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Epitope-Blocking Enzyme-Linked Immunosorbent Assays for the Detection of Serum Antibodies to West Nile Virus in Multiple Avian Species

Bradley J. Blitvich,[1] Nicole L. Marlenee,[1] Roy A. Hall,[2] Charles H. Calisher,[1] Richard A. Bowen,[3] John T. Roehrig,[4] Nicholas Komar,[4] Stanley A. Langevin,[4] and Barry J. Beaty[1]*

Arthropod-Borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology and Pathology,[1] and Animal Reproduction and Biotechnology Laboratory, Equine Center,[3] Colorado State University, Fort Collins, Colorado 80523; Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado 80522[4]; and Department of Microbiology and Parasitology, University of Queensland, St. Lucia, Queensland, Australia 4072[2]

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We report the development of epitope-blocking enzyme-linked immunosorbent assays (ELISAs) for the rapid detection of serum antibodies to West Nile virus (WNV) in taxonomically diverse North American avian species. A panel of flavivirus-specific monoclonal antibodies (MAbs) was tested in blocking assays with serum samples from WNV-infected chickens and crows. Selected MAbs were further tested against serum samples from birds that represented 16 species and 10 families. Serum samples were collected from birds infected with WNV or Saint Louis encephalitis virus (SLEV) and from noninfected control birds. Serum samples from SLEV-infected birds were included in these experiments because WNV and SLEV are closely related antigenically, are maintained in similar transmission cycles, and have overlapping geographic distributions. The ELISA that utilized MAb 3.1112G potentially discriminated between WNV and SLEV infections, as all serum samples from WNV-infected birds and none from SLEV-infected birds were positive in this assay. Assays with MAbs 2B2 and 6B6C-1 readily detected serum antibodies in all birds infected with WNV and SLEV, respectively, and in most birds infected with the other virus. Two other MAbs partially discriminated between infections with these two viruses. Serum samples from most WNV-infected birds but no SLEV-infected birds were positive with MAb 3.67G, while almost all serum samples from SLEV-infected birds but few from WNV-infected birds were positive with MAb 6B5A-5. The blocking assays reported here provide a rapid, reliable, and inexpensive diagnostic and surveillance technique to monitor WNV activity in multiple avian species.

West Nile virus (WNV) is a single-stranded, positive-sense RNA virus that has been placed in the genus *Flavivirus*, family *Flaviviridae* (2). It is a member of the Japanese encephalitis virus complex, which also includes Saint Louis encephalitis virus (SLEV), Murray Valley encephalitis virus, and Koutango virus (14). These viruses are maintained in cycles between mosquitoes and birds. The principal vectors for WNV are *Culex* species mosquitoes (13). Many different wild bird species act as reservoir hosts for WNV, whereas humans, horses, and other mammals are usually incidental hosts. Clinical manifestations associated with WNV infections in humans include fever, headache, rash, fatigue, myalgia, and arthralgia and, occasionally, acute hepatitis, encephalitis, and meningitis (13).

WNV was first isolated in 1937 from the blood of a febrile adult woman in the West Nile district of Uganda (25). This virus has since been reported in Africa, the Middle East, Asia, southern Europe, Australia (subtype Kunjin virus), and more recently North America (8, 13, 16, 18, 21). The initial outbreak of WNV in North America took place in New York City in 1999, with 62 confirmed human cases and seven deaths reported (8, 20). This outbreak coincided with extensive mortality in birds, particularly crows, in the same geographic area (6). WNV rapidly spread to neighboring states, with 21 human cases (two deaths) reported in New York, New Jersey, and Connecticut in 2000 (18) and a further 66 human cases (nine deaths) reported in 10 states in 2001 (21). WNV activity has now been detected in most states in the eastern half of the United States.

Diagnosis of WNV infections in birds is complicated by the presence of other flaviviruses in the same geographic area. Of particular relevance to North American surveillance programs is SLEV, as this virus has a close antigenic and genetic relationship to WNV, is maintained in nature in similar transmission cycles, and has been isolated from wild birds (2, 7, 27). Furthermore, SLEV is endemic throughout the United States, particularly in the central and eastern states and in the southwest. This virus has also been responsible for two major outbreaks in Central America and a number of sporadic cases in Central and South America. Other avian flaviviruses found in the Americas include Ilheus virus (ILHV) and Bussuquara virus (BSQV) (2).

Serologic diagnosis of WNV in birds can be achieved by

* Corresponding author. Mailing address: Arthropod-Borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523. Phone: (970) 491-2988. Fax: (970) 491-8323. E-mail: bbeaty@colostate.edu.

