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Enzyme Immunoassay for Detection of Antibodies against Eastern Equine Encephalomyelitis Virus in Sentinel Chickens

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We developed an enzyme immunoassay (EIA) for the detection of immunoglobulin M (IgM) and IgG subclass antibodies directed against eastern equine encephalomyelitis (EEE) virus in chickens. The assays were compared with the serum plaque reduction neutralization test (PRNT) and the hemagglutination inhibition (HI) test for ability to detect antibodies against EEE virus in laboratory-infected birds. No cross-reactivity was detected in serum from chickens inoculated with St. Louis encephalitis or Highlands J virus. The interval after infection when EEE virus-specific antibodies were first detected by IgM and IgG EIAs was found to be similar to that determined by the PRNT and HI tests: 2 to 4 days. The IgG EIA, PRNT, and HI test detected antibodies to EEE virus for at least 27 to 30 days after inoculation. In contrast, serum from five of seven chickens did not contain detectable IgM 30 days after infection. Similarly, in all three naturally infected sentinel chickens from Maryland, IgM class antibody was undetectable 1 to 5 weeks after IgM was initially detected. EIAs provide simple and rapid alternatives to traditional tests for monitoring EEE virus infections in sentinel chicken flocks. Moreover, the IgM EIA provides a means to separate recently infected chickens from those infected ≥1 month earlier.

The use of sentinel chicken flocks to monitor the activity of arthropod-borne viruses is widespread among public health and mosquito control agencies (1, 5, 16). A variety of techniques are used to determine when chickens become infected with virus, the hemagglutination inhibition (HI) test being the most common. The HI test is a standard serologic assay for several reasons: it is easy to perform, state diagnostic laboratories regularly use it for a variety of etiologic agents and are familiar with its performance and the interpretation of results, reagents are commercially available or can be obtained from the Centers for Disease Control, and the test is inexpensive.

Although frequently used, the HI test has several characteristics that limit its effectiveness for use in an arboviral surveillance program. Laboratories that perform HI tests ensure reproducibility and reduce costs by testing a large number of serum specimens accumulated over time. Most laboratories that are performing these serological tests are state diagnostic facilities and are often located far from the site of surveillance. Both factors delay testing and reporting of results to the local agencies that depend upon the test results to initiate or intensify control measures.

In this study, we developed and evaluated enzyme immunoassays (EIAs) for the detection of antibodies against eastern equine encephalomyelitis (EEE) virus in sentinel chickens. The EIAs we evaluated incorporate many of the advantages of the HI test but are adaptable to decentralized use by a field laboratory. The antibody detection EIA would complement antigen detection EIAs that have already been developed for mosquitoes (6, 7, 13), avians (11), and equines (12). Together, antibody and antigen EIAs would provide a means for rapid diagnosis and surveillance of EEE virus infections. Our studies on rapid detection of EEE virus infections may also serve as a model for assessing the utility of EIAs for diagnosis and surveillance of other arbovirus infections.

MATERIALS AND METHODS

Laboratory-infected chickens. Seven 4-week-old White Leghorn chickens purchased from Truslow Farms, Chestertown, Maryland, were inoculated intramuscularly with $10^{4.8}$ Vero cell 50% tissue culture infective doses ($TCID_{50}$s) of EEE virus (strain ME-77132). Control chickens, six in each group, were inoculated with $10^{4.3}$ Vero cell $TCID_{50}$ of St. Louis encephalitis (SLE) virus (strain TNM4-212), $10^{4.5}$ Vero cell $TCID_{50}$s of Highlands J (HJ) virus (strain Ct An-B8-74), or 0.1 ml of diluent. The descriptions of these viruses, including date and place of isolation, passage history, and source of isolation, are provided by Scott and Olson (11). Chickens were bled daily for 31 days, including the day before inoculation. Blood specimens were collected by venipuncture, mixed with diluent, and centrifuged after the blood was allowed to clot (11). After centrifugation, serum fractions were stored at −20°C until they were tested. Immunoglobulin G (IgG) and IgM antibody titers were determined for all blood specimens collected. Plaque reduction neutralization test (PRNT) antibody titers were determined for specimens collected on days 0 to 7, 12, 14, 17, 22, 27, and 30. HI titers were done with the same specimens as for the PRNT, except day 30 was not included in that analysis. Viremic responses of chicks during days 0 to 7 postinoculation were determined by assaying 10-fold dilutions of sera in baby hamster kidney (BHK) cells. Titers were expressed as BHK $TCID_{50}$s per milliliter of blood (11).

