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JOURNAL OF CLINICAL MICROBIOLOGY, June 1983, p. 1026–1031 Vol. 17, No. 6
0095-1137/83/061026-06$02.00/0

Detection of Rift Valley Fever Virus Antigen by Enzyme-Linked Immunosorbent Assay

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Received 2 December 1982/Accepted 22 February 1983

A double-antibody (sandwich) enzyme-linked immunosorbent assay (ELISA) was adapted to detect Rift Valley fever virus antigen. Antibodies were purified from hyperimmune mouse and rabbit sera by affinity chromatography, using CNBr-activated Sepharose 4B coupled to a beta-propiolactone-inactivated sucrose-acetone-extracted suckling mouse liver antigen. In the assay, antigen was captured by mouse antibody adsorbed to polystyrene plates and then detected by reacting sequentially with rabbit anti-Rift Valley fever virus antibody and swine anti-rabbit immunoglobulin G conjugated to alkaline phosphatase. ELISA proved to be useful in measuring viral antigen in different animal systems. However, great variation was found in the amount of antigen per PFU encountered in different circumstances. The ELISA system was optimized using supernatant fluids from infected Vero cell cultures and had a sensitivity of 10^5 PFU/ml. Hamsters develop progressive viremia, much as seen in susceptible domestic animals, such as lambs; ELISA could reliably detect 10^6 PFU/ml of viremic hamster serum. Rhesus monkeys with Rift Valley fever infection were positive by ELISA even when viremias were only 5×10^3 PFU/ml. ELISA also proved to be useful in measuring viral antigen in infected mosquitoes.

Rift Valley fever (RVF) is an economically important arthropod-borne virus disease in Africa, principally affecting sheep, goats, and cattle (8, 9). In humans, RVF has been considered to be a benign febrile illness. However, in 1975 the occurrence of fatal hemorrhagic fever and encephalitis after RVF infection was documented in South Africa and Zimbabwe (12, 14). During RVF epidemics in Egypt in 1977 and 1978, a large number of human fatalities were reported (6, 7). Vaccines are available for both animal and human use (2, 3, 10). There are also laboratory animal data which suggest that convalescent-phase plasma or the antiviral drug ribavirin might be useful in treating those with life-threatening hemorrhagic fever (3).

In regions where RVF is known to be a potential problem, the clinical diagnosis of an epizootic disease may be relatively easy, as most pregnant ewes and cows abort due to the infection, and high mortality is often seen among newborn lambs (8, 9). Human disease, particularly in exposed veterinarians and slaughterhouse workers, should occur simultaneously. Nevertheless, diagnosis is often delayed when RVF extends into new regions, and laboratory confirmation is necessary even in the presence of suspicious disease activity. Since prophylactic measures are available, the need for rapid diagnosis of the disease is obvious. Specific RVF virus (RVFV) antibodies may not be detectable during the first few days of the disease. There are also serological cross-reactions to be considered between RVFV and other viruses in the phlebotomus fever virus group (11).

Since the RVF viremia often reaches very high titers (in many species 10^4 to 10^9 PFU/ml for several days, a viral antigen assay may be the method of choice for diagnosis (7–9). The enzyme-linked immunosorbent assay (ELISA) has been successfully used for the detection of other viruses and viral antigen and found to be a rapid, sensitive, and specific method (4, 15). The ELISA system was therefore applied for RVFV antigen detection.

MATERIALS AND METHODS

Virus. Zagazig Hospital (ZH) 501 strain of RVFV was originally isolated by James Meegan, Naval Medical Research Unit-3, Cairo, Egypt, from a fetal human hemorrhagic fever case in Egypt in 1977. The second fetal rhesus monkey lung cell culture passage of the ZH 501 strain was used to infect the animals (hamsters and monkeys) and mosquitoes used in this study.

The Entebbe strain of RVFV isolated in Uganda from a mosquito pool in 1944 (10) and passaged in mice was used for the production of human RVF vaccines and for the production of an inactivated antigen for laboratory use (3, 10, 13).

The following antigenically related phleboviruses grown in cell cultures (until cytopathogenic effect occurred) or in suckling mouse brain (10% homogenate) were also tested undiluted in ELISA for cross-reactions: sand fly Naples, sand fly Sicilian, Arumowat, Punta Toro, Gordil, Karimabad, Gabek Forest, and Saint Floris. All of these viruses were tested in a plaque assay (described below and see reference 11).

Antisera. Hyperimmune antibodies against RVFV were produced in rabbit sera and mouse ascitic fluids. Rabbits were immunized with the Entebbe strain of RVFV (13). Mice received 0.1 ml of a 10% suckling mouse liver homogenate of strain ZH 501 antigen intraperitoneally and were treated 24 h later with 3 mg of polyriboinosinic-polyribocytidylic acid per kg complexed with poly-L-lysine and carboxymethyl cellulose subcutaneously to prevent lethal liver disease (3). Subsequently, they received four to six injections of antigen emulsified in complete Freund adjuvant before ascites were induced with sarcoma 180.

Affinity chromatography. To increase the sensitivity and to decrease the background activity of ELISA, affinity-purified antibodies were used (5). A beta-propiolactone-inactivated sucrose-acetone-extracted mouse liver antigen has been manufactured by Government Services Division, Salk Institute, Swiftwater, Pa., for RVF hemagglutination (HA) inhibition serology, using the Entebbe strain of RVFV (13). In the present study the HA antigen was coupled to CNBr-activated Sepharose 4B, using 1 g of gel and 2 ml of undiluted HA antigen (1). One milliliter of rabbit serum or mouse ascites fluid was passed through the column and incubated for 30 m i at room temperature. The gel was then washed with phosphate-buffered saline, and the adsorbed RVFV immunoglobulins were eluted from the column with 0.2 M glycine, pH 2.8. The eluate was immediately neutralized by Tris buffer; the immunoglobulins were then dialyzed overnight against phosphate-buffered saline and concentrated 10 times in a Minicon concentrator. The final protein concentrations calculated from the optical density (OD) at 280 nm were 1.00 and 0.52 mg/ml for the rabbit antibodies and the mouse antibodies, respectively.

Hamsters. The development of RVF viremia was determined in 25 golden Syrian hamsters. The hamsters were infected with graded doses of the ZH 501 strain of RVFV by intraperitoneal inoculation and then bled every 4 h until all of the animals succumbed to the infection. All hamsters died within 4 days.

All 104 serum samples were tested by ELISA, plaque assay, and agar gel diffusion (AGD) tests. Twelve noninfected hamster serum samples were tested by ELISA to determine background activity. All serum specimens were tested undiluted in ELISA.

Monkeys. Twelve rhesus monkeys were inoculated with RVFV and bled daily for 4 days (Table 1). Monkeys 1 through 5 were unmanipulated before infection. Monkeys 6 through 8 had participated in a passive protection experiment with RVFV several months before rechallenge to determine their immune status and had antibodies against RVFV in their pre-inoculation sera. Monkeys 9 through 12 were passively protected with rhesus monkey hyperimmune serum (0.25 ml/kg of body weight) 2 days before infection. All 12 monkeys were infected subcutaneously on day 0 with 1.1×10^6 PFU of RVFV per ml.

All 60 serum specimens were tested in a plaque assay for viremia. Fifty of these serum specimens were available and tested by ELISA and AGD. The 8 pre-inoculation sera and another 10 normal rhesus monkey sera were tested in parallel for a determination of background activity. All samples were tested undiluted in ELISA.

Mosquitoes. Viral replication was monitored in an Egyptian strain of *Culex pipiens* L. fed on infected hamsters. Individual insects were collected at 2 h and at days 2, 4, and 6 postinfectious blood meal. Mosquitoes were triturated in 1 ml of Hanks balanced salt solution and tested for virus by plaque assay on Vero E-6 monolayers. A total of 22 infected and 10 noninfected mosquito homogenates were tested undiluted in ELISA.

Humans. Ninety-six human sera, randomly selected from one battalion of Swedish United Nations soldiers were tested undiluted in ELISA. Five acute-phase sera from patients with hepatitis A were tested to analyze reactions with liver-specific antigens.

Sheep. Twenty sheep used as laboratory animals in Sweden were bled, and the sera were tested undiluted.

Mice. A normal mouse liver pool (10% homogenate) and a normal or phlebovirus-infected mouse brain pool (10% homogenate) were tested undiluted.

