Complement-Dependent Cytotoxicity Assay for Differentiating West Nile Virus from Japanese Encephalitis Virus Infections in Horses

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A complement-dependent cytotoxicity (CDC) assay was established to measure antibodies to the West Nile virus (WNV) nonstructural protein 1 (NS1) in horses. Sera collected from a WNV-infected horse mediated lysis of WNV NS1-expressing cells in a dose-dependent manner at higher percentages than sera from a Japanese encephalitis virus (JEV)-infected horse. The percentages of specific lysis for sera diluted 1:10 to 1:80 were <19.8% (assay cutoff) for almost all of the 100 JEV-infected or uninfected horses tested, in contrast to 55 to 76% in WNV-infected horses. Experimental infection revealed that horses became anti-WNV NS1 antibody positive 10 days after WNV infection. This study demonstrated the utility of this assay for differentiating WNV from JEV infections in horses.

West Nile virus (WNV) and Japanese encephalitis virus (JEV) are included in the Japanese encephalitis (JE) serological group of the Flavivirus genus of the Flaviviridae family (4, 13). JEV is distributed mostly in Asia, whereas WNV is spread worldwide, apart from most areas of Asia (3, 14, 17). There is a possibility that WNV may be introduced into Asia including Japan, similar to its introduction and rapid expansion in the Western Hemisphere. Since the clinical features of WNV infection in humans and horses (6, 16) are similar to those caused by JEV (2, 16), the differential diagnosis of WNV from JEV infections is reliant on laboratory tests. The neutralization test provides the highest specificity among currently available serodiagnostic tests (11). However, even with the neutralization test, cross-reactivity among members of the JE serological group can affect a differential diagnosis, making it difficult to differentiate between WNV and JEV infections (11). Experiments using mice (12), pigs (18), and horses (15) have indicated that upon infection with WNV, animals preimmune to JEV by vaccination or infection induce strong anamnestic responses to JEV. Specifically, neutralizing antibody levels against JEV are commonly equivalent to or even higher than those against WNV.

Antibody-mediated complement-dependent cytotoxicity (CDC) is a mechanism whereby complement activation triggered by specific antibody binding to an antigen on a cell surface causes the formation of the C5b-9 membrane attack complex, which can lyse the target cell (1). We have previously shown the usefulness of this mechanism for measuring antibodies to the nonstructural protein 1 (NS1) of JEV in equine sera (7). In the present study, we examined if the CDC assay could be applied for the detection of antibodies to NS1 of WNV and if this was able to differentiate WNV from JEV infections in horses.

Sera obtained from horses experimentally infected with WNV have been described previously (5). Briefly, two 1-year-old thoroughbred horses (yearlings; horse numbers 1 and 2) were infected subcutaneously with $1 \times 10^7$ PFU of the NY99 strain of WNV. Serum collected from horse 1 at 28 days postinfection was used as a positive control in the present CDC assay and the conventional enzyme-linked immunosorbent assay (ELISA). Sera from 100 individual thoroughbred horses born and kept in Japan were used as negative controls for antibodies to WNV NS1, as in our previous study (5). Of the 100 sera, 40 were negative and 60 positive for anti-JEV NS1 antibodies as determined by ELISA (8). The 40 sera that were negative for JEV NS1 antibodies were collected from 20 yearlings vaccinated with inactivated JE vaccine and 20 without vaccination. All 40 of these horses were born and kept in an area of northern Japan where JEV is not endemic. The 60 sera positive for JEV NS1 antibodies were collected from horses aged 3 to 12 years, as used in our earlier survey (9). All animal experiments were conducted according to the Guidelines for Animal Experimentation at the Equine Research Institute.

The CDC assay previously established for measuring JEV NS1 antibodies (7) was modified to detect WNV NS1 antibodies. The antigen used for the present assay was a stably transfected 2G2 cell line that constitutively expresses the NS1 protein of the WNV Eg101 strain (5). Fifty microliters of serum-free minimal essential medium (SF-MEM) containing $5 \times 10^4$ 2G2 recombinant cells was mixed with an equal volume of heat-inactivated test serum diluted in SF-MEM and incubated on ice for 30 min. This mixture was then mixed with 11 µl of commercial rabbit complement (Low-Tox-M rabbit complement; Cedarlane, Hornby, Canada) for a final concentration of 10% and incubated at 37°C for 2 h. Following centrifugation, 50 µl of the supernatant was mixed with 50 µl of a lactose dehydrogenase (LDH) substrate (Cytotoxicity Detection Kit Plus; Roche, Mannheim, Germany) and incubated at room temperature for 15 min. The resulting color reaction was