Sentinel chickens. Four chickens were exposed to natural EEE viral infection from biting mosquitoes by placing them in cages located in the Pocomoke Swamp, Maryland, a field study area in which EEE viral activity has been previously

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shown to be endemic (9, 13, 19). Chickens were bled approximately once each week from July through October 1986. Blood specimens were handled as described above for laboratory-infected chickens.

Additional sentinel chickens were maintained by the East Volusia Mosquito Control District, Florida, as part of a state surveillance program for SLE and EEE virus transmission activity. Aliquots of the sera collected from these birds at 2-week intervals between April and November 1986 were shipped to our laboratory in Maryland and then stored at −20°C until they were assayed for antibodies.

Serologic tests. HI tests for EEE, SLE, and HJ virus antibodies were done with standard methods (4). Sucrose-acetone-extracted (15) and betapropriolactone-inactivated (14) hemagglutinating antigens were obtained from the Yale Arbovirus Research Unit, New Haven, Connecticut, and from the Centers for Disease Control, Fort Collins, Colorado. Twofold dilutions were tested beginning with a 1:10 dilution. Neutralizing antibodies were measured in sera by using the PRNT as described by Scott et al. (10). Briefly, tests were done with Vero cells contained in 6-well plates. Tenfold dilutions of serum, beginning with a 1:10 dilution, that caused an 80% or greater reduction in the number of plaques compared with controls were considered antibody positive. Virus stocks used in the PRNTs were the same strains used to infect experimental chickens.

The following reagents and procedures were used in both EIAs. Coating buffer was phosphate-buffered saline, pH 7.4 (PBS); wash buffer was PBS containing 0.1% Tween 20 (PBST). EIA diluent consisted of PBST containing 5% horse serum and 1 g of dextran sulfate (molecular weight, 37,000 to 43,000; Sigma Chemical Co., St. Louis, Mo.) per liter. Blocking solution contained PBST and 5% horse serum. EEE and control antigens, specimens to be tested, and enzyme-conjugated detection antibody were diluted in EIA diluent. The substrate was 2,2′-azino-di-[3-ethyl-benzthiazoline sulfonate] (ABTS) (6). Positive controls were sera from infected chickens that had measurable levels of antibody when tested by HI and the PRNT. Negative-control sera were from chickens inoculated only with diluent and that lacked EEE antibody when tested by HI and the PRNT. Box titrations were done to optimize concentrations of antigen and antibodies used in the assay. Unless otherwise noted, 100 μl of a solution was added to each well of the EIA plate.

In all our EIAs, a positive serum dilution was one in which the difference in absorbance between that serum specimen when tested with EEE antigen and the negative-control antigen exceeded the mean plus 3 standard deviations of at least five negative-control serum specimens tested with both positive and negative antigens. Each dilution of serum was tested in triplicate, and the highest dilution that gave a positive reading in all three wells was recorded as the end point. The highest dilution of serum that was tested was 1:12,800. Therefore, sera that were positive at 1:12,800 were considered to have a dilution of ≥1:12,800.

Our IgG EIA consists of the following six steps, which are similar to the procedure described by Voller et al. (18) with some modifications: (i) round-bottom, 96-well polystyrene Immunolon II plates (Nunc, Inc., Newbury Park, Calif.) were coated with purified capture antibody (EEE hyperimmune ascitic fluid prepared by Douglas Watts, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland [USAMRIID]) diluted in coating buffer, and plates were incubated at 4°C overnight in a moist chamber; (ii) the following morning, blocking buffer was added, and plates were incubated for 1 h at 37°C; (iii) EEE virus antigen (expired vaccine prepared in chick embryo fibroblast cells obtained from James Meegan, USAMRIID) and negative-control antigens (cell culture fluid) were added to appropriate wells of the microtiter plates and incubated for 1 h at 37°C; (iv) twofold dilutions of test chicken sera, beginning with a 1:100 dilution, were added to appropriate wells and plates were incubated for 1 h at 37°C; (v) goat anti-chicken IgG conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) in EIA diluent was added to the plates and incubated 1 h at 37°C; and (vi) the substrate ABTS was added to each well of the plates and incubated for 30 min at 37°C. The plates were read at 414 nm with an EIA reader (Multiskan; Flow Laboratories, Alexandria, Va.). After each of the six steps, plates were washed three times with EIA wash buffer (200 μl per well).

