

Ebola hemorrhagic fever (EHF) patients treated at Kikwit General Hospital during the 1995 outbreak were tested for viral antigen, IgG and IgM antibody, and infectious virus. Viral antigen could be detected in virtually all patients during the acute phase of illness, while antibody was not always detectable before death. Virus was also isolated from patients during the course of their febrile illness, but attempts to quantify virus in Vero E6 cells by standard plaque assay were often unsuccessful. IgG and IgM antibody appeared at approximately the same time after disease onset (8–10 days), but IgM persisted for a much shorter period among the surviving convalescent patients. IgG antibody was detectable in surviving patients through about 2 years after onset, the latest time that samples were obtained. Detection of Ebola virus antigens or virus isolation appears to be the most reliable means of diagnosis for patients with suspected acute EHF, since patients with this often-fatal disease (80% mortality) may not develop detectable antibodies before death.

Most outbreaks of Ebola (EBO) hemorrhagic fever (EHF) have occurred in remote areas, and the delayed recognition of the outbreaks has often meant that virus transmission has ceased before scientists could reach the area and safely collect, store, and systematically investigate clinical materials from the patients. Indeed, the medical facilities involved in EHF outbreaks have often been incriminated in the spread of virus from patient to patient and even among medical staff. Several EHF patients have been cared for after transport to larger medical facilities or investigated following the occurrence of laboratory infection [1–3]. However, these instances are uncommon, and only a small number of EHF patients have been tested for virus, virus antigen, and antibody response.

The 1995 epidemic in Kikwit, Democratic Republic of the Congo (DRC), appeared to offer unique opportunities for conducting clinical virology studies since the site was relatively accessible and the outbreak was still active when the etiologic diagnosis was established. However, the situation was far from ideal, and much of the effort initially planned for virologic and immunologic study of patients was necessarily diverted to restoration of hospital services and the institution of barrier-nursing practices. This shift was necessary to prevent further infection of medical personnel and family members of patients, who already represented a major proportion of the EHF patients [4]. Nevertheless, it was possible to collect and preserve a moderate number of clinical specimens for later analyses at the Centers for Disease Control and Prevention (CDC) and the National Institute for Virology (NIV).

The results of the subsequent laboratory investigations are presented here. Tests included antigen-detection, IgM-capture, and IgG ELISAs and virus quantitation. Reverse transcriptase–polymerase chain reaction (RT-PCR) and nucleotide sequencing were used to establish the initial diagnosis and to identify the virus as being nearly identical to the prototype virus from the 1976 epidemic in DRC [5] (see Sanchez et al., this issue [6]). We also show the evolution of the viral parameters mentioned above and assess the diagnostic utility of these assays in various scenarios in which they might be used for laboratory diagnosis of clinically suspected EHF patients.

Materials and Methods

Collection and handling of clinical specimens. Initial specimens were collected from 14 patients in Kikwit and sent via the Institute for Tropical Medicine in Belgium to CDC as frozen blood [5]. Following the arrival of the CDC response team on 10 May 1995, acutely ill EHF patients were cared for in a supervised, restricted-access, barrier-nursing environment in a pavilion of Kikwit General Hospital [7]. Blood samples were collected in vacuumed containers and allowed to clot at ambient temperature for no more than 2 h, and the serum was separated in a limited-access field laboratory in which personnel wore protective clothing and positive-pressure air-purifying respirators. Convalescent patients were restricted to the hospital compound, and samples were obtained from them at less frequent intervals [8].
Figure 1. Ebola antigen (Ag) and EBO-Z IgM and IgG antibody findings from first samples from patients with laboratory-confirmed Ebola hemorrhagic fever. Data are limited to first samples that were obtained between 5 days before and 10 days after onset. A. Adjusted optical density (OD; measured at 410 nm) sums for 4 dilutions (1:4–1:256 for antigen detection, 1:100–1:6400 for IgG and IgM tests). B. Titers assigned to each serum specimen. Horizontal bars represent cutoff or positive-negative threshold for tests. Each “petal” on “sunflower” (□) represents data point; sunflowers without petals represent single data points.

