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Epitope-Blocking Enzyme-Linked Immunosorbent Assays for Detection of West Nile Virus Antibodies in Domestic Mammals

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We evaluated the ability of epitope-blocking enzyme-linked immunosorbent assays (ELISAs) to detect West Nile virus (WNV) antibodies in domestic mammals. Sera were collected from experimentally infected horses, cats, and pigs at regular intervals and screened in ELISAs and plaque reduction neutralization tests. The diagnostic efficacies of these techniques were similar.

West Nile virus (WNV) is a single-stranded, positive-sense RNA virus in the genus *Flavivirus*, family *Flaviviridae* (3). It is a member of the *Japanese encephalitis virus* (JEV) complex, which also includes JEV, *St. Louis encephalitis virus* (SLEV), *Murray Valley encephalitis virus*, and *Koutango virus* (KOUV) (12). WNV is transmitted in natural cycles between mosquitoes and birds, and humans and horses are incidental hosts (11). WNV has a wide geographic distribution, recently including North America. The initial outbreak of WNV in North America took place in New York in 1999, with mortality observed in humans, horses, and numerous species of wild birds (6, 8, 15). WNV activity has now been detected throughout most of the United States, and clinical infections in thousands of humans and horses have been reported. WNV is known to infect many other mammals, including cats, dogs, donkeys, goats, sheep, pigs, cows, rabbits, squirrels, and bats (7, 11, 13).

Serologic diagnosis of WNV infections in vertebrates can be achieved by the plaque reduction neutralization test (PRNT) and the hemagglutination inhibition assay (1). However, these assays are laborious and therefore not ideal for large-scale routine testing of sera. In contrast, enzyme-linked immunosorbent assays (ELISAs) provide rapid diagnostic and surveillance techniques to monitor WNV activity. We have previously reported the application of epitope-blocking ELISAs for the detection of WNV antibodies in multiple avian species (2, 9). Here, the ability of the blocking ELISAs to detect WNV antibodies in selected species of domestic mammals was evaluated.

Protocols used to prepare ELISA coating antigen and perform blocking ELISAs have been described previously (2). Five monoclonal antibodies (MAbs) were tested in blocking assays, and the production and characterization of these MAbs have been described elsewhere (10, 16, 17). Briefly, MAb 2B2 is WNV and KOUV specific, MAb 3.1112G is WNV specific, MAb 6B5A-2 is SLEV specific, and MAbs 3H6 and 6B6C-1 are flavivirus group reactive. All MAbs detect E protein epitopes, with the exception of MAb 3.1112G, which detects an NS1 epitope. MAb 6B6C-1 was labeled with horseradish peroxidase; all other MAbs were unlabeled. To calculate the percent inhibition of MAb binding in blocking assays, the following formula was used: $100 - [(TS - B)/(CS - B)] \times 100$. TS denotes the optical density (OD) of the test sample, CS denote the OD of the control serum, and B denotes the background OD. Previously, an inhibition value ≥30% was shown to indicate the presence of viral antibodies in avian sera (2), and the same diagnostic criterion was used here.

Nine horses (H1 to H9), four cats (C1 to C4), and four pigs (P1 to P4) were experimentally inoculated with WNV (NY-99) via infected *Aedes albopictus* mosquitoes. Prior to inoculation, all animals were screened by PRNT for neutralizing antibodies against WNV and SLEV and were shown to be negative. Animals were then relocated to a BL-3 containment facility, where virus inoculations were performed. Horses were sampled at 7, 14, 21, and 28 days postinfection (p.i.), unless they were euthanized earlier. Cats were sampled at 7, 14, and 28 days p.i., and pigs were sampled at 7, 14, and 21 days p.i. Animals were also bled immediately before inoculation (0 days p.i.). These animals are being used in WNV experimental infection trials. Details of that study, including the viremia profile and course of clinical disease (if any) of each animal, will be presented elsewhere (D. R. Bowen, unpublished data). Serum was obtained from an additional horse (H10) that was immunized with a recombinant DNA vaccine expressing the WNV prM and E genes prior to WNV challenge (5). Fifteen weeks postimmunization, the horse was challenged with WNV

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TABLE 1. Percent inhibition of MAbs by blocking ELISA using sera from WNV-infected horses[c]

