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Original Contribution

Failure to Detect Simian Immunodeficiency Virus Infection in a Large Cameroonian Cohort with High Non-human Primate Exposure

Cyrille F. Djoko,[2,3] Nathan D. Wolfe,[1,4] Avelin F. Aghokeng,[5,8] Matthew LeBreton,[2] Florian Liegeois,[5] Ubald Tamoufe,[2] Bradley S. Schneider,[1] Nancy Ortiz,[1] Wilfred F. Mbacham,[3] Jean K. Carr,[6] Anne W. Rimoin,[7] Joseph N. Fair,[1] Brian L. Pike,[1] Eitel Mpoudi-Ngole,[8] Eric Delaporte,[5] Donald S. Burke,[9] and Martine Peeters[5]

[1]*Global Viral Forecasting, San Francisco, CA*
[2]*Global Viral Forecasting, Yaoundé, Cameroon*
[3]*Biotechnology Centre & Department of Biochemistry, University of Yaoundé I, Yaoundé, Cameroon*
[4]*Program in Human Biology, Stanford University, Stanford, CA 94305*
[5]*Laboratoire Retrovirus, UMI 233, Institute for Research and Development (IRD) and University of Montpellier 1, Montpellier, France*
[6]*Institute of Human Virology, University of Maryland School of Medicine, Baltimore, MD*
[7]*Department of Epidemiology, UCLA School of Public Health, University of California, Los Angeles, CA*
[8]*Virology Laboratory CREMER/IMPM/IRD, Yaoundé, Cameroon*
[9]*School of Public Health, University of Pittsburg, Pittsburg, PA*

Abstract: Hunting and butchering of wildlife in Central Africa are known risk factors for a variety of human diseases, including HIV/AIDS. Due to the high incidence of human exposure to body fluids of non-human primates, the significant prevalence of simian immunodeficiency virus (SIV) in non-human primates, and hunting/butchering associated cross-species transmission of other retroviruses in Central Africa, it is possible that SIV is actively transmitted to humans from primate species other than mangabeys, chimpanzees, and/or gorillas. We evaluated SIV transmission to humans by screening 2,436 individuals that hunt and butcher non-human primates, a population in which simian foamy virus and simian T-lymphotropic virus were previously detected. We identified 23 individuals with high seroreactivity to SIV. Nucleic acid sequences of SIV genes could not be detected, suggesting that SIV infection in humans could occur at a lower frequency than infections with other retroviruses, including simian foamy virus and simian T-lymphotropic virus. Additional studies on human populations at risk for non-human primate zoonosis are necessary to determine whether these results are due to viral/host characteristics or are indicative of low SIV prevalence in primate species consumed as bushmeat as compared to other retroviruses in Cameroon.

Keywords: transmission, simian immunodeficiency virus, primates, humans, Central Africa, bushmeat

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Correspondence to: Bradley S. Schneider, e-mail: bschneider@gvfi.org

Hunting and butchering of wild animals in Central Africa is associated with a variety of human diseases including monkeypox, Ebola, and human immunodeficiency virus (HIV) (Wolfe et al. 2007). HIV is now recognized as having

emerged via multiple cross-species transmissions of simian immunodeficiency viruses (SIV) from chimpanzees and gorillas, giving rise to HIV-1 groups M, N, O, and P, and from sooty mangabeys, giving rise to HIV-2 (A–H) (Hahn et al. 2000; Van Heuverswyn et al. 2006; Plantier et al. 2009). Given the high levels of reported exposure to blood and body fluids of non-human primates (NHPs) (Wolfe et al. 2004a), the high prevalence of SIVs in certain NHPs (Peeters et al. 2002; Aghokeng et al. 2010), and hunting and butchering associated cross-species transmission of at least two other groups of retroviruses in central Africa (Wolfe et al. 2004b, 2005; Calattini et al. 2007, 2009), it may be that SIV is actively transmitted to humans from primate species other than mangabeys, chimpanzees, and/or gorillas. Here, we looked for SIV infections in a large group of individuals who hunt and butcher primates in rural Central Africa with SIV lineage-specific serological assays and polymerase chain reaction (PCR). This cohort is composed of highly exposed individuals as demonstrated by their high-risk behavior and infection with other zoonotic retroviruses (Wolfe et al. 2004a, b, 2005).

