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Identification of Ebola virus sequences present as RNA or DNA in organs of terrestrial small mammals of the Central African Republic

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ABSTRACT – The life cycle of the Ebola (EBO) virus remains enigmatic. We tested for EBO virus in the organs of 242 small mammals captured during ecological studies in the Central African Republic. EBO virus glycoprotein or polymerase gene sequences were detected by reverse transcription PCR in RNA extracts of the organs of seven animals and by PCR in DNA extract of one animal. Neither live virus nor virus antigen was detected in any organ sample. Direct sequencing of amplicons identified the virus as being of the Zaire/Gabon subtype. Virus-like nucleocapsids were observed by electron microscopy in the cytoplasm of the spleen cells of one animal. The animals belonged to two genera of rodents (Muridae; *Mus setulosus*, *Praomys* sp1 and *P.* sp2) and one species of shrew (Soricidae; *Sylvisorex ollula*). These preliminary results provide evidence that common terrestrial small mammals living in peripheral forest areas have been in contact with the EBO virus and demonstrate the persistence of EBO virus RNA and DNA in the organs of the animals. Our findings should lead to better targeting of research into the life cycle of the EBO virus. © 1999 Éditions scientifiques et médicales Elsevier SAS

Ebola virus / RT-PCR / terrestrial small mammals / electron microscopy / DNA form

1. Introduction

The Ebola (EBO) and Marburg (MBG) viruses (Filoviridae) have negative non-segmented RNA genomes. Filoviruses cause haemorrhagic fever in humans, with mortality rates of up to 23% for the MBG virus and 88% for the EBO virus [1, 2]. Since 1976, the EBO virus has been responsible for several epidemics in Central Africa, particularly in Sudan (1976), the Democratic Republic of Congo (1976, 1995), and Gabon (1994, 1996).

The natural reservoirs of the EBO and MBG viruses are unknown. Experimental studies have shown that the EBO virus replicates in several animals including monkeys, rodents and bats [3–5]. Since 1976, thousands of vertebrates and invertebrates captured around the epidemic foci in the Democratic Republic of Congo have been tested. However, EBO has not been detected in, or isolated from, these animals, mostly rodents, shrews, and bats [6] and tests for viral antigens have all been negative [7]. From these results and the epidemiology of Ebola fever which suggests that humans have only intermittent contact with the virus, it was concluded that viruses should be present in the heart of the rainforest environment. Monath [8] stressed the possible importance of arboreal animals such as bats and suggested that there may be reservoirs in the canopy.

However, historical biogeographic analysis, based on the locations of the eight sites of known epidemics, indicates that the epidemics did not occur in the forest refuges that resulted from the fragmentation of the forest during the Upper Pleistocene, but rather at the present periphery of the Guinean-Congolese forest block, in habitats currently undergoing fragmentation including mosaics of forests and open environments [9]. This led us to suggest that

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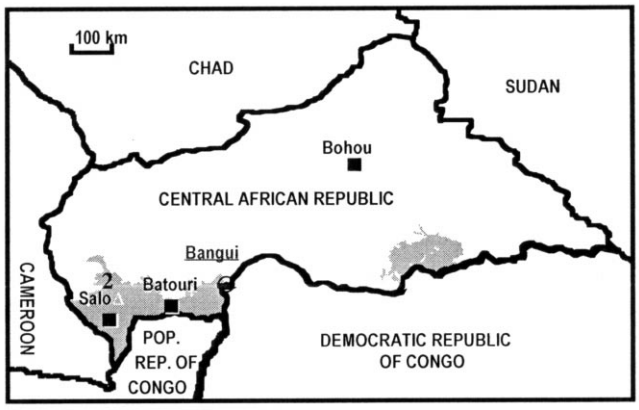

Figure 1. Map of the Central African Republic. The Guinean-Congolese forest block is indicated in grey. Black squares show the principal capture sites.

the EBO virus may circulate in an environment other than mature forest and may not be restricted to the tree canopy. In addition, several serological studies have shown that the virus is present in the Central African Republic (CAR) [10–12] and a high seroprevalence was found between 1994–1997 both in forest-dwelling populations (13.2% in Pygmy populations) and populations in degraded habitats (4% in Bantu villagers) [13]. Here we report further evidence supporting these hypotheses.

