Naturally acquired simian retrovirus infections in central African hunters

Nathan D Wolfe, William M Switzer, Jean K Carr, Vinod B Bhullar, Vedapuri Shanmugam, Ubald Tamoufe, A Tassy Prosser, Judith N Torimiro, Anthony Wright, Eitel Mpoudi-Ngole, Francine E McCutchan, Deborah L Birx, Thomas M Folks, Donald S Burke, Walid Heneine

Summary

Background Hunting and butchering of wild non-human primates infected with simian immunodeficiency virus (SIV) is thought to have sparked the HIV pandemic. Although SIV and other primate retroviruses infect laboratory workers and zoo workers, zoonotic retrovirus transmission has not been documented in natural settings. We investigated zoonotic infection in individuals living in central Africa.

Methods We obtained behavioural data, plasma samples, and peripheral blood lymphocytes from individuals living in rural villages in Cameroon. We did serological testing, PCR, and sequence analysis to obtain evidence of retrovirus infection.

Findings Zoonotic infections with simian foamy virus (SFV), a retrovirus endemic in most Old World primates, were identified in people living in central African forests who reported direct contact with blood and body fluids of wild non-human primates. Ten (1%) of 1099 individuals had antibodies to SFV. Sequence analysis from these individuals revealed three geographically-independent human SFV infections, each of which was acquired from a distinct non-human primate lineage: De Brazza's guenon (*Cercopithecus neglectus*), mandrill (*Mandrillus sphinx*), and gorilla (*Gorilla gorilla*), two of which (De Brazza's guenon and mandrill) are naturally infected with SIV.

Interpretation Our findings show that retroviruses are actively crossing into human populations, and demonstrate that people in central Africa are currently infected with SFV. Contact with non-human primates, such as happens during hunting and butchering, can play a part in the emergence of human retroviruses and the reduction of primate bushmeat hunting has the potential to decrease the frequency of disease emergence.

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Departments of Epidemiology (N D Wolfe ScD, F E McCutchan PhD, Prof D S Burke MD)**, International Health** (N D Wolfe, A T Prosser PhD, D S Burke)**, and Molecular Microbiology and Immunology** (N D Wolfe)**, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, USA; HIV and Retrovirology Branch, Division of AIDS, Sexually Transmitted Disease, and Tuberculosis Laboratory Research, National Center for HIV/AIDS, Sexually Transmitted Disease, and Tuberculosis Prevention, Centers for Disease Control and Prevention, Atlanta, GA, USA** (W M Switzer MPH, V B Bhullar BS, V Shanmugam PhD, A Wright BS, T M Folks PhD, W Heneine PhD)**; Henry M Jackson Foundation, Rockville, MD, USA** (J K Carr PhD, F E McCutchan)**; Army Health Research Centre, Yaounde, Cameroon** (U Tamoufe MSc, J N Torimiro MSc, E Mpoudi-Ngole MD)**; and Walter Reed Army Institute of Research, Rockville, MD, USA** (D L Birx MD)

Correspondence to: Dr Nathan Wolfe, Department of Epidemiology, Bloomberg School of Public Health, Johns Hopkins University, 615 North Wolfe Street E7132, Baltimore, MD 21205, USA (e-mail: nwolfe@jhsph.edu)

Introduction

In the 20 years since its discovery, HIV-1 has caused morbidity and mortality in man on a previously unimaginable scale. Phylogenetic studies[1] of HIV-1 and HIV-2, alongside their counterpart simian immunodeficiency viruses (SIVs), show that the pandemic has resulted from as many as eight independent transmissions from African monkeys and apes. Contact with the blood and body fluids of animals during hunting or butchering can lead to transmission of many diseases[2] and has been proposed as the primary mechanism for HIV origins.[1] Non-human primate bushmeat from central Africa has a high frequency of SIV infection, confirming that individuals who hunt or butcher these animals are at risk for zoonosis.[3] Although SIV and other primate retroviruses are known to infect laboratory and zoo workers,[4–9] transmission of these viruses to human beings who are in regular contact with non-human primates in natural settings has not been documented.