In a previous pilot study involving a limited number of hunters ($n = 76$) in Cameroon we used multiple SIV peptides in an enzyme-linked immunosorbent assay (ELISA) (Ndongmo et al. 2004) to screen for SIV infection and found a positive correlation between SIV seroreactivity and exposure to primates (Kalish et al. 2005). In this study, we extended the investigation to a larger population at risk for NHP zoonosis using a broader panel of SIV lineage-specific peptides covering greater SIV diversity.

In accordance with approved human subject protocols and following informed consent, blood samples and behavioral data were collected from 2,436 individuals living in rural areas where ongoing natural transmission of at least two other simian retroviruses from NHPs to humans has been documented (Wolfe et al. 2004b, 2005). Among them, 1,910 were from HIV-negative healthy adults and 437 samples were collected from HIV-negative hospital patient [312 sexually transmitted infection (STI) outpatients and 125 non-traumatic hospitalized patients] in two district hospitals (Fig. 1). The third subgroup of 89 samples was collected from 21 hospital and 68 healthy rural adults with HIV-indeterminate western blot profiles (HIV Blot 2.2 Genelabs Diagnostics, Singapore). HIV-indeterminate samples were chosen because of the possibility that their HIV test status could be due to infection with a divergent HIV variant or a new SIV. Among the entire study population, 33.8% (823/2436) were females and 42.6% were young adults between 16 and 30 years of age. All STI outpatients were over 16 years of age, while hospitalized

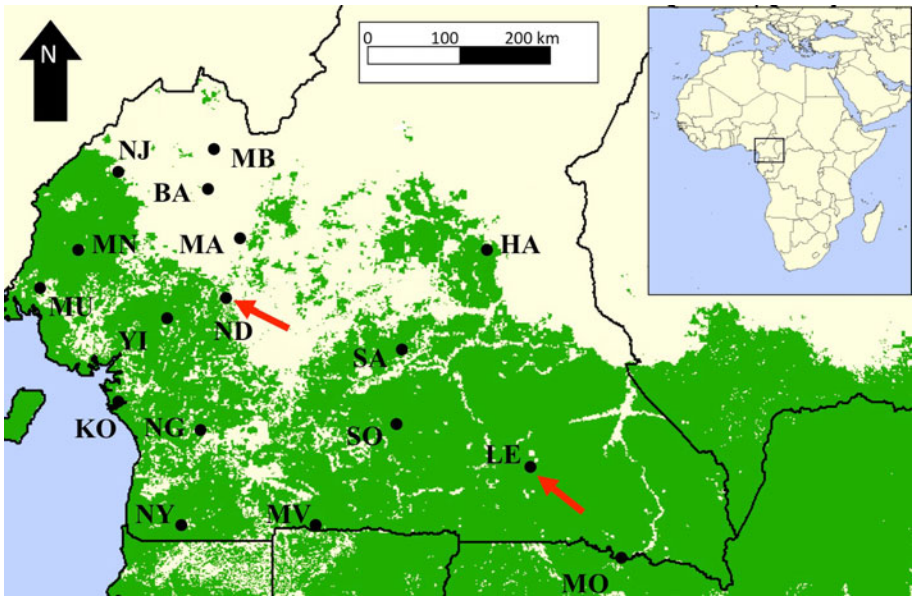

Fig. 1. Map of southern Cameroon showing the distribution of study sites. HIV-negative healthy adult samples from all 17 sites (*black dots*) were analyzed in this study, 2 of the sites (*red arrows*) provided samples from healthy adults and patients. *Green shading* represents vegetation canopy cover (Mayaux et al. 1997). *LE* Lomie, *MO* Moloundou, *MA* Makomssap, *BA* Bangourein, *NY* Nyabessan, *MV* Mvangan, *SA* Sobia, *SO* Somalomo, *KO* Mouanko, *MB* Mayo Binka, *ND* Ndikinimeki, *HA* Hamann, *MU* Mundemba, *NJ* Njikwa, *NG* Ngovayang, *YI* Yingui, *MN* Manyemen.

