Am. J. Trop. Med. Hyg., 78(1), 2008, pp. 63–69

Rapid Active Assay for the Detection of Antibodies to West Nile Virus in Chickens

Stephanie S. Groves, Michael J. Turell,* Charles L. Bailey, and Victor N. Morozov

The National Center for Biodefense and Infectious Diseases, George Mason University, Manassas, Virginia; US Army Medical Research Institute of Infectious Diseases, Frederick, Maryland; Institute of Theoretical and Experimental Biophysics, Russian Academy of Science, Pushchino, Moscow Region, Russia

Abstract. To reduce the assay time for detecting virus-specific antibodies in serum, we developed microarray-based active immunoassay techniques for detecting West Nile virus (WNV)-specific IgM molecules in chicken blood. The assay uses electrophoretic concentration of IgM molecules onto WNV antigens arrayed on a dialysis membrane followed by detection of bound IgM molecules with functionalized magnetic beads as active labels. This assay takes only 15 minutes and has the same sensitivity as a commercially available human WNV IgM antibody-capture enzyme-linked immunosorbent assay (commonly called a MAC-ELISA) modified for use with chicken sera.

INTRODUCTION

West Nile virus (WNV), a member of the Japanese encephalitis antigenic complex (genus *Flavivirus*, family *Flaviviridae*), can cause a spectrum of human disease ranging from mild febrile illness to fatal neuroinvasive disease.[1–3] Since its introduction into the Western Hemisphere in 1999, WNV has spread rapidly across the entire continental United States, Canada, Mexico, Latin America, and the Caribbean in only 5 years.[4–7] It is believed that WNV is still establishing its geographic distribution, suggesting that large epidemics will continue to occur during the next several years.[8] During epidemics, it is essential that clinically relevant diagnostic tests are available to quickly and accurately diagnose WNV infection. Furthermore, recent findings have shown that the virus can be transmitted by blood and organ donations,[9] breast feeding,[10] intrauterine exposure,[11] and laboratory procedures,[12] emphasizing the importance of developing a rapid and accurate serologic assay for diagnosing WNV infection. Currently, the IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) is the primary serologic assay routinely used for laboratory diagnosis of WNV infection.[13–15] The MAC-ELISA is a valuable tool for the diagnosis of infection because IgM antibody titers are detectable early, rise rapidly in the disease course, and are usually less virus cross-reactive than IgG.[16] However, the MAC-ELISA has two serious limitations. It is a time-consuming test, requiring 2–3 working days to complete because overnight incubations are necessary to enhance sensitivity.[13] Furthermore, the assay requires a great deal of material. Thus, commercially available WNV MAC-ELISA kits for humans range from $610 to $660 dollars for a 24-sample test.[17,18] Considering these problems, alternative immunoassay techniques have been developed such as a microsphere-based immunoassay, in which IgM or IgG antibodies are bound from serum to fluorescent beads, tagged with fluorescently labeled anti-antibodies, and analyzed in a dual-laser cytometer.[19,20] However, this assay still takes 3–4.5 hours to complete, with most of this time spent on incubations.

To reduce the assay time for detecting WNV-specific antibodies in serum even further, we used the recently emerged analytical concept of active assays. In active assays, the slow diffusion-controlled reactions, which make incubation times long, are dramatically accelerated by active transport of soluble analytes to immobilized probe molecules. Electrophoretic delivery of analytes can reduce incubation times from hours to minutes,[21–23] whereas active delivery of labels (functionalized magnetic beads) can reduce detection time to seconds while increasing sensitivity.[24] Use of microarrays in such assays can provide additional advantages of assay multiplication and miniaturization, which reduces cost of valuable consumable materials. Although antigen and antibody microarrays have been used already to profile immune response to pathogens[25] and in diagnostics of infectious diseases,[26,27] such microarrays have never been used in active assays of pathogen-specific antibodies to the best of the authors' knowledge.

To date, elements of the active microarray-based assay have only been shown in model systems made up of solutions of pure antigens and antibodies.[21–24] In this paper, we report application of these methods to the development of an assay for pathogen-specific antibodies in serum samples to show their advantages and limitations. Our primary goal was to test several combinations of microarrays and magnetic bead detection techniques to determine the most efficient combination in terms of sensitivity and assay time for detecting WNV-specific IgM antibodies.

MATERIALS AND METHODS

Preparation of proteins for manufacturing arrays. Anti-chicken IgM antibody (Bethyl Laboratories, Montgomery, TX) was dialyzed overnight against milli-Q water at 4°C until electrical conductivity of the solution reached 25–50 μS cm.[1] The conductivity was determined using a home-made microcell with a volume of 4 μL. The protein concentration of the dialyzed sample was determined by measuring the optical density (OD) at 280 nm using a known extinction coefficient.[28] Concentrated trehalose (Sigma-Aldrich, St Louis, MO) solution was added to the antibody solution to give a 10-fold excess (wt/wt) over the protein concentration. The sample was distributed in 10-μL aliquots and stored at –25°C.

