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Evaluation of Widely Used Diagnostic Tests To Detect West Nile Virus Infections in Horses Previously Infected with St. Louis Encephalitis Virus or Dengue Virus Type 2▽†

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Primary West Nile virus (WNV) infections can be diagnosed using a number of tests that detect infectious particles, nucleic acid, and specific IgM and/or IgG antibodies. However, serological identification of the infecting agent in secondary or subsequent flavivirus infections is problematic due to the extensive cross-reactivity of flavivirus antibodies. This is particularly difficult in the tropical Americas where multiple flaviviruses cocirculate. A study of sequential flavivirus infection in horses was undertaken using three medically important flaviviruses and five widely utilized diagnostic assays to determine if WNV infection in horses that had a previous St. Louis encephalitis virus (SLEV) or dengue virus type 2 (DENV-2) infection could be diagnosed. Following the primary inoculation, 25% (3/12) and 75% (3/4) of the horses mounted antibody responses against SLEV and DENV-2, respectively. Eighty-eight percent of horses subsequently inoculated with WNV had a WNV-specific antibody response that could be detected with one of these assays. The plaque reduction neutralization test (PRNT) was sensitive in detection but lacked specificity, especially following repeated flavivirus exposure. The WNV-specific IgM enzyme-linked immunosorbent assay (IgM ELISA) was able to detect an IgM antibody response and was not cross-reactive in a primary SLEV or DENV response. The WNV-specific blocking ELISA was specific, showing positives only following a WNV injection. Of great importance, we demonstrated that timing of sample collection and the need for multiple samples are important, as the infecting etiology could be misdiagnosed if only a single sample is tested.

One of the classic challenges in flavivirus diagnostics is the issue of cross-reactivity among flavivirus antibodies with heterologous viral antigens. Accurate identification of an infecting agent can be problematic and depends upon the diagnostic assay as well as the infection history and immune status of the vertebrate host. For example, greater levels of cross-reactivity are found among flaviviruses within the same antigenic complex (7). In addition, when performing flavivirus diagnostics on samples from hosts in areas where multiple flaviviruses are circulating, repeated and sequential infections are common and the ability of any particular diagnostic test to accurately implicate the infecting agent depends upon the ability of that assay to distinguish among the various and often antigenically similar flaviviruses.

While this issue has been important for years in Asia, where multiple flaviviruses cocirculate, this problem has become increasingly significant recently in the Western Hemisphere with the spread of West Nile virus (WNV). In the subtropical latitudes (Canada and the continental United States), there are only limited geographic pockets where other flaviviruses, particularly St. Louis encephalitis virus (SLEV), are known to exist. Therefore, diagnosis of WNV infection has predominantly occurred for individuals with no preexisting flavivirus antibody. However, in the tropical Americas (Central America, South America, and the Caribbean), individuals are likely to have been repeatedly exposed to multiple enzootic flaviviruses, including the dengue viruses (dengue virus type 1 [DENV-1] to DENV-4), SLEV, Ilheus virus, T'Ho virus, and yellow fever virus (8, 12, 13, 15, 27, 29, 32, 33, 43). This not only complicates diagnosis but suggests the possibility of cross-protection or, conversely, antibody-dependent enhancement (ADE) of the immune response, thus modulating the course of disease (34). Only a few cases of human WNV infection and limited equine disease in tropical America have been diagnosed (11, 36). Whether this limited amount of disease is due to unknown viral or vertebrate host factors, the presence of antibodies to alternate flaviviruses that induce cross-protection, or limitations of diagnostic capacity for differential diagnosis of multiple infections is unknown. The lack of information concerning the vertebrate host antibody response following repeated flavivirus infection further complicates the diagnosis of WNV disease in

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TABLE 1. Horse infection summary

Cohort (infection course[a])	Horses	Virus strain used	Dose (PFU/ml)
1 (SLEV-WNV)	9, 10	V4285	$10^{3.4}$
		NY99	$10^{4.3}$
	11, 12	V4285	$10^{3.9}$
		NY99	$10^{4.3}$
	19, 20	V4285	$10^{6.0}$
		NY99	$10^{4.0}$
2 (SLEV-SLEV-WNV)	5, 6	TBH 28	$10^{4.0}$
		TBH 28	$10^{4.0}$
		NY99	$10^{4.0}$
	7, 8	V4285	$10^{3.4}$
		V4285	$10^{3.3}$
		NY99	$10^{4.0}$
	17, 18	TBH 28	$10^{6.0}$
		TBH 28	$10^{6.0}$
		NY99	$10^{4.0}$
3 (DENV-WNV)	13, 14	TR1751	$10^{5.3}$
		NY99	$10^{4.7}$
	21, 22	TR1751	$10^{4.0}$
		NY99	$10^{4.0}$

[a] Sequential inoculations were given at 21-day intervals.

tropical America. This is mainly due to the inability to obtain paired and/or multiple serum specimens from animals with completely documented infection histories.