TABLE 1. Flavivirus MAbs used in blocking ELISAs

MAb	Virus (isolate) to which MAb was raised[a]	Isotype	Reactivity[b]
2B2	WNV (subtype KUNV) (isolate MRM16)	IgG2a	Reacts with WNV, KOUV (A, B), no reaction with SLEV, MVEV (C)
3.1112G	WNV (subtype KUNV) (isolate OR393)	IgM	Reacts with WNV (B), No reaction with KOUV (D)
3.67G	WNV (subtype KUNV) (isolate OR393)	IgG_1	Reacts with WNV, KOUV (B)
3.91D	WNV (subtype KUNV) (isolate OR393)	IgG_3	Reacts with WNV, KOUV (B)
6B5A-2	SLEV (isolate MSI-7)	IgG2a	Reacts with SLEV (B); no reaction with WNV, MVEV, JEV, YFV, DENV 1–4 (D)
6B6C-1	SLEV (isolate MSI-7)	IgG2a	Reacts with SLEV, WNV, MVEV, JEV, YFV, DENV 1–4 (D)
3H6	MVEV (isolate MVE3/51)	IgG2a	Reacts with MVEV, SLEV, WNV, JEV, YFV, KOKV, DENV 1–3 (E)

[a] Abbreviations: West Nile virus (WNV), Kunjin virus (KUNV), Koutango virus (KOUV), Saint Louis encephalitis virus (SLEV), Murray Valley encephalitis virus (MVEV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), Dengue virus (DENV), Kokobera virus (KOKV).

[b] Letters in parentheses indicate how reactivity was determined. A, determined by hemagglutination assay and neutralization test (10); B, determined by direct ELISA (24); C, determined by indirect immunofluorescence antibody assay (17); D, determined by indirect immunofluorescence antibody assay (23); E, determined by hemagglutination assay and neutralization test (11). Details of virus strains are described in the references cited above.

plaque reduction neutralization tests (PRNTs). However, PRNTs for type-specific diagnosis are laborious, and expensive and require live virus and for these reasons are not ideal for large-scale routine testing. Hemagglutination inhibition assays are also labor-intensive, as bird serum samples must first undergo multiple treatments for nonspecific inhibitors with acetone extraction and perhaps protamine sulfate treatment (1, 15). Furthermore, hemagglutination inhibition assays are not specific in that, with antigen for WNV, antibodies that cross-react with other closely related flaviviruses may also be detected. In contrast, direct enzyme-linked immunosorbent assays (ELISAs) provide a rapid, sensitive, and inexpensive screening test for the detection of antibodies to WNV in birds (3). However, this technique is not suitable when screening samples from a wide variety of bird species, as different secondary (reporter) antibodies are required depending on the species being tested.

An indirect immunoglobulin G (IgG) ELISA was recently reported that detected WNV antibodies in all 23 avian species and 12 orders tested (5). This assay also recognized antibodies in SLEV-infected chickens. Antibodies were detected with a pooled suspension of horseradish peroxidase-labeled IgG antibodies from birds representing four orders. Another alternative is an epitope-blocking ELISA, which does not require the use of multiple reporter or capture antibodies and has previously been shown to be an effective method to specifically identify Kunjin virus infections in sentinel chickens (9). The development of epitope-blocking ELISAs to detect antibodies to WNV in diverse bird species would be a significant advance not only in WNV surveillance studies, but in all flavivirus surveillance studies.

MATERIALS AND METHODS

Cell culture. C6/36 and ATC-15 cell lines (both derived from *Aedes albopictus* larvae) were cultured at 28°C with 5% CO_2 in minimum essential medium (Gibco, Grand Island, N.Y.) containing 10% heat-inactivated fetal bovine serum (Gemini Bio-Products, Woodland, Calif.), 0.1 mM nonessential amino acids, 0.15% sodium bicarbonate, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). African green monkey kidney (Vero) cells were cultured at 37°C with 5% CO_2 in the same medium.

Viruses. All virus strains were obtained from the World Health Organization Reference Center maintained at the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colo. The viruses used in this study were WNV NY99-35261-11, SLEV TBH-28, ILHV (original), and BSQV BeAn-4073. Virus stocks were prepared in Vero and/or C6/36 cells, and virus titers determined by endpoint assay in Vero cells.