WNV antigens for arrays were obtained from a formalin-inactivated WNV vaccine for equines (Fort Dodge Animal Health, Overland Park, KS). WNV viral particles were first concentrated 100-fold by ultracentrifugation at 70,000 rpm for 1 hour at 4°C. The presence of viral particles in the pellet was verified using atomic force microscopy. The pellet was dialyzed overnight against milli-Q water at 4°C, and concen-

* Address correspondence to M. J. Turell, Virology Division, USAMRIID, 1425 Porter Street, Fort Detrick, MD 21702-5011. E-mail: mturell@erols.com

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 JAN 2008	**N/A**	

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Rapid active assay for the detection of antibodied to West Nile virus in chickens. American Journal of Tropical Medicine and Hygiene 78:63-69	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Groves /S Turell, MJ Bailey, C Morozov, VN	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD	**TR-07-018**
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release, distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

To reduce the assay time for detecting virus-specific antibodies in serum, we developed microarray-based active immunoassay techniques for detecting West Nile virus (WNV)-specific IgM molecules in chicken blood. The assay uses electrophoretic concentration of IgM molecules onto WNV antigens arrayed on a dialysis membrane followed by detection of bound IgM molecules with functionalized magnetic beads as active labels. This assay takes only 15 minutes and has the same sensitivity as a commercially available human WNV IgM antibody-capture enzyme-linked immunosorbent assay (commonly called a MAC-ELISA) modified for use with chicken sera.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**7**	

trated trehalose solution was added to the solution to give a 10-fold excess over the protein concentration. The latter was determined by using a quartz crystal microbalance.[29] The WNV-trehalose mixture was distributed in 10-μL aliquots and stored at –25°C. Part of the viral pellet that was used in non-array experiments was resuspended in phosphate-buffered saline (PBS) and stored at 4°C.

Preparation of substrate. A dialysis membrane from regenerated cellulose (Fisher Scientific, Pittsburgh, PA) was treated as described.[22] Briefly, wet membrane was glued to a plastic O-ring with cyanoacrylate glue. To enhance adhesion, the ring was pre-treated for 20 seconds in a low-pressure discharge in a home-built radio frequency (RF) plasma apparatus. The glued membrane was dried in a stream of air. To avoid occasional cracking of the membrane at low room humidity, prepared dialysis membranes were stored in a closed chamber with a humidity of 85% provided by damp Na_2SO_4. Before electrospray deposition, the dialysis membrane was subjected to a 30-Watt RF plasma discharge for 20 seconds at a pressure of 1–2 Torr.

Fabrication of microarrays by electrospray deposition. The electrospray deposition technique[29,30] was used to fabricate arrays of probe molecules on a plasma-activated dialysis membrane. The protein/trehalose mixture (5–10 μL) was sucked into a capillary, and the latter was placed into an apparatus, similar to that previously described.[30] A voltage of ~4–5 kV was applied to the platinum electrode inside the capillary, with the current never exceeding 100 nA. Deposition was performed at a relative humidity of 20–40%. These conditions were shown to maintain the highest functional activity of the electrospray-deposited proteins.[29] A nylon mesh was used to fabricate protein microarrays with protein spots 30–50 μm in size, spaced by 150 μm.

Immobilization of proteins on a microarray. After protein deposition, the microarray was placed in a chamber at 100% humidity for 30 minutes. Solid dry deposits were converted to microdroplets from which dissolved protein molecules were chemically linked to membrane surface carbonyl groups that had been generated in the plasma discharge. To reduce Schiff bases and block any unreacted carbonyl groups, the membrane was incubated for 45 minutes in 50 mmol/L Tris-HCl, pH = 7.5, containing 1% bovine serum albumin (BSA) and 1% $NaBH_3CN$. The microarrays were washed and stored in a blocking solution (20 mmol/L Tris-HCL, 0.15 mol/L NaCl, 0.1% Tween-20, 1% BSA, and 0.02% NaN_3, pH 7.5) at 4°C.

Functionalization of magnetic beads. To bind anti-chicken IgM and anti-mouse IgG antibodies (Sigma-Aldrich) to 1-μm magnetic beads (New England BioLabs, Ipswich, MA), 0.2 mL of a 0.6% suspension of protein A–coated magnetic beads were washed three times with 0.1 mol/L sodium phosphate buffer, pH 8.0, and mixed with 200 μg of either anti-chicken IgM antibody or anti-mouse IgG antibody. After gentle stirring for 1 hour at room temperature, the suspension was washed three times with 0.1 mol/L sodium phosphate buffer, pH 8.0. Beads were incubated with a solution of 0.2 mol/L triethanolamine and 25 mmol/L dimethyl pimelidate dihydrochloride (DMP), pH 8.2, for 45 minutes at room temperature with gentle agitation. To block excess DMP, the beads were collected and incubated with 0.1 mol/L ethanolamine, pH 8.2, for 1 hour at room temperature with gentle agitation. The beads were washed twice with PBS and resuspended in 200 μL of PBS containing 0.1% Tween-20 and 0.02% NaN_3 and stored at 4°C.

To bind anti-WNV antibody to the magnetic beads, 100 μL of a 0.5% suspension of streptavidin-coated magnetic beads was washed three times with 0.3 mL of PBS and mixed with 30 μg of anti-WNV E protein monoclonal antibody (catalog no. 8150; Chemicon International, Temecula, CA) that was biotinylated as previously described.[31] After overnight incubation at 4°C, the beads were washed three times with PBS and resuspended in 100 μL of PBS containing 0.1% Tween-20 and 0.02% NaN_3 and stored at 4°C.

