New Sensitive Competitive Enzyme-Linked Immunosorbent Assay Using a Monoclonal Antibody against Nonstructural Protein 1 of West Nile Virus NY99

Jiro Hirota, Yoshihiro Shimoji, and Shinya Shimizu
National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan

West Nile virus (WNV) is an enveloped single-stranded RNA virus that belongs to the genus *Flavivirus*, family *Flaviviridae* (23). WNV is maintained and amplified in birds and mosquitoes. WNV belongs to the Japanese encephalitis virus (JEV) serocomplex group, which includes JEV, St. Louis encephalitis virus (SLEV), and Murray Valley encephalitis virus (MVEV) (23). WNV can infect various animals (6); however, in most of the infected animals, viremia lasts only a few days (9, 19). Consequently, the isolation of WNV or its detection in living animals using reverse transcription-PCR (RT-PCR) is difficult, particularly in the field. Hence, serodiagnosis is used mainly for clinical diagnosis or field surveillance of living animals.

*Flaviviruses* are classified into serocomplex groups by cross-neutralization using hyperimmunized mouse or rabbit sera (8, 10). Structural and nonstructural proteins of *flaviviruses* belonging to the same serocomplex group exhibit highly shared antigenicity and cross-react well in many serodiagnoses. Serodiagnoses of *flaviviruses* are performed using indirect enzyme-linked immunosorbent assays (ELISAs), hemagglutination inhibition (HI) tests, IgM capture ELISAs (MAC-ELISAs), epitope-blocking ELISAs (B-ELISAs), and neutralization tests (4, 15, 12, 25). Of these tests, neutralization tests, HI tests, and indirect ELISAs show cross-reactivity for sera infected or immunized with closely related *flaviviruses* (4, 15). Currently, cocirculation of multiple closely related *flaviviruses* is observed. JEV, MVEV, and WNV are prevalent in North Australia (20), and SLEV and WNV are prevalent in North America (27). In areas where closely related *flaviviruses* do not coexist, like France or Italy, WNV serodiagnosis can be performed without considering these cross-reactivities (3, 24). However, cross-reactivity in areas where multiple *flaviviruses* circulate needs to be considered.

To overcome cross-reactivity in serodiagnosis, virus-specific tests have been developed. Of these tests, B-ELISA and competitive ELISA (c-ELISA) are useful because they detect virus-specific antibodies in sample sera by utilizing the competition between these antibodies and monoclonal antibodies (MAbs). B-ELISA and c-ELISA differ in the incubation time in the serum reaction step. In general, in a c-ELISA, a MAb solution is added to the wells just after diluted sample sera are aliquoted. On the other hand, in a B-ELISA, the diluted sample sera are incubated for several hours before the MAb solution is added. In both ELISAs, the amount of immobilized MAbs is measured. There is no need to prepare secondary antibodies for each animal species. This is advantageous for serosurveillance of *flaviviruses*, because they can infect many species of animals, including horses, humans, bats, and many species of birds and reptiles (6). The characteristics of the B-ELISA and c-ELISA fully depend on the type of MAb used, and therefore, suitable MAbs for virus-specific B-ELISA or c-ELISA are required. A B-ELISA has been developed for the following *flaviviruses*: dengue virus (7), MVEV (13), WNV (12, 17), and JEV (7). Relatively low dilutions (5 to 10 times) of sera are required for B-ELISAs. As a consequence, relatively large volumes of sera are required.

As mentioned above, WNV is maintained and amplified in birds and mosquitoes. Therefore, WNV infection among wild birds starts earlier than WNV infections in humans and horses (26). Hence, sentinel chicken and wild bird surveillance are used to determine the prevalence of WNV. In small birds, however, it is difficult to collect enough blood without killing them. The availability of small amounts of sample sera has been one of the limitations in serosurveillance of small animals. A more sensitive assay would allow a larger range of animals to be used for WNV surveil-
lance. Here, we report a novel anti-nonstructural protein 1 (anti-NS1) MAb and the development of a MAB-based c-ELISA that can detect WNV infections with sera diluted 100 times (c-ELISA100).

MATERIALS AND METHODS

Viruses. WNV (NY99-A301, g2266, Eg101, and Kunjin MRM61C), JEV (Nakayama NIH and JaNaR0102), MVEV (MVE-51), and SLEV (Par- ton) were used. Virus culture supernatants were prepared using Vero cells. The virus culture supernatant was precentrifuged (5,000 × g, 30 min, 4°C) to remove cell debris and inactivated with β-propiolactone (Nacalai Tesque, Kyoto, Japan) (14). Viruses were purified by ultracentrifugation (80,000 × g, 14 h, 4°C) using 20% to 55% (wt/wt) linear gradients of sucrose in TAN buffer (0.02 M triethanolamine, 0.13 M NaCl [pH 8.0]). The solution that contained viruses was fractionated, diluted in TAN buffer, and ultracentrifuged (50,000 × g, 2 h, 4°C) to pellet the viruses. The pellets were resuspended in TAN buffer. The purified viruses were used as antigens for immunization, indirect ELISA, competitive ELISA, and Western blotting. All experiments using infectious viruses were approved by the Biosafety Committee of the National Institute of Animal Health in Japan and were performed in a biosafety level 3 laboratory.

MAbs. MAbs were developed in accordance with Current Protocols in Immunology (33) with slight modifications. Briefly, 4-week-old female BALB/c mice were immunized intraperitoneally 2 or 3 times with 20 μg of WNV NY99 strain antigen mixed with aluminum hydroxide gel. The final immunization was performed by intravenous injection of 20 μg of WNV antigen. The mice were anesthetized and exsanguinated 3 days after the final immunization. The spleen cells of the mice were fused with P3-X63- A8-6-1 myeloma cells using 50% polyethylene glycol (Hampton Research, CA). The hybridoma cells were diluted in hypoxanthine-aminopterin-thymidine (HAT) selection medium and incubated for 7 to 10 days in a CO2 incubator at 37°C for 1 h. The next day, the samples were centrifuged at 2,500 g, late at room temperature for 4 to 6 h and then kept at 4°C overnight. Then, the samples were centrifuged at 2,500 × g for 20 min, and the isolated sera were frozen at −80°C until use.

J EV-, MVEV-, SLEV-, and WNV-immunized chicken sera. Five-week-old specific-pathogen-free chickens were used. Six chickens were used for WNV NY99, five chickens were used for SLEV, and four chickens were used for the other viruses. Each chicken was immunized intramuscularly with 25 μg of each viral antigen mixed with aluminum hydroxide gel, 3 times biweekly. The chickens were bled 2 weeks after the final immunization. The blood samples were permitted to coagulate at room temperature for 4 to 6 h and then kept at 4°C overnight. The next day, the samples were centrifuged at 2,500 × g for 20 min. The isolated serum was frozen at −80°C until use. All serum samples had a neutralizing effect on immunized virus in a plaque reduction neutralization test (PRNT).

Competitive ELISA. A 96-well plate (Nunc, Roskilde, Denmark) was coated with 150 ng/well of WNV NY99 antigen diluted in carbonate-bicarbonate-buffered saline (50 mM; pH 9.6), and the plate was incubated for one night at 4°C. The plate was then washed three times with 350 μl of PBS containing 0.02% Tween 20 (PBST). The plate was blocked with 20% BlockAce (Dainippon Pharmaceutical) in PBST. One hundred microliters of diluted sera in PBST containing 2% BlockAce (PBST-BA) was added to each well, followed by the addition of 100 μl of MAB diluted in PBST-BA. Subsequently, 100 μl of diluted peroxidase-labeled anti-mouse IgGs (Zymed) in PBST-BA was added to each well. For each step, the incubation condition was 1 h at 37°C, and washing was done three times with 350 μl of PBST after each step. Finally, 100 μl of substrate solution containing 1.0 mM 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma) and 4 mM H2O2 in 50 mM sodium citrate buffer (pH 4.0) was added. The absorbance of each well was measured at 405 nm after incubation for 2 h at 37°C. Results were expressed as percent inhibition (PI), calculated using the following formula: 100 − [(absorbance value of test sample)/(absorbance value of control serum)] × 100.