Bird serum samples. Birds were experimentally inoculated with WNV (NY99) via infected *Culex tritaeniorhynchus* mosquitoes except for fish crows, which were subcutaneously inoculated with 3,500 PFU of virus. All birds were sampled at 14 days postinfection, and domestic chickens were also sampled at 19, 28, and 31 days postinfection. fish crows were vaccinated with recombinant WNV prior to infection unless otherwise stated; all other birds were not vaccinated. Serum samples were also obtained from SLEV-infected birds captured during the 2001 St. Louis encephalitis epidemic in Monroe, La. Normal chicken serum was purchased from Vector Laboratories, Burlingame, Calif.

Serum samples were obtained from the following birds (with species and family names denoted in parentheses): American crows (*Corvus brachyrhynchos*, Corvidae), American robins (*Turdus migratorius*, Turdidae), a blue jay (*Cyanocitta cristata*, Corvidae), Canada geese (*Branta canadensis*, Anatidae), domestic chickens (*Gallus gallus*, Phasianidae), a domestic turkey (*Meleagris gallopavo*, Phasianidae), European starlings (*Sturnus vulgaris*, Sturnidae), fish crows (*Corvus ossifragus*, Corvidae), graylag geese (*Anser anser*, Anatidae), a house finch (*Carpodacus mexicanus*, Fringillidae), house sparrows (*Passer domesticus*, Passeridae), Japanese quails (*Coturnix japonica*, Phasianidae), killdeers (*Charadrius vociferus*, Charadriidae), monk parakeets (*Myiopsitta monachus*, Psittacidae), mourning doves (*Zenaida macroura*, Columbidae), a northern bobwhite (*Colinus virginianus*, Phasianidae), ring-necked pheasants (*Phasianus colchicus*, Phasianidae), and rock doves (*Columba livia*, Columbidae).

Mouse hyperimmune ascitic fluids. Mouse hyperimmune ascitic fluids containing antibodies against various flaviviruses (WNV, SLEV, ILHV, and BSQV) were obtained from the World Health Organization Reference Center maintained at the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colo.

MAbs. The seven MAbs used in this study are described in Table 1. Briefly, our panel of antibodies consisted of four WNV MAbs (3.1112G, 3.67G, 3.91D, and 2B2), two SLEV MAbs (6B6C-1 and 6B5A-2), and one Murray Valley encephalitis virus MAb (3H6). All MAbs detect E protein epitopes except MAb 3.1112G, which detects an NS1 epitope. MAb 6B6C-1 was labeled with horseradish peroxidase; all other MAbs were unlabeled. The production and characterization of these MAbs have been described elsewhere (10, 11, 23, 24).

Preparation of ELISA coating antigen. ATC-15 cell monolayers (grown in 150-cm^2 culture flasks) were inoculated with the specified flavivirus at a multiplicity of infection of 0.01. WNV- and ILHV-infected cells were scraped from flasks at 5 days postinfection, and SLEV- and BSQV-infected cells were scraped from flasks at 7 days postinfection and then clarified by centrifugation at 7,000 × *g* for 10 min at 4°C (Hermle Labnet centrifuge, model Z383K). Cell pellets were resuspended in 6 ml of ice-cold borate saline buffer (120 mM sodium chloride, 50 mM boric acid, 24 mM sodium hydroxide, pH 9.0), clarified by centrifugation at 7,000 × *g* for 20 min at 4°C, and washed three more times. Cell pellets were resuspended in 900 μl of 0.1% sodium dodecyl sulfate, then 100 μl of Triton X-100 and 2 ml of borate saline buffer were added to the suspension. Samples were sonicated on ice (20% output for 30 s) and clarified by centrifugation at

15,000 × *g* for 10 min at 4°C, and supernatants were collected and stored at −70°C. Control antigen was also prepared from uninfected cell monolayers.

Blocking ELISAs. Blocking ELISAs were performed following the protocol of Hall et al. (9), with several modifications. Briefly, the inner 60 wells of 96-well microtiter plates (Dynatech Industries Inc.) were coated with 100 μl of antigen diluted in carbonate-bicarbonate buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6). Control antigen prepared from uninfected cell monolayers was also used. Coated plates were incubated overnight at 4°C. Plates were washed six times with 250 μl of wash buffer (phosphate-buffered saline [pH 7.5] containing 0.1% Tween 20) with an automatic plate washer. Two hundred microliters of blocking buffer (phosphate-buffered saline containing 5% skim milk) was added to each well and incubated for 40 min at 37°C. After six washes, 50 μl of serum diluted 1:10 was added to each well and incubated for 2 h at 37°C, and the wells were washed again six times.