The IgM EIA was an antibody capture assay that included the following seven steps: (i) goat anti-chicken IgM (Bethyl Laboratories, Inc., Montgomery, Tex.) diluted in coating buffer was incubated overnight at 4°C is a moist chamber; (ii) plates were blocked as in the IgG assay; (iii) twofold dilutions of chicken sera in EIA diluent beginning at a 1:50 dilution were added and allowed to incubate for 2 h at 37°C; (iv) positive and negative antigens were added and incubated for 1 h at 37°C; (v) anti-EEE virus hyperimmune ascitic fluid was added and incubated for 1 h at 37°C; (vi) goat anti-mouse IgG conjugated with horseradish peroxidase (Cooper Biomedical, Malvern, Pa.) was added and incubated for 1 h at 37°C; and (vii) the substrate ABTS was added and incubated for 30 min at 37°C. Plates were washed after each step, and end-point dilutions were determined as in the IgG assay.

RESULTS

Figure 1 shows the acquisition and duration of antibody against EEE virus in experimentally infected chickens. Panel C shows that IgG class antibody to EEE virus measured by EIA appeared as early as day 4 after inoculation, peaked between days 7 and 24, and was detectable for the remainder of the 30 days that we bled the chickens. Panels A and B show that HI and neutralizing antibodies were first detectable on days 4 and 2, respectively, postinoculation and persisted through the end of the study period. Panel D shows that IgM class antibody to EEE virus measured by EIA was first detectable at 3 to 4 days after inoculation, rose rapidly to peak levels between days 4 and 11, and then diminished.

There was considerable variation among the chickens in IgM class antibody response to EEE virus inoculation. All birds had detectable IgM by day 4, and three of seven birds had detectable IgM on day 3. The duration of detectable IgM varied from 12 days postinoculation to detection during the entire 30-day study period. There was a correlation between the duration of detectable IgM antibody and the level of viremia in the chicken. Chickens that had high viremias ($>10^{3.8}$) had IgM antibody titers that persisted longer than they did in chickens with lower ($<10^{3.0}$) viremic responses. Serum from only two of seven chickens contained detectable IgM 30 days after inoculation. The titers of both positive sera were 1:100.

All chickens inoculated with EEE virus had detectable viremias ($10^{2.1}$ to $10^{5.6}$) for 1 to 2 days. None of the SLE virus- or diluent-inoculated birds had detectable viremias. Three of the six HJ virus-inoculated chickens had detectable viremias ($10^{2.1}$ to $10^{3.6}$) for 1 day.

All chickens inoculated with HJ virus and four of five inoculated with SLE virus developed antibodies detectable by HI and PRNT to the infecting viruses. These kinds of