ELISA procedure. A double-antibody (sandwich) ELISA was used to quantify RVF antigen. Affinity-purified mouse anti-RVF antibodies (100 μl) were diluted 1:160 in coating buffer (0.05 M sodium carbonate, pH 9.5 to 9.7) and added to 60 of the 96 wells (excluding the outer rows) of polystyrene microtiter plates (Cooke M 29 AR; Dynatech Laboratories, Inc., Alexandria, Va.). After 1 h of incubation at 37°C, the plates were washed four times in rinsing solution (0.9% saline with 0.05% Tween 20). The test sample (100 μl) was then added to the well, undiluted or diluted in ELISA buffer (phosphate-buffered saline with 0.05% Tween 20 and 0.5% bovine serum albumin).

After another hour of incubation of 37°C and four washes in rinsing solution, 100 μl of the affinity-purified rabbit RVF antibodies diluted 1:640 in ELISA buffer (containing 1% normal mouse serum) was added as a second antibody (detector antibody), and the plate was again incubated for 1 h at 37°C. After washing four times in rinsing solution, 100 μl of the alkaline phosphatase-labeled swine anti-rabbit immunoglobulin G (Orion Diagnostica, Helsinki, Finland) was added, diluted 1:100 in ELISA buffer. After another incubation (1 h at 37°C) and washing, 100 μl of the substrate *p*-nitrophenylphosphate (Sigma Chemical Co., St. Louis, Mo.) diluted in diethanolamine buffer (1 M diethanolamine [pH 9.8], 0.5 mM $MgCl_2$), was added. The reaction was read after 30 min at room temperature by a spectrophotometer at 405 nm. A sample was considered positive if the OD was more than the mean background + 2 standard deviations. Optimal dilutions of affinity-purified rabbit antibodies and mouse antibodies were determined for each batch prepared by box titration.

Virus plaque assay. Serial 10-fold dilutions of monkey or mosquito samples were tested for the presence of virus in a plaque assay with tissue culture plates containing 2- to 4-day-old monolayers of uncloned or

TABLE 1. Rhesus monkeys infected with RVFV

Rhesus monkey[a]	Day 0 preinoculation			Day 1		Day 2		Day 3		Day 4	
	Log_{10} PFU/ml	PRNT[b] antibodies	ELISA OD	Log_{10} PFU/ml	ELISA OD	Log_{10} PFU/ml	ELISA OD	Log_{10} PFU/ml	ELISA OD	Log_{10} PFU/ml	ELISA OD
1	<0.7	<1:10	—[c]	<0.7	0.010	5.24	0.167	3.48	0.025	<0.7	0.536
2	<0.7	<1:10	0.049	<0.7	0.009	5.00	0.245	5.34	0.629	<0.7	0.729[d]
3	<0.7	<1:10	0.033	<0.7	0.000	3.70	0.549	5.08	0.835	<0.7	0.216
4	<0.7	<1:10	0.059	2.85	0.006	4.00	0.140	4.40	0.583	<0.7	0.233
5	<0.7	<1:10	—	1.25	0.073	0.7	0.034	0.7	0.080	<0.7	0.017
6	<0.7	<1:80	0.041	<0.7	0.017	<0.7	0.001	<0.7	0.029	<0.7	—
7	<0.7	1:160	0.059	<0.7	0.070	<0.7	0.009	<0.7	—	<0.7	—
8	<0.7	1:40	0.050	<0.7	0.071	3.70	0.108	<0.7	—	<0.7	0.030
9	<0.7	<1:10	—	<0.7	0.053	<0.7	0.088	<0.7	0.044	<0.7	0.017
10	<0.7	<1:10	0.074	<0.7	0.018	<0.7	0.111	<0.7	—	<0.7	0.038
11	<0.7	<1:10	0.043	<0.7	0.020	<0.7	0.003	<0.7	0.022[e]	<0.7	0.021
12	<0.7	<1:10	—	<0.7	0.000	<0.7	0.010	<0.7	0.013[e]	<0.7	—

[a] Monkeys 1 through 5 were unmanipulated before infection on day 0. Monkeys 6 through 8 were passively immunized and challenged with ZH 501 RVFV several months before rechallenge on day 0. Monkeys 9 through 12 were passively protected with monkey hyperimmune serum (0.25 ml/kg of body weight) on day −2.

[b] PRNT, Plaque reduction neutralization test.

[c] No sample available for testing.

[d] The sample was run in a different test from the other. The value was adjusted with the same control.

[e] Citrate-treated sera were available only. (Other viremic and nonviremic citrate sera were run together with duplicate sera without citrate, with no difference in ELISA OD.)