MAbs were diluted in blocking buffer at the specified dilution, added to each well (50 μl), and incubated for 1 h at 37°C. For non-horseradish peroxidase-labeled MAbs, plates were washed, and 50 μl of horseradish peroxidase-conjugated rabbit anti-mouse IgG (Zymed Laboratories, San Francisco, Calif.) was added at a dilution of 1:2,000 to each well and again incubated for 1 h at 37°C, followed by six more washes. Equal volumes of ABTS (2,2′ azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]) and peroxidase solutions from the ABTS Microwell peroxidase substrate system (KPL, Gaithersburg, Md.) were mixed, and 75 μl was added to each well. The optical density at a wavelength of 415 nm was determined with an automated plate reader. The percent inhibition of MAb binding was calculated (9) as $100 - [(TS - B)/(CS - B)] \times 100$, where TS is the mean optical density of the test serum, CS is the mean optical density of the control serum (from uninfected chickens), and B is the background optical density.

Test samples were typically analyzed in duplicate or triplicate, whereas at least three wells of control chicken serum were included on each 96-well plate. The percent inhibition was calculated once the mean optical density in the wells containing the control serum samples exceeded 0.3. An inhibition value of ≥30% was considered to indicate the presence of viral antibodies (as explained later).

Plaque reduction neutralization test. The ability of serum samples from birds to neutralize WNV and SLEV was determined by PRNT. Serum samples were heat inactivated at 56°C for 30 min and then serially diluted twofold in BA-1 diluent (Hanks M-199 salts, 50 mM Tris [pH 7.6], 1% bovine serum albumin, 0.35 g of sodium bicarbonate per liter, 100 U of penicillin per ml, 100 mg of streptomycin per ml, 1 mg of fungizone per ml), starting at a dilution of 1:5. Next, 100 μl of diluted serum was mixed with an equal volume of diluent containing 200 PFU of virus. One hundred microliters of serum-virus suspension was transferred onto confluent Vero cell monolayers in six-well plates and incubated at 37°C for 60 min. Three milliliters of neutral red-deficient Earl's balanced salt solution containing 20 g of yeast extract per liter, 100 g of lactalbumin hydrolysate per liter, 25% fetal bovine serum, 1% fungizone-gentamicin, and 0.5% agarose were added to each well, and the plates were incubated at 37°C for 48 h. Then 3 ml of the same medium containing 0.22% neutral red was added to each well at 2 or 6 days postinfection for the WNV and SLEV PRNTs, respectively. Plaques were counted 24 h later, and titers were expressed as the reciprocal of the serum dilution yielding ≥90% reduction in the number of plaques ($PRNT_{90}$). All serum samples were tested in duplicate.

RESULTS

Determination of MAb flavivirus specificity. The cross-reactivities of the seven MAbs against WNV, SLEV, ILHV, and BSQV were determined by direct ELISA. The panel of MAbs consisted of four that were raised against WNV (MAbs 2B2, 3.1112G, 3.67G, and 3.91D), two raised against SLEV (MAbs 6B5A-2 and 6B6C-1), and one raised against Murray Valley encephalitis virus (MAb 3H6). The specificities of these MAbs against selected flaviviruses were characterized previously (Table 1). Four antibodies (MAbs 2B2, 3.1112G, 3.67G, and 3.91D) reacted with WNV antigen but not with SLEV, ILHV, or BSQV antigens (data not shown). MAb 6B5A-2 specifically reacted with SLEV antigen, whereas MAbs 6B6C-1 and 3H6 reacted with the four flaviviruses tested. For those MAbs that failed to recognize a particular antigen, ELISAs were repeated with multiple MAb and antigen dilutions to ensure that antibody binding did not occur. As expected, antibodies to WNV, SLEV, ILHV, and BSQV in mouse hyperimmune ascitic fluids reacted with their respective antigens but not with uninfected mosquito cells.

TABLE 2. Inhibition of WNV-reactive MAbs by blocking ELISA with sera from chickens infected with WNV[a]

MAb	Optimal MAb dilution	% Inhibition of MAb binding at time between infection and serum collection (days)[b]:			
		14	19	28	31
2B2	1:40	79.3 ± 1.8	69.0 ± 3.7	49.7 ± 5.4	61.0 ± 5.7
3.1112G	1:2,000	85.3 ± 0.7	87.8 ± 0.5	71.6 ± 2.0	82.3 ± 0.3
3.67G	1:80	58.6 ± 4.7	67.7 ± 0.3	49.2 ± 4.3	51.8 ± 2.2
3.91D	1:40	47.2 ± 6.6	NT	NT	NT
6B6C-1	1:16,000	71.8 ± 2.6	84.4 ± 1.9	72.4 ± 2.6	79.3 ± 2.1
3H6	1:4	60.3 ± 1.8	NT	NT	NT

[a] MAbs were tested in triplicate. All sera were diluted 10-fold.
[b] Values are means ± standard deviations. NT, not tested.

