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MAJOR ARTICLE

Extensive Serological Survey of Multiple African Nonhuman Primate Species Reveals Low Prevalence of Immunoglobulin G Antibodies to 4 Ebola Virus Species

Ahidjo Ayouba,[1] Steve Ahuka-Mundeke,[2,3] Christelle Butel,[1] Placide Mbala Kingebeni,[2] Severin Loul,[4] Nikki Tagg,[5] Christian-Julian Villabona-Arenas,[1] Audrey Lacroix,[1] Simon-Pierre Ndimbo-Kumugo,[2] Alpha K. Keita,[1,6] Abdoulaye Toure,[6,7] Emmanuel Couacy-Hymann,[8] Sebastien Calvignac-Spencer,[9] Fabian H. Leendertz,[9] Pierre Formenty,[10] Eric Delaporte,[1] Jean-Jacques Muyembe-Tamfum,[2,3] Eitel Mpoudi Ngole,[11] and Martine Peeters[1]

[1]Recherches Translationelles sur VIH et Maladies Infectieuses/Institut national de la santé et de la recherche médicale 1175, Institut de Recherche pour le Développement and University of Montpellier, France; [2]Institut National de Recherche Biomédicales, Kinshasa, Democratic Republic of the Congo (DRC); [3]Service de Microbiologie, Cliniques Universitaires de Kinshasa, DRC; [4]Ministry of Livestock, Fisheries and Animal Industries, Yaoundé, Cameroon; [5]Projet Grands Singes, Centre for Research and Conservation, Royal Zoological Society of Antwerp, Belgium; [6]Centre de Recherche et de Formation en Infectiologie de Guinée, and [7]Chaire de Santé Publique, Université Gamal Abdel Nasser de Conakry, Guinea; [8]Laboratoire National D'appui au Développement Agricole/Laboratoire Central de Pathologie Animale, Bingerville, Ivory Coast; [9]Epidemiology of Highly Pathogenic Microorganisms, Robert Koch Institute, Berlin, Germany; [10]Emerging and Dangerous Pathogens Laboratory Network, World Health Organization, Geneva, Switzerland; and [11]Centre de Recherches sur les Maladies emergentes, ré-émergentes et la médecine nucleaire/Institut de Recherches Médicales et d'études des plantes médecinales, Yaoundé, Cameroon

Bats are considered a reservoir species for Ebola viruses, but nonhuman primates (NHPs) have represented a source of infection in several outbreaks in humans. Here we report serological screening of blood or fecal samples from monkeys (n = 2322) and apes (n = 2327). Thirty-six NHP species from Cameroon, Democratic Republic of the Congo, and Ivory Coast were tested with a sensitive and specific Luminex-based assay for immunoglobulin G antibodies to 4 Ebola virus species. Using the simultaneous presence of antibodies to nucleoproteins and glycoproteins to define positivity, we showed that specific Ebola virus antibodies are not widespread among NHPs. Only 1 mustached monkey (*Cercopithecus cephus*) from Cameroon was positive for *Sudan ebolavirus*. These observations support that NHPs are most likely intermediate hosts for Ebola viruses. With the increasing frequency of Ebola outbreaks, it is crucial to identify the animal reservoir and understand the ecology of Ebola viruses to inform disease control.

Keywords. monkey; ape; Ebola; Africa.

Ebola virus disease (EVD) is a complex zoonosis, and each reported outbreak is most likely the result of an independent zoonotic event [1]. Today it is believed that bats constitute a reservoir and that they infect humans directly or via intermediate hosts, such as nonhuman primates (NHPs), duikers, or other mammals [2, 3]. Although without direct evidence, exposure to bat bushmeat is suggested in the 2007 outbreak in Luebo, Democratic Republic of the Congo (DRC), and bats are also suspected to be at the origin of the major EVD outbreak in West Africa in 2014 [4, 5]. For several outbreaks, recent contact with blood of NHPs through hunting or butchering of carcasses by the index individual was reported [1, 6]. Apes represented a source of infection in humans in Gabon, Republic of Congo, and Ivory Coast. Moreover, these human outbreaks coincided temporally and geographically with EVD outbreaks in apes, associated with high mortality rates [7–10]. Contact with monkeys is suspected in at least 2 outbreaks; in Boende, DRC in 2014, and in the Republic of the Congo in 2003 [11, 12]. Interestingly, in the EVD outbreak among chimpanzees in the Tai forest in Ivory Coast in 1994, an association was also observed between the consumption of monkeys (western red colobus) by chimpanzees and their Ebola virus infection rates [10]. Between the different EVD outbreaks of 1994 and 2003 in Gabon, 35 mortality and morbidity episodes were reported in wild animals in areas where previous EVD epidemics occurred, involving a wide diversity of NHP species (gorilla, chimpanzee, greater spot-nosed and mustached monkeys, black colobus, mandrill), but also bush pigs, sitatungas, and duikers [13]. Unfortunately, no laboratory tests were performed to identify the causes of death.

Today, the role of reservoir and intermediate species in EVD outbreaks is still unclear and better knowledge on circulation of Ebola viruses in different wildlife species is thus necessary.

Despite high mortality rates of Ebola in apes, antibodies have been observed in several wild-born but captive NHPs including chimpanzees, gorillas, mandrills, drills, De Brazza monkeys, and baboons [14, 15]. These observations suggest that Ebola could be widespread among NHPs and that nonlethal and asymptomatic or pauci-symptomatic infections occur in

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Correspondence: M. Peeters, PhD, TransVIHMI, IRD-INSERM, University of Montpellier, 911 Ave Agropolis, 34394 Montpellier, France (martine.peeters@ird.fr).

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certain NHP species, as seen in humans [16, 17]. Moreover, a recent study showed that 10% of gorillas from an area with a high lethal EVD outbreak possess antibodies, suggesting that some infected animals can survive from the disease. More importantly, these observations also suggest that Ebola viruses circulate in areas where no outbreaks have yet been reported, such as Cameroon or Kenya [14, 15]. This is also the case for bats where antibodies have been detected in animals from West Africa (Ghana, Nigeria), West-Central Africa (Cameroon), East Africa (Zambia), and Asia (Bangladesh, China) [18–22].