Serum source[a]	Day post-challenge	WNV $PRNT_{90}$ titer	% Inhibition of binding of MAb[b]:			
			2B2	3.1112G	3H6	6B6C-1
H1	0	<10	0	4.5	3.8	4.1
H2	0	<10	3.4	2.7	3.4	5.0
H3	0	<10	0	1.3	1.7	3.8
H4	0	<10	0	2.5	4.7	5.6
H5	0	<10	5.2	0.6	2.6	2.6
H6	0	<10	0	2.9	4.0	4.2
H7	0	<10	11.7	11.0	1.2	0
H8	0	<10	1.4	0	3.0	0
H9	0	<10	3.3	5.2	0.3	0
Mean			2.8	3.4	2.7	2.8
H1	7	<10	0	0	0.8	0.2
H2	7	<10	4.9	0	0	0
H3	7	<10	4.2	1.6	0	16.2
H4	7	<10	6.4	5.6	10.3	13.9
H5	7	<10	10.9	0	0	18.6
H6	7	<10	10.0	7.4	1.3	4.1
H7	7	<10	0	0.8	0	0
H8	7	<10	13.0	6.6	0	0
H9	7	<10	0	3.0	3.3	0
Mean			5.5	2.8	1.7	5.9
H1	14	**160**	**45.3**	**34.3**	**34.8**	**34.6**
H2	14	**80**	**31.0**	**34.1**	20.9	**39.9**
H3	14	**160**	24.1	**55.6**	**34.5**	**56.9**
H4	14	**80**	18.0	**64.3**	**35.0**	**57.5**
H5	14	**160**	**57.0**	**57.6**	**39.6**	**65.8**
H6	14	**160**	**36.6**	**58.7**	**35.7**	**58.1**
H7	14	**80**	8.7	**46.0**	27.1	**56.0**
H8	14	**≥160**	**30.6**	**69.0**	29.6	**49.8**
H9	14	**≥160**	**45.0**	**80.5**	**47.8**	**59.1**
Mean			**32.9**	**55.6**	**33.9**	**53.1**
H1	21	**160**	**58.0**	**69.7**	**51.5**	**57.9**
H2	21	**40**	**36.0**	**60.7**	**38.7**	**57.5**
H3	21	**40**	**34.4**	**64.7**	**50.2**	**61.9**
H4	21	**40**	**41.1**	**75.2**	**52.7**	**72.3**
H5	21	**40**	**76.7**	**69.3**	**43.9**	**68.0**
Mean			**49.2**	**67.9**	**47.4**	**63.5**
H1	28	**≥160**	**48.5**	**74.0**	**54.5**	**65.6**
H2	28	**≥160**	**60.5**	**72.8**	**53.4**	**73.6**
H4	28	**80**	**40.0**	**79.5**	**54.2**	**70.8**
Mean			**49.7**	**75.4**	**54.0**	**70.0**
H10[d]	31	**320**	**77.2**	28.8	**44.3**	**66.2**

[a] All sera were diluted 10-fold and tested in duplicate. Control serum consisted of commercially available normal horse serum (Vector Laboratories, Burlingame, Calif.) or a pooled suspension of sera collected from H1 to H9 at day 0.
[b] MAb 2B2 is WNV and KOUV specific; MAb 3.1112G is WNV specific; MAbs 3H6 and 6B6C-1 are flavivirus group reactive.
[c] Boldface inhibition values (≥30%) and boldface PRNT titers (≥20) were considered significant.
[d] Horse was vaccinated prior to virus challenge.

via infected *A. albopictus* mosquitoes, and serum was collected 31 days later.

First, the ability of the blocking ELISA to detect antibodies in the nonvaccinated WNV-infected horses was evaluated. Most-promising results were obtained in assays utilizing MAbs 3.1112G and 6B6C-1, with antibodies detected in all sera collected at ≥14 days p.i. (Table 1). These findings correlated well with the PRNT data. That is, all sera exhibiting neutralizing titers at a 90% plaque reduction level ($PRNT_{90}$) were positive in ELISAs. For MAb 3.1112G, the mean inhibition values for sera collected at 14, 21, and 28 days p.i. were 55.6, 67.9, and 75.4%, respectively. For MAb 6B6C-1, the mean inhibition values for sera collected at 14, 21, and 28 days p.i. were 53.1, 63.5, and 70.0%, respectively. ELISAs that utilized MAbs 2B2 and 3H6 were also effective, with antibodies detected in most horses at 14 days p.i. and in all horses at ≥21 days p.i. Serum from the vaccinated horse was positive in all assays using E-specific MAbs, revealing that the horse had generated an immune response prior to WNV challenge (Table 1). In contrast, the serum was negative in the ELISA that utilized MAb 3.1112G. However, the inhibition value was just below the diagnostic criterion, suggesting that a limited amount of MAb-binding inhibition occurred. It is possible that vaccination did not provide sterile immunity and that a low level of WNV replication occurred after challenge.