To bind WNV antigens to magnetic beads, 20 μL of a 0.5% suspension of anti-WNV IgG magnetic beads was washed three times with PBS and mixed with 10 μL of WNV antigen suspension in PBS prepared as described above. After overnight incubation at 4°C, the beads were washed three times with PBS and resuspended in 20 μL of PBS containing 0.1% Tween-20 and 0.02% NaN_3 and stored at 4°C

Infection of chickens with WNV. Chicken serum specimens used in this study were prepared at the US Army Medical Research Institute for Infectious Diseases (USAMRIID). Five 1-day-old male leghorn chickens (Meyer Hatchery, Polk, OH) were inoculated subcutaneously with 0.1 mL of a solution containing $10^{5.1}$ plaque-forming units (PFUs) of WNV (strain: Crow 397-99). This strain of virus had been isolated from a crow that died in Bronx, NY, in 1999 and had been passed twice in Vero cells before use in these studies.[32] Age-matched, uninfected chickens were held in a separate cage as negative controls. In addition, five 1-day-old chickens were inoculated similarly with 10^5 PFU of the ZH501 strain of Rift Valley fever virus (RVFV) to serve as virus-infected, negative controls for the WNV assay. A second cohort of seven 3-day-old male chickens was similarly inoculated with WNV 2 days later. A blood sample (0.2 mL/bleed) was drawn from the jugular vein approximately every other day from each bird up until it died or was euthanized. Of the five 1-day-old birds infected with WNV, only one bird survived to Day 6, at which point it was sick and euthanized. Only two of the older chickens (inoculated when they were 3 days old with WNV) survived for 24 days. Larger samples (~1 mL) were obtained from these individual birds on Days 20 and 24 postinfection (PI). Serum was separated from whole blood. An aliquot of each serum sample was diluted 1:10 in diluent (10% fetal bovine serum in medium 199 with Earle salts, $NaHCO_3$, and antibiotics) and tested by plaque assay to determine the viral titer for each sample. A total of 64 serum samples were transferred to George Mason University. Twenty-three specimens contained histories of infecting virus, virus titers, and Day PI. The remaining 41 samples were blinded. Before removal from USAMRIID, all samples were subjected to 1–2 million rads of gamma radiation to destroy any infectious materials. Samples were stored at –70°C until use.

Microarray-based assay techniques for detecting WNV-specific IgM in serum with magnetic bead detection. Three assay techniques used to detect WNV-specific IgM in serum were specific to the type of microarrays and functionalized magnetic beads used and are summarized in Table 1. Chains of bonds holding magnetic beads on arrays in each assay are schematized in Figure 1.

Passive microarray-based screening. All the assay techniques were first tested and compared in screening of serum samples using a passive format. Droplets of reagent solutions

TABLE 1
Description of microarray-based assay techniques with magnetic bead detection

Assay	Abbreviation	Microarray	Magnetic beads
Reverse array assay*	RAA	WNV antigen (vaccine)	Polyclonal anti-(chicken-IgM)-IgG
IgM capture assay†	MCA	Polyclonal anti-(chicken-IgM)-IgG	Polyclonal anti-mouse-IgG
Modified IgM capture assay‡	MMCA	Polyclonal anti-(chicken-IgM)	Monoclonal anti-IgG WNV-IgG

* In this assay, WNV-specific IgM from serum was captured on an array of WNV antigens. The bound IgM was detected with anti-chicken IgM magnetic beads.
† In this assay, IgM from serum was captured on an array of anti-chicken IgM antibodies. WNV antigen was added to the array. If any of the bound IgM was specific for WNV, the WNV antigen bound to it. Monoclonal anti-WNV antibody was added to bind with the bound WNV antigen. Bound anti-WNV antibody was detected with anti-mouse IgG magnetic beads.
‡ The assay is similar to the MCA assay but required fewer steps. After capturing IgM on an array of anti-chicken IgM antibodies and the addition of WNV antigen, bound WNV antigen was detected directly with anti-WNV magnetic beads.

were placed on a small piece of an array (5 × 5 mm typically) and allowed to react without stirring for a period of time depending on the assay design. Specifics for each assay are presented below. A special holder was designed to accommodate multiple samples and to wash them simultaneously. The holder was composed of a plastic plate with an array of 69 holes 1 mm in diameter. A second plate with an array of grooves was glued to the bottom of the first one. When air was pumped from the space between the plates, the array pieces were strongly pressed to the holes by external pressure.

Reverse assay array. Serum was diluted 1:100 in blocking solution (1% BSA, 20 mmol/L Tris-HCl, 0.15 mol/L NaCl, and 0.05% Tween-20, pH 7.5). Twenty microliters of each sample was incubated on a piece of a WNV antigen array overnight at 4°C. Arrays were washed three times with wash solution (20 mmol/L Tris-HCL, 0.15 mol/L NaCl, and 0.05% Tween-20, pH 7.5) and tested with anti-chicken IgM magnetic beads using the push-pull technique described in the section on detection with magnetic beads below.

IgM capture assay. Serum was diluted 1:100 in blocking solution. Twenty microliters of each sample was incubated on a piece of an anti-chicken IgM array overnight at 4°C. Arrays were washed three times with wash solution and incubated for 2 hours with 20 μL of WNV pellet prepared as described above and diluted 1:100 with blocking solution. Preconcentrated commercial WNV antigen was used to ensure purity of the sample (i.e., to remove preservatives or other components of the vaccine that could inhibit the assay). After washing three times with the wash solution, monoclonal anti-WNV E protein antibody, diluted 1:1,000 in blocking solution, was incubated on the arrays for 1 hour at room temperature. The arrays were tested with anti-mouse IgG magnetic beads using the push-pull technique described in the section on detection with magnetic beads below.