2. Materials and methods

2.1. Collection of material and description of sites

The study was conducted in the CAR and included field work on the ecology of rodents (family Muridae), shrews (family Soricidae) and bats (order Chiroptera). Animals of the three taxa were captured at three sites *(figure 1):* i) The Batouri River site in the Ngotto forest (3°56′ N, 16°56′ E; Lobaye District; 180 km SW of Bangui) consists of a dense semi-deciduous forest. This primary forest is often affected by storms such that there are numerous tree falls creating openings in the canopy; ii) Salo[2] (and Adibori, located 7 km from Salo[2]) is located 310 km southwest of Bangui (right bank of the Sangha; 3°31′ N, 16°02′ E; Dzanga-Sangha District). Its vegetation is a mosaic of primary and secondary forest and village fields. These two sites lie at the northern limit of the Guineo-Congolese forest block; iii) The Bohou River site (Sangba; 8°25′ N, 20°38′ E; Bamingui-Bangoran District, 490 km north of Bangui) is a gallery forest located in the Sudano-Sahelian savannah, 400 km north of the forest block.

Sherman traps and metal snap traps were used to capture rodents. Pitfall traps were used for shrews (Bohou and Batouri) and nets for bats (Salo[2] and Bohou). Six hundred and thirty-three animals were killed at the site and an autopsy performed for 242 of them *(table I)*. The organs of these animals were placed in Nunc cryotubes and frozen immediately in liquid nitrogen.

2.2. Testing for the EBO virus by antigen capture

Various organs from the captured mammals were homogenised in phosphate-buffered saline (PBS). The immunocapture test used a mixture of monoclonal antibodies selected for their ability to capture EBO antigens followed by ELISA to detect virus antigens using an anti-EBO virus (Zaire-Sudan-Reston) rabbit serum as previously described [14].

2.3. Inoculation of cell cultures and mice with specimens from mammals

Attempts were made to isolate EBO virus in the containment laboratory of the Institut Pasteur in Bangui from samples testing positive by reverse transcription (RT)-PCR. The organs were homogenised in PBS and a 1 in 10 dilution of the suspension was used to inoculate cultures of various cell types: Vero E6 (monkey kidney cells), C6/36 (*Aedes albopictus*) and BHK 21 (hamster kidney cells). The suspension was also injected intracerebrally into newborn mice and subcutaneously into guinea pigs using standard techniques. Viral antigens were detected, on the one hand, by antigen immunocapture and ELISA of cell culture supernatants, brain homogenates from baby mice and guinea pig sera as described above, and on the other hand, by direct immunofluorescence of cells using anti-EBO polyclonal rabbit serum (kindly supplied by Dr P. Rollin).

2.4. RNA and DNA extraction from organs of mammals

Two protocols were used to extract EBO virus nucleic acids from the organs (spleen, liver, and kidney) of the animals. In the procedure carried out in Bangui, RNA was isolated from the organs of the animals with the RNeasy kit (Quiagen) according to the manufacturer's protocol. Briefly, frozen tissue samples were homogenised in RLT buffer containing guanidium salt and centrifuged at 12 000 *g* for 3 min at room temperature. One volume of 70% ethanol was added to the cleared lysate and the mixture was transferred to an RNeasy column. The nucleic acids fixed on the column were washed with specific buffer provided by the manufacturer and eluted with diethylpyrocarbonate (DEPC)-treated distilled water.

In the procedure performed in Paris, organ samples were homogenised in TRIZOL® reagent (Life Technologies) and incubated for 5 min at room temperature. Chloroform (0.2 volumes) was added and the mixture was incubated for 3 min at room temperature. The samples were centrifuged at 12 000 *g* for 15 min at 4 °C and the RNA in the aqueous phase was precipitated with an equal volume of isopropyl alcohol for 10 min at room temperature. RNA precipitate was rinsed with 70% ethanol and dried. DNA in the interphase from the initial homogenate was isolated by precipitation with two volumes of 100% ethanol in 0.3 M ammonium acetate overnight at -20 °C. The DNA was pelleted by centrifugation at 12 000 *g* for 5 min at 4 °C, rinsed with 70% ethanol and dried.

RNA and DNA were dissolved in DEPC-treated distilled water. In some experiments, DNase treatment was performed before PCR by incubating a fraction of the RNA or DNA sample for 1.5 h at 37 °C with 21 units of DNase I in sample buffer (Pharmacia). DNase I was inactivated by heating at 95 °C for 5 min.

2.5. Amplification of EBO virus gene fragments

RNA sequences encoding fragments of EBO glycoprotein and polymerase were amplified by RT-PCR followed

Table I. List of mammals collected and tested for EBO virus.

