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Research Paper

Detection of West Nile Virus Infection in Birds in the United States by Blocking ELISA and Immunohistochemistry

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ABSTRACT

A blocking ELISA targeting an immunodominant West Nile epitope on the West Nile Virus NS1 protein was assessed for the detection of West Nile-specific antibodies in blood samples collected from 584 sentinel chickens and 238 wild birds collected in New Jersey from May–December 2000. Ten mallard ducks (*Anas platyrhynchos*) experimentally infected with West Nile virus and six uninfected controls were also tested. The ELISA proved specific in detecting WNV antibodies in 9/10 chickens and 4/4 wild birds previously confirmed as positive by Plaque Reduction Neutralization test (PRNT) at the Center for Disease Control, Division of Vector Borne Diseases, Fort Collins, CO, USA (CDC). Nine out of the ten experimentally infected mallard ducks also tested positive for WN antibodies in the blocking ELISA, while 6/6 uninfected controls did not. Additionally, 1705 wild birds, collected in New Jersey from December 2000–November 2001 and Long Island, New York between November 1999 and August 2001 were also tested for WN antibodies by the blocking ELISA. These tests identified 30 positive specimens, 12 of which had formalin-fixed tissues available to allow detection of WN specific viral antigen in various tissues by WNV-specific immunohistochemistry. Our results indicate that rapid and specific detection of antibodies to WN virus in sera from a range of avian species by blocking ELISA is an effective strategy for WN Virus surveillance in avian hosts. In combination with detection of WN-specific antigens in tissues by immunohistochemistry (IHC) the blocking ELISA will also be useful for confirming WN infection in diseased birds. Key Words: West Nile Virus—Blocking ELISA—Immunocytochemistry—Wild birds—Arbovirus—Serum-blood spot. Vector-Borne Zoonotic Dis. 3, 99–110.

INTRODUCTION

West Nile virus (WNV) is a mosquito-borne flavivirus in the Japanese encephalitis antigenic virus complex (Heinz 2000). It is active in many parts of the world, including Africa, the Middle East, Europe, Russia, India, parts of South East Asia, Australia, and North America (Murgue et al. 2002, Roehrig et al. 2002, Hall et al. 2002). Two major genetic lineages of the virus have been identified (Scherret et al. 2001). Lineage I viruses include the strain of West Nile (WN) introduced into North America and the viruses responsible for recent outbreaks of disease in humans, horses, and domestic birds in Russia, Europe,

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and the Middle East. Lineage II viruses include the Ugandan prototype virus and isolates from Africa, Madagascar, and the Middle East (Scherret et al. 2001). The Australian *Flavivirus* Kunjin (KUN) has also been recognized as a subtype of WNV within Lineage I (Heinz et al. 2000, Scherret et al. 2001). Numerous mosquito species are the primary vectors, but the role of ticks as a facilitator of virus transport by birds has been documented (Abbassy et al. 1993, L'vov et al. 2002) and needs further consideration.

The role of resident and migratory birds in virus transmission (Work et al. 1955) underlines the need to have a reliable antibody screening test for avian specimens. Since numerous mosquito-borne flaviviruses are antigenically closely related, the distinction of specific antibodies is difficult, if not impossible by conventional hemagglutination-inhibition (HI) or fluorescent antibody (FA) tests. When testing against a battery of related strains, a high titer will not necessarily reflect a current infection, but may rather indicate an anamnestic response to a previous infection with another *Flavivirus*. Similar problems are encountered with the ELISA test, whether or not an antibody capture scenario is used. The use of monoclonal antibodies in blocking or competitive formats has enhanced the specificity of this test, but cross-reactions can still occur, particularly if the monoclonal antibodies are directed toward epitopes common to many flaviviruses. Steric hindrance from adjacent highly conserved epitopes may also be a problem.

A blocking ELISA targeting an immunodominant epitope common to the NS1 protein of KUN and WN viruses was previously shown to be effective for detecting KUN-specific antibodies in sera from naturally infected chickens (Hall 1995). In the wake of the outbreak of WNV in New York in 1999 and observations that the New York virus was genetically and antigenically very similar to KUN (Lanciotti et al. 1999), preliminary studies on sera from sentinel chickens and wild birds (Pham et al. 2001) infected during the initial outbreak in New York indicated that the method was potentially useful for detecting WNV-specific antibodies in naturally infected birds. Here we report the use of the blocking ELISA test in four different serological studies of wild and domestic birds. The purpose of the study was twofold: (1) to evaluate the ability of the blocking ELISA to detect WN antibodies in avian sera previously confirmed by plaque reduction neutralization test (PRNT) at the Centers for Disease Control and Prevention, Division of Vector-Borne Diseases, Fort Collins, Colorado (CDC) and (2) to assess the value of an immunohistochemical technique using two WN-specific monoclonal antibodies to provide a confirmatory diagnosis of WNV infection in formalin-fixed tissues of deceased birds.