Modified IgM capture assay. Serum was diluted 1:100 in blocking solution. Twenty microliters of each sample was incubated on a piece of an anti-chicken IgM array overnight at 4°C. Arrays were washed three times with wash solution and 20 μL of concentrated killed WNV pellet diluted 1:100 in blocking solution was incubated on the arrays for 2 hours at room temperature. The arrays were tested with anti-WNV IgG magnetic beads using the push-pull technique described in the section on detection with magnetic beads below.

Electrophoresis-assisted active reverse assay array. Electrophoretic concentration of WNV-specific IgM molecules among other charged molecules was performed as recently described.[22–24] Briefly, 7 × 7-mm^2 pieces of WNV antigen microarrays were clamped between two rubber O-rings in an electrophoretic apparatus,[24] and the upper and lower electrode chambers were filled with an electrode solution of 5 mmol/L Gly-Gly buffer, pH 8.5. Serum was diluted 1:10,000 in an electrophoretic buffer consisting of 1% poly vinyl alcohol and 1% poly vinyl pyrrolidone in 5 mmol/L Gly-Gly, pH 8.5. At this pH, IgM molecules became negatively charged. Approximately 300 μL of each sample was loaded into the electrophoretic cells, and electrophoresis was performed at a current of 1.5 mA/cell with 180–260 V applied for 10 minutes. The application of electric field brings all charged IgM molecules to the array surface. Other similarly charged proteins are also brought to the microarray surface, but they are unable to establish strong bonds and are removed with washing (data not shown). After electrophoretic capture, the arrays were washed three times with wash solution and tested with anti-chicken IgM magnetic beads using the push-pull technique described in the section on detection with magnetic beads below.

In all of the assay techniques, sera from uninfected chickens or chickens infected with RVFV otherwise identically treated

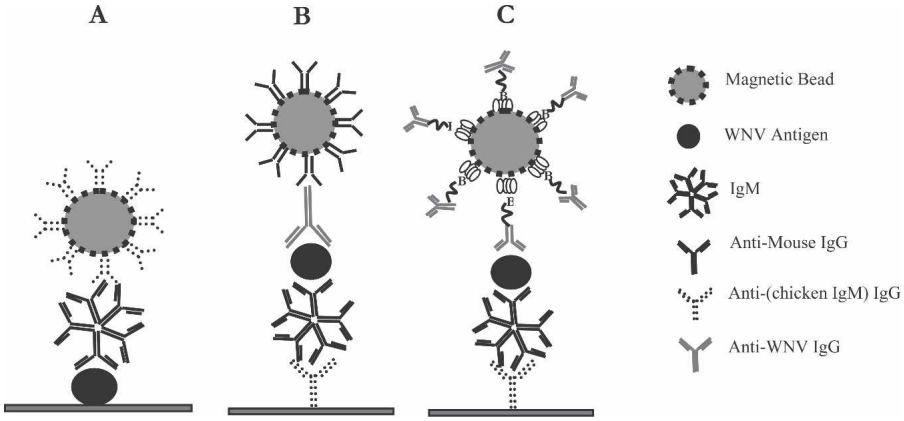

FIGURE 1. Schematic of assay techniques designed to detect WNV-specific IgM in serum. **A**, RAA. **B**, MCA. **C**, MMCA.

(radiation, storage) were used as negative controls. A negative control was deemed successful only if no array of spots marked with beads was visible on assay completion. Sera from chickens infected with WNV and bled on Day 4 or more PI were used as positive controls in the assay development.

Detection with magnetic beads. In the bead-based assays used here, the so-called "push-pull" detection technique was used as described.[24] Briefly, a droplet of blocking solution was applied onto the array, and 0.5 μL of bead suspension in the same solution was distributed over the array surface. A rare-earth permanent magnet (5–10 mm in diameter) was placed underneath the array for 5–10 seconds to push the beads to the array surface. The magnet was removed, and another magnet equipped with a stainless steel magnetic concentrator was approached from above to pull unbound and weakly bound beads to the top of the droplet. The beads were removed from the droplet by touching the droplet surface with the concentrator tip. The stock suspension of magnetic beads was washed twice with the blocking solution before use to remove any free antibody molecules that leaked from the beads on storage. Images of arrays with bound beads were acquired with a digital camera attached to an optical microscope equipped with a dark-field illuminator. To quantify the results, SCION/NIH program, freely available on the Internet, was used to measure the spot brightness from the acquired images. The spot brightness of several spots on the array with bound beads was measured as a difference between the gray level of the spots and that of the background between the spots. Samples were considered positive when a pattern of spots decorated by beads was observed visually. When measured at the 8-bit resolution grayscale (255 levels), this minimum resolution corresponded to three units or 5–10 beads on a spot 50×50 μm^2.