All $PRNT_{90}$-positive cat sera were positive in blocking assays that utilized MAbs 3H6 and 6B6C-1 (Table 2). ELISAs using these MAbs also detected antibodies in two $PRNT_{90}$-negative sera. However, both sera exhibited low PRNT titers at a plaque reduction level of 80% (data not shown). Assays using MAbs 3.1112G and 2B2 were also effective, with antibodies detected in three and four cats, respectively, at 21 days p.i. Analysis of the pig sera revealed that all specimens collected at 28 days p.i. were positive in ELISAs utilizing MAbs 3.1112G and 6B6C-1, while three were positive in assays using MAbs 2B2 and 3H6 (Table 2). In contrast, only two sera collected on day 28 were $PRNT_{90}$ positive, although one of the $PRNT_{90}$-negative samples exhibited a low $PRNT_{80}$ titer (data not shown). All $PRNT_{90}$-positive pig sera collected at 14 days p.i. were positive in assays using MAb 6B6C-1, while the two $PRNT_{90}$-positive sera collected at 7 days p.i. were negative in all assays.

To further validate the diagnostic efficacy of the blocking ELISAs, sera from five SLEV-infected cows (CO1 to CO5) were tested. Specimens were collected from domestic cows during a serological survey in Chiapas, Mexico, in 2001. All sera contained detectable levels of neutralizing antibodies against SLEV (Table 2). Antibodies were detected in four cows in assays using the flavivirus group-reactive MAbs 3H6 and 6B6C-1 and in three cows with the SLEV type-specific MAb 6B5A-2. All sera were negative when the WNV MAbs, 2B2 and 3.1112G, were used. One serum sample failed to block MAb binding in all assays.

The ability of the blocking-ELISA technique to detect antibodies against various other flaviviruses of the Americas was determined. Hyperimmune mouse ascitic fluids (HIMAFs) containing antibodies against WNV, SLEV, Ilheus virus (ILHV), Bussuquara virus (BSQV), or Powassan virus (POWV) were tested in ELISAs using MAb 6B6C-1. All HIMAFs significantly inhibited MAb binding. The inhibition values were 58.9% for the WNV HIMAF, 70.9% for the SLEV HIMAF, 68.1% for the ILHV HIMAF, 38.7% for the BSQV HIMAF, and 60.2% for the POWV HIMAF. The ability to detect POWV antibodies is of particular interest, as phylogenetic studies have shown that the tick-borne and mosquito-borne flaviviruses are evolutionarily diverse and separated into two distinct clades (3). Therefore, this assay can potentially be exploited to identify antibodies against many different flavivi-

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TABLE 2. Percent inhibition of MAbs by blocking ELISA using sera from WNV-infected cats and pigs and SLEV-infected cows[d]

Serum source[a]	Day post-challenge	$PRNT_{90}$ titer for:		% Inhibition of binding of MAb[b]:				
		WNV	SLEV	2B2	3.1112G	3H6	6B6C-1	6B5A-2
Cat								
C1	7	<10	—[c]	3.8	0	1.6	1.8	—
C2	7	<10	—	1.8	8.4	0	11.0	—
C3	7	<10	—	3.8	6.4	0	0	—
C4	7	<10	—	0	0	0	0	—
C1	14	**10**	—	23.5	0	**50.8**	**55.7**	—
C2	14	**40**	—	**36.8**	7.5	**48.1**	**49.0**	—
C3	14	**10**	—	10.3	28.4	**47.1**	**47.2**	—
C4	14	<10	—	12.9	26.5	**47.1**	**39.6**	—
C1	21	**40**	—	**58.7**	17.9	**57.1**	**66.2**	—
C2	21	**160**	—	**61.8**	**41.1**	**61.7**	**60.9**	—
C3	21	<10	—	**31.0**	**41.7**	**55.2**	**63.6**	—
C4	21	**40**	—	**49.0**	**43.4**	**55.2**	**56.5**	—
Pigs								
P1	0	<10	—	4.4	0	0	0	—
P2	0	<10	—	0	0	0	2.8	—
P3	0	<10	—	0	0	0	1.8	—
P4	0	<10	—	0	0	0	2.3	—
P1	7	**20**	—	0	3.1	1.9	8.0	—
P2	7	<10	—	8.5	11.0	4.0	2.3	—
P3	7	**20**	—	2.6	8.5	0	6.2	—
P4	7	<10	—	3.5	7.7	14.2	0	—
P1	14	**40**	—	13.7	12.5	24.8	**36.5**	—
P2	14	**10**	—	29.6	**32.3**	22.0	**38.2**	—
P3	14	**20**	—	17.9	**34.4**	13.4	**37.4**	—
P4	14	<10	—	0	19.8	7.6	15.5	—
P1	28	**40**	—	**38.6**	**42.6**	**46.5**	**66.2**	—
P2	28	**10**	—	**48.7**	**50.3**	**43.2**	**66.1**	—
P3	28	<10	—	**46.9**	**53.4**	**43.9**	**71.8**	—
P4	28	<10	—	6.8	**36.8**	22.0	**33.6**	—
Cows								
CO1	NA[e]	<10	**≥320**	0	0	**64.6**	**71.9**	**48.5**
CO2	NA	<10	**20**	0	0	0	0	0
CO3	NA	<10	**40**	9.7	0	**71.7**	**76.6**	**54.4**
CO4	NA	<10	**160**	0	0	**85.3**	**86.0**	**52.8**
CO5	NA	<10	**40**	2.8	0	**65.1**	**73.1**	0

