Detection by Enzyme-Linked Immunosorbent Assay of Antibodies to West Nile virus in Birds

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We adapted an indirect immunoglobulin G enzyme-linked immunosorbent assay to facilitate studies of West Nile virus (WNV) and evaluated its application to taxonomically diverse avian species. Anti-WNV antibodies were detected in 23 bird species, including many exotic species, demonstrating its value in studies of WNV epizootiology.

West Nile virus (WNV) is transmitted in an enzootic cycle between *Culex* spp. mosquitoes and their avian hosts (1–4). Sentinel birds have long been used in arbovirus surveillance (5–9), and serologic surveys of wild and captive birds are valuable in determining whether an arbovirus is present in a particular locality (10). While plaque-reduction neutralization tests (PRNT) are the standard for arbovirus serologic testing, they are frequently unavailable in many laboratories for several reasons: They generally require high levels of biocontainment; they are time-, labor-, and cost-intensive; and they require specialized technical expertise. A rapid serologic diagnostic assay suitable for screening large numbers of specimens and posing minimal biohazard would facilitate large-scale avian-based serologic surveillance for WNV. Accordingly, we sought to determine whether an indirect enzyme-linked immunosorbent assay (ELISA) designed to detect seroreactivity against *St. Louis encephalitis virus* (SLEV) and *Western equine encephalomyelitis virus* (WEEV) (11) could be modified to detect anti-WNV antibodies in taxonomically diverse wild-caught and captive avian species.

To produce ELISA antigen, Vero cells were infected with WNV and processed into antigen as described (12), with New York–derived reference stocks of WNV (31000365; see Ebel et al. [13] for source and sequence information). Fifty microliters of antigen diluted 1:100 in fresh coating buffer (0.015M Na₂CO₃, 0.035M NaHCO₃, pH 9.6) was applied to each well. Plates with test specimens were returned to a humid chamber and incubated at 37°C for 1 h. Following incubation, blocking solution was discarded and test samples, diluted 1:100 in PBS with 0.05% Tween, and 0.5% bovine albumin (PBS-T-BA), were applied to one negative and two positive antigen-containing wells. Plates with test specimens were returned to a humid chamber and incubated at 37°C for 1 h. Following incubation, plates were removed, washed as above, and 50 µL of horseradish peroxidase–conjugated goat anti-wild bird immunoglobulin (Ig) G (Bethyl Laboratories, Inc., Montgomery, TX), diluted 1:1000 in PBS-T-BA, was applied to each well. After incubation and washing as above, plates were developed with 50 µL of tetrathymethylbenzidine (TMB)-peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) for 7 min. The reactions were stopped with 50 µL of 1:20 H₂PO₄, and the optical density (OD) of each well was read at 450 nm. Blank (no test sera), positive, and negative controls were included on each plate. To compute the positive/negative (P/N) value of each sample, we divided the mean OD of positive antigen-containing wells by the OD of the negative antigen-containing wells. Samples with a P/N value ≥2 were considered positive and were tested further by PRNT(14). Specimens were confirmed positive if their 90% neutralization titer against WNV was at least fourfold greater than against SLEV, a closely related flavivirus that may cross-react with WNV antigens in screening assays (15,16).

Optimum concentrations of antigens for the ELISA were determined by applying known positive and negative chicken samples to wells containing serial twofold dilutions of antigen. Optimal concentrations were defined as those yielding the highest mean P/N value for known positive samples and P/N values closest to unity (one) for known negative samples. Generally, a 1:100 dilution of the crude antigens was optimal. Using a similar strategy, we then determined the optimal serum dilutions for pigeon and wild bird sera.

Specimens for testing were either donated from the collection at the Bronx Zoo or collected during an avian surveillance project conducted in New York City during 2001. Avian blood samples were collected as whole blood and stored at 4°C, centrifuged for 10 min at maximum speed in a microcentrifuge, and serum was separated. In some cases, samples were collected and heparinized, and plasma was separated and stored as described previously.