Inhibition of MAb binding with serum samples from WNV-infected chickens. The six WNV-reactive MAbs were characterized in blocking ELISAs performed with a pooled suspension of serum samples collected 14 days postinfection from several WNV-infected chickens (anti-WNV serum samples). WNV coating antigen and MAbs were tested at multiple dilutions, at dilutions that resulted in the greatest inhibition of MAb-binding used for all subsequent ELISAs. Optimal antibody dilutions ranged from 1:4 to 1:80 for MAbs produced as hybridoma culture supernatants and 1:2,000 to 1:16,000 for MAbs that were mouse ascitic fluids (Table 2). For all MAbs, the optimal dilution of WNV antigen was 1:100. Under these conditions, we were able to achieve >47% inhibition of MAb binding in all assays. The most promising results were obtained with MAbs 3.1112G, 2B2, and 6B6C-1, yielding 85.3%, 79.3%, and 71.8% inhibition, respectively. Assays that utilized these MAbs were also effective in detecting antibodies in anti-WNV chicken serum samples collected at 19, 28, and 31 days postinfection. The SLEV-specific MAb 6B5A-2 was optimized in blocking assays with SLEV antigen and a pooled suspension of serum samples from multiple avian species infected with SLEV (anti-SLEV serum samples; data not shown). Optimal antigen and MAb dilutions were 1:100 and 1:1,000, respectively.

Inhibition of MAb binding with serum samples from WNV-infected crows. Next, the diagnostic efficacy of each blocking assay was characterized for its ability to detect WNV antibodies in serum samples from corvids. Eight fish crows were experimentally infected with WNV (six of which had been vaccinated against this virus prior to infection), and serum samples were collected at 14 days postinfection. All serum samples were positive in blocking assays performed with MAbs 2B2, 3.1112G, and 3.67G, with mean inhibition values of 87.1%, 69.4%, and 60.5%, respectively (Table 3). As explained below, an inhibition value of ≥30% was chosen as the diagnostic criterion to indicate the presence of flavivirus-specific antibodies. Six serum samples tested positive with WNV MAb 3.91D; the mean inhibition value in this assay was 40.9%. The MAbs to SLEV and Murray Valley encephalitis virus were less effective, with five, three, and two specimens being positive with MAbs 6B6C-1, 3H6, and 6B5A-2, respectively. No major difference in MAb inhibition was observed when comparing

TABLE 3. Inhibition of MAbs by blocking ELISA with sera from crows infected with WNV

Sample serum no.	Crow vaccinated	% Inhibition of MAb[a]						
		2B2	3.1112G	3.67G	3.91D	6B6C-1	6B5A-2	3H6
1	Yes	98.4 ± 0.4	67.2 ± 6.8	74.4 ± 4.5	52.4	49.9	74.1	50.0
2	Yes	96.4 ± 0.8	62.3 ± 5.5	89.8 ± 2.0	75.0	15.0	11.5	0
3	No	89.3 ± 4.2	71.3 ± 4.7	52.8 ± 4.0	30.2	35.0	26.6	14.3
4	Yes	69.2 ± 8.5	70.0 ± 5.6	44.8 ± 5.2	28.4	38.5	0	28.7
5	Yes	93.0 ± 1.1	65.5 ± 6.2	77.5 ± 5.2	48.8	76.5	66.1	69.2
6	Yes	87.3 ± 2.9	85.1 ± 3.2	47.8 ± 6.3	19.6	28.0	16.1	19.3
7	Yes	69.1 ± 7.5	56.0 ± 5.2	40.1 ± 10.8	39.1	56.0	0	40.5
8	No	93.9 ± 3.2	78.1 ± 5.7	57.1 ± 8.0	33.8	0	12.8	0
Mean		87.1	69.4	60.5	40.9	37.4	25.9	27.8

[a] Values are means ± standard deviations. Inhibition values of ≥30% are considered significant. MAbs 2B2, 3.1112G, and 3.67G were tested in triplicate. All other MAbs were tested in duplicate. All sera were diluted 10-fold.

serum samples from vaccinated and nonvaccinated birds (Table 3).