[a] All sera were diluted 10-fold and tested in duplicate. Control serum was obtained from an uninfected animal(s) of the same species. Control cat sera consisted of a pooled suspension of sera collected from C1 to C4 on day 0. Control pig sera consisted of commercially available normal pig serum (Vector Laboratories) or a pooled suspension of sera collected from P1 to P4 on day 0. Control cow serum was obtained from a cow that was PRNT negative for SLEV and WNV.

[b] MAb 2B2 is WNV and KOUV specific; MAb 3.1112G is WNV specific; MAbs 3H6 and 6B6C-1 are flavivirus group reactive; MAb 6B5A-2 is SLEV specific, and assays utilizing this MAb were performed with coating antigen prepared from SLEV-infected cells.

[c] —, not tested.

[d] Boldface inhibition values (≥30%) and boldface PRNT titers (≥20) were considered significant.

[e] NA, not applicable.

ruses. Serum from an uninfected mouse was used as control serum when calculating the percentages of inhibition.

To ascertain the sensitivity of the ELISAs, 15 randomly selected samples were tested at multiple dilutions against MAbs 3.1112G and 6B6C-1. Sera were serially twofold diluted by using a starting dilution of 1:10, and HIMAFs were serially twofold diluted by using a starting dilution of 1:5,000. Both MAbs worked particularly well when used to screen sera from nonvaccinated horses. The six specimens tested exhibited ELISA end point titers between 80 and 320 (Table 3). In assays that utilized MAb 6B6C-1, all cat, pig, and cow sera tested displayed ELISA end point titers ≥160. The WNV and SLEV HIMAFs significantly inhibited the binding of MAb 6B6C-1 at dilutions ≥40,000.

TABLE 3. Determination of antibody titers in sera and ascitic fluids by blocking ELISA

Sample source	Day post-challenge	Blocking-ELISA titer for MAb:		PRNT titer for:	
		3.1112G	6B6C-1	WNV	SLEV
H1	21	80	160	160	—[a]
H2	21	160	80	40	—
H4	21	160	160	40	—
H1	28	160	160	≥160	—
H2	28	160	320	≥160	—
H4	28	160	80	80	—
H10	31	<10	10,240	320	—
C2	21	80	≥640	160	—
C3	21	20	≥640	<10	—
P1	28	40	160	40	—
P3	28	80	320	<10	—
CO4	NA[b]	<10	160	<10	160
CO5	NA	<10	320	<10	40
WNV HIMAF	NA	—	80,000	—	—
SLEV HIMAF	NA	—	40,000	—	—

[a] —, not tested.

[b] NA, not applicable.

Therefore, the blocking ELISA reliably detected flavivirus antibodies in several evolutionarily diverse species of mammals. Indeed, similar assays have been exploited to detect serum antibodies against WNV (subtype Kunjin virus) in laboratory-infected rabbits (9). The present study demonstrated the concordance of ELISA and PRNT. Occasional disparities were observed, but this was not unexpected because the two assays do not necessarily detect the same antibody types. Furthermore, neutralizing antibodies have a greater longevity than nonneutralizing antibodies (4), and this may explain why one cow tested negative by ELISA. Comparative analyses of immunoglobulin M antibody capture ELISAs and PRNTs revealed occasional discrepancies in arbovirus diagnosis (14). However, blocking ELISAs provide a more rapid and less expensive means to detect WNV infections than PRNTs, so these assays would greatly facilitate WNV surveillance studies in the United States.

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