To help evaluate the accuracy in diagnosing secondary or tertiary WNV infection in areas where multiple exposures to flaviviruses are likely, we performed sequential flavivirus infection studies with equines and then compared the abilities of commonly used diagnostic assays to determine infection etiology. Equines were selected because they are important vertebrate hosts of WNV and little is known about their responses to sequential flavivirus infections (40). In addition, equines are frequently part of surveillance programs, and thus, the information obtained from our study would be useful to public health officials (1). We chose two widely distributed and prevalent flaviviruses known in tropical America, SLEV and DENV-2, for the primary infections in our study (19, 29). SLEV is known to infect horses (1, 14, 29, 32, 33, 36), but the resulting temporal antibody profiles are not documented. There is no literature on dengue virus infection of equines, but DENV-2 is present throughout the tropical Americas and the mosquito vectors that transmit this virus will feed upon horses (41). In this study, samples obtained from these subjects were tested for virus, viral nucleic acid, and antibodies to flaviviruses. Detection of antibodies is most commonly performed using enzyme-linked immunosorbent assay (ELISA) platforms or neutralization assays. These tests not only detect specific antigens but can also detect specific antibody types (i.e., IgM, IgG, neutralizing antibody [Nt Ab], etc.). Therefore, we employed the IgM ELISA, IgG ELISA, blocking ELISA, and plaque reduction neutralization test (PRNT) to monitor for the development and presence of specific antibodies in response to flavivirus infections. We provide here the kinetics and cross-reactivity of antibody development in equines following infection with multiple flaviviruses using multiple diagnostic assays.

MATERIALS AND METHODS

Horse inoculations and infections. Six- to nine-month-old horses were screened by ELISA for flavivirus antibodies prior to inclusion in this study. Only antibody-free animals were used in the study. Two days before infections, animals were moved to a biocontainment building at Colorado State University and maintained under animal biosafety level 3 laboratory conditions for the duration of the study. Cohorts of 4 or 6 horses were subcutaneously inoculated with SLEV (cohorts 1 and 2) or DENV-2 (cohort 3) at doses ranging from $\log_{10}$ 3.3 to $\log_{10}$ 6.0 PFU/ml (Table 1). Twenty-one days after the primary injection, the same horses were inoculated with either SLEV (cohort 2) or WNV (cohorts 1 and 3). Twenty-one-day intervals were chosen to allow for and ensure sufficient time for antibody development. Cohort 2 horses that had been twice injected with SLEV received an injection of WNV at 42 days after the initial inoculation. Blood was collected every 3 days throughout the course of the study for all animals, with the day zero time point occurring immediately after inoculation. Clinical signs were monitored daily.

Viruses. The viruses used in this study were WNV (strain NY99-356262-11), SLEV (strains TBH-28 and V4285), and DENV-2 (strain TR1751). The viruses were obtained from the Arbovirus Reference Collection at the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention (CDC), Fort Collins, CO, and Colorado State University, Fort Collins, CO.

RNA extraction and real-time reverse transcription-PCR (RT-PCR) assay. A TaqMan real-time PCR assay was performed to test acute-phase serum samples for viral nucleic acid. First, viral RNA was isolated from serum using the QiaAmp viral RNA protocol (Qiagen, Valencia, CA). Total RNA was extracted from 140 μl of the serum sample and eluted from the kit columns into a final volume of 60 μl of elution buffer. The RNA was stored at −80°C until use.

The WNV-specific 3′ noncoding (3′NC) region and envelope (ENV) region primers and probe sets were used for the detection of WNV (23). The SLEV and DENV-2 oligonucleotide sets were designed with the Primer Select software program (DNAStar, Madison, WI) (Table 2) and were based on the available published GenBank full-length sequences. The real-time probes were labeled with a 5′-end 6-carboxyfluorescein (FAM) reporter dye and a 3′-end black hole quencher 1 (BHQ1) dye. A QuantiTect probe RT-PCR kit (Qiagen, Valencia, CA) was used for the real-time (TaqMan) assay. A 50-μl total reaction volume consisted of kit components, 10 μl of RNA, 400 nM primer, and 150 nM probe. The reactions were subjected to 45 cycles of amplification in an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA) according to the recommended conditions. The previously described positivity limits were used for the WNV assay (24). The SLEV and DENV limits of detection were found to be threshold cycle (C_T) values of 38.5 and 40.0, which are equivalent to 0.1 and 1.0 PFU/ml,

