ELISA for the Detection of Antibodies to Ebola Viruses

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EIAs for IgG and IgM antibodies directed against Ebola (EBO) viral antigens have been developed and evaluated using sera of animals and humans surviving infection with EBO viruses. The IgM capture assay detected anti-EBO (subtype Reston) antibodies in the sera of 5 of 5 experimentally infected animals at the time they succumbed to lethal infections. IgM antibodies were also detected in the serum of a human who was infected with EBO (subtype Reston) during a postmortem examination of an infected monkey. The antibody was detectable as early as day 6 after infection in experimentally infected animals and persisted for <90 days. The IgG response was less rapid; however, it persisted for >400 days in 3 animals who survived infection, and it persisted for ~10 years after infection in the sera of 2 humans. Although these data are limited by the number of sera available for verification, the IgM assay seems to have great promise as a diagnostic tool. Furthermore the long-term persistence of the IgG antibodies measured by this test strongly suggests that the ELISA will be useful in field investigations of EBO virus.

The ecology and epidemiology of Ebola (EBO) virus subtypes have been the topic of considerable study since the occurrence of high-mortality EBO epidemics [1–3]. Such studies have been hampered by the lack of effective serologic tools with which to survey for individuals, populations, or other vertebrate hosts with specific serologic evidence of past infection. The indirect fluorescent antibody test (IFAT) [4, 5] has been the most commonly used serologic technique for EBO virus serology. It has shown good sensitivity for antibodies during early convalescence and has been used extensively in serologic surveys for EBO virus antibody prevalence [6–14]; however, it has been criticized for a perceived lack of specificity in populations with no apparent probability of infection with the African filoviruses [15] and because of an inability to confirm its results with those of other immunologic techniques [16].

Other serologic tests for EBO viral antibody have been described: They include ELISA [17], RIA [18], RIPA (radioimmunoprecipitation assay), and Western blot analysis (immunoassay of viral proteins separated by polyacrylamide gel electrophoresis and transferred to a paper medium) [19]. ELISA and RIA are procedures that have been used commonly for the screening of antibodies to other viral diseases; however, they have not been commonly applied in filovirus research, and their practical value remains largely unevaluated. The RIPA and the Western blot measure antibody responses to individual viral proteins in slightly different ways and offer the advantage of showing the molecular specificity of the response; however, both techniques are most commonly used as confirmatory or research tools to dissect the immune response—the tests are too cumbersome to be used as primary serologic tools in studies of an epidemiologic scale.

The duration of antibodies measured by serologic tests used for epidemiologic studies is a central question in their use: How rapidly do antibodies become detectable and how long do they remain measurable? The rapid decline of IFAT antibodies in persons infected with EBO virus has been reported [2]; however, the use of the various tests (including the IFAT) on sera documented to be from genuine cases is limited, thus handicapping (for epidemiologic purposes) the interpretation of results from existing serologic tests for filoviruses.

Herein, we present the use of an ELISA test for both IgG and IgM antibodies. We also present data that support the sensitivity and longevity of these tests in a limited number of experimentally infected primates and humans who survived EBO infections.