No noticeable pattern was observed when the percent inhibition values of each MAb were compared except for MAbs 3.67G and 3.91D. For each individual serum sample, the percent inhibition value reported with MAb 3.67G was approximately 20% higher than the corresponding value for MAb 3.91D. It is noteworthy to mention that, of all the antibodies used in these studies, only MAbs 3.67G and 3.91D were raised against the same virus strain and protein (Tables 1 and 3). To ascertain the sensitivity of the blocking assays, all anti-WNV crow serum samples were serially diluted twofold, starting at a dilution of 1:10, and tested against MAbs 3.67G, 2B2, and 3.1112G. The last two antibodies worked particularly well, with all eight serum samples significantly inhibiting the binding of these MAbs at dilutions of ≥1:320 (Table 4). Correlation of the ELISA endpoint titers to the $PRNT_{90}$ endpoint titers revealed that the former technique showed superior sensitivity in detecting antibodies in these particular serum samples.

Analysis of serum samples from WNV- and SLEV-infected birds of multiple species. The ability of blocking assays to detect flavivirus antibodies in serum samples from many bird species and to distinguish between WNV and SLEV infections was determined. ELISAs were conducted with serum samples from 13 WNV-infected laboratory birds that represented eight species and seven families and seven field-captured SLEV-infected birds (five species, five families). Control serum samples from 12 birds (nine species, eight families) with no previous exposure to either virus were also included. Overall, serum samples were collected from 32 individual birds that belonged to 16 species and 10 families. ELISAs were performed with the WNV MAbs 2B2, 3.1112G, and 3.67G and the SLEV MAbs 6B6C-1 and 6B5A-2.

TABLE 4. Determination of serum antibody titers in anti-WNV crow sera by blocking ELISA and PRNT

Anti-WNV crow serum sample no.	$PRNT_{90}$ titer	ELISA titer		
		2B2	3.1112G	3.67G
1	≥320	10,240	640	1,280
2	≥320	5,120	640	10,240
3	160	320	1,280	80
4	≥320	640	640	80
5	160	5,120	640	2,560
6	40	640	640	40
7	80	320	640	160
8	160	1,280	2,560	1,280

Analysis of control serum samples obtained from uninfected birds revealed that the mean nonspecific inhibition value was 6.7% for MAb 2B2, 7.0% for MAb 3.1112G, 3.5% for MAb 3.67G, 5.2% for MAb 6B6C-1, and 4.1% for MAb 6B5A-2 (Table 5). The highest nonspecific inhibition value was 24.3%, and therefore the diagnostic criterion selected was ≥30%. Percent inhibition values were calculated by comparing the optical density of the test serum to that of the control serum from uninfected chickens. In some instances, when percent inhibitions could be calculated with a control serum from an uninfected bird of the same species, similar inhibition values were obtained (data not shown).

The most effective assays for the detection of WNV serum antibodies were those performed with MAbs 3.1112G and 2B2 (Table 5). All 13 anti-WNV serum samples were positive with these antibodies. The efficiencies of these assays were similar, with a mean inhibition value of 66.5% for MAb 3.1112G and 59.6% for MAb 2B2. There was no apparent correlation between the MAb inhibition value and PRNT titer, in that a high inhibition value did not necessarily correspond to a high PRNT titer. For MAb 3.1112G, the mean inhibition value for anti-WNV serum samples with PRNT titers of <160 and ≥160 was 65.1% and 68.2%, respectively. For MAb 2B2, the mean inhibition value for anti-WNV serum samples with PRNT titers of <160 and ≥160 was 56.9% and 62.8%, respectively. Analysis of the seven anti-SLEV serum samples revealed that five were positive with MAb 2B2 but none were positive with MAb 3.1112G. Assays performed with MAb 6B6C-1 were also effective when tested against anti-WNV serum samples. The mean inhibition value for this MAb was 64.6%, and all serum samples except one were positive for viral antibodies. MAb 3.67G worked reasonably well, with viral antibodies detected in most (10 of 13) anti-WNV serum samples.

