JOURNAL OF VIROLOGY, Apr. 2004, p. 4330–4341 Vol. 78, No. 8
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.8.4330–4341.2004

Rapid Diagnosis of Ebola Hemorrhagic Fever by Reverse Transcription-PCR in an Outbreak Setting and Assessment of Patient Viral Load as a Predictor of Outcome

Jonathan S. Towner,[1] Pierre E. Rollin,[1] Daniel G. Bausch,[1] Anthony Sanchez,[1] Sharon M. Crary,[1,2] Martin Vincent,[1] William F. Lee,[3] Christina F. Spiropoulou,[1] Thomas G. Ksiazek,[1] Mathew Lukwiya,[4] Felix Kaducu,[5] Robert Downing,[6] and Stuart T. Nichol[1]*

Special Pathogens Branch[1] and Department of Pathology,[3] Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; Depauw University, Greencastle, Indiana[2]; and Saint Mary's Lacor Hospital[4] and Gulu Regional Hospital, Ministry of Health,[5] Gulu, and Uganda Virus Research Institute, Centers for Disease Control and Prevention Uganda, Entebbe,[6] Uganda

Received 12 September 2003/Accepted 3 December 2003

The largest outbreak on record of Ebola hemorrhagic fever (EHF) occurred in Uganda from August 2000 to January 2001. The outbreak was centered in the Gulu district of northern Uganda, with secondary transmission to other districts. After the initial diagnosis of Sudan ebolavirus by the National Institute for Virology in Johannesburg, South Africa, a temporary diagnostic laboratory was established within the Gulu district at St. Mary's Lacor Hospital. The laboratory used antigen capture and reverse transcription-PCR (RT-PCR) to diagnose Sudan ebolavirus infection in suspect patients. The RT-PCR and antigen-capture diagnostic assays proved very effective for detecting ebolavirus in patient serum, plasma, and whole blood. In samples collected very early in the course of infection, the RT-PCR assay could detect ebolavirus 24 to 48 h prior to detection by antigen capture. More than 1,000 blood samples were collected, with multiple samples obtained from many patients throughout the course of infection. Real-time quantitative RT-PCR was used to determine the viral load in multiple samples from patients with fatal and nonfatal cases, and these data were correlated with the disease outcome. RNA copy levels in patients who died averaged 2 $\log_{10}$ higher than those in patients who survived. Using clinical material from multiple EHF patients, we sequenced the variable region of the glycoprotein. This Sudan ebolavirus strain was not derived from either the earlier Boniface (1976) or Maleo (1979) strain, but it shares a common ancestor with both. Furthermore, both sequence and epidemiologic data are consistent with the outbreak having originated from a single introduction into the human population.

Ebolavirus is a single-stranded, negative-sense RNA virus that can produce high-mortality disease in humans and nonhuman primates and has caused sporadic outbreaks of Ebola hemorrhagic fever (EHF) in Central Africa and Southeast Asia. The virus genome is almost 19 kb long and encodes seven viral proteins, namely, nucleoprotein (NP), phosphoprotein (VP35), matrix protein (VP40), glycoprotein (GP), replication-transcription protein (VP30), matrix protein (VP24), and polymerase (L), with an additional soluble glycoprotein produced from an edited GP mRNA (19, 21, 22). The genes are arranged in the order 3′-NP-VP35-VP40-GP-VP30-VP24-L-5′. Currently, there are four known species of ebolavirus, three of which are found in Africa (Zaire, Sudan, and Tai Forest), and a fourth species, Reston, found in Asia. Outbreaks associated with these viruses are often large and can cause high levels of mortality, sometimes reaching 50 to 90% of infected individuals. In humans, death typically occurs 7 to 10 days after the onset of symptoms and can be preceded by mucosal hemorrhages, visceral hemorrhagic effusions, diffuse coagulopathy, shock, and central nervous system complications, such as convulsions. Early viral amplification is thought to occur in mononuclear phagocytes and is followed by massive liver, spleen, and lung infection, endothelial cell leakage (hemorrhage), and ultimately death. There is no known specific therapy for EHF, and due to the severity of the disease, the rapid onset of symptoms, and the ease of human-to-human transmission, the ebolaviruses are classified as biosafety level 4 (BSL-4) viruses and are on the Centers for Disease Control and Prevention (CDC) Category A list of potential bioterrorist agents.

While much publicity and research have been devoted to Zaire ebolavirus, the most lethal of the ebolaviruses, the outbreak that ended with the largest number of human EHF cases was caused by Sudan ebolavirus. This outbreak occurred from August 2000 to January 2001 and was centered in the Gulu district of northern Uganda, with spread to two of the country's southern districts through the movement of infected individuals and subsequent secondary contact transmissions (4). In total, there were 425 cases with 224 deaths (53% case fatality). This level of case fatality was similar to that seen for two previous EHF outbreaks associated with Sudan ebolavirus (in 1976 and 1979), in which 53% of 284 cases and 66% of 34 cases had fatal outcomes (6). As has been the case with most EHF epidemics, there was not an established diagnostic laboratory nearby during the Gulu outbreak; thus, a field laboratory was established on-site in rural northern Uganda at a local missionary hospital (St. Mary's Lacor Hospital) for the purpose of identifying acute EHF cases. The single previous attempt to

* Corresponding author. Mailing address: MS G-14, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333. Phone: (404) 639-1115. Fax: (404) 639-1118. E-mail: stn1@cdc.gov.

establish a field laboratory in an epidemic setting (in 1976 in Zaire) relied upon an immunofluorescence assay (IFA) for acute case identification, but the results were poor (2, 9, 14). During the Uganda outbreak, an antigen-capture diagnostic assay (16) along with a newly developed nested reverse transcription-PCR (RT-PCR) assay were used, which together proved very effective as field diagnostic tools for the detection of ebolavirus antigen and nucleic acid in patient serum, plasma, and whole blood. During the outbreak, almost 1,800 samples were tested by antigen capture and about 1,100 were tested by RT-PCR. The epidemic represented the first reemergence of Sudan ebolavirus in over 20 years and provided a rare opportunity to collect multiple specimens from patients throughout the course of the disease and thus to gain a better understanding of the clinical virology of Sudan ebolavirus and to meaningfully assess the available diagnostic assays.

After the outbreak, we retrospectively measured the viral load throughout the course of disease in a subset of 45 patients, using real-time quantitative RT-PCR (Q-RT-PCR). For some of these samples, the viral load was also measured by plaque assay. Collectively, these findings represent the first comprehensive determination of viral load profiles in humans infected with Sudan ebolavirus, and these measurements ultimately allow the retrospective prediction of patient outcome on the basis of viral load. This study also provides a unique side-by-side comparison of newer technologies, such as real-time 5′-nuclease RT-PCR, and more established EHF diagnostic tests. Lastly, we determined the sequence of the variable portion of GP from samples collected throughout the outbreak and determined that the ebolavirus outbreak was likely the result of a single introduction of the virus into the human population.

MATERIALS AND METHODS

Specimen collection and handling. Patients diagnosed with EHF were cared for in a supervised, restricted-access, barrier-nursing environment in a designated pavilion of either St. Mary's Lacor Hospital or Gulu General Hospital. A limited-access field laboratory was set up in St. Mary's Lacor Hospital, in which all laboratory personnel wore adequate protective clothing and, if necessary, battery-operated, positive-pressure, air-purifying respirators. In brief, laboratory operations were performed as follows. Blood samples were obtained daily from suspect patients at each of the isolation wards, and when possible, samples were obtained every other day from patients confirmed to have EHF. The blood samples were allowed to clot at ambient temperature, and the sera were isolated and separated into multiple aliquots. Initial sample processing was performed in a laminar-flow biosafety cabinet made available in the laboratory from the hospital. One aliquot was used for antigen-capture and immunoglobulin G (IgG) enzyme-linked immunosorbent assays (ELISAs), and a duplicate aliquot was mixed with a phenol-guanidinium-based chaotrope and then decontaminated and passed to a separate room that was designated for RNA purification and RT-PCR. RT-PCR products were analyzed in a third room to avoid potential cross-contamination. The goal of the field laboratory was to provide next-day results for patients suspected of having EHF. Samples that arrived late in the day were stored overnight at 4°C and processed the next day. Antigen-capture assays with separated sera were performed on-site as previously described (15, 16). Remaining blood, sera, and clots were labeled and stored in liquid nitrogen. Because of limited space within the liquid nitrogen containers at the field laboratory, samples were periodically transported to the Uganda Virus Research Institute for temporary storage in mechanical freezers at −80°C. At the end of the outbreak, all samples were transported on dry ice in International Airline Transport Association-compliant safety shippers to the BSL-4 laboratory at CDC (Atlanta, Ga.), where they were catalogued and stored in liquid nitrogen.

Total RNA purification. Total RNAs were purified by mixing 100 μl of sample (serum, blood, or plasma) with 500 μl of a monophasic solution of 4 M guanidine thiocyanate and phenol (TriPure; Roche). After being mixed, the samples were transferred to clean 1.7-ml microcentrifuge tubes, and the outsides of the tubes were decontaminated with 3% Lysol. Samples were then passed out of the high-containment-level laboratory to a room that was designated for RNA isolation and RT-PCR setup. After a brief centrifugation, 200 μl of chloroform-isoamyl alcohol (24:1) was added and each sample was extensively vortexed. Samples were then centrifuged at 16,000 × *g* for 15 min at ambient temperature in a Microfuge (Eppendorf). Occasionally, when the interface was thick, the samples were centrifuged an additional 15 min. The aqueous phase was carefully extracted and added to 12 μl of RNA Matrix (Q-Biogene/Bio101), and the mixture was vortexed and then allowed to incubate, with occasional mixing, for 5 min at room temperature. Each sample was then spun at 16,000 × *g* for 1 min to pellet the RNA matrix, and the resulting supernatant was discarded. Residual liquid was removed after an additional pulse centrifugation. Samples were washed with 900 μl of wash buffer (Q-Biogene/Bio101), and after the removal of residual wash buffer, were resuspended in 50 μl of nuclease-free H_2O. Each sample was incubated for 5 min at 55°C, and the aqueous RNA was recovered after a 1-min centrifugation at 16,000 × *g* and stored at −80°C. Note that when RNAs were extracted in the field, they were stored at −40°C.

Field diagnostic nested RT-PCR. First-round RT-PCRs were set up in a laminar-flow biosafety cabinet according to the manufacturer's directions, using Access RT-PCR kits (Promega) and 5 μl of purified total RNA. Initially, 50-μl reactions were used, but later, to conserve reagents, 25-μl total volume reactions were used. The first-round primers were designed to recognize and amplify a 185-nucleotide fragment of the NP open reading frame (ORF) from either Sudan or Zaire ebolavirus RNA. The second-round (nested) primers similarly would recognize either Sudan or Zaire ebolavirus, generating a 150-nucleotide fragment. The sequences of the primers used were as follows: SudZaiNP1(+), 5′-GAGACAACGGAAGCTAATGC-3′, and SudZaiNP1(−), 5′-AACGGAAGATCACCATCATG-3′, for the first round; and SudZaiNP2(+), 5′-GGTCAGTTTCTATCCTTTGC-3′, and SudZaiNP2(−), 5′-CATGTGTCCAACTGATTGCC-3′, for the second round. The underlined nucleotides designate parts of the sequence that are different between the Sudan and Zaire ebolaviruses, where the actual nucleotide shown represents the sequence of Sudan ebolavirus at that position. Nested PCRs were set up according to the manufacturer's instructions, using *Taq* DNA polymerase (Roche) and a reaction buffer that yielded 1.5 mM Mg^{2+}. The conditions for the first-round RT-PCRs were to initially incubate the reaction mixtures for 30 min at 50°C to allow RT, followed by 2 min at 94°C to allow enzyme inactivation and denaturation. This was then followed by 38 cycles of denaturing at 94°C for 30 s, annealing at 50°C for 30 s, and elongation at 68°C for 1 min. The thermocycling conditions for the nested reactions were identical to those for the first-round reactions, with the exception that there was no RT step and the elongation temperature was 72°C. All amplification products were analyzed in 2% agarose–Tris-acetate-EDTA gels stained with ethidium bromide.

Two-step Q-RT-PCR analysis. Because of cold-chain lapses during the transport of field-isolated RNAs to the CDC laboratory, RNAs for use in the Q-RT-PCR study were reisolated from unthawed aliquots of each frozen serum. The cold chain for the transfer of the serum samples to CDC was continuous, and RNAs were isolated as described above. The two-step Q-RT-PCR-based fluorescence assay (for the detection of genomic-sense RNA) was set up by first converting the ebolavirus negative-strand RNA to cDNA in a separate RT reaction containing a positive-sense primer specific for the NP ORF region of the Gulu strain of Sudan ebolavirus. The positive-sense RT primer used was 5′-GAAAGAGCGGCTGGCCAAA-3′. In a 10-μl reaction volume, the following components were mixed: 2 μl of 5× RT buffer, 0.2 μl of 10 mM deoxynucleoside triphosphates, 4.6 μl of nuclease-free H_2O, 0.2 μl (10 pmol) of RT primer, 1 μl of RNA, and 2 μl of Moloney murine leukemia virus reverse transcriptase (diluted 1:400 in 1× reaction buffer). Note that the reverse transcriptase was added last and only after the sample had been preheated to 55°C for 2 to 3 min to minimize nonspecific priming. Reactions were then incubated for 15 min at 55°C and then diluted fivefold with nuclease-free H_2O prior to the inactivation of reverse transcriptase by incubation at 95°C for 30 min. Samples were then pulse centrifuged to concentrate all of the liquid to the bottom of the tube.

For this single-strand-specific two-step real-time PCR-based quantification assay, 5 μl of each cDNA reaction mixture was mixed with 12.5 μl of 2× TaqMan Universal Master mix (Applied Biosystems), 25 pmol each of forward and reverse primers, 5 pmol of a fluorogenic probe, and nuclease-free H_2O to a total volume of 25 μl. The forward primer was the same as that used for the RT step, while the reverse primer was 5′-AACGATCTCCAACCTTGATCTTT-3′. The fluorogenic probe was 5′-TGACCGAAGCCATCACGACTGCAT-3′ and was labeled at the 5′ end with the reporter dye FAM and at the 3′ end with the quencher QSY7. The primers and the fluorogenic probe were specific for the NP region of the Gulu strain of Sudan ebolavirus and together generated an amplicon of 69 nucleotides. The probe and primer combinations were designed by Primer Express software from Applied Biosystems. The reactions were thermo-

cycled in an Applied Biosystems 7700 instrument by heating to 50°C for 2 min, followed by heating to 95°C for 10 min to activate the AmpliTaq polymerase. The reactions were then subjected to 40 cycles of amplification by alternately incubating at 95°C for 15 s and 60°C for 1 min. All PCRs were performed in triplicate, with each run containing control reactions in which either RNA, RT primer, or reverse transcriptase was omitted.

For the generation of RNA for use as a standard curve with the Q-RT-PCR assay, an approximately 1-kb portion of the NP ORF containing the primer and probe target sequences was amplified from virus RNA isolated from the Gulu strain of Sudan ebolavirus. The fragment was then cloned into a bidirectional transcription vector so that either positive- or negative-strand RNA could be generated by in vitro transcription. Following in vitro transcription, the DNA template was digested three times with DNase (RNase and protease free) (Roche). The transcribed RNA was then phenol-chloroform extracted and ethanol precipitated two times, followed by further purfication over two RNA-easy columns (Qiagen) to remove unincorporated nucleotides and small remaining undigested DNA. The transcribed NP RNA was quantitated by standard methods, using an experimentally measured optical density at 260 nm (OD_{260}) and a calculated molar extinction coefficient based upon the exact NP fragment sequence. In vitro-transcribed and -quantitated negative-sense NP RNA was then serially diluted and used to generate a standard curve for the Q-RT-PCR analysis of genomic-sense RNA in patient samples. For each Q-RT-PCR series, the threshold cycle (C_t) value was set within the linear range of DNA amplification for all productive reactions. Because the RNA used in the Q-RT-PCR assay was extracted from a noncellular environment (i.e., serum), no comparison to a "housekeeping" gene, such as glyceraldehyde-6-phosphate dehydrogenase, was performed.

One-step Q-RT-PCR. One-step real-time Q-RT-PCRs (for the detection of both positive- and negative-sense RNAs) were set up by mixing 12.5 μl of 2× TaqMan one-step RT-PCR master mix reagents without AmpErase UNG, 25 pmol each of forward and reverse primers, 5 pmol of fluorogenic probe, 1 μl of total RNA, 0.62 μl of 40× Multiscribe reverse transcriptase, and nuclease-free H_2O to a total volume of 25 μl. Reactions were incubated for 15 min at 50°C followed by heating to 95°C for 10 min. The reaction mixtures were then subjected to 40 cycles of amplification by alternately incubating them at 95°C for 15 s and 60°C for 1 min. All PCRs were performed in triplicate along with control reactions in which either RNA or reverse transcriptase was omitted.

Antigen detection and IgG ELISAs. Antigen detection ELISAs were performed as previously described (15, 16). Specimens were tested at four dilutions (1/4, 1/16, 1/64, and 1/256). Titers and the cumulative sum (four dilutions) of the optical density (OD_{sum}) were recorded. IgG ELISAs were performed in the field and at CDC as previously described (16), using ebolavirus lysates. Specimens were tested at four dilutions (1/100, 1/400, 1/1600, and 1/6400). Titers and the OD_{sum} were recorded. Sera were considered positive if the titer was ≥400 and the sum of the adjusted ODs was >0.6.

Virus plaque assay. Plaque assays were set up in a laminar-flow safety cabinet in a BSL-4 laboratory (at CDC) on confluent monolayers of Vero E6 cells in six-well plastic tissue culture plates. The virus was diluted in serial 10-fold dilutions in Dulbecco's modified Eagle's medium. Dilutions of 10^{-1} through 10^{-5} were adsorbed to the cells by plating 200 μl of a diluted specimen in duplicate onto the monolayer and incubating it for 1 h at 37°C. The inoculum was removed and the monolayers were overlaid with a solution of 1% agarose (SeaKem ME; FMC), 2% fetal bovine serum, 2 mM L-glutamine, 15 mM HEPES (pH 7.5), 1× minimal essential medium without phenol red (GIBCO, Invitrogen, Life Technologies), and 1× antibiotic-antimycotic (GIBCO, Invitrogen, Life Technologies). The plates were then incubated for 7 to 8 days at 37°C and then fixed overnight with 2 ml of stock (37%) formaldehyde per well. The agarose overlays were removed and the wells were rinsed with H_2O. The plates were then double-bagged in heat-sealed pouches, and the external surfaces of the pouches were decontaminated with 3% Lysol before they were removed from the BSL-4 laboratory according to standard procedures. The plates were then gamma irradiated with 2×10^6 rads. Virus plaques were revealed by a 1-h incubation with a 1:1,000 dilution of a rabbit anti-ebolavirus antibody followed by a second 1-h incubation with a 1:1,500 dilution of a horseradish peroxidase-labeled goat anti-rabbit secondary antibody (Bio-Rad). After thorough rinsing with deionized H_2O, 500 μl of True-Blue (KPL) substrate was added, and after 10 to 15 min, virus plaques were counted.

Nucleotide sequencing and phylogenetics. For determination of the sequence of the variable portion of the virus glycoprotein, the region was amplified by RT-PCR using Access RT-PCR kits (Promega) (as described above) from RNAs isolated directly from patient sera, using primers designed on the basis of the Maleo strain (GenBank accession number U23069) of Sudan ebolavirus. The primers used for first-round amplification were SudGP1(+) (5′-CGAGAGGC AGCAAACTACAC-3′) and SudGP4(−) (5′-GTGTATATGCCTTCTGCACC-3′). The thermocycling conditions for the first-round RT-PCRs were as follows: 30 min at 48°C to allow RT and 2 min at 94°C for enzyme inactivation and denaturation, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 68°C for 2 min. The first-round amplifications yielded single-band products of the expected size (1,032 nucleotides) but of insufficient quantity for effective DNA sequencing. Therefore, these DNA amplification products were gel purified and used as templates for second-round nested PCRs, using the same Access PCR kits but no reverse transcriptase. The primers used for the nested reactions were SudGP5(+) (5′-ATCAAGTTACT ATGCCACATCC-3′) and SudGP6(−) (5′-ATCCAGGCAATCCCAGC-3′), which together amplify an ~970-nucleotide fragment. The thermocycling conditions for the nested reactions were identical to those for the first-round reactions, with the exception that there was no RT step and the annealing temperature was raised to 55°C. The second-round amplification products required only minimal processing with filter cartridges (Qiagen) to remove buffer, unincorporated deoxynucleotide triphosphates, and primers prior to sequencing. The primers used for sequencing were SudGP(2+) (5′-ACTACAAAGGGAAGAATCTC-3′), SudGP(3+) (5′-AACCAACAACACCACCGAGA), and SudGP(3−) (5′-TCT CGGTGGTGTTGTTGGTT-3′), in addition to SudGP5(+) and SudGP6(−) (described above).

DNA products produced by PCR were purified in Qiaquick spin columns (Qiagen) and sequenced directly by use of Big Dye terminator cycle sequencing ready reaction mix (ABI) and an ABI Prism 3100 genetic analyzer. The obtained sequence chromatograms were analyzed with Sequencher, version 4.0.5, software (Gene Codes). Filovirus sequences were aligned by the PILEUP program of the Wisconsin Package, version 10.2 (Accelrys, Inc., Burlington, Mass.). Phylogenetic analysis was done with PAUP4.0b10 (Sinauer Associates Inc. Publishers). The phylogenetic analysis was performed by comparing 875 nucleotides of the experimentally determined GP nucleotide sequence described above (corresponding to nucleotides 821 to 1696 of the Maleo strain of Sudan ebolavirus) with the corresponding known sequences of the other indicated filoviruses.

RESULTS

Field diagnosis of EHF by antigen-capture ELISA and nested RT-PCR. The initial confirmation that the hemorrhagic fever outbreak in Uganda was indeed caused by ebolavirus came from the National Institute for Virology (R. Swanepoel, personal communication) by means of RT-PCR fragments generated from patient specimens. The RT-PCR fragments corresponded to the polymerase region and yielded Sudan-like ebolavirus nucleotide sequences. On the basis of this confirmation of EHF caused by the Sudan species, we designed a nested set of primers to anneal to the NP domain in regions that are conserved between the Sudan and Zaire species so that the primers would amplify RNA from either virus. We chose regions with the most conservation for the target sequence so that the primers would have the highest likelihood of recognizing the reemergent Sudan-like ebolavirus. We chose the NP region because the NP mRNA is the most abundant virus-specific RNA generated in infected cells and thereby would allow for the highest potential sensitivity. In addition, we used a more generic "Filo A and B" primer set that was utilized in previous outbreaks (20) to detect ebolavirus RNA and has since been used by Leroy et al. (17) and Drosten et al. (10) for ebolavirus diagnostic assays.

In the Gulu district, suspect cases were directed to one of two isolation wards established within the Gulu township, one at St. Mary's Lacor Hospital and the other at Gulu General Hospital. Patients were assessed (by clinicians) and blood samples were obtained for diagnostic testing in the field laboratory. Each sample was processed into multiple aliquots for use in RT-PCR and in serologic (IgG) and antigen-capture assays (15, 16). The RT-PCR assay was initially performed with the Filo A and B primers mentioned above under previously de-

TABLE 1. Summary of diagnostic testing by antigen-capture ELISA and RT-PCR during the EHF outbreak in Gulu, Uganda

Assay	No. of samples tested	No. of positive samples	No. of false positives[a]	No. of false negatives[b]	No. of PCR^+ Ag^+ samples	No. of PCR^+ Ag^- samples	
						Early detection	Late detection
Antigen-capture ELISA	1,771	282	1	0			
RT-PCR	1,083	246	3[e]	4			
Total					196	17[c]	20[d]

[a] False positives were defined as samples that initially tested positive by a particular assay but tested negative with a subsequent sample from the same patient and for which all other diagnostic tests were and continued to be negative.
[b] False negatives were defined as samples that tested negative by nested RT-PCR and positive by antigen capture.
[c] Represents 13 patients for which the maximum time between PCR^+ Ag^- samples was 72 to 96 h.
[d] Represents 18 patients for which the maximum time between PCR^+ Ag^- samples was 48 to 72 h.
[e] There were an additional 10 potential false positives that were unresolvable.

scribed conditions (20). Unfortunately, the sensitivity of these primers was less than that of the antigen-capture assay that was performed in parallel. Of the first 49 samples that tested positive by antigen capture, only 30 (61%) tested positive with the Filo A and B primer set, and none of the PCR-positive samples were antigen negative. For this reason, the newly developed nested set of NP primers, which should have greatly improved the sensitivity, was used exclusively for all remaining RT-PCR analyses. In addition, serum rather than whole blood was used whenever possible in an effort to minimize the presence of RT-PCR inhibitors in the extracted RNAs.

In total, 1,771 samples were tested by antigen-capture ELISA and 282 were identified as positive for ebolavirus according to the criteria that were previously published for this assay (16). The results of the field laboratory diagnostic testing are summarized in Table 1. Including those samples tested by the Filo A and B primer set, a total of 1,083 specimens were analyzed by RT-PCR, and of those, 246 samples were identified as positive. Of the 246 PCR-positive (PCR^+) samples, 196 were concordant with the antigen-capture assay (i.e., PCR^+ Ag^+), leaving 50 samples that were discordant with the antigen-capture assay (PCR^+ Ag^-). Among the 50 discordant samples, 17 (representing 13 patients) were obtained very early in the acute phase of disease, just after the onset of symptoms but prior to testing positive by antigen capture (early detection) in subsequent samples. Another 20 PCR^+ Ag^- specimens (representing 18 patients) were obtained during the convalescent phase, coincident with IgM (data not shown) and/or IgG responses. These convalescent-phase patients often remained PCR positive for 24 to 48 h (the maximum interval was 72 h) after clearing detectable antigen. The remaining 13 discordant samples represented potential false positives, as determined by using the antigen-capture ELISA as the reference standard (assuming no antigen false positives) to which the RT-PCR assay was compared. Three of the potential false-positive samples were later proven to be PCR^- Ag^- by testing of duplicate samples from the same patient, and 10 were unresolvable due to the lack of additional confirmatory samples. The earliest virus detection by which a time frame could be established between two PCR^+ Ag^- samples from the same patient was 72 h prior to testing positive by antigen-capture ELISA. Collectively, these data demonstrate the high sensitivity of the nested RT-PCR assay and its field use for early EHF case identification. The RT-PCR assay was substantially more time-consuming than the antigen-capture ELISA, and therefore in an effort to maintain pace with the ongoing outbreak, later samples from established laboratory-positive patients were often not further tested by RT-PCR until the antigen levels had peaked and had subsequently diminished to the ELISA detection limit. In addition, there were four samples that were PCR^- Ag^+, not including the false negatives with the Filo A and B primer set. All four of these PCR^- Ag^+ samples were close to the threshold detection limit of the antigen-capture ELISA.

Determination of viral load in serum samples from patients who died and patients who survived. After the outbreak and upon return to CDC in Atlanta, we sought to determine viral load profiles of released virus in the sera of a subset of patients who died and patients who survived, using Q-RT-PCR, and to correlate these data with levels of antigen and IgG and with patient outcome. The serum samples chosen for this analysis met the criterion of being from patients for which the time of onset of symptoms was well established, and each blood specimen contained sufficient material for all subsequent analyses.

Three sets of 5′-nuclease primer-probe combinations were originally designed, two for NP and one for GP, and all three primer-probe sets were tested for sensitivity and specificity on total RNAs isolated from infected cells (data not shown). Ultimately, one primer-probe combination for NP was found to be slightly more sensitive than the other two combinations. This set, yielding a slope of -3.4 when C_t values were graphed versus $\log_{10}$ RNA dilutions (data not shown), was subsequently used for all experiments described below. For further validation of the quantitative assay, serial $\log_{10}$ RNA dilutions of either positive- or negative-sense NP RNA were analyzed with either negative- or positive-strand primers during the RT step. As shown in Fig. 1A, reactions with RT primers of the opposite sense as the target RNA produced threshold (C_t) values that were 15 to 18 units lower than that for reactions programmed with the same sense RT primer. On the basis of the slopes of the standard curves, 15 to 18 C_t values translates to an ~10,000-fold difference in RNA copy number, indicating that 1 RNA copy in ~10,000 is due to mispriming during the RT step. Therefore, we considered the contribution to the total signal by mispriming to be negligible and considered the signal generated to be representative of levels of authentic genomic-sense ebolavirus RNA when the positive-strand RT primer was used.

Our observation when handling blood specimens in the field was that hemolysis was often pronounced, particularly for acute EHF patients. Therefore, the effects of nonviral RNA found in either serum or whole blood on the efficiency of the Q-RT-PCR assay were determined. As shown in Fig. 1B, only a very slight inhibition was observed when serial 100-fold di-

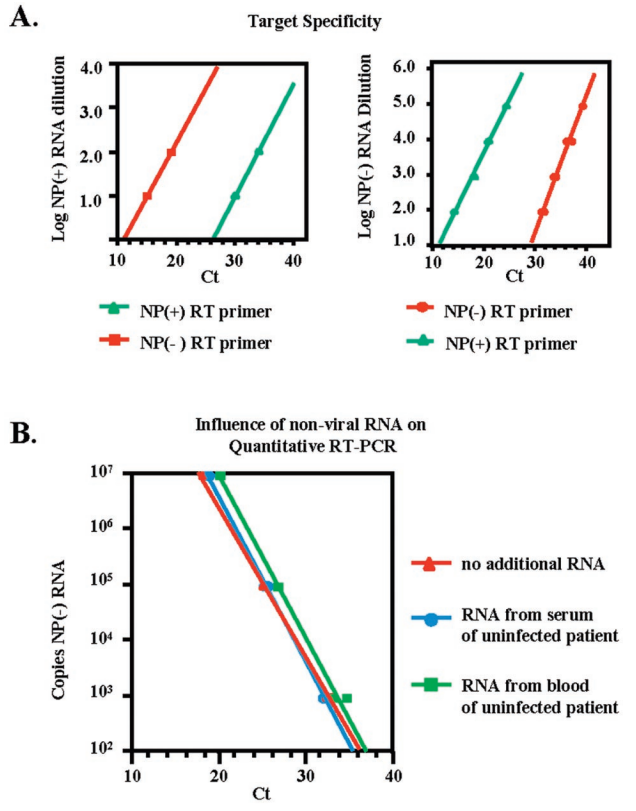


FIG. 1. Target specificity of NP primer-probe set designed for the Gulu strain of Sudan ebolavirus. (A) In vitro-transcribed genomic- or antigenomic-sense NP RNA was serially diluted and amplified by using either positive- or negative-sense primers during the RT step. (B) In vitro-transcribed genomic-sense NP RNA was serially diluted and tested either alone or in the presence of RNA isolated from serum or whole blood of an uninfected person.

luted negative-sense RNA was analyzed in the presence or absence of total RNA isolated from either serum or whole blood. We concluded that RNA copy number measurements would not be significantly affected by the cell lysis often found with EHF infections and that the variability of virus-induced cell lysis between different patient samples should not greatly alter the viral load values.

We next selected 27 patients with fatal outcomes and 18 with nonfatal outcomes, all but 4 of whom had two or more samples taken throughout the course of the infection, and measured the genomic-sense RNA copy number per milliliter of serum. In addition, we used the serum antigen levels determined in the field and plotted these values with the corresponding RNA copy numbers measured from the same samples. IgG levels, which were initially measured in the field, were reassayed at CDC by use of a cell lysate generated from Vero E6 cells infected with the Gulu strain of Sudan ebolavirus. With each set of samples tested by Q-RT-PCR, three control reactions were included (containing no reverse transcriptase, no template, or no RT primer) and found to be negative. Figure 2 shows a total of six viral load profiles, representing three patients with fatal outcomes (Fig. 2A, panels 1, 2, and 3) and another three patients with nonfatal outcomes (Fig. 2B, panels 1, 2, and 3). Collectively, they are representative of the spectrum of profiles observed for patients in the Gulu outbreak. One of the striking features is the very high level of genomic-sense RNA detected in samples from the patients who died, sometimes reaching 10^{10} RNA copies per ml of serum. While some nonsurvivors (e.g., those represented in Fig. 2A, panels 2 and 3) had RNA copy levels (10^7) similar to those seen in patients who survived, by far the majority had levels similar to that seen in Fig. 2A, panel 1, which represents a patient who had a considerably more rapid and acute disease course (often only one or two blood samples could be obtained from such patients before death). Figure 2B shows the viral load profiles for three survivors who ultimately showed IgG responses of various degrees, although often not until the clearance of antigen (and RNA) was well under way. The difference in the RNA copy levels between the patients with fatal and nonfatal outcomes can best be seen in Fig. 3A, which shows that the levels (from days 1 to 9 after the onset of symptoms) for nonsurvivors averaged at least 2 $\log_{10}$ higher than those for survivors. Furthermore, during the first 2 days of symptoms, the early rate of increase of genomic-sense RNA was considerably higher in patients with fatal outcomes. The viral RNA levels in nonsurvivors reached, on average, 10^8 to 10^9 RNA copies per ml of serum by day 2, a level that predicts a poor outcome when compared with that observed for patients who survived. Of the 82 samples tested from patients who survived, only 2, representing 2 (11%) of 18 patients, reached the 10^8 RNA copies/ml plateau, whereas nearly half of the samples tested (34 of 73) from patients who died, representing 21 (78%) of 27 patients, reached the same level or higher. Notably, 20 (91%) of 22 nonsurvivors had viral loads that reached $\geq 10^8$ RNA copies/ml within the first 8 days after the onset of symptoms, thus suggesting that 10^8 RNA copies/ml can be considered an approximate threshold that predicts a fatal outcome with a positive predictive capability of >90%. The results of the antigen-capture assay performed on the same set of samples are shown in Fig. 3B. The data obtained with the antigen-capture assay show large standard errors from the mean for each time point. For this reason, antigen-capture data from an additional 62 patients who died and 35 who survived were added to the data already presented in Fig. 3B. With the results of this larger data set (shown in Fig. 3C), a moderate difference between antigen levels was seen for nonsurvivors and survivors, with the former tending to have levels that were 1 to 2 OD units higher than those for patients who survived. These data are similar to those seen for human infections with Zaire ebolavirus (16). Overall, however, the measurement of genomic-sense RNA by Q-RT-PCR seems to be the more effective prognosticator of a fatal outcome.

An analysis of patient IgG levels revealed some interesting results. Six of 27 persons who died mounted a positive IgG response within 15 days after the onset of symptoms, which was also seen for humans infected with Zaire ebolavirus (16), for which 4 of 7 persons, whose samples were obtained on the day of death, had positive IgG responses. For survivors of the recent Gulu outbreak, 4 of 18 patients never mounted a positive IgG response by the time antigen was cleared, with 2 of the 4 IgG nonresponders having specific IgG responses that were virtually undetectable, in one case up to 14 days after clearing of the antigen. A possible explanation is that IgG, as measured by this assay, may not be required for virus clearance

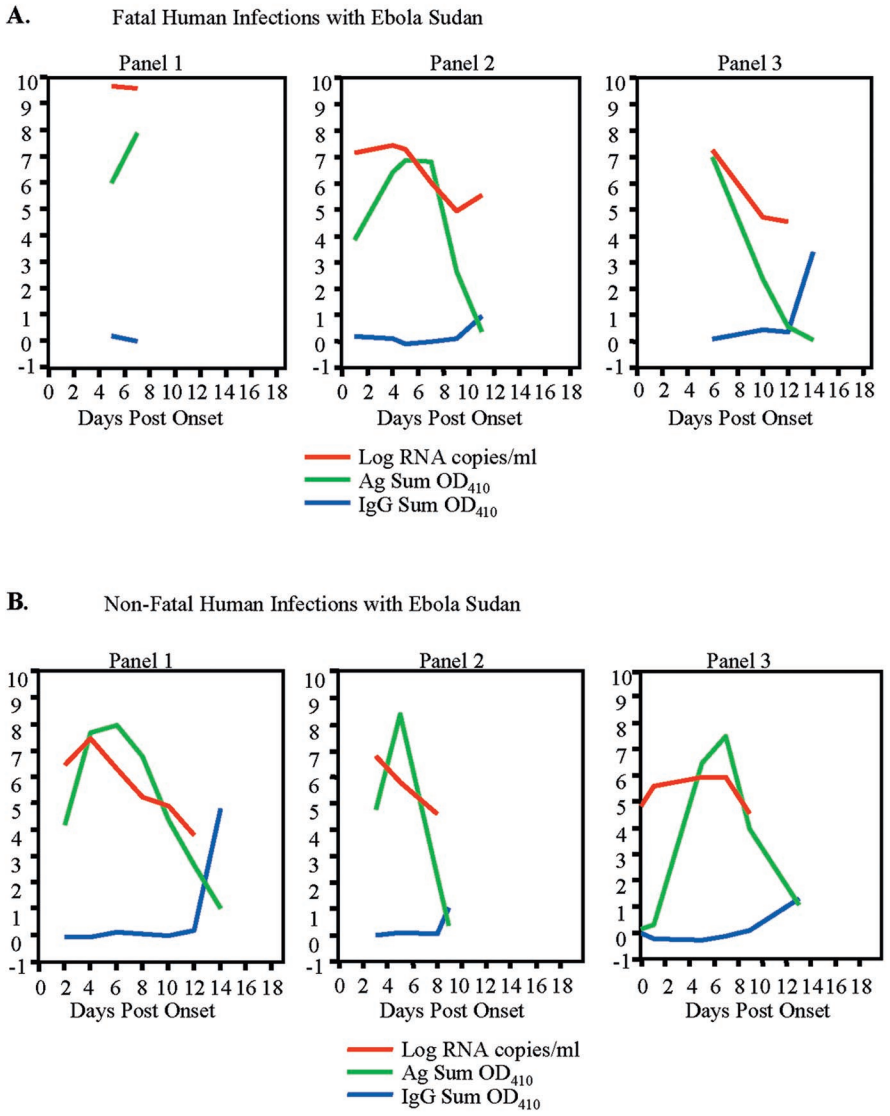


FIG. 2. Representative viral load profiles of EHF patients as determined by Q-RT-PCR analysis and compared with antigen-capture and IgG levels determined for the same samples. (A) Fatal case profiles. (B) Nonfatal case profiles.