Separated sera and clots were labeled and stored in liquid nitrogen until transported on dry ice in International Airline Transport Association–compliant safety shippers to the CDC and NIV, where the samples were sorted on dry ice and then stored in either gas-phase liquid nitrogen freezers or mechanical −70°C freezers. For antigen-detection and serologic tests, the serum samples were taken into a biosafety level 4 (BSL-4) laboratory and thawed, an aliquot was placed into a designated well of a microtiter format tube holder (Bio-Rad, Emeryville, CA) and sealed with paraffin film, and the holder and contents were double heat–sealed in plastic bags. The sealed samples were passed out through a 3% Lysol (PRO Brands, Wayne, NJ) immersion tank and frozen on dry ice before being irradiated (while chilled with dry ice) with 2 × 10⁶ rads (20,000 Gy) of gamma in a cobalt irradiator (Gammacel 220; Nordion International, Kanata, Canada). Specimens to be used for virus isolation and enumeration by virus assays (see below) remained in the BSL-4 laboratory and were handled in the same manner. The assays were initiated within a BSL-4 laboratory at the same time as the samples were thawed to make aliquots for serologic tests.

Tests were initially performed as sample batches as they arrived at CDC. To examine temporal patterns, we chose a subset of patients, each with ≥4 specimens, for simultaneous testing. Tests of samples not used in the temporal analyses were not repeated unless warranted by quality-control procedures.

Antigen-detection ELISA. The antigen-detection test was fundamentally as described previously [9]. Modification from the published procedure included testing of clinical materials at dilutions of 1:4 through 1:256 in a 4-fold dilution series and substitution of the previously published detection antibody with a new rabbit serum that was used as the antigen detector. The rabbit serum was a polyclonal hyperimmune serum made to the EBO virus subtypes that were known at the time it was made in 1991: EBO Zaire (EBO-Z), EBO Sudan, and EBO Reston. The test was performed on materials that had been irradiated as mentioned above. The results for each sample are presented as both the titer and as the sum of the adjusted (specific capture less mock capture well) optical density (OD) values from all of the four dilutions. Samples were considered positive if the antigen titer was 1:16 and the sum adjusted OD was >0.45. For calculation of mean titers for the antigen-detection ELISA, a value of 1 was assigned to those specimens that had titers <1:4, the first dilution tested.

IgM and IgG ELISAs. The EBO-Z IgM antibody in patient sera was measured with an IgM capture format that is often used for arboviruses and hemorrhagic fevers. The test follows one that was originally described for hepatitis A virus [10] and as adapted for EBO virus [11]. A modified antigen was used in the assay. It was prepared in a BSL-4 laboratory from Vero E6 cells grown in roller bottles, infected with the prototype (Mayinga) EBO-Z virus, and harvested when virtually all the cells had become infected.
**Figure 2.** Ebola antigen (Ag) and EBO-Z IgM and IgG antibody findings from samples obtained on day of death from 7 patients with laboratory-confirmed Ebola hemorrhagic fever. A, Adjusted optical density (OD; measured at 410 nm) sums for 4 dilutions (1:4–1:256 for antigen detection, 1:100–1:6400 for IgG and IgM tests). B, Titers assigned to each serum specimen. Horizontal bars represent cutoff or positive-negative threshold for tests. Each “petal” on “sunflower” (□) represents data point; sunflowers without petals represent single data points.

The roller bottles with infected cells were frozen and thawed, the cells and medium were decanted, and 0.1 M Tris, pH 8.5, was added to buffer the medium. The medium and cells were then refrozen in plastic bottles, heat-sealed in double bags in the BSL-4 laboratory, passed through a 3% lysol immersion tank, and frozen on dry ice before being irradiated with $5 \times 10^6$ rads (50,000 Gy) of gamma in a cobalt irradiator. The material was then thawed, sonicated, and dispensed into small containers for use as the antigen in IgM capture assays. A mock-infected Vero E6 antigen was similarly prepared.

Sera were tested against both the EBO-Z antigen and the mock-infected antigens at 4-fold dilutions of 1:100 through 1:6400. The rabbit serum that was used in the antigen-detection test also served as the antigen detector in the IgM capture format test. The substrate H$_2$O$_2$-ABTS (Kirkegaard & Perry, Gaithersburg, MD) was used after the anti-rabbit conjugate. The wells were measured on a microplate reader at 410 nm, and the ODs were recorded. The adjusted OD measured at 410 nm was calculated by subtracting the OD of the mock-infected antigen wells from the corresponding EBO-Z–infected antigen wells. An adjusted OD of $>0.10$ was required for each dilution to be considered positive and for a titer to be assigned accordingly. Sera were considered positive if the titer was $>1:400$ and the sum of the adjusted OD was $>0.6$.

