Persistence and Genetic Stability of Ebola Virus during the Outbreak in Kikwit, Democratic Republic of the Congo, 1995

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Ebola virus persistence was examined in body fluids from 12 convalescent patients by virus isolation and reverse transcription–polymerase chain reaction (RT-PCR) during the 1995 Ebola hemorrhagic fever outbreak in Kikwit, Democratic Republic of the Congo. Virus RNA could be detected for up to 33 days in vaginal, rectal, and conjunctival swabs of 1 patient and up to 101 days in the seminal fluid of 4 patients. Infectious virus was detected in 1 seminal fluid sample obtained 82 days after disease onset. Sequence analysis of an RT-PCR fragment of the most variable region of the glycoprotein gene amplified from 9 patients revealed no nucleotide changes. The patient samples were selected so that they would include some from a suspected line of transmission with at least three human-to-human passages, some from 5 survivors and 4 deceased patients, and 2 from patients who provided multiple samples through convalescence. There was no evidence of different virus variants cocirculating during the outbreak or of genetic variation accumulating during human-to-human passage or during prolonged persistence in individual patients.

Ebola (EBO) and Marburg viruses are members of the family Filoviridae and cause severe hemorrhagic fever in human and nonhuman primates [1, 2]. Mortality rates of 80%–90% were documented during the 1976 and 1995 outbreaks of EBO hemorrhagic fever (EHF) in the Democratic Republic of the Congo (DRC) [3, 4]. The major routes of transmission during outbreaks appear to be contact with blood or infectious fluids from deceased patients and from persons in the acute phase of disease [1]. Since outbreaks usually occur in remote places where health care resources are scarce, only limited numbers of clinical samples have been available for analysis. Therefore, few data exist concerning virus clearance, persistence, and shedding during the postclinical phase of the disease. There is evidence suggesting that filoviruses can persist in convalescent patients. Marburg virus has been isolated from seminal fluid and from the anterior chamber of the eye of convalescent patients recovering from hemorrhagic fever up to 80 days after onset of disease [5, 6]. In addition, EBO virus was found in seminal fluid of a convalescent patient 61 days after onset of disease [7], but it is unknown whether such patients can contribute to filovirus transmission.

During the 1995 EBO outbreak, patients admitted to Kikwit General Hospital, DRC, with a clinical diagnosis of EHF were isolated in a single hospital pavilion. Entrance to this pavilion was restricted, and strict barrier-nursing techniques were implemented to prevent transmission to medical personnel and family members caring for the patients [8, 9]. Patients who recovered from the disease were released from the isolation unit into an adjacent building under more relaxed barrier-nursing conditions. Whether convalescent patients were shedding virus at the time of release was unknown. One of the objectives of this study was to determine whether EBO virus is still present in body fluids of convalescent patients after clinical symptoms subside, and if so, what the duration of virus persistence is.

Another aspect of the current study was to determine the genetic stability of EBO virus during the outbreak. Viruses with RNA genomes have the potential for rapid evolution due to the high error rate of their RNA polymerase [10]. As soon as clinical material from the outbreak was available, it was determined that the EBO virus strain causing disease in Kikwit (EBO, subtype Zaire [EBO-Z] 1995) was genetically very similar to the strain that caused the 1976 EHF outbreak in Yambuku, DRC (EBO-Z 1976) [11]. Nucleotide (nt) divergence between the glycoprotein (GP) genes of these viruses was <2%, despite the fact that they were isolated almost 20 years and >1000 km apart [12, 13]. An objective of this study was to determine if the genetic stability observed between 1976 and 1995 EBO-Z isolates could also be demonstrated in the

Informed consent was obtained from all patients included in this study. The study was performed according to guidelines of the US Department of Health and Human Services.

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Kikwit outbreak during repeated human-to-human passages and during virus persistence within individual patients. In addition, we attempted to determine whether additional strains of EBO virus were circulating during the outbreak.

Material and Methods

Patients and samples. Samples were obtained from 7 patients (A–G, table 1) recovering from EHF in the isolation pavilion at Kikwit General Hospital, Bandundu region, DRC, during 2–13 July 1995. Specimens included blood, urine, and saliva samples and vaginal, conjunctival, rectal, and skin swabs. Blood samples were obtained by venipuncture, using sterile tubes; urine samples were collected in individual sterile cups. Swab samples were collected using the Viral Culturette System (Becton Dickinson Microbiological Systems, Cockeysville, MD). Duplicate samples were obtained from patients A–F (table 1): One aliquot was kept on ice for 2–6 h and then frozen in liquid nitrogen for transport to the Centers for Disease Control and Prevention (CDC), and the

Table 1. Persistence of Ebola virus in convalescent-phase samples from patients in Kikwit, Democratic Republic of the Congo.