Simian foamy virus (SFV), or spumaretrovirus, is transmitted at a higher rate (about 2·5%) than other primate retroviruses to zoo workers and research

Panel 1: **Abbreviations for non-human primate species**

Asterisks indicate species endemic to Cameroon
Cmo*=*Cercopithecus mona* (Mona monkey),
Cal=*Cercopithecus albogularis* (Sykes monkey),
Cce=*Cercopithecus cephus* (red-eared guenon),
Clh=*Cercopithecus lhoesti* (L'Hoest's monkey),
Cne*=*Cercopithecus neglectus* (De Brazza's guenon),
Cha=*Cercopithecus hamlynii* (Hamyln's guenon),
Epa*=*Erythrocebus patas* (Patas monkey),
Cpy=*Chlorocebus pygerythrus* (vervet),
Lal*=*Lophocebus albigena* (grey-cheeked mangabey),
Pan=*Papio anubis* (olive baboon),
Pcy=*Papio cynocephalus* (yellow baboon),
Pur=*Papio ursinus* (Chacma baboon),
Cto*=*Cercocebus torquatus* (red-capped mangabey),
Cag*=*Cercocebus agilis* (agile mangabey),
Mta*=*Miopithecus talapoin* (talapoin monkey),
Mle*=*Mandrillus leucophaeus* (drill),
Msp*=*Mandrillus sphinx* (mandrill),
Cgu*=*Colobus guereza* (mantled guereza),
Mmu=*Macaca mullata* (rhesus macaque),
Mcy=*Macaca cyclopsis* (Formosan rock macaque),
Ppy=*Pongo pygmaeus* (Bornean orang-utan),
Hpi=*Hylobates pileatus* (pileated gibbon),
Ggo*=*Gorilla gorilla* (Western lowland gorilla),
Ppn=*Pan paniscus* (bonobo),
Pvl*=*Pan troglodytes vellerosus* (Nigerian chimpanzee),
Ptr*=*Pan troglodytes troglodytes* (central African chimpanzee),
Pvr=*Pan troglodytes verus* (West African chimpanzee),
Asp=*Ateles spp* (spider monkey)

Panel 2: **Genbank accession numbers**

The 20 new SFV *int* sequences are AY278774–AY278792 and AY442339. The *int* sequences used for phylogenetic comparison are X83295 (Cpy[SFV3agm]), AF049081 (Pan[SFVbab]), AF049083 (Pan[SFV10]), X83292 (Mmu[SFVmac]), X83290 (Mcy[SFV2]), X54482 (Mmu[SFV-1a]), X58484 (Mmu[SFV-1b]), AY195689 (Ppy[SFV11]), AF516486 (Hpi[Sam106]), AY195688 (Ggo[SFVggo]), AF049086 (Ppn[Bo]), AY195686 (Pvl[Cpz2]), AY195681 (Ptr[B1]), AY195682 (Pvr[C1138]), X83296 (Pvr[SFV6]), and X83298 (Asp[SFV8spm]). All primate SFV LTR sequences were available at Genbank. Genbank accession numbers for the three new SFV LTR sequences from infected people are AY390392–AY390394

workers.[7–9] SFV is also endemic in most Old World primates.[10,11] For these reasons, SFV infection can serve as a sensitive marker for the potential for natural infection with other, less transmissible simian retroviruses, such as SIV. Furthermore, SFV is genetically diverse and shows host-specific viral lineages, which facilitate the identification of the non-human primate source species in SFV-infected people.[7,10] Although global populations have been screened for evidence of natural SFV infection, studies have not focused on individuals reporting contact with non-human primates in the wild, and have so far failed to present evidence of natural infection.[12] Here, we combine evaluation of behaviours, such as the hunting and butchering of non-human primates, that may place individuals at risk for the acquisition of simian retroviruses with examination of blood samples for evidence of SFV infection.

Methods

We did the studies in the context of a community-based HIV-prevention campaign designed to provide information and decrease transmission. Participation was voluntary and participants gave informed consent. The study protocol was approved by the Johns Hopkins Committee for Human Research, the Cameroon National Ethical Review Board, and the HIV Tri-services Secondary Review Board. We made the questionnaires and matching samples anonymous by removing all personal identifiers to provide an unlinked study population.