MATERIALS AND METHODS

Blood and tissue collection

Study 1 consisted of 584 blood samples collected from 68 sentinel chickens in 22 locations in New Jersey between June 2000 through September 2001, as well as an additional 162 American crow and 76 Fish crow samples collected in New Jersey from December 2000 to January 2001.This serum collection included eight specimens that were previously confirmed positive for WN antibodies when tested by standard PRNT against both SLE and WN viruses at CDC (Beaty and Calisher 1989). Study 2 was composed of 1560 blood spot samples that were collected from wild birds (including 176 corvid samples) captured in WN endemic foci in New Jersey during 2001and 2002.

Sentinel chicken blood samples were obtained from the wing vein using 3-cc syringes. Drops of blood were absorbed onto a 1×5 cm strip of blotter paper which was allowed to dry, placed in a plastic storage bag with appropriate data, and stored at 4°C until shipment to Orange County Vector Control District (OCVCD). Blood samples were obtained by cardiac puncture using 10-mL syringes. A blood-spot aliquot was made at the time of collection, and the remainder of the blood sample was transferred to a sterile 10-mL tube and allowed to clot at room temperature. Samples were then spun down and the resulting sera stored in cryovials at −70°C. Blood-spot specimens were coded and sent to OCVCD for testing by blocking ELISA.

Wild birds were caught in New Jersey using Japanese mist nets or modified crow traps. Each bird was identified to species, age, and sex, and then banded with a USGS band. For all species other than corvids, a drop of blood obtained by lancet prick of the brachial vein was absorbed onto Whatman no. 1 filter paper, which was allowed to dry, placed in a plastic bag with appropriate data, and stored at 4°C until shipment to OCVCD. In American crows (*Corvus brachyrhyncos*) and Fish crows (*Corvus ossifragus*), a blood sample was obtained from the jugular vein. Three hundred microliters were placed onto each blotter paper and sent to OCVCD. The remaining sample in study 1 was centrifuged at 2500 rpm for 30 min at 4°C and the serum sent to CDC for PRNT testing. All samples were collected according to the Biohazard Safety Guidelines Rutgers University.

In Studies 1 and 2, only chicken and corvid avian sera were screened for antibodies to WNV by the HI assay (Clarke and Casals, 1958), at the USDA National Veterinary Services Laboratory, Ames, Iowa. Suspect positive samples were confirmed by the standard PRNT method against both SLE and WN viruses at CDC (Beaty at al. 1989).

Study 3 consisted of blood samples collected from 145 birds between June 1999 and January 2002 on Long Island, New York. These birds were either euthanized or had died following admission to a wildlife rehabilitation center with a history of severe neurological disease. Whole sera were collected and stored at −20°C. Tissues from 12 of these birds had been gross-trimmed to no more then 6 mm in thickness and fixed in 10% neutral, buffered formalin for later histologic and WN-antigen immunohistochemistry examination.

Formalin-fixed tissues were microtome sectioned at 5 μm and stained with hematoxylin and eosin for routine histopathologic examination. Additional 5-μm serial sections were made for immunohistochemistry (IHC) staining with the DAKO EnVision System® Immunohistochemistry Kit (#K1390, DAKO Corporation, Carpinteria, CA) as previously reported (Steele et al. 2000). In brief, sections were deparaffinized in xylene, subjected to proteolytic enzyme digestion at room temperature with Proteinase K (#S3020, DAKO Corporation), peroxidase blocked, treated with primary antibody, and then exposed to a peroxidase-labeled, polymer-conjugated secondary antibody. Color was developed with DAB, and sections were counterstained with hematoxylin and mounted in Permount® (Fischer Scientific, Fair Lawn, NJ).

For primary antibodies, polyclonal rabbit anti-WN (BioReliance #80-533, Rockville, MD) was used for initial screening with positive confirmation by anti-WN mab 3A3 (1:200) and 7H2 (1:20) (#80-524, BioReliance). Positive controls were formalin-fixed tissue from WN experimentally infected crows (*Corvus brachyrynchos*; R.G. McLean, National Wildlife Health Center, Madison, WI), while negative controls were tissue sections stained with WN-negative rabbit primary antibody (#80-534, BioReliance).

Study 4 was a blind serological study of 10 WN experimentally infected and six control Mallard ducks (*Anas platyrhynchos*). Thirteen-week-old Mallard ducks held in BL-3 containment facility at the USGS National Wildlife Health Center in Madison, Wisconsin, were infected by subcutaneous inoculation with 0.1 mL of a virus suspension containing approximately 5000 pfu of a vero cell culture infected with WNV isolated from an infected American crow from Suffolk County, New York. The identity of the isolate used for experimental infection was confirmed by Lanciotti et al. (1999) and found identical to the original New York isolate. Six control ducks were inoculated with an equal volume of saline and treated similarly. Blood samples were taken 5 weeks post-inoculation and the experiment concluded. The blood was centrifuged at 2500 rpm for 20 min at 10°C, and the serum was stored in cryovials at −70°C. Aliquots of serum were heat treated at 56°C for 30 min prior to serological testing. Mallard ducks developed mean daily viremia exceeding 10^5 pfu per mL for 5–6 days. (R.G. McLean, unpublished data).

