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Methods for Detection of West Nile Virus Antibodies In Mosquito Blood Meals

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Abstract

We describe and compare 2 qualitative serologic techniques for detecting West Nile virus (WNV)-specific antibodies in mosquito blood meals. The techniques are the biotin microsphere immunoassay (b-MIA) and the inhibition platform of the VectorTest™ WNV antigen assay (VecTest-inhibition). To demonstrate the ability of these tests to detect WNV-neutralizing antibodies, we experimentally exposed feeding mosquitoes to blood containing 5 concentrations of 6B6C-1, a flavivirus-neutralizing monoclonal antibody. Antibody concentrations were quantified using the 90% plaque-reduction neutralization test ($PRNT_{90}$). After 24 h of blood-meal digestion at 22.5°C, the threshold $PRNT_{90}$ titer of detection was ≤18 for b-MIA and ≤50 for VecTest-inhibition. Both tests reliably detected antibodies in 3 of 3 blood meals that had been digested for up to 30 h, or were about 25% digested. The b-MIA was also applied to mosquitoes that had engorged on avian blood in Arizona following a WNV epidemic in 2010. There was no significant difference in the WNV antibody prevalence determined by b-MIA (52% of 71 avian blood meals) compared to the WNV-neutralizing antibody prevalence in birds determined by direct sampling (49% of 234 birds). VecTest-inhibition requires fewer resources and may be used in the field without a laboratory, but consumes the entire blood meal and relies on subjective interpretation of results. The b-MIA requires a laboratory and sophisticated equipment and reagents. Results for b-MIA are analyzed objectively and can be applied to mosquito blood meals with greater confidence than the VecTest-inhibition method and thus can contribute substantially to research and surveillance programs that would benefit from the detection of specific WNV antibodies in mosquito blood meals.

Keywords

Mosquito; blood meal; antibody; West Nile virus; surveillance

Introduction

West Nile virus (WNV) (*Flaviviridae*; *flavivirus*) is an important pathogen of humans, domestic animals, and wildlife (Kilpatrick 2011). As such, this virus is the subject of research, surveillance, and public health intervention. Specific antibody detection methods are often used to determine infection rates in humans and other vertebrate animals as part of the intelligence-gathering effort employed during surveillance and research activities (Komar 2001). Traditionally, to capture these data, large numbers of vertebrate hosts must be sampled. Obstacles to this approach include the requirement for expertise in animal

capture, handling, and identification and the erroneous assumption that animals sampled are equally exposed to infectious mosquito bites.

Mosquitoes are known to be biased in their host utilization (Klowden 1996). This bias has a significant impact on WNV transmission dynamics (Simpson et al. 2012). The force of transmission of any mosquito-borne pathogen is exponentially proportional to the rate of contact between vector and host. This is due to the requirement of vector–host contact for pathogen transmission from both an infectious vector to a virus-amplifying vertebrate host, and an infectious vertebrate to a susceptible vector (Macdonald 1957). Mosquito blood meals result from these contacts, so knowing the potential for each contact to result in transmission is useful for understanding the local force of transmission. The presence of virus-specific antibodies in the host's blood inhibits transmission, but most serologic techniques for detecting these antibodies require a sample volume larger than the quantity of blood consumed in a mosquito blood meal. Therefore serologic evaluation of vertebrate populations has relied on direct sampling of the vertebrates. The resulting estimate for seroprevalence has been used for inferring the probability that a vector encounters an immune blood meal.

Direct sampling of the blood meals acquired by local mosquito vectors would provide a more precise estimate of the probability (ϕ) that a vector encounters an immune host, or the complementary probability ($1 - \phi$) that a vector encounters a susceptible host. Technological advances now permit assessment of specific WNV reactivity in small volumes of blood, such as are found in mosquito blood meals, which typically contain 1–3 μl. We provide proof-of-principle for 2 qualitative serologic techniques for the detection of WNV-specific antibodies in mosquito blood meals. The techniques are the biotin microsphere immunoassay (b-MIA) and the inhibition platform of the VectorTest™ WNV antigen assay (VecTest-inhibition).