TABLE 2. Oligonucleotide sets used in the TaqMan real-time PCR assay

Oligonucleotide[a]	Sequence (5′–3′)	Product size (bp)
SLEV 1992(+)	ACCACCTTTCGGCGATTCTTAC	90
SLEV 2018(+) probe	FAM-TCGTCGGAAGAGGCACCACCCAGATTA-BHQ1	
SLEV 2028(-)	CTTCCCAATGCTGCTTCCCTCTT	
DENV 1085(+)	CCAAACAACCCGCCACTCTAAG	159
DENV 1145(+) probe	FAM-AACAGACTCGCGCTGCCCAACACA-BHQ1	
DENV 1244(-)	TTTCCCCATCCTCTGTCTACCATA	

[a] The annealing temperatures were 55 to 58°C for primers and 65 to 68°C for probes.

respectively (unpublished data), using previously described techniques (26). Briefly, the C_T cutoff value was determined by first making several RNA dilutions, with the aim of progressing from detection to no detection with the use of the optimized oligonucleotides (primers and probe) under standard real-time RT-PCR conditions. The average C_T of the last dilution set that yields 10 out of 10 detection events (C_T of 45 or less) is the limit of detection for that set of oligonucleotides. In addition, each run includes a standard RNA curve. The standard curve was completed by serially diluting the virus stock and extracting the RNA from each dilution according to the previously mentioned RNA extraction protocol while simultaneously titrating each dilution in a standard plaque assay (PFU/ml). A curve correlation coefficient of ≥0.950 and a 90 to 100% PCR efficiency was used to validate each detection assay, and the RNA amounts were correlated with numbers of PFU equivalents per milliliter as previously reported (22, 45). While an alternative approach is to calculate numbers of RNA copies per ml, we chose the presentation of PFU equivalents per ml as this is more relevant in a diagnostic setting.

IgM ELISA. To detect the presence of WNV and SLEV immunoglobulin M (IgM) in the serum samples, the IgM ELISA was performed as previously described, with the following modifications (10, 30). A 96-well Immulon II HB plate (Dynatech Industries, Chantilly, VA) was coated with 75 μl of goat anti-horse IgM (Kirkegaard and Perry Laboratories, Gaithersburg, MD), which was diluted (1:3,000 for WNV and 1:1,000 for SLEV) in carbonate/bicarbonate buffer. Fifty microliters/well of 1:400 wash buffer-diluted sample sera and control sera were added to the wells and allowed to incubate for 1 h at 37°C in a humidified chamber. Normal or viral antigen was diluted 1:160 in wash buffer, and 50 μl/well was added in triplicate to the appropriate wells, where they were allowed to incubate overnight at 4°C. A horseradish peroxidase-conjugated monoclonal antibody (MAb), 6B6C-1, produced by Jackson Immunological Laboratories (West Grove, PA) diluted 1:16,000 for WNV and 1:6,000 for SLEV was then used as a detector antibody (38). The protocol was continued with no additional modifications. Calculations of the optical density (OD) reading of the sample on the viral antigen divided by the OD reading of the normal control sera on the viral antigen (*P*/*N* values) were performed by following the guidelines of previous studies (10, 30). For a specimen to be considered IgM positive for the test virus, the *P*/*N* must be ≥3 and the value of *P* for the test specimen must be greater than or equal to twice the mean OD of the test specimen reacted on normal antigen. Percents sensitivity and specificity were calculated for the modified WNV IgM ELISA based on the PRNT results (true positive or negative) for this sample set. The results for the WNV-specific blocking ELISA were used in those situations where a 4-fold difference was not observed between PRNT results. The sensitivity and specificity were 83.6% and 93.4%, respectively.

IgG ELISA. To detect the presence of WNV immunoglobulin G (IgG) in serum samples, a previously described IgG ELISA protocol was conducted (21). Briefly, a 96-well Immulon II HB plate was coated with 75 μl/well of the broadly cross-reactive flavivirus 4G2 MAb, which was diluted 1:2,000 in carbonate/bicarbonate buffer. Blocking buffer was added and allowed to incubate as described above. Normal or viral antigen was added after a 1:160 dilution in wash buffer and allowed to bind to the 4G2 MAb. The sample sera (diluted 1:400), positive control (diluted 1:800), and negative control (diluted 1:400) were added to triplicate wells and allowed to incubate for 1 h at 37°C. Goat anti-horse IgG alkaline phosphatase-conjugated antibody was then added at a 1:1,600 dilution. The protocol was continued with no additional modifications.

Calculations of *P*/*N* values were performed by following the guidelines of previous studies (10, 30). For a specimen to be considered IgG positive for the test virus, the *P*/*N* must be ≥3, and the value of *P* for the test specimen must be greater than or equal to twice the mean OD of the test specimen reacted on normal antigen.

Blocking ELISA. Sera were tested for antibodies to flaviviruses by blocking ELISA as previously described (3). ELISAs were performed using the WNV-specific MAb 3.1112G (Chemicon International, Temecula, CA) or the flavivirus-specific MAb 6B6C-1, obtained from the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO. The ability of the test sera to block the binding of the MAbs to WNV antigen was compared with the blocking ability of control horse serum without antibody to WNV (Vector Laboratories, Burlingame, CA). Data were expressed as relative percentages, and inhibition values of ≥30% were considered indicative of the presence of viral antibodies.