Patient, IDNUM	Sex, age (years)	Last viremia (no. of days after disease onset)	Samples			
			First	Second	Third	Fourth
A, 2209	F, 15	13	Urine, vaginal, rectal, saliva, skin, serum, blood clot, conjunctival (33)	NA	NA	NA
B, 2240	F, 50	4	Urine, vaginal, rectal, skin, blood clot, serum, conjunctival (30)	NA	NA	NA
C, 2261	M, 22	6	Urine, rectal, saliva, skin, conjunctival (11)	Urine, saliva, skin, conjunctival, serum, blood clot (14)	Urine, rectal, saliva, conjunctival, skin, serum, blood clot (18)	NA
D, 2254	F, 45	13	Urine, vaginal, rectal, saliva, conjunctival, skin (20)	Urine, vaginal, rectal, saliva, conjunctival, skin, serum, blood clot (23)	Urine, vaginal, rectal, saliva, conjunctival, skin, serum, blood clot (27)	Urine, vaginal, rectal, saliva, conjunctival, skin, serum, blood clot (31)
E, 2247	F, 50	15	Urine, vaginal, rectal, saliva, conjunctival, skin (24)	Urine, vaginal, rectal, saliva, conjunctival, skin, serum, blood clot (27)	Urine, vaginal, rectal, saliva, conjunctival, skin, serum, blood clot (31)	Urine, vaginal, rectal, saliva, conjunctival, skin, serum, blood clot (33)
F, 2248	F, 25	12	Urine, **vaginal**, **rectal**, saliva, **conjunctival**, skin (22)	Urine, **vaginal**, rectal, saliva, conjunctival, skin, serum, blood clot (25)	Urine, vaginal, **rectal**, saliva, conjunctival, skin, serum, blood clot (29)	Urine, **vaginal**, rectal, saliva, conjunctival, skin, serum, blood clot (33)
G, 2253	F, 12	4	Rectal, saliva, skin, conjunctival (25)	Urine, vaginal, rectal, saliva, conjunctival, skin, serum, blood clot (28)	NA	NA
H, 2060	M, 27	8	**Seminal fluid (51)**	PBL (57)	**Seminal fluid‡ (82)**	Seminal fluid (704)
I, 2032	M, 25	21	**Seminal fluid (57)**	PBL (62)	**Seminal fluid (101)**	NA
J, 96	M, 29	15	**Seminal fluid (63)**	**Seminal fluid (97)**	Seminal fluid (700)	NA
K, 11	M, 33	6	**Seminal fluid (63)**	PBL (68)	Seminal fluid (707)	NA
L, 2110	M, 32	ND	Urine, rectal, skin, saliva, conjunctival, serum, blood clot, seminal fluid (62)	NA	NA	NA

NOTE. All samples were obtained during June–August 1995, except for 3 seminal fluid samples obtained in April 1997, and tested by reverse transcription–polymerase chain reaction (RT-PCR) and virus isolation. Nos. in parentheses are days after disease onset. IDNUM = identification no.; NA = not applicable; ND = not determined; PBL = peripheral blood lymphocytes. Bold indicates samples positive by RT-PCR. Bold indicates positive samples.

* Last instance in which Ebola virus was detected in blood.

† Virus isolated in tissue culture.

‡ Virus isolation.