Materials and Methods

Blood-engorged colonized mosquitoes

Defibrinated goose blood (Colorado Serum Co., Denver, CO) was spiked with 300 μl, 150 μl, 75 μl, 37.5 μl, and 18.75 μl (in a total volume of 3 μl blood) of a concentrated 5 mg/ml solution of 6B6C-1, a mouse-derived monoclonal antibody with strong flavivirus-neutralizing activity (Roehrig et al. 1983), obtained from the diagnostic laboratory of the World Health Organization Collaborating Center on Arbovirus Diagnostics and Reference located at the Centers for Disease Control and Prevention (CDC) Division of Vector-Borne Diseases, in Fort Collins, CO. Each concentration of antibody-spiked blood was fed to several hundred colonized female *Culex quinquefasciatus* Say (Sebring strain) mosquitoes using a Hemotek membrane feeding system (Hemotek Membrane Feeding Systems, Accrington, Lancashire, United Kingdom) that was placed over caged mosquitoes. Mosquitoes were given approximately 30 min to obtain a blood meal. After 30 min the blood was removed and the cage was placed in an environmentally controlled chamber for the engorged mosquitoes to digest their blood meals. The chamber was set to 22.5°C to simulate average nightly temperatures during the arbovirus transmission season in northern

Colorado. Nine engorged mosquitoes were collected for each antibody concentration and frozen at −80°C at 6-h intervals postfeeding beginning at 12 h and continuing through 54 h.

Field-collected mosquitoes

Blood-engorged mosquitoes were collected by CDC mosquito resting traps beneath a house sparrow *Passer domesticus* L., communal roost in Maricopa County, AZ (Panella et al. 2011). Mosquitoes were identified to species, and size of undigested blood meal was recorded as full, ¾, ½, or less. Mosquitoes with ½ to full blood meals were chosen for maceration and host species identification by polymerase chain reaction amplification of the mitochondrial cytochrome oxidase I gene and/or cytochrome B gene and nucleotide sequencing following previously described methods (Kent et al. 2009), except that maceration followed the protocol described below. Those blood meals that were sized as ¾ or full were selected for additional testing by b-MIA to detect WNV-specific antibodies.

Biotinylation of blood meals

Engorged mosquito abdomens were removed with forceps and placed individually in a grinding tube with 500 µl of 10× phosphate-buffered saline (PBS) and a zinc-coated BB pellet. Abdomens were homogenized using a MixerMill® MM300 (Retsch-Allee 1-5, Haan, Germany) set at 20 cycles/sec for 3 min. Homogenates were clarified by centrifugation at 10,000 rpm for 3 min. Antibodies in these samples were then labeled with biotin to provide a means of virus-specific antibody detection, following the protocol described by Basile et al. (2010) with minor modifications. Briefly, 55 µl of mosquito abdomen homogenate or control media was loaded into each well of a 100,000-molecular-weight-cutoff filter plate (Acroprep 96 Omega 100K; VWR Scientific, San Francisco, CA) and supplemented with 5 µl of 5.55 mg/ml sulfo-LC-biotin (Pierce, Rockford, IL). The filter plate was incubated at room temperature for 30 min on a rotary plate shaker (Lab-Line Instruments, VWR Scientific) at 800 rotations/min (rpm). Biotinylated antibodies were retained in the wells and unwanted components were removed by vacuum filtration. Samples/controls were subsequently washed in the filter plate using 100 µl PBS and then resuspended in 60 µl PBS. The entire volume (60 µl) of each sample/control was added to a low-binding 96-well plate and diluted with 60 µl of Candor Low Cross buffer (Boca Scientific, Boca Raton, FL).

Biotin microsphere immunoassay

In a 96-well filter plate (Millipore Corp., Billerica, MA), a 50-µl volume of each diluted biotinylated sample was added to its corresponding well containing 50 µl suspension of washed microspheres, prepared as previously described (Basile et al. 2010). We used microsphere set 32 (Radix Biosolutions, Georgetown, TX) conjugated to either West Nile viral antigen or its corresponding normal control antigen (Hennessey Research, Kansas City, MO). Samples were allowed to react with the antigens/microspheres on the plate shaker set at 800 rpm for 45 min at room temperature, then washed twice with 100 ml PBS–bovine serum albumin (BSA) 1% solution using a vacuum manifold, and resuspended in 50 ml of streptavidin–phycoerythrin (Jackson Immunoresearch, West Grove, PA). Plates were again shaken for 15 min at 800 rpm at room temperature, washed twice, and finally resuspended in 100 µl of PBS–BSA 1%. Presence of antibodies was measured with a BioPlex instrument