E-6 cloned Vero cells. Hamster sera were tested on LLC-MK_2 monolayers. There were no significant differences among the three cell types in comparative assays. Duplicate 35-mm wells were inoculated with diluted sera, absorbed for 1 h at 37°C in a 5% CO_2 atmosphere, and overlaid with a 45°C mixture of 1 part 1% agarose and 1 part 2× basal Eagle medium with Earle salts, 17 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 8% heated calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. After incubation at 37°C in a 5% CO_2 atmosphere for 3 days, a second overlay was applied which was identical to the first but contained neutral red stain (1:9,000). Plaques were enumerated on the following day.

All dilutions were performed in Hanks balanced salt solution buffered to pH 7.2 with 10 mM HEPES and containing 2% heated calf serum, 50 U of penicillin per ml and 50 μg of streptomycin per ml. All specimens run in the plaque assay had been frozen once and stored at −70°C until tested.

AGD. All monkey and hamster sera tested by ELISA were also tested by the AGD test. The test was performed using trays and templates from Miles Laboratories, Inc., Elkhart, Ind. Hexagonal patterns (8 mm on a side) of 5-mm wells were punched in approximately 1.8-mm-deep gels formed by solidifying 9 ml of 0.8% agarose (Seakem; Microbiological Associates, Walkersville, Md.) in borate-buffered saline, pH 8.3, on plates (9.5 by 4.5 cm).

Specific antiserum (25 μl of RVF immune mouse serum) was placed in the center well. Samples (25 μl each) of beta-propiolactone-inactivated sucrose-acetone-extracted suckling mouse liver RVF antigen were placed in opposite outer wells; 25-μl samples of the test sera were then placed undiluted in the remaining four outer wells. The gels were examined daily for 3 days. A sample was considered positive if it formed a precipitation line showing identity with the positive control line.

RESULTS

Standardization. ELISA was developed and optimized using Vero cell culture-grown RVFV. The OD was linear with $\log_{10}$ PFU of virus per milliliter in the interval from 5.8 to 7.3 when supernatant fluids from infected Vero cells were used, and the ELISA system had a sensitivity of 10^5 PFU/ml (Fig. 1).

The beta-propiolactone-inactivated sucrose-acetone-extracted mouse liver antigen (HA antigen) (13) used to prepare the affinity column was tested with twofold dilutions (starting with a dilution of 10^{-2}) and proved to be linear in the interval from 1:800 to 1:25,600.

Since there is a serological relationship between RVFV and some members of the sand fly fever virus group, six of these viruses were also tested in the RVFV ELISA. Suckling mouse brain or cell culture-grown Naples (cell culture, 1×10^6 PFU/ml), Sicilian (cell culture, 7.4×10^4 PFU/ml; suckling mouse brain, 5.0×10^7 PFU/ml), Arumowat (cell culture, 3.1×10^4 PFU/ml), Punta Toro (cell culture, 2.9×10^7 PFU/ml), Gordil (cell culture, 8.2×10^6 PFU/ml), Karimabad (cell culture, 5.7×10^6 PFU/ml), Gabek Forest (suckling mouse brain, 2.0×10^5 PFU/ml), and Saint Floris (suckling mouse brain, 2.5×10^7 PFU/ml) phleboviruses tested undiluted were negative by ELISA. Unin-

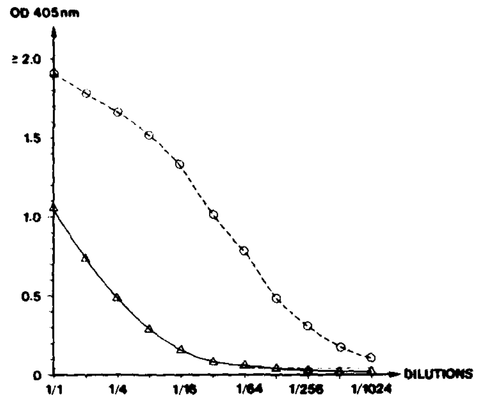

FIG. 1. Standardization of ELISA, using Vero cell culture-grown RVFV (△) and HA antigen (○) in different dilutions. The starting dilution for the Vero cell culture antigen was 1:1 (2×10^7 PFU/ml) and that for the HA antigen was 1:100.

fected cells and normal mouse brain were used as negative controls in all of these tests.

