antibody titer was the highest serum dilution that neutralized at least 80% or 95% of the viral plaques.

A subset of 48 horse sera from subjects naturally exposed to the WNV (group A, 19 serum samples), vaccinated for WNV (group B, 20 serum samples), or from animals with no exposure to the WNV (negative controls, 9 serum samples) also was analyzed by a standard WNV neutralization assay using a microtiterplate method at the CVMDL following the National Veterinary Services Laboratory (Ames, Iowa, USA) 2004 protocol. Briefly, sera were heat inactivated at 56°C for 30 minutes and subsequently diluted 1 in 5 in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, Missouri, USA) containing antibiotics and antimycotics. Two-fold serial dilutions were made in duplicate rows of a 96-well plate. A serum control well was included for each sample. Twenty five μL of WNV, at a working dilution of $10^{3.9}$ and representing a 200 tissue culture infecting dose ($TCID_{50}$), was diluted in DMEM with antibiotics, antimycotics and 10% guinea pig complement and then added to each well except the serum control wells. The $TCID_{50}$ virus titer is defined as the reciprocal of the highest dilution of virus that infects 50% of the inoculated host cells in culture. Serum control wells received 25 μL of the DMEM containing 10% guinea pig complement. After the virus was added, plates were incubated for one hour at 37°C with 5% CO_2. Vero 76 cells were prepared by growing in a 75 cm^2 flask for 3 days until fully confluent. These cells were then split into 40 ml of cell culture media. One hundred μL of the prepared Vero cells were then added to all wells. Plates were incubated for four to five days at 37°C with 5% CO_2, and the wells were examined for cytopathic effect using an inverted microscope. Positive and negative control sera were included in test trials. The endpoint titer was the highest serum dilution in which no cytopathic effect was observed in both duplicate samples.

Indirect fluorescent antibody (IFA) staining

Analyses were conducted to further screen any horse sera obtained from ill subjects prior to the 1999 outbreak of WNV in New York or Connecticut and found to be positive by our modified ELISA and negative by PRNT for antibodies to this virus. Commercially prepared slides, containing fixed virus-infected Vero cells, were purchased (Panbio, Columbia, Maryland, USA) and used following the manufacturer's directions to detect total antibodies. Slides contained viruses from the following groups: Flavivirus (family *Flaviviridae*: WNV, *St. Louis encephalitis virus*, and *Powassan virus*); Alphavirus (family *Togaviridae*: *eastern equine encephalitis* and *western equine encephalitis viruses*); and Bunyavirus (family *Bunyaviridae*; *La Crosse virus*). The second antibody used was a fluorescein isothiocyanate-conjugated rabbit anti-horse immunoglobulin (Sigma-Aldrich) diluted to 1 in 64 with PBSS. Since background reactivity was evident at a serum dilution of 1 in 40 when a pre-immune serum was tested, a serum dilution of 1 in 320 was used to judge reactivity of selected horse sera.

Specificity tests

Additional tests were conducted to assess specificity. To determine if there was cross-reactivity due to equine antibodies produced to common tick-associated bacterial infections, 43 horse sera (group C) were analyzed by the modified ELISA for total antibodies to *A. phagocytophilum* and *B. burgdorferi* (Magnarelli et al., 2000, 2001, 2004; Magnarelli & Fikrig, 2005). Humoral responses to these pathogens in horses can be robust with high concentrations of IgM and IgG antibodies being produced. It was important to assess assay specificity because the presence of broadly reactive IgM antibodies can sometimes confuse ELISA and other serologic test results, even though these antibodies are directed to antigens of unrelated pathogens. A recombinant p44 antigen of *A. phagocytophilum*, expressed and purified as a maltose-binding fusion protein, was used in a polyvalent ELISA, whereas in analyses for *B. burgdorferi* antibodies, whole-cell antigen (strain 2591) was incorporated in a polyvalent ELISA. Both bacterial agents are transmitted by *Ixodes scapularis* ticks in northeastern United States and *Ixodes ricinus* ticks in Europe.

Statistical analyses

To assess concordance of assay results, significant differences in percentages of positive results were determined by using a z - test with Yate's correction (Sigmastat; SPSS). Values of $P < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

ELISA and PRNT concordance

Analyses by an ELISA and PRNT (at 95% plaque reduction) confirmed the presence of serum antibodies to WNV in 21 (91%) of 23 serum specimens, representing natural infections (group A) in horses. At an 80% plaque reduction rate, all 23 sera were positive. Similarly, 85% of the 20 vaccinated horses (group B) were positive in both tests (Table 1). Although the seropositivity value in the PRNT (95% positive at the 95% plaque reduction rate) exceeded that of an ELISA (85% positive) for vaccinated subjects, the difference was not statistically significant ($z = 0.53$, $p = 0.60$). Overall serologic results for both of these study groups were highly concordant (91% agreement).