(Bio-Rad Laboratories, Hercules, CA), which uses a duality of lasers to identify the microsphere sets and their associated biological reactions based on the phycoerythrin-labeled streptavidin that binds biotin. The results were generated in terms of median fluorescent intensities (MFIs) representing 100 microspheres per sample. A positive sample had a mean antibody-induced MFI (MFI from WNV antigen-coated microspheres minus MFI from normal antigen-coated microspheres) derived from triplicate testing that was ≥ mean MFI of 3 negative controls (mosquitoes fed on unspiked blood) plus 3 standard deviations. Each assay used an undigested mosquito blood meal spiked with the flavivirus-neutralizing monoclonal antibody 6B6C-1 (highest concentration) as a positive control.

VectorTest West Nile Virus Antigen Assay inhibition platform

The VecTest-inhibition assay follows the protocol for antigen detection provided by the manufacturer for the VectorTest WNV antigen assay (Vector Test Systems, Inc., Thousand Oaks, CA). This protocol uses a paper-based wicking test for rapid (15-min) detection of WNV antigen in mosquito pools or other biological samples. The protocol was modified to detect WNV-reactive antibody in biological samples by adding a known quantity of WNV antigen into the assay buffer, such that the antigen detection function of the assay now signaled a negative result rather than a positive result (Cheng and Su 2011). Inactivated WNV cell-lysate–derived antigen was added to the assay buffer at a 1:4,000 dilution, determined empirically. An antibody-negative control assay developed 2 purple bars on the test strip: a control line indicated that the gold-labeled monoclonal WNV-specific detector antibody on the test strip was functional, and a test line indicated that WNV antigen was detected in the assay buffer. Antigen was produced by sonication of WNV-infected Vero cells as previously described (Pauvolid-Corrêa et al. 2014). For detection of WNV-reactive antibodies in mosquito blood meals, the engorged abdomen was crushed onto a Nobuto strip between a glass microscope slide and a glass cover slip so that any undigested blood was absorbed onto the filter paper. The Nobuto strip was allowed to dry at ambient conditions for at least 1 h. The blood spot was cut off the tip of the strip and placed into a snap-cap tube (supplied by the manufacturer) containing 120 μl of VectorTest assay buffer supplemented with WNV antigen, vortexed 30 sec, and incubated at 37°C for 30 min prior to adding the VectorTest strip. If present in the sample, WNV-reactive antibodies competed with the detector antibody and thus inhibited the deposition of gold on the test line. We used a conservative criterion for a positive result. The presence of any purple coloration at the test line was considered negative, even if it was considerably less intense than the control.

Plaque-reduction neutralization test

Defibrinated goose blood containing different concentrations of 6B6C-1 antibody as described above for feeding colonized mosquitoes was centrifuged briefly for separation of plasma. Plasma samples were diluted 1:50 (for titrations starting at a 1:100 dilution) or 1:5 (for titrations starting at a 1:10 dilution) in BA-1 solution (Hank's M-199 salts, 1% BSA, 350 mg/liter sodium bicarbonate, 100 units/ml penicillin, 100 mg/liter streptomycin, 1 mg/liter amphotericin B in 0.05 M Tris, pH 7.6) and then serially diluted using 6 2-fold dilutions on a 96-well polypropylene test plate. These dilutions were incubated for 1 h at 37°C with an equal volume of virus suspension containing approximately 150 plaque-forming units/0.1 ml of a reference strain of lineage 1 WNV originally isolated in New York in 1999. The

mixtures were then inoculated onto monolayers of Vero cells adhered to 6-well polystyrene culture plates, with each dilution (1:10, 1:20, 1:40, 1:80, 1:160, 1:320, and 1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200) tested in duplicate. Titers of neutralizing antibody were determined by measuring the highest dilution that neutralized 90% of the challenge virus, compared with BA-1 controls. For low-titered preparations of 6B6C-1-spiked blood (with titers between 10 and 40), additional dilutions were prepared to precisely determine the titer. The inoculated cultures were overlayed with 0.5% agarose in nutrient media (10 g/liter yeast extract, 50 g/liter lactalbumin hydrolysate), and incubated for 2 days at 37°C, 5% CO_2. They were then stained using a second overlay of the same formula with 0.004% neutral red dye added for plaque visualization. Plaques were recorded after an additional day of incubation.