PRNT. Neutralizing antibodies (Nt Ab) against WNV, SLEV, and DENV-2 antigen in the equine serum samples were detected by the plaque reduction neutralization test (PRNT) (9, 25). The samples were heat inactivated for 30 min at 56°C and then serially diluted 2-fold in Dulbecco's minimum essential medium (DMEM) diluent (DMEM with 10% fetal bovine serum [FBS], 100 U/ml of penicillin, 100 mg/ml of streptomycin [Gibco, Carlsbad, CA]), starting at a 1:10 dilution. A suspension of 100 PFU virus/125-μl diluent was then mixed with the diluted serum samples and the suspension incubated for 1 h at 37°C. The virus strains used in the PRNT are those listed in Table 1. The serum-virus suspension was then transferred onto a Costar 6-well cell culture plate (Corning, Corning, NY) containing a semiconfluent monolayer of Vero cells and incubated for 1 h at 37°C. The plates were rocked every 15 min during this incubation. Then, each well was covered with a 0.4% Genepure LE agarose–DMEM layer (ISC BioExpress, Kaysville, UT) and allowed to incubate for the appropriate duration (3 days for WNV and 6 to 7 days for SLEV and DENV-2) at 37°C. After incubation, the agarose layer was removed and the wells were covered with a fixative/staining solution (40% methanol and 0.25% crystal violet). Plaques were counted, and titers were calculated and expressed as the reciprocal of the serum dilution yielding a ≥80% reduction ($PRNT_{80}$) in the number of plaques. All samples were tested in duplicate.

RESULTS

Viremia and clinical signs of illness. None of the serum samples yielded infectious virus when analyzed by a plaque assay (data not shown), nor was there evidence of SLEV or DENV-2 by virus-specific real-time RT-PCR. However, WNV nucleic acid was detected by real-time RT-PCR in 68.8% (11/16) of serum samples for up to 6 days postinfection (dpi) (Tables 3 to 5). The levels detected corresponded to 1 to 100 PFU equivalents per ml of serum. Heterologous real-time RT-PCR assays performed on sera from horses exposed to more than one virus (>18 dpi) resulted in no detection to the initial virus (data not shown). None of the horses showed any clinical signs of illness.

Primary SLEV injection. Two cohorts of horses were examined, one receiving a single injection of SLEV and one receiving two doses of SLEV prior to WNV exposure (Tables 3 and 4). Only 5 of 12 (41.7%) horses with SLEV exposure had detectable levels of specific antibody in any assay prior to WNV infection. The SLEV-specific IgM ELISA, SLEV PRNT, and flavivirus-reactive blocking ELISA all correctly identified SLEV antibodies at multiple time points prior to WNV infection. However, in one animal (no. 17), WNV-reactive neutralizing (Nt) antibodies were identified prior to WNV exposure at 27 and 42 days. When SLEV-specific antibody was detectable (prior to WNV exposure), it was most likely to be detected by PRNT (5/12 animals) or the flavivirus-specific blocking ELISA (4/12 animals) as early as day 9 or 12, respectively. The SLEV-specific IgM ELISA detected antibodies in only 3 animals prior to WNV exposure, and the results for the flavivirus IgG and WNV-specific blocking ELISAs were negative for all 12 horses until after exposure to WNV.

All horses initially exposed to SLEV except one (no. 6) developed a WNV-specific antibody response after WNV exposure. While one animal had antibodies at 3 days, most of the animals developed detectable levels of WNV-specific antibodies on days 9 to 12 postinjection. Peak WNV Nt-antibody titers occurred between days 12 and 18 and, while highly variable in titer, were typically higher than SLEV Nt-antibody titers. SLEV Nt-antibody titers did increase as WNV Nt-antibody titers developed after exposure, and this increase in titer was greater than 4-fold in 7 of 12 (58%) horses. Additionally, titers generally shifted from the SLEV titers being 2- to 4-fold greater than the WNV Nt-antibody titers to the WNV Nt-antibody titers being 2- to 8-fold greater than the SLEV titers after WNV exposure. However, there was still considerable cross-reactivity, with 14/19 samples (74%) having <4-fold dif-

ferences between SLEV and WNV starting at the first WNV antibody detection. This would lead to an indeterminate detection of the infecting etiology. Two samples had SLEV Nt-antibody titers that were 4-fold greater than the WNV titers, which would lead to an incorrect diagnosis that the most recently infecting agent was SLEV.