MAC-ELISA. In the absence of a commercially available WNV MAC-ELISA kit for birds, a kit for human serum was purchased from Focus Diagnostics (Cypress, CA) and modified to make it applicable for chicken serum. Nunc MaxiSorp flat-bottomed 96-well plates (Fisher Scientific, Hampton, NH) were coated with 100 μL/well of anti-chicken IgM in coating buffer (50 mmol/L sodium carbonate, pH 9.5) at a 1:100 dilution. Plates were incubated overnight at 4°C, after which the coating solution was removed by vacuum suction and 300 μL/well of blocking solution (3% dry milk in 20 mmol/L Tris-HCl, 0.15 mol/L NaCl, and 0.05% Tween-20, pH 7.5) was applied to the plates, followed by incubation at room temperature for 30 minutes. Use of such plates coated with anti-chicken IgM instead of anti-human IgM was the only difference between our modified WNV MAC-ELISA and the commercial one. The plates were washed twice with wash buffer, and 100 μL of each chicken serum test specimen, diluted 1:400 in blocking buffer, was added in quadruplet. One hundred microliters of uninfected chicken serum purchased from Sigma Aldrich, diluted in the same manner, was added to four wells to be used as a cut-off calibrator. Plates were incubated for 1 hour at room temperature and washed three times with wash buffer. Recombinant WNV E/preM protein was diluted as recommended by the vendor (Focus Diagnostics) in PBS, and 100 μL was added to two wells per each specimen. Similarly, 100 μL of blocking solution was added to two wells per each specimen. Plates were incubated overnight at 4°C, after which they were washed four times with wash buffer. Then, 100 μL of ready-to-use from the vendor anti-flavivirus–horseradish peroxidase antibody was added to each well. Plates were incubated for 1 hour at room temperature and were washed four times with wash buffer before adding 100 μL of 3,3′5,5′-tetramethylbenzidine. The plates were incubated for 10 minutes at room temperature in the dark, and the colorimetric reactions were stopped by addition of 100 μL of stop reagent. The ODs were read at A_{450} with an automated microplate reader. Positive samples were determined as recommended by the vendor.[17] Briefly, the net sample OD was first calculated by subtracting the average OD value obtained when antigen was added from the average OD value obtained without antigen. The index for each sample was calculated by dividing the net sample OD by the average cut-off calibrator OD. Samples were considered positive if the index value was > 1.10 and negative if the value was < 0.90.

RESULTS

Passive screening of samples for the presence of WNV-specific IgM molecules. All three assays were first tested in a passive mode using the set of 23 known samples. All samples obtained from chickens infected with WNV and bled on Day 4 or greater PI were positive. All other samples including serum from uninfected controls, chickens infected with RVFV, and chickens infected with WNV and bled between Days 1 and 3 PI were negative. Therefore, we tested the remaining 41 blinded samples passively using each of the three techniques (reverse assay array [RAA], IgM capture assay [MCA], and modified IgM capture assay [MMCA]). Figure 2 shows the spot brightness of the samples that tested positive for WNV-specific IgM when screened passively in any of the three protocols described above. A positive cut-off value of 3 was used to distinguish between positive and negative samples. Of all the serum samples (64), 11 tested positive for WNV-specific IgM in all three assays. In addition, one specimen (407) was positive for WNV-specific IgM in the RAA and the MCA but not the MMCA, and one specimen

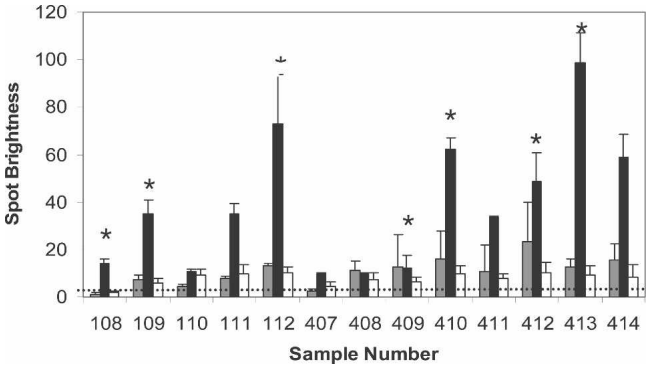

FIGURE 2. Spot brightness of serum samples that registered as positive for WNV-specific IgM in any of the three assays preformed passively. Results are shown as gray bars for the MMCA, black bars for the MCA, and white bars for the RAA. Each sample was tested in triplicate in each assay. Bars indicate SD between the three replicate tests. Spot brightness means the difference in the gray levels of spots and the area between the spots measured in images with 8-bit resolution and averaged over four to five spots. Samples were considered positive when spot brightness was ⩾ 3 as indicated by the dashed horizontal line. Asterisks mark the samples with known history.

(108) tested positive for WNV-specific IgM only in the MCA. All other 51 samples were negative for WNV-specific IgM in all three assays, meaning that no visible array emerged on assay completion.

Using a home-developed MAC-ELISA, Johnson and others[33] showed that mature chickens (20 and 60 weeks old) infected with WNV virus by needle developed detectable IgM levels beginning at the Day 4 PI, and the level maintained detectable for more than 1 month PI. All such chickens developed detectable IgM by Day 6 PI. Because most 2-day-old chickens that were infected with WNV died before Day 6 PI and no WNV-specific IgM was found in chickens bled before Day 4 PI, we chose the latter as a threshold for calculating sensitivity. The sensitivity of each assay was defined as the percentage of samples obtained Day 4 or more PI that tested positive for WNV-specific IgM. Of the 14 serum samples from chickens infected with WNV that were obtained Day 4 or later PI, 12 tested positive in the RAA (86%), 13 tested positive in the MCA (93%), and 11 tested positive in the MMCA (79%). Although the MCA provided notably larger signals (Figure 2) and the greatest sensitivity in passive format, it required the greatest time and materials of the three assays. The RAA had a sensitivity of 86% and required the least amount of time and materials. Therefore, this assay was chosen as a candidate for the active assay.