Serum recovery from blood spots and blocking ELISA

Each blood spot was eluted overnight with 2 mL of PBS–Tween–0.5% bovalbumin at 4°C. Samples were then centrifuged at 1000 rpm for 10 min at 8°C, with the final dilution being approximately 1:20. An aliquot was stored at 4°C

TABLE 1. STUDY 1: DISTRIBUTION OF WEST NILE ANTIBODIES IN 13 BLOOD SAMPLES FROM WILD AND DOMESTIC BIRDS COLLECTED IN NEW JERSEY BY RUTGERS UNIVERSITY, 2000–2001

Laboratory number	*Species*	*Collection date*	*Location county*	*Broad flavivirus monoclonal % inhibition*	*WN-specific monoclonal % inhibition*	*WNV end point titer*	*Blocking ELISA results*	*CDC PRNT*
AV-036	Chicken	12/21/00	Essex	21%	12%	N/A	Negative	Positive
AV-039	Chicken	12/21/00	Middlesex	94%	93%	1:80	Positive	Positive
AV-044	Chicken	12/21/00	Morris	97%	96%	1:160	Positive	Positive
AV-467	Chicken	09/28/00	Morris	79%	59%	1:40	Positive	Positive
AV-481	Chicken	09/21/00	Essex	21%	92%	1:80	Positive	Positive
AV-487	Chicken	09/29/00	Middlesex	35%	68%	>1:80	Positive	Positive
AV-530	Chicken	10/10/00	Morris	45%	94%	1:20	Positive	N/D
AV-572	Chicken	10/26/00	Morris	62%	77%	1:80	Positive	Positive
AV-581	Chicken	10/27/00	Middlesex	43%	90%	>1:80	Positive	Positive
AV-833	Fish Crow	01/25/00	Middlesex	20%	79%	1:80	Positive	Positive
AV-631	Fish Crow	11/22/00	Middlesex	46%	91%	1:80	Positive	Positive
AV-682	American Crow	11/24/00	Middlesex	85%	62%	≥1:80	Positive	Positive
AV-814	American Crow	01/22/01	Middlesex	55%	93%	1:40	Positive	Positive

N/A, nonapplicable; N/D, not done.

for testing, while the remaining sample was stored at −20°C for future reference or retesting.

The blocking ELISA procedure was performed as described by Hall et al. (1995), with minor modifications noted below. The viral antigen was WN (strain HB6343), which has been shown to be genetically and antigenically closely related to the WN-NY99 strain (Scherret et al. 2001). ELISA antigen was prepared as detergent lysates of WN-infected C6/36 cells. Briefly, confluent monolayers of C6/-36 cells in 150-cm^2 flasks were infected with WN virus at a multiplicity of infection of 1. At 3 days postinfection, the cells from each flask were harvested and washed three times in 6 mL of borate saline, pH 9 (BS9). The final pellet was resuspended in 0.9 mL of 0.1% SDS, followed by the addition of 0.1 mL of 10% Triton-X 100. The lysate suspension was briefly sonicated on ice, and then clarified by centrifugation at 13000*g* for 10 min at 4°C. The supernatant was then aliquoted and stored −20°C. The optimal dilution for adsorbtion to ELISA plates was determined by titration and usually found to be between 1:500 and 1:2000. WN-specific monoclonal antibody (WN mab) 31112G and broad flavivirus monoclonal antibody (BF mab) 3H6 were prepared as crude hybridoma culture supernatants or murine ascitic fluids, as previously described (Hall et al. 1995).

Specimens were tested in duplicate at a dilution 1:20 for initial screening, and positive reactors were retested at further dilutions. All sera were tested against the mock antigen (uninfected cell lysates). Controls, which were repeated on each plate, included a WN-negative serum (chicken), two WN-positive sera (chicken and wild bird), one SLE-positive serum (chicken), mock antigen, viral antigen, and diluent (TENTC). An OD value was recorded for each test and control on a Microsoft Excel® spreadsheet, and percent inhibition of mab binding for each test sample was calculated using the formula ([100 − (TS-B/TC-B × 100)]). A percent inhibition of 45% or more was the threshold chosen to interpret a specimen as positive for either broad flavivirus-specific or WN-specific antibodies.

RESULTS

Serologic analysis of blood spots or sera from wild, domestic, or experimentally infected birds

Study 1. Of 584 chicken blood spots tested, eight sera had previously tested positive for WN antibodies by PRNT (CDC). As shown in Table 1, the blocking ELISA detected seven of the positive samples, but failed in one instance—Chicken AV-036, which was reported as negative in the blocking ELISA because of low levels of inhibition (21% for BF and 12% for WN-specific antibodies). An additional chicken (Chicken AV-530) not tested by PRNT was found positive by blocking ELISA , showing strong inhibition levels of 45% and 94% for BF mab and WN mab, respectively. Similarly, two American crow specimens and two Fish crow specimens, which had tested WN-positive by PRNT at CDC, were correctly identified as such by the blocking ELISA.