Interlaboratory concordance of PRNT results

To compare results obtained independently in different laboratories, virus neutralization analyses also were conducted at the CVMDL. All 20 vaccinated horses (group B) included in the test panel were positive (≥ 1 in 10 titer), compared to the 19 positives identified by this method at the CAES. The CVMDL neutralization titer for the remaining serum sample, in reference to the discrepant result, was 1 in 25. Parallel tests performed on a subset of 19 horse sera representing natural exposure to WNV (group A) revealed 100% concordance in neutralization test results at the CVMDL and CAES. Moreover, all nine negative control sera were non-reactive in both laboratories.

ELISA vs. PRNT testing

All three serologic tests were useful in detecting antibodies to WNV in naturally exposed and vaccinated horses. It was particularly helpful to have PRNT results to interpret ELISA findings. Although laborious and time consuming, the PRNT yields more definitive results and is a reliable confirmatory method, while an ELISA is advantageous as a rapid prescreening procedure in surveillance programs (Castillo-Olivares & Wood, 2004). However, many variations exist in ELISA methodologies to detect antibodies to the WNV, and there is potential for false positive results when whole-cell viral antigens are used to detect IgM antibodies (Davidson et al., 2005). Our modified ELISA with recombinant WNV E protein antigens used with peroxidase-labeled goat anti-horse immunoglobulins yielded sensitive and reproducible results. The recombinant WNV E protein antigens were sufficiently reactive in the present study and were likewise used effectively by other investigators in a suspended microsphere immunoassay to detect WNV antibodies in human and equine sera (Wong et al., 2004; Balasuriya et al., 2006). Therefore, the use of recombinant viral antigens is an acceptable alternative to the use of whole-cell antigens.

Naturally infected vs. vaccinated horse sera

Antibody titers, measured by our modified ELISA, for naturally infected (group A) and vaccinated (group B) horses were comparable. However, a wide range of titration endpoints was noted for both groups (Table 2), with skewness toward higher titers. In each case, more than 77% of the antibody titers were greater than 1 in 1280. Geometric means of 2243 and 2004 were calculated for naturally infected and vaccinated horses, respectively. For the latter group, the geometric mean for horses boosted with the recombinant E protein (mean = 3225) exceeded that calculated (geometric mean = 1174) for the Fort Dodge vaccine, as determined by the ELISA containing recombinant E protein antigen.

Neutralizing antibody titers for natural infections (group A) and vaccinations (group B) varied. At a 95% plaque reduction rate for group A, horse sera diluted from 1 in 10 to ≥ 1 in 320 decreased viral plaque numbers (Tables 1 & 2). At 80% neutralization of plaque-forming units, all 23 sera in group A were considered positive with a PRNT titer of ≥ 1 in 320 being most frequently recorded (17 samples); the remaining sera had neutralizing antibodies at concentrations of 1 in 40 (1 sample), 1 in 80 (4 samples), and 1 in 160 (1 sample). The frequency distribution for neutralizing antibody titers in group B vaccinated subjects paralleled those recorded for natural infections (Table 2). All 10 horses that were boosted with the Fort Dodge vaccine developed neutralizing antibodies that decreased viral plaque numbers by 95%; serum dilutions ranged from 1 in 10 to ≥ 1 in 320. Similarly, 9 of 10 other horses boosted with the experimental recombinant subunit vaccine had concentrations of neutralizing antibodies in the same range. Serum from the remaining vaccinated horse was negative by ELISA and the PRNT at both the 95% and 80% plaque reduction rates.