In general, results for all ELISAs were negative until at least day 9 after WNV injection in both SLEV/WNV cohorts. Some animals never generated detectable ELISA antibodies. For example, horse no. 6 never produced any ELISA antibodies but did have SLEV Nt antibodies at days 21 to 30. Other examples include horses 18 and 19, which were positive for IgG-, flavivirus-, and WNV-specific blocking ELISA but never demonstrated any IgM-specific response. Additionally, horse no. 10 developed antibodies as shown by positive results for WNV IgM, IgG, and blocking ELISA but never generated Nt antibodies or SLEV-specific IgM. The remaining horses did develop IgM responses, but this appeared to be cross-reactive, as the SLEV IgM data mirrored the WNV data in most cases and were typically found only after WNV exposure. IgG ELISA was a good method for diagnosis of WNV infection; in cohort 1, all 6 horses developed IgG antibody between days 6 and 15 after WNV injection, with these antibodies remaining until the end of the experiment. Three of five (60%) animals in cohort 2 showed similar results. The WNV-specific blocking ELISA was also a good indicator of recent WNV infection, with positive results starting between days 9 and 12 after WNV injection in both cohorts. Notably, neither the WNV-specific blocking ELISA nor the flavivirus IgG ELISA gave a positive result prior to WNV exposure, and both assays gave consistently positive results once the horse seroconverted. The flavivirus-specific blocking ELISA also gave positive results for most subjects after WNV exposure, but this assay also detected SLEV (albeit inconsistently).

Primary DENV-2 injection. The final cohort consisted of horses injected with DENV-2 followed by WNV 21 days later (Table 5). Following primary injection of DENV-2, specific antibody was not detected by any of the ELISAs but was detected by an Nt-antibody assay in 3 of 4 horses. Following secondary exposure to WNV, all 4 animals had 4-fold increases in DENV Nt-antibody titer. Similar to what was seen in cohorts with initial SLEV injection, all ELISA formats, including those developed for SLEV, detected antibodies typically starting by 9 days after WNV exposure. These data clearly demonstrate cross-reactivity between WNV antibody and SLEV antigen because none of these horses had been injected with SLEV. WNV-specific and SLEV-cross-reactive Nt antibodies were also detected between 6 and 9 days after WNV injection. Both WNV and SLEV Nt-antibody titers were among the highest titers detected in any cohort, with SLEV titers equal to or less than the WNV titers in all samples.

WNV or SLEV antibody-positive sera from cohorts 1 and 2 were evaluated to determine if cross-reactivity with DENV-2 antigen occurred. No DENV-2 Nt antibodies were detected after a single infection; however, DENV-2 Nt antibodies were observed in both SLEV-WNV cohorts after WNV exposure.

TABLE 3. Real-time PCR, PRNT, and ELISA results for cohort 1 (SLEV-WNV series)[a]

Inoculum and day	Detection in indicated sample by indicated assay																																											
	Real-time PCR[b]						$PRNT_{80}$														ELISA[c]																Blocking ELISA									
							WNV						SLEV						DENV		WNV IgM						SLEV IgM						WNV IgG						WNV						Flavivirus					
	9	10	11	12	19	20	9	10	11	12	19	20	9	10	11	12	19	20	19	20	9	10	11	12	19	20	9	10	11	12	19	20	9	10	11	12	19	20	9	10	11	12	19	20
SLEV																																													
0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
9							−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
12							−	−	−	−	−	−	−	−	−	−	20	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−
15							−	−	−	−	−	−	−	−	−	−	10	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−
18							−	−	−	−	−	−	−	−	−	−	20	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−
WNV																																													
21	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
24	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	10	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
27	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	20	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+/−	−	−	−	−	+	+
30			−	−			10	−	−	−	40	40	20	−	−	−	80	10	40	−	+	−	+	−	−	+/−	−	−	−	−	−	+	−	−	−	−	+	+	+	−	−	−	+	+
33							80	−	80	40	40	160	40	−	40	20	160	20	40	−	+	−	+	+	−	+	+	−	+	−	−	+	−	−	−	−	+	+	−	+/−	+	+	+	+	+	+
36							160	−	80	40	40	80	40	−	40	80	80	10	40	10	+	+/−	+	+	−	+	+	−	+	−	−	−	+	+/−	+	+	+	+	+	+	−	+	+	+	+	+
39							80	−	160	40	80	40	40	−	20	−	40	10	40	−	+	+	+	+	−	+/−	+	−	+	+	−	−	−	+	+/−	+	+	+	+	+	+	+	+	+	+	+
42							80	−	80	20	NA	NA	80	−	20	−	NA	NA	NA	NA	+	−	+	−	NA	NA	+	−	+	+/−	NA	NA	+	+/−	+	+	NA	NA	+	+	+	+	NA	NA	−	+	+	+	NA	NA

[a] NA, no sample; blank, not tested.
[b] The real-time PCR results are virus specific. The positive-detection cutoff values for WNV, SLEV, and DENV were 37.0, 38.5, and 40.0, respectively.
[c] *P*/*N* values of ≥3.0 for the IgG ELISA and IgM ELISA and a ≥30% inhibition value for the blocking ELISA were considered indicative of a positive test result. *P*/*N* values of ≥2 and <3 and 27 to 29% are considered indicative of equivocal samples.