Active screening of serum samples using RAA. Figure 3 shows a comparison of spot brightness from all the samples that tested positive for WNV-specific IgM when tested actively and passively in the RAA. When serum samples were screened for the presence of WNV-specific IgM in the RAA using electrophoretic capture, the same 12 samples that tested positive for WNV-specific IgM in the passive format of this assay, which required 24 hours to complete, also tested positive in this format, which required only 15 minutes to yield results. In addition, the signal produced in the active assay was significantly greater than that seen in the passive assay. This is a result of active concentration of all IgM molecules in the sample to the vicinity of the membrane surface arrayed with probe molecules. Spot brightness in the passive assay plotted against the spot brightness in the active assay for each sample resulted in a correlation coefficient of 0.87.

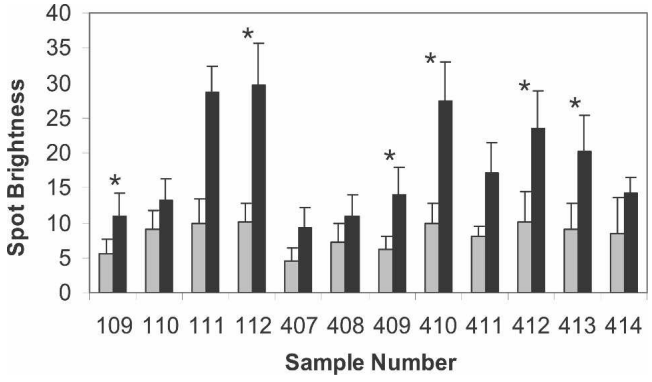

FIGURE 3. Comparison of spot brightness of serum samples that registered as positive for WNV-specific IgM in passive and active formats of the RAA. Results are shown as gray bars for the passive testing and black bars for the active testing. Each sample was tested in triplicate in each assay, with four to five spots averaged in each array. Bars indicate SD between the three replicate tests. Asterisks mark the samples with known history.

Correlation of the active RAA with a WNV MAC-ELISA. Using the criteria described above, the sensitivity of the active RAA was calculated to be 86% (95% confidence interval [CI], 60, 96). However, note that if not all chickens develop IgM at Day 4 PI, the estimate of sensitivity is underscored. It may be more appropriate to compare the newly developed assays to the conventional assay used. Currently, the WNV MAC-ELISA is the most widely used diagnostic method for early diagnosis of acute WNV infection in humans.[13–15] In the absence of a commercially available WNV MAC-ELISA kit for birds, a commercially available WNV MAC-ELISA kit for human serum purchased from Focus Diagnostics and slightly modified to make it applicable for chicken serum, was used for comparison with our active RAA. Twelve of the 64 samples were positive for WNV-specific IgM in the MAC-ELISA (data not shown). These 12 samples were the same samples that tested positive for WNV-specific IgM in the active RAA. Thus, our active RAA assay has the same sensitivity as the MAC-ELISA, while requiring significantly less time to yield results: 2–3 days for MAC-ELISA compared with 15 minutes for the active RAA. Figure 4 shows the correlation between the active RAA and the MAC-ELISA. A correlation coefficient of 0.96 was obtained when the spot brightness of the array in the active RAA was plotted against the OD value in the MAC-ELISA for each sample.

The sensitivity and assay time of the different assay techniques developed to detect WNV-specific IgM in chicken serum are summarized in Table 2.

DISCUSSION

Using electrophoretic capturing and magnetic bead detection technology, we developed a diagnostic assay for detecting WNV-specific IgM in chicken serum. When compared with the conventional diagnostic assay for WNV (MAC-ELISA), the active RAA had 100% sensitivity (95% CI: 76, 100) yet yielded results in 15 minutes as opposed to the 2–3 days needed for the MAC-ELISA. Although this assay time does not consider the time needed to prepare the different components of the assay, if such assay were to be made commercially available, all components would be manufactured and ready-to-use, resulting in a total assay time of ~15 min-

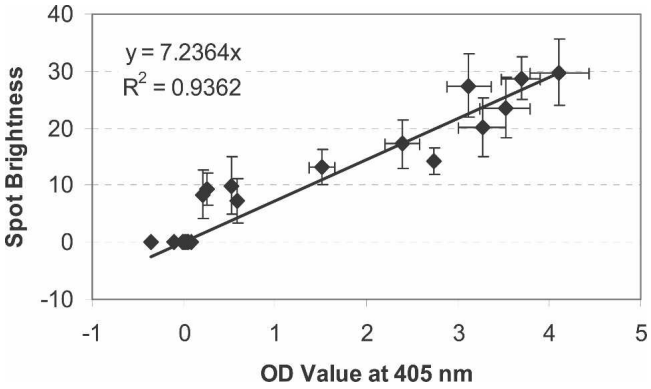

FIGURE 4. Correlation between the active RAA and the MAC-ELISA. Spot brightness of the array in the active RAA plotted against OD values in the MAC-ELISA for each sample. Each sample was tested in triplicate in the RAA and in duplicate in the MAC-ELISA. Bars indicate SD between the tests.