Plaque reduction neutralization test. PRNT for WNV was performed as described in the Office International des Epizooties (OIE) manual using a 6-well plate format with Vero cells (25). Briefly, test sera were heat inactivated at 56°C for 30 min. The sera were then diluted and mixed with an equal volume of virus preparation (105 PFU/ml) in minimal essential medium containing 10% guinea pig serum and kept for 1 h at 37°C. One hundred microliters of the mixtures was inoculated into each well of a Vero cell monolayer and kept at 37°C for 1 h in a CO2 incubator with occasional agitation. Nutrients containing tragacanth gum (Nacalai Tesque) were added, and the cells were kept in the CO2 incubator for 3 to 5 days before fixing with formalin and staining with crystal violet (Nacalai Tesque). The highest serum dilution with plaque reductions of 90% was defined as the titration endpoint. Serum samples that showed 90% plaque reduction at more than 1:10 dilution were considered positive.

Animal welfare. All experiments using living animals were approved by the Ethics Committee of the National Institute of Animal Health in Japan.

RESULTS

Characterization of the monoclonal antibody SHW-7A11. We developed a new anti-WNV MAB, SHW-7A11 (IgG1, kappa
It reacted with NS1 (45 kDa) in Western blot analysis with WNV particles as antigens (Fig. 1). Next, we determined the cross-reactivity of SHW-7A11 with other JEV serocomplex flaviviruses by indirect ELISA. SHW-7A11 was relatively specific for WNV NY99, Kunjin, and Eg101 strains. However, SHW-7A11 did not react with WNV g2266, JEV Nakayama, JEV JaNAr0102, SLEV, and MVEV (Fig. 2).

**Cross-reactivity in competitive ELISA.** Both c-ELISAs were optimized for sera diluted 10 and 100 times using SHW-7A11 and were called c-ELISA10 and c-ELISA100, respectively. The only difference between the two c-ELISAs was the SHW-7A11 concentrations, 5.9 μg/ml for c-ELISA10 and 0.19 μg/ml for c-ELISA100. The specificity of these newly developed c-ELISAs was studied using a series of flavivirus-immunized chicken sera.

In c-ELISA10, the mean PIs for WNV NY99-, Kunjin-, Eg101-, and g2266-immunized sera were 76%, 72%, 61%, and 43%, respectively. The mean PI for JEV- and SLEV-immunized sera was lower than 10%. The PI for MVEV-immunized sera was 25% (Fig. 3).

In c-ELISA100, the mean PI for WNV NY99- and Kunjin-immunized sera was approximately 35%. The mean PI for JEV- and SLEV-immunized sera was lower than 3%, while the MVEV-immunized sera showed a PI of 8% (Fig. 3). The cutoff point was calculated as the mean PI plus 3 standard deviations of heterologous flavivirus-immunized sera (Table 1).

Most of the WNV NY99 clusters cocirculate with SLEV in North, Central, and South America. Therefore, as an example, the cutoff points for SLEV were used in the following experiments to validate the c-ELISAs that we developed.

**Positive conversion ratio of WNV-infected chickens in c-ELISAs and PRNT.** Sera collected serially from 9 chickens experimentally infected with WNV were used to compare the time course change of positive conversion ratios in PRNT and in the developed c-ELISAs. A positive conversion ratio can be calculated using the following formula: (number of positive converted chickens)/(total number of chickens) × 100. In the c-ELISA, chickens that showed a higher PI than the SLEV cutoff point (23.2% for c-ELISA10 and 8% for c-ELISA100) were considered positive. In PRNT, chickens that showed 90% plaque reduction at more than 1:10 dilution were considered positive. All WNV-infected chickens showed positive results 21 days after infection with both c-ELISA10 and c-ELISA100. With PRNT, it took 35 days after infection to obtain positive results for all chickens, although over 40% of the chickens were positive 7 days after infection (Fig. 4).