but is more a marker of the overall immune response. Our data are in slight contrast with the results of Baize et al. (5), for which none of the fatal EHF cases infected with Zaire ebolavirus during the 1996 outbreaks in Gabon showed specific IgG responses while all the survivors did, at least to the nucleoprotein.

Relationship of genomic-sense RNA copy number to infectious titer. Because RNA copy number is only a measure of genomic-sense RNA molecules and not actual infectious virus, we sought to correlate the two by analyzing a subset of samples by Q-RT-PCR and plaque assay. Our experience was that the measurement of virus load in clinical specimens by a plaque assay was often inconsistent, a finding that was reported previously for Zaire ebolavirus clinical specimens from the 1995 outbreak in Kikwit, Zaire (16). Therefore, we also included in the analysis an ebolavirus stock propagated from an isolate from a Gulu EHF patient. Clinical specimens for which plaque assays generated countable plaques are shown in Table 2. The data together demonstrate a clear correlation between the Q-RT-PCR assay and the plaque assay results. Throughout a 4-$\log_{10}$ range, the virus load measured (in RNA copies per milliliter of serum) by Q-RT-PCR was consistently 3 to 4 $\log_{10}$ higher than the corresponding measurement by plaque assay (in PFU/ml). Similar results were observed with nonclinical material, indicating that the difference in results between the two assays used to measure virus load in clinical specimens was consistent. Q-RT-PCR assays have been effectively designed for other RNA viruses, such as dengue virus (11) and the newly discovered severe acute respiratory syndrome (SARS) coronavirus, by which the levels of genomic-sense RNA are also several orders of magnitude higher than the corresponding numbers of infectious virus particles (H.-S. H. Houng, personal communication). Attempts to obtain an ebolavirus particle count for clinical specimens were unsuccessful. The protein concentrations were so high that fixation by formaldehyde, paraformaldehyde, or glutaraldehyde resulted in a gelatinous

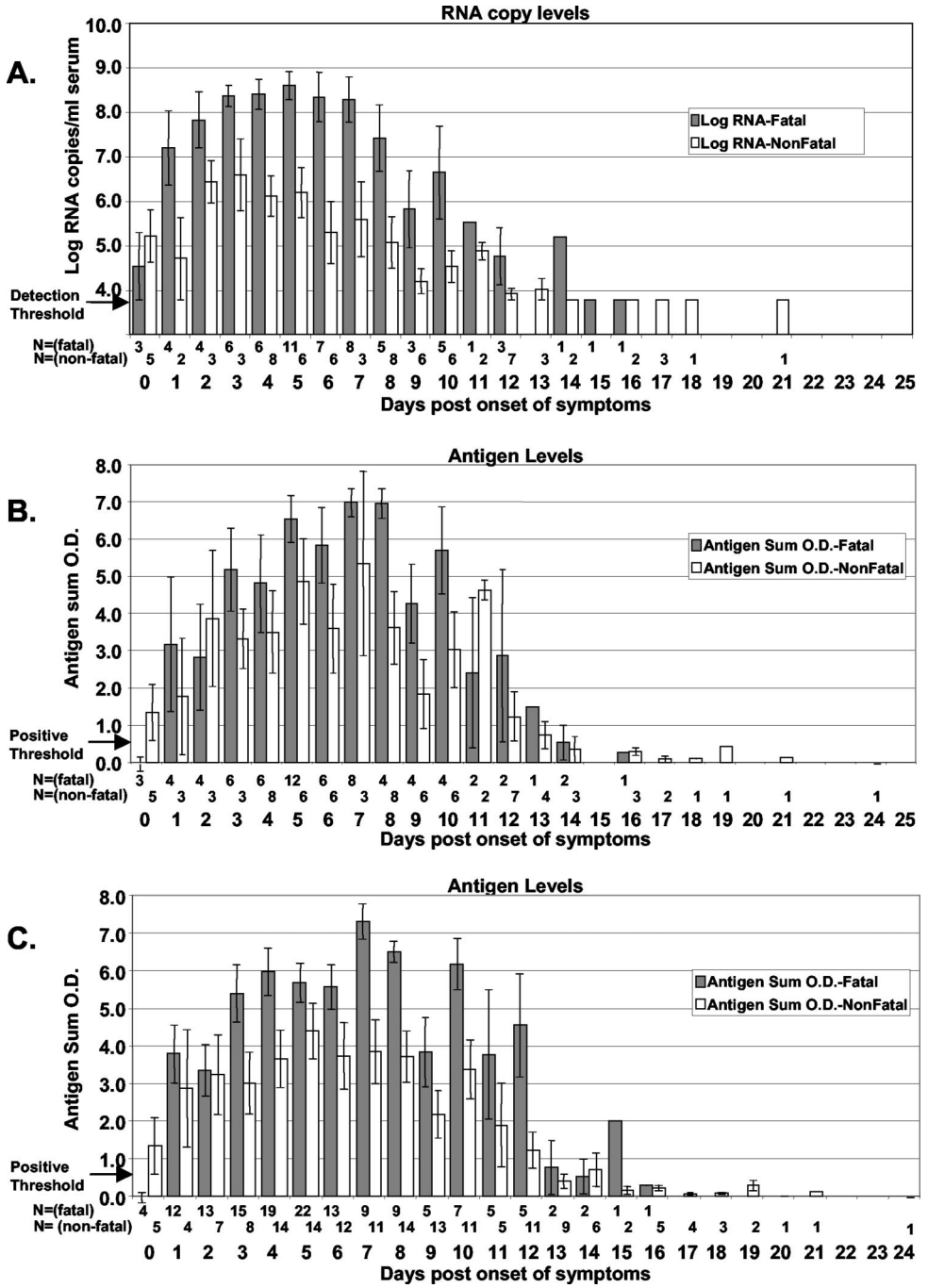


FIG. 3. Summary of RNA copy and antigen levels of EHF cases with fatal and nonfatal outcomes. Each bar represents the arithmetic mean value, and the error bars represent 1 standard error of the mean for each time point. (A) Mean $\log_{10}$ RNA copies per milliliter of serum from 18 survivors and 27 nonsurvivors. The threshold of detection was 6,200 RNA copies per ml of serum and was the lowest level detected in any of the patients analyzed. For the purposes of the graph, this value was assigned to samples of laboratory-confirmed patients that had C_t values of 40. (B) Mean antigen levels determined in duplicate aliquots of those described in panel A. The positive threshold was 0.45 adjusted OD_{sum} units at 410 nm. For samples to be considered positive, they must have had a titer of at least 1:16 and an OD_{sum} of >0.45. (C) Mean antigen levels determined for the specimens described in panel B combined with antigen levels measured in samples from an additional 35 survivors and 62 nonsurvivors that were not tested by Q-RT-PCR (total = 89 fatal and 53 nonfatal cases).

TABLE 2. Summary of clinical serum specimens from a single patient tested by plaque assay, Q-RT PCR, and antigen-capture ELISA

Sample no.	Plaque assay result (PFU/ml)	Q-RT-PCR result (RNA copies/ml)	Antigen-capture ELISA result (OD_{sum})
1469	5×10^1	2.0×10^5	0.11
1522	1.5×10^4	1.8×10^7	0.82
1527	4.3×10^5	4.0×10^8	3.54
1591	1.6×10^6	3.3×10^9	7.38
1612	5.3×10^5	2.5×10^8	7.46
Stock virus	3.7×10^5	3.5×10^9	ND[a]

[a] ND, not determined.

mix that was unsuitable for electron microscopy, and attempts at centrifuging the virus to remove the serum protein resulted in virus pellets that could not be reliably resuspended for accurate quantitation.