		Southwest Bangui				*Northeast Bangui*			
		Salo[2] - Adibori		*Batouri*		*Bohou*		*Total*	
		Dzanga-Sangha		*Ngotto*		*Sangba*			
		May-June 1994		*Oct. 1998-Jan. 1999*		*June 1998*			
Genus and species	*Habitat*	*n captured*	*n tested*	*n captured*	*n tested*	*n captured*	*n tested*	*n captured*	*n tested*
Muridae		187	(70)	101	(67)	30	(26)	318	(163)
Mus sp1/sp2	S/A	75	(40) [a]			11	(11)		
Hylomyscus complex	F	6	(3)	75	(60)	3	(2)		
Praomys complex	F	32	(4) sp1 [a]	22	(6)	15	(12) sp2[a, b]		
Hybomys univittatus	F	28	(8)	1					
Grammomys rutilans	F	2	(1)						
Lemniscomys striatus	S	4	(1)						
Lophuromys sp1/sp2	F/S	14	(5)			1	(1)		
Stochomys longicaudatus	F	11	(4)	2					
Deomys ferrugineus	F	6	(2)						
Rattus rattus	A	6	(2)						
Malacomys longipes	F	1		1	(1)				
Oenomys hypoxanthus	F	2							
Soricidae		36	(8)	81	(14)	59	(34)	176	(56)
Crocidura attila	F/A	2	(1)			1	(1)		
Crocidura crenata	F			1					
Crocidura denti	F					4	(3)		
Crocidura denti group	F	16	(2)						
Crocidura dolichura	F			11					
Crocidura goliath	F	1							
Crocidura hildegardeae	F/A	2	(1)						
Crocidura ludia	F					41	(23)		
Crocidura mutesae	F	12	(3)						
Crocidura nigrofusca	F			5	(1)				
Crocidura nigrofusca group	F			4	(2)				
Crocidura olivier	F/A			9		6	(4)		
Crocidura poensis group	F	2	(1)	4	(2)				
Crocidura roosevelti	F/S					5(3)			
Paracrocidura schoutedeni	F			9	(1)				
Sylvisorex johnstoni	F			11					
Sylvisorex ollula	F	1		25	(7) [a]				
Suncus infinitesimus	S					2			
Suncus remyi	F			1	(1)				
Congosorex sp	F			1					
Chiroptera		113	(1)			26	(22)	139	(23)
Epomophorus gambianus	S					4	(3)		
Myonycteris torquata	F	37				14	(13)		
Micropteropus pusillus	F/S					2	(2)		
Epomops franqueti	F	34	(1)						
Megaloglossus woermanni	F	37							
Roussetus egyptiacus	F/S	3							
Hypsignatus monstrosus	F	1							
Vespertilionidae	F/S	1				5	(4)		
Hipposideridae	F/S					1			
Total		336	(79)	182	(81)	115	(82)	633	(242)

[a]Detection of EBO virus RNA by RT-PCR performed in Bangui using EBO primers (see text): *M. setulosus* (R13154, R13208, R13452, R13459); *Praomys* sp1 (R13204); *Praomys* sp2 (NC76); *S. ollula* (R18857). [b]Observation of filovirus-like nucleocapsid in medular spleen cells: *Praomys* sp2 (NC76). Habitat: F = Forest, S = Savannah, A = Anthropic. n = number of individuals.

by nested PCR, according to standard protocols [15]. Two protocols were used to detect EBO virus glycoprotein and polymerase gene sequences in the organs of the animals.

The first-strand cDNA in the glycoprotein gene was synthesised: a mixture of 10 µL of 10× RT buffer (Boehringer Mannheim), 8 µL dNTPs (2.5 mM, Promega), 3.5 µL each of the EBO-1 and EBO-2 primers at 100 ng/µL *(table II)* and 68.5 µL sterile distilled water was exposed to UV light for 20 min and then 0.5 µL of AMV reverse transcriptase (Boehringer Mannheim), 1 µL *Taq* polymerase (5 U/µL, Boehringer Mannheim) and 5 µL of RNA were added. The mixture was incubated at 41 °C for 1 h

Table II. Oligonucleotide primers used in RT-PCR and nested PCR of EBO virus sequences in the glycoprotein gene with predicted sizes of amplicons.