participants included 36 (28.8%) individuals between 5 and 15 years of age (mean age of 11 years). The population cohort included a high number of individuals at risk for NHP zoonosis. Among the healthy and hospital patients 1020/1910 (53.4%) and 168/437 (38.4%) reported hunting; 1551/1910 (81.2%) and 313/437 (71.6%) reported butchering, 358/1910 (18.73%) and 131/437 (29.9%) reported keeping NHPs as pets, respectively. Scratch or bite by NHPs was also reported by 24 (5.49%) individuals in the hospital sub-group and by 89/1910 (4.7%) of healthy adults. Though only the HIV-negative samples were selected based on their level of exposure to the body fluids of NHPs through hunting, butchering, and pet keeping, behavioral data were also looked for the HIV-indeterminate samples at and indicate that 37 (41.57%) reported hunting, 65 (73.03%) reported butchering, and 21 (23.59%) reported keeping NHPs as pets. Also, 4 (4.49%) individuals with HIV-indeterminate profile reported having been scratched or bitten by NHPs.

SIV antibodies were detected in human plasma by an indirect ELISA using synthetic biotinylated gp41 peptides (Neosystems, Strasbourg, France) representing the following SIV lineages (SIVsmm, SIVlho, SIVrcm, SIVtal, SIVsyk, SIVdeb, SIVgsn, SIVmon, SIVmus, SIVcpz-ant). For SIVmnd and SIVcol, only V3 loop peptides were available, as described previously (Aghokeng et al. 2006, 2010). The combination of these assays detects SIV antibodies in NHPs with an overall sensitivity and specificity of 96 and 97.5%, respectively (Aghokeng et al. 2010). Samples that tested positive in synthetic gp41 SIV lineage-specific ELISAs were validated by re-testing with the V3-loop peptides of the corresponding SIV lineages for confirmation of gp41 reactivity and SIV lineage discrimination as previously reported (Aghokeng et al. 2006). Samples were considered seroreactive when optical density (OD) was greater or equal to cut-off value that was set at 0.3 based on previous evaluation of known SIV-negative and SIV-positive NHP control samples, i.e., for each test, the mean OD of known NHP negative controls plus five standard deviations was used to define the cut-off value and was uniformized at 0.3 for all peptides (Aghokeng et al. 2010). For all seroreactive samples, DNA was extracted from peripheral blood mononuclear cells (PBMCs) using Qiagen DNA Extraction Kit (Qiagen, Courtaboeuf, France). The presence of SIV DNA was evaluated by PCR using various combinations of degenerate *pol* gene primers known to amplify all existing SIV lineages and by SIV lineage-specific primers on samples reactive with peptides of the following lineages or groups: SIVtal/deb/syk, SIVmnd, SIVmus/mon/gsn, and SIVcpz (Clewley et al. 1998; Courgnaud et al. 2001; Liegeois et al. 2006; Aghokeng et al. 2006, 2007) (Supplementary Table 1). Any DNA fragment of appropriate size was immediately purified and sequenced.

289 Samples (11.8%) were reactive with one or more gp41 SIV peptide. Subsequent testing with a corresponding V3-loop ELISA assay from the same SIV lineage did not generate significant signals. The proportion of SIV seroreactive samples was higher in western blot indeterminate samples (21/89, 23.6%) compared to the HIV-negative group (268/2357, 11.4%) ($\chi^2 = 12.3$, $P < 0.001$). Within the HIV-negative group, more healthy adult samples (242/1910, 12.7%) reacted with SIV peptides as compared to the samples from hospital patients (26/447, 5.8%) ($\chi^2 = 16.9$, $P < 0.001$).

Behavioral data for the 289 seroreactive individuals demonstrate that 95.6% of these individuals reported behaviors that expose humans to NHP pathogens including exposure to NHP blood or body fluids through hunting, butchering and/or owning pets. Comparison with the seronegative group reveals that fewer (85.2%) individuals reported risky behaviors ($\chi^2 = 24.6$, $P < 0.001$), and only 4.9% of individuals in the seronegative group reported participating in all three risky behaviors compared to 21.4% individuals in the seroreactive group ($\chi^2 = 109$, $P < 0.001$). About 23.8% of seropositive individuals were female and their age varied from 16 to 88 years with a mean age of 38 years. The age range for the seronegative group was between 3 and 93 years with a mean age of 36 years; the proportion of females (35.7%) was higher in the seronegative group.