Materials and Methods

Viral Antigens

Viral antigens for the IgG test were made by detergent basic buffer extraction of infected tissue culture cells. The viruses employed were EBO (subtype Zaire [EBO-Z], Mayinga strain; passage: MRC5 + 2, Vero + 1, CV7 + 1); EBO (subtype Sudan [EBO-S], Boniface strain; passage: guinea pig + 1, Vero + 2,
MRC5 + 1; Vero + 2, suckling mouse brain + 1, Vero + 1, CV7 + 1; and EBO (subtype Reston [EBO-R], strain H28; passage: MA104 + 4, CV7 + 1). In brief, MA104 cells in roller bottles were infected with EBO virus strains and allowed to proceed until advanced cytopathic effect was observed. Cells remaining attached to the walls of the roller bottles were scraped free with a rubber policeman, and cells and supernatant were separated by centrifugation (10,000 g) at 4°C for 10 min. Cells were washed once in 0.01 M borate saline, pH 9.0, and pelleted as above. The cell pellet was resuspended with a vortex in borate saline, pH 9.0, with 1% (vol/vol) Triton X-100 (Sigma, St. Louis) in a polycarbonate Oak Ridge tube (Nalge, Rochester, NY). The resuspended pellet was then sonicated in a chilled cup horn for 10 min, with the device set at a 50% duty cycle. This sonicated suspension was centrifuged at 10,000 g for 5 min at 4°C, and the supernatant was collected as stated above for the antigen. The supernatant was gamma-irradiated with 2 × 10^6 rad (20,000 Gy) to ensure virus inactivation (repeated infectivity tests have shown complete inactivation of the preparation due to the combined effect of the detergent and the irradiation). Following irradiation, the antigen was aliquoted into small portions and stored at −20°C until used. Uninfected MA104 cells were similarly prepared and used as a control or comparison antigen.

Viral antigens for the IgM test were made by centrifuging (25,000 g at 4°C) infected supernatant from infected CV7 cell cultures for 120 min. The virus pellet was resuspended in 0.01 M PBS, pH 7.4, of about a 100th of the original volume from which it had been spun, gamma-irradiated with 5 × 10^6 rad (50,000 Gy), and safety tested for residual virus infectivity.

**Sera**

The sera of animals infected experimentally with the EBO-R virus [20] were used for IgG and IgM tests. Two of the animals were Macaca mulatta that had been infected with a virus seed inadvertently contaminated with simian hemorrhagic fever virus (SHFV); however, both animals were also clearly infected with EBO-R. The third animal, a Macaca fascicularis, was infected with another isolate (H28) of EBO-R that was not contaminated with SHFV. Periodic blood sampling and separation of serum were performed over the ensuing 400+ days. Sera from 548 primates who were imported under quarantine requirements with no evidence of active filoviral infection were also tested by IFAT and IgG ELISA.

Human sera were from an animal handler who had become infected during the 1989–1990 epizootic in a quarantine facility [20, 21] or from persons who survived either laboratory [22] or field infections [23] with EBO-Z.

**IFAT Procedures**

The procedure was done as previously described with some small modifications [4]. EBO-infected and uninfected Vero cells were prepared in a biosafety level 4 laboratory, washed in PBS, and resuspended in a freezing medium (Eagle MEM with Earle’s salts and 10% fetal calf serum plus 5% DMSO). The frozen cell suspensions were inactivated with gamma irradiation (5 × 10^6 rads [50,000 Gy]) to allow for safe handling. A PBS suspension of infected and uninfected (30%-70%) cells was put on 12-spot Teflon-coated slides, dried, fixed in acetone, and stored at −70°C until used. Sera (2-fold dilutions) were incubated in the wells of the slides for 30 min at 37°C in a humidified container and then washed in PBS followed by distilled water for 10 min each. Bound anti-EBO antibodies were detected by anti-human IgG fluorescein-labeled rabbit anti-human conjugate for 30 min at 37°C in a humidified container. After a final wash, the coverslips were mounted with glycerol-PBS (50:50), and the slides were read by use of a fluorescent microscope with appropriate barrier and excitation filters for fluorescein isothiocyanate visualization.

**ELISA Procedures**

**General procedures.** All reagents were added to wells of polyvinyl chloride microtiter plates (Dynatech, Vienna, VA) in 100-μL volumes. Antigens and antibodies were adsorbed to wells after dilution in 0.01 M PBS, pH 7.4, and adsorbed overnight. Wash buffer was comprised of PBS with 0.1% (vol/vol) Tween 20 (Bio-Rad, Richmond, CA) (PBS-T). All subsequent serum and reagent dilutions were in 5% nonfat milk (Difco, Detroit) in PBS-T. Incubations, except for substrate, were done for 60 min at 37°C in a humid chamber; between reagent steps, plates were washed 3 times with PBS-T. Substrate was H_2O_2-ABTS (Kirkegaard & Perry, Gaithersburg, MD). Plates with substrate were incubated for 30 min in a humid chamber at 37°C before the plates were read spectrophotometrically and optical density (OD) values at 410 nm were captured on a microcomputer. Adjusted OD values at 410 nm were computed using a commercial spreadsheet package (Lotus 123; Lotus, Cambridge, MA).