One difficulty regarding interpretation and comparison of data from the different studies on NHPs, bats, or other wildlife species is the diversity of antibody assays employed, criteria used to define positivity, and the Ebola viruses that they target. For example, among the 5 studies that reported today on Ebola in NHPs, antibody detection was done using immunofluorescence, enzyme-linked immunosorbent assay (ELISA), or Western blotting [14, 15, 23, 24]. To date, 4 different Ebola virus species have been reported in humans in Africa, initially apparently limited to certain geographic regions: *Zaire ebolavirus* (EBOV) in West-Central Africa (western part of DRC, Gabon, and Republic of Congo); *Sudan ebolavirus* (SUDV) in Sudan and Uganda; *Bundibugyo ebolavirus* (BDBV) in East Africa (DRC and Uganda); and *Tai ebolavirus* in West Africa (Ivory Coast) [1]. However, the recent EVD outbreak in West Africa was confirmed as EBOV, showing thus a wider geographical spread of EBOV in Africa. EBOV was also identified in the last outbreak in eastern DRC (North Kivu), although previous outbreaks in this area were due to BDBV and SUDV [1] (Mbala et al, unpublished data). The majority of existing data on wildlife report only on EBOV, but in analogy to EBOV it cannot be excluded that the other Ebola viruses have a larger geographical spread than actually observed. Therefore, we developed a high-throughput Luminex-based assay that included antigens from EBOV, SUDV, and BDBV from Africa as well as from *Reston ebolavirus* (RESTV), which has to date only been reported in macaques and pigs from Asia and is apparently not harmful to humans [25].

The frequency of EVD outbreaks seems to have recently increased; for example, between May 2017 and July 2018, 3 independent EVD outbreaks occurred in 3 different provinces in DRC, and 2 outbreaks reached densely populated cities [26]. Therefore, studies on the ecology and animal reservoir of Ebola viruses are now urgently needed to quantify risks for future outbreaks and implement prevention measures. Although NHPs have been a source of infection in several outbreaks, only limited and disparate information is available on Ebola in NHPs, especially in monkeys. Here, we focused on the potential role of NHPs and studied to what extent antibodies to 4 different Ebola species can be detected in NHPs from DRC and Ivory Coast, 2 countries that have experienced EVD outbreaks, and from Cameroon, considered to be at high risk for future outbreaks [1].

MATERIALS AND METHODS

Samples From Monkeys and Apes

Samples were collected from bushmeat and pet monkeys between 1999 and 2016 as part of previous and ongoing studies on retroviral infections [27–30]. Bushmeat samples were collected at 14 different forest sites in southern Cameroon and DRC (Figure 1). Samples from pets were collected at 18 different sites in Cameroon (Supplementary Figure 1). Whole blood was collected from monkey bushmeat, either by intracardiac puncture and subsequent storage at –20°C, or by whole blood collection at the points of hunting injury and spotting, as a dried blood spot (DBS) on Whatman 903 filter paper (GE Healthcare) as described previously [28, 29]. Blood was drawn on ethylenediaminetetraacetic acid tubes from pet monkeys by venipuncture after tranquilization with ketamine [27]. Species were visually identified in the field and confirmed on a subset of samples by sequence analysis, as previously described [27–30].

Fecal samples were collected between 2005 and 2017 from wild ape populations at 11 different sites in Cameroon and DRC as part of previous studies on retroviral infections [31–33] (Figure 1). Samples were collected from central chimpanzees (*Pan troglodytes troglodytes*), western lowland gorillas (*Gorilla gorilla gorilla*), and bonobos (*Pan paniscus*). Most samples were collected around night nests and feeding sites, but also opportunistically. Samples were stored in RNA*later* (Ambion), kept at ambient temperature in the field for a maximum of 3 weeks, and then stored at –20°C or –80°C. In the framework of a long-term veterinary follow-up program approved by the competent local authorities, fecal samples were obtained in the research area of the Tai National Park (Côte d'Ivoire) in 2001 and blood samples between 2004 and 2015 from either immobilized or necropsied NHPs that died of natural causes. The study was approved by the respective ministries of environment, research, and/or health and the national ethics committees.

Screening for Ebola Virus Antibodies

All samples were tested using our previously described serological assay based on Luminex technology [34]. Recombinant of nucleoprotein (NP), viral protein 40 (VP40), and/or glycoprotein (GP) for different Ebola virus species (EBOV, SUDV, BDBV, and RESTV) are used in this assay [34]. Whole blood, plasma, and DBS samples were tested at a final dilution of 1:1000 in assay buffer, taking into account the hematocrit of 50% in reconstituted plasma from DBS or whole blood. For all fecal samples, RNA*later*-precipitated immunoglobulins were resolubilized by diluting the fecal/RNA*later* mixture (2 mL) with phosphate-buffered saline (PBS)–Tween 20 (7 mL), followed by incubation for 1 hour at 60°C, centrifugation (3900*g* for 10 minutes) to clarify the solution, and dialysis against PBS overnight at 4°C [31–33]. The reconstituted extracts were then tested in the Luminex assay as previously described [34].