Comparison of sensitivity between standard RT-PCR and real-time Q-RT-PCR. The data presented thus far demonstrate the usefulness of RT-PCR in an outbreak setting for early EHF case identification. In an effort to improve our molecular diagnostic RT-PCR assay(s), we sought to determine in side-by-side comparisons what type of PCR technology provides the highest sensitivity while minimizing the potential for false positives or negatives. To do this, we directly compared standard (positive- and negative-strand detection) single-round and nested RT-PCR (as fielded in the Gulu outbreak) and one-step (positive- and negative-strand detection) and two-step (negative-strand detection) real-time Q-RT-PCR (TaqMan). The experimental design was to extract the total RNA, as described above, from a single serum sample from an acute EHF patient and to analyze serial 10-fold dilutions of the total RNA, using each of the four RT-PCR assays. The results of this analysis are summarized in Fig. 4A. The specific RNA dilutions are listed in column 1, while column 2 shows the numbers of genomic-sense RNA copies per milliliter for each sample, as determined by the two-step Q-RT-PCR assay already used above to retrospectively determine patient viral loads. Column 3 shows the number of genomic-sense RNA copies used to program each cDNA reaction. The results show that the threshold for detection by single-round standard RT-PCR is approximately 10^5 genomic-sense RNA copies per ml (or 100 copies per cDNA reaction) and that the sensitivity of the nested RT-PCR assay utilized in the Gulu outbreak is about 10-fold higher at a limit of 10^4 copies per ml (10 copies per cDNA reaction). The results of the single-round nested RT-PCR assay are additionally presented in Fig. 4B, in which electrophoresis of the reactions showed single intense bands of the expected sizes with a minimal background.

The two-step Q-RT-PCR protocol, summarized in Fig. 4A, has a threshold sensitivity that is roughly equal to that of the single-round standard RT-PCR. Given the sensitive probe technology inherent in the two-step Q-RT-PCR assay, one might have expected an increase in detection sensitivity. The reason for the lack of increase is likely twofold. First, the two-step Q-RT-PCR assay detects only negative (genomic)-sense RNA, whereas the single-round standard RT-PCR detects both positive and negative RNA strands. Second, the two-step Q-RT-PCR protocol uses only 1/10 of the initial cDNA synthesis to program the subsequent real-time PCRs, thereby building in a further 10-fold dilution of the target molecules.

The most sensitive method tested was the one-step Q-RT-PCR assay, which detected ebolavirus-specific RNA in the range of 10^3 genomic-sense copies per ml, or roughly one genomic-sense RNA per cDNA reaction. This increased sensitivity was expected, as the one-step Q-RT-PCR protocol has both forward and reverse primers present during the cDNA synthesis step, thereby allowing the additional detection of antigenomic RNA (NP mRNA).

Given the ability in a one-step protocol to amplify both genomic and antigenomic RNA, it is not surprising that ebolavirus RNA was occasionally detected in samples diluted to less than one genomic-sense copy per cDNA reaction. Unlike nested RT-PCR, the one-step Q-RT-PCR assay does not require additional sample manipulations beyond the initial setup, thus reducing the risk of cross-contamination. Because of this simplicity, combined with a higher sensitivity than nested RT-PCR, we envision the use of one-step Q-RT-PCR in future outbreak responses.

Genetic analysis of ebolavirus circulating within the human population. In an effort to determine if there were multiple ebolavirus genetic lineages circulating within this outbreak, we analyzed multiple specimens from EHF patients. The samples analyzed were obtained from EHF patients with fatal and nonfatal outcomes found throughout the temporal span of the outbreak and representing all three geographic locations within Uganda where EHF cases occurred (Fig. 5A). While the entire GP sequence was determined for the reference strain (GenBank accession number AY344234), an ~970-nucleotide region containing the variable portion of the glycoprotein ORF was amplified by RT-PCR directly from each of the clinical serum specimens. No nucleotide sequence changes were found among the five sequences amplified. This genetic homogeneity was consistent with the epidemiologic data that linked the EHF cases found in the townships of Mbarara and Masindi to those found in Gulu, the initial and major site of the outbreak (4). The fact that the sequences were all identical to each other indicates that this outbreak was likely the result of a single introduction of the virus into the human population from the unknown natural reservoir.

When the Gulu 2000-2001 glycoprotein variable region sequence was compared with that of the first (known) Sudan ebolavirus isolate, Boniface 1976, 58 nucleotide and 32 amino acid differences were observed (6% and 10.7%), respectively. We used the sequence information to perform a maximum parsimony analysis to estimate the evolutionary relationship of this Gulu strain relative to other known ebolavirus isolates. Glycoprotein sequence data from multiple marburgvirus isolates were used as the outgroup. This analysis is presented in Fig. 5B, in which the Gulu isolate is clearly placed in the Sudan clade, consistent with initial results by R. Swanepoel (National Institute for Virology), using polymerase gene sequence data obtained from RT-PCR products generated from samples from the beginning of the outbreak. The previous Sudan strains of 1976 (Boniface) and 1979 (Maleo) are not ancestral to the Gulu 2000-2001 strain, but all three share a common ancestor. This relationship is well supported by the indicated bootstrap analysis.

A.

Sample Dilution	RNA copies per ml	RNA copies per cDNA reaction	Single-round RT-PCR	Nested PCR	Two-step Q-RT-PCR	One-step Q-RT-PCR
Neat	10^9	10^6	+	+	+ {21.6, 21.4, 21.8}	+ {16.0, 16.0, 16.0}
10^{-1}	10^8	10^5	+	+	+ {23.2, 23.5, 23.3}	+ {19.1, 19.1, 19.1}
10^{-2}	10^7	10^4	+	+	+ {27.2, 27.3, 27.2}	+ {23.1, 23.1, 23.1}
10^{-3}	10^6	10^3	+	+	+ {31.2, 30.1, 30.9}	+ {27.2, 27.1, 27.2}
10^{-4}	10^5	10^2	+	+	+ {34.5, 36.1, 34.4}	+ {29.1, 29.2, 29.2}
10^{-5}	10^4	10	−	+	− {40.0, 40.0, 40.0}	+ {33.3, 35.2, 33.0}
10^{-6}	10^3	1	−	−	− {40.0, 40.0, 40.0}	+ {36.0, 35.9, 40.0}
10^{-7}	10^2	.1	−	−	− {40.0, 40.0, 40.0}	− {40.0, 40.0, 36.1}
10^{-8}	10	.01	−	−	− {40.0, 40.0, 40.0}	− {40.0, 40.0, 40.0}

B.

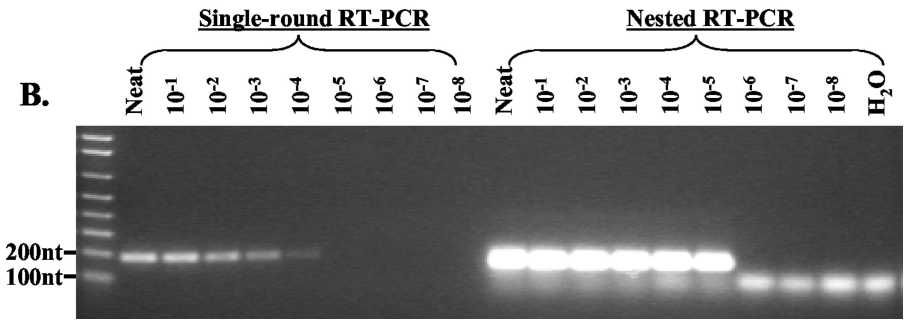


FIG. 4. Comparison between standard single-round and nested RT-PCR and one-step and two-step real-time Q-RT-PCR. All cDNA reactions were programmed with 1 μl of total RNA and were analyzed side-by-side in the indicated RT-PCR assays. (A) Summary chart of RNA dilutions and the subsequent classification (+ or −) by each assay. For the Q-RT-PCR assays, the corresponding triplicate C_t values associated with each RNA dilution are shown. For a result in the Q-RT-PCR assay to be considered positive, at least two of the three triplicate samples had to register a C_t of <40. (B) One percent agarose gel showing the amplification products present in 5 μl of reaction mix for each of the RNA dilutions after standard single-round and nested RT-PCR. The corresponding sizes of the expected DNA amplicons are 185 and 150 nucleotides, respectively.

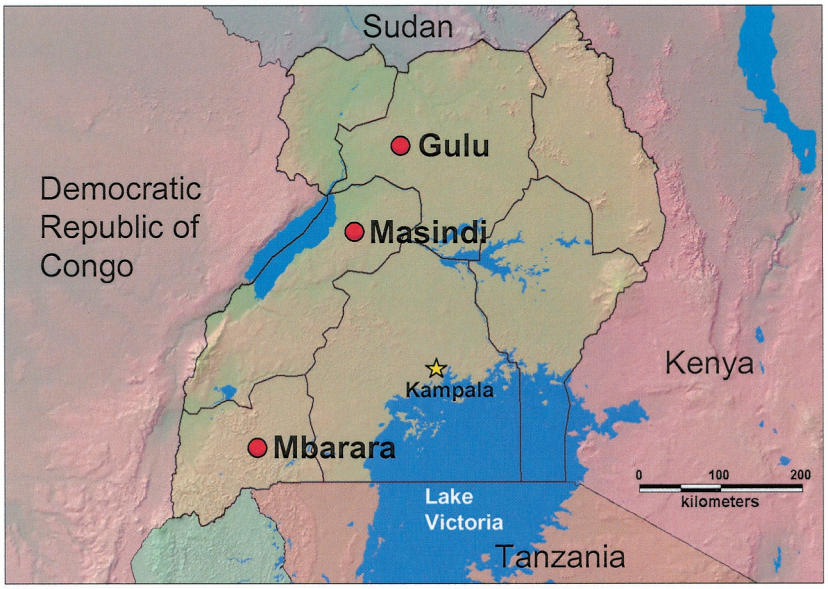
DISCUSSION

In this report, we demonstrate the utility of a nested RT-PCR assay for use in a field setting for the purpose of rapid and accurate diagnosis of early acute-phase EHF cases. This assay was used in combination with and was directly compared to a reliable and rapid antigen-capture ELISA. Clearly, the greatest value for the RT-PCR-based assay is early case identification, as demonstrated by its ability to identify 13 patients up to 72 h prior to identification by any other available test. In addition, another 18 patients were shown to remain PCR positive up to 3 days after clearing detectable antigen, a result that was also seen for infections with Zaire ebolavirus, in which virus-specific RNAs were detected in semen up to 101 days after the onset of symptoms (18). In addition to earlier detection, a consequence of a more sensitive detection assay is that a more informed decision can be made when releasing convalescent-phase patients back into the community.