When WN-positive sera were titrated in the blocking ELISA, titers ranged from 1:20 to ≥1:80. When a single dilution of serum was tested (1:20), the percent inhibition of mab ranged from 59% to 94%. Table 2 compares the

TABLE 2. STUDY 2: DISTRIBUTION OF BROAD FLAVIVIRUS AND WEST NILE ANTIBODIES IN POSITIVE AVIAN BLOOD SPOTS COLLECTED IN NEW JERSEY BY RUTGERS UNIVERSITY FROM JUNE 2000 TO JANUARY 2001

			Broad Flavivirus mab 3H		*West Nile epitope mab 31112G*	
Species	*Total tested*	*Total positive*	*Number reactors*	*Average inhibition*	*Number reactors*	*Average inhibition*
American Crow	162	2	2	70%	2	78%
Fish Crow	76	2	2	43%	2	80%
Chicken	584	8	6	70%	8	69%
Total	822	12	10	61%	12	76%

TABLE 3. STUDY 2: SPECIES DISTRIBUTION OF AVIAN SERA COLLECTED IN NEW JERSEY IN 2001 AND PREVALENCE OF WEST NILE ANTIBODIES AS DETECTED BY BLOCKING ELISA

Species	*Scientific name*	*Number collected*	*Species*	*Scientific name*	*Number collected*
Red-winged Blackbird	*Agelaius phoeniceus*	33	Song Sparrow	*Melospiza melodia*	71
Eastern Bluebird	*Sialia sialis*	2	Swamp Sparrow	*Melospiza georgiana*	18
Indigo Bunting	*Passerina cyanea*	11	Unidentified Sparrow		6
Northern Cardinal	*Cardinalis cardinalis*	27	White-throated Sparrow	*Zonotrichia albicollis*	26
Gray Catbird	*Dumetella carolinensis*	151 (2*)	European Starling	*Sturnus vulgaris*	32
Yellow-breasted Chat	*Icteria virens*	2	Northern Waterthrush	*Seirus noveboracensis*	5
Carolina Chickadee	*Poecile carolinensis*	7	Swainson's Thrush	*Catharus ustulatus*	1
American Crow	*Corvus brachyrhyncos*	78 (4*)	Wood Thrush	*Hylocichla mustelina*	8
Blue Jay	*Cyanocitta cristata*	11	Eastern Tufted Titmouse	*Baeolophus bicolor*	9
Fish Crow	*Corvus ossifragus*	49 (1*)	Brown Thrasher	*Toxostoma rufum*	6
Brown-headed Cowbird	*Molothrus ater*	33	Blue-headed Vireo	*Vireo solitarius*	1
Mourning Dove	*Zenaida macroura*	6	Red-eyed Vireo	*Vireo olivaceus*	5
Rock Dove	*Columba livia*	141	White-eyed Vireo	*Vireo griseus*	2
American Goldfinch	*Carduelis tristis*	32	Black-and-white Warbler	*Mniotilta varia*	1
House Finch	*Carpodacus mexicanus*	16	Blackpoll Warbler	*Dendroica striata*	1
Unidentified Finch		1	Black-throated Blue Warbler	*Dendroica caaerulescens*	4
Northern Flicker	*Colaptes auratus*	4	Black-throated Green Warbler	*Dendroica virens*	1
Yellow-shafted Flicker	*Colaptes auratus*	1	Canada Warbler	*Wilsonia canadensis*	1
Least Flycatcher	*Empidonax minimus*	2	Common Yellow-throat	*Geothlypis trichas*	43 (1*)
Common Grackle	*Quiscalus quiscula*	20	Kentucky Warbler	*Oporornis formosus*	2
Ruby-throated Hummingbird	*Archilochus colubris*	2	MacGillivray's Warbler	*Oporornis tolmiei*	2
Dark-eyed Junco	*Junco hyemalis*	6	Mourning Warbler	*Oporornis philadelphia*	1
Slate-colored Junco	*Junco hyemalis*	28	Myrtle Warbler	*Dendroica coronata coronata*	3
Ringed Kingfisher	*Ceryle torquata*	1	Nashville Warbler	*Vermivora ruficapilla*	1
Golden-Crowned Kinglet	*Regulus satrapa*	2	Newfoundland Yellow Warbler	*Dendroica petechia amnicola*	1
Northern Mockingbird	*Mimus polyglottos*	15	Palm Warbler	*Dendroica palmarum*	22
Baltimore Oriole	*Icterus cucullatus*	5	Prairie Warbler	*Dendroica discolor*	2
Orchard Oriole	*Icterus spurius*	1	unidentified Warbler		1
Ovenbird	*Seiurus aurocapillus*	1	Yellow Warbler	*Dendroica petechia*	7
American Redstart	*Setophaga ruticella*	6	Yellow-rumped Warbler	*Dendroica coronata*	171
American Robin	*Turdus migratorius*	45	Downy Woodpecker	*Picoides pubescens*	3
Chipping Sparrow	*Spizella passerina*	25	Red-bellied Woodpecker	*Melanerpes carolinus*	3
Field Sparrow	*Spizella pusilla*	3	Eastern Wood-Peewee	*Contopus virens*	2
Fox Sparrow	*Passerella iliaca*	1	Carolina Wren	*Thryothorus ludovicianus*	10
Grasshopper Sparrow	*Ammodramus savannarum*	1	House Wren	*Troglodytes aedon*	11
House Sparrow	*Passer domesticus*	252	Sharp-shinned Hawk	*Accipiter striatus*	1
Lincoln Sparrow	*Melospiza lincolnii*	4	Others		6
Savannah Sparrow	*Passerculus sandwichensis*	45		Total	1560