The IgG ELISA was performed using gamma-inactivated basic buffer detergent extracts of Vero E6 cells infected with the same prototype strain (Mayinga) of EBO-Z [11]. Mock antigens were prepared in the same manner from uninfected E6 cells. Each serum sample was tested at the same dilution series as mentioned above for the IgM capture ELISA. An anti-human IgG (gamma chain–specific) horseradish peroxidase conjugate (Accurate Chemical, Grand Island, NY) and H$_2$O$_2$-ABTS substrate system were used to detect bound immunoglobulins. Adjusted OD values, measured at 410 nm, were calculated in the same manner as given above for the IgM test; however, cutoff values $>0.25$ greater were required for each dilution to be considered positive, and titer values were assigned accordingly. Sera with titers $>1:400$ through the dilution series and adjusted OD sums of $>1.25$ were considered IgG positive. For calculation of mean titers for both the IgM and IgG ELISAs, a value of 50 was assigned to those specimens that had titers $<1:100$.

**Virus isolation.** Virus isolation was attempted on selected specimens to qualitatively determine if infectious virus was present. Isolation attempts were performed in a BSL-4 laboratory by using confluent monolayers of Vero E6 cells in 25-cm$^2$ plastic tissue culture flasks. Each flask was inoculated with 100 $\mu$L of a 1/10 dilution of the sample being tested for virus. The flask was then continuously rocked in an incubator (37°C) for $\sim 1$ h before 7 mL of maintenance medium (Eagle MEM with Earle’s salts, 2% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 $\mu$g/mL streptomycin, and 50 $\mu$g/mL gentamycin) was added. The caps
Figure 3. EBO-Z IgM mean of adjusted optical density (OD; measured at 410 nm) sum (□; A) and log₁₀ titers (□; B) from sera and blood of patients with laboratory-confirmed Ebola hemorrhagic fever. Bars represent 1 SE of mean for each time point. Time is shown in days after onset and is divided into 2-day intervals up to day 30 and into 30-day intervals after 30 days. N = no. of data points in each time group. Ag = antigen.

on the flasks were closed tightly, and the flasks were incubated at 37°C.

For the first few days, flasks were observed daily for cytopathic effect (CPE), and then they were observed every 2–3 days. The maintenance medium was changed at day 7, and if CPE did not affect >50% of the cells, cultures were harvested at 14 days and tested for EBO virus antigen as detailed below. Cultures that became contaminated with bacteria or mold were frozen, thawed, passed through a 0.22-μM filter, and reinoculated onto another flask of confluent Vero E6 cells. At 14 days, or if CPE was obvious and affected >50% of the cells, the cultures were harvested and tested for EBO viral antigen by indirect fluorescent antibody test. Cells were detached from the flask by agitation with sterile 3-mm glass beads, and ~4 mL of this cell suspension was labeled and
stored at −70°C for further use. The remaining ~3 mL of cell
suspension was centrifuged in aerosol guard tube carriers, and the
cells were resuspended in ~0.5 mL of borate saline, pH 9. Using a
plastic Pasteur pipette, we placed small drops of the cell suspen-
sion onto four wells on each of two Teflon-coated slides (Cell-
Line Associates, Newfield, NJ) and allowed them to air dry.

The slides were placed in slide boxes, heat-sealed in double bags
in the BSL-4 laboratory, passed through a 3% lysol immersion tank,
and frozen on dry ice before being irradiated with 2 × 10⁶ rads of
gamma in a cobalt irradiator. Following fixation with acetone, the
cells were stained with a 1:100 dilution of the polyclonal hyperim-
mune rabbit serum (2 wells) that was used in the antigen-detection
assay and with normal rabbit serum (2 wells) followed by a fluores-
cence isothiocyanate–conjugated anti-rabbit IgG (ICN, Irvine, CA);
they were then observed with a fluorescent microscope for specific
EBO viral antigens. Slides with inactivated and fixed cells that were
infected with EBO-Z virus were used to control each batch of slides
that was tested for EBO virus antigen. If specimens were virus
isolation positive but had no detectable plaques, they were assigned
a value of 1.0 log₂₀ pfu/mL for analyses.

**Virus plaque assay.** Plaque assays were performed in a BSL-
4 laboratory according to the method of Earley et al. [12] and Moe
et al. [13] on confluent monolayers of Vero E6 cells in 6-well
plastic tissue culture plates. The initial samples of the prototype
virus from the 1995 Kikwit outbreak that were isolated in Vero
E6 cells were passaged one more time (Vero E6/2) and used to
establish and control the plaque assay. Samples were diluted 10-
fold in Hanks’ balanced salt solution with 5% heat-inactivated
fetal bovine serum. Initially, we tested the clinical samples at
dilutions of 10⁻¹ through 10⁻⁶ in duplicate wells, with 0.2 mL
inoculated onto each well and adsorbed with constant rocking at
37°C in an incubator. After it became evident that virus titers were
either low or undetectable using this procedure, specimens were
tested by this method only at dilutions of 10⁻¹ through 10⁻³. All
specimens were handled in a laminar flow safety cabinet, and virus
control plates were handled at the end of each procedure in a
separate laminar flow safety cabinet in another room in the BSL-
4 laboratory. Plates were stained at day 6 by the addition of a
second agar overlay containing neutral red and observed for several
days to obtain optimal plaque counts; titers were expressed as
plaque-forming units per milliliter. Again, if the plaque assay
yielded no detectable virus plaques and the isolation attempt on
the same specimen was positive, we recorded the virus titer for
the specimen as 1.0 log₂₀ pfu/mL.