However, the data presented here also illustrate the drawback of the reliance on any single diagnostic assay alone, including the RT-PCR assay, for EHF diagnosis. The most serious shortcoming of the RT-PCR assay is the greater ease with which false positives and false negatives can be generated. A nested assay is especially prone to template contamination because there is the extra high-risk step of physically opening the first-round reactions, thus increasing the potential exposure to high concentrations of DNA amplicons. This risk may increase as the outbreak continues, as more and more positive samples are analyzed. In this outbreak, there were three PCR^+ Ag^- samples that by analysis of duplicate samples were later shown to be falsely positive by PCR. Most of the potentially false-positive samples occurred in the later stages of the outbreak. The individuals from whom the 10 unresolved samples were taken were unavailable for subsequent sampling to verify or disprove the initial results. While a false positive can clearly

A.

Sample #	Origin of Specimen	Date of Sample	Outcome
83	Gulu	23 Oct 2000	Fatal
343	Mbarara	28 Oct 2000	Fatal
671	Masindi	11 Nov 2000	Fatal
981	Gulu	19 Nov 2000	Fatal
2300	Gulu	15 Jan 2001	Non-fatal



B.

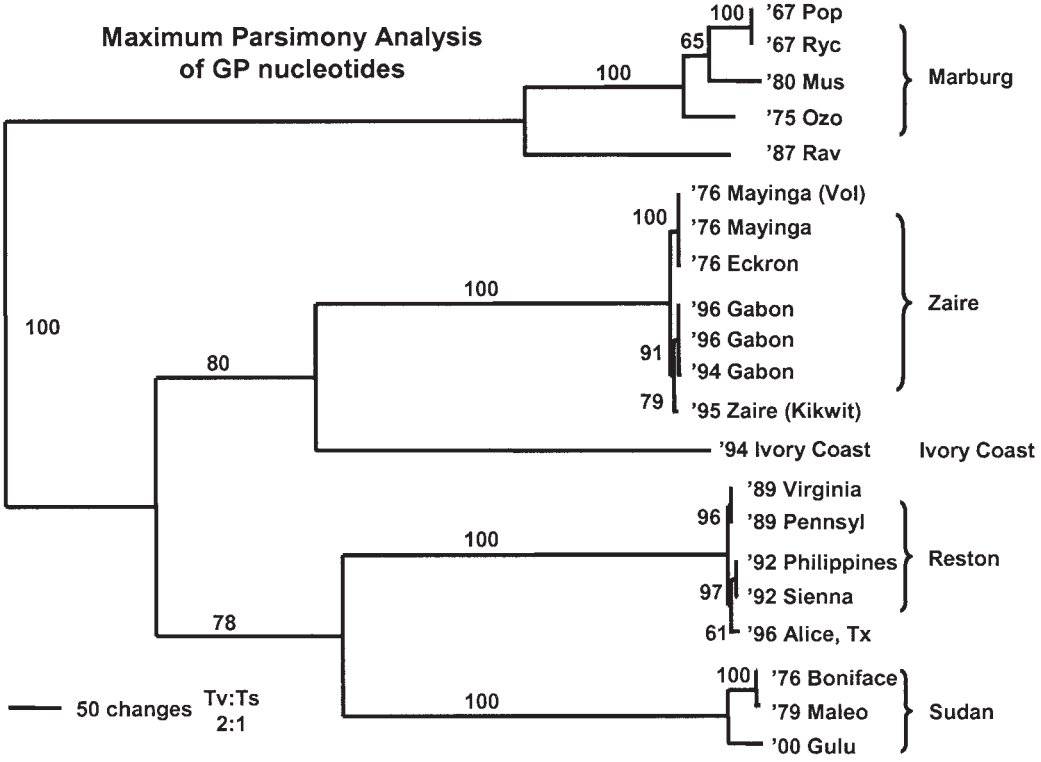


FIG. 5. Summary of glycoprotein variable region nucleotide sequence analysis (GenBank accession number AY344234). (A) Nucleotides 821 to 1696 (nucleotide numbering of Sudan ebolavirus, Maleo strain [GenBank accession number U23069]) were determined for five EHF patients representing (i) all three geographic distributions of the EHF outbreak within Uganda, (ii) cases from the beginning, middle, and end of the outbreak, and (iii) fatal and nonfatal outcomes. (B) Maximum parsimony analysis of nucleotide sequence differences among the GP variable regions (analogous to nucleotides 821 to 1696 of Sudan ebolavirus, Maleo strain) of representative filoviruses was performed by using a heuristic search option and a 2:1 weighting of transversions over transitions. Bootstrap analysis was conducted with 500 pseudoreplicates of the data set, and values above 50% are shown at branch points. Horizontal distances represent nucleotide step differences (see bar scale), while vertical branches are for visual clarity only.

put an individual at unnecessary risk by causing the person to be placed in a high-risk environment (e.g., an Ebola isolation ward), more serious consequences can occur from false negatives. With a false-negative result, a person may be released into the community with the understanding that they do not have EHF, when in fact they have the potential to become highly contagious and, at least initially, assume their symptoms are not due to EHF.

Because of the serious potential for false positives and negatives, we do not rely solely on a single diagnostic test but instead on a collection of tests that together establish a laboratory diagnosis. For instance, sole reliance on the first RT-PCR assay (using the Filo A and B primers) used in the beginning phase of the outbreak would have led to the initial misclassification of 19 of 46 samples. The false negatives could have resulted for a number of reasons, which include but are not limited to inhibitor contamination of the RNA preps, a low copy number of the target sequence (particularly in the first days after the onset of symptoms in nonfatal cases), and nucleotide mismatches between primer and target sequences that are a result of the unique genetic identity of the particular ebolavirus strain. In addition, the RNA extractions were performed under suboptimal conditions, as ice was not available and the ambient temperature was often ≥30°C. The first point, regarding inhibitor or protein contamination, can easily be dealt with in the laboratory by repurification of the sample and/or dilution of the RNA prior to analysis (10). The second point with respect to a lower copy number at the onset of symptoms may be more relevant in outbreaks of Sudan ebolavirus, in which there are more patients who survive and whose viral loads are lower. The third point, nucleotide mismatches between primer and target sequences causing inefficient amplification, will always present a potential problem at the onset of an outbreak before definitive nucleotide sequence information can be gained and a more exact primer design can be implemented. The first generation of Q-RT-PCR primers, originally designed to anneal to a sequence conserved between the 1976 and 1979 isolates, were redesigned because of single nucleotide mismatches with the 2000 Gulu sequence that would have led to decreased amplification efficiencies. These points, in combination with the risk for false positives, illustrate why an RT-PCR assay, while extremely useful, should not be relied upon as the sole diagnostic assay but should always be utilized in conjunction with other reliable assays, such as the antigen-capture ELISA.

The retrospective analysis of 45 cases demonstrates a number of interesting aspects of clinical virology that highlight some general features of ebolavirus infections. The most obvious feature revealed by the Q-RT-PCR analysis was the extremely rapid accumulation (days 0 to 2 after the onset of symptoms) of genomic-sense RNA in patients with fatal outcomes. The RNA copy number (per milliliter of serum) throughout the course of the disease averaged 2 $\log_{10}$ higher than that in patients who survived. The average peak titer of cases with a fatal outcome was 3.4×10^9, while that for cases with a nonfatal outcome was 4.3×10^7 RNA copies/ml. A marked difference in viral load was previously observed with Zaire ebolavirus (5, 16) and Lassa fever virus (13), for which high levels of viremia, especially early in the course of disease, were associated with poor outcomes. From the standpoint of the predictive capability of Q-RT-PCR, a correct prediction of disease outcome can be correctly assigned >90% of the time if a patient's maximum RNA titer reaches $\geq 10^8$ RNA copies/ml within 8 days after the onset of symptoms.

This analysis of the Uganda outbreak allows for some comparisons between human infections with Sudan and Zaire ebolaviruses. The most obvious difference is the lower mortality seen with Sudan ebolavirus infections. In this outbreak in Uganda, ~53% of the cases were fatal (4), compared with 80 to 90% with Zaire ebolavirus (1, 3). Interestingly, though, the number of nonsurvivors of the Gulu outbreak with IgG responses was not appreciably higher than that seen for the 1995 Kikwit outbreak (14). Of the 27 fatal Sudan ebolavirus cases examined, 6 (24%) had IgG titers above 400, with two patients having titers of 1,600 at the time of death. The mean times from the onset of symptoms to death between persons infected with either Sudan or Zaire ebolavirus were also very similar (8.6 and 9.6 days, respectively). Furthermore, in this recent Gulu outbreak, 4 of 18 survivors did not mount positive specific IgG responses, in one case at 14 days after antigen clearance. Given the variability of the IgG responses by EHF patients having both fatal and nonfatal outcomes, this suggests at least two things: first, IgG may not play an important role in the clearance of virus, as suggested by studies in nonhuman primates that demonstrated the ineffectiveness of passive transfer of neutralizing IgG prepared from hyperimmune horse serum (12), and second, human infections with Sudan ebolavirus, given the lower mortality rate, may not show as high a degree of immunological suppression, thus resulting in a more effective cell-mediated response. The immunological suppression associated with Zaire ebolavirus has recently been demonstrated in vitro by the discovery of a powerful gamma interferon antagonistic domain present in VP35 (7).