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read by spectrophotometry at 490 nm. All procedures were performed in duplicate in 96-well microplates. The percentage of specific cell lysis was calculated according to the manufacturer’s instructions using the following formula: $100 \times \frac{(A - C)}{(B - C)}$, where $A$ represents the absorbance value obtained with test serum (experimental release), $B$ represents the absorbance value obtained by lysing all of the target cells with 1% Triton X-100 (maximum release), and $C$ represents the absorbance obtained with target cells incubated in SF-MEM containing rabbit complement at 10% (minimum release). When this calculation provided a negative value, 0.0% was assigned as the result. Test sera with a specific lysis percentage greater than the cutoff value (19.8% of specific lysis) were determined to be positive for WNV NS1 antibodies. For the one-dilution method, the percentages of specific cell lysis obtained in 1:10 to 1:80 dilution series of sera were used as the WNV NS1 antibody level. In the end point method, the WNV NS1 antibody titer was expressed as the highest serum dilution giving greater than 19.8% of specific lysis.

The dose-response curve obtained in the present CDC assay using serum from WNV-infected horses and WNV NS1-expressing cells (Fig. 1, left panel, closed circles) was consistent with previous reports using serum from JEV-infected horses and JEV NS1-expressing cells (7). This provided validation of the CDC assay for measuring WNV NS1 antibodies in horse sera. Percentages of specific lysis were higher than those obtained with serum from JEV-infected horses at most serum dilutions, although two sera showed equivalent WNV NS1 antibody levels in the ELISA (Fig. 1, right panel).

The usefulness of the present CDC assay for differentiating WNV from JEV infections was determined using a total of 100 samples collected from individual thoroughbred horses regarded as free of antibodies to WNV (Fig. 2A). Serum dilutions of 1:10 to 1:80 showed that most of these sera had less than 20% specific lysis, which was lower than the 54.5 to 75.5% levels obtained with the positive control serum (horse 1 at 28 days after infection) in five separate experiments. We determined that 19.8% was an appropriate cutoff value to differentiate positive from negative samples for antibodies to WNV NS1. This value was calculated by the mean (4.08%) plus 2 times the standard deviation (7.85%) of the percentage of specific lysis obtained at the 1:10 dilution. Under this cutoff, all of the 40 horses without antibodies to JEV NS1 were negative for WNV NS1 antibodies, even in a 1:10 dilution of sera, irrespective of whether these horses were vaccinated. Of the 60 horses with antibodies to JEV NS1, only 5 were positive at a 1:10 dilution and 1 at 1:20; there were no positive sera at 1:40 and 1:80 dilutions. The end point method, which overcomes the limitations of the one-dilution method, which cannot correctly measure high antibody levels (Fig. 2B), indicated that there were antibody titers of <1:10 in 95 of 100 negative samples, in contrast to 1:160 in 95 of 100 positive samples.
The time courses obtained by the end point method at serum dilutions of 1:40, 1:80, both horses became positive within 18 days after WNV infection. The time courses obtained by the one-dilution method of the CDC assay, percentages of specific lysis obtained in a 1:10 dilution of serum started increasing on day 10 and then plateaued at approximately 70 to 80% on day 12 for both horses. Even at serum dilutions of 1:20, zero at 1:40 and 1:80 dilutions. Thus, the 1:40 or 1:80 dilution used in the one-dilution method with a cutoff for specific lysis of 19.8%, or in the end point method as a borderline dilution, is considered to provide stringent criteria for the CDC assay, with false-positive results unlikely to occur.

In conclusion, the present study showed that the CDC assay can measure WNV NS1 antibodies in horses. Thus, this assay would be a useful tool for differentiating WNV from JEV infections in serological diagnoses.

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FIG. 3. Time courses of WNV NS1 antibodies in two horses experimentally infected with WNV. Sera were tested by the one-dilution method at serum dilutions of 1:10, 1:20, 1:40, and 1:80 and the end point method of the CDC assay. For comparison, data of the blocking ELISA, conventional ELISA to measure WNV NS1 antibodies, and neutralizing test were included from our earlier study (5). Each datum of the CDC assay represents an average obtained in two separate experiments (standard deviations are indicated by bars). Dotted lines indicate the cutoff values for the one-dilution (19.8%) or the end point (1:10) method of the CDC assay. Dashed lines indicate the cutoff value in the blocking ELISA (27.6%). The week zero time point on the abscissa indicates the sample taken immediately before the WNV infection.

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