Vaccine-induced humoral responses

Humoral responses of naturally exposed horses (group A) were similar to those of vaccinated subjects (group B). Seropositivity rates and antibody titers recorded for the two groups were similar, as were the neutralizing titers. When the two vaccinated groups were compared, the virus neutralization titers were likewise comparable. Relatively low ELISA and PRNT titers recorded after the use of a killed virus vaccine in other mammals (Tesh et al., 2002; Kutzler et al., 2004) may, along with cellular immune activity, provide protection against WNV. One vaccinated horse in the present study, however, did not have detectable antibodies to the WNV recombinant E protein antigen. Nonetheless, performance of the two vaccines was considered comparable; the difference in detectable responses (100% vs. 90%) can be attributed to variable immune responses in a diverse population. In other studies, antibodies were not detected in some horses immunized with the killed WNV vaccine (Davidson et al., 2005). Further, in horses challenged with inactivated eastern equine encephalitis virus, antibodies did not persist in some subjects beyond two months after vaccination (Barber et al., 1978). Viremia in horses can be relatively low and of short duration (Castillo-Olivares & Wood, 2004), particularly in horses infected by mosquito bites (Bunning et al., 2002). Moreover, IgM antibodies may rise slowly during early infection, remain detectable for limited periods of time (< 3 months) following natural infection, and, in some horses, may not even be produced in measurable amounts following WNV vaccination (Castillo-Olivares & Wood 2004). It should also be noted that in tests of human sera from persons diagnosed with *La Crosse virus* infections (Calisher et al., 1986), IgM antibody produced early in illness does not always show neutralizing activity. Therefore, careful consideration should be given to the time blood samples are taken from subjects relative to onset of clinical signs of illness and to the selection of sensitive antibody tests to minimize false negative results. Further, in view of the variable immune responses among horses, due to age, breed, and other factors (Davidson et al., 2005), and the abundance of infected mosquitoes that can occur in certain locations, vaccination for WNV may be needed every six months for some horses (Davidson et al., 2005). These authors reported that vaccine-induced antibody responses decreased with age of horses. Therefore, it is an acceptable option to test horse sera post vaccination to determine if antibodies have been produced.

Assay specificity

In specificity studies, three (13%) of the horses naturally exposed to WNV (group A) contained antibodies to this pathogen and also had antibodies to *A. phagocytophilum* or *B. burgdorferi*. Two of the 43 sera representing horses with undiagnosed illnesses prior to 1999 (group C) and tested at the CAES contained relatively high concentrations of antibodies to all three pathogens (Table 3). Three (11%) of the 28 sera (group C) containing antibodies to one or more bacterial pathogens had antibodies to the WNV. Single reactions to separate antigens (n=15 sera; 35%) nearly equaled multiple reactions to different disease organisms (n=14 sera; 33%). Based on these results, there was no convincing evidence of cross-reactivity in our ELISA for WNV antibodies being caused by high concentrations of antibodies to *A. phagocytophilum* or *B. burgdorferi*.

In analyses of 43 horse sera in group C, obtained from ill animals prior to the 1999 WNV outbreak, four (9%) specimens were positive by our modified ELISA but negative in the PRNT (Table 1). These horses resided in southwestern Connecticut in the following towns: Bethany, Newtown (two horses), and Weston. In an ELISA, antibody titers ranged from 1 in 640 to 1 in 1280. All nine sera from a negative control group were non-reactive by both assay methods (Table 1). Based on these preliminary results, we suspected that the four positive sera obtained prior to 1999 might have antibodies to other flaviviruses related to WNV, such as *St. Louis encephalitis virus* or *Powassan virus*. The latter human pathogen occurs in northeastern and western United States and Ontario, Canada (Gritsun et al., 2003), where *Ixodes cookei*, an important vector, and other hard-bodied ticks occur. Exposure to the “deer tick virus”, a subtype of *Powassan virus*, is also possible (Ebel et al., 2000). In serological tests of human and horse sera for *Flavivirus* antibodies, cross-reactivity among closely related viruses in this serogroup is well documented (Castillo-Olivares & Wood, 2004;Wong et al., 2004;Martin et al., 2000;Johnson et al., 2005), regardless of antigens incorporated in assays. The high degree of structural relatedness of E proteins among flaviviruses is an important factor that can lead to false positives when antibodies are present to such agents as *Powassan*, *St. Louis* *encephalitis*, *Japanese encephalitis*, or *dengue viruses*. However, there are no reports of the latter three viruses occurring naturally in Connecticut or Europe. Further, in more than ten years of statewide surveillance, conducted at CAES for encephalitis viruses in Connecticut mosquitoes, *St. Louis encephalitis virus* has never been isolated. Although travel histories for these four positive horses are unknown, it is not uncommon for these animals to be moved nationally or internationally. We recognize that horses that travel to midwestern or southern United States may be exposed to *St. Louis encephalitis* virus there. Plaque reduction neutralization assays, kindly conducted at the Arbovirus Laboratory, Wadsworth Center, New York State Department of Health (Albany, New York) and following the methods of Lindsey et al., 1976, indicated antibody titers of < 1 in 10 against *St. Louis encephalitis virus*, WNV, and *Powassan virus*. In addition, results of IFA staining methods revealed a pattern of uniform, diffuse cytoplasmic staining that was not specific to any particular family of viruses tested. Therefore, we conclude that reactivity of the four horse sera from ill subjects in our modified ELISA was probably due to binding of non-specific antibodies. Based on results of our modified ELISA and the negative PRNT findings for the four sera reactive in this ELISA, we also conclude that none of the 43 horses tested in the present study (group C) had exposure to WNV prior to the 1999 outbreak in northeastern United States. Although it is unknown how the WNV entered North America, there was a rapid emergence and spread of the virus over a large geographic region of the western hemisphere during and after that year. It is, therefore, important to monitor for the introduction of exotic viruses and other pathogens in conjunction with surveillance and pathogen isolation programs for mosquitoes, ticks, and other hematophagus arthropods. Serological assays, such as the ELISA described in the present study, can be used as aids in laboratory diagnosis and ecological studies.