The most efficient assay for the detection of SLEV serum antibodies utilized MAb 6B6C-1. All anti-SLEV serum samples inhibited the binding of this MAb by >64%, and the mean inhibition value was 79.8% (Table 5). MAb 6B5A-2 also readily detected flavivirus antibodies in anti-SLEV serum samples; all specimens except one were positive. The serum sample that failed to demonstrate significant MAb inhibition was from a rock dove with a low PRNT titer. However, low PRNT titers

TABLE 5. Inhibition of MAbs by blocking ELISA with anti-WNV, anti-SLEV, and normal sera from multiple avian species[a]

Serum type and avian species	WNV $PRNT_{90}$ titer	SLEV $PRNT_{90}$ titer	% Inhibition of MAb:				
			2B2	3.1112G	3.67G	6B6C-1	6B5A-2
Anti-WNV							
American robin	40		84.8	86.4	45.8	24.4	0
Blue jay	≥320		56.1	70.0	33.4	58.1	0
Canada goose-1	≥320		59.7	65.0	44.1	63.2	22.0
Canada goose-2	≥320		65.1	67.4	40.1	70.3	2.2
Japanese quail-1	80		51.0	60.8	18.1	65.1	40.7
Japanese quail-2	40		51.6	38.5	35.0	64.1	12.3
Killdeer-1	≥320		85.1	76.8	36.8	77.0	0
Killdeer-2	160		44.5	57.4	35.1	68.1	28.4
Monk parakeet	80		43.3	56.5	27.9	68.4	0
Mourning dove	40		41.6	88.6	2.7	62.4	0
Ring-necked pheasant-1	80		46.5	62.0	35.7	77.6	0
Ring-necked pheasant-2	160		66.0	72.6	40.5	80.5	0
Ring-necked pheasant-3	40		79.4	62.9	44.8	60.5	2.2
Mean			59.6	66.5	33.8	64.6	8.3
Anti-SLEV							
Domestic turkey		640	49.3	17.7	16.8	91.4	94.1
European starling-1		≥40	20.2	6.3	7.2	75.4	47.4
European starling-2		20	45.4	0.7	7.7	64.9	93.9
Graylag goose		160	44.5	13.1	15.2	91.9	83.3
House sparrow		160	57.0	7.5	18.0	93.2	85.0
Rock dove-1		320	86.0	0	15.4	76.8	87.7
Rock dove-2		40	12.2	0	9.3	65.1	11.8
Mean			44.9	6.5	12.8	79.8	71.9
Normal							
American crow-1			9.6	15.7	13.8	0	15.0
American crow-2			5.5	11.1	5.6	0	7.0
American crow-3			4.2	5.0	0	0	0
American robin-1			0	8.2	0.6	0	14.6
American robin-2			0	9.5	0	0	5.3
Graylag goose			10.1	5.0	5.5	21.9	0.6
House finch			15.2	0	0	4.1	0
House sparrow			3.6	10.8	2.2	12.7	7.2
Japanese quail			11.6	5.6	3.2	0	0
Monk parakeet			0	0	1.6	0	0
Mourning dove			4.5	2.0	5.7	0	0
Northern bobwhite			15.8	5.8	3.7	24.3	0
Mean			6.7	7.0	3.5	5.2	4.1

[a] Titers of <10 are not shown. Inhibition values of ≥30% are considered significant. All sera were diluted 10-fold.

did not necessarily correlate to low percent inhibition values. For example, there was 93.9% inhibition with MAb 6B5A-2 when testing an anti-SLEV serum sample from a European starling with a $PRNT_{90}$ titer of 20. There was 87.7% inhibition with MAb 6B5A-2 when testing anti-SLEV serum from another rock dove with a $PRNT_{90}$ titer of 320. Interestingly, the single sample that was negative was also one of the two anti-SLEV samples that were negative with MAb 2B2. One of two anti-WNV serum samples from a Japanese quail was positive with MAb 6B5A-2.

To further assess the sensitivity of the assays, three randomly selected serum samples were tested at multiple dilutions against MAbs 2B2, 3.1112G, 6B5A-2, and 6B6C-1. We tested one anti-WNV serum sample from a Japanese quail and two anti-SLEV serum samples from a rock dove and graylag goose. Serum samples were serially twofold diluted, with a starting dilution of 1:10 and a final dilution of 1:5,120. Best results were obtained with MAb 6B6C-1, with significant inhibition of MAb binding observed with all three serum samples at a dilution of 1:5,120 (data not shown).