*Specimens positive for West Nile Virus antibodies by blocking ELISA (total positive is 8).

average percent inhibition obtained against BF mab and WN mab, respectively, according to species. Ten out of 12 positive sera inhibited BF mab with a range of 43–61%. For the 12 positive reactors, the WN mab was inhibited with a range of 69–80%. Two hundred and seventy sera found negative by PRNT exhibited low level inhibition of both mabs, 18–26% for BF mab and 20–23% for WN mab. These reactions were considered negative according to the ELISA cutoff criteria noted above.

Study 2. This study examined 1560 blood spots representing 69 avian species (Table 3). Eight specimens tested positive for WN antibodies by blocking ELISA: four American crows, one Fish crow, one common yellow-throat (*Geothlypis trichas*), and two gray catbirds (*Dumetella carolinensis*). CDC had previously found three of these American crows and one Fish crow positive by PRNT. The average percent inhibition of WN-specific mab was 68%.

Study 3. The third study included 145 sera with a species distribution as shown in Table 4. Twenty-nine birds presented with clinical syndromes compatible with central nervous system disease. Of these, 24 had WN-specific antibodies and 22 had BF-reactive antibody when tested by blocking ELISA.

Study 4. In this study, sera from 10 experimentally infected Mallard ducks and six uninfected control ducks were tested for WN antibody in the blocking ELISA. Nine of the 10 specimens from experimentally infected birds tested positive. The tenth specimen, XAP407, showed an inhibition of 48% with a titer of 1:40 for the BF mab, but failed to inhibit the WN mab, and therefore was reported as WN-negative. The six control birds tested negative by blocking ELISA (Table 6).

Confirmation of WN infection by immunohistochemistry

Formalin-fixed tissues available from 12 (one Red-tailed Hawk [*Buteo jamaicensis*), one Pigeon (*Columba livia*] and 10 American crows) of the 24 ELISA-positive birds in Study 3 were strongly positive for WN antigen by IHC (Table 5).

In all cases, moderate to large sized cytoplasmic clumps of antigen were visualized in circulating monocytes, periarteriolar sheath histiocytes of the spleen, and fixed histiocytes within many tissues. Antigen was also common in Kupffer cells (80% of specimens) of the liver, but very occasionally in hepatocytes (Fig. 1). In all specimens, antigen was also detected in interstitial macrophages as well as tubular

TABLE 4. SPECIES DISTRIBUTION AND WEST NILE ANTIBODIES IN AVIAN SERA COLLECTED ON LONG ISLAND, NY, 1999–2001

Species	*Scientific name*	*Number collected*	*Number with CNS clinical signs*	*IHC positive for West Nile*
American Crow	*Corvus brachyrhyncos*	28	16	12
American Robin	*Turdus migratorius*	1	0	0
Blue Jay	*Cyanocitta cristata*	2	1	1
Canada goose	*Branta canadensis*	3	2	0
Cardinal	*Cardinalis cardinalis*	1	0	0
Cedar Waxwing	*Bombycilla cedrorum*	1	0	0
Domestic goose	*Anser domesticus*	2	2	0
Grackle	*Quiscalus quiscula*	1	0	1
House Sparrow	*Passer domesticus*	37	0	2
Kestrel	*Falco sparverius*	2	1	0
Mallard	*Anas platyrhynhcos*	1	1	0
Mockingbird	*Mimus polyglottos*	1	0	0
Mourning Dove	*Zenaida macroura*	3	2	0
Pigeon	*Columba livia*	33	0	3
Red-tailed Hawk	*Buteo jamaicensis*	4	3	2
Starling	*Sturnus vulgaris*	25	1	3
Total		145	29	24

TABLE 5. STUDY 3: DISTRIBUTION OF BROAD FLAVIVIRUS AND WEST NILE ANTIBODIES IN WILD BIRDS COLLECTED IN LONG ISLAND, NEW YORK, FROM JUNE 2000 TO AUGUST 2001