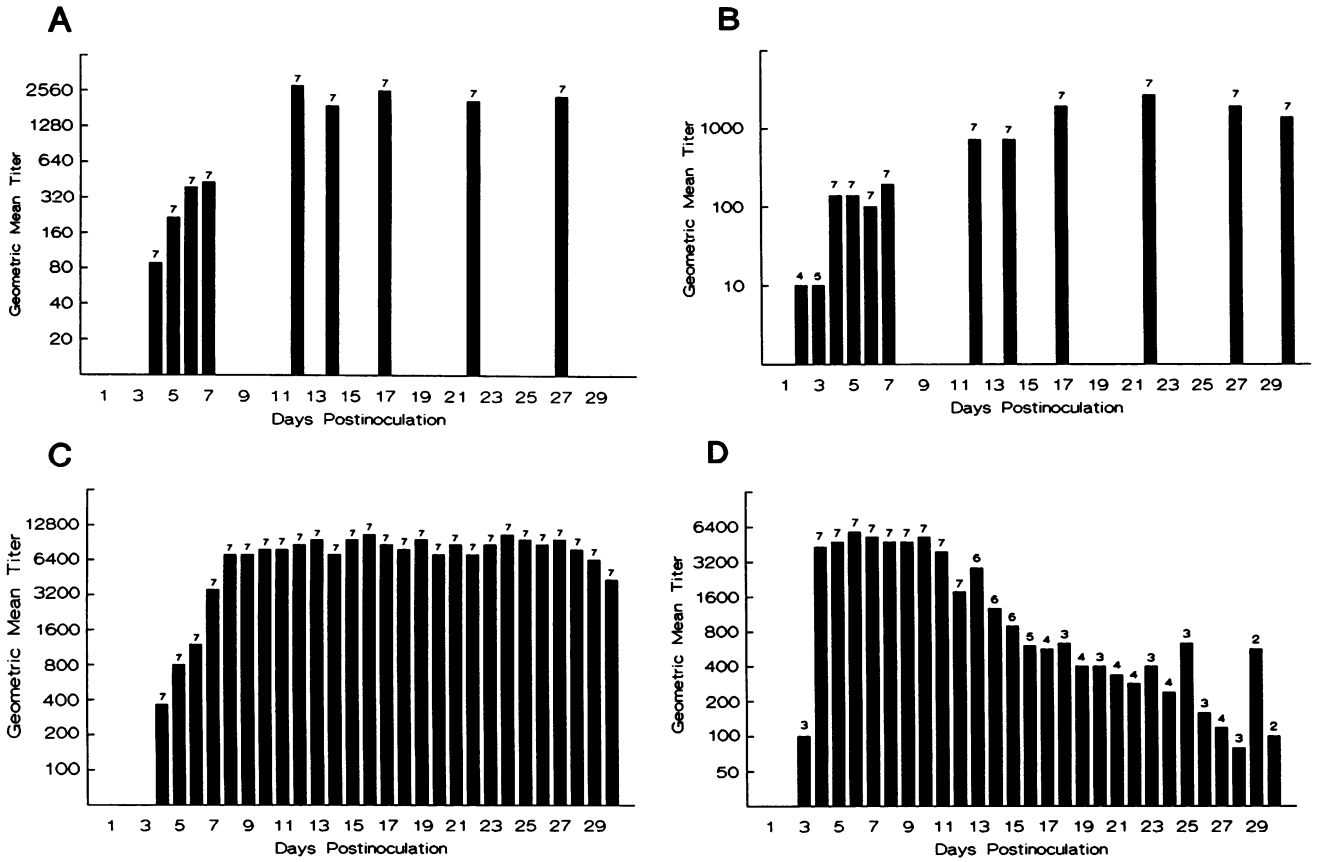

FIG. 1. Detection of antibody in chickens experimentally inoculated with $10^{4.8}$ $TCID_{50}$s of eastern equine encephalomyelitis virus. (A) Hemagglutination inhibition antibody; (B) plaque reduction neutralization antibody; (C) immunoglobulin G detected by EIA; (D) immunoglobulin M detected by EIA. Titers are expressed as reciprocals of geometric means for only those birds with detectable antibody. Numbers above bars are the number of birds with a detectable antibody titer. Seven birds were inoculated with virus.

antibodies were continuously detectable from the time they were first identified until our study ended, 27 to 30 days after inoculation. In birds inoculated with SLE virus, HI antibody was first detected 10 to 30 days after inoculation and PRNT antibody was first detected 6 to 16 days after inoculation. For HJ virus-inoculated chickens, HI antibody was first detected 5 to 11 days after inoculation and PRNT antibody was first detected 1 to 7 days after inoculation. No cross-reactions were observed when sera from these birds were tested by EIA for IgM or IgG directed against EEE virus. None of the sera from diluent-inoculated chickens contained detectable antibody.

Three of the four sentinel chickens located in the Pocomoke swamp seroconverted during the 1986 field study (Table 1). Results of EIAs for IgG and IgM class antibodies to EEE virus are shown in Table 1. Antibody was initially detectable in two birds on 29 August and in a third on 5 September. IgG class antibodies to EEE virus were detected until the project ended (7 to 8 weeks). IgM antibody was detected for only 1 to 5 weeks, after which it was no longer detectable.

A total of 290 blood specimens were tested from sentinel chickens maintained in Volusia County, Florida, between April and November 1986. Nineteen of the specimens were found positive for EEE virus by both the HI test and the IgG EIA. The sensitivity and specificity of the IgG EIA for EEE antibodies, compared with the HI test, were both 100%.

DISCUSSION

Ogata and Byrne (8) showed that the HI antibody response in chickens experimentally infected with EEE virus was initially detectable 7 days after inoculation and remained detectable for at least 100 days. Our findings for initial detection of antibody are similar to theirs except that we detected HI antibody earlier, on day 4. HI antibody to EEE virus remained detectable for our entire study period. Similarly, Calisher et al. (3) reported that in experimentally infected chickens, HI and PRNT antibodies to EEE virus remained detectable for at least 250 and 90 days, respectively.