Procedures

Blood was obtained from participants, transported to a central laboratory, processed into plasma and peripheral blood lymphocyte samples, and stored at –80°C until used. We first screened plasma samples for antibodies to SFV using EIA following standard procedures. We diluted samples 1 in 100 and tested them in duplicate in separate microplates containing antigen from either uninfected canine thymocytes or combined SFV antigen from canine thymocytes infected with SFV from ape (SFVcpz, chimpanzee) or monkey (SFVagm, African green monkey). We averaged the replicate sample optical density values, and calculated optical density ratios of reactivity to SFV over the uninfected antigens. An optical density ratio of greater than 1·32 was set as a cutoff value for seroreactive samples on the basis of assay validation with PCR-confirmed infected and uninfected non-human primates and human beings (data not shown). We further tested EIA-reactive samples by two western blot assays as previously described.[10] Criteria for western blot positivity were reactivity in the SFV blot to Gag p68 and p72, or p70 and p74 proteins (characteristic of monkey-type or ape-type SFV infection, respectively) and absence of similar

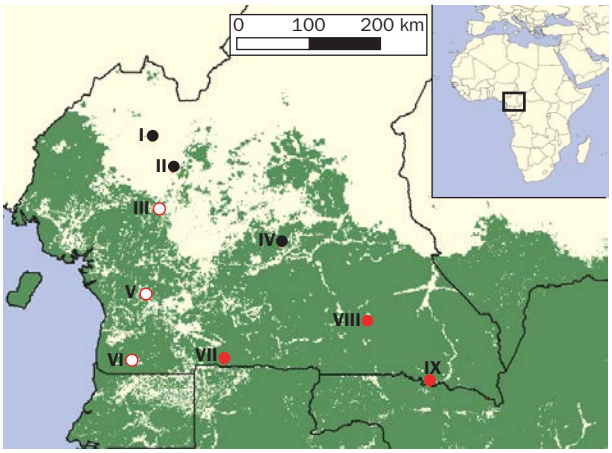

Figure 1: **Map of study sites**
Study sites I–IX shown in relation to distribution of lowland tropical forest in central Africa (green). Black dots, no individuals with evidence of SFV infection. Red dots with white centres, individuals with serological evidence but no PCR evidence of SFV infection. Red dots, people with both serological and PCR evidence of SFV infection.

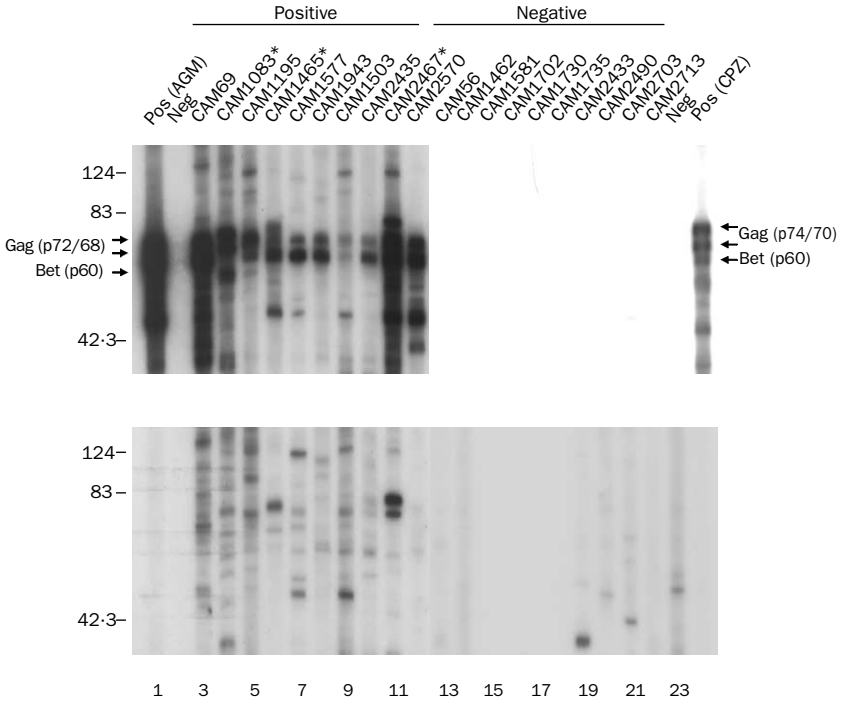

Figure 2: **Detection of antibodies to SFV with a combined antigen (SFVagm and SFVcpz) western blot assay**
Upper, combined SFV antigens. Lower, uninfected cell lysate antigen. Lanes 1 and 24, positive-control serum samples from SFV-infected African green monkey and chimpanzee, respectively; lanes 2 and 23, negative-control serum samples from an uninfected person. The molecular weight protein marker sizes indicated on the left. Western-blot positive samples with positive PCR results are indicated by an asterisk.

