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Enzyme Immunosorbent Assay for Ebola Virus Antigens in Tissues of Infected Primates

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A sandwich enzyme immunosorbent assay (EIA) using a mixture of mouse monoclonal antibodies for antigen capture and polyclonal hyperimmune rabbit anti-Ebola virus serum for antigen detection was developed and evaluated on the tissues of monkeys naturally or experimentally infected with strains of Ebola viruses. When compared with virus isolation, the antigen detection EIA was both sensitive and specific: 44 of 45 (97.7%) liver homogenates and 38 of 41 (92.7%) spleen homogenates that were culture positive and tested by both techniques were positive for viral antigen, while 85 of 87 (97.7%) culture-negative liver homogenates and 66 of 66 culture-negative spleen homogenates were found to be antigen negative. The assay, initially developed to detect antigens of prototype African strains of Ebola virus, reliably detected related strains of Ebola virus found during two recent outbreaks of Ebola virus infection among imported, quarantined *Macaca fascicularis* monkeys in the United States. The assay allows economical and rapid testing of large numbers of tissue specimens. Antigen was found in homogenates of spleen and liver and in serum.

Ebola viruses emerged as the etiological agents of high-mortality (88% in Zaire and 55% in the Sudan) viral hemorrhagic fever during two outbreaks in Africa in 1976 (1, 6, 13). The two geographically proximate outbreaks, later shown to be caused by related but distinct viral agents (Ebola virus Zaire [EBO-Z] and Ebola virus Sudan [EBO-S] [7, 12]), were concentrated in hospital settings and associated with the use of needles and syringes without proper sterilization between patients.

The high mortality rate associated with these outbreaks and the risk of importation of other viruses causing hemorrhagic fevers have prompted us to seek a means of viral diagnosis more rapid than traditional virus isolation and identification. The presence, demonstrated by electron microscopy, of plentiful Ebola (3) and Marburg (11) virus structures in tissues of experimentally infected primates and the presence of high titers of infectious virus in blood and tissues of experimentally infected primates (2, 4) suggest that antigen detection would have a good probability of success for early laboratory diagnosis of these viral diseases. Therefore, an antigen detection enzyme immunosorbent assay (EIA) was designed and constructed to test the effectiveness of this technique.

Two Ebola virus epizootics occurring in 1989 and 1990 among *Macaca fascicularis* monkeys held in a quarantine facility in the United States (5) offered a unique opportunity to examine the sensitivity and practical usefulness of the antigen detection EIA to identify Ebola virus infection in ill or dying animals.

In this report, we describe the EIA, its usefulness for diagnosis in Ebola virus infections of primates, and other cogent aspects of its use.

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MATERIALS AND METHODS

Antisera. Anti-Ebola virus antisera were prepared as mouse monoclonal antibodies (MAb) and hyperimmune polyclonal rabbit serum. MAb produced against the prototype viruses EBO-Z and EBO-S were screened for their ability to capture EBO-S and EBO-Z virus antigens on polyvinyl chloride microtiter plates (Dynatech, Vienna, Va.) in a sandwich antigen detection format. A pool of eight MAb, each yielding high optical density values at 410 nm (OD_{410}) with either virus strain, was prepared and used in the antigen detection EIA. Polyclonal rabbit anti-Ebola virus hyperimmune serum was prepared by purification of EBO-Z on potassium tartrate gradients and hyperimmunization of rabbits with viable virus harvested from these gradients and combined with complete, and then incomplete, Freund's adjuvant. Serum was collected, evaluated by an indirect immunofluorescent antibody test, and then tested for its ability to detect antigen captured by the pooled MAb.