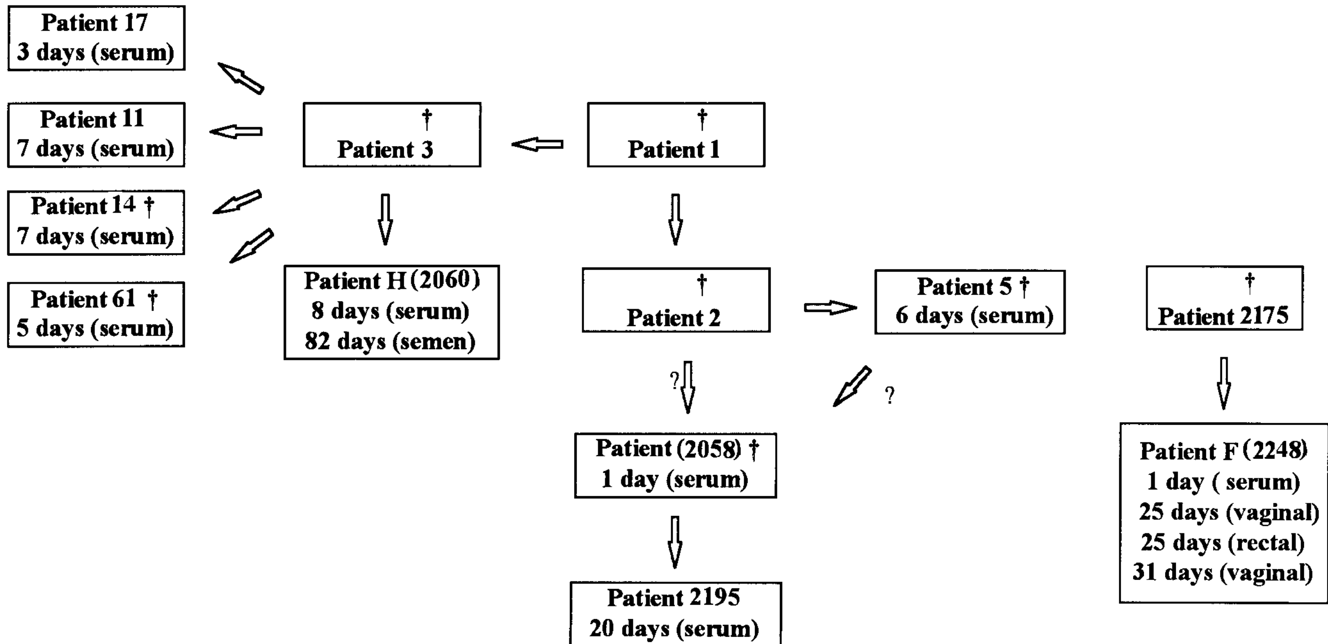


Figure 1. Suspected lines of transmission between patients selected for glycoprotein gene sequencing. Bold indicates sequenced samples, † Indicates deceased patient. Days = no. of days after onset of disease. Types of samples are indicated.

other was placed in a 1.5-mL microcentrifuge tube containing 500 μL of 4 *M* guanidine thiocyanate solution.

Additional samples included blood (obtained during the acute phase of disease) from 9 patients (figure 1). Among these samples were some from a suspected line of transmission that included at least three human-to-human passages, some from 5 survivors and 4 deceased patients, and 2 from patients who provided multiple samples through convalescence (figure 1). In addition, we also tested seminal fluid samples that were obtained from 5 convalescent patients (H–L, table 1) between 51 and 101 days after onset of disease as well as follow-up seminal fluid samples obtained from 3 of these patients on days 700–707 after onset in April 1997 (table 1).

RNA extraction. All field work with potentially infectious material was performed using a HEPA filter–fitted positive air purifying respirator (Racal Systems, Frederick, MD) in a restricted-access laboratory of Kikwit General Hospital. Total RNA was extracted using the acid-guanidine-phenol extraction procedure as previously described [14]. Tubes containing the aqueous phase (RNA in 4 *M* guanidine thiocyanate) were kept on ice for up to 2 weeks and then transported at room temperature (3 days) to CDC. RNA was precipitated from these samples by adding 1 vol of isopropanol, incubating overnight at −20°C, and centrifuging at 14,000 *g* for 20 min. RNA pellets were washed twice with 70% ethanol, dried under vacuum, and resuspended in 50 μL of diethyl pyrocarbonate–treated water. In the case of sera or blood, the RNaid kit (Bio 101, La Jolla, CA) was used instead of isopropanol precipitation. RNA samples were kept at −70°C until tested.

Amplification and sequencing of nucleic acids. A one-tube reverse transcription–polymerase chain reaction (RT-PCR) was performed using a thermostable DNA polymerase from *Thermus thermophilus* (GeneAmp EZ rTth RNA PCR kit; Perkin-Elmer, Norwalk, CT). This enzyme performs both RT and DNA amplification. Amplification was done using a thermocycler (model 9600; Perkin-Elmer) with a temperature profile of 50°C for 30 min (RT step) followed by 35 cycles at 94°C for 15 s and 50°C for 60 s and a final elongation step at 60°C for 10 min, for a total cycle time of 2.4 h. Products were analyzed by electrophoresis on 2.5% agarose gels and visualized by ethidium bromide staining. We used two sets of primers that targeted the nucleocapsid protein (NP) and the GP genes of EBO virus. Primers REM2 and REM5, which target the NP gene of EBO virus, are described by Sanchez et al. [15] (in this issue). Two additional primers were designed for amplification of the most variable region of the GP gene of EBO virus. The nt sequence of these primers and their 5′ and 3′ positions relative to the GP gene of 1976 strain of EBO-Z are as follows: GPZAI1201F = 1201 5′-ACAGTCAAGGAAGGGAAG-3′ 1218; GPZAI1485R = 1502 5′-GTTTTGGGGACTTGTTGT-3′ 1485.