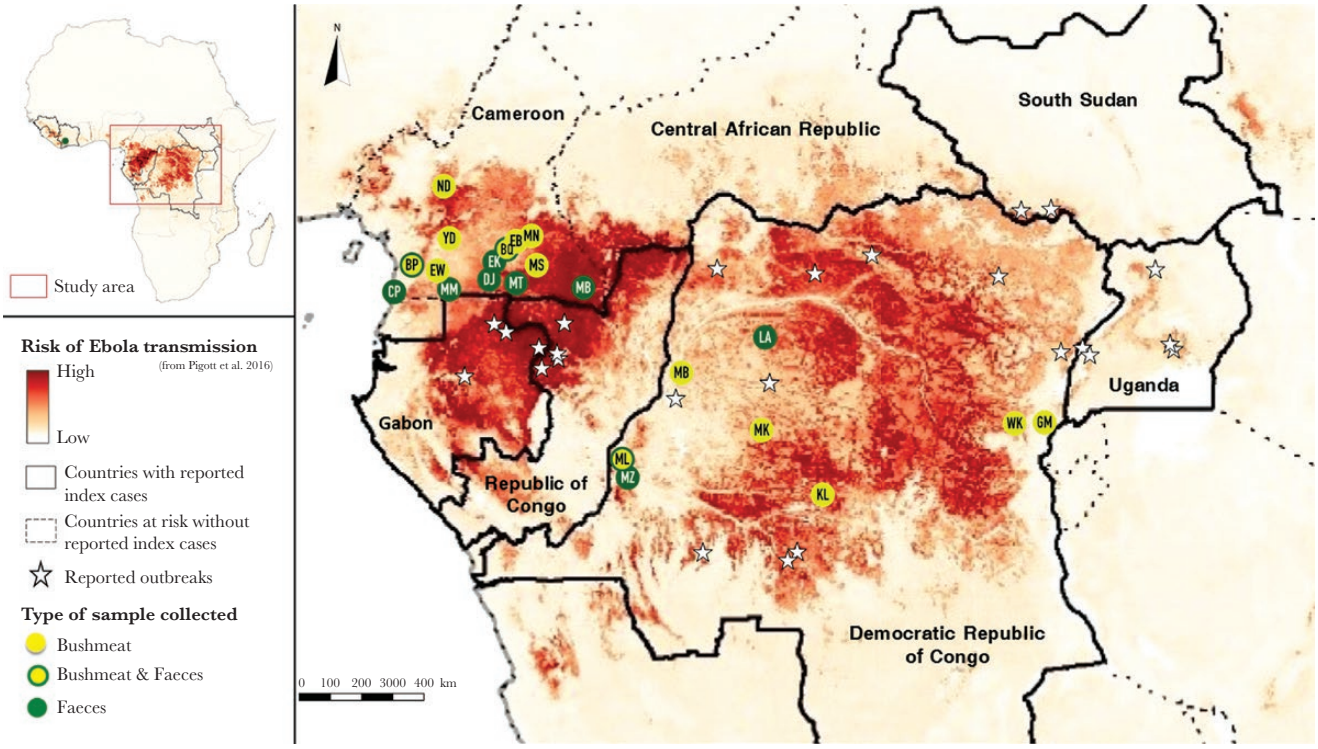

Figure 1. Collection sites. Sites where samples from nonhuman primates were collected are highlighted with circles on the maps, as follows: yellow indicates sites where bushmeat samples from monkeys were collected; green, sites where fecal samples from apes were collected; yellow and green, sites where bushmeat samples from monkeys and fecal samples from apes were collected. The samples from Tai forest in Ivory Coast are not shown. Maps are adapted from Pigott et al [50]; areas closer to dark red are estimated at highest risk for Ebola virus spillover events, and areas in light yellow are least at risk. Abbreviations: BP, Bipindi; BQ, north of Dja; CP, Campo; DJ, Djoum; EB, Eboumetoum; EK, Ekom; EW, Ebolowa; GM, Goma; KL, Kole; LA, Lomako-Yokokala; MB, Mambele for green dot, Mbandaka for yellow dot; ML, Malabo; MM, Mengame; MN, Mindourou; MS, Messok; MT, Mintom; MZ, Manzana; ND, Nditam; WK, Walikale; YD, Yaoundé.

In brief, tests were performed in 96-well flat-bottomed filter plates (Millipore), and 100 µL of samples (final plasma dilution 1:1000; final fecal sample dilution 3:4) was incubated with 50 µL of beads for 16 hours at 4°C in the dark on a plate shaker at 300 rpm/minute. After washing with assay buffer, 50 µL of antihuman immunoglobulin G (IgG) biotin labeled (BD Pharmingen) was added at a concentration of 4 µg/mL in each well and incubated for 30 minutes in the dark while shaking at 300 rpm. After washing, 50 µL streptavidin-R-phycoerythrin (Fisher Scientific/Life Technologies) at 4 µg/mL was added per well and incubated for 10 minutes while shaking at 300 rpm. Antigen/antibody reactions were subsequently read on BioPlex-200 equipment (Bio-Rad); at least 100 events were read for each bead set, and the results were expressed as median fluorescence intensity (MFI) per 100 beads.

In the absence of positive control samples for NHPs, we analyzed our data obtained from plasma and DBS samples with different statistical methods to determine MFI cutoff values for each antigen as reported in our previous study on bats [21, 35, 36]. We used a change-point analysis [37] with the R package "changepoint" [38] and calculated 1 single shift in the arithmetic mean with the AMOC (at most 1 change) method [39]. In analogy with other studies on Ebola virus serology in bats or wildlife, we also fitted univariate distributions to our data and defined the cutoff based on a 0.001 risk of error [40]. The set of candidate distributions was reduced with a bootstrapped skewness-kurtosis analysis [41]. Maximum likelihood estimation was performed to select the best-fit distribution based on the Akaike information criterion using the R library "fitdistrplus" [42]. The best-fit distribution was the negative binomial, but the negative exponential distribution was also used as in other studies on serology in wildlife [40]. Data were bootstrapped 10 000 times and averaged for each antigen. Analyses were done with R software version 3.3.2. We then compared the cutoff values identified by the 3 different methods and calculated their mean as a consensus cutoff that we used in this study (Supplementary Table 1). We considered a sample antigen reactive if MFI was above the cutoff value. Reactivity to both GP and NP proteins indicated specific EBOV and SUDV positivity [34].

For fecal samples, we first evaluated to what extent Ebola virus antibodies can be detected in feces from EBOV survivors from the Postebogui cohort in Guinea [43]. We also spiked different dilutions of EBOV survivors' plasma samples in gorilla fecal dialysates to test the persistence of reactivity in this media. We compared MFI values in paired plasma and fecal samples and adapted the cutoff values in accordance with MFI values observed in feces as compared to plasma.