Antigen capture EIA. Optimal dilutions of capture MAb ascitic fluids and rabbit detector antibodies were determined by serial cross-titration of reagents. In the procedure used to detect antigen in this study, the MAb capture pool was diluted 1:1,000 in 0.01 M phosphate-buffered saline (PBS) (pH 7.4), and 100 μl was adsorbed overnight at 4°C onto polyvinyl chloride microtiter plates. Control wells were similarly adsorbed with a 1:1,000 dilution of ascitic fluid induced with the parent myeloma line. The capture antibodies and control antibody were removed by three rinses with 200 μl of a mixture of 0.01 M PBS (pH 7.4) and 0.1% Tween 20 (PBS-Tw). All wells of the plates then received 100 μl of 5% (wt/vol) Bacto skim milk (Difco) in PBS-Tw. Unclarified tissue suspensions and sera were tested by adding 33 μl of tissue suspension or serum to the 100-μl skim milk–PBS-Tw mixture in the first row of the plate and serially moving 33 μl through the subsequent three rows after mixing; thus, dilutions of 1:4, 1:16, 1:64, and 1:256 were tested. Tissue suspensions or sera were similarly diluted in wells coated with control ascitic fluid. Microtiter plates with antigen dilutions were incubated in a humidified chamber for 1 h at 37°C and again washed three times with PBS-Tw, and rabbit

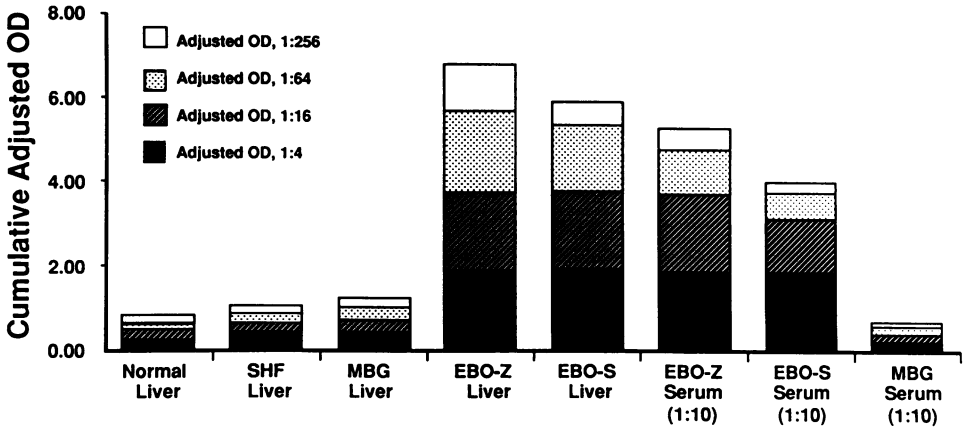

FIG. 1. Results of antigen detection tests of the liver and serum of animals experimentally infected with the prototype African strains of Ebola virus, EBO-Z and EBO-S. Tissues of animals infected with Marburg virus (MBG) or SHF virus and tissues of uninfected animals are included as negative controls. The total height of the bar represents the cumulative OD_{410}, and each component of the bar represents the adjusted OD_{410} at the dilution indicated.

anti-Ebola virus detector antibody was added at a dilution of 1:1,500 to all wells. A further 1-h incubation at 37°C was followed by three washes with PBS-Tw. Affinity-purified goat anti-rabbit antibody conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added and incubated for 1 h at 37°C. After three additional washes, 100 μl of 2-2′-azino-di-[3-ethyl-benzthiazoline sulfonate] substrate (ABTS; Kirkegaard & Perry Laboratories) was added to all wells, the mixture was incubated for 30 min, and the OD_{410} was read. The OD_{410} values of wells coated with the anti-Ebola virus MAb mix were adjusted by subtracting the OD_{410} value for the corresponding well that had been coated with normal ascitic fluid. This adjusted OD_{410} was taken as a measure of the amount of antigen specifically bound. All OD_{410} values were transmitted directly from a Dynatech MR600 reader to a microcomputer, and adjustments were performed with commercial spreadsheet software. The criterion for a positive result was defined by measuring the mean and standard deviation of the OD_{410} from negative control tissues run with each assay. We considered specimens positive if their OD values exceeded the mean plus three standard deviations of the ODs of the negative controls.