Although the majority of the gp41 seroreactive samples did not react with the corresponding homologous V3 peptide ELISA, suggesting non-specific reactivity, Table 1 shows 23 gp41 seroreactive samples that displayed either a high OD value (≥ 1) corresponding to signals of positive controls used in the assay or cross-reactivity to gp41 peptide combinations as previously observed among well-documented SIV infected samples (Table 1) (Aghokeng et al. 2006). These results were obtained within HIV-negative (21/23) and HIV western blot indeterminate (2/23) samples, and cross-reactivity to SIVgsn/mus/mon, SIVmnd, SIVlho, SIVsmm, and SIVdeb peptides was seen. With the exception of SIVlho from l'hoest monkeys and SIVsmm from sooty mangabeys, SIV antigens associated with sample reactivity corresponded to locally occurring NHP species, i.e., SIVgsn/mus/mon from greater spot nosed, mustached

Table 1. Serological Profiles and Exposure Data of Individuals with Suspected SIV Exposure

Sample code	Peptide								Behavioral data			
	SIVcpz-ant gp41	SIVmus gp41	SIVmon gp41	SIVsmm gp41	SIVrcm gp41	SIVmnd v3	SIVlho gp41	SIVdeb gp41	Hunts NHPs	Butchers NHPs	Keeps NHP Pets	Exposure accident
CAM0079NY	0.07	0.13	**1.22**	**0.32**	0.09	0.08	0.08	**0.68**		Yes		
CAM0200NY	**0.39**	**0.69**	**0.43**	0.14	0.11	0.19	0.11	0.10	Yes	Yes	Yes	Yes
CAM0921MO	0.10	**0.58**	**0.61**	0.11	0.12	0.10	0.09	**0.35**	Yes	Yes		
CAM0942MO	0.13	0.22	0.21	0.26	0.19	**1.37**	0.11	0.24	Yes	Yes		
CAM1016MO	0.09	0.09	0.09	**0.61**	**0.53**	0.10	0.17	0.07	Yes	Yes	Yes	Yes
CAM1035MO	0.10	0.09	0.08	0.15	0.11	**0.65**	0.13	**0.67**	Yes	Yes		Yes
CAM1059MO	0.11	0.08	0.09	**0.86**	0.13	0.11	0.12	**0.44**	Yes	Yes		Yes
CAM1060MO	0.09	0.11	0.09	0.12	0.23	**1.62**	0.08	0.08	Yes	Yes		
CAM1106MO	0.18	0.08	0.10	0.23	**0.75**	0.11	0.16	0.35	Yes	Yes		
CAM1119MO	0.10	0.06	0.07	**0.76**	0.24	0.08	0.07	**1.00**	Yes	Yes		
CAM1309NG	0.08	0.08	0.10	**0.56**	0.09	0.13	0.08	**0.64**	Yes	Yes	Yes	Yes
CAM1339NG	0.06	0.14	0.12	**0.71**	0.08	0.09	0.07	**1.02**	Yes	Yes		Yes
CAM1563MV	0.13	**0.99**	**1.11**	0.13	0.10	0.10	0.16	0.12		Yes		
CAM2029ND	0.08	0.10	0.08	**1.01**	0.10	0.07	0.07	**1.13**		Yes		Yes
CAM2579ND	0.12	0.09	0.08	**0.89**	0.09	0.09	0.08	**0.75**	Yes	Yes		
CAM3083HA	0.12	0.10	0.11	**0.51**	0.13	0.11	0.12	**1.01**	Yes			Yes
CAM3838KO	0.06	**0.87**	0.08	**0.79**	0.05	0.05	0.07	**1.02**				
CAM4106MU	0.05	0.06	0.06	**1.26**	0.06	0.07	0.10	0.06				
CAM4124HA	0.09	0.09	0.08	0.08	0.06	0.25	**1.15**	0.10		Yes		
CAM4298STN*	0.16	0.19	0.19	0.19	0.19	**0.76**	**0.93**	0.28	Yes	Yes		
CAM4487HAL*	0.08	0.21	**1.00**	0.07	0.07	0.08	0.06	0.07				
CAM1737LE §	0.21	**0.33**	**0.54**	0.22	0.19	0.21	0.12	0.16	Yes	Yes	Yes	
CAM1789LE §	**0.73**	0.16	0.24	0.13	**0.63**	0.08	0.07	0.12				