Table 1. Number of Samples Collected for Each Species at the Different Collection Sites in Cameroon, Democratic Republic of the Congo, and Ivory Coast

Genus	Species	Common Name	CM Pets	CM BP	CM ND	CM YD	CM BQ	CM EW	CM EB	CM MS	CM MN	DRC MB	DRC ML	DRC MK	DRC KL	DRC WK	DRC GM	IC TAI	No. of Samples
Allenopithecus	*nigroviridis*	Allen swamp monkey	…	…	…	…	…	…	…	…	…	44	…	2	1	…	…	…	47
Cercocebus	*agilis*	Agile mangabey	14	…	…	1	9	…	39	13	55	3	…	…	…	…	…	…	134
	atys	Sooty mangabey	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	21	21
	torquatus	Red-capped mangabey	2	…	…	…	…	4	…	…	…	…	…	…	…	…	…	…	6
Colobus	*angolensis*	Angolan colobus	…	…	…	…	…	…	…	…	…	…	…	4	21	…	…	…	25
	guereza	Mantled guereza	1	…	15	8	…	…	2	…	8	1	…	…	1	…	…	…	36
	polykomos	King's colobus	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	11	11
	satanus	Black colobus	…	…	…	…	…	8	…	…	…	…	…	…	…	…	…	…	8
Piliocolobus	*badius*	Western red colobus	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	23	23
	tholloni	Tsuapa red colobus	…	…	…	…	…	…	…	…	…	…	…	2	84	…	…	…	86
Procolobus	*verus*	Olive colobus	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	2	2
Cercopithecus	*ascanius*	Red-tailed monkey	…	…	…	…	…	…	…	…	…	41	42	12	106	7	33	…	241
	campbelli	Campbell's monkey	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	1	1
	cephus	Mustached monkey	29	6	10	32	25	122	197	14	129	…	…	…	…	…	…	…	564
	diana	Diana monkey	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	3	3
	hamlyni	Hamlyn's monkey	…	…	…	…	…	…	…	…	…	…	…	…	…	…	6	…	6
	lhoesti	l'Hoest monkey	…	…	…	…	…	…	…	…	…	…	…	…	…	6	31	…	37
	mitis	Blue monkey	…	…	…	…	…	…	…	…	…	…	…	…	…	20	31	…	51
	mona	Mona monkey	9	…	…	1	…	…	…	…	…	…	…	…	…	…	…	…	10
	neglectus	De Brazza monkey	5	…	…	4	8	2	13	…	2	18	2	1	6	…	…	…	61
	nictitans	Greater spot-nosed monkey	42	10	22	66	…	71	35	36	158	9	…	…	…	…	…	…	449
	petaurista	Lesser spot-nosed monkey	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	3	3
	pogonias	Crested mona monkey	5	2	11	19	11	24	31	17	66	…	…	…	…	1	1	…	188
	preussi	Preuss monkey	1	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	1
	wolfi	Wolf's monkey	…	…	…	…	…	…	…	…	…	21	1	5	31	6	8	…	72
Chlorocebus	*tantalus*	Tantalus monkey	15	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	15
Erythrocebus	*patas*	Patas monkey	17	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	17
Lophocebus	*albigena*	Gray-cheeked mangabey	7	…	10	…	…	…	2	16	68	…	…	1	…	…	2	…	106
	aterrimus	Black mangabey	…	…	…	…	…	…	…	…	…	…	…	2	31	…	…	…	33
Mandrillus	*leucophaeus*	Drill	1	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	1
	sphinx	Mandrill	16	1	…	…	…	10	…	…	…	…	…	…	…	…	…	…	27
Miopithecus	*talapoin*	Northern talapoin	8	3	…	1	…	8	…	…	…	…	…	…	…	…	…	…	20
Papio	*anubis*	Olive baboon	16	…	…	…	…	1	…	…	…	…	…	…	…	…	…	…	17
Total			188	22	68	132	53	250	319	96	486	137	45	29	281	40	112	64	2322

Collection sites are shown in Figure 1.

Abbreviations: BP, Bipindi; BQ, North Dja; CM, Cameroon; DRC, Democratic Republic of the Congo; EB, Eboumetoum; EW, Ebolowa; GM, Goma; IC, Ivory Coast; KL, Kole; MB, Mbandaka; MK, Monkoto; ML, Malebo; MN, Mindourou; MS, Messok; ND, Nditam; TAI, Tai National Park; YD, Yaoundé.

RESULTS

Diversity of NHP Species Tested

A total of 4649 samples from 36 different NHP species were analyzed: 2322 were from monkeys and 2327 from apes. The species in sampling sites reflects the NHP distribution according to the biogeographic areas. The numbers of each monkey species collected at the different sites, illustrated in Figure 1, are shown in Table 1. In Cameroon, 1614 samples were tested from 17 different monkey species. The predominant species were *Cercopithecus cephus* (34.9%) and *Cercopithecus nictitans* (27.3%), followed by *Cercopithecus pogonias* (11.5%), *Cercocebus agilis* (8.1%), and *Lophocebus albigena* (6.4%). Among the 644 samples from DRC, the predominant monkey species was *Cercopithecus ascanius* (37.4%), followed by *Piliocolobus tholloni* (13.4%), *Cercopithecus wolfi* (11.2%), *Cercopithecus mitis* (7.9%), *Allenopithecus nigroviridis* (7.3%), and *Cercopithecus lhoesti* (5.8%). In Ivory Coast, among the 64 monkey samples, *Piliocolobus badius* (35.9%), *Cercocebus atys* (32.8%), and *Colobus polykomos* (17.2%) predominated.

Among the 2327 samples from apes, 1182 (51%) were from western lowland gorillas (*G. g. gorilla*) in Cameroon, 353 (15%) from bonobos (*P. paniscus*) in DRC, and 792 (34%) from chimpanzees across Africa including samples from the western (*Pan troglodytes verus*, n = 57) and central chimpanzee (*P. t. troglodytes*, n = 735). Table 2 displays for each ape species or subspecies numbers collected at each site, illustrated in Figure 1.