**Fluorescent focus assay (FFA).** The FFA was based on the
technique of Smith et al. [14]. Ten-fold dilutions of samples were
prepared in medium (Eagle MEM with Hanks’ balanced salt solu-
tion, 2% heat-inactivated fetal bovine serum, 100 μg/mL streptomycin,
100 μg/mL penicillin, and 50 μg/mL gentamycin), and 0.1-
ml volumes were inoculated into duplicate wells of Vero E6
monolayers in Lab-Tek 8-chamber culture slides (Nunc, Naperv-
ille, IL). The inocula were adsorbed for 1 h at 37°C, and the
volume of medium in each well was brought up to 0.4 mL. Fluores-
cent foci were detected and counted by indirect immunofluores-
cence with hyperimmune rabbit anti-EBO serum in replicate cul-
tures following 3–5 days of incubation. Titers were expressed as
fluorescent focus-forming units/mL.

**Results**

A total of 533 blood or serum samples were collected from
89 patients and tested for virus, virus antigen, or antibodies.
In some instances, there was not enough material to perform
fying acute cases of EHF during the Kikwit epidemic. The data assay did not yield recordable counts. Nevertheless, 

Figure 4. The IgM values appeared to peak among patients 

IgG test results for the first samples obtained from EHF pa-

were only rarely positive after day 12.

selected for simultaneous testing for virus antigen and antibody; first group of Kikwit patients and that had been passaged only 

when considering the adjusted OD (measured at 410 nm) sums, 

we were able to isolate virus from all samples that had measurable 

virus by the FFA. Although our attempts to quantify vire-

mias by plaque assay appear to be less successful than we had 

anticipated, controls that were included with each assay yielded 

consistent titers with a virus that had been isolated from the 

first group of Kikwit patients and that had been passaged only 

twice to obtain sufficient working stocks. Virus isolation was 

also attempted in flasks from each specimen chosen for virus 

assay, and the results were frequently positive when the plaque 

assay did not yield recordable counts. Nevertheless, figure 4 

plainly shows that virus isolation attempts from serum samples 

were only rarely positive after day 12.

The evolution of IgM antibody in EHF patients is presented 

in figure 6. The IgM values appeared to peak among patients 

~18 days after onset; this time point included very few patients 

who did not eventually survive their infections. IgM antibody, 

as shown in figure 2, was found in fewer than half of the 

patients (3/7) at the time of their deaths. Mean IgG antibody 

titers (figure 7) appear to reach maximum levels slightly later 

than the mean for EBO IgM. The EBO IgM antibodies in 

survivors are relatively short lived and seem to decline more 

rapidly than other IgM class viral antibodies measured by the 

IgM-capture ELISA.

We also attempted to follow a group of survivors, for about 

2 years, to obtain more information about the persistence of 

antibody. While the numbers of patients are small, individuals 

still had detectable IgG antibody titers when samples were 

collected nearly 2 years after onset of illness (figure 7). While 

these results are encouraging, the specific signal, particularly 

when considering the adjusted OD (measured at 410 nm) sums, 

would be better if it provided more specific signal. Some of 

the individual patient values diminish toward the numeric cut-

off value, which was necessitated by the relatively high ad-

justed OD sums seen in the general population (higher ratios
of signal to noise). Again, detectable IgM antibody was much more ephemeral, largely disappearing after ~60 days after the recorded onset of disease (figure 6).

The mortality outcome of other viral hemorrhagic fevers has been associated with the amplitude of viremias during the early course of the disease [15]. We attempted to examine if there was a correlation between viral antigenemia and disease outcome among the patients for whom multiple samples were available. These data show only moderate differences between those who survived and those who succumbed to the disease (figure 8). The lack of discrimination may be partially due to antigenemias having reached maximum scale, particularly when using the titer values. However, the viremia data, while subject to some complication because of the performance of

![Figure 6.](image-url)
the plaque assay, seem to suggest that there were higher mean virus titers in patients who died than in those who survived (figure 9).