Direct sequencing of the amplimers was done by the dideoxy chain termination method on a fluorescent-dye automated sequencer (Applied Biosystems, Foster City, CA), using the same primers as used for RT-PCR. In some cases in which direct sequencing was not possible, PCR products were cloned using the TA cloning kit (InVitrogen, San Diego), and at least 3 clones of each sample were sequenced. Sequences were aligned and compared using the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, WI).

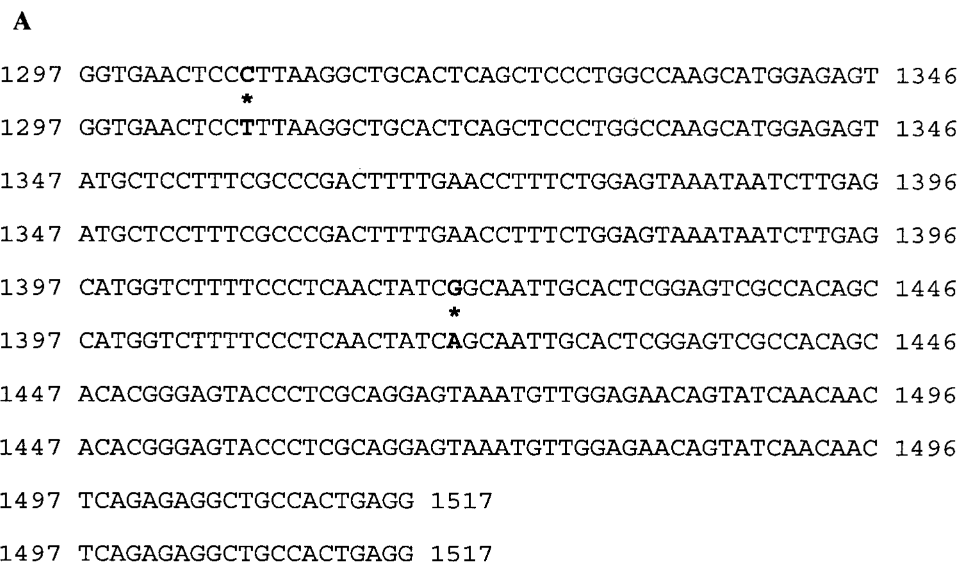
Virus isolation. All procedures involving potentially infectious samples were done in a biosafety level 4 facility at CDC. Confluent monolayers of Vero E6 cells in T-25 flasks were inoculated with 0.05–0.1 mL of undiluted patient serum or plasma or with material resuspended from swabs diluted in 1 mL of Hanks' balanced salt

solution containing 5% heat-inactivated fetal bovine serum and antibiotics. After adsorption with constant rocking for 1 h at 37°C, maintenance medium (7 mL) was added to the flasks, which were maintained at 37°C with tightened lids. Cells were observed every other day for signs of cytopathic effect (CPE). If no CPE appeared, cells were fed again with maintenance medium at 7 days and observed for a further 7 days. Fourteen days after inoculation (or when CPE developed), the cells were removed from the culture flask with glass beads, and spot slides were made, fixed, and tested for the presence of EBO antigens by IFA. The detection antibody used was pooled sera from 2 rabbits, each hyperimmunized by sequential inoculation of live EBO-Z and EBO subtypes Sudan and Reston.

Results

A total of 148 samples were obtained from 7 EHF patients in the isolation unit at Kikwit General Hospital on four sampling dates between 2 and 13 July 1995 (table 1, patients A–G). All patients were recovering and were at least 1 week past their last EBO antigen–positive blood test. Several samples of vaginal, rectal, and conjunctival swabs from patient F were positive by RT-PCR for the presence of EBO virus NP RNA for up to 33 days after onset of disease (table 1). However, no infectious virus was recovered in tissue culture when duplicate samples taken from patients A–G were tested, possibly as a consequence of inactivation during storage. In addition, seminal fluid samples obtained as late as 101 days after onset of disease tested positive by RT-PCR in 4 of 5 convalescent patients (patients H–L, table 1). One of these samples, which was obtained 82 days after onset of disease, yielded an EBO virus isolate on E6 Vero cells (see Rowe et al. [16], in this issue). An additional follow-up seminal fluid sample obtained from patients H, J, and K in April 1997 (>700 days after onset of disease) tested negative by RT-PCR (table 1). All RT-PCR products for the NP gene were sequenced and shown to be identical to one another and to the EBO-Z 1995 reference strain; however, they differed by 2 nt (1 amino acid change) from the EBO-Z 1976 reference strain (figure 2A, B).