Low Prevalence of Ebola Virus IgG Antibodies in Monkeys

The 2322 monkey samples were tested for Ebola antibodies with the multiplex antibody assay. The results are summarized for each antigen and for each Ebola virus species in Tables 3 and 4. No sample reacted simultaneously with GP and NP proteins from EBOV, and we therefore considered that none of the samples had specific antibodies to EBOV. Only a single sample, derived from a mustached monkey (*C. cephus*) from Cameroon, had antibodies to both NP and GP proteins from SUDV. Reactivity to another combination of 2 antigens was also low: 1 baboon (*Papio anubis*) was reactive with VP and GP proteins from EBOV, SUDV, and BDVB and 1 greater spot-nosed monkey (*C. nictitans*) with VP and GP from BDBV. All the other samples reacted only with 1 antigen: 0.6% for NP and 0.5%–1% reactivity for the different VP40 proteins. Highest reactivity was observed with GP proteins, and ranged from 2.2% to 2.6% with GP proteins derived from the African Ebola virus species (EBOV, SUDV, and BDBV) and was 1.5% for the Asian Ebola virus species (RESTV). Almost all GP-reactive samples were simultaneously reactive to GP proteins from >1 Ebola virus species. Highest reactivities (>2%) to GP proteins were observed in the following species: *C. nictitans*, *C. cephus*, *C. agilis*, *Chlorocebus tantalus*, and *Colobus polykomos*.

Table 2. Number of Samples Collected for Each Ape Species, by Site

Species	Country and Site	No.
Pan troglodytes verus	Ivory Coast	
	Tai	57
	Subtotal	57
Pan troglodytes troglodytes	Cameroon	
	BQ	120
	CP	114
	DJ	45
	EK	120
	MB	316
	MT	20
	Subtotal	735
Gorilla gorilla gorilla	Cameroon	
	BP	143
	BQ	239
	CP	289
	DJ	167
	EK	72
	MB	161
	MT	22
	MM	89
	Subtotal	1182
Pan paniscus	DRC	
	LA	137
	MZ	166
	ML	50
	Subtotal	353
	Total	2327

Collection sites are shown in Figure 1.

Abbreviations: BP, Bipindi; BQ, North Dja; CP, Campo; DJ, Djoum; DRC, Democratic Republic of Congo; EK, Ekom; LA, Lomako-Yokokala; MB, Mambele; ML, Malebo; MM, Mengame; MT, Mintom; MZ, Manzana.

No Evidence of IgG Ebola Virus Antibodies in Apes

As previously observed for human immunodeficiency virus (HIV)/simian immunodeficiency virus (SIV) cross-reactive antibody detection in fecal samples from apes, sensitivity is lower and interpretation criteria needed to be adapted [31–33]. Therefore, we first tested 57 paired feces (preserved in RNA*later*) and plasma samples from EBOV survivors to evaluate the feasibility and sensitivity of Ebola virus antibody detection in fecal samples. In contrast with the antibody profile observed in plasma (ie, simultaneous positivity against NP + GP or NP + GP + VP), antibody reactivity in feces was more frequently directed against a single protein (ie, 57/57 [100%] in plasma vs 18/57 [31.6%] in fecal samples; Supplementary Table 2). However, 29 of 57 (50.9%) of fecal samples were reactive with at least 1 antigen, but a mean decrease of 0.67 to 1.37 $\log_{10}$ of MFI values was observed compared to plasma (Supplementary Table 2). Thus, we adapted the interpretation criteria in fecal samples to antibody reactivity with a single antigen and decreased the above used cutoff values with 1 $\log_{10}$ value. The lower sensitivity in fecal samples is comparable to that observed for HIV/SIV cross-reactive antibody detection in fecal samples from apes.

Table 3. Number and Percentage of Samples for Each Species Reactive With the Different Antigens Used in the Luminex Assay for *Zaire ebolavirus*