**IgG ELISA.** The IgG ELISA was performed by coating the plate directly with the basic buffer detergent extract described above. Dilutions of antigens used to coat polyvinyl chloride microtiter plates were determined by checkerboard titration with reference sera. In this instance, antigens for EBO-R, EBO-S, and EBO-Z were diluted 1:1000 in PBS, and 0.1 mL was adsorbed to the microtiter plates overnight at 4°C. An uninfected MA104 cell culture antigen was also coated to plates and used to determine the specific binding of antibody to viral antigens. Antigen was removed from the wells by washing 3 times with PBS-T. Sera were diluted 1:100 and 4-fold through 1:6400 in 5% nonfat milk in PBS-T and allowed to react with the antigen-coated wells. Bound IgG was detected with mouse anti-human IgG (γ-chain specific; Accurate Chemical, Westbury, NY) conjugated to horseradish peroxidase. ODs at 410 nm were recorded on a microplate spectrophotometer, and the OD of the uninfected antigen-coated well was subtracted from its corresponding viral antigen–coated well to yield the adjusted OD at 410 nm. A panel of 5 to 6 normal sera are run each time the assay was used. The mean and SD of the value of the adjusted ODs were accumulated and used to calculate a value equal to the mean ± 3 SD. This represents the cutoff value for the assay and is usually about an OD (at 410 nm) of 0.2 at a 1:100 dilution.

**IgM capture ELISA.** A virus-specific IgM test was done by capturing the IgM from the serum with goat anti-human antibody (μ-chain specific; Tago, Burlingame, CA) adsorbed to the wells of the microtiter plates and then allowing the captured IgM to react with viral antigen [24]. Captured viral antigen was detected...
with a polyclonal hyperimmune, polyvalent anti-EBO mouse ascitic fluid prepared by immunization with the 3 EBO virus strains. Bound anti-EBO antibodies were detected by anti-mouse IgG (heavy and light chain specific; Boehringer Mannheim, Indianapolis) conjugated to horseradish peroxidase, followed by H$_2$O$_2$-ABTS substrate. A negative CV7 cell antigen was used to adjust for the specific reaction of the reagents with captured viral antigen, and the adjusted OD for each dilution at 410 nm was calculated as above. Optimal dilutions of all reagents were determined by checkerboard titration with early convalescent serum from 1 of the experimentally infected monkeys.

Results

IgG ELISA. The results of the IgG ELISA and the IFAT using sera of experimentally infected primates are illustrated in figure 1. Detectable levels of IgG antibody were reliably detectable at about days 10–12. Once the antibodies became detectable, the adjusted OD at the 1:100 dilution remained high (>1.0 OD at 410 nm), and titers remained moderate (800–1600) for the remaining time the primates were monitored. As shown in figure 2, the antibody levels to the homologous EBO-R strain appeared to be consistently higher during early convalescence. However, with time, the antibody levels became approximately equivalent for antigens of all 3 strains of EBO virus. There was no cross-reaction of the sera of these EBO virus–infected monkeys with a similarly prepared antigen of Marburg virus.

The IFAT response of these same animals was also compared with the results of the IgM capture ELISA (figure 3). The IFAT titers rose at the same time or later than titers for the IgM capture ELISA, but they also diminished once peak titers were reached. The IFAT titers in 2 of the 3 monkeys who were followed >400 days fell below titers that would be considered reliable (figure 1).

We also tested the sera of a number of humans who had been infected with strains of EBO virus isolated from the 1976 epidemic in the Democratic Republic of the Congo (DRC). The sera from 2 of these individuals were collected ~10 years
Figure 2. IgG response of primates experimentally infected with EBO-R virus, as measured by IgG ELISA for homologous, EBO-Z, and EBO-S antigens.

after they were infected with EBO. In both instances, the adjusted OD and titers were similar to those seen in the late convalescent sera of the experimental monkeys (table 1). Also shown in table 1 are ELISA data that were collected for other individuals in association with the 1976 epidemic in DRC, but specific time intervals for the collections were not known.