			Antibodies in West Nile positive sera						
			Broad Flavivirus reactors		*West Nile epitope specific reactors*		*Number with clinical signs*	*Number with tissues available*	*Number positive by IHC*
Species	*Total tested*	*Total positive*	*Number reactors*	*Average inhibition*	*Number reactors*	*Average inhibition*			
American Crow	28	12	11	44%	12	91%	9	9	9
House Sparrow	37	2	2	63%	2	97%	0	0	NA
Pigeon	33	3	3	73%	3	59%	0	0	NA
Red-tailed Hawk	4	2	2	51%	2	79%	2	0	NA
Starling	25	3	2	87%	3	67%	1	1	1
Blue Jay	2	1	1	95%	1	81%	1	1	1
Grackle	1	1	1	76%	1	97%	1	1	1
Mallard	1	0	NA	NA	NA	NA	NA	NA	NA
Mockingbird	1	0	NA	NA	NA	NA	NA	NA	NA
Others	13	0	NA	NA	NA	NA	NA	NA	NA
Total	145	24	22	70%	24	82%	14	12	12

and collecting duct epithelia of the kidney, but in only a few cases in the glomerular epithelia. Antigen was also commonly detected, but to varying degrees within the pancreatic acinar cells, adrenal cortical and medullary cells, oocytes and stromal cells of the ovary, interstitial cells in the testis, and pneumocytes of the lungs.

In the brains of 50% of the birds, antigen was noted as fine to moderately granular staining in widely scattered Purkinje cells and their dendrites (Fig. 2) within the molecular layer and occasionally in basket cells and granule cells of the granular layer of the cerebellum. In about 50% of these cases, nonsuppurative encephalitis or menigoencephalitis was noted with antigen present in neurons and glial cells.

Multifocal, mild to severe mononuclear myocarditis with antigen in necrotic myocytes and active macrophages was observed in 86% of the birds, while in birds without histologic lesions in the heart (14%), antigen was noted multifocally as dense fine granular bands within myocytes. In about 15% of the above cases, antigen was also detected within vascular smooth muscle cells. In three birds with myocarditis, mononuclear pericarditis was also noted with antigen not uncommonly found with infiltrating macrophages.

In 50% of the birds, segmentally within the jejunum, moderate to marked amounts of antigen were noted throughout large expanses of the crypt and villar epithelia as well as in macrophages scattered throughout the interstitial spaces (Fig. 3) and in perivascular aggregates. Additionally, antigen could variably be found in at least some neurons of the myenteric plexuses. In the cloaca of 60% of the birds, antigen was detected in epithelia as varying sized multifocal areas.

Validity of percent inhibition cutoff value

A cutoff value of 45% inhibition for either WN or BF mab was based on the range of inhibition levels exhibited by negative control sera (0% − X%, mean of Y%). Figures 4 and 5 show the distribution of inhibition levels for each of the first 962 specimens examined. Thirty-six specimens were found WN positive according to our preset cutoff value of 45%; 86% of these specimens showed an inhibition of ≥60% for WN-specific mab (Fig. 4). Among the 926 remaining specimens, 342 showed some inhibition for BF and WN mabs, but 90% of these reactions were ≤30% (Fig. 5). This contrasts clearly with the distribution of inhibition in WN-positive specimens (Fig. 4), and therefore the 342 specimens can be considered negative. The cutoff of 45% inhibition for a positive interpretation is therefore conservative and under epidemic circumstances could prob-

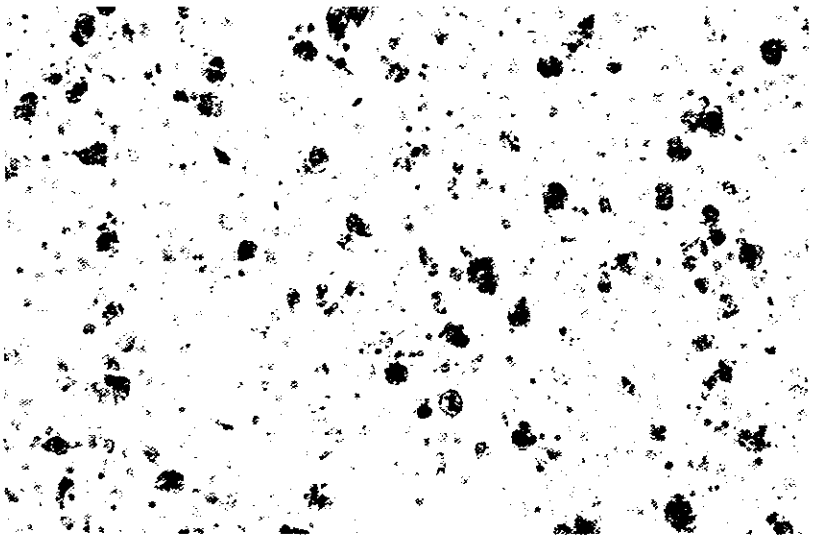

FIG. 1. Study 3: West Nile Virus–specific antigen detected by immunohistochemistry in Kupffer cells in liver of an American crow (*Corvus brachyrhyncos*).