	Sex	Age	SFV	Site	Hunting technique	Exposure to non-human primates		
						Hunting	Butchering	Pet
1	Male	44	NK	VI	Gu, W	M, C, G	M, C, G	M, G
2	Male	45	SFVggo	IX	Gu, W, B	M, C, G	M, C, G	
3	Male	38	NK	V	W, H, Ma	M	M	
4	Female	48	SFVmsp	VII	W	M	M	
5	Female	75	NK	VII	NR		M, C, G	
6	Male	20	NK	VII	W, H, Ma	M		
7	Male	72	NK	VIII	NR	M, C, G		
8	Male	33	NK	VIII	W	M	M	
9	Male	25	SFVcne	VIII	NR		M, C	M
10	Female	44	NK	III	NR		M	M

NK=SFV origin not known. Study sites are shown in figure 1. Gu=gun. W=wire snare. H=by hand. B=bow and arrow. Ma=machete. NR=techniques not reported. M=monkey. C=chimpanzee. G=gorilla.

Demographic data and risk profiles for ten individuals with confirmed SFV-seropositive results

reactivity in the blot from the uninfected control antigen.[10] Specimens with very weak reactivity to two bands between the p68 and p72 kD, and p70 and p74 kD ranges were regarded as atypical. We undertook serotyping of antibody reactivity by separate western blot testing with either SFVcpz or SFVagm antigens as previously described.[9]

We took fresh EDTA or sodium-citrate-treated whole blood specimens on an opportunistic basis as part of initial or yearly physical examinations from wild captured or captive bred non-human primates, in accordance with the animal care and use committees at each institution. We obtained peripheral blood lymphocytes by Ficoll-hypaque centrifugation and prepared DNA lysates as described previously.[7] To avoid contamination, human and primate samples were processed separately and tested in laboratories in different buildings.

SFV isolates from a mandrill, drill, and olive baboon were propagated on Cf2Th cells and DNA lysates were prepared as previously described[7,10] and used to generate SFV sequences for the phylogenetic analyses. We did SFV cultures and DNA preparation in a biosafety level three laboratory that was physically separated from the processing of either human or non-human primate biological specimens.

To avoid contamination, we did PCR analysis of DNA from uncultured human or non-human primate peripheral blood lymphocytes and DNA from cultured SFV-infected cells separately on different days in physically-isolated laboratories. We prepared DNA from peripheral blood lymphocytes and confirmed its integrity by β-actin PCR as previously described.[13,14] We amplified 1 μg of peripheral blood lymphocyte DNA from all people with western blot positive and atypical results with generic nested PCR of a 465 bp integrase (*int*) sequence using methods previously described.[10] We chose the *int* gene because it is a highly-conserved region of the polymerase (*pol*) gene of the SFV genome, and PCR primers in this region have been used successfully to amplify diverse SFVs.[10]

Long-terminal repeat (LTR) sequences between 300 bp and 330 bp were also amplified with primers PBF1 (5′CAC TAC TCG CTG CGT CGA GAG TGT 3′) and PBR2 (5′GGA ATT TTG TAT ATT GAT TAT CC 3′) in the first round of PCR, and FVLGF1 (5′ TGT TCG AGA CTC TCC AGG ITT GGT AAG 3′) and PBR2 in the second amplification.[15] These primers are located within the variable R/U5 region of the LTR and hence give different size amplification products with certain SFV lineages.[16] We did 40 cycles of amplification in each round of the LTR PCR under standard conditions with a 45°C annealing temperature. Positive controls for both the *int* and LTR PCRs were dilutions of DNA lysates prepared from cells infected with an Asian macaque SFV (SFVmac) isolate. SFVmac is specific to Asian macaques and should not be found in African primates, and it therefore controls for cross-contamination from positive controls.

We analysed sequences from PCR-amplified products as previously reported[10] using the neighbour-joining and maximum-likelihood methods and 1000 bootstrap replicates or puzzling steps, respectively, to test reliability of final tree topologies. All *int* trees were rooted with the New World (ie, South and Central American) SFV-8 sequence from a spider monkey (Asp[SFVspm8]). We used the orang-utan sequence (SFVPpy) as an outgroup for the LTR phylogenetic analysis. We calculated percentage identities between the Cameroonian and selected primate SFV sequences using the Bestfit program (version 10.2) in the Genetics Computer Group's Wisconsin sequence analysis package on a UNIX workstation.