Hamsters. A total of 104 serum specimens from 25 RVFV-infected hamsters were tested by plaque assay, AGD, and ELISA. The comparison between the plaque assay and ELISA results is shown in Fig. 2. Twelve noninfected hamster sera were tested to determine background activity in ELISA and mean + 2 standard deviations = 0.044 was used as the border value between negative and positive. All sera with 10^6 PFU/ml or more (with one exception) were positive by ELISA. Three hamsters with early low viremia were positive by ELISA even though their viremias were between $10^{1.7}$ and $10^{4.4}$ PFU/ml.

AGD was only positive in 2 of 15 samples with viremia levels of 10^8 to $10^{8.9}$ and in 28 of 31 with 10^9 PFU/ml or greater.

Monkeys. Twelve rhesus monkeys, including five susceptible and seven preimmunized animals infected with RVFV and bled daily during 4 days, were tested by plaque assay, ELISA, and AGD (Table 1). Eighteen normal monkey sera were tested to determine the background activity, and mean + 2 standard deviations = 0.071 was determined to be the border between negative and positive. Two of the five monkeys developed a low viremia on day 1 (less than 10^3 PFU/ml) which was not detected by ELISA. Four of five monkeys were viremic on day 2, ranging between $10^{3.7}$ and $10^{5.2}$ PFU/ml; all four were positive by ELISA. Three of four viremic monkeys on day 3 (viremia, $10^{3.5}$ to $10^{5.3}$ PFU/ml) were positive. None of the monkeys had detectable virus on day 4, but four continued to be positive by ELISA. Of the seven monkeys immunized before RVFV challenge, one developed detectable viremia ($10^{3.7}$ PFU/ml) and was positive by ELISA. To confirm the specificity of ELISA, we performed a blocking test. All ELISA-positive sera, including the samples with undetectable infectivity in the plaque test, became negative when premixed with RVFV immune mouse sera but not when premixed with normal mouse sera before testing by ELISA. ELISA could detect RVFV-specific antigen, even though viremia was only 5×10^3 PFU/ml.

All 50 serum specimens tested by ELISA were also tested by AGD and were negative.

Mosquitoes. Viral replication in an Egyptian *C. pipiens* strain is shown in Fig. 3. Individual mosquito samples were examined by both plaque assay and ELISA. An eclipse phase, or drop in viral plaque titer, was observed by day 2 postinfectious blood meal. This titer decrease was followed by an increase, with a maximum by day 6 postinfectious blood meal. Mosquitoes collected at 2 h and at day 6 postinfectious blood meal had similar virus titers (PFU per mosquito sample). However, differences in ELISA OD readings indicated a difference in the amount of antigen. ELISA could detect antigen in mosquitoes containing only sufficient virus to yield 10^3 PFU/ml of homogenate.

Ten uninfected *C. pipiens* homogenates were also tested by ELISA for the estimation of background activity, and 0.140 (mean + 2 standard deviations) was used as the border value between negative and positive.

Other specimens (humans, sheep, and mice). A total of 96 normal human and 20 normal sheep sera were tested undiluted; all were negative.

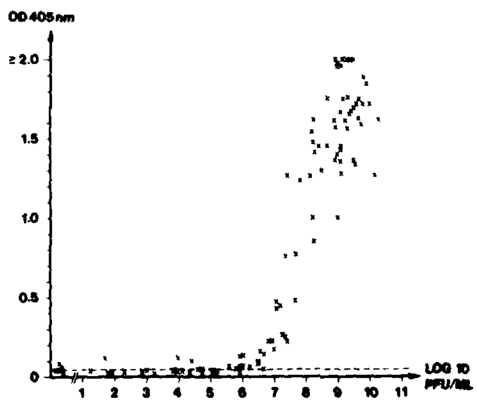

FIG. 2. Viremic hamster sera tested both by ELISA (OD) and by virus plaque assay ($\log_{10}$ PFU per milliliter).