				NP	GP	GP	VP40	NP + GP
Genus	Species	Country	No. Tested	EBOV	EBOV-K	EBOV-M	EBOV	EBOV
Allenopithecus	*nigrovidis*	DRC	47	0 (0; .0–7.5)	0 (0; .0–7.5)	0 (0; .0–7.5)	0 (0; .0–7.5)	0 (0; .0–7.5)
Cercocebus	*agilis*	Cam, DRC	134	0 (0; .0–2.8)	4 (3.0; 1.1–7.4)	4 (3.0; 1.1–7.4)	0 (0; .0–2.8)	0 (0; .0–2.8)
	atys	IC	21	0 (0; .0–15.5)	0 (0; .0–15.5)	0 (0; .0–15.5)	0 (0; .0–15.5)	0 (0; .0–15.5)
	torquatus	Cam	6[a]	0	0	0	0	0
Colobus	*angolensis*	DRC	25	0 (0; .0–13.3)	0 (0; .0–13.3)	(0; .0–13.3)	1 (4.0; .7–19.5)	0 (0; .0–13.3)
	guereza	Cam, DRC	36	1 (2.8; .5–14.2)	0 (0; .0–9.6)	0 (0; .0–9.6)	1 (2.8; .5–14.2)	0 (0; .0–9.6)
	polykomos	IC	11	0 (0; .0–25.9)	1 (9.1; 1.6–37.7)	1 (9.1; 1.6–37.7)	0 (0; .0–25.9)	0 (0; .0–25.9)
	satanus	Cam	8[a]	0	0	0	0	0
Piliocolobus	*badius*	IC	23	0 (0; .0–14.3)	0 (0; .0–14.3)	0 (0; .0–14.3)	0 (0; .0–14.3)	0 (0; .0–14.3)
	tholloni	DRC	86	0 (0; .0–4.3)	0 (0; .0–4.3)	0 (0; .0–4.3)	0 (0; .0–4.3)	0 (0; .0–4.3)
Procolobus	*verus*	IC	2[a]	0	0	0	0	0
Cercopithecus	*ascanius*	DRC	241	0 (0; .0–1.6)	1 (0.4; .07–2.3)	2 (0.8; .2–2.9)	2 (0.8; .2–2.9)	0 (0; .0–1.6)
	campbelli	IC	1[a]	0	0	0	0	0
	cephus	Cam, DRC	565	1 (0.2; .03–1.0)	29 (5.1; 3.6–7.3)	34 (6.0; 4.3–8.3)	3 (0.5; .2–1.6)	0 (0; .0–.7)
	diana	IC	3[a]	0	0	0	0	0
	hamlyni	DRC	6[a]	0	0	0	0	0
	lhoesti	DRC	37	1 (2.8; .5–13.8)	0 (0; .0–9.4)	0 (0; .0–9.4)	0 (0; .0–9.4)	0 (0; .0–9.4)
	mitis	DRC	51	0 (0; .0–7.0)	0 (0; .0–7.0)	0 (0; .0–7.0)	0 (0; .0–7.0)	0 (0; .0–7.0)
	mona	Cam	10	1 (10.0; 1.8–40.4)	0 (0; .0–27.8)	0 (0; .0–27.8)	0 (0; .0–27.8)	0 (0; .0–27.8)
	neglectus	Cam, DRC	61	1 (1.6; .3–8.7)	1 (1.6; .3–8.7)	0 (0; .0–5.9)	0 (0; .0–5.9)	0 (0; .0–5.9)
	nictitans	Cam, DRC	448	7 (1.6; .7–3.2)	17 (3.8; 2.4–6.0)	16 (3.6; 2.2–5.7)	3 (0.7; .2–1.9)	0 (0; .0–.9)
	petaurista	IC	3[a]	0	3	3	0	0
	pogonias	Cam, DRC	188	1 (0.5; .1–2.9)	1 (0.5; .1–2.9)	0 (0; .0–2.0)	0 (0; .0–2.0)	0 (0; .0–2.0)
	preussi	Cam	1[a]	0	0	0	0	0
	wolfi	DRC	72	0 (0; .0–5.1)	0 (0; .5–.1)	0 (0; .0–5.1)	0 (0; .5–.1)	0 (0; .0–5.1)
Chlorocebus	*tantalus*	Cam	15	0 (0; .0–20.4)	1 (6.7; 1.2–29.8)	1 (6.7; 1.2–29.8)	0 (0; .0–20.4)	0 (0; .0–20.4)
Erythrocebus	*patas*	Cam	17	0 (0; .0–18.4)	0 (0; .0–18.4)	0 (0; .0–18.4)	1 (5.9; 1.0–26.9)	0 (0; .0–18.4)
Lophocebus	*albigena*	Cam	106	1 (0.9; .2–5.2)	0 (0; .0–3.5)	0 (0; .0–3.5)	0 (0; .0–3.5)	0 (0; .0–3.5)
	aterrimus	DRC	33	0 (0; .0–10.4)	0 (0; .0–10.4)	0 (0; .0–10.4)	0 (0; .0–10.4)	0 (0; .0–10.4)
Mandrillus	*leucophaeus*	Cam	1[a]	0	0	0	0	0
	sphinx	Cam	27	0 (0; .0–12.5)	0 (0; .0–12.5)	0 (0; .0–12.5)	0 (0; .0–12.5)	0 (0; .0–12.5)
Miopithecus	*talapoin*	Cam	20	0 (0; .0–16.1)	0 (0; .0–16.1)	0 (0; .0–16.1)	0 (0; .0–16.1)	0 (0; .0–16.1)
Papio	*anubis*	Cam	17	1 (5.9; 1.4–26.9)	0 (0; .0–18.4)	0 (0; .0–18.4)	1 (5.9; 1.4–26.9)	0 (0; .0–18.4)
Total			2322	15 (0.6; .4–1.1)	58 (2.5; 1.9–3.2)	61 (2.6; 2.1–3.4)	12 (0.5; .3–.9)	0 (0; .0–.2)

Data are presented as No. positive (%; 95% confidence interval) unless otherwise indicated.

Abbreviations: Cam, Cameroon; DRC, Democratic Republic of the Congo; EBOV, *Zaire ebolavirus*; GP, glycoprotein; IC, Ivory Coast; K, Kissoudougou strain; M, Mayinga strain; NP, nucleoprotein; VP40, viral protein 40.

[a]Percentages were not calculated when number of samples tested was <10.

Using these adapted criteria, none of the 2316 fecal samples reacted with any of the recombinant proteins in the Luminex assay. In addition, the 11 blood samples from western chimpanzees from the Tai forest in Ivory Coast were also negative for all antigens.

DISCUSSION

Although there is evidence that NHPs, and especially apes, play a role in zoonotic transmission of EVD outbreaks, few data are available on Ebola infection in NHPs. Here we report an extensive serological survey to 4 different Ebola virus species in 4649 NHP samples representing 36 different species. We applied a Luminex-based sensitive and specific antibody assay for the simultaneous detection of antibodies to 4 of the 5 species previously described in humans [34]. To identify specific Ebola virus antibodies, we used the same algorithm to NHPs as that developed for human EBOV survivors (ie, simultaneous positivity to NP and GP recombinant proteins); this was deemed appropriate because a recent study in cynomolgus macaques (*Macaca fascicularis*) naturally infected with RESTV revealed also that convalescent macaques having cleared the virus presented antibodies to NP and GP proteins, both by ELISA and immunofluorescence [44]. As such, we observed only 1 mustached monkey (*C. cephus*) from southern Cameroon with antibodies to GP and NP from SUDV. Only 2 other samples had antibodies to 2 different antigens from the same Ebola virus species; 1 *P. anubis* sample was reactive to VP40 and GP proteins from EBOV, SUDV, and BDBV, suggesting nonspecific or

Table 4. Number and Percentage of Samples for Each Species Reactive With the Different Antigens Used in the Luminex Assay for *Sudan ebolavirus*, *Bundibugyo ebolavirus*, and *Reston ebolavirus*