Raw ELISA data obtained with 23 samples suspected to contain SIV-specific antibodies; 21 HIV negative [including two samples from hospital patients (marked with *) and two western blot indeterminate (marked with a § sign). ODs > 0.30 are in boldface type. Eleven of those, all HIV negative, including one sample collected from a non-traumatic hospitalized adult, displayed ODs ≥ 1, i.e., in the same range as most positive controls in our SIV lineage-specific ELISA test. Exposure data relative to contacts with NHP fluids are also displayed.

and mona monkeys, SIVmnd from mandrills and SIVdeb from De Brazza monkeys.

To avoid overlooking any potential SIV infection, SIV PCR was performed on 232 samples for which an OD ≥ 0.3 was observed for at least one peptide and for which PBMCs were available. Although several combinations of universal and specific primers (Supplementary Table 1) with different amplification conditions were used, SIV-specific nucleic acid could not be detected in the SIV seroreactive samples.

As the attempts to amplify viral DNA were unsuccessful, we cannot conclude whether the SIV seroreactivity of these individuals is due to SIV infection or whether they constitute false positives. Nonetheless, seroreactivity in the absence of virus could suggest exposure to SIV antigens. Antibodies to SIVmnd from mandrills and SIVcol from mantled guerezas (also without virus amplification) have been previously reported in two Cameroonian individuals (Souquiere et al. 2001; Kalish et al. 2005) with high OD values and cross-reactivity patterns have previously been associated with SIV exposure in humans (Ndongmo et al. 2004). However, it should be noted that non-specific antibody reactivity is also frequently observed with commercial HIV screening assays, particularly in Central Africa (Aghokeng et al. 2010). The synthetic V3 loop peptide ELISA failed to detect V3 antibodies in gp41-reactive samples. Such discrepancies between the gp41 assay and corresponding V3 loop peptide ELISA have been reported previously (Aghokeng et al. 2006) and can be related to false positivity with the gp41 peptide or the characteristics

of the V3 loop peptide ELISA. In fact the latter is known to display low sensitivity and high specificity and may in some cases lead to false negative results (Simon et al. 2001; Aghokeng et al. 2006). Despite this performance issue, the V3 loop peptide assay has been helpful in differentiating between SIV lineages, and has exhibited valid signals with the positive controls used in this study.

Compared with previous studies, we analyzed samples from a larger number of individuals at risk of NHP zoonosis (Wolfe et al. 2004b, 2005) and used a more extensive panel of SIV peptides. Although we found 23 SIV highly seroreactive samples, SIV nucleic acids were not detected. The failure to detect SIV infection is surprising given the high NHP exposure of these individuals and the relatively large cohort that we tested.