Discussion

Despite the large number of EHF cases during the Kikwit epidemic of 1995, the systematic collection of patient material was delayed until the epidemic had been virtually controlled and EBO virus transmission in the community had largely ceased. The control of the epidemic was a major accomplishment, but because efforts were diverted to that task rather than to sample collection, the number of patients from whom serial samples were obtained to establish the natural evolution of the detectable antigen and IgG and IgM antibodies was small. Nevertheless, these data do validate the use of the antigen-detection test as a means of detecting acute infections with
Figure 8. Comparison of Ebola antigen (Ag) detection adjusted optical density (OD; measured at 410 nm) sums (A) and log_{10} titers (B) among Ebola hemorrhagic fever patients who survived and those who died. Symbols represent mean; bars represent 1 SE of mean for each time point. Time is shown in days after onset and is divided into 2-day intervals up to day 30 and into 30-day intervals after 30 days. N = no. of data points in each time group.
EBO virus. The data suggest that while antibody is present in many of the patients during the acute phase of the disease, the antigen OD values and titers are high and the IgM and IgG values are low, thus making interpretation of the antigen test much more certain. Indeed, the antigen-detection ELISA has been used to detect EBO virus infections in patients in Côte d’Ivoire [16], South Africa [3], and Kikwit [5] and among primates in the United States in 1989, 1990 [9], and 1996 [17].

While the RT-PCR assay probably has greater absolute sensitivity in terms of the quantity of the virus that the test can detect, the antigen-detection ELISA has provided results that have agreed in virtually 100% of the specimens obtained from acutely ill EHF patients tested in parallel [6]. Despite this concordance, the antigen-detection ELISA did not detect EBO virus antigens in semen samples from convalescent patients who were positive by RT-PCR [18]; however, the same samples also failed to yield infectious virus in our isolation attempts, including repeated efforts with extended culture.

Our ability to quantitate virus in EHF patient serum or blood was limited in attempts using a standard plaque assay [12, 13] employing neutral red staining; the FFA was better able to quantitate virus, perhaps because the assay depends upon microscopic identification of infected cells. This may allow visualization of small viral foci that were not visible as plaques in the neutral red plaque assay. This is intriguing because the control, an isolate of virus from an early EHF patient in the Kikwit epidemic, yielded consistent plaque titers throughout the testing period. Perhaps there were inhibitors in many of the patient samples that limited the size of viral foci to less than visible size.

When comparing patients who died and those who survived, we noted apparent differences between the mean values for EBO antigen detected and virus titer. This finding is similar to that described for Lassa fever [15], in which early higher viremias were associated with poor prognosis. The utility of transfusions of convalescent blood is the topic of an accompanying paper [19], and a similar comparison among those who received transfusions of whole convalescent blood and those who did not showed no apparent differences in either virus antigen or virus titers (data not shown).

The RT-PCR and the EBO antigen—detection ELISA both offer the advantage of testing samples that have been rendered noninfectious by the use of chaotrope or gamma irradiation, respectively, and thus allow safe handling of materials outside of the confines of a BSL-4 laboratory. Because blood samples may not be obtained from patients who have already expired and because, in some remote areas, there may not be readily available means for refrigerating and transporting frozen materials, the use of immunohistochemical antigen detection in fixed tissues should also be considered [20].
Measurement of antibody appears to be of limited utility in the diagnosis of acute EHF cases. Our data suggest that many patients do not have antibody early in the course of their illness, and many die without developing detectable antibodies; however, they do have antigen and virus in their blood. Nevertheless, antibody remains the principal means of testing sera from patients who have recovered and who are being diagnosed retrospectively. The amplitude and duration of the IgM antibody is somewhat disappointing when compared with results obtained by similar IgM assays we have tested in other hemorrhagic fever infections, such as Crimean-Congo hemorrhagic fever or Lassa fever (Ksiazek TG and Rollin PE, unpublished data). However, the assay clearly does have utility for the diagnosis of convalescent EHF patients in the early stages of recovery.

The primary utility of the IgG test remains in assessing populations who are thought to have been infected at some time in the more distant past, such as in studies of populations for evidence of filovirus infections. Our experience with long-term follow-up of known patients is limited: Results for the few patients who were followed for ~2 years in this study represent the majority of such data currently available. It would be desirable to have a test that has higher ratios of signal to noise and longer duration; however, in similar limited experience with EHF patients tested with the indirect fluorescent antibody test, serum titers appeared to persist, but they did wane over extended periods.

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