TABLE 4. Real-time PCR, PRNT, and ELISA results for cohort 2 (SLEV-SLEV-WNV series)[a]

Inoculum and day	Detection in indicated sample by indicated assay																																							
	Real-time PCR[b]						$PRNT_{80}$														ELISA[c]														Blocking ELISA									
							WNV						SLEV						DENV		WNV IgM						SLEV IgM						WNV IgG						WNV						Flavivirus					
	5	6	7	8	17	18	5	6	7	8	17	18	5	6	7	8	17	18	17	18	5	6	7	8	17	18	5	6	7	8	17	18	5	6	7	8	17	18	5	6	7	8	17	18	5	6	7	8	17	18
SLEV																																																	
0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
9							−	−	−	−	−	−	−	−	−	−	10	80	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	−	−	−	−
12							−	−	−	−	−	−	−	−	−	−	40	80	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	−	−	−	−
15							−	−	−	−	−	−	−	−	−	−	40	40	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	−	−	−	−
18							−	−	−	−	−	−	−	−	−	−	20	40	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SLEV																																																	
21	−	−	−	−	−	−	−	−	−	−	−	−	−	20	−	−	20	40	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	+	−	−	−
24	−	−	−	−	−	−	−	−	−	−	−	−	−	20	−	−	20	20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	+	−	−	−
27	−	−	−	−	−	−	−	−	−	−	10	−	−	20	−	−	10	40	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	+	−	−	−
30							−	−	−	−	−	−	−	40	−	−	10	10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	+	+	−	−
33							−	−	−	−	−	−	−	−	20	−	20	10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	+	+	−	−
36							−	−	−	−	−	−	−	−	20	−	10	20	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	+	+	−	−
39							−	−	−	−	−	−	−	−	20	−	10	20	−	−	−	−	−	−	−	+/−	−	−	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	+	+	−	−
WNV																																																	
42	−	−	−	−	+	−	−	−	−	−	10	−	−	−	20	−	10	20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	+	+	−	−
45	−	−	−	−	+	+	−	−	−	−	−	10	−	−	20	−	40	10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	+	+	−	−
48	−	−	+	+	−	−	−	−	−	−	160	160	−	−	20	−	160	160	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NA	−	−	−	−	−	−	−	−	−	−	+	+	−	−
51			−	−			160	−	−	−	320	1,280	20	−	20	−	160	320	40	80	+	−	−	−	+	−	−	−	−	−	−	−	−	−	+	NA	−	−	−	+/−	+	+	+	−	+	−	+	+	−	+
54							160	−	20	80	320	1,280	80	−	40	−	160	640	80	80	+	−	+/−	+	+	−	+	−	+/−	−	−	−	+	−	+	−	+	NA	−	−	+	−	+	+	+	−	+	+	+	+	−	+
57							160	−	40	≥320	160	1,280	80	−	160	20	160	640	80	80	+	−	+/−	+	+	−	+	−	+	+	−	−	+	−	+	−	+	NA	+	−	+	+	+	+	+	−	+	+	+	+	−	+
60							320	−	40	≥320	160	1,280	80	−	80	10	160	640	80	160	+	−	−	+	+	−	+	−	+/−	+	−	−	+	−	+	−	+	NA	+	−	+	+	+	+	+	−	+	+	+	+	−	+
63							320	−	20	160	NA	NA	80	−	40	10	NA	NA	NA	NA	+	−	−	−	+	NA	NA	+	−	+/−	+	−	−	+	−	+	−	NA	NA	+	−	+	+	NA	NA	+	−	+	+	NA	NA

[a] NA, no sample; blank, not tested.
[b] The real-time PCR results are virus specific. The positive-detection cutoff values for WNV, SLEV, and DENV were 37.0, 38.5, and 40.0, respectively.
[c] *P*/*N* values of ≥3.0 for the IgG ELISA and IgM ELISA and a ≥30% inhibition value for the blocking ELISA were considered indicative of a positive test result. *P*/*N* values of ≥2 and <3 and 27 to 29% are considered indicative of equivocal samples.

DISCUSSION

In the tropical Americas, where multiple flaviviruses are endemic, it is critical to evaluate the efficacy of WNV diagnostic assays to differentially detect infecting flavivirus agents. While other studies have looked at cross-reactivity with a limited number of tests and single-time-point samples (17, 46), our sequential infection studies provided a complete set of serum samples collected every 3 days for over 2 months from animals that were known to be free of any previous flavivirus exposure. Furthermore, our study was geographically relevant for our objective and incorporated all the widely available and used equine diagnostic assays. In many countries in Central and South America, horses are both surveillance tools for and susceptible to WNV, making the information generated from this controlled study useful for surveillance programs and for understanding the course of illness and recovery in a biologically important system.