Oligonucleotide primer	*Gene target*[a]	*Primer sequence (sense)*	*T° Hybridization*	*Target gene (product size)*
EBO-1	GP	5'-TGGGTAATYATCCTYTTCCA (+)		
EBO-2	GP	5'-ACGACACCTTCAGCRAAAGT (-)	38 °C	(479 bp)
EBO-3	GP	5'-GTTTGTCGKGACAAACTGTC (+)		
EBO-4	GP	5'-TGGAARGCWAAGTCWCCGG (-)	43 °C	(308 bp)
FILO-A	POL	5'-ATCGGAATTTTTCTTTCTCATT (+)		
FILO-B	POL	5'-ATGTGGTGGGTTATAATAATCACTGACATG (-)	37 °C	(419 bp)
GAB-1	POL	5'-GAATGTAGGTAGAACCTTCGG (+)		
GAB-2	POL	5'-GCATATAACACTGTGGGATTG (-)	55 °C	(353 bp)

[a]Glycoprotein (GP) or polymerase (POL) genes targeted by the primers. EBO-1, EBO-2, EBO-3 and EBO-4 oligonucleotide sequences were kindly provided by H. Feldmann. FILO-A and FILO-B oligonucleotide sequences were from Sanchez et al. [16]. The reactivity of the oligonucleotides was tested by RT-PCR and nested PCR on Gabon 1994 EBO virus RNA. Oligonucleotides were degenerated with abbreviation codes: R = A, G; Y = C, T; K = G, T; W = A, T.

and then at 94 °C for 1 min. A Perkin Elmer 9700 thermocycler was used for amplification as follows: 40 cycles of denaturation at 94 °C for 40 s, annealing at 38 °C for 40 s and extension at 72 °C for 1 min, with a final extension phase at 72 °C for 2 min. The products of the first PCR were used for nested PCR. The reaction mixture consisted of 3 µL of amplified cDNA, 10 µL of 10× buffer (Boehringer Mannhein), 8 µL dNTPs, 71 µL distilled water, 3.5 µL (each) of the EBO-3 and EBO-4 primers at 100 ng/µL *(table II)* and 1 µL of *Taq* polymerase. The mixture was denatured at 94 °C for 1 min and subjected to 40 cycles of denaturation at 94 °C for 40 s, annealing at 43 °C for 40 s and extension at 72 °C for 1 min. It was then subjected to a final extension phase at 72 °C for 2 min. Negative controls were included in each test, in which the RNA or DNA extracted from organs was replaced with water.

A slightly different protocol was used to amplify a fragment of the polymerase gene using consensus filovirus primers [16] for RT-PCR and EBO Gabon subtype-specific primers for nested PCR *(table II)*. RNA (10 µL) was mixed with 1 µL (each) of the FILO-A and FILO-B (50 ng/µL) primers and heated to 95 °C for 2 min. The tube was then chilled on ice and a mixture containing 4 µL of 5× RT buffer, 1 µL RNasin (Promega), 0.8 µL of 5 mM dNTPs and 0.5 µL AMV reverse transcriptase (Boehringer Mannheim) made up to 9 µL with distilled water was added. The first-strand DNA was synthesised by incubating the mixture at 42 °C for 1 h. The products of this reaction (5 µL) were placed in a thin-walled 0.2-mL tube and 45 µL of a mixture containing 33 µL of distilled water, 1.5 µL of 50 mM $MgCl_2$, 5 µL of 5× *Taq* polymerase (Boehringer Mannheim), and 5 µL (each) of the FILO-A and FILO-B (50 ng/µL) primers was added. The DNA was amplified as follows: three cycles of denaturation at 94 °C for 1 min, annealing at 37 °C for 30 s and extension at 74 °C for 2 min followed by 35 cycles at 94 °C for 1 min, 42 °C for 30 s and 74 °C for 2 min. Aliquots (1 µL) of the products were used for nested PCR in a final volume of 50 µL containing the GAB-1 and GAB-2 primers *(table II)*. The PCR cycling pattern was identical to that for EBO-3 and EBO-4 except that the annealing temperature used for GAB primers was 55 °C.

2.6. Detection and characterization of EBO virus-amplified nucleotide sequences

The amplification products were analysed by electrophoresis in 2% agarose gels containing 0.5 µg/mL ethidium bromide, in TAE (10 mM Tris-Cl, 50 mM Na acetate, 1 mM EDTA) buffer. The gels were viewed under UV transillumination and treated for Southern blotting as previously described [17]. The probes consisted of RT-PCR products from Vero E6 cells infected with the Gabon 1994 EBO virus strain [18] amplified with glycoprotein- or polymerase-specific oligonucleotide primers. The positive PCR products were purified with the Wizard PCR Preps kit (Promega) and radiolabelled with α^{32}P-dATP using a nick-translation kit (Boehringer Mannheim).