To test the genetic stability of the GP gene of EBO virus among different patients during the course of the outbreak in Kikwit and also during persistence within the same patient, we selected 13 specimens from 9 patients for GP sequence analysis. These specimens included the earliest (4 May 1995) and latest (19 June 1995) available samples from acutely infected patients and samples from fatal and non-fatal cases (figure 1). Included were samples from a suspected line of transmission with at least three known human-to-human passages and an



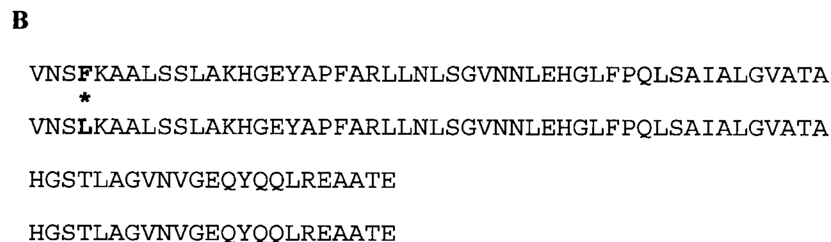


Figure 2. Alignment of partial nucleotide (**A**) and amino acid (**B**) sequences of nucleocapsid protein (NP) gene of EBO-Z 1976 (top) and representative sequence derived from all patients who were positive for NP gene by reverse transcription–polymerase chain reaction (bottom). Bold (*) indicates nucleotide or amino acid substitutions.

A

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1223 AGTGTCGCATCTAACAACCCTTGCCACAATCTCCACGAGTCCCCAATCCC 1272
                 *                             *   *
1223 AGTGTCGCATCTGACAACCCTTGCCACAATCTCCACGAGTCCTCAACCCC 1272

1273 TCACAACCAAACCAGGTCCGGACAACAGCACCCATAATACACCCGTGTAT 1322
     *                                 *
1273 CCACAACCAAACCAGGTCCGGACAACAGCACCCACAATACACCCGTGTAT 1322

1323 AAACTTGACATCTCTGAGGCAACTCAAGTTGAACAACATCACCGCAGAAC 1372

1323 AAACTTGACATCTCTGAGGCAACTCAAGTTGAACAACATCACCGCAGAAC 1372

1373 AGACAACGACAGCACAGCCTCCGACACTCCCTCTGCCACGACCGCAGCCG 1422
                                    * *
1373 AGACAACGACAGCACAGCCTCCGACACTCCCCCCGCCACGACCGCAGCCG 1422

1423 GACCCCCAAAAGCAGAGAACACCAACACGAGCAAGAGCACTGACTTCCT 1471
           *                            * *  *   *
1423 GACCCCTAAAAGCAGAGAACACCAACACGAGCAAGGGTACCGACCTCCT 1471
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B

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VSHLTTLATISTSPQSLTTKPGPDNSTHNTPVYKLDISEATQVEQHHRRT
               **
VSHLTTLATISTSPQPPTTKPGPDNSTHNTPVYKLDISEATQVEQHHRRT

DNDSTASDTPSATTAAGPPKAENTNTSKSTDF
          *       *         *  *
DNDSTASDTPPATTAAGPLKAENTNTSKGTDL
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Figure 3. Alignment of partial nucleotide (**A**) and amino acid (**B**) sequences of glycoprotein gene of EBO-Z 1976 (top) and sequence representative of all patients' acute- and convalescent-phase samples (bottom). Bold (*) indicates nucleotide or amino acid substitutions.

EBO virus isolated from seminal fluid of a patient 82 days after onset of disease (figure 1). The region of the GP gene sequenced was that known to be most divergent between EBO-Z 1976 and EBO-Z 1995 [11]. Sequence analysis of 249 nt of this region found no nt differences among any of the samples mentioned above (figure 3). As expected, 12 nt (6 amino acid) differences were observed between all the patients' sequences and the EBO-Z 1976 virus (figure 3A, B).