Nomenclature and geographic range were as previously described.[17] We coded non-human primates using the first letter of the genus name and the first two letters of the species or subspecies names with their house names or codes within parentheses (panel 1). GenBank accession numbers for the 20 new SFV *int* sequences, the *int* sequences used for phylogenetic comparison, and the three new SFV LTR sequences from infected humans are shown in panel 2.

Role of the funding source

JKC, FM, and DLB at the US Military HIV Research Program, one of the sponsoring organisations, contributed to the study design, data interpretation, and writing of the report.

Results

We examined 200 individuals from each of nine villages in southern Cameroon, close to natural non-human primate habitats, both forested and non-forested (figure 1). Individuals were asked to identify and quantify their exposure to a range of non-human primates, which were classified into categories that could be reliably distinguished by this population: chimpanzee, gorilla, and monkey. 1099 (61%) of 1800 participants reported direct exposure to fresh non-human primate blood and body fluids, mainly through hunting and butchering. We screened these 1099 exposed individuals for SFV antibodies using an EIA capable of detecting divergent monkey and ape SFVs, followed by confirmation with a

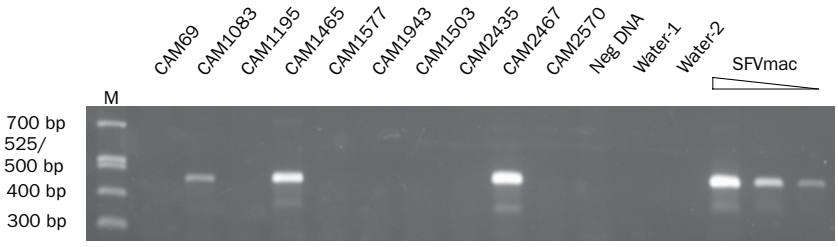

Figure 3: **Detection of SFV integrase sequences by nested PCR**
M=molecular weight marker. Neg DNA=uninfected human peripheral blood lymphocyte DNA control. Water-1 and water-2=negative reagent controls for primary and nested PCR tests respectively. SFVmac=DNA from 15, 1·5, and 0·15 canine thymocytes infected with an SFV macaque isolate.