				NP	GP	VP40	NP + GP	GP	VP40	GP
Genus	Species	Country	No. Tested	SUDV	SUDV	SUDV	SUDV	BDBV	BDBV	RESTV
Allenopithecus	*nigrovidis*	DRC	47	0 (0; .0–7.5)	0 (0; .0–7.5)	0 (0; .0–7.5)	0 (0; .0–7.5)	0 (0; 0–7.5)	0 (0; .0–7.5)	1 (2.1; .4–11.1)
Cercocebus	*agilis*	Cam, DRC	134	0 (0; .0–2.8)	4 (3.0; 1.1–7.4)	2 (1.5; .4–5.3)	0 (0; .0–2.8)	5 (3.7; 1.6–8.4)	2 (1.5; .4–5.3)	4 (3.0; 1.1–7.4)
	atys	IC	21	2 (9.5; 2.6–28.9)	0 (0; .0–15.5)	0 (0; .0–15.5)	0 (0; .0–15.5)	0 (0; .0–15.5)	0 (0; .0–15.5)	0 (0; .0–15.5)
	torquatus	Cam	6[a]	0	0	0	0	0	0	0
Colobus	*angolensis*	DRC	25	0 (0; .0–13.3)	0 (0; .0–13.3)	0 (0; .0–13.3)	0 (0; .0–13.3)	0 (0; .0–13.3)	0 (0; .0–13.3)	0 (0; .0–13.3)
	guereza	Cam, DRC	36	0 (0; .0–9.6)	0 (0; .0–9.6)	0 (0; .0–9.6)	0 (0; .0–9.6)	0 (0; .0–9.6)	0 (0; .0–9.6)	0 (0; .0–9.6)
	polykomos	IC	11	0 (0; .0–25.9)	0 (0; .0–25.9)	0 (0; .0–25.9)	0 (0; .0–25.9)	0 (0; .0–25.9)	0 (0; .0–25.9)	0 (0; .0–25.9)
	satanus	Cam	8[a]	0	1	0	0	0	0	0
Piliocolobus	*badius*	IC	23	0 (0; .0–14.3)	0 (0; .0–14.3)	0 (0; .0–14.3)	0 (0; .0–14.3)	0 (0; .0–14.3)	0 (0; .0–14.3)	0 (0; .0–14.3)
	tholloni	DRC	86	0 (0; .0–4.3)	0 (0; .0–4.3)	0 (0; .0–4.3)	0 (0; .0–4.3)	0 (0; .0–4.3)	0 (0; .0–4.3)	0 (0; .0–4.3)
Procolobus	*verus*	IC	2[a]	0	0	0	0	0	0	0
Cercopithecus	*ascanius*	DRC	241	2 (0.8; .2–2.9)	1 (0.4; .07–2.3)	1 (0.4; .07–2.3)	0 (0; .0–1.6)	2 (0.8; .2–2.9)	0 (0; .0–1.6)	1 (0.4; .07–2.3)
	campbelli	IC	1[a]	0	0	0	0	0	0	0
	cephus	Cam, DRC	565	5 (0.9; .4–2.1)	34 (6.0; 4.3–8.3)	4 (0.7; .3–1.8)	1 (0.2; .03–1.0)	30 (5.4; 3.7–7.5)	6 (1.1; .5–2.3)	19 (3.4; 2.2–5.2)
	diana	IC	3[a]	0	0	0	0	0	0	0
	hamlyni	DRC	6[a]	0	0	0	0	0	0	0
	lhoesti	DRC	37	0 (0; .0–9.4)	0 (0; .0–9.4)	0 (0; .0–9.4)	0 (0; .0–9.4)	0 (0; .0–9.4)	0 (0; .0–9.4)	0 (0; .0–9.4)
	mitis	DRC	51	0 (0; .0–7.0)	0 (0; .0–7.0)	0 (0; .0–7.0)	0 (0; .0–7.0)	0 (0; .0–7.0)	0 (0; .0–7.0)	0 (0; .0–7.0)
	mona	Cam	10	1 (10.0; 1.8–40.4)	0 (0; .0–27.8)	0 (0; .0–27.8)	0 (0; .0–27.8)	0 (0; .0–27.8)	0 (0; .0–27.8)	0 (0; .0–27.8)
	neglectus	Cam, DRC	61	0 (0; .0–5.9)	1 (1.6; .3–8.7)	1 (1.6; .3–8.7)	0 (0; .0–5.9)	1 (1.6; .3–8.7)	0 (0; .0–5.9)	1 (1.6; .3–8.7)
	nictitans	Cam, DRC	448	2 (0.5; .1–1.6)	8 (1.8; .9–3.5)	5 (1.1; .5–2.6)	0 (0; .0–.9)	13 (2.9; 1.7–4.9)	5 (1.1; .5–2.6)	6 (1.3; .6–2.9)
	petaurista	IC	3[a]	0	1	0	0	3	0	0
	pogonias	Cam, DRC	188	0 (0; .0–2.0)	0 (0; .0–2.0)	1 (0.5; .1–2.9)	0 (0; .0–2.0)	1 (0.5; .1–2.9)	0 (0; .0–2.0)	0 (0; .0–2.0)
	preussi	Cam	1[a]	0	0	0	0	0	0	0
	wolfi	DRC	72	0 (0; .0–5.1)	0 (0; .0–5.1)	0 (0; .0–5.1)	0 (0; .0–5.1)	0 (0; .0–5.1)	1 (1.4; .2–7.5)	0 (0; .0–5.1)
Chlorocebus	*tantalus*	Cam	15	0 (0; .0–20.4)	1 (6.7; 1.2–29.8)	0 (0; .0–20.4)	0 (0; .0–20.4)	1 (6.7; 1.2–29.8)	0 (0; .0–20.4)	1 (6.7; 1.2–29.8)
Erythrocebus	*patas*	Cam	17	0 (0; .0–18.4)	0 (0; .0–18.4)	1 (5.9; 1.0–26.9)	0 (0; .0–18.4)	0 (0; .0–18.4)	1 (5.9; 1.0–26.9)	0 (0; .0–18.4)
Lophocebus	*albigena*	Cam	106	2 (1.9; .5–6.6)	0 (0; .0–3.5)	3 (2.9; .9–8.0)	0 (0; .0–3.5)	0 (0; .0–3.5)	1 (0.9; .2–5.2)	0 (0; .0–3.5)
	aterrimus	DRC	33	0 (0; .0–10.4)	0 (0; .0–10.4)	0 (0; .0–10.4)	0 (0; .0–10.4)	0 (0; .0–10.4)	0 (0; .0–10.4)	0 (0; .0–10.4)
Mandrillus	*leucophaeus*	Cam	1[a]	0	0	0	0	0	0	0
	sphinx	Cam	27	0 (0; .0–12.5)	0 (0; .0–12.5)	1 (3.7; .6–18.3)	0 (0; .0–12.5)	0 (0; .0–12.5)	0 (0; .0–12.5)	0 (0; .0–12.5)
Miopithecus	*talapoin*	Cam	20	0 (0; .0–16.1)	0 (0; .0–16.1)	3 (15.0; 5.2–36.1)	0 (0; .0–16.1)	0 (0; .0–16.1)	2 (10.0; 2.7–30.1)	0 (0; .0–16.1)
Papio	*anubis*	Cam	17	0 (0; .0–18.4)	0 (0; .0–18.4)	1 (5.9; 1.4–26.9)	0 (0; .0–18.4)	0 (0; .0–18.4)	2 (11.8; 3.3–34.3)	2 (11.8; 3.3–34.3)
Total			2322	14 (0.6; .4–1.0)	51 (2.2; 1.7–2.9)	23 (1.0; .7–1.5)	1 (0.04; 0–.3)	56 (2.4; 1.9–3.1)	20 (0.9; .6–1.3)	35 (1.5; 1.1–2.1)

Data are presented as No. positive (%; 95% confidence interval) unless otherwise indicated.