The amplified products were purified and sequenced directly using the Big Dye Terminator sequencing kit (Perkin Elmer). The sequencing reaction products were analysed on an automated ABI377 sequencer (Perkin Elmer).

The supernatant of Vero E6 cells infected with the Gabon 1994 EBO virus strain was amplified and sequenced separately, at the Institut Pasteur in Paris. The sequences obtained were aligned with published viral glycoprotein gene sequences [19] or compared with the sequences of the Gabon 1994 strain, using the sequence analysis program CLUSTAL V.

2.7. Electron microscopy

Small fragments of the spleen were fixed by incubation in 2.5% gluteraldehyde, 0.1M sodium cacodylate pH 7.4 for 2 h at room temperature. The samples were rinsed in 2.5% gluteraldehyde, 0.1M sodium cacodylate pH7.4 and were postfixed by incubation in 2% osmium tetraoxide, 0.1M sodium cacodylate for 2 h. The fragments were rinsed with distilled water, dehydrated in a graded series of acetone solutions and embedded in Epon. Ultrathin sections were cut and contrast stained with uranyl acetate and lead citrate by standard methods. They were observed in standard conditions with a Phillips CM12 microscope.

3. Results

Two hundred and forty-two of the 633 animals captured during the ecological studies were killed at the trapping sites. They included 163 rodents, 56 shrews, and 23 bats *(table I)*. The Muridae and Soricidae communities captured at Batouri consisted entirely of forest-dwelling species *(table I)*. *Sylvisorex ollula* was the dominant shrew (0.31 relative abundance) and the genus *Hylomyscus* accounted for 0.72 (relative abundance) of the rodents. The rodent community at Bohou comprised both savannah and forest species. The savannah *Mus setulosus* and the forest *Praomys* sp2 were the most common forms (0.37 and 0.5 relative abundance, respectively). Most (relative abundance 0.69) of the shrews were *Crocidura ludia*, a forest species. At Salo[2], ten genera of forest rodents were captured in primary and secondary forest areas and six were caught in fields, along with the savannah species, *M. setulosus*, which was the most abundant species (0.79).

Samples were taken from the kidney, liver and spleen tissues of the 242 animals. Organ homogenates from each animal were tested for the virus by antigen capture. No EBO virus antigen was detected (data not shown).

We first used serial RT-PCR to assess whether the organs of the animals contained EBO virus RNA. Viral RNA fragments were amplified by RT-PCR followed by nested PCR using probes EBO-1 to -4 that had been tested in the Virology Institute in Marburg, on tissue culture-derived RNA, for their specificity to amplify a region in the glycoprotein gene of subtypes Zaire, Sudan, and Reston of the EBO virus *(table II)*. RT-PCR and nested PCR were carried out at the Institut Pasteur in Bangui with no positive control, so as to avoid cross-contamination. Fragments 308 bp in size, corresponding to the predicted size of the fragment of the glycoprotein gene from the EBO virus, were obtained from the spleen samples of seven animals (*figure 2* and *table I*). The sequences obtained were identical to those of the EBO Zaire 1976, Zaire 1995, and Gabon 1994 strains [19]. No EBO virus strain was present in the laboratory at the time of testing, and contamination during amplification was therefore unlikely.

Samples from the spleen, liver and/or kidney of the seven animals were tested at the Institut Pasteur in Paris following a slightly different protocol for RNA extraction and RT-PCR (see Materials and methods). In addition to the EBO primers targeting the glycoprotein gene, we also used recently designed primers specific for polymerase gene sequences in the filovirus genus [16]. We then carried out nested PCR using primers specific for the Gabon subtype of EBO virus *(table II)*. These tests confirmed the presence of EBO virus nucleotide sequences in six of the seven animals testing positive in the CAR *(table III)*. However, only two animals (nos. R13452 and NC76) tested positive with the primers targeting the glycoprotein gene of EBO virus, and five contained sequences for the polymerase. The spleen of animal no. NC76 contained both sequences. Only the amplicons from the spleens of animals nos. NC76 and R18857 were detected directly by ethidium bromide staining, whereas the other animals were found to be positive only after Southern blotting *(table III)*.