We assessed the specificity of the IgG assay by testing sera of primates imported under quarantine requirements and which had no evidence of active filoviral infection, as determined by IFAT of paired blood samples. Table 2 compares results for parallel IFATs and ELISAs. No animals among the 548 tested had positive ELISA results, but 68 reacted at ≥1:64 in the IFAT using mixed filoviral antigens. We interpret these results as being a good indication of the specificity (no false positives) of the ELISA IgG test, not as a lack of sensitivity (false negatives: IFAT positive, ELISA negative). There were no cross-reactions in the ELISA or IFAT of Marburg- or EBO-positive sera with the heterologous antigens.

Collectively, these data, which were derived from tests on sera of experimental primates and 2 humans, provide strong support for the measurement of long-lasting IgG antibodies with the IgG ELISA we describe. The ELISA data derived
from quarantined animals could be interpreted as an indication of an insensitive test, but when considered with the data mentioned above, it seems more likely that the IFAT-ELISA comparison suffers from the lack of specificity reported by others for the IFAT.

**IgM ELISA.** Figure 3 presents data from an IgM capture assay that was done on sera of experimentally infected monkeys. Although early sampling was infrequent, these data clearly show that the IgM antibody was acquired early, was measurable by day 6, rose rapidly, peaked by ~3 weeks, and diminished to undetectable levels by 84 days following infection. The test appeared to be specific for antigen prepared from EBO-R because no IgM antibody could be detected using antigens for either EBO-Z or EBO-S (data not shown).

One animal handler, who had been infected following an accident during a postmortem examination of an infected monkey, was positive for IgM antibody 28 days following the incident. His serum had an adjusted OD of 1.68 (at 410 nm) at a dilution of 1:100. A subsequent serum sample, obtained 60 days after the above sample, had nearly returned to baseline levels, having an adjusted OD of 0.20 at a 1:100 dilution.

**Discussion**

We interpret these data as strong evidence that the IgG ELISA is a specific test, giving adjusted OD values (at 410 nm) in excess of 1.0 at 1:100 dilutions of sera from infected primates. Although the number of animals and persons who we tested who were known to have been infected was small, the duration of IgG antibody among them was such that the test holds excellent promise for use as a serologic tool for epidemiologic and epizootiologic investigation of the EBO vi-
Table 1. Results of ELISAs and indirect fluorescent antibody tests (IFATs) for anti-Ebola (EBO) viral IgG in the sera of persons with previous EBO virus infections.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Infecting virus subtype</th>
<th>Time since infection</th>
<th>1:100 OD*</th>
<th>ELISA titer</th>
<th>IFAT titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EBO-R</td>
<td>28 days</td>
<td>1.32</td>
<td>≥6400</td>
<td>2048</td>
</tr>
<tr>
<td>1</td>
<td>EBO-R</td>
<td>84 days</td>
<td>1.65</td>
<td>400</td>
<td>512</td>
</tr>
<tr>
<td>2</td>
<td>EBO-Z</td>
<td>Years</td>
<td>1.81</td>
<td>1600</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>EBO-Z (?)</td>
<td>Years</td>
<td>1.96</td>
<td>800</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>EBO-Z (?)</td>
<td>?</td>
<td>1.94</td>
<td>1600</td>
<td>1280</td>
</tr>
<tr>
<td>5</td>
<td>EBO-Z (?)</td>
<td>?</td>
<td>1.97</td>
<td>1600</td>
<td>160</td>
</tr>
<tr>
<td>6</td>
<td>EBO-Z (?)</td>
<td>?</td>
<td>1.94</td>
<td>≥6400</td>
<td>320</td>
</tr>
<tr>
<td>7</td>
<td>EBO-Z (?)</td>
<td>?</td>
<td>1.95</td>
<td>1600</td>
<td>640</td>
</tr>
<tr>
<td>8</td>
<td>EBO-Z (?)</td>
<td>?</td>
<td>1.94</td>
<td>1600</td>
<td>80</td>
</tr>
<tr>
<td>9</td>
<td>EBO-Z (?)</td>
<td>?</td>
<td>1.92</td>
<td>≥6400</td>
<td>320</td>
</tr>
<tr>
<td>10</td>
<td>EBO-Z (?)</td>
<td>?</td>
<td>1.96</td>
<td>≥6400</td>
<td>640</td>
</tr>
</tbody>
</table>

NOTE. EBO-R = Reston subtype, EBO-Z = Zaire subtype.
* Adjusted OD (optical density at 410 nm) of antigen indicated to left, with OD of normal cell antigen subtracted from it.
1 ? indicates that it was not known when individuals were infected; sera were collected from persons in area of epidemic in Democratic Republic of the Congo (DRC) in 1976 or 1977.
2 ? indicates that it was not known when individuals were infected; sera were identified during investigation of DRC outbreak in 1976 but were not from hospital cases who had recovered from disease identified as being EBO virus infection.

vors might contribute to a high prevalence of IgG antibodies. Only one human infection with EBO-R was followed; the patient’s serum was IgM positive at day 28 and near baseline 2 months later, thus approximating the data available from the experimental primates. The antibody was specific for the EBO-R, which probably necessitated the use of multiple or mixed viral antigens in areas where >1 strain of EBO virus might be suspected as the cause of hemorrhagic fevers. Equivalent results were published recently concerning a patient infected with a new serotype of EBO (subtype Côte d’Ivoire) [25]. Using antigens obtained from the 3 different EBO subtypes, some cross-reactions were observed with the IFAT and the IgG ELISA, but the IgM capture ELISA was only positive using the homologous antigen.

Table 2. Results of indirect fluorescent antibody test and ELISA of sera from primates quarantined and tested in a mandated quarantine program.

<table>
<thead>
<tr>
<th>ELISA results</th>
<th>IFA results</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>&lt;64</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>480</td>
<td>68</td>
</tr>
<tr>
<td>Total</td>
<td>480</td>
<td>68</td>
</tr>
</tbody>
</table>

Compared with data from the IFAT, which is used by most investigators in previous outbreak and epidemiologic investigations, these data seem to offer several distinct advantages. First, the apparent specificity and enduring sensitivity of the IgG ELISA for EBO virus antibodies combine to make the test an excellent tool for investigation of the ecology of these viruses. The poor specificity of IFAT antibodies is an impediment in interpreting epidemiologic studies; the availability of a specific and durably sensitive test would greatly facilitate investigation of the natural history of this group of viruses. Second, the ease of performance of this assay lends itself to investigation on a population scale. The antigens themselves are easy to produce in quantities amenable to this scale, and they can be easily inactivated using the same techniques that have already been proven for other viral preparations for this group of viruses. Our data comparing IFAT and ELISA results among quarantined primates (table 2) strongly support the specificity of the ELISA; similarly, tests of 449 humans with no contact with primates
and randomly selected from primary-care outpatients in the United States were also found negative by the IgG ELISA (Ksiazek TG, unpublished data), while 12 were found positive by the IFAT [26].

The IgM assay seems to provide another tool useful during the investigation and diagnosis of outbreaks of hemorrhagic fevers. Detection of virus-specific antigens or nucleic acids coupled with antigen detection [27, 28] and polymerase chain reaction, would make possible rapid and specific diagnosis of acute or recent cases, even in areas where EBO viruses are endemic.

Although another ELISA test for detection of IgG has been described [17], the dynamics and persistence of the measurable response and the specificity of the test were not rigorously evaluated. Even though our ability to evaluate the sera of known infections with this group of viruses was limited by the availability of appropriate sera, we believe that the results are encouraging and strongly suggest that this assay will provide a preferable alternative to the IFAT for measurement of IgG antibodies to EBO viruses.

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References