Preparation of tissues. Tissue suspensions (10%, wt/vol) were made in Eagle's minimal essential medium with Earle's salts and 10% heat-inactivated fetal bovine serum by trituration of the weighed tissues with a sterile mortar and pestle or a mechanical stomacher device (Tekmar, Cincinnati, Ohio). Positive tissues from monkeys experimentally infected by parenteral inoculation with prototype African strains or the newly isolated Reston strain (5) (EBO-R), as well as tissues from uninfected monkeys, were run with each assay. Tissues from monkeys infected with Marburg and simian hemorrhagic fever (SHF) viruses were also run with each assay to ensure the specificity of the assay for Ebola viruses.

Tissue preparation and virus isolation were performed in a maximum-containment biosafety level 4 (BL4) laboratory. Initial antigen detection tests were also performed under BL4 containment. However, later antigen detection tests were performed under BL2 conditions with tissue suspensions which had been gamma irradiated after their preparation in the BL4 laboratory.

Virus isolations and virus titration. Viruses were isolated in 25-cm^2 flasks of Vero and MA104 cells by adsorption with clarified 10% tissue suspensions for 1 h at 37°C. Cells were observed for cytopathic effect for 2 weeks. All cell cultures were tested for viral antigen by immunofluorescent staining with fluorescein isothiocyanate-conjugated convalescent-phase serum from an experimentally infected monkey; those with visible cytopathic effect were harvested, fixed, and stained, and the remaining cultures were similarly immunostained at the end of a 2-week cultivation.

Virus infectivity was assayed by counting plaques on monolayers of MA104 cells in multiwell plastic plates according to a standard method (10).

Laboratory animal care standards. In conducting the research described in this report, we adhered to the recommendations of the *Guide for the Care and Use of Laboratory Animals* (2a). The animal facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

RESULTS

The results of the antigen detection EIA for tissues from monkeys experimentally infected with EBO-Z, EBO-S, Marburg virus, or SHF virus, as well as uninfected animals, are presented in Fig. 1. Not only can one clearly distinguish the EBO-Z- and EBO-S-infected tissues from uninfected tissues, but the adjusted OD_{410} values are high, suggesting that Ebola virus antigen is abundant. The Ebola virus test did not detect antigen in animals experimentally infected with Marburg or SHF virus. These results encouraged us to apply this test to the related filovirus EBO-R.

Comparisons of antigen detection for EBO-R antigens and virus isolation results among animals from this epizootic are presented in Tables 1 and 2. Table 1 details the findings for the livers and spleens obtained from animals dying or killed during the first Ebola virus epizootic at a Reston, Va., quarantine facility (5). Table 2 presents data from a second epizootic that occurred after the first epizootic had ended, the building had been decontaminated, and the facility had been repopulated with newly imported *M. fascicularis*. The tested animals from this facility had died within the previous 12 h or were sacrificed when ill.

The sensitivity of the antigen detection EIA for tissue specimens was good. Antigen was detected in 44 of 45 (98%)

TABLE 1. Comparison of Ebola virus antigen detection and Ebola virus isolation attempts in the first (1989) Reston epizootic

Tissue	Antigen detection	No. of samples with indicated results of virus isolation in MA104 cells		
		Positive	Negative	Total
Liver	Positive	14[a]	0	14
	Negative	0	67	67
	Total	14	67	81
Spleen	Positive	12[b]	0	12
	Negative	2	60	62
	Total	14	60	74

[a] Includes three livers from which both SHF virus and Ebola virus were isolated.
[b] Includes five spleens from which both SHF virus and Ebola virus were isolated.

TABLE 2. Comparison of Ebola virus antigen detection and Ebola virus isolation attempts in the second (1990) Reston epizootic

Tissue	Antigen detection	No. of samples with indicated results of virus isolation in MA104 cells		
		Positive	Negative	Total
Liver	Positive	30[a]	2	32
	Negative	1	18	19
	Total	31	20	51
Spleen	Positive	26[b]	0	26
	Negative	1	6	7
	Total	27	6	33

[a] Includes six livers from which both SHF virus and Ebola virus were isolated.
[b] Includes nine spleens from which both SHF virus and Ebola virus were isolated.

virus culture-positive livers and 38 of 41 (93%) virus culture-positive spleens. The specificity of the antigen detection EIA was also good. No antigen was detected in 85 of 87 (98%) virus culture-negative livers or 66 of 66 (100%) virus culture-negative spleens. Another finding, illustrated in Tables 1 and 2, is the ability of the Ebola virus antigen detection EIA to ascertain the presence of Ebola virus antigens in the presence of SHF virus, which complicated isolation of EBO-R.

Results of the detection of Ebola virus antigen in the sera of a smaller number of animals from the second epizootic are presented in Table 3. Among the sera taken from animals during the 1990 epizootic, Ebola virus antigen was found in 11 of 15 (73%) serum specimens from which Ebola virus was isolated. No viral antigen was detected in the 18 serum specimens from animals from which no virus was isolated. Data from a more quantitative comparison of antigen detection and virus isolation, with a limited number of experimentally inoculated monkeys from which serial serum specimens were drawn, are presented in Fig. 2. These data clearly reveal parallel increases for both antigenemia and viremia. It appears that antigen levels may have a threshold of detection on the order of $\sim 10^{2.0}$ PFU/ml. Thus, it is likely that the antigen content of sera taken early during the course of Ebola virus infection, or late in the course of infection in animals that are destined to survive, will fall below the threshold of sensitivity of the antigen detection EIA. Nonetheless, the amplitude and duration of viral antigenemia among experimentally inoculated animals suggest that the antigen detection EIA offers good promise for laboratory diagnosis of Ebola virus infection before the death of an animal.

The specificity of the Ebola virus antigen detection EIA has been further assessed by the performance of antigen detection tests on tissues of monkeys that have died during quarantine at other facilities at which there has been no serologic evidence of active Ebola virus transmission. Livers of 52 animals from such facilities have been tested for Ebola virus antigen with no positive findings, thus further confirming the specificity of the assay with respect to monkeys dying in import quarantine conditions.

DISCUSSION

The Ebola virus antigen detection EIA, when applied during two Ebola virus epizootics of *M. fascicularis*, was able to rapidly and reliably demonstrate the presence of Ebola virus antigens in the liver and spleen of animals that had died or were killed in a moribund condition. The sensitivity of the antigen detection assay was 98% for livers and 93% for spleens of animals dead or dying with Ebola virus infection. Sera from a smaller number of animals were also tested for antigen, and although the assay yielded a positive result in only 73% of virus isolation-positive sera, it still offers promise of an earlier diagnosis of infection in animals before death.

In the context of an Ebola virus epizootic occurring in a primate facility, the sensitivity demonstrated in our studies should ensure the identification of Ebola virus etiology among the affected primate population. The most compelling advantage of the antigen detection EIA is the rapidity with which results are available. Including time for tissue preparation, the assay can be performed in approximately 5 h. While the assay is not 100% sensitive compared with virus isolation, results are available within hours; this provided a great advantage in monitoring the status of outbreaks in the primate colony from which the specimens used in this study came. Continued use of the antigen detection test to monitor animals dying during quarantine with no serologic evidence of Ebola virus infection among quarantined cohorts has been uniformly negative, thus confirming the specificity of the assay when used for this purpose.

An additional advantage of the antigen detection assay is its immunologically based specificity for discriminating Ebola virus infections from SHF virus infections. MA104 cells, while proving to be the most favorable system for isolation of Ebola virus, also support growth of SHF virus. In the outbreaks described, considerable efforts were necessary to determine the presence of dual infections of the tissues of individual animals or to guard against overgrowth of SHF virus in animals with low levels of EBO-R in their tissues. It

TABLE 3. Comparison of Ebola virus antigen detection and attempts to isolate Ebola virus from sera of monkeys during the 1990 Reston epizootic

Antigen detection	No. of samples with indicated result of virus isolation in MA104 cells		
	Positive	Negative	Total
Positive	11[a]	0	11
Negative	4	18	22
Total	15	18	33

[a] Includes six serum samples from which both Ebola and SHF viruses were isolated.

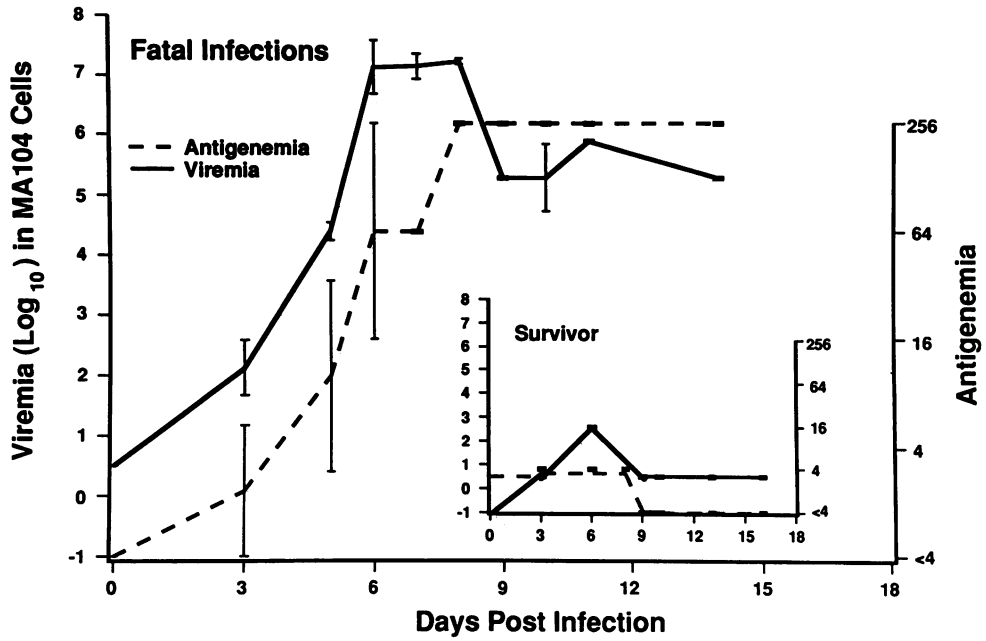

FIG. 2. Results of antigen detection and infectivity assays on MA104 cells for Ebola virus in the serum of *M. fascicularis* experimentally infected with Ebola virus (strain EBO-R [Reston-H28]). Datum points are mean values, and error bars represent the standard error of the mean.

is still not entirely clear whether viral interference may have occurred during the isolation of Ebola virus from the tissues of animals also infected with SHF virus, but this complication increased the time and effort necessary to recover Ebola virus from tissues of dually infected animals.

The potential usefulness of the Ebola virus antigen detection test for early diagnosis of Ebola virus infection in animal serum was demonstrated with *M. fascicularis* experimentally infected with EBO-R and serially bled and tested for both antigen and Ebola virus infectivity. The correlation of antigen and virus titers appears to have been good once a minimal virus threshold of between 10^2 and 10^3 PFU/ml was exceeded.

While the potential use of this assay in diagnosis of Ebola virus infection in humans remains unevaluated, the assay was formulated with antibodies directed at viral antigens from the prototype African strains, EBO-Z and EBO-S. High titers of viral antigen were detected in the serum and liver of single *M. fascicularis* monkeys infected with either of the prototype strains. Therefore, it appears that the assay should perform well with human specimens. Furthermore, the format of the test is robust, and similar assays for other agents have been repeatedly implemented in field laboratories by members of our group (8, 9). We believe that this assay offers great promise for rapid laboratory diagnosis in any further epidemics of African Ebola viruses.

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