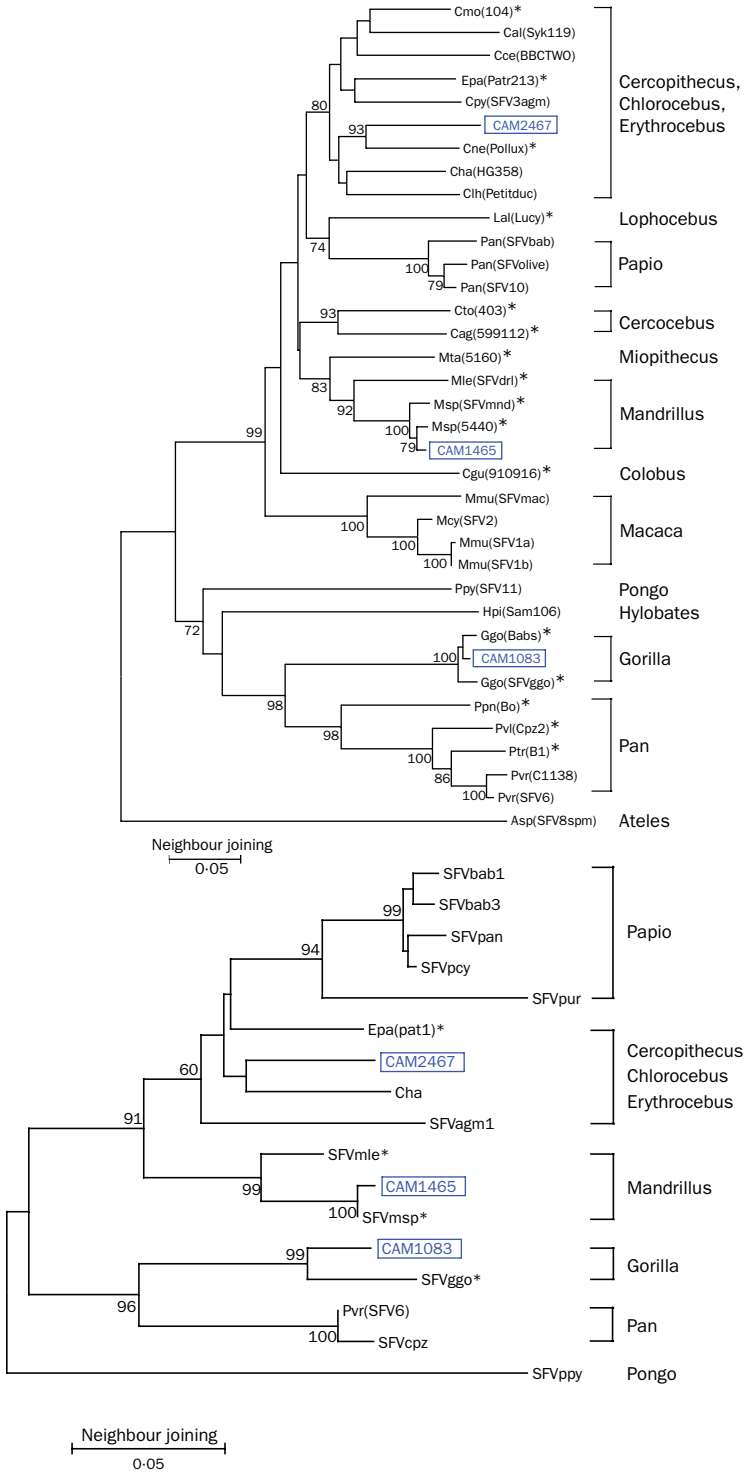

Figure 4: **Phylogenetic analysis of SFV sequences from infected hunters (boxed) and non-human primates**

Upper, integrase (425 bp) gene; lower, LTR (202 bp) gene. Branch lengths are drawn to scale with reference to the horizontal scale bar. Numbers at nodes indicate bootstrap values (%); only values greater than 60% are shown. Asterisks indicate species native to Cameroon.

validated SFV western blot assay.[10] 179 (16·3%) of the exposed people were EIA reactive, and the test results of ten (1%) of the 1099 were confirmed as seropositive by western blot analysis. All ten samples were reactive to the diagnostic SFV Gag doublet proteins and were unreactive to bands of the same molecular weight in the control blot from the uninfected antigen (figure 2). 12 additional EIA-reactive samples showed weak, atypical western blot patterns (data not shown). Individuals with western-blot-positive results were exclusively from lowland forest sites and included both men and women who reported frequent opportunities for contact with the blood and body fluids of non-human primates (table).

We prepared DNA from peripheral blood lymphocytes matched with the ten western blot positive plasma samples. Three of these ten (CAM2467, CAM1465, and CAM1083) tested positive for both SFV *int* and LTR sequences by PCR analysis using generic primers based on available Old World monkey and ape SFV sequences (figure 3). We sequenced the *int* and LTR amplicons and analysed them phylogenetically using SFVs from non-human primates native to Cameroon and from other Old World monkeys. The *int* sequences from monkey and ape species all clustered into separate lineages, suggesting co-speciation of host and SFV (figure 4).

We identified identical tree-topologies using maximum likelihood analysis (data not shown), lending further support to the genetic relations from the neighbour-joining analysis. The sequences from all three Cameroonian hunters were SFV-related and distinct from each other. The SFV *int* sequences all clustered with a different central African non-human primate SFV lineage with high bootstrap support. CAM1083 clustered tightly with gorilla (SFVggo) in the ape SFV group. CAM1465 and CAM2467 both fell within the monkey SFV clades. CAM1465 clustered tightly with SFV from mandrills (SFVmsp), whereas CAM2467 clustered with the sequences formed by *Cercopithecus* spp (De Brazza's guenon and Hamlyn's guenon) and had the highest relatedness to SFV from De Brazza's guenon (SFVcne) (figure 4).

Similar phylogenetic relations were also apparent in the analysis of the LTR sequences (figure 4). Both CAM1083 and CAM1465 LTR sequences clustered with high bootstrap support with the clades from gorilla and mandrill, respectively, whereas CAM2467 clustered with a cercopithecus sequence from Hamlyn's guenon. LTR sequences from De Brazza's guenons are not available to confirm the closer phylogenetic relatedness seen in *int* between these sequences and that of CAM2467. This study extends the known range of non-human primates capable of transmitting SFV to man, which before this report included only animals common to zoos and laboratories (baboons, African green monkeys, macaques and chimpanzees[7,9,18–21]).

