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 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 10⁶ PFU/ml. Hamsters develop progressive viremia, much as seen in susceptible domestic animals, such as lambs; ELISA could reliably detect 10⁶ PFU/ml of viremic hamster serum. Rhesus monkeys with Rift Valley fever infection were positive by ELISA even when viremias were only 5 × 10⁵ 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 10⁶ to 10⁷ 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 fatal human hemorrhagic fever case in Egypt in 1977. The second fatal 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 cytopathogen effect occurred) or in suckling mouse brain (10% homogenate) were also tested undiluted in ELISA for cross-reactions: sand fly Naples, sand fly Sicilian, Arumowal, Punta Toro, Gordil, Kamrabad, 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 E-6 monolayers. A total of 22 infected and 10 noninfected RVFV was produced in rabbit sera and mouse ascitic fluids, respectively. Reference 11). Egyptian strain of phleboviruses All 60 serum specimens were tested in a plaque assay on Vero E-6 monolayers. A total of 22 infected and noninfected mosquito homogenates were tested undiluted in ELISA.

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**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 RVFV antigens. Affinity-purified mouse anti-RVFV 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 (0.1% saline with 0.05% Tween 20). The test sample (100 μl) was then added to the wells, 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 at 37°C and four washes in rinsing solution, 100 μl of the affinity-purified rabbit RVFV 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 phosphate-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 MgCl₂), was added. The reaction was read after 30 min 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.

**Viruses 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
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 10^3 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, 7.4 x 10^6 PFU/ml), Sicilian (cell culture, 1 x 10^6 PFU/ml), Gabek Forest (suckling mouse brain, 2.0 x 10^7 PFU/ml), and Saint Floris (suckling mouse brain, 2.5 x 10^8 PFU/ml) phleboviruses tested undiluted were negative by ELISA. Unin-
developed a low viremia on day 1 (less than 10⁵ 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 × 10⁴ 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 10⁵ 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 SPECIES (HUMANS, SHEEP, AND MICE). A total of 96 normal human and 20 normal sheep sera were tested undiluted; all were negative.

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 10⁵ PFU/ml) which was not detected by ELISA. Four of five monkeys were viremic on day 2, ranging between 10⁵.⁵ and 10⁶.² PFU/ml; all four were positive by ELISA. Three of four viremic monkeys on day 3 (viremia, 10⁵.⁵ to 10⁵.⁷ 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 (10⁵.⁵ 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 × 10⁴ PFU/ml.

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 = 0.064 was used as the border value between negative and positive. All sera with 10⁶ 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 10⁵.² and 10⁶ PFU/ml.

AGD was only positive in 2 of 15 samples with viremia levels of 10⁶ to 10⁸.⁹ and in 28 of 31 with 10⁹ 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 10⁵ PFU/ml) which was not detected by ELISA. Four of five monkeys were viremic on day 2, ranging between 10⁵.³ and 10⁶.² PFU/ml; all four were positive by ELISA. Three of four viremic monkeys on day 3 (viremia, 10⁵.³ to 10⁵.⁷ 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 (10⁵.⁵ 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 × 10⁴ PFU/ml.
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 $10^4$ 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-41) 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.

LITERATURE CITED