Statistical analysis

Exact binomial confidence intervals around proportions (Clopper and Pearson 1934) were calculated using an on-line calculator (Soper 2014). Proportions were compared for detection of significant differences at a significance level of $\alpha = 0.05$ using the chi-square test.

Results

Colonized *Cx. quinquefasciatus* mosquitoes were fed goose blood containing 5 different concentrations of flavivirus group–reactive monoclonal antibody 6B6C-1 under laboratory conditions. The antibody preparations were assayed for WNV-neutralizing activity by plaque-reduction neutralization test (PRNT), and 90% PRNT ($PRNT_{90}$) titers were determined to be 800, 200, 50, 18, and 12. For each titer, a time series of engorged mosquitoes was prepared so that cohorts represented different lengths of blood meal digestion starting at 12 h of digestion and continued at 6-h intervals until digestion was complete at 54 h. Groups of 3 mosquitoes from each digestion-time cohort were tested for WNV reactivity by b-MIA and VecTest-inhibition. Antibodies at all 5 titers were detected by b-MIA, but less reliably after longer digestions (Table 1A). VecTest-inhibition reliably detected antibodies at $PRNT_{90}$ titers of 50 and higher (Table 1B). At 24 h digestion, the threshold titer of detection was ≤18 for b-MIA and ≤50 for VecTest-inhibition. Both tests reliably detected antibodies in blood meals that had been digesting for up to 30 h, or approximately 25% digested (Fig. 1).

To determine whether application of b-MIA to mosquito blood meals yielded similar results to serologic testing of vertebrate blood collected directly from birds trapped close to mosquito collection sites, we compared the prevalence of WNV-neutralizing blood samples collected from house sparrow and other bird species to corresponding prevalence of WNV-reactive antibodies in mosquito blood meals, detected by b-MIA. Sampling of birds and mosquitoes coincided in time and space following a WNV outbreak in Maricopa County, AZ, in 2010. There was no significant difference in the neutralizing antibody prevalence in birds of all ages determined by direct sampling (49% of 234 birds) and the antibody prevalence determined by b-MIA of mosquito blood meals (52% of 71 birds) derived from the same selection of species (species origins of engorged blood was determined by DNA

sequencing of individual blood meals). These results are presented for *P. domesticus* and 6 other species combined, with similar results obtained for both groups (Table 2).

Resources required for b-MIA testing included a biosafety cabinet for safe maceration of a mosquito abdomen, a centrifuge, a BioPlex or similar instrument, associated reagents, microspheres, WNV antigens, and a laboratory to house the equipment. The assay time required for testing a mosquito abdomen using b-MIA was approximately 3 h, which included 10 min for homogenization and clarification by centrifugation. The protocol leaves material available for retesting or for additional testing such as nucleic acid extraction, which is necessary for blood donor (i.e., vertebrate host) identification and for determining the virus infection status of the mosquito abdomen. Throughput capacity is high given that a mixer mill easily handles 48 samples, and the BioPlex unit handles 48 samples for each bead–antigen combination. Erroneous results are unlikely because measurements are determined objectively and testing replicates is feasible.

Resources required for VecTest-inhibition included Nobuto filter strips, microscope slides and cover slips, a temperature-controlled incubator, WNV antigen, and VectorTest WNV antigen assay test strips. Assay time was approximately 2 h, which included absorbing the blood onto a Nobuto filter strip, 1 h for drying, 30 min for elution of antibodies, and 15 min for colorimetric development of the VectorTest WNV antigen assay test strip. No material is available for additional tests. A laboratory is desirable but not required. Throughput capacity is low. Erroneous results can occur because of the inability to test a single mosquito abdomen more than once and because the signal for a positive test result is determined subjectively.