Discussion

This study was done to better understand the potential secretion or excretion of EBO virus from infected patients during early convalescence. Viral RNA sequences were found in mucosal swab samples from a convalescent patient, indicating that EBO virus may be capable of persisting in mucosal membranes for at least 33 days after onset of disease. The inability to isolate virus in tissue culture from duplicates of the patient's mucosal swab samples could be due to loss of viability of the virus during storage or transport from Kikwit to Atlanta or to the presence of virus at levels below the level of detection in tissue culture. It is also possible that virus was sequestered inside cells, such as macrophages, or was bound to mucosal antibodies.

An alternative explanation is that only viral RNA or viral nucleocapsids were present in these samples. The persistence of viral RNA (presumably in the form of replicating intracellular nucleocapsids) in the absence of infectious virus has been reported in negative-strand RNA viruses, such as human respiratory syncytial virus and vesicular stomatitis virus [17–19]. However, in these systems, RNA was present in low copy numbers, and very sensitive nested PCR assays were necessary for detection. In this study, strong PCR products were obtained using single-tube, non-nested RT-PCR assays, suggesting that a large number of RNA molecules were present.

Whether patient F could shed or transmit EBO virus during this time or for how long virus was present in her mucosal membranes is unknown. Samples of seminal fluid were positive for viral RNA in 4 of 5 convalescent patients tested as late as 101 days after onset of disease, and infectious virus was recovered from a sample 82 days after onset of disease (see Rowe et al. [16], in this issue). However, seminal fluid samples from 3 patients $\geqslant$700 days after onset of disease were RT-PCR negative even though earlier samples were positive. Taken to-

gether, these data suggest that these patients were probably shedding virus in the early convalescence phase following their release from the hospital but not at later times. Although no secondary cases were associated with convalescent patients during this outbreak, our evidence for virus persistence in mucosal membranes and in seminal fluid should be carefully considered in the refinement of recommendations for release of patients recovering from EHF. At a minimum, these precautions should include condom use by patients for at least 3 months after onset of disease.

The genetic stability of EBO virus was tested by sequencing the region of the GP gene previously found to be most divergent when the 1976 and 1995 strains of EBO-Z were compared [11]. The samples selected for this purpose included those from patients who represented at least three human-to-human passages. In addition, there were samples from fatal cases and survivors and samples from individual patients in the acute and convalescent periods of disease (up to 82 days apart). None of these samples had any nt substitutions over the 249-nt region analyzed. We did not find any evidence of >1 strain of EBO virus circulating during this outbreak. This is consistent with the view of a single-source outbreak, with patients linked back to 1 human case (see Khan et al. [20], in this issue). It has been speculated that EBO virus may rapidly accumulate mutations during passages in the human host, resulting in attenuation of the virus by the later stages of an outbreak. The lack of genetic changes observed here suggests this is an unlikely case; however, attenuating mutations elsewhere in the genome cannot be ruled out.

The genetic stability observed here contrasts with the general perception that most RNA viruses evolve rapidly because of the high error rate of their RNA polymerases [10]. By analogy with vesicular stomatitis virus, the half-life of EBO virus intracellular nucleocapsids is likely to be <24 h [21]. Assuming that EBO virus must replicate during viral persistence in a living organism, several virus replicative cycles must have occurred during the 82 days of virus persistence in patient H. Other examples of virus genetic stability during extensive replication in nature can be seen with other members of the Mononegavirales (negative-sense, single-strand RNA viruses). For instance, vesicular stomatitis virus, despite its ability to rapidly evolve during growth under various tissue culture conditions, is genetically very stable in nature within disease-endemic regions. Viruses isolated as much as 30 years apart but within the same disease–endemic zone are almost identical in their nt sequence, showing an adaptation to a particular ecologic niche where the virus is maintained in nature [22, 23]. Genetic stability in nature has also been observed for rabies and measles viruses [24–26]. The significance of the genetic stability of EBO virus is unknown, but it likely reflects the powerful purifying selective forces constraining the potential rapid evolution of the virus.

Other RNA viruses, such as influenza virus and foot-and-mouth disease virus, undergo rapid evolution within the host [27–29]. These viruses elicit strong host immunologic responses, and neutralizing antibodies are thought to be important in driving their rapid evolution. In the case of EBO virus, this seems not to be the case, since nt changes were not detected in the most variable region of the GP gene even after 82 days of persistence within patient H. One possible explanation for this is the reported lack of a neutralizing antibody response in convalescent-phase sera from patients or experimental animals who survived infection with EBO virus [1]. Postclinical persistence of filoviruses certainly warrants further study.

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