PRNT testing was conducted according to standard protocols (14). Briefly, where sample quantities permitted, test sera were serially diluted from 1:5 through 1:160 in BA-1 diluent (M199H, 0.05M Tris pH 7.6, 1% bovine serum albumin, 0.35g/L sodium bicarbonate, 100 units/mL penicillin, 100 µg/mL streptomycin, and 1 µg/mL fungizone) and 100 µL was...
incubated overnight at 4°C with 100 μL of virus containing approximately 200 PFU of WNV (strain 31000365) or SLEV virus strain no. 59268 (Parton). If insufficient sample was available, higher starting dilutions (usually 1:10) were used. In the morning, 100 μL of each serum-virus mixture was added onto confluent monolayers of Vero cells and allowed to adsorb at 37°C for 1 h. Following incubation, a nutrient-agar overlay was added, and the plates were returned to the incubator. PRNT testing for WNV used a single basal medium Eagle–based overlay containing neutral red, while SLEV testing required application of a double overlay, the first without and the second with neutral red, applied 3 days after the first. Plaques were counted on the 3rd (WNV) or 5th (SLEV) day after the test was initiated. The highest dilution of serum neutralizing 90% of the inoculum as determined by back-titration was considered the neutralizing titer.

All statistical analyses were done with Microsoft Excel (Microsoft Corp., Redmond, WA).

The predictive value of a positive test (PVP) and of a negative test (PVN) were determined by using the sera of birds caught in mist nets during a WNV serologic survey conducted during the summer of 2001 (manuscript in preparation). Of 3,581 specimens tested, 233 (7%) were ELISA positive. Of these positive specimens, 163 (70%) were also positive by PRNT, for a PVN of 100%. Further, all ELISA-negative specimens were tested for neutralizing antibody by PRNT. PRNT-positive specimens were confirmed by PRNT. The high values in the reactive well may have occurred as a result of technical error (e.g., splashing). Alternatively, the ELISA may be more sensitive than neutralization and may detect anti–WNV antibodies that PRNT does not. We always performed a confirmatory test to resolve true from false positives; nonetheless, this ELISA dramatically reduced the number of confirmatory tests we conducted during WNV surveillance in 2000 and 2001. Use of the ELISA described here yielded substantial cost reduction and time savings compared with screening specimens by PRNT.

This ELISA detected anti–WNV antibody in a taxonomically diverse array of captive and wild birds. In 23 species from 12 avian orders, IgG antibodies were detectable by using commercially available anti-wild bird horseradish peroxidase–conjugated antibodies. The breadth of the reactivity of this conjugate was surprising, given that it was generated by using IgG isolated from the sera of four species representing only four avian orders: Passeriformes, Columbiformes, Galliformes, and Anseriformes (11). Although this protocol has been documented to react broadly in an ELISA to detect SLEV antibody in 13 species representing seven orders (11), known positive sera from three orders (Ciconiiformes, Gruiformes, and Charadriiformes) were not detected. We obtained positive results for each of these orders. The reasons for this discrepancy in our results are not clear but may be related to differences in the antibody titer of the specimens we tested or to general differences in the immune response to WNV compared with SLEV. Alternatively, some of the measures we took to optimize our test (e.g., the substitution of tetramethylbenzidine peroxidase substrate for 2,2′-azino-di-[3-ethylbenzthiazoline-6-sulfonate]) may have increased the assay’s sensitivity, allowing detection of fewer bound conjugated antibodies, as may occur with test sera derived from divergent avian species. The lack of correlation between P/N values with PRNT titers is not surprising given that the P/N value was obtained from a single serum dilution and does not represent an endpoint titer. Although this serologic method should be evaluated for each avian order tested, our results demonstrate that this testing protocol is appropriate for WNV serologic surveys of free-ranging and captive sentinel birds.

Conclusions

The PVP of this assay appears to be somewhat lower than that of another reported ELISA protocol (17) and some other flavivirus serologic assays, such as PRNT, but is higher than that reported for the assay from which it was derived (11). The PVP of our test might have been higher had we more stringently evaluated our ELISA-positive specimens: a number of specimens had P/N values ≥2 because one of the two positive antigen wells was highly reactive. None of these specimens were confirmed by PRNT. The high values in the reactive well may have occurred as a result of technical error (e.g., splashing). Alternatively, the ELISA may be more sensitive than neutralization and may detect anti–WNV antibodies that PRNT does not. We always performed a confirmatory test to resolve true from false positives; nonetheless, this ELISA dramatically reduced the number of confirmatory tests we conducted during WNV surveillance in 2000 and 2001. Use of the ELISA described here yielded substantial cost reduction and time savings compared with screening specimens by PRNT.