**Comparison between the increases in c-ELISA PI and the increase of PRNT titer in WNV-infected chickens.** Sequentially collected sera of experimentally WNV-infected chicken were used to compare the increases in the c-ELISA PI and the PRNT titer. In PRNT, the chickens inoculated with 10^6 and 10^7 virus showed seroconversion at later time points compared with the chickens inoculated with 10^3 and 10^4 virus. In PRNT, chickens 6 to 9 (inoculated with 10^3 and 10^4 virus) were found to be positive on day 7 after infection, but chickens 1, 3, and 4 (inoculated with 10^6 and 10^7 virus) showed a delay and were not found to be positive until 28 days to 35 days after infection. Conversely, no crucial differences were found in the increasing value of c-ELISA PI among these chickens. Most of the c-ELISA PIs increased with time. Although PIs obtained with c-ELISA100 were lower than those obtained with c-ELISA10, both assays showed a very similar trend in the increase of PI (Fig. 5).
DISCUSSION

WNV can infect humans, rodents, horses, birds, and reptiles (6). Of these animals, birds are the most sensitive to WNV infection. In particular, the Corvidae species is very sensitive to WNV and the mortality among these birds is relatively high, which has resulted in a decrease in their population in North America (18). With swabs or internal organs of dead birds, isolation of WNV or detection of WNV using RT-PCR is relatively easy. However, it is difficult to detect WNV in living animals (16). WNV infection in most other birds and animals is not lethal and quickly eliminated from the body (9, 19, 32). For these reasons, serodiagnosis and serosurveillance of living animals are carried out.

WNV circulates and amplifies in birds and mosquitoes. Therefore, WNV infection occurs earlier in birds than in other animals (26). Therefore, sentinel and wild bird surveillance is a suitable method for the early detection of WNV prevalence. Blood can be easily collected from big birds by venipuncture, but not from medium or small birds, and only a small amount of sera is obtained. Virus species-specific serodiagnosis using small serum samples is useful for surveillance of small animals. c-ELISA100 can detect WNV infections using sera diluted 100 times; therefore, only a few microliters of sera is required for analysis. Thus, our c-ELISA should be effective for WNV serosurveillance of small animals.

In some cases, filter paper is used to collect blood from birds (11, 28). After the filter paper is dried, the antibodies are eluted by immersing the paper in buffered saline. Drawing blood by this method is a common practice in serosurveillance of diseases in humans, particularly in developing countries (22). It is a simple way to collect the blood sample, and the filter paper need not be kept cold during transport to the laboratory. A disadvantage is that upon extraction from the paper, the sera are diluted at least 16-fold (28, 31). The previously developed

---

TABLE 1: Cross-reactivity of JEV serogroup-immunized sera in the newly developed c-ELISAs. The MAb SHW-7A11-based c-ELISA optimized for sera diluted 10 times was called c-ELISA10, and the c-ELISA optimized for sera diluted 100 times was called c-ELISA100. The numbers of chickens in the different groups were as follows: 6 for WNV NY99, 5 for SLEV and Parton, and 4 for the other viruses. Data represent means ± standard deviations (SD) (error bars).

| Virus Strain | Cutoff % inhibition | c-ELISA10 | c-ELISA100 |
|--------------|---------------------|-----------|------------|
| JEV Nakayama |                     | 4.3       | 12.9       |
| JEV JaNAr0102 |                    | 28.9      | 2.4        |
| SLE Parton   |                     | 23.2      | 8.0        |
| MVEV MVE1-51 |                     | 68.3      | 31.0       |

* The cutoff point is the mean percent inhibition plus 3 standard deviations.
WNV B-ELISAs work with sera diluted 5 to 10 times. Therefore, it is difficult to analyze samples derived from filter paper. Our newly developed c-ELISA could be applied to sera diluted 10 to 100 times by changing the MAb concentration. Therefore, the filter paper elution method could be used in combination with the c-ELISA that we developed. This would allow a more efficient surveillance of WNV infections.