DISCUSSION

We developed epitope-blocking ELISAs that reliably detect WNV serum antibodies in numerous North American avian species. The most efficient assays utilized MAbs 2B2 and 3.1112G; antibodies were detected in all WNV-infected birds when these MAbs were used. However, most serum samples from birds infected with the closely related SLEV were also positive with MAb 2B2. In contrast, all anti-SLEV serum samples failed to block the binding of MAb 3.1112G, suggesting that this assay can be exploited in North American surveillance studies to differentiate between WNV and SLEV infections. Indeed, this assay is routinely used to monitor sentinel chickens in northwestern Australia, as it differentiates between Kunjin virus infections and those caused by other flaviviruses found in this region, namely Murray Valley encephalitis virus and KOKV (9). Moreover, this assay has been exploited to screen >2,500 serum samples from numerous avian species captured throughout the United States and shown to specifically identify infections with WNV (R. H. Evans, M. Jozan, F. Stebbins, C.

Fogarty, K. Abeyta, R. A. Hall, and B. Tangredi, Abstr. 70th Annu. Conf. Mosquito Vector Control Assoc. California, 2002).

ELISAs that utilized MAb 3.67G were specific for WNV antibodies, albeit several anti-WNV serum samples failed to test positive, while MAb 6B5A-2 generally detected antibodies in SLEV-infected but not WNV-infected birds. The most efficient assay for the detection of serum antibodies to SLEV utilized MAb 6B6C-1. Serum antibodies were detected in all SLEV-infected birds and most WNV-infected birds with this assay. The blocking assays reported here, unlike PRNTs or hemagglutination inhibition assays, provide a rapid and inexpensive serologic technique suitable for large-scale screening of serum specimens.

MAbs 2B2 and 6B5A-2 did not recognize SLEV and WNV antigens, respectively, by direct ELISA. However, blocking assays performed with MAb 2B2 detected antibodies in most anti-SLEV serum samples, and the binding of MAb 6B5A-2 was blocked by several anti-WNV serum samples. Presumably, the inability of these MAbs to react with their epitopes in the presence of serum antibodies against the other virus is a consequence of steric hindrance. That is, antibodies common to both viruses are binding to nearby epitopes, thereby blocking the binding of the MAb. Flavivirus cross-reactive and type-specific MAbs have been shown to compete with one another in competitive binding assays (22).

Assays performed with MAbs 2B2 and 3.67G were more efficient in detecting antibodies in fish crows than in the other WNV-infected bird species tested. In contrast, antibodies were detected in only five of eight fish crows in the assay that utilized MAb 6B6C-1, whereas almost all other birds infected with either WNV or SLEV tested positive. These findings may reflect the different inoculation methods and/or virus doses used in these experiments. Fish crows were subcutaneously inoculated, but all other anti-WNV serum samples were from birds that had been experimentally inoculated via infected mosquitoes, and all anti-SLEV serum samples were obtained from wild birds. However, it is unlikely that the assay performed with MAb 3.1112G simply discriminated between experimental and naturally acquired infections rather than WNV and SLEV infections. Earlier studies have shown that this assay discriminates between WNV and SLEV antibodies in naturally infected chickens (12).

Recently, antibody capture ELISAs were designed for the routine diagnosis of WNV infections in humans, and these assays were shown to be highly sensitive (19, 26). Similarly, the blocking ELISAs reported here displayed high levels of sensitivity. No obvious correlation between ELISA percent inhibition values and PRNT titers was observed, although this was not unexpected, as these tests do not necessarily detect the same antibody types. That is, blocking assays can detect serum antibodies that have no neutralizing ability. Comparison of antibody titers determined by blocking ELISA and $PRNT_{90}$ revealed that the former technique was superior in sensitivity when examining serum samples collected at 14 days postinfection. However, it is important to note that neutralizing antibodies have a greater longevity than nonneutralizing antibodies (4). Therefore, blocking assays may not necessarily display superior sensitivity over PRNTs when used to test serum samples from field-captured birds. Titration experiments indicated that the assay performed with MAb 6B6C-1 was the most sensitive in detecting serum antibodies in birds that had been experimentally infected by mosquitoes or naturally infected. It is possible that this assay displayed greater sensitivity because MAb 6B6C-1 is horseradish peroxidase labeled, unlike the other antibodies used in these experiments.

The blocking assays reported here are currently being used to monitor potential WNV activity in migratory and resident avian populations in Mexico. We are screening bird serum samples in ELISAs with MAb 3.1112G to specifically identify WNV infection and MAb 6B6C-1 to detect flavivirus cross-reactive antibodies. Additional studies in our laboratories will determine whether these assays can also detect antibodies to other flaviviruses found in the Americas, such as ILHV and BSQV. Recent studies have shown that our ELISAs can be exploited to detect WNV infections in various species of domestic mammals (unpublished data).

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