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### Table. Comparison of serologic assay results with reactivity across a range of avian orders and species

| Common Name               | Species          | Order          | Indirect ELISA P/N<sup>a</sup> | PRNT titer |
|---------------------------|------------------|----------------|----------------------------------|------------|
|屋燕 #1                     | *Passer domesticus* | *Passeriformes* | 3.0                              | 40         |
|屋燕 #2                     | *P. domesticus*   | *Passeriformes* | 3.0                              | 20         |
|屋燕 #3                     | *P. domesticus*   | *Passeriformes* | 7.2                              | 80         |
|北方模翅鸟                   | *Mimus polyglottos* | *Passeriformes* | 2.1                              | >10        |
|欧洲星燕                   | *Sturnus vulgaris* | *Passeriformes* | 3.0                              | 40         |
|美国乌鸦 #1                 | *Corvus brachyrhynchos* | *Passeriformes* | 2.8                              | 80         |
|美国乌鸦 #2                 | *C. brachyrhynchos* | *Passeriformes* | 2.0                              | 20         |
|老鹰鸽 #1                  | *Columba livia*   | *Columbiformes* | 2.9                              | 80         |
|老鹰鸽 #2                  | *C. livia*        | *Columbiformes* | 2.5                              | 320        |
|老鹰鸽 #3                  | *C. livia*        | *Columbiformes* | 7.6                              | 10         |
|白额雕          | *Grus vipio*      | *Gruiformes*    | 5.3                              | >640       |
|斑嘴鹳 #1                  | *Gerontica eremita* | *Ciconiiformes* | 9.4                              | 320        |
|斑嘴鹳 #2                  | *G. eremita*      | *Ciconiiformes* | 11.4                             | 640        |
|斑嘴鹳 #3                  | *G. eremita*      | *Ciconiiformes* | 4.0                              | 80         |
|黑头夜鹭                   | *Nycticorax nycticorax* | *Ciconiiformes* | 5.2                              | 160        |
|火烈鸟 #1                  | *Phoenicopterus chilensis* | *Phoenicopteriformes* | 3.7                              | 160        |
|火烈鸟 #2                  | *P. chilensis*    | *Phoenicopteriformes* | 4.2                              | >640       |
|大蓝鹭                   | *Argusianus argus* | *Galliformes*   | 7.3                              | 40         |
|肯尼亚冠豚      | *Guttera edouardi* | *Galliformes*   | 6.7                              | 80         |
|火烈鸟                   | *Meleagris gallopavo* | *Galliformes*   | 6.0                              | 320        |
|楔嘴雁                   | *Podiceps melodus* | *Galliformes*   | 3.5                              | >640       |
|黑头野鸡                   | *Pelecanus erythrorhynchos* | *Pelecaniformes* | 5.7                              | >640       |
|黑头鸥                   | *Phalacrocorax bougainvillii* | *Pelecaniformes* | 2.1                              | 80         |
|棕鹈                     | *Pelecanus occidentalis* | *Pelecaniformes* | 3.8                              | 160        |
|普通野鸡                   | *Anser sp.*       | *Anseriformes*  | 3.6                              | 40         |
|大白鹈                     | *Cygnus buccinator* | *Anseriformes*  | 4.0                              | 160        |
|楔尾鹭                   | *Strix varia*     | *Strigiformes*  | 2.6                              | 160        |
|鸵鸟                     | *Struthio camelus* | *Struthioniformes* | 3.9                              | 640        |
|麦哲伦企鹅               | *Spheniscus magellanicus* | *Sphenisciformes* | 4.3                              | >640       |
|黑颈鹤                   | *Grus nigricollis* | *Gruiformes*    | 2.4                              | 80         |
|笑鸥                     | *Larus atricilla*  | *Charadriiformes* | 6.9                              | 320        |
|美洲野鸭                   | *Anas sp.*        | *Anseriformes*  | 1.1                              | <10        |
|加拿大鹅                   | *Branta canadensis* | *Anseriformes*  | 1.2                              | <20        |
|美洲鸡                   | *Gallus gallus*   | *Galliformes*   | 0.9                              | <10        |
|SLEV-阳性鸡             | *G. gallus*       | *Galliformes*   | 2.5                              | 20         |
|WEEV-阳性鸡               | *G. gallus*       | *Galliformes*   | 1.3                              | <20        |

<sup>a</sup>ELISA, enzyme-linked immunosorbent assay; P/N, positive/negative ratio; PRNT, plaque-reduction neutralization tests; SLEV, St. Louis encephalitis virus; WEEV, Western equine encephalomyelitis virus.

WNV-negative specimens shown below bold line
Acknowledgments

The authors thank Paul Calle and the Wildlife Conservation Society, Nick Komar, Joseph Burns, and Carrie Dean for generous donation of the specimens included in this study. In addition, we thank the two anonymous reviewers for their helpful and thoughtful comments.

This work was supported by the Wadsworth Center, New York State Department of Health through a grant from the Centers for Disease Control and Prevention.

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