Using the most commonly performed diagnostic assays, we determined that all tests had both advantages and disadvantages; knowing the properties of each particular system provides each user the ability to decide which assay or combination of assays best suits their needs. While we did consider alternative assays (e.g., cross-reactive reduced antigen ELISA or microsphere immunoassay [MIA] bead formats [20, 37]), we found that the results were no better than the more broadly available assays or that reagents were not available for equine testing (data not shown).

Real-time RT-PCR has proven to be an effective method for detection of WNV, SLEV, and DENV nucleic acid from a variety of sample types (22, 23). In this report, positive results were obtained only from sera of WNV exposed horses. This corresponds with previous studies that have shown that a small percentage (<10%) of horses develop clinical disease and low but detectable viremia (5, 18, 40). Therefore, this test is an important tool for diagnosing early infection, especially with viruses that cause minimal viremia. For comparing acute-phase-sample assays, virus isolation is not likely to be the most fruitful approach, as it is more expensive than other techniques and requires both specialized facilities and training. However, if the objective is to obtain virus stocks for future studies, virus isolation is essential.

Serological assays are the most commonly used diagnostic assays due to their simplicity, comparably low cost, and requirements for few specialized apparatus or facilities. Our tests fell into two distinct methods categories, ELISA and PRNT, with each having both advantages and disadvantages. ELISA formats are inexpensive, are safe to perform without specialized containment, as no live virus is used, and can rapidly screen large numbers of samples but can have variable sensitivity and specificity. PRNT assays require containment facilities along with well-trained technical staff, are more time-consuming and expensive, and may provide the most conservative interpretation of etiology. PRNT assays may also yield different titer values, depending upon the cutoff value used. In this study, we report the titers using an 80% cutoff but also calculated the titers for both 50% and 90% (see the supplemental table) and, as expected, noted a change in the reported antibody titer. However, in virtually all samples,

TABLE 5. Real-time PCR, PRNT, and ELISA results for cohort 3 (DENV-WNV series)[a]

Inoculum and day	Detection in indicated sample by indicated assay																															
	Real-time PCR[b]				$PRNT_{80}$												ELISA[c]												Blocking ELISA					
					WNV				DENV				SLEV				WNV IgM				SLEV IgM				WNV IgG				WNV				Flavivirus			
	13	14	21	22	13	14	21	22	13	14	21	22	13	14	21	22	13	14	21	22	13	14	21	22	13	14	21	22	13	14	21	22	13	14	21	22
DENV																																				
0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
6	−	−	−	−	−	−	−	−	10	160	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
9	−	−	−	−	−	−	−	−	80	≥320	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
12	−	−	−	−	−	−	−	−	80	≥320	20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
15					−	−	−	−	40	≥320	80	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
18					−	−	−	−	20	≥320	80	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
WNV																																				
21	−	−	−	+	−	−	−	−	40	160	160	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
24	+	+	+	+	−	−	−	−	40	160	20	20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
27	−		−	−	40	20	−	−	40	≥320	20	10	−	40	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
30					≥320	1,280	40	40	40	160	20	20	20	640	−	40	+	+	−	−	+	+	−	−	+	+	−	−	−	+	−	+	−	−	−	+	+
33					≥2,560	1,280	160	≥320	320	160	80	40	40	640	160	40	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
36					2,560	1,280	320	160	160	80	40	40	40	640	40	40	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
39					1,280	640	80	160	160	80	20	40	80	320	80	20	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
42					1,280	640	NA	NA	160	80	NA	NA	40	320	NA	NA	+	+	NA	NA	+	+	NA	NA	+	+	NA	NA	+	+	NA	NA	+	+/−	NA	NA

[a] NA, no sample; blank, not tested.
[b] The real-time PCR results are virus specific. The positive-detection cutoff values for WNV, SLEV, and DENV were 37.0, 38.5, and 40.0, respectively.
[c] *P*/*N* values of ≥3.0 for the IgG ELISA and IgM ELISA and a ≥30% inhibition value for the blocking ELISA were considered indicative of a positive test result. *P*/*N* values of ≥2 and <3 and 27 to 29% are considered indicative of equivocal samples.

the interpretation of results and determination of infecting etiology were not affected.

The WNV IgM ELISA is the test of choice for diagnosis of recent infection in humans and was modified for horses in this study (30). Curiously, while this is typically considered to be an early appearing antibody, in this study, it was not detected any earlier than IgG antibody. Furthermore, although WNV IgM was detected in most instances where WNV Nt-antibody titers were present, there were cases of IgM presence in the absence of Nt-antibody titers. This could be a false-positive IgM detection, or more likely, the IgM generated early in infection was not neutralizing, as has been shown in humans (6). There were other instances where IgM was not observed until after Nt antibodies were detected, making the IgM ELISA less sensitive than some of the other assays.