Calisher et al. (3) concluded that the IgM capture enzyme immunoassay provided an acceptable alternative to the "HI test for detecting antibody in sentinel chickens and in young, wild birds used for arbovirus surveillance." Their results differed from ours, however, in that they were able to detect IgM class antibody against EEE virus for at least 250 days after chickens were experimentally infected. Our data suggest that in the majority of chickens, IgM is not detectable 30 days after infection (Fig. 1). Only two of seven chickens in our study had detectable levels of EEE virus-specific IgM at 30 days postinoculation, and both had low titers: 1:100. Moreover, in all three of the chickens that were naturally infected in the Pocomoke Swamp, IgM against EEE virus was undetectable 5 weeks after it was initially detected

TABLE 1. Antibody detection by EIA in sentinel chickens naturally infected with eastern equine encephalomyelitis virus in the Pocomoke Swamp, Maryland, during 1986

Date	Reciprocal of serum dilution detectable[a] for IgM or IgG							
	IgM[b] from chicken no.:				IgG[c] from chicken no.:			
	18	19	20	21	18	19	20	21
5 July	0	0	0	0	0	0	0	0
13 July	0	NT[d]	0	0	0	0	0	0
17 July	0	0	0	0	0	0	0	0
24 July	0	0	0	0	0	0	0	0
1 August	0	0	0	0	0	0	0	0
8 August	0	0	0	0	0	0	0	0
14 August	0	0	0	0	0	0	0	0
22 August	0	0	0	0	0	0	0	0
29 August	3,200	400	0	0	800	800	0	0
5 September	400	400	50	0	800	800	800	0
12 September	400	400	0	0	800	800	100	0
19 September	NT	NT	NT	NT	NT	NT	NT	NT
27 September	0	400	0	0	800	800	800	0
3 October	0	0	0	0	800	800	800	0
10 October	NT	NT	NT	NT	NT	NT	NT	NT
18 October	0	0	0	0	800	800	800	0
28 October	0	0	0	0	800	800	800	0

[a] A value of 0 indicates that antibody was undetectable by EIA at the lowest dilution assayed. Nonzero values are reciprocals of the highest serum dilution detectable by EIA.

[b] Serum specimens assayed for IgM were initially screened at a 1:50 dilution.

[c] Serum specimens assayed for IgG were initially screened at a 1:100 dilution.

[d] NT, not tested.

(Table 1). We conclude, therefore, that detection of IgM against EEE virus in sentinel chickens demonstrates a recent infection—probably within the last 30 days.

Other workers have observed results similar to ours when measuring IgM responses to arboviruses in other vertebrate species. Vernon and Webb (17) found that the IgM class antibody to vesicular stomatitis virus was initially detectable in an experimentally infected equine 6 days after inoculation, reached peak levels between days 8 and 12, and was detected last at a 1:100 dilution between days 36 and 63 postinoculation. The last day that IgM was detectable could not be determined exactly because no sera were tested between days 37 and 63. On day 64 postinoculation IgM was not detectable. An experimentally infected bovine responded to vesicular stomatitis virus infection with an IgM response that was initially detected 6 days after inoculation and that reached peak levels on days 9 and 10. IgM was last detected at a 1:10 dilution 19 days after inoculation, which was when the experiment concluded.

Similarly, Burke et al. (2) measured IgM class antibody to Japanese encephalitis virus in sentinel swine exposed to natural infection. IgM was detected 2 to 3 days following the first day of the viremia, reached peak levels 9 to 10 days after the viremia was detected, and then rapidly decreased to low levels within 2 to 3 weeks of the viremias.

Our studies with chickens infected in the laboratory define the ability of the IgM and IgG EIAs to detect EEE virus-specific antibodies compared with two traditional antibody assays, the PRNT and HI test. Studies with sentinel chickens in Maryland and Florida show that the EIAs are applicable to naturally infected birds.

Although the use of the IgM capture EIA allowed us to detect an EEE virus infection 1 day earlier than the HI test or IgG EIA, this advantage would not be a significant one for arbovirus surveillance programs as they are currently operated. Sentinel chicken flocks are bled at 1- or 2-week intervals; the potential advantage of detecting an infected chicken 1 day earlier by IgM capture EIA is therefore negligible. The principal advantages of both the IgM and IgG EIAs are that they are inexpensive (6) and can be done rapidly and relatively easily in a field laboratory. Blood specimens collected in the field could be tested the same day. The time required from the beginning of the test to its completion is 5 h for the IgG EIA and 6 h for the IgM EIA. The IgM EIA has the additional advantage over the HI test, the PRNT, and the IgG EIA of being able to identify birds that were recently infected, i.e., within 1 month of being bled.

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