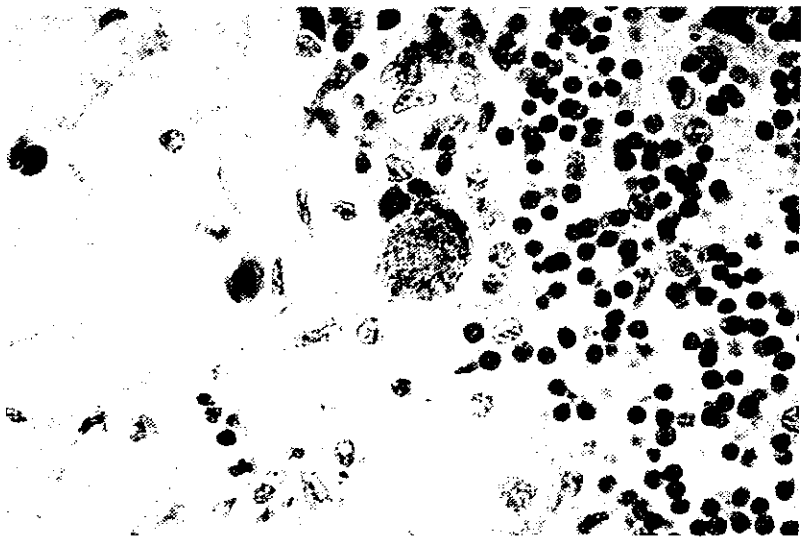

FIG. 2. Study 3: West Nile Virus–specific antigen detected by immunohistochemistry in Purkinje cell in cerebellum of Red-tailed Hawk (*Buteo jamaicensis*).

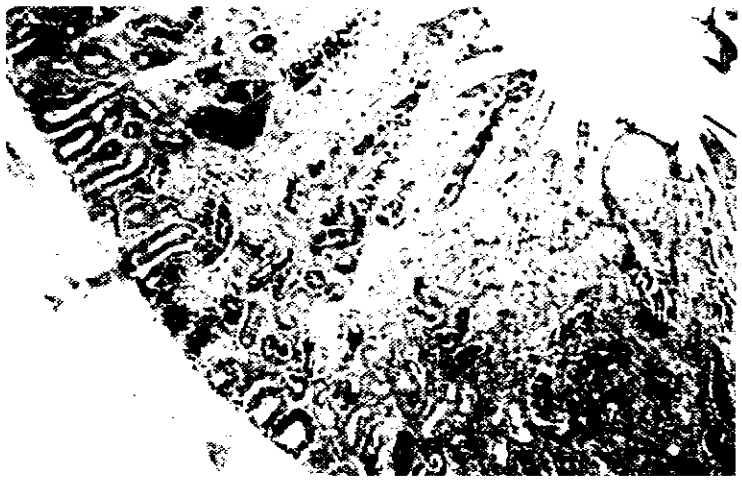

FIG. 3. Study 3: West Nile Virus–specific antigen in epithelia and macrophages in small intestine of American Crow (*Corvus brachyrhyncos*)

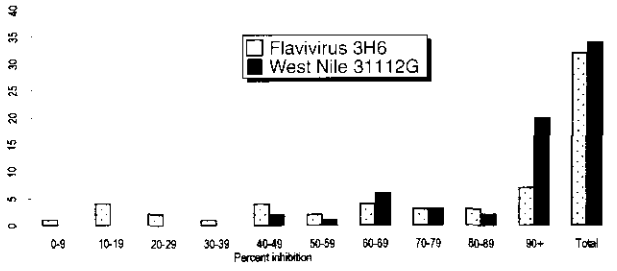

FIG. 4. Percent inhibition by Flavivirus and West Nile antibodies in 36 positive avian blood samples.

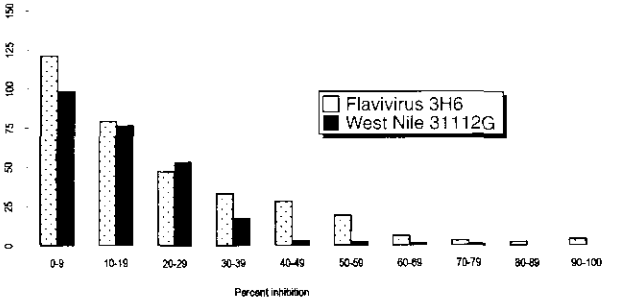

FIG. 5. Percent inhibition by Flavivirus and West Nile antibodies in 342 negative avian blood samples.

ably be lowered to 35% in order to increase the sensitivity of the test.