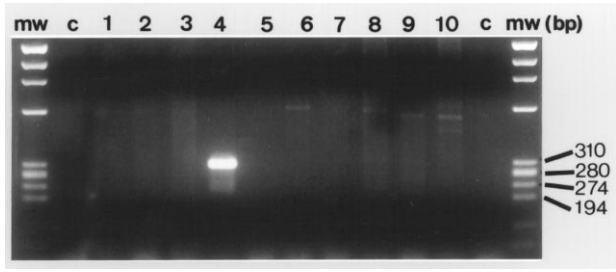

Figure 2. Agarose gel showing specific EBO virus-derived RT-PCR products. Spleen samples of 10 (1 to 10) animals tested were analysed by RT-PCR and nested PCR using EBO glycoprotein-specific oligonucleotide primers (test performed in Bangui, CAR). The amplification products were analysed by electrophoresis on a 2% agarose gel containing 0.5mg/mL ethidium bromide. The sizes of the amplified DNA fragments viewed under UV transillumination were estimated by comparison with molecular weight (mw) standards (X174 DNA cut with *Hae*III). Line 4 shows an amplicon with the expected size (308 bp) obtained from mammal no. R13452 (*M. setulosus*). (C) shows negative controls included in the reaction in which the RNA extracted from organs was replaced by water.

Attempts were made to isolate the virus from spleen samples testing positive by RT-PCR. All attempts were unsuccessful after three passages for each of the samples (data not shown).

It has been suggested that the lymphocytic choriomeningitidis virus persists in mammals either as defective particles with deleted RNA sequences [20] or as virally determined DNA [21]. Two animals only, nos. R13452 and R18857, from which organs were still available, were tested for the presence of EBO DNA sequences for both glycoprotein and polymerase genes. Both RNA and DNA extract were treated or not by DNase I and were tested by PCR and nested PCR. The DNA extract of the spleen, but not of the liver and the kidneys, of mammal no. R13452 tested positive with the EBO glycoprotein-specific primers *(figure 3)*. All organs tested negative if treated with DNase I before amplification. All negative controls were negative. There was only one mismatch between the sequence of the EBO virus DNA fragment from animal no. 13452 and that of the cDNA from Gabon in the 1994 strain (data not shown).

We examined spleen samples from *Praomys* sp. 2 no. NC76 and *M. setulosus* no. R13452 by electron microscopy. In the spleen of rodent no. NC76, tubular structures, 40 to 45 nm in diameter and of various lengths, were present in the cytoplasm of some of the medullar cells *(figure 3)*. These structures were morphologically very similar to the filovirus nucleocapsids described in the cytoplasm of Vero cells infected experimentally with the EBO virus [22]. No enveloped viral particles were observed. No EBO virus-like structures were observed in the spleen of rodent no. R13452. The procedure of fixation of the samples did not enable immunohistochemistry analysis.

Table III. Detection of EBO virus RNA and DNA gene fragments in organs of seven small mammals.

Organ		*RT-PCR*[a]					
		EB staining		*Southern-blot*		*PCR*[b]	
Identification number		*GP*	*POL*	*GP*	*POL*	*GP*	*POL*
NC76	Spleen	+	–	+	+	NT	NT
R13154	Liver	–	–	–	+	NT	NT
	Kidney	–	–	–	+	NT	NT
R13204	Spleen	–	–	–	+	NT	NT
R13208	Spleen	–	–	–	+	NT	NT
R13452	Liver	–	–	+	–	–	–
	Kidney	–	–	+	–	–	–
	Spleen	–	–	NT	NT	+	–
R13459	Liver	–	–	–	NT	NT	
R18857	Spleen	–	+	–	+	–	–

[a]RT-PCR on RNA extracts using primers targeting glycoprotein (GP) or polymerase (POL) genes (see table II). Amplicons were detected by electrophoresis and ethidium bromide staining (EB staining) or hybridization using radiolabelled probes (Southern blot). [b]PCR on DNA extracts of the organs. Amplicons were detected by ethidium bromide staining after agarose gel electrophoresis (see *figure 4*). NT: not tested

4. Discussion

The transmission cycle of the EBO virus remains unknown, and its reservoirs have not been identified despite studies of these issues [6, 7]. We therefore investigated the ecology of the virus and the tools available for its detection. Our study was multidisciplinary such that various aspects of the natural history of the virus could be investigated.