Abbreviations: BDBV, *Bundibugyo ebolavirus*; Cam, Cameroon; DRC, Democratic Republic of the Congo; GP, glycoprotein; IC, Ivory Coast; NP, nucleoprotein; RESTV, *Reston ebolavirus*; SUDV, *Sudan ebolavirus*; VP40, viral protein 40.

[a]Percentages were not calculated when the number of samples tested was <10.

cross-reactivity, and 1 *C. nictitans* sample from Cameroon was reactive with GP and VP40 from BDBV.

This is the first study to have evaluated a large number of monkeys using the same assay and interpretation criteria. We tested 2322 monkeys compared to the previous total of 930 from studies in Cameroon (n = 177), Gabon (n = 318), DRC (n = 251), and Kenya (n = 184) using a wide diversity of antibody detection assays [14, 15, 23, 24]. In these previous studies, EBOV-positive samples were seen in 3 of 184 captive baboons in a primate center in Kenya and in captive monkeys from Cameroon: in 1 of 8 De Brazza monkeys, 1 of 25 baboons, 1 of 18 mandrills, and 5 of 34 drills [14, 15]. In our study, the 27 mandrills and 61 De Brazza monkeys were all negative and 1 of 17 baboon samples was reactive with VP40 and GP proteins of different Ebola virus species. The different results may be due to the low numbers tested per species, the different geographic areas, and the different tests used.

Antibody detection in fecal samples has been validated and used for Ebola using Western blot in gorillas that live in areas in Republic of Congo with previous EBOV outbreaks, showing that 8 of 80 (10%) had EBOV antibodies [16]. In our study, no evidence for Ebola virus antibodies was observed in 2316 fecal samples, corresponding to around 1362 apes taken into account from previous resampling estimates [31–33]. Our samples are mainly from areas without previously documented outbreaks; however, the majority were obtained in areas considered at risk for outbreaks [1]. Moreover, some sites like Djoum, Mambele, Mintom, or Mengame (Figure 1), which account for >650 samples in southern Cameroon, are located within a 100- to 300-km flight distance from areas of previous outbreaks in Gabon. Moreover, the samples from Ivory Coast were from the Tai National Park where an Ebola outbreak was documented among chimpanzees in 1994 and all fecal samples were collected in 2001 in the specific outbreak area. In contrast, a previous study using blood samples from wild-born but captive chimpanzees and gorillas revealed high EBOV antibody levels in apes: 21 of 119 (17.7%), 3 of 71 (4.2%), and 5 of 35 (14.3%) chimpanzees from Cameroon, Gabon, and Republic of the Congo, respectively, and 2 of 17 (11.2%) gorillas from Cameroon [15]. If EBOV prevalence in apes in Cameroon is as high as suggested in the previous study, we would have expected some reactive samples. As our assay has been shown to have a >95% sensitivity on human samples [34] and a 50% sensitivity on fecal samples, we would expect to have observed some reactivity in at least 100 fecal samples from chimpanzees or gorillas.

Reactivity to a single Ebola virus antigen, especially GP, in NHPs has to be further explored, for example with neutralization assays, to ascertain whether it corresponds to nonspecific antibody reactivity or cross-reactivity with another pathogen, or different kinetics of antibodies to the different Ebola proteins. Recent studies have shown that bats can be infected with Ebola viruses that are different from the species known to infect humans, which could induce cross-reactivity with GP antigens [45, 46]. NHPs and bats share habitats and fruits in their diet. Virus transmission from bats to NHPs is suggested to occur when primates come in contact with fruit that is contaminated with feces, urine, or saliva from infected bats [2, 3]. Interestingly, a recent study reported that *Cercopithecus* species hunt roosting bats for consumption, which could be another, and a particularly efficient, route for Ebola virus transmission [47]. Preying on bats has been reported in *C. ascanius* and *C. mitis* species in East Africa, and also in bonobos in DRC [47, 48]. It is also possible that different modes of exposure to Ebola virus could lead to different antibody profiles, that is, contaminated fruit vs contact with infected bats during hunting.

Whereas the majority of the outbreaks have been limited in terms of geographic spread and number of people infected, the 2014–2015 EVD outbreak in West Africa clearly illustrates the epidemic potential of a single zoonotic transmission in the presence of certain factors in favor of epidemic spread [49]. The recent EVD outbreaks in 2018 in the Equateur and Kivu provinces of DRC also illustrate that the virus can reach urban centers, even in a context of weak mobility infrastructure [26].

In conclusion, combining results on NHPs from our (4649 samples) and previous studies (~1300 samples), it is clear that Ebola virus antibodies are not widespread among NHPs, which confirms that NHPs are not reservoir species and that if Ebola virus infection in NHPs occurs, few animals survive. More samples from NHPs, bats, and other animal species from different regions across Africa should be studied to define which animals play a role in EVD outbreaks by acting as a reservoir species or as an amplifying host species. Nevertheless, with the increasing frequency of Ebola virus outbreaks (3 outbreaks in a 1-year period in DRC), it becomes extremely urgent to identify the animal reservoir(s) and to understand the ecology of Ebola viruses. It is estimated that transmission from animals to humans is possible in 23 countries across Central and West Africa with a total of >300 million inhabitants, and that at least 22 million of these people live in high-risk areas [50].

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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