We used purified virus particles, which were prepared by sucrose gradient ultracentrifugation, as the antigen to produce MAbs. In general, E, PrM/M, and C proteins are present in purified flavivirus particles. MAb SHW-7A11 reacted with NS1, as determined by Western blot analysis. NS1 binds to E protein (5, 21) and is associated with intracellular organelles and transported to the surfaces of flavivirus-infected cells (1). Thus, NS1 in our purified virus would be derived from the envelope membrane, resulting in the presence of NS1 in the purified virus antigen. The NS1 protein was also identified in purified JEV, SLEV, and MVEV using SDS-PAGE analysis (data not shown). Five of the eight established hybridomas reacted with NS1. We conclude that NS1, which was present in our purified antigen, has enough antigenicity to elicit an immune response.

Styer et al. reported that WNV infection through a mosquito bite is equivalent to a needle infection with 10^7 PFU in chickens (30). Chickens infected with WNV by mosquito bites showed early viremia and a high virus titer. A similar tendency was observed in our preliminary experimental data on WNV infections in young chickens. The WNV inoculation of chickens 1 to 4 resembles a mosquito infection more than the infection in chickens that received smaller amounts of virus. Chickens 1 to 4 showed a late increase in neutralizing antibodies, but the newly developed c-ELISA detected the infection, as was seen in other chickens.

In comparison, the mean PI of WNV-infected chicken was lower in c-ELISA100 than in c-ELISA10. However, the cross-reactivity with antibodies against related flaviviruses was lower in c-ELISA100 than in c-ELISA10. The cutoff point for c-ELISA100 was also lower. As a result, WNV infection in chicken was detected earlier in c-ELISA100.

SLEV- and JEV-immunized sera showed low PIs in both c-ELISAs; therefore, the newly developed c-ELISAs are specific for WNV and do not recognize both SLEV and JEV. MVEV-immunized chicken sera showed higher cross-reactivity. Cross-reactivity should be taken into account when sera are collected from areas where MVEV is present. Among WNV-immunized sera, NY99- and Kunjin-immunized sera displayed a high PI in both c-ELISAs, but Eg101- and g2266-immunized sera showed a lower PI particularly in the c-ELISA100. This c-ELISA can detect anti-WNV NY99 and anti-Kunjin strain antibodies but cannot detect all WNV strains, such as the g2266 strain.

MAb SHW-7A11 did not react with the WNV g2266 antigen in indirect ELISA; however, the mean PI of WNV g2266-immunized sera was >40% in the c-ELISA10. In the c-ELISA, there would be two competition mechanisms. One is direct competition; another is spatial competition. In direct competition, serum antibodies and MAbs recognize the same epitope, and serum antibodies inhibit MAb binding. In spatial competition, serum antibodies that recognize the neighboring epitope to the MAb epitope would spatially cover the latter, thereby preventing MAb binding. There are few serum antibodies that directly compete with SHW-7A11 in WNV g2266-immunized sera; therefore, the major competition mechanism is thought to be spatial competition between serum antibodies and MAbs. In our experiment, there was a tendency for the spatially competed sera (e.g., WNV g2266- and MVEV-immunized sera) to show a higher standard deviation compared

![Comparison of the positive conversion ratios for c-ELISAs and PRNT. Nine specific-pathogen-free chickens were inoculated with the WNV NY99. The chickens were bled 1 week, 10 days, 2 weeks, 3 weeks, 4 weeks, 5 weeks, and 6 weeks after virus infection. In c-ELISAs, chickens that showed higher PI than the SLEV cutoff point (23.2% for c-ELISA10 and 8% for c-ELISA100) were considered positive. In PRNT, chickens that showed 90% plaque reduction at more than 1:10 dilution were considered positive. A positive conversion ratio can be calculated using the following formula: (number of positive converted chickens)/(total number of chickens) × 100.](http://cvi.asm.org/281)
to directly competed sera (e.g., WNV NY99-, Kunjin-, and Eg101-immunized sera). Spatial competition can be influenced by many factors other than direct competition; thus, the individual difference becomes larger.