The likelihood of successful detection and identification of virus depends on the virological tests used, especially if the rate of replication in the host is unknown and probably low. Previous studies with vertebrates captured around epidemic sites in Zaire after the 1976 [6] and 1995 [7] epidemics used antigen capture and cell culture techniques. These tests gave negative results in all cases, as they did in our study. We found that only a procedure based on RT-PCR and nested PCR and in some cases, Southern blotting, known to be the most sensitive techniques [16, 17], detected viral RNA in the organs of animals captured in the CAR, thereby demonstrating the value of these techniques. One animal (no. R13459) that tested positive for the glycoprotein gene by RT-PCR at the Institut Pasteur in Bangui tested negative at the Institut Pasteur in Paris, for both the glycoprotein and polymerase genes. Moreover, in Paris, only two animals tested positive for the glycoprotein gene and five for the polymerase gene. There are three major reasons for this difference: i) the organs tested in Paris had been subjected to several cycles of freezing and thawing before RNA extraction; ii) the protocols for RNA extraction and for RT-PCR-nested PCR were different; and iii) fragments of genomic DNA are often found associated with RNA preparations and we could not exclude the possibility that in some preparations, the EBO fragment we identified in Bangui or in Paris was present as DNA in the positive animals. The lymphocytic choriomeningitidis virus (a segmented negative-stranded RNA virus) has been shown to persist as either RNA or DNA in spleen or lymph nodes after clearance of acute infection [21]. This may occur in animals containing endogenous reverse transcriptase activity [23]. As we did not have enough tissue for most of the organs of tested mammals, we were able to test for EBO DNA in only two animals (nos. R13452 and R18857). Rodent no. R13452 tested positive for the EBO glycoprotein gene with the DNA extract from the spleen. The liver and kidneys of this animal tested positive for EBO RNA sequences by Southern blotting but not for EBO DNA. These results suggest that viral material may be present in some forms in lymphoid tissues [21] and that DNA fragments may be only poorly transcribed to give a few RNA molecules. We were unable to determine whether the DNA fragment was inte-

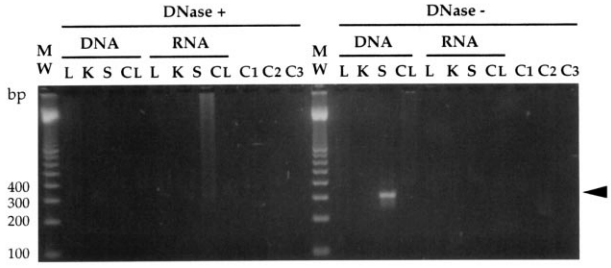

Figure 3. Agarose gel showing specific EBO virus-derived amplified products. DNA and RNA were extracted from liver (L), kidney (K) and spleen (S) samples of *M. setulosus* animal no. R13452. The extracts were treated (DNase$^+$) or not (DNase$^-$) by DNase I before amplification by PCR and nested PCR, using EBO glycoprotein-specific oligonucleotide primers. Several controls were included in the tests: (CL), liver organ from an animal tested negative by RT-PCR on spleen extract (see *figure 2*); C1, negative control for PCR (see legend *figure 2*); C2 and C3, negative controls for PCR and nested PCR. The amplification products were analysed by electrophoresis on a 2% agarose gel containing 0.5mg/mL ethidium bromide. The sizes of the amplified DNA fragments viewed under UV transillumination were estimated by comparison with molecular weight (MW) standards. The arrow shows an amplicon with the expected size (308 bp).