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Abbreviations

WNV, West Nile virus; PRNT, plaque reduction neutralization test.

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Table 1

Results of analyses for antibodies to *West Nile virus* in horse sera from Connecticut and New York State by plaque reduction neutralization tests (PRNT) and a polyvalent enzyme-linked immunosorbent assay (ELISA) containing recombinant envelope protein antigen.

	PRNT*		ELISA*	
Study groups	**Total sera tested**	**Number and (%) positive**	**Total sera tested**	**Number and (%) positive**
Natural infection (A)	23	21 (91)	23	21 (91)
Vaccinated (B)	20	19 (95)	20	17 (85)
Unknown history (C)	34	0 (0)	43	4 (9)
Negative control	9	0 (0)	9	0 (0)

*Positive by PRNT when at least 95% neutralization of plaque-forming units was detected. Positive ELISA titers (≥ 1 in 160). Note: unknown history refers to blood samples drawn from ill horses in 1995−1996 prior to the 1999 outbreak of human and horse cases of West Nile encephalitis in northeastern United States. All tests were performed on one serum sample per animal at The Connecticut Agricultural Experiment Station.

Table 2

Frequency distributions of serum antibody titers for horse sera positive by a polyvalent enzyme-linked immunosorbent assay (ELISA) or in plaque reduction neutralization tests (PRNT).

	Number sera with positive antibody titers*			
	ELISA		PRNT	
Reciprocal antibody titers	Natural infection	Vaccinated	Natural Infection	Vaccinated
10	NA	NA	1	2
20	NA	NA	5	1
40	NA	NA	1	2
80	NA	NA	2	4
160	0	1	9	3
320	1	1	3	7
640	1	2	NA	NA
1280	4	4	NA	NA
2560	10	3	NA	NA
5120	5	3	NA	NA
10240	0	3	NA	NA
Total positive horse sera	21	17	21	19

*Positive antibody titers by an ELISA were 1 $\geq$ 160. Seropositive PRNT indicate 95% neutralization of plaque-forming units at all specified serum dilutions but not tested beyond 1 in 320. NA = not applicable. All tests were performed at The Connecticut Agricultural Experiment Station.

Table 3

Comparison of serologic test results for 43 horse sera (group C) from subjects with undiagnosed illnesses prior to 1999 and tested for antibodies to *West Nile virus*, *Anaplasma phagocytophilum*, and/or *Borrelia burgdorferi* by separate polyvalent enzyme-linked immunosorbent assays.

		Reciprocal antibody titers (range)		
Antigen groups*	**Number positive**	**WNV**	**AP**	**BB**
WNV	1	1280	--	--
WNV+AP	0	--	--	--
WNV+AP+BB	2	1280	(640−5120)	(2560−5120)
WNV+BB	1	640	--	2560
AP	2	--	(160−320)	--
AP+BB	11	--	(160−10240)	(640−40960)
BB	12	--	--	(640−40960)
Total (%) positive	29 (67%)			

* Recombinant envelope E protein antigen was used in an ELISA for *West Nile virus* (WNV) antibodies, whereas recombinant protein 44 and whole-cell antigens of *A.. phagocytophilum* (AP) and *B. burgdorferi* (BB), respectively, were incorporated separately in an ELISA. All tests were performed at The Connecticut Agricultural Experiment Station.