Our results, 0.94% seroreactivity without antigen detection, suggest that SIV infections could occur less frequently than SFV (1% seropositive and 0.33% PCR positive) infections (Wolfe et al. 2004b, 2005); however, differences are small. In the same population, new HTLV-3 and HTLV 4 strains were also reported in 2 patients (2/930), but it is less evident to determine which proportion of the HTLV infected patients (9.7% seropositive and 1.4% PCR positive) acquired their infection by a recent zoonotic transmission, because in contrast to SFV this virus is also transmitted among humans. Frequent exposure and high prevalence most likely play a role in the probabilities of transmission of certain SIVs and other simian retroviruses to humans. For example, SIVcpz*Ptt* and SIVsmm prevalence is highest in West Central and West Africa (30 and 50%, respectively), where the precursors of HIV-1 M (M and N) and HIV-2 (A and B) have been identified in chimpanzees and mangabeys (Keele et al. 2006; Van Heuverswyn et al. 2007; Santiago et al. 2005). We recently showed in 2,500 samples derived from primate bushmeat collected from seven different sites in southern Cameroon that the overall primate SIV seroprevalence was relatively low, 2.93% (76/2586), and ranged from 0 to >30% depending on the species (Aghokeng et al. 2010). Interestingly, the absence of SIV infection or low SIV prevalence (around 1%) was observed in the species that are most frequently hunted in the study sites, i.e., greater spot nosed (*Cercopithecus nictitans*), mustached (*C. cephus*), and crested mona monkeys (*C. pogonias)* together with agile (*Cercocebus agilis*) and grey cheeked mangabeys (*Lophocebus albigena*) represent 90% of NHP bushmeat in southern Cameroon (Aghokeng et al. 2010). SIV prevalence in primate bushmeat in Cameroon (2.9%) is lower than STLV prevalence (8.1%) (Aghokeng et al. 2010; Liegeois et al. 2006; Sintasath et al. 2009) and in general 10–80% of wild primates are SFV infected (Mouinga-Ondémé et al. 2011; Leendertz et al. 2010; Goldberg et al. 2009; Morozov et al. 2009; Calattini et al. 2004; Liu et al. 2008). The variation in prevalence in primate bushmeat could in part explain why no cross-species transmission to humans with other SIVs has been demonstrated previously in Cameroon. However, SIV prevalence varies by geographic region and species (Aghokeng et al. 2006, 2010), therefore it cannot be excluded that increased exposure and risk of SIV cross-species transmission could exist in other areas of Africa where humans are hunting and butchering NHP with higher SIV infection rates. Finally, it is also possible that absence of SIV antigen detection is related to SIV infection with viral loads below the detection limits of the tools utilized to detect infection with highly divergent SIV strains, as has been previously observed in an SIVsmm-infected lab worker with persistent presence of antibodies in the absence of detectable nucleic acids or viral isolates during chronic infection (Khabbaz et al. 1994).

The finding of SIV seroreactivity in four persons who did not report hunting, butchering, or keeping NHP pets may represent false positive results, as has been observed in specimens originating in Central Africa. These behaviors are known to increase contacts between humans and NHPs and the risk for acquiring new infectious agents from those animals (Wolfe et al. 2004a, b, 2005). Nonetheless, the absence of exposure through these behaviors does not necessarily rule out all potential contact. Alternative exposure routes may exist that could explain this finding. Although contacts with contaminated fruits would generally result in food poisoning, contacts with infected feces could potentially lead to low concentrations exposure to viral pathogens and, hypothetically, to infection (Peeters et al. 2002; Van Heuverswyn et al. 2006).

High SIV prevalence and frequent trans-species contact do not automatically suggest that significant levels of active human infection should be present. It is possible that transmission of SIVs to humans is common at the human/NHP interface, but only a limited proportion of the transmitted strains adapt to the human host to generate a chronic infection (Wolfe et al. 2007). It is striking and, perhaps, informative that in our highly NHP-exposed cohort (including individuals previously found infected with other retroviruses of simian origin), no SIV infections could be detected. Additional studies are needed to examine whether the absence of SIV infection in populations at

risk for NHP zoonosis is due to low SIV prevalence, variations in regional bushmeat infection rates, or a result of specific viral/host factors. Given the potential pathogenicity of these lentiviruses, as illustrated by the HIV pandemic that resulted from a single cross-species transmission, it remains important to determine the extent that humans are exposed to SIVs, and whether other SIVs have crossed or are crossing the species barrier. Emerging viruses can remain unrecognized for several years due to weak health infrastructure, poor diagnostic capacity, and low sensitivity of commercial HIV screening assays to detect SIV antibodies. Thus, these viruses can spread locally and, due to increasing travel, rapidly expand to other geographical areas with favorable conditions for epidemic spread. Continuous surveillance with increased sample sizes and longitudinal studies aimed at identifying acute infections in hunters and other people exposed to wild NHPs are required to further explore SIV transmission, as well as the transmission of yet unknown pathogens to humans, and potentially prevent entry and spread of novel viruses in the human population.

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