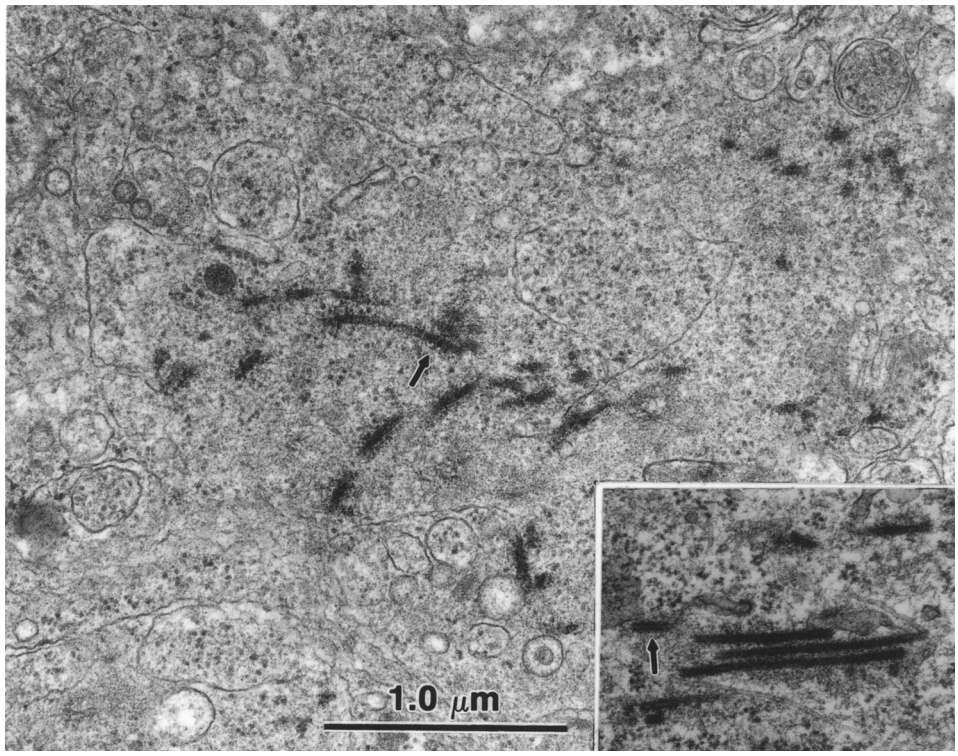

Figure 4. Electron micrograph. Spleen tissue of a captured animal (*Praomys* sp2 NC76). Groups of viral nucleocapsids in spleen cells are indicated by arrows. In the insert: EBO virus nucleocapsids in experimentally infected Vero E6 cells (magnification × 29 500).

grated or extrachromosomal because too little material was available.

Infectious EBO viruses are produced when the virus leaves the cell, by the budding off of nucleocapsids from the plasma membrane [3]. In the organs in which viral glycoprotein and polymerase gene fragments were detected by RT-PCR and PCR, viral antigen capture by ELISA and the isolation of the virus from cells or baby mice were unsuccessful. None of the animals showed signs of disease when they were caught. Thus, live EBO virus disappeared in animals that had been in contact with the virus. We therefore examined these cells under the electron microscope. Structures very similar to viral structures were observed, at low density, in the cytoplasm of spleen cells from one (no. NC76) of the captured animals. Interestingly, this animal tested positive for both glycoprotein and polymerase genes. No enveloped viral particles were observed by electron microscopy, suggesting that the virus may be maintained in this animal in the form of viral nucleocapsids, presumably indicating the presence of defective particles.

The seven EBO nucleic acid-positive animals belonged to two orders, three genera and four species. Six of the animals were rodents (*M. setulosus, Praomys* sp.1 and *P.* sp.2) and the last was a shrew (*S. ollula*) *(table I)*. All were captured at ground level. None of the bats tested EBO-positive by RT-PCR. The shrew came from a naturally disturbed primary forest (Batouri). The *Praomys* sp.2 was captured in an isolated gallery forest (Bohou). At the Salo[2] site, the *Praomys* sp.1 and the *M. setulosus* no. R13459 were caught in an extensively damaged secondary forest and the three other *M. setulosus* were caught in fields.

The trapping sites in the CAR, at which we collected EBO nucleic acid-positive animals, are located at the periphery of the forest block, a few kilometers away from the savannah-forest mosaic (the case of the two southern sites) or 400 km away from this block (northern site). Both forest species and savannah species were included among the animals testing positive for EBO. The savannah species *M. setulosus* is currently anthropic and commonly present around villages. Thus, virus reservoirs/vectors may not necessarily live in predominantly forest habitats and humans may be continuously in contact with them. The reason why no human cases of Ebola fever have been reported in the CAR is unknown but may be due to an attenuated strain circulating in this country [8].

This study detected EBO nucleic acid in animals, although only a relatively small number of animals were tested (1 EBO-positive /4 *Praomyes* sp.1; 1/12 *Praomys* sp.2; 1/7 *Sylvisorex*, and 4/40 *Mus* sp.). All animal species in which EBO sequences were detected belonged to the dominant species in their communities during the sampling period.

The similarity between the glycoprotein-specific sequences obtained for animals tested during this study in the CAR and the same regions of the genomes of the Zaire and Gabon strains defines a common EBO virus subtype for these three countries. This subtype differs from that of Ivory Coast [19]. These results fit well with our knowledge of the history of fauna in tropical Africa. Recent studies

have recognised a complex of faunal regions centred around the Congo basin (including the Congo River tributaries) that clearly differs from the complex of West African regions [24]. The evolution of virus therefore follows that of the mammalian fauna, suggesting a common ancient history. This confirms that the genetic evolution of the EBO virus has been limited in both space and time [25].

Our discovery of EBO virus sequences in terrestrial small mammals provides evidence that these animals have been in contact with the virus and that viral sequences may persist in RNA and DNA forms. These results should help direct future research into the natural history of this virus.

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