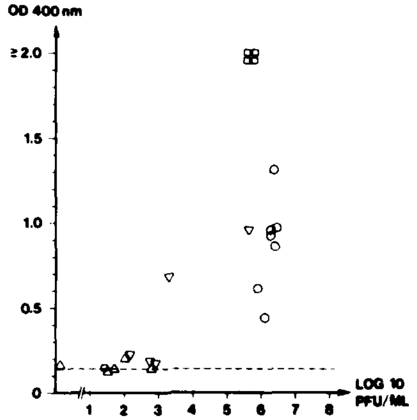

FIG. 3. Mosquitoes (*C. pipiens*) tested after feeding on viremic hamsters. Individual insects tested by ELISA (OD) and by virus plaque assay ($\log_{10}$ PFU per milliliter). Symbols: ○, 2 h; △, day 2; ▽, day 4; □, day 6.

Since RVFV may cause extensive liver damage and since the antibodies used in ELISA had been purified by affinity chromatography, using antigen prepared from mouse liver, we tested normal mouse liver (10% homogenate) and normal mouse brain (10% homogenate). No false-positive reactions were found. Five acute-phase sera from hepatitis A patients were also negative when tested undiluted.

DISCUSSION

The recent RVF epidemic in Egypt has demonstrated the potential for this disease to extend its geographic boundaries and cause serious human and animal disease. There is a need for rapid diagnostic tools to detect RVF in both humans and animals in known enzootic regions and potentially receptive extension zones. Since RVF antibodies may not be detectable during the first few days of disease and since the viremia often reaches high titers for several days, a viral antigen assay may be the method of choice.

The ELISA system in this study was developed with supernatant fluids from infected Vero cell cultures as the antigen and could detect antigen in dilutions containing 10^5 PFU of infectious virus per ml. This ELISA was then applied to several laboratory systems of potential relevance to field applications. Hamsters were used to mimic the progressive viremia seen in susceptible domestic animals. The assay reliably detected antigen when 10^6 PFU of virus per ml was present in hamster serum, a level of viremia often reached by sheep, cattle, and goats.

There were marked differences among species in the relation between antigen levels and infectivity. Rhesus monkey sera which were used as a model of human viremia had detectable antigen by ELISA when viremias rose above $10^{3.4}$ PFU/ml. Antigen was usually still detected on day 4 postinfection when detectable viremia had disappeared. This antigen could be present in the form of nonvirion antigens or neutralized virus-antibody complexes. This finding has potential relevance to the pathogenesis of the biphasic fever course, hemorrhagic fever, or retinal vasculitis in humans.

ELISA also proved to be useful in measuring viral antigen in infected mosquitoes. The 2-h samples presumably represented viremic hamster blood, and the ratio of viral antigen to infectivity was only slightly higher than that measured for hamster serum. When substantial infectivity titers reappeared in the mosquitoes on day 6, however, there was a 10-fold-higher OD in ELISA. Thus, the relationship between antigen as measured by ELISA and infectivity evaluated by PFU varied among the different species tested and during the course of infection of a single species.

A Formalin-inactivated monkey cell culture vaccine for RVF virus was also tested in the ELISA (data not shown). Five lots of vaccine with various abilities to induce neutralizing antibodies in humans (3) were compared and varied about twofold in their antigen contents. Unfortunately, there was no correlation with their potency in humans. A lot which produced a geometric mean titer of only 1:70 in recipients had significantly more ELISA reactivity than one resulting in a geometric mean titer of 1:1,000. It should be noted that the differences in ELISA results were not great and that the interlot differences in immunogenic effects were tested only on a limited number of recipients. However, these results could also indicate that the vaccine antigens responsible for stimulation of plaque reduction neutralizing antibodies are not detected well by ELISA or do not dominate the ELISA results because of the presence of other antigens in higher concentrations. Because of the discrepancies among antigen measurements, infectivity titers, and the immunogenicity of vaccines, one of the immediate priorities in further ELISA development should include tests to allow the measurement of specific viral antigens.

This ELISA test for RVF antigens merits further consideration for field evaluation since it provides a rapid, specific, and sensitive test which can detect viral antigen present in diagnostically relevant concentrations. Furthermore, it can be prepared, standardized, and performed entirely with inactivated reagents, increasing its utility in surveillance in nonendemic areas. The test could also be useful for confirmation of severe forms of RVF infections in humans since therapy, although experimental and potentially toxic (3), may be available in the near future.

ACKNOWLEDGMENTS

This project was supported by the Swedish National Defense Research Institute (FOA-4) and the U.S. Army.

We thank Joel Dalrymple, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md., for scientific advice and Erling Norrby, Karolinska Institute, Stockholm, Sweden, for helpful criticism of the manuscript.

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