As previously noted, the WNV and SLEV IgM assays had significant cross-reactivity in this study (31). For example, in cohort 3 horses, the results for the SLEV IgM assay were positive for most WNV IgM-positive samples, even though these animals were never exposed to SLEV. The IgM ELISA does have the advantage of being the only assay able to state that a WNV infection was recent. In one animal, WNV IgM persisted for as few as 7 days (equine no. 12), conclusively indicating a recent WNV infection. Furthermore, the WNV-specific IgM ELISA never produced a positive result prior to WNV exposure, indicating that this assay is indeed WNV specific.

The WNV-specific blocking ELISA was also extremely specific and never generated a false positive, even after repeated flavivirus exposure, making it an excellent option for diagnosing WNV infection in equines with a history of previous flavivirus infection. This assay has proven to be effective in detecting total IgM and IgG from birds and domestic animals (3, 4) but less effective for humans from regions where multiple flaviviruses cocirculate (28). In contrast to the virus-specific blocking ELISA, the flavivirus group blocking ELISA had some sensitivity limitations. In cohort 1, the assay was able to detect all of the initial samples that were SLEV Nt antibody positive but it also produced some false positives. In cohort 3, none of the samples receiving only DENV-2 were positive with the flavivirus-specific blocking ELISA, and in cohort 2, many of the samples with SLEV Nt-antibody titers were negative in this test. This result is particularly interesting considering that the monoclonal antibody used was developed using an SLEV antigen.

Because the IgG assay is designed to work more broadly on flaviviruses and has previously been shown to detect DENV-2, SLEV, WNV, Japanese encephalitis virus, Murray Valley encephalitis virus, Powassan virus, and yellow fever virus antibodies in humans (2, 10, 21), it was unexpected that this assay generated results similar to those of the WNV-specific blocking ELISA. Possible explanations for this deviation from human studies include the options that IgG responses are different in humans and horses and that the equine-adapted WNV assay is more specific for WNV than the human assay.

Traditionally, the PRNT is the gold standard for serological diagnosis and confirmation. In our study, the PRNT was a conservative test, often resulting in a diagnosis of "recent flavivirus infection." Additionally, cross-reactivity between different serogroups was observed. This was most clearly seen in cohorts 1 and 2 when DENV Nt-antibody titers were detected after sequential infections. Interestingly, it does not appear that SLEV Nt antibodies cross-react with WNV antigen in instances of single infection. Given the low SLEV antibody titers, it is likely that the SLEV antibody response is simply too low to elicit WNV cross-reactivity. Due to concerns that the SLEV viral dose administered was insufficient to elicit an immune response, a higher dose was also administered. The results were similar with the two dosages, suggesting that SLEV is a poor immunogen.

Previous field reports found seroconversion to SLEV in domestic and sentinel horses in Central and South America, reinforcing the idea that undocumented SLEV infection could affect diagnosis (1, 14, 29, 32, 33, 36). We found only low levels of antibody against SLEV even after 2 sequential injections, but these antibodies persisted to day 39 and through the subsequent heterologous exposure to WNV. Based on the high WNV Nt-antibody titers observed at the later time points for cohort 2, it can be theorized that the WNV antibodies present are cross-reacting with SLEV antigens. This phenomenon was seen even more clearly with horses initially receiving a DENV-2 injection, where the development of antibody was even more robust. The degree of cross-reactivity between WNV and DENV-2 was somewhat unexpected since these viruses are in distinct serogroups. This finding suggests the possibility that humans with febrile illness in areas where dengue is endemic may indeed have WNV infections even when serological assays suggest a dengue virus etiology, particularly when the patient has had previous dengue virus infection. However, while our results clearly demonstrate this possibility for equines, it is important to point out that we cannot be certain how our results for equines will correlate with the data from human infections. Furthermore, it is significant to note the timing of sample collection as it relates to testing outcome. As our data show, antibody levels can rise and fall rapidly, particularly when only low levels exist. Thus, a single sample may give misleading or inaccurate results of the true etiology, underscoring the importance of testing both acute- and convalescent-phase samples. Furthermore, while numerous protocols with minor technical variations exist (30, 44), testing all the protocol variants published was not a feasible option; rather, our objective was to evaluate each technique overall.

A final question is whether previous exposure to a flavivirus can modulate disease following a subsequent WNV infection. At least partial protection was seen in hamsters immunized with SLEV and subsequently challenged with WNV (16, 42). Because of the close antigenic relationship between WNV and SLEV, this result may not be unexpected. More intriguing are the reports that hamsters immunized with DENV were protected against lethal WNV infection (35, 39). While none of the horses in our study developed clinical illness, the antibody responses developed in horses could prevent subsequent disease which would support earlier studies with rodents. This finding may provide one plausible reason for the absence of WNV epidemics in areas where dengue viruses and SLEV are endemic. Further studies will be necessary to examine this phenomenon more fully.

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