The determination of viral load profiles by Q-RT-PCR provided consistent results that correlated well with other ebolavirus quantitation assays. What was surprising, however, was the approximately 4-$\log_{10}$ disparity between genomic RNA levels and the numbers of PFU measured from the same respective samples. A number of features of ebolavirus biology could easily account for the difference. First, the determined 50% lethal dose for a mouse-adapted stain of Zaire ebolavirus was previously calculated to be 0.025 to 0.04 PFU (8), or roughly one virion, indicating that only 3 to 4% of this ebolavirus actually leads to plaque formation. Second, negative-sense RNA viruses are especially prone to the generation of deletion-type and copy-back replicons, which can replicate very efficiently and thus provide large quantities of targets for PCR amplification. Third, the antigenomic promoter of a negative-strand RNA virus is very powerful, generating many copies of genomic-sense RNA, not all of which are effectively packaged. These features, combined with the lytic nature of ebolavirus, could release into the blood thousands of genomic-sense RNA targets per milliliter of blood in addition to the RNAs that are properly packaged into infectious virions. Also worth noting is the fact that the DNA amplicons generated in the Q-RT-PCR assay are only ~100 nucleotides long, and therefore virions along with their RNAs can undergo a substantial amount of degradation that would lower the number of infectious virions while not affecting the genomic-sense RNA copy measurements. The results shown in this study are consistent with those

in a previous report of RNA copy levels in patient sera from a single fatal case and a single nonfatal case from the same outbreak (10). These earlier data, however, were generated from single-step RT-PCRs that would not discriminate between genomic- and antigenomic (mRNA)-sense RNA.

This report describes the utility of a newly developed nested RT-PCR assay that, together with an antigen-capture ELISA, was successfully used to diagnose hundreds of acute EHF cases in an outbreak setting. It should be noted that no cases were identified by PCR or antigen capture prior to the onset of symptoms, an important fact that needs to be considered when screening at-risk personnel or patient contacts that do not present any clinical symptoms. Should the opportunity and need present itself for a field laboratory in future outbreaks, we envision the use of the antigen-capture ELISA combined with a higher throughput and highly sensitive one-step fluorogenic Q-RT-PCR assay appropriate for portable real-time PCR machines. With this technology, a degree of prognostication capability could be achieved that may prove valuable for the assessment of risk potential for EHF patients. Patients with high viral loads would be assumed to be especially contagious, thus demanding extreme vigilance in following barrier-nursing guidelines by healthcare workers and family members charged with feeding and general patient care.

ACKNOWLEDGMENTS

We thank Kent Wagoner and Laura Morgan for their excellent help with databases and graphics.

REFERENCES

1. **Anonymous.** 1978. Ebola hemorrhagic fever in Sudan, 1976. Report of a W.H.O./International Study Team. Bull. W. H. O. **56:**247–270.
2. **Anonymous.** 1978. Ebola hemorrhagic fever in Zaire, 1976. Bull. W. H. O. **56:**271–293.
3. **Anonymous.** 1995. Outbreak of Ebola viral hemorrhagic fever—Zaire, 1995. Morb. Mortal. Wkly. Rep. **44:**381–382.
4. **Anonymous.** 2001. Outbreak of Ebola hemorrhagic fever—Uganda, August 2000-January 2001. Morb. Mortal. Wkly. Rep. **50:**73–77.
5. **Baize, S., E. M. Leroy, M. C. Georges-Courbot, M. Capron, J. Lansoud-Soukate, P. Debre, S. P. Fisher-Hoch, J. B. McCormick, and A. J. Georges.** 1999. Defective humoral responses and extensive intravascular apoptosis are associated with fatal outcome in Ebola virus-infected patients. Nat. Med. **5:**423–426.
6. **Baron, R. C., J. B. McCormick, and O. A. Zubeir.** 1983. Ebola virus disease in southern Sudan: hospital dissemination and intrafamilial spread. Bull. W. H. O. **61:**997–1003.
7. **Basler, C. F., X. Wang, E. Muhlberger, V. Volchkov, J. Paragas, H. D. Klenk, A. Garcia-Sastre, and P. Palese.** 2000. The Ebola virus VP35 protein functions as a type I IFN antagonist. Proc. Natl. Acad. Sci. USA **97:**12289–12294.
8. **Bray, M., K. Davis, T. Geisbert, C. Schmaljohn, and J. Huggins.** 1999. A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. J. Infect. Dis. **179**(Suppl. 1)**:**S248–S258.
9. **Courtois, D., and M. A. D. B. Isaacson.** 1978. Plasmaphérèse dans le foyer épidémique de Yambuku, Zaire, p. 213–216. *In* S. R. Pattyn (ed.), Ebola virus hemorrhagic fever. Elsevier/North-Holland Biomedical Press, Amsterdam, The Netherlands.
10. **Drosten, C., S. Gottig, S. Schilling, M. Asper, M. Panning, H. Schmitz, and S. Gunther.** 2002. Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR. J. Clin. Microbiol. **40:**2323–2330.
11. **Houng, H.-S. H., D. Hritz, and N. Kanesa-thasan.** 2000. Quantitative detection of dengue 2 virus using fluorogenic RT-PCR based on 3′-noncoding sequence. J. Virol. Methods **86:**1–11.
12. **Jahrling, P. B., T. W. Geisbert, J. B. Geisbert, J. R. Swearengen, M. Bray, N. K. Jaax, J. W. Huggins, J. W. LeDuc, and C. J. Peters.** 1999. Evaluation of immune globulin and recombinant interferon-alpha2b for treatment of experimental Ebola virus infections. J. Infect. Dis. **179**(Suppl. 1)**:**S224–S234.
13. **Johnson, K. M., J. B. McCormick, P. A. Webb, E. S. Smith, L. H. Elliott, and I. J. King.** 1987. Clinical virology of Lassa fever in hospitalized patients. J. Infect. Dis. **155:**456–464.
14. **Johnson, K. M., P. A. Webb, and D. L. Heymann.** 1978. Evaluation of the plasmapheresis program in Zaire, p. 213–216. *In* S. R. Pattyn (ed.), Ebola virus hemorrhagic fever. Elsevier/North-Holland Biomedical Press, Amsterdam, The Netherlands.
15. **Ksiazek, T. G., P. E. Rollin, P. B. Jahrling, E. Johnson, D. W. Dalgard, and C. J. Peters.** 1992. Enzyme immunosorbent assay for Ebola virus antigens in tissues of infected primates. J. Clin. Microbiol. **30:**947–950.
16. **Ksiazek, T. G., P. E. Rollin, A. J. Williams, D. S. Bressler, M. L. Martin, R. Swanepoel, F. J. Burt, P. A. Leman, A. S. Khan, A. K. Rowe, R. Mukunu, A. Sanchez, and C. J. Peters.** 1999. Clinical virology of Ebola hemorrhagic fever (EHF): virus, virus antigen, and IgG and IgM antibody findings among EHF patients in Kikwit, Democratic Republic of the Congo, 1995. J. Infect. Dis. **179**(Suppl. 1)**:**S177–S187.
17. **Leroy, E. M., S. Baize, C. Y. Lu, J. B. McCormick, A. J. Georges, M. C. Georges-Courbot, J. Lansoud-Soukate, and S. P. Fisher-Hoch.** 2000. Diagnosis of Ebola haemorrhagic fever by RT-PCR in an epidemic setting. J. Med. Virol. **60:**463–467.
18. **Rodriguez, L. L., A. De Roo, Y. Guimard, S. G. Trappier, A. Sanchez, D. Bressler, A. J. Williams, A. K. Rowe, J. Bertolli, A. S. Khan, T. G. Ksiazek, C. J. Peters, and S. T. Nichol.** 1999. Persistence and genetic stability of Ebola virus during the outbreak in Kikwit, Democratic Republic of the Congo, 1995. J. Infect. Dis. **179**(Suppl. 1)**:**S170–S176.
19. **Sanchez, A., M. P. Kiley, B. P. Holloway, and D. D. Auperin.** 1993. Sequence analysis of the Ebola virus genome: organization, genetic elements, and comparison with the genome of Marburg virus. Virus Res. **29:**215–240.
20. **Sanchez, A., T. G. Ksiazek, P. E. Rollin, M. E. Miranda, S. G. Trappier, A. S. Khan, C. J. Peters, and S. T. Nichol.** 1999. Detection and molecular characterization of Ebola viruses causing disease in human and nonhuman primates. J. Infect. Dis. **179**(Suppl. 1)**:**S164–S169.
21. **Sanchez, A., S. G. Trappier, B. W. Mahy, C. J. Peters, and S. T. Nichol.** 1996. The virion glycoproteins of Ebola viruses are encoded in two reading frames and are expressed through transcriptional editing. Proc. Natl. Acad. Sci. USA **93:**3602–3607.
22. **Volchkov, V. E., S. Becker, V. A. Volchkova, V. A. Ternovoj, A. N. Kotov, S. V. Netesov, and H. D. Klenk.** 1995. GP mRNA of Ebola virus is edited by the Ebola virus polymerase and by T7 and vaccinia virus polymerases. Virology **214:**421–430.