TABLE 2
Comparison of specificity and assay time of different assay techniques

	Assay				
	MAC-ELISA	RAA		MCA	MMCA
Active/passive	Passive	Passive	Active	Passive	Passive
Sensitivity*	86%	86%	86%	93%	79%
95% CI	60, 96	60, 96	60, 96	69, 99	52, 92
Time (min)	4,320 (3 days)	1,440	15	1,440	1,440

* Sensitivity was defined as the percentage of samples obtained Day 4 or more PI that tested positive for WNV-specific IgM.

utes. The active RAA had a specificity of 100% (95% CI: 93, 100) when tested with serum samples from uninfected chickens and chickens infected with RVFV. Additional specificity tests using serum from chickens exposed to a related *flavivirus* need to be conducted to determine whether the assay can differentiate a response to WNV from that of a similar pathogen. When dealing with patient care, it is important to confirm a diagnosis as quickly as possible. The assay described in our study can provide results in 15 minutes compared with the 2–3 days required for a MAC-ELISA, and yet the sensitivity and specificity were essentially identical. Thus, physicians would be able to confirm a diagnosis of WNV infection or know to seek an alternative diagnosis within hours of taking a sample rather than having to wait for days.

In addition, the diagnostic assay developed for detecting WNV-specific IgM is simple and straight forward. The active RAA does not require any special or intensive training of the operator. The assay does not require any unique or expensive equipment and could be reproduced in any laboratory.

There are several potential problems with this assay. These include the possible interference of other immunoglobulins. This would be of particular importance if the assay were developed for use for humans where anti-WNV specific IgG can present within a few days of symptom onset when blood samples for testing are usually taken.[17] Samples being tested in this assay may benefit from being IgG-depleted. Also, there is a necessity of desalting biologic samples by dialysis or by exclusion chromatography when using the technology. Although such desalting can be performed during electrophoretic capturing, the presence of salt notably increases capturing procedure time because lower voltages must be applied to keep the sample heating low.

In conclusion, this research clearly shows how conventional immunoassay protocols can be converted into microarray-based active formats with magnetic bead detection, resulting in reduced cost and assay time while maintaining or increasing sensitivity. The active RAA developed for rapid detection of WNV-specific IgM molecules in chicken serum is clearly more advantageous than the current convention assays for WNV detection as discussed above. It is important to mention that the technology described here can be used to detect a variety of different pathogens, simply by using the appropriate antibodies and/or antigens. Like all immunoassays, the sensitivity and specificity of the assay will depend on the quality of the antibodies and antigens used.

Received August 22, 2007. Accepted for publication October 21, 2007.

Acknowledgments: The authors gratefully acknowledge the valuable suggestions and editorial comments of T. Born and T. Y. Morozova.

Financial support: This study was supported by DOE Grant DE-F C52-04NA25455.

Disclaimer: In conducting research using animals, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication 86-23, Revised 1996). The facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

Authors' addresses: Stephanie S. Groves, The National Center for Biodefense and Infectious Diseases, George Mason University, Manassas, VA 20110, Telephone: 709-993-9493, E-mail: sgroves1@gmu.edu. Michael J. Turell, Virology Division, US Army Medical Research Institute of Infectious Diseases, 1425 Porter St., Fort Detrick, MD 21702-5011, Telephone: 301-619-4921, E-mail: michael.turell@amedd.army.mil. Charles L. Bailey, The National Center for Biodefense and Infectious Diseases, George Mason University, Manassas, VA 20110, Telephone: 703-993-4271, E-mail: cbailey2@gmu.edu. Victor N. Morozov, The National Center for Biodefense and Infectious Diseases, George Mason University, Manassas, VA 20110, Telephone: 703-993-4294, E-mail: vmorozov@gmu.edu, and Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region 142290, Russia.

Reprint requests: Victor N. Morozov, The National Center for Biodefense and Infectious Diseases, George Mason University, Manassas, VA 20110. E-mail: vmorozov@gmu.edu.

REFERENCES

1. Chowers MY, Lang R, Nassar F, Ben-David D, Giladi M, Rubinshtein E, Itzhaki A, Mishal J, Siegman-Igra Y, Kitzes R, Pick N, Landau Z, Wolf D, Bin H, Mendelson E, Pitlik SD, Weinberger M, 2001. Clinical characteristics of the West Nile fever outbreak, Israel, 2000. *Emerg Infect Dis 4:* 675–678.
2. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steel K, Crise B, Volpe KE, Crabtree MB, Scherret JH, Hall RA, MacKenzie JS, Cropp CB, Panigrahy B, Ostlund E, Schmitt B, Malkinson M, Banet C, Weissman J, Komar N, Savage HM, Stone W, McNamara T, Gubler DJ, 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science 286:* 2333–2337.
3. Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summers MD, 1995. Virus taxonomy, classification and nomenclature of viruses. *Arch Virol 10 (Suppl):* 1–586.
4. Centers for Disease Control, 2007. Statistics CDC, Surveillance, and Control. Available at: http://www.cdc.gov/NCIDOD/DVBID/WESTNILE/surv&controlhtm. Accessed August 14, 2007.
5. Elizondo-Quiroga D, Davis CT, Fernandez-Salas I, Escobar-Lopex R, Velasco Olmos D, Soto Gastalum LC, Aviles Acosta M, Elizondo-Quiroga A, Gonzalez-Rojas JI, Contreras Cordero JF, Guzman H, Travassos da Rosa A, Blitvich BJ, Barrett AD, Beaty BJ, Tesh RB, 2005. West Nile Virus isolation in human and mosquitoes, Mexico. *Emerg Infect Dis 11:* 1449–1452.
6. Weir E, Shapiro H, 2004. West Nile virus: round five. *CMAJ 170:* 1669–1670.
7. Komar N, Clark G, 2006. West Nile virus activity in Latin America and the Caribbean. *Rev Panam Salud Publica 19:* 112–117.
8. Campbell GL, Marfin AA, Lanciotti RS, Gubler DJ, 2002. West Nile virus. *Lancet Infect Dis 2:* 519–529.
9. Centers for Disease Control and Prevention, 2002. West Nile virus activity—United States, September 26-October 2, 2002, and investigations of West Nile virus infections in recipients of blood transfusion and organ transplantation. *Morb Mortal Wkly Rep 51:* 884–895.
10. Centers for Disease Control and Prevention, 2002. Possible West Nile virus transmission to an infant through breast-feeding—Michigan, 2002. *Morb Mortal Wkly Rep 51:* 879.

