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High prevalences and a wide genetic diversity of simian retroviruses in non-human primate bushmeat in rural areas of the Democratic Republic of Congo

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Abstract

Like the majority of emerging infectious diseases, HIV and HTLV are of zoonotic origin. Here we assess the risk of cross-species transmissions of their simian counterparts, SIV and STLV, from nonhuman primates (NHP) to humans in the Democratic Republic of Congo (DRC). A total of 331 samples, derived from NHP bushmeat, were collected as dried blood spots (DBS, n=283) or as tissue samples (n=36) at remote forest sites in northern and eastern DRC. SIV antibody prevalences in DBS were estimated with a novel high throughput immune assay with antigens representing the actual known diversity of HIV/SIV lineages. Antibody positive samples were confirmed by PCR and sequence analysis. Screening for STLV infection was done with universal primers in *tax* and new strains were further characterized in LTR. SIV and STLV infection in tissue samples was done by PCR only. Overall, 5% and 15.4% of NHP bushmeat was infected with SIV and STLV respectively. A new SIV lineage was identified in Allen's swamp monkeys (*Allenopithecus nigroviridis*). Three new STLV-1 subtypes were identified in Allen's swamp monkeys (*Allenopithecus nigroviridis*), blue monkeys (*Cercopithecus mitis*), red tailed guenons (*Cercopithecus ascanius schmidt*) and agile mangabeys (*Cercocebus agilis*). SIV and STLV prevalences varied according to species and geographic region. Our study illustrates clearly, even on a small sample size from a limited number of geographic areas, that our knowledge on the

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genetic diversity and geographic distribution of simian retroviruses is still limited and that humans continue to be exposed to relative high proportions on infected NHP bushmeat.

Keywords

non-human primate; bushmeat; retrovirus; SIV; STLV; Democratic Republic of Congo

Introduction

AIDS is one of the most important infectious diseases to have emerged in the 20th century. The HIV/AIDS epidemic was first recognized around 1980 in the United States, but retrospective studies on stored serum and biopsy samples showed that HIV-1 (Human Immune Deficiency Virus Type 1) was already present in Kinshasa, the capital city from the Democratic Republic of Congo (DRC), in 1959/1960 (Zhu et al. 1998; Worobey et al. 2008). In 1985, another virus, HIV-2, was observed in patients with AIDS from West Africa (Clavel et al. 1986). Subsequently, several studies showed that HIV-1 started to diverge in the human population since the beginning of the 20th century in Kinshasa, DRC, and HIV-2 around 1940 in Guinea-Bissau (Faria et al. 2014; Lemey et al. 2003; Wertheim et al. 2009).

Like the majority of emerging infectious diseases, HIV-1 and HIV-2, are also of zoonotic origin and their closest relatives are Simian Immune Deficiency Viruses (SIV). Today, SIVs have been described in at least 45 different African nonhuman primate (NHP) species, and in general each NHP species are infected with species-specific SIV lineages (Peeters et al. 2014). SIVcpzPtt from common chimpanzees (*Pan troglodytes troglodytes*) and SIVgor from western lowland gorillas (*Gorilla gorilla gorilla*) in west central Africa are most closely related to HIV-1 (Hahn et al. 2000; Keele et al. 2006; Vanheeuverswyn et al. 2006). SIVsmm from sooty mangabeys (*Cercocebus atys*) in West Africa are the closest relatives of HIV-2 (Gao et al. 1992; Hirsch et al. 1989). The four HIV-1 groups (M, N, O and P) and the nine HIV-2 groups (A-I) are each the result of independent cross-species transmissions. Large scale studies on fecal samples from chimpanzees and gorillas across Africa showed that chimpanzee populations from south-east and south-central Cameroon are infected with the ancestors of HIV-1 group M and N, respectively, and gorilla populations from south-central and south-west Cameroon harbor the ancestors of HIV-1 group O and P (D'Arc et al. 2015; Keele et al. 2006). Cross-species transmissions from sooty mangabeys to humans occurred in Sierra Leone, Liberia and Ivory Coast (Chen et al. 1996; Chen et al. 1997; Santiago et al. 2005). These 13 HIV variants have different epidemiological histories, some have only been observed in a handful of individuals, like HIV-1 N and P or HIV-2 C to I. Others were able to spread to some extent in humans, HIV-1 O in West Central Africa and HIV-2 A and B in West Africa. Only one, HIV-1 group M, has spread worldwide and is responsible for the global pandemic, with 60 million infections worldwide since the description of the first AIDS cases in the 1980's (Peeters et al, 2014).

Although less pathogenic than HIV, Human T-cell lymphotropic viruses, have also a simian origin. The four HTLV types, 1 to 4, in humans have each simian counter-parts (STLV-1 to 4) (Le Breton et al. 2014; Gessain 2011; Gessain et al. 2013). HTLV-1 and 2 are relatively

widespread and are estimated to infect around 20 million people, but HTLV-3 and HTLV-4 have only been documented in a handful of individuals in central Africa (Gessain et al. 2012; Mahieux et al. 2011; Richard et al. 2016). A high genetic diversity is seen among the different HTLV/STLV types, especially 1 and 3. In contrast to SIV, STLV strains from different NHP species cluster by geography and not according to species origin (Peeters et al. 2014). In certain HTLV subtypes, human and simian viruses are interspersed, suggesting many independent cross-species transmission events with STLVs from numerous NHP species to humans, eg. gorillas, chