A Search for Ebola Virus in Animals in the Democratic Republic of the Congo and Cameroon: Ecologic, Virologic, and Serologic Surveys, 1979–1980

Joel G. Breman, Karl M. Johnson,* Guido van der Groen, C. Brian Robbins, Mark V. Szczeniowski,* Kalisa Ruti,† Patricia A. Webb,* Florian Meier,* David L. Heymann,* and Ebola Virus Study Teams‡

Smallpox Eradication Unit, World Health Organization, and Musée d'Histoire Naturelle, Geneva, Switzerland; Special Viral Pathogens Branch, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium; Division of Mammals, National Museum of Natural History, Smithsonian Institution, Washington, DC; World Health Organization and Programme Elargi de Vaccination, Ministry of Public Health, Kinshasa, Democratic Republic of the Congo; l'Organisation de Lutte contre les Endémies en Afrique Centrale, Yaoundé, Cameroon

More than 30 years after the first outbreak of Marburg virus disease in Germany and Yugoslavia and 20 years after Ebola hemorrhagic fever first occurred in central Africa, the natural history of filoviruses remains unknown. In 1979 and 1980, animals in the Democratic Republic of the Congo and Cameroon were collected during the dry season near the site of the 1976 Ebola hemorrhagic fever epidemic. The study objectives were to identify local animals and search for evidence of Ebola virus in their tissues. A total of 1664 animals representing 117 species was collected, including >400 bats and 500 rodents. Vero and CV-1 cells and IFA and RIA were used for virus and antibody detection, respectively. No evidence of Ebola virus infection was found. This study was limited in time and animal collections and excluded insects and plants. Long-term, prospective, multidisciplinary comparative studies will yield more information than will repeat short forays on the ecology of filoviruses.

From August to November 1976, the first outbreaks of Ebola hemorrhagic fever (EHF) occurred almost simultaneously in the northwestern part of the Democratic Republic of the Congo (DRC) and southern Sudan. Both epidemics were explosive, with 318 cases and 280 deaths (79%) occurring in DRC [1] and 284 cases and 151 deaths (53%) in Sudan [2].

Phylogenetic analysis of isolates from each area indicated that there were 2 subtypes of Ebola (EBO) virus, designated as Zaire and Sudan [3]; this was evidence that the source of the DRC outbreak was likely local. In DRC, at the time of the initial investigations in 1976, >800 bedbugs and 147 mammals (mainly wild rodents) were collected, and EBO virus was not detected in these samples [4]. In Sudan, a warehouse in which cotton was stored and bats roosted was implicated [2]; none of 100 specimens from 501 vertebrates collected in 1977 from this site had EBO virus [5].

One year after the outbreak of EHF in DRC, a patient with EBO virus infection presented to a mission hospital in the Tandala area, Gemena Zone, DRC. This led to an epidemiologic investigation, which retrospectively indicated that 2 cases of EHF had occurred in 1972 in the same hospital [6]. In 1979, a collection of serum samples from a seminomadic pygmy population living in the Congo Basin of southeastern Cameroon indicated an EBO virus seropositivity rate of 8% as determined by IFA, a test now known to have low specificity [7].

Three years after the epidemics, there were virtually no known leads on where EBO virus was lurking in nature. This situation existed despite the fact that outbreaks of Marburg virus (the first filovirus detected) had occurred in Germany and Yugoslavia in 1967 in association with nonhuman primates imported for biomedical research [8] and in southern Africa in 1975 in a tourist who had been bitten by an insect while trekking in Zimbabwe [9].

As resources were not available to mount an ecologic study for EBO virus alone, arrangements were made to couple the investigations with those addressing human monkeypox. The first cases of human monkeypox had been identified in DRC in 1971 [10]. By the late 1970s, an active monkeypox surveillance and research program coordinated by the World Health Organization (WHO) indicated that a large concentration of cases had been occurring in the Equateur Province of DRC. This was the same general area where the large outbreak of EHF occurred in 1976, where patients became ill with EHF in 1972 and 1977, and where IFA antibodies to EBO virus were found in domestic guinea pigs during an investigation of suspected human cases in the Tandala area [11].

Because the natural reservoirs of human monkeypox and the filoviruses were unknown and because diseases caused by both

Reprints or correspondence (new affiliation): Dr. Joel Breman, Fogarty International Center, NIH, Bldg. 31, Room B2C39, 31 Center Dr., MSC 2220, Bethesda, MD 20892-2220 (jbreman@nih.gov).

* Current affiliations: Department of Biology, University of New Mexico, Albuquerque (K.M.J.); Division of Emerging and Other Communicable Diseases Surveillance and Control, WHO, Geneva (M.V.S. and D.L.H.); Argyll and Bute, United Kingdom (P.A.W.); World Conservation Union, Genolier, Switzerland (F.M.).

† Deceased.

‡ A list of team members follows the Acknowledgments.

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classes of pathogens were occurring in the same area of DRC and possibly in eastern Cameroon (∼500 km distant; figure 1), field surveys for the two infections in DRC and Cameroon were initiated. The studies were done from June to August 1979 in DRC and in February and March 1980 in Cameroon; these are dry season months in central Africa.

Methods

DRC Studies

Site. The DRC study was confined to two rural areas in Equateur Province in northwestern DRC that comprise farming villages, each with a few hundred to a thousand residents; one area encompassed sections of Bumba Zone and Lisala Zone, and the other was in Gemena Zone (figures 2, 3). The first study area (2800 km^2) was located around the village of Yalosemba. The population of 50,000 was mainly of the Budja tribe and was involved in production of palm oil, rice, coffee, cocoa, and rubber. The second area centered around Tandala in Gemena Zone, with a population of ∼80,000, which belonged mainly to the Gwaka tribe.

Both study sites were within an area of the tropical rainforest that was altered by agricultural activities. Hunting and trapping of animals in the nearby primary and secondary forest was common. Men and adolescent boys were responsible for hunting, using homemade traps and antiquated rifles, and some dressing of game in the field; women prepared the animals for cooking and had extensive contact with animal tissues. It was common to boil or smoke the game before consumption. Most animal organs were consumed. Health care in the area was provided by poorly equipped and staffed government clinics, better equipped mission hospitals, and traditional healers.

Animal collections. Three methods were used to obtain animals in DRC: hunting and trapping by 7 hunting teams, trapping by project zoologists, and purchase of live animals captured by local villagers. Using a list of the local names of animals desired, project team members visited villages, announcing the prices to be paid for certain animals. After this initial publicity, team members went to the villages twice daily for animal collection. The initial goal was to collect 50 specimens of each species that was designated as a priority, mainly on the basis of reports of possible contact with monkeypox and EHF patients. The reports were obtained from the patients, their families, and nearby residents. A nonrandom collection method was used, so the number 50 was based on the expectation that if an animal had been infected, serologic or virologic evidence might reasonably appear in this number of animals. This number also limited the number of specimens from an abundant species that would be sent to the overburdened collaborating laboratories. Hunting permits were issued by the Institut Zairois de la Conservation de la Nature. Permits to use imported hunting firearms were issued by the Département de l'Administration du Territoire.

Sampling of blood and visceral organs from wild animals. Live animals were anesthetized with metofane or, for larger animals, by an injection of ketamine hydrochloride. Blood was then collected by direct heart puncture and diluted in collection vials. Animal number, genus, species, sex, age, condition, weight, type of preservation, blood dilution, total length, and other measurements, depending upon the animal, were noted. Immediately after these procedures were completed, specimens of liver, kidney, and spleen were collected under sterile conditions, put in vials, and placed in a freezer compartment of the refrigerator at −10°C. At the end of the day when the harvest of specimens was complete, the samples were placed in a liquid nitrogen and carbon dioxide slush, using nylon stockings as vial holders. If a harvested organ showed abnormalities, an addi-

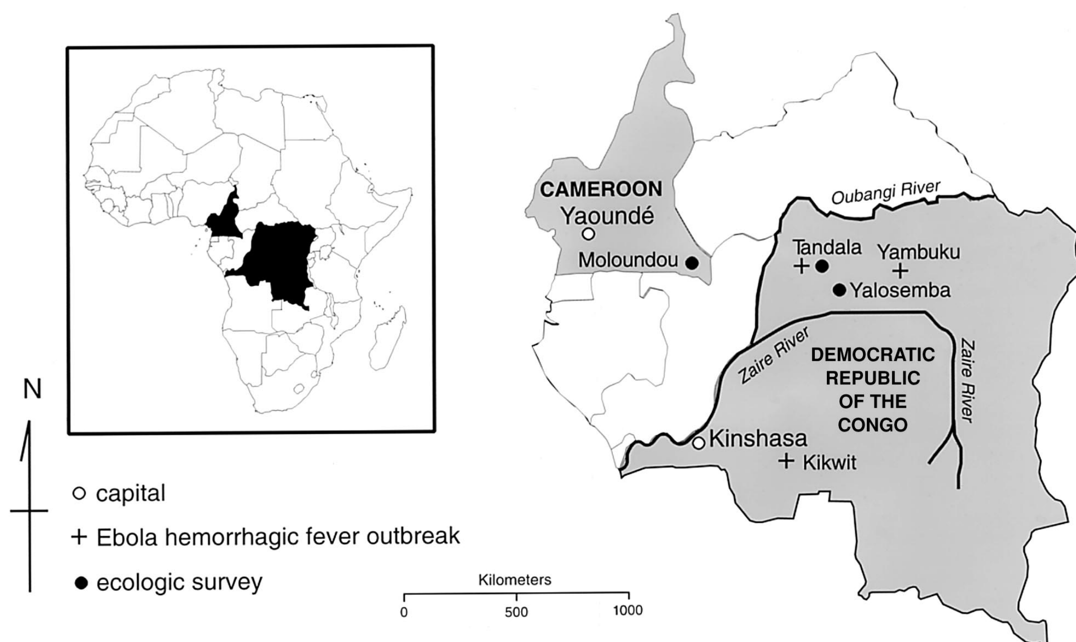

Figure 1. Ecologic surveys for Ebola virus in Democratic Republic of the Congo and Cameroon, 1979 and 1980.

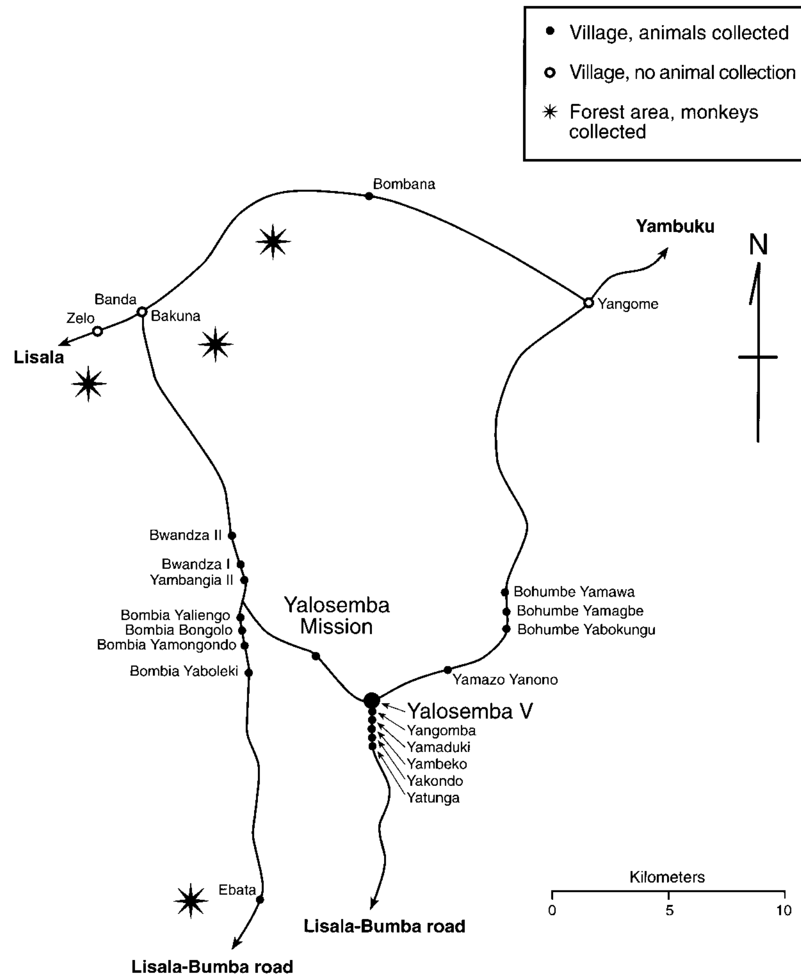

Figure 2. Ecologic survey for Ebola virus among villages visited in Banda-Yowa collectivity, Bumba Zone, Equateur Province, Democratic Republic of the Congo, 1979.

tional organ section was stored in 10% formaldehyde for later histologic investigations.

Since there was danger that ⩾1 species might have harbored EBO virus, all field laboratory work involving exposure to blood, urine, or animal tissue was done with disposable gloves, gowns, and surgical masks. Instruments were rinsed in a detergent solution, flamed in alcohol between each organ dissection, and autoclaved at 20 lb for 20 min each day.

Preservation of animal skins and skulls for identification. Large mammals were preserved for identification mostly as a salted and dried skin, with the skull removed. Other animals were preserved as whole animals pickled in formol solution; as stuffed skin covered with borax or sodium silicofluoride, plus skull; as flat, salted skin plus skull; as skull only; or as complete skeletons. To dry the skull, the brain and fleshy tissue were removed; the skull was then placed in the sun out of reach of dogs for ∼48 h. Animals were identified by mammalogist and naturalist team members using standard references [12–17].

Virus isolation and serology. At the Centers for Disease Control and Prevention (CDC), portions of frozen tissues from animals were removed from vials, using individual sterile instruments, triturated in a sterile glass grinder, and prepared as 10% whole virus suspensions in PBS containing 10% fetal calf serum and antibiotics. Drained tubes of E6 Vero cell cultures were inoculated with 0.1 mL of tissue suspension (two tubes per sample), and fluid culture medium containing 2% serum was replaced after 30 min. Cultures were maintained at 36°C for 7 days without a change of culture medium. Cells were scraped from tubes, and the cells and supernatants were pooled for each sample. Cells were applied as spots on standard microscope slides, air dried, fixed for 10 min in cold acetone, and then stored at −70°C until tested for the presence of EBO virus–specific antigens.

Plasma obtained from a DRC patient who survived EHF in 1976 was diluted (1:32) to contain eight antibody units and applied to the spots on slide cell harvests. This was washed off after 30 min at 37°C, and a fluorescein-tagged goat anti-human globulin was applied, incubated, and washed in a similar fashion. Positive and negative antigen and antibody controls were used in each test batch. Slides were examined by use of a microscope (Leitz Instruments, Luton, UK) fitted with a fluorescent light source and filters. Apple-green fluorescent granules located in the cytoplasm of cells were considered as evidence of presumptive EBO virus–specific antigens. Frozen material from such samples was also passaged twice in Vero cells to try to establish that the sample contained replicating virus.

Sera from animals were tested for EBO virus antibodies by IFA as described previously [7]. Vero cells infected with EBO virus, subtype Zaire (Mayinga strain), were mixed with normal cells, and

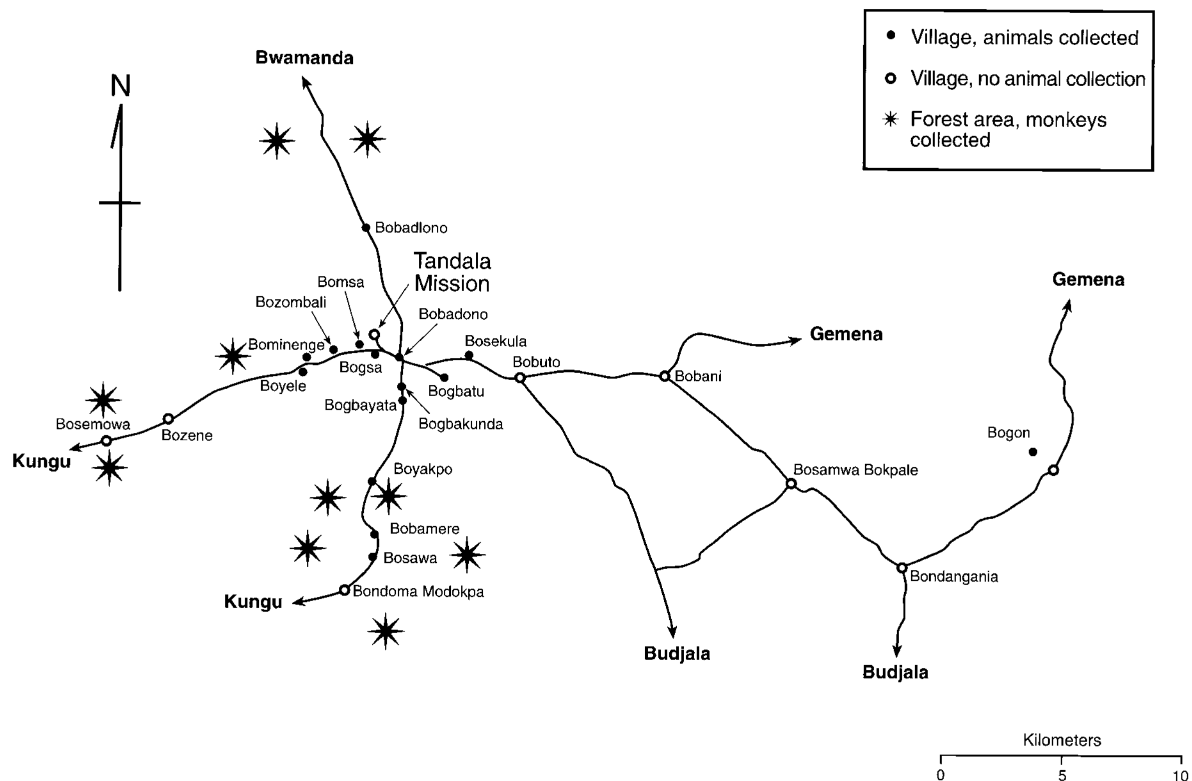

Figure 3. Ecologic survey for Ebola virus among villages visited in Tandala area, Germena Zone, Equateur Province, Democratic Republic of the Congo, 1979.

a rhodamine counterstain was used to reduce nonspecific green staining of the cells. Sera were examined at a 1:4 dilution, and candidate positive sera were repeat tested at 4-fold dilutions of 4–256. If positive, samples were then tested by an RIA [18] for confirmation. All monkey sera were assayed using an anti-human globulin conjugate that was shown by immunodiffusion to react strongly with sera from these species.

Anti-species sera were not available for many of the other animal taxa, notably bats, squirrels, and certain other animals unique to tropical Africa, such as the pangolin, *Manis tricuspis.* To address this problem, 0.5-mL aliquots of serum were prepared from 26 species for which at least 4 samples were available. Three pools containing sera from 8 or 9 species, which were grouped by taxonomic relationship to the degree possible, were assembled, and gamma globulins were obtained from them by ammonium sulfate precipitation. Individual goats received 1.0 mL of globulins in Freund's complete adjuvant intramuscularly followed by two monthly injections of incomplete adjuvant. Goat sera were harvested, shown to react without dilution to each of the species globulins in the appropriate pool, prepared as globulin solutions, and conjugated with fluorescein isothiocyanide [19]. These conjugates were used at the least dilution that did not cause strong nonspecific staining of the EBO virus antigen–containing Vero cells, usually 1:4.

Cameroon Studies

Site. In southeastern Cameroon, near Moloundou (figure 1), the studies centered around the pygmy village of Mbatika-Sasse, where serum samples for EBO virus and poxvirus antibodies were collected previously (figure 4). The pygmies were mainly forest dwellers and highly skilled hunters; they supplied antelope, monkey, and other animals to persons in permanent villages. Their leaf-covered houses (''mongaloos'') were moved as the population traveled through the forest in small groups. The pygmies hunted with snare traps, crossbows and arrows, and guns and fished by building a series of successively smaller dams. More recently, they had become more sedentary for economic security and had begun to grow yams, plantain, bananas, peppers, and tobacco.

Animal collections. Animals for study were captured by pygmy hunters, who were rewarded monetarily for animals caught, with the goal of collecting as many live animals as possible. Photographs or skulls (or both) were taken of representative animals unfamiliar to the project mammalogist. As in DRC, animals were identified by mammalogist and naturalist team members using standard references [12–17]. Blood samples were obtained for hemoglobin electrophoretic comparison with specimens identified and cataloged previously in Sierra Leone. Organ and tissue samples of animals were collected and preserved in liquid nitrogen as done in DRC.

Virus isolation and serology. At the Institute of Tropical Medicine (Antwerp, Belgium), animal tissues were tested for virus and viral antigens in a manner similar to that used at the CDC, except that CV-1 cells (normal kidney, African green monkey, *Cercopithecus aethiops*) were used as primary cul-

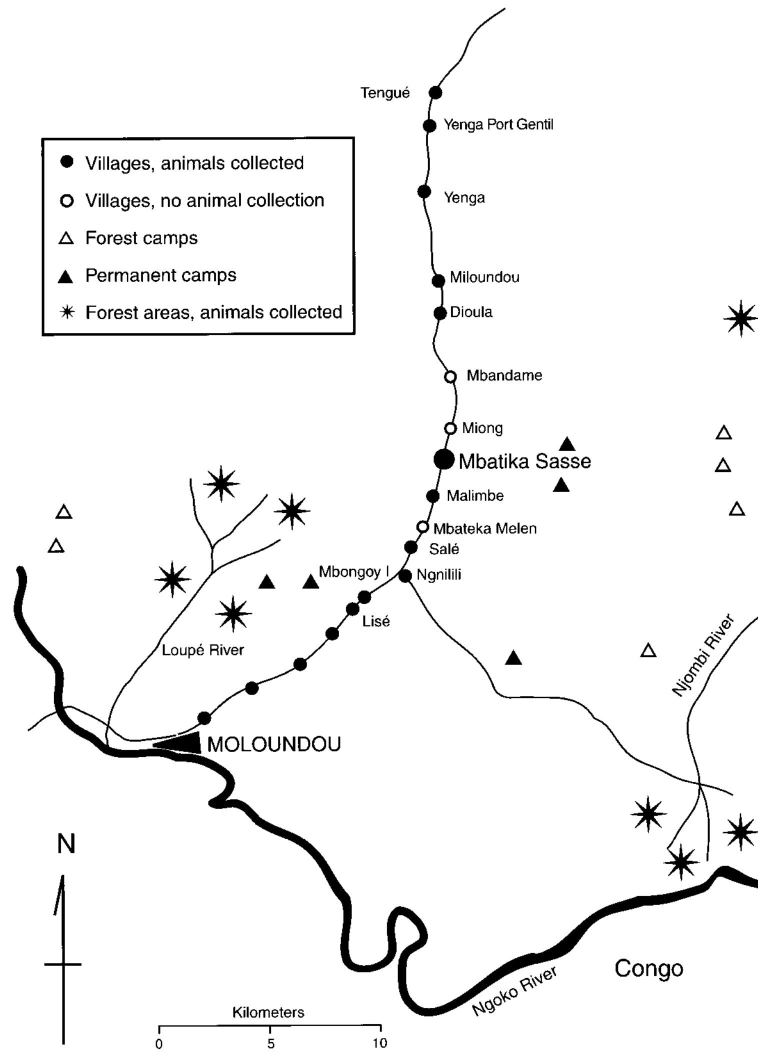

Figure 4. Ecologic survey for Ebola virus among villages visited in southeastern Cameroon, 1980.

tures. Specimens that showed weak cytopathic effect or were positive by immunofluorescence staining were retested in Vero cells obtained from the CDC.

The EBO virus–positive control serum was from a patient who had recovered from infection with virus from Sudan. Antibody tests in wild and domestic animals were done using staphylococcal protein A conjugate in lieu of species-specific anti-globulin sera. Affinity of protein A for different animal globulins varied greatly (1:1666 for human, guinea pig, and rabbit; 1:1 for rat; 1:22 for common mouse).

Results

Animal collections. A total of 1664 animals was collected: 1478 (89%) in DRC and 186 (11%) in Cameroon (table 1). These animals represented 93 species in DRC and 32 species in Cameroon. In Cameroon, 103 animals (55% of specimens) were mice, rats, and other small rodents. Fifty or more specimens were captured of 16 species, 3 of which were primates. Rodents (including squirrels) and bats were two-thirds of all animals captured (table 2). Overall, 117 species were tested.

Animal virologic and serologic surveys. One serum sample from a flying squirrel, *Anomalurus derbianus,* collected in the Tandala area, DRC, was repeatedly positive by IFA to EBO virus antigens at a titer of 1:16. The RIA, however, failed to confirm this reactivity, and virus was not recovered from any of the tissue samples obtained from this or the other 3 members of this species. All remaining sera were antibody-negative. EBO virus was not isolated from any animal specimen in either laboratory. However, 10 tissue samples from Cameroon were seemingly positive by cytopathic effect or immunofluorescence staining of cells taken from the primary culture. After reisolation was attempted, 3 samples were again positive by immunofluorescence, but virus could not be recovered after further passage on Vero cells.

Hunting and eating patterns. In DRC, monkeys were reported to be by far the most frequently hunted and eaten animals, with the duiker antelope *(Cephalophus monticola)* being second. Women did not consume the ''gambala'' (*Colobus*

Table 1. Animals collected in the Democratic Republic of the Congo and Cameroon, 1979–1980.

Genus and species	Identification	No. captured
Primates		
Cercopithecus ascanius	White-nosed monkey	94
Cercopithecus pagonias	Mona monkey	55
Cercopithecus nictitans	White-nosed monkey	51 (2)
Procolobus pennantii	Red colobus	14
Cercopithecus neglectus	Brazza's monkey	12
Cercocebus galeritus	Mongabey	11
Allenopithecus nigroviridis	Allen's monkey	10
Perodicticus potto	Potto	8 (2)
Other (⩽5 captured)*		12 (1)
Subtotal, primates		267 (5)
Chiroptera (bats)		
Pipistrellus nanus		73
Mops thersites		69
Mops condylurus		54
Hipposideros cyclops		52
Chaerephon major		26
Eptesicus tenuipinnis/Eptesicus rendalli		22
Epomops franqueti		21
Mops congicus		20
Hipposideros ruber		17
Myotis bocagei		17
Chalinolobus species		15
Mops namulus		15
Nycteris species		14
Scotophilus species		10
Taphozous peli		9
Eidolon helvum		6
Other (⩽5 captured)†		23
Subtotal, bats		463 (0)
Squirrels		
Funisciurus anerythrus	Redless squirrel	58
Heliosciurus rufobrachium	Sun squirrel	58
Funisciurus limniscatus	Four-striped squirrel	6 (6)
Paraxerus species	Bush squirrel	6
Other (⩽5 captured)‡		9 (2)
Subtotal, squirrels		137 (8)
Other rodents		
Oenomys hypoxanthus	Rufous-nosed rat	82 (27)
Lophuromys sikapusi	Rusty-bellied rat	81
Mastomys species	Multimammate rat	72
Praomys jacksoni	Jackson's soft-furred rat	60
Rattus species	Rat	52
Hybomys univittatus	Peters' striped mouse	50 (45)
Lemniscomys striatus	Typical spotted grass mouse	30
Cricetomys emini	Gambian rat	19 (1)
Graphiurus species	African dormouse	19
Thamnomys rutilans	Shining thicket rat	13 (12)
Hylomyscus stella	Stella wood mouse	11
Stochomys species	Target rat	8 (8)
Praomys tullbergi	Tullberg's soft-furred rat	7 (7)
Other (⩽5 captured)§		10 (3)
Subtotal, other rodents		514 (103)
Other mammals		
Manis tricuspis	Tree pangolin	66 (17)
Rhynchocyon cirnei	Elephant shrew	28
Cephalophus monticola	Blue duiker	17
Canis domesticus	Dog (domestic)	14 (14)
Atherurus africanus	Brush-tailed porcupine	10
Crocidura poensis	Shrew	9
Crossarchus alexandri	Dark mongoose	7

Table 1. (*Continued*)

Genus and species	Identification	No. captured
Other mammals (*Continued*)		
Dendrohyrax dorsalis	Tree hyrax	7
Neotragus pygmaeus	Royal antelope	6 (6)
Scutisorex somereni	Hero shrew or armored shrew	6
Other (≤5 captured)[‖]		16 (9)
Subtotal, other mammals		186 (46)
Birds		
Corythaeola cristata	Great blue turaco	15
Tropicranus albocristatus	White-crested hornbill	13
Ceratogymna atrata	Black-casqued hornbill	6
Other (≤5 captured)[¶]		33 (18)
Subtotal, birds		67 (18)
Reptiles		
Snake (genus and species not identified)	Snake	8
Tortoise (genus and species not identified)	Tortoise	7
Others (≤5 captured)**		15 (6)
Subtotal, reptiles		30 (6)

NOTE. Data are total no. of animals collected (no. captured in Cameroon).

* *Cercopithecus cephus* (mustached monkey), *Colobus guereza* (black-and-white colobus), *Galagoides demidoff* (galago), *Lophocebus albigena* (mangabey), *Miopithecus talapoin* (talapoin).

† *Chaerophon chapini, Hipposideros commersoni, Hipposideros* species, *Hypsignathus monstrosus, Kerivoula lanosa, Megaloglossus woermanni, Mops* species, *Myonycteris torguata, Taphozous mauritianus.*

‡ *Anomalurus derbianus* (Fraser's flying squirrel), *Anomalurus pusillus* (lesser flying squirrel), *Funisciurus isabella* (four-striped squirrel), *Funisciurus pyrrhopus* (red-footed squirrel).

§ *Colomys goslingi* (African water rat), *Malacomys longipes* (long-footed rat), *Malacomys verschureni* (long-footed rat), *Mus* species (typical or pygmy mouse).

‖ *Cephalophus dorsalis* (black-striped duiker), *Crocidura flavescens* (shrew), *Crocidura* species (shrew), *Dendrohydrax arboreus* (tree hyrax), *Felis cattus* (domestic cat), *Genetta tigrina* (large-spotted genet), *Herpestes ichneumon* (Egyptian mongoose), *Hyemoschus aquaticus* (chevrotain), *Nandinia binotata* (two-spotted palm civet), *Potamogale velox* (otter shrew or giant water shrew), *Viverra civetta* (African civet).

¶ *Andopadus virens* (little greenbul), *Barbet, Bycanistes cylindricus* (brown-checked hornbill), *Ceuthmochares aereus* (yellow bill), *Ciccaba woodfordi* (African wood-owl), *Floceus* species, *Francolinus* species (Francolin's partridge), *Gallina domesticus* (chicken), *Gallinula* species (mud hen), *Malimbus nitens* (Gray's malimbe), *Nectarina olivacea* (olive sunbird), *Nectarina* species (sunbird), *Nicator chloris* (nicator), *Otus* species (scops owl), *Pogoniulus* (tinkerbirds), *Polyboroides radiatus* (Harrier hawk), *Spermophaga hacmatgin* (bluebill), *Tockus* species (hornbills), *Turdus pelios* (olive thrush), unknown nestling.

** *Bitis gabonica* (viper), *Bitis nasicornis* (viper), *Chamaeleo* species (lizard), *Chelonia* species (turtle), *Dendroaspis* species (snake), *Osteolemus* species (crocodile), *Varanus niloticus* (reptile), *Varanus nioticus* (monitor).

monkey), ''mokomboso'' (chimpanzee), and ''akange'' (mongoose), because of the odor of the animal and fear of spontaneous abortion in case of unknown pregnancy.

Discussion

These studies failed to identify an animal reservoir of EBO virus. The methods used and results obtained, however, have importance for ongoing efforts to solve the biologic and ecologic riddle of filoviruses. A monetary reward brought many samples, but most of these were from species that were both common, peridomestic, and relatively easy to capture. It is unlikely that these animals would harbor an enzootic of EBO virus; it does not seem fruitful to concentrate on animals with which humans have frequent and close contact unless there is incriminating epidemiologic evidence. In human monkeypox, for example, careful analysis of age and gender of persons infected with virus, and their interrogation, led to investigational concentration on precisely the species in closest contact with humans in rural DRC and to the isolation of the agent from a squirrel [20–22].

More than half of the animals sampled in our collection were from species in closest contact with village residents; indeed, 10 of the 16 species with sample sizes of at least 50 individuals were of peridomestic animals. Recent human EBO virus infections in Côte d'Ivoire and Gabon have been caused by contact with, or consumption of rainforest-dwelling dead and infected chimpanzees [23–25]. Chimpanzees may suffer fatal clinical disease similar to that in humans and are, therefore, likely not the reservoir. In a recent study in South Africa, fruit and insectivorous bats had EBO virus–related viremia for up to 4 weeks without mortality [26]. This finding provides a specific clue that should be followed up vigorously in the wild.

In the current study, we must question whether the samples were collected in a nonrandom manner and whether the methods used to test them for EBO virus infection were adequate

Table 2. Total number of animals collected in the Democratic Republic of the Congo and Cameroon.

	No.	%	No. of species
Primates	267 (5)	16	13 (2)
Bats	463 (0)	28	25
Squirrels	137 (8)	8	8 (2)
Other rodents	514 (103)	31	17 (7)
Other mammals	186 (46)	11	21 (7)
Birds	67 (18)	4	23 (11)
Reptiles	30 (6)	2	10 (2)
Total	1664 (186)	100	117 (31)

NOTE. Data are total no. of animals collected (no. captured in Cameroon).

to conclude that the species with at least 50 samples can be regarded as not playing a role in the life cycle of this virus. The number 50 was a rough goal, used as an administrative and scientific guide. Low-frequency events require very large samples. In addition, seasonal occurrence must be considered; it is most desirable to undertake ecologic studies during the same time period when human disease is occurring or has occurred and to track secular trends of the presence and habits of incriminated animals. While these studies were done in the dry season, the 1976 outbreak occurred mainly during the rainy season. The serologic tests used in this study are recognized to lack precision, particularly specificity. Because positive controls were not used in nonhuman primate IFA testing, it cannot be said definitively that antibodies were not present in these specimens.

The genetic stability of EBO virus in DRC over the interval of 19 years is remarkable [27]. Molecular studies of filoviruses have shown this family of viruses to be most closely related to paramyxoviruses [28]. One of the latter agents, Sendai virus, a murine parainfluenza virus type 3, has been detected on occasion in the brains of chronically infected mice, and only then by recourse to polymerase chain reaction (PCR) probes. This was because infection was marked by the absence of viral protein expression and antibody formation [29]. Thus, collection of brain samples and use of PCR detectors in other organs with a set of well-designed primers, including primers that would detect gene sequences of nonstructural proteins, may be advisable in further field work on animal reservoirs.

Another incompletely resolved problem relates to the binding affinity of various anti-species or other conjugates to serum samples in the antibody tests. This question is critical in view of the fact that sensitive virus neutralization methods have not been developed for filoviruses. While the negative data reported herein are valid for the primate and rodent species involved, there is less than total confidence that this is true for many other taxa studied. Protein A conjugates have very different species-specific binding power, and the conjugates prepared in goats were not rigorously titrated against globulins of the many genera and families represented in the study. This problem must be resolved in future studies of candidate vertebrate reservoir species.

The diversity of vertebrates, both exo- and endothermic, in tropical African ecosystems poses a formidable challenge to discovery of a filovirus reservoir. Progress may be most efficient if decisions are taken in advance of field studies such that specific hypotheses are forwarded and some rejected, the animals involved are carefully listed, the best methods for their capture are selected, and the collection and testing of all samples utilize the best technology and the principal biologic mechanisms that might be utilized by the virus.

Within major animal classes and habitats, it should be valuable to compare the fauna of the Philippine Islands with that of central Africa and give priority to those in which closely related pairs exist. This notion is based on the observation that genetic distances among 3 African subtypes of EBO virus are roughly equal to distances between that of EBO virus (subtype Reston [originating from the Philippines]) and any 1 of these African agents [27, 28]. It seems likely that vertebrate virus reservoirs in these disparate parts of the world will turn out to belong to the same subclass or more closely related taxa. Finally, the report that the plant-feeding insect *Psammotettix alienus* (Dahlbom) may have filovirus-like structures [30] would add further complexity to the problem that is already the longest-running search for the true home of a zoonotic virus for humans in the past half century.

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Ebola Virus Study Team Members

In DRC: Thomas A. Leonard and Joseph B. McCormick, Special Viral Pathogens Branch, National Center for Infectious Diseases (NCID), CDC, Atlanta (current residence for T.A.L., Atlanta [retired]; current affiliation for J.B.M., Institute Pasteur, Paris); Gasana Ndoli, Centre de Recherche Scientifique, Ministry of Education and Science, Bukavu, and Egidio Zanotto, WHO, Kinshasa, DRC (current residence for E.Z., Padova, Italy [retired]); and L. W. Robbins, Carnegie Museum of Natural History, Pittsburgh (current affiliation, Southwest Missouri State University, Springfield, MO).

In Cameroon: Jean-François Bergmann and Ethleen Smith Lloyd, l'Organisation de Lutte contre les Endémies en Afrique Centrale (current affiliations, respectively, Hôpital Lariboisière, Paris, and Division of Viral and Rickettsial Diseases [DVRD], NCID, CDC, Atlanta) and Robert Kamanu, Ministry of Health, Yaoundé, Cameroon; Joan Mitchell Hackett, Catholic Mission, Diocese of Doumé, Mouloundou, Cameroon (current affiliation, Catholic Relief Services, Columbia, Maryland); and John W.

Krebs, Special Viral Pathogens Branch, NCID, CDC, Atlanta (current affiliation, DVRD, NCID, CDC).

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