In conclusion, we developed the anti-WNV NY99 MAb SHW-7A11. The newly designed c-ELISAs, which are based on the use of MAb SHW-7A11, can detect anti-WNV NY99 antibodies with sera diluted 10 times and 100 times.
ACKNOWLEDGMENTS

We thank K. Morita of the University of Nagasaki for kindly providing Japanese encephalitis virus (JaNAr0102). We are grateful to Masaji Mase and Katsushi Kanehira (NIAH, NARO, Japan) for technical advice.

This study was partially supported by a grant-in-aid from the Zoonoses Control project of the Ministry of Agriculture, Forestry and Fisheries of Japan.

REFERENCES

1. Alcorn-LePoder S, et al. 2006. Secretion of flaviviral non-structural protein NS1: from diagnosis to pathogenesis. Novartis Found. Symp. 277:233–253.
2. Andrew SM, Titus JA. 1991. Unit 2.7. Purification of immunoglobulin G, p 1–12. In Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W (ed), Current protocols in immunology. John Wiley and Sons, New York, NY.
3. Autorino GL, et al. 2002. West Nile virus epidemic in horses, Tuscany region, Italy. Emerg. Infect. Dis. 8:1372–1378.
4. Beaty BJ, Calisher CH, Shope RE. 1995. Arboviruses, p 189–212. In Lennette EH, Lennette DA, Lennette ET (ed), Diagnostic procedures for viral, rickettsial, and chlamydial infections, 7th ed. American Public Health Association, Washington, DC.
5. Blitvich BJ, Mackenzie JS, Coelen RJ, Howard MJ, Hall RA. 1995. A novel complex formed between the flavivirus E and NS1 proteins: analysis of its structure and function. Arch. Virol. 140:145–156.
6. Bowen RA, Nemeth NM. 2007. Experimental infections with West Nile virus. Curr. Opin. Infect. Dis. 20:293–297.
7. Burke DS, Nisalak A, Gentry MK. 1987. Detection of flavivirus antibodies in human serum by epitope-blocking immunoasay. J. Med. Virol. 23:165–172.
8. Calisher CH, et al. 1989. Antigenic relationship between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. J. Gen. Virol. 70:37–43.
9. Castillo-Olivares J, Wood J. 2004. West Nile virus infection of horses. Vet. Res. 35:467–483.
10. De Madrid AT, Porterfield JS. 1974. The flaviviruses (group B arboviruses): a cross-neutralization study. J. Gen. Virol. 23:91–96.
11. Graczyk TK, Cranfield MR, Shiff CJ. 1993. ELISA method for detecting anti-Plasmodium relictum and anti-Plasmodium elongatum antibody in infected duckling sera using Plasmodium falciarpin antigens. J. Parasitol. 79:879–885.
12. Hall RA, Broom AK, Hartnett AC, Howard MJ, Mackenzie JS. 1995. Immunodominant epitopes on the NS1 protein of MVEV and KUN viruses serve as targets for a blocking ELISA to detect virus-specific antibodies in sentinel animal serum. J. Virol. Methods 51:201–210.
13. Hawkes RA, et al. 1990. Defined epitope blocking with Murray Valley encephalitis virus and monoclonal antibodies. J. Med. Virol. 32:31–38.
14. Hierholzer JC, Killington RA, Stokes A. 1996. Preparation of antigens, p 47–70. In Mahy BWJ, Kangro HO (ed), Virology methods manual, 1st ed. Academic Press, London, United Kingdom.
15. Hirota J, Nishi H, Matsuda H, Tsunemitsu H, Shimiz S. 2010. Cross-reactivity of Japanese encephalitis virus-vaccinated horse sera in serodiagnosis of West Nile virus. J. Vet. Med. Sci. 72:369–372.
16. Johnson DJ, Ostlund EN, Pedersen DD, Schmitt BJ. 2001. Detection of North American West Nile virus in animal tissue by a reverse transcription-nested polymerase chain reaction assay. Emerg. Infect. Dis. 7:739–741.