DISCUSSION

Of 2543 blood samples from wild and domestic birds reported in this study, the blocking ELISA identified 52 samples as positive. In Study 1, 11/12 samples (7/8 chickens and 4/4 corvids) were previously found positive at CDC for WN-specific antibodies by PRNT. In Study 4, 9/10 experimentally infected Mallard ducks were correctly identified by the WN-specific blocking assay (Table 6). These numbers were too small to claim definitively that the specificity of the assay is 90%, but these are encouraging results, considering that many of the blood spots tested had been in cold storage for 4–6 months prior to testing. These results as well as those previously reported by Hall (2000) with chicken sera and by Blitvich et al. (2003) in wild birds clearly demonstrate that the blocking ELISA is highly specific for WNV.

In one instance (Study 1), the PRNT was positive while the blocking ELISA was negative. This is not uncommon since antibodies produced during the course of infection are of different isotypes and are directed toward different antigenic epitopes. Such tests as HI and ELISA will detect antibodies directed at some but not all antigen epitopes, whereas the PRNT,

TABLE 6. STUDY 4: DISTRIBUTION OF BROAD FLAVIVIRUS AND WEST NILE ANTIBODIES IN EXPERIMENTALLY INFECTED MALLARD DUCKS (*ANAS PLATYRHYNCHOS*)

Laboratory number	*Species number*	*Treatment*	*Broad flavivirus % inhibition*	*End point titer*	*West Nile epitope % inhibition*	*End point titer for West Nile*	*Elisa result as reported prior to disclosure*
02-AV-0001	XAP00-402	WNV-16399-3	60%	1:80	59%	1:40	Positive
02-AV-0002	XAP00-403	Control	No inhibition	NA	37%	1:20	Negative
02-AV-0003	XAP00-404	WNV-16399-3	39%	1:20	57%	1:20	Positive
02-AV-0004	XAP00-405	Control	No inhibition	NA	26%	1:20	Negative
02-AV-0005	XAP00-406	Control	No inhibition	NA	No inhibition	NA	Negative
02-AV-0006	XAP00-407	WNV-16399-3	48%	1:40	No inhibition	NA	Negative*
02-AV-0007	XAP00-408	WNV-16399-3	70%	1:80	72%	1:160	Positive
02-AV-0008	XAP00-409	Control	No inhibition	NA	No inhibition	NA	Negative
02-AV-0009	XAP00-410	WNV-16399-3	60%	1:160	60%	1:40	Positive
02-AV-0010	XAP00-411	WNV-16399-3	51%	1:160	64%	1:20	Positive
02-AV-0011	XAP00-412	WNV-16399-3	68%	1:320	45%	1:320	Positive
02-AV-0012	XAP00-413	WNV-16399-3	81%	1:320	65%	1:320	Positive
02-AV-0013	XAP00-414	WNV-16399-3	65%	1:320	50%	1:320	Positive
02-AV-0014	XAP00-415	WNV-16399-3	65%	1:80	53%	1:40	Positive
02-AV-0015	XAP00-416	Control	No inhibition	NA	19%	1:20	Negative
02-AV-0016	XAP00-417	Control	No inhibition	NA	No inhibition	NA	Negative

*Reported as negative for West Nile; NA, nonapplicable.

which involves a viral replication scenario, is more likely to detect all possible types of antibodies. Depending on the timing of serum collection, the PRNT thus will detect antibodies not yet detectable by conventional serological methods. In some cases, the neutralization may not be successful because of a variety of variables such as the sample itself, which may require the addition of accessory factor to restore its neutralizing capacity (Chappel et al. 1971). In such a case, a serum will be positive by ELISA and negative by PRNT (Study 2, one American crow).

The range of percent inhibition obtained for the 2543 sera in this study (Figs. 4 and 5) illustrates clearly that an inhibition of ≥45% is indicative of the presence of WN-specific antibody and allows the elimination of other sera as non-specific reactions.

Our study also reaffirms the confirmatory value of detecting WN-specific antigen in bird tissues by WN-specific immunohistochemistry (Steele et al. 2000, Ellis et al. 2002). Indeed, complete correlation of clinical signs of WN infection, detection of WN virus-specific antibody by blocking ELISA, and visualization of WN antigen in selected tissues further reinforced the diagnostic value of the blocking ELISA.

It could be argued that the blocking ELISA, which does not discriminate between IgG and IgM antibodies, will not provide evidence of a recent infection. However, the duration and fate of avian IgM antibodies following an infection with a neurotropic virus like WN remains to be assessed in various species of wild birds. Indeed, the interpretation of IgM-positive results might be problematic in non-epidemic years. In the scenario of birds infected with WN in areas where SLE is known to reoccur, the determination of IgM in avian sera might be irrelevant.

The blocking ELISA format has proven useful in testing a wide variety of avian species, without the need for species-, genus- or family-specific antibody-enzyme conjugates (Blitvich et al. 2003). In addition, ELISA plates coated with inactivated WN antigen can safely be stored at −20°C for as long as 6 months without deleterious effects. This assay fits well in our repertoire of serological techniques for WN diagnosis.

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