Each of the three people who tested positive by PCR was from a different rural village in the lowland forest of southern Cameroon, a region of high primate-biodiversity. The SFVggo-infected person was a 45-year-old man. He reported that he butchered and consumed monkey, chimpanzee, and gorilla meat, and hunted all these groups, using at various times, guns, bows, and wire snares. The SFVmsp-infected person was a 48-year-old woman who reported that she consumed and butchered monkeys, and hunted monkeys with wire

snares. The participant infected with SFVcne was a 25-year-old man who butchered both monkeys and chimpanzees, but who may also have been exposed through contact with a pet monkey. All three species implicated in these zoonotic SFV infections inhabit the geographic range of the study.

We did not detect SFV sequences in seven of the ten individuals who were western-blot positive or in all 12 with atypical western-blot profiles. To distinguish monkey-type from ape-type SFV infection, plasma samples from the seven people who tested positive by western blot and negative by PCR were serotyped by western blot with either ape or monkey SFV antigen. All seven showed stronger reactivity to monkey SFV antigen than to ape antigen, suggesting monkey-type infection (data not shown). The reasons for the negative SFV PCR results in these seven samples are unknown. They might be due to the presence of low proviral loads, divergent viruses, or they may indicate non-specific reactivity with the SFV Gag proteins. Previous findings in primates show similar serological and PCR reactivity in monkeys infected with divergent SFV strains.[10] Additional studies with virus isolation and PCR are needed to confirm SFV infection in these people.

Discussion

SFVs are known to have the potential to infect laboratory and zoo workers who are occupationally exposed to captive non-human primates. Our findings show that individuals reporting direct contact with primates are also infected with SFV under natural conditions. They show that people can be naturally infected with SFV originating from many non-human primate hosts (gorillas, mandrills, and De Brazza's guenon). Of note, mandrills and De Brazza's guenons, and other monkeys and apes from this geographic region, are infected with unique SIVs,[3] some of which replicate in human cells in vitro.[21] Therefore the results suggest that the opportunity for cross-species transmission of other retroviruses, such as SIV, also exists in the same exposed population.

SFV infections in this study were from several geographically isolated locations, suggesting that—contrary to conventional wisdom—retroviral zoonosis is widespread, arising in various locations where people are naturally exposed to non-human primates. Although we cannot estimate the total number of such infections, widespread contact with such primates throughout rural forested regions of central Africa suggests that many such infections probably exist.

The scarce information that exists suggests that there is no secondary transmission or morbidity and mortality in people with SFV infections.[7,20] However, since previous studies have been restricted to very few occupationally infected people, and because SFV is not screened for in blood banks, naturally acquired SFV might have spread undetected both within and outside central Africa, or it might be pathogenic. Awareness of this possibility calls for increased follow-up of SFV-infected individuals and further surveillance, since population-level spread raises the potential for viral adaptation and the evolution of pathogenicity.

Our results show simian retroviral zoonosis in people who have direct contact with fresh non-human primate bushmeat, and suggest that such zoonoses are more frequent, widespread, and contemporary than previously appreciated. The increased amount of hunting in central Africa that has resulted from a combination of urban demand for bushmeat and greater access to primate habitats provided by logging roads,[22] has increased the frequency of human exposure to primate retroviruses and other disease-causing agents. In addition to helping conserve endangered species, the reduction of non-human primate hunting activities has the potential to reduce the frequency of cross-species transmission of simian retroviruses and other pathogens.

Contributors

N Wolfe, D Burke, W Switzer, T Folks, and W Heneine contributed to planning and design of the study. N Wolfe acquired and analysed primary data and contributed to the writing of the report. W Switzer, V Bhullar, V Shanmugam, A Wright, T Folks, and W Heneine designed and undertook laboratory studies, analysed laboratory results, and contributed to the writing of the report. J Carr, F McCutchan, and D Birx contributed to the design of the study, interpretation of results, and writing of the report. J Torimiro and E Mpoudi-Ngole assisted in the design of the study and interpretation of results. A Prosser and U Tamoufe designed behavioural questionnaires and contributed to the interpretation of results. All authors approved the final version of the report.

Conflict of interest statement

None declared.

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