17. Kitai Y, Shoda M, Kondo T, Konishi E. 2007. Epitope-blocking enzymelinked immunosorbent assay to differentiate West Nile virus from Japanese encephalitis virus infections in equine sera. Clin. Vaccine Immunol. 14:1024–1031.
18. LaDeau SL, Kilpatrick AM, Marra PP. 2007. West Nile virus emergence and large-scale declines of North American bird populations. Nature 447:710–713.
19. Langevin SA, Bunning M, Davis B, Komar N. 2001. Experimental infection of chickens as candidate sentinel for West Nile virus. Emerg. Infect. Dis. 7:726–729.
20. Mackenzie JS, Williams DT. 2009. The zoonotic flaviviruses of Southern, South-Eastern and Eastern Asia, and Australasia: the potential for emergent viruses. Zoonoses Public Health 56:338–356.
21. Matveeva VA, Bugrysheva IV, Bakhvalova VN, Morozova OV. 1997. Secretion of tick-borne encephalitis virus glycoproteins E and NS1 heterocomplex in the late stage of infection. Vopr. Virusol. 42:179–182. (In Russian.)
22. Mei JV, Alexander JR, Adam BW, Hannon WH. 2001. Use of filter paper for the collection and analysis of human whole blood specimens. J. Nutr. 131:1631S–1636S.
23. Monath TP, Heinz FX. 1996. Flaviviruses, p 961–1034. In Fields BN, Howley PM (ed), Fields virology, vol. 1, 3rd ed. Lippincott-Raven Publishers, Philadelphia, PA.
24. Murgue B, et al. 2001. West Nile outbreak in horses in southern France, 2000: the return after 35 years. Emerg. Infect. Dis. 7:692–696.
25. Office International des Epizooties. 2004. West Nile virus, p 1064–1071. In Manual of diagnostic tests and vaccines for terrestrial animals: mammals, birds and bees, 5th ed. Office International des Epizooties, Paris, France.
26. O’Leary DR, et al. 2004. The epidemic of West Nile virus in the United States, 2002. Vector Borne Zoonotic Dis. 4:61–70.
27. Reisen W, Brault AC. 2007. West Nile virus in North America: perspectives on epidemiology and intervention. Pest. Manag. Sci. 63:641–646.
28. Roy P, Nachimuthu K, Koteswaran A, Albert A, Venugopalan AT. 1997. Postvaccinal immune response to regimens of Newcastle disease vaccination by filter paper sampling technique. Trop. Anim. Health Prod. 29:20–24.
29. Shimizu S, et al. 1988. Isolation of Theileria sergenti piroplasms from infected erythrocytes and development of an enzyme-linked immunosorbent assay for serodiagnosis of T. sergenti infections. Res. Vet. Sci. 45:206–212.
30. Styer LM, Bernard KA, Kramer LD. 2006. Enhanced early West Nile virus infection in young chickens infected by mosquito bite: effect of viral dose. Am. J. Trop. Med. Hyg. 75:337–345.
31. Thangavelu A, Rai GD, Elankumaran S, Koteswaran A. 2000. Evaluation of a filter paper blood sampling technique for quantitative assessment of antibodies to infectious bursal disease virus. Trop. Anim. Health Prod. 32:179–182.
32. Xiao SY, Guzman H, Zhang H, Travassos da Rosa AP, Tesh RB. 2001. West Nile virus infection in the golden hamster (Mesocricetus auratus): a model for West Nile encephalitis. Emerg. Infect. Dis. 7:714–721.
33. Yokoyama WM. 1991. Unit 2.5. Production of monoclonal antibodies, p 1–17. In Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W (ed), Current protocols in immunology. John Wiley and Sons, New York, NY.