11. Centers for Disease Control and Prevention, 2002. Intrauterine West Nile virus infection—New York. *Morb Mortal Wkly Rep 51:* 1135–1136.
12. Centers for Disease Control and Prevention, 2002. Laboratory-acquired West Nile virus infections—United States, 2002. *Morb Mortal Wkly Rep 51:* 1133–1135.
13. Martin DA, Muth DA, Brown T, Johnson AJ, Karabatsos N, Rochrig JT, 2000. Standardization of immunoglobulin M capture enzyme-linked immunosorbent assays for routine diagnosis of arboviral infections. *J Clin Microbiol 38:* 1823–1826.
14. Malan AK, Martins TB, Hill HR, Litwin CM, 2004. Evaluations of commercial West Nile virus immunoglobulin G (IgG) and IgM enzyme immunoassays show the value of continuous validation. *J Clin Microbiol 42:* 727–733.
15. Prince HE, Lape-Nixon M, Moore RJ, Hogrefe WR, 2004. Utility of the focus technologies West Nile virus immunoglobulin M capture enzyme-linked immunosorbent assay for testing cerebrospinal fluid. *J Clin Microbiol 42:* 12–15.
16. Kuby J, 1997. *Immunology*. Third edition. New York: WH Freeman and Co.
17. Focus Diagnostics, 2007. West Nile virus IgM Capture DxSelect. Available at: http://www.focusdx.com/. Accessed August 14, 2007.
18. PanBio, 2007. West Nile virus IgM Capture ELISA. Available at: http://www.panbio.com/. Accessed August 14, 2007.
19. Wong SJ, Demarest VL, Boyle RH, Wang T, Ledizet M, Kar K, Kramer LD, Fikrig E, Koski R, 2004. Detection of human anti-flavivirus antibodies with a West Nile virus recombinant antigen microsphere immunoassay. *J Clin Microbiol 42:* 65–72.
20. Johnson AJ, Noga AJ, Kosoy O, Lanciotti RS, Johnson AA, Biggerstaff BJ, 2005. Duplex microsphere-based immunoassay for detection of anti-West Nile virus and anti-St. Louis encephalitis virus immunoglobulin m antibodies. *Clin Diag Lab Immunol 12:* 566–574.
21. Ewalt KL, Haigis RW, Rooney R, Ackley D, Krihak M, 2001. Detection of biological toxins on an active electronic microchip. *Anal Biochem 289:* 162–172.
22. Morozov VN, Morozova TY, 2003. Electrophoresis-assisted active immunoassay. *Anal Chem 75:* 6813–6819.
23. Morozov VN, Evanskey M, Tan YK, Shaffer D, Morozova TY, Bailey C, 2003. Ultrafiltration membrane for electrophoretic capturing of pathogens for AFM imaging. *Langmuir 22:* 1742–1748.
24. Morozov VN, Morozova TY, 2006. Active bead-linked immunoassay on protein microarrays. *Anal Chim Acta 564:* 40–52.
25. Neuman de Vegvara HE, Robinson WH, 2004. Microarray profiling of antiviral antibodies for the development of diagnostics, vaccines, and therapeutics. *Clin Immunol 111:* 196–201.
26. Mezzasoma L, Bacarese-Hamilton T, Di Cristina M, Rossi R, Bistoni F, Crisanti A, 2002. Antigen microarrays for serodiagnosis of infectious diseases. *Clin Chem 481:* 121–130.
27. Bacarese-Hamilton T, Mezzasoma L, Ardizzoni A, Bistoni F, Crisanti AJ, 2004. Serodiagnosis of infectious diseases with antigen microarrays. *App Microbiol 96:* 10–17.
28. Johnstone A, Thorpe R, 1988. *Immunochemistry in Practice.* Oxford: Blackwell Scientific Publications.
29. Morozov VN, Morozova TY, 1999. Electrospray deposition as a method to fabricate functionally active protein films. *Anal Chem 71:* 1415–1420.
30. Morozov VN, Morozova TY, 1999. Electrospray deposition as a method for mass fabrication of mono- and multicomponent microarrays of biological and biologically active substances. *Anal Chem 71:* 3110–3117.
31. Singh R, Maloney EK, 2002. Labeling of antibodies by in situ modification of thiol groups generated from selenol-catalyzed reduction of native disulfide bonds. *Anal Biochem 304:* 147–156.
32. Turell MJ, O'Guinn M, Oliver J, 2000. Potential for New York mosquitoes to transmit West Nile virus. *Am J Trop Med Hyg 62:* 413–414.
33. Johnson AJ, Langevin S, Wolff KL, Komar N, 2003. Detection of anti-West Nile virus immunoglobulin M in chicken serum by an enzyme-linked immunosorbent assay. *J Clin Microbiol 41:* 2002–2007.