Discussion

Determining the WNV-reactive antibody content in the blood imbibed by a mosquito is of value for a variety of research and surveillance applications. However, the low volume of vertebrate blood in a typical mosquito blood meal (1–3 μl) prohibits using traditional antibody detection methods such as PRNT or enzyme-linked immunosorbent assay. We evaluated 2 alternative antibody detection systems that have been utilized for WNV surveillance and research. Both of these systems, b-MIA and VecTest-inhibition, have been used for detecting WNV-reactive antibodies in vertebrate blood samples, but neither of them had been applied to mosquito blood meals (Basile et al. 2010, Cheng and Su 2011). Both systems are independent of the species of vertebrate blood examined, measure total antibody, and could be modified for use with other viruses beside WNV. Both systems, and particularly b-MIA, successfully detected neutralizing antibody titers within a range (50–800) that is typically found in birds with detectable WNV-neutralizing antibody titers. Komar et al. (2005) cited $PRNT_{90}$ titers detected in wild birds to fall within a range of 40–320. Because we were successful in demonstrating the ability of both these techniques to detect WNV-neutralizing antibody in the small volumes of blood found in a mosquito blood meal, their applicability extends to other arboviruses as well.

The VecTest-inhibition assay presents significant limitations, such as the inability to retest a specimen, the subjectivity inherent in the assay and the potential for erroneous results. For

example, very weak lines observed in the VecTest-inhibition assay are herein considered negative for detection of WNV-specific antibodies, but they could be considered equivocal, and may in fact indicate very low antibody titers (i.e., lower than our detection limit of 50). Yet, the low resource requirement, rapid speed of the assay, and ability to apply the test in the field without the need for a laboratory makes this a potentially highly desirable test. However, when a laboratory and sufficient resources are available, the b-MIA appears to be the antibody detection test that can be applied to mosquito blood meals with greatest confidence and thus can contribute substantially to research and surveillance programs that would benefit from the detection of specific antibodies in mosquito blood meals. In particular, the b-MIA would be the test of choice if acquisition of additional data derived from the blood meal is desirable, such as identification of the vertebrate donor species and estimation of the viral infection rate of the mosquitoes being tested.

Although we demonstrated that WNV-reactive antibody prevalence rates in field-collected mosquitoes were similar to those derived by direct sampling from the associated population of birds, we note also that these measures are biologically distinct. The latter is often utilized to represent the seroprevalence of a pathogen. However, this measurement in mosquito blood meals is a biased representation of the seroprevalence rate. Rather, it measures the immunity rate encountered by vector mosquitoes. It differs from seroprevalence because individual vertebrate hosts are not necessarily fed upon by equal numbers of mosquitoes. Multiple vectors may in fact derive their blood from a subset of one or more hosts that are most accessible to these vectors for blood meal acquisition. For modeling purposes, this measurement is in many ways more useful than seroprevalence. For example, if a sick, defenseless vertebrate becomes available to host-seeking vectors, these vectors may primarily feed from this one host. The epidemiological outcome could depend greatly on the immune status or the infectious status of this one individual host. Models that depend on seroprevalence derived from direct sampling of vertebrate populations would not take into account the skewed effects on transmission due to the availability of this important subset of accessible blood meal hosts. Furthermore, this measure of immunity in the blood imbibed by vectors will reflect the contribution of cryptic hosts that are difficult to sample by traditional serological surveys.

A final consideration is the relative difficulty of sampling blood directly from vertebrates versus sampling freshly engorged mosquitoes. Both sampling efforts present challenges. Obstacles to direct sampling are the need for biologists and/or veterinarians with experience in handling and identification of a variety of wildlife. Numerous layers of permissions are required for direct sampling, including from property owners, state and federal authorities, and institutional animal use committees. Adding humans to the list of targeted vertebrates requires a separate set of professionals involved in collecting blood, such as clinicians and epidemiologists, and a separate set of permissions, including consent forms and human use review boards. Obstacles to sampling engorged mosquitoes include the need for experienced field entomologists who can locate and trap engorged mosquitoes, and trained technicians or taxonomists who can identify mosquito species. The determination of which sampling effort is more feasible depends on the resources available.

