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 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 tropical America.
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 commonly used diagnostic assays to determine infection etiology. 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 (Ct) values of 38.5 and 40.0, which are equivalent to 0.1 and 1.0 PFU/ml,

| Oligonucleotide* | Sequence (5′–3′) | Product size (bp) |
|------------------|------------------|-------------------|
| SLEV 1992 (+)    | ACCACCTTTTCGCGATATTAC | 90               |
| SLEV 2018 (+)    | FAM-TGTCGGAAGAGGCCACCCAGATTA-BHQ1 | |
| SLEV 2028 (-)    | CTTCCTCAATGTCTACTCTCTCCTT | |
| DENV 1085 (+)    | CCAAACACCCGCACACTCTCTAAG | 159              |
| DENV 1145 (+)    | FAM-AACAGACTCGCGCTGCGCCAACACA-BHQ1 | |
| DENV 1244 (-)    | TTTCCCGATCCTGCTACCATA | |

* 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 CP 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 CP of the last dilution set that yields 10 out of 10 detection events (CP 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 $\geq 0.950$ 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 (20, 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-

**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. 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 ELISA 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-
In general, results for all ELISAs were negative until the 9th day post-WNV infection in both SLEV/WNV cohorts. Some examples include horses 18 and 19, which were positive for SLEV IGM and WNV-specific IgM responses. Additionally, horses 10 and 11 did not develop antibodies against SLEV but did have SLEV IGM. The remaining horses developed IgM responses, but this appeared to be cross-reactive, as the SLEV IgM data mirrored the WNV data in most cases. In general, results for all ELISAs were negative until the 9th day post-WNV infection in both SLEV/WNV cohorts. Some examples include horses 18 and 19, which were positive for SLEV IGM and WNV-specific IgM responses. Additionally, horses 10 and 11 did not develop antibodies against SLEV but did have SLEV IGM. The remaining horses developed IgM responses, but this appeared to be cross-reactive, as the SLEV IgM data mirrored the WNV data in most cases.

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

| Inoculum and day | Real-time PCR⁵ | PRNT<sub>50</sub> | ELISA* | 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 | 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 | NA | NA | NA |
|                  | 3 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 4 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 9 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 12 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 15 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 18 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
| WNV              | 21 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 24 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 27 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 30 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 33 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 36 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 39 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 42 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 45 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 48 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 51 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 54 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 57 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 60 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |
|                  | 63 - - - - - - - - - - - - - - - - - - - - - - - | - - - - - - - - - - - - - - - | - - - | - | NA | NA | NA | NA |

* NA, no sample; blank, not tested.

⁵ 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.

* 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. In this study, we concurrently performed 11 diagnostic assays on serum samples from horses infected with WNV, DENV, and SLEV, allowing for a comprehensive assessment of the effectiveness of these assays. While other studies have focused on detecting flaviviruses with single assays, we employed a multi-assay approach to provide a more complete evaluation of diagnostic performance.

Our results demonstrated that the most commonly used diagnostic assays, such as ELISA and PRNT, were effective in detecting WNV, DENV, and SLEV. However, we also observed limitations in these assays, particularly with respect to cross-reactivity and sensitivity, especially in the early stages of infection. For example, the PRNT assay, while highly specific, may not be the most sensitive method for the early detection of flavivirus infection, as it requires the cultivation of virus in cell cultures.

On the other hand, the more rapid and sensitive RT-PCR assay was able to detect flaviviruses in serum samples collected 3 days after the onset of clinical signs, which is consistent with findings from previous studies. Nevertheless, the sensitivity of RT-PCR can vary depending on the target region, and selection of the appropriate primer sets is crucial to ensure accurate detection.

Overall, our study highlights the importance of using a combination of diagnostic assays to optimize the detection and identification of flaviviruses in equine populations. This approach not only enhances the accuracy of diagnosis but also facilitates the timely implementation of control measures to prevent the transmission of these arboviruses.

In conclusion, the concurrent evaluation of multiple diagnostic assays in a controlled manner is essential for understanding the efficacy and limitations of these tools. Our findings underscore the need for ongoing surveillance and research to develop more effective and efficient diagnostic strategies for flavivirus infections.
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 WNV 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 infections, 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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