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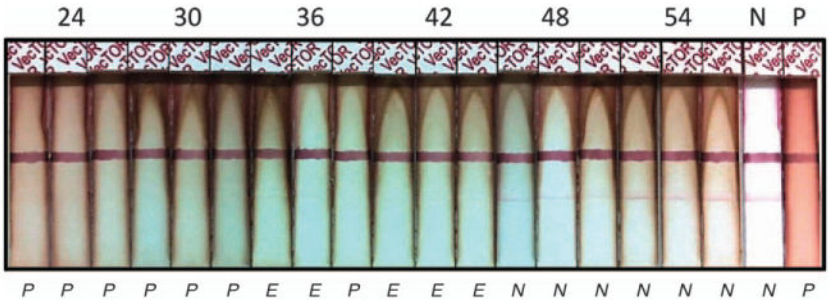

Fig. 1.

Results of VectorTest inhibition for mosquito blood meals containing 6B6C-1 monoclonal antibody ($PRNT_{90}$ titer = 200) in goose blood following mosquito digestion at 22.5°C at 6-h intervals from 24 to 54 h (12 and 18 h tests not shown). Interpretation of the result is provided below each test strip with *P* for positive, *N* for negative, and *E* for equivocal. N, negative control; P, positive control.

Table 1

Detection of WNV[1]-reactive monoclonal antibody 6B6C-1 in mosquito blood meals, following temperature-controlled digestion, by (A) b-MIA and (B) VecTest-inhibition. Three mosquitoes were tested at each time point. +/+/+ indicates that all three mosquitoes tested positive. Time points were at 6-h intervals from 12 to 54 h post–blood ingestion. Antibody titers in the blood fed to mosquitoes was determined by $PRNT_{90}$.

	Blood meal digestion at 22.5°C (h)							
$PRNT_{90}$ antibody titer	**12**	**18**	**24**	**30**	**36**	**42**	**48**	**54**
(A) b-MIA								
800	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/−
200	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
50	+/+/+	+/+/+	+/+/+	+/+/+	+/+/−	+/+/−	−/−/−	−/−/−
18	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	−/−/−	−/−/−
12	+/+/+	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−
(B) VecTest-inhibition								
800	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	−/−/−	−/−/−	−/−/−
200	+/+/+	+/+/+	+/+/+	+/+/+	+/−/−	−/−/−	−/−/−	−/−/−
50	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	−/−/−	−/−/−
18	−/−/−	+/−/−	−/−/−	−/−/−	−/−/−	−/−/−	−/−/−	+/−/−
12	−/−/−	−/−/−	−/−/−	+/−/−	−/−/−	+/−/−	−/−/−	+/−/−

[1] WNV, West Nile virus; b-MIA, biotin microsphere immunoassay; VecTest-inhibition, the inhibition platform of the VectorTest™ WNV antigen assay; $PRNT_{90}$, 90% plaque-reduction neutralization test.

Table 2

Comparison of WNV[1] seroprevalence by neutralization testing[2] ($PRNT_{90}$) to WNV-reactive antibody rate in mosquito blood meals (determined by b-MIA) for house sparrow (*Passer domesticus*) and all other bird species[3] at a WNV outbreak location in Maricopa County, AZ, in 2010.

Species	Antibody detection method	Positive/total (%)	95% confidence interval (%)	*P*-value (chi-square statistic)
House sparrow *Passer domesticus* L.	$PRNT_{90}$	56/107 (52.3)	[42.5, 62.1]	$P = 0.52$ (0.4042)
	b-MIA	30/52 (57.7)	[43.2, 71.3]	
All other birds[3]	$PRNT_{90}$	59/127 (46.5)	[37.6, 55.5]	$P = 0.43$ (0.6168)
	b-MIA	7/19 (36.8)	[16.3, 61.6]	

[1] WNV, West Nile virus; b-MIA, biotin microsphere immunoassay; VecTest-inhibition, the inhibition platform of the VectorTest™ WNV antigen assay; $PRNT_{90}$, 90% plaque-reduction neutralization test.

[2] The neutralizing antibody data had been published previously (Komar et al. 2013).

[3] "All other birds" includes house finch (*Haemorhous mexicanus* Müller), mourning dove (*Zenaida macroura* Linnaeus), great-tailed grackle (*Quiscalus mexicanus* Gmelin), Eurasian collared-dove (*Streptopelia decaocto* Frivaldsky), curve-billed thrasher (*Toxostoma curvirostre* Swainson) and brown-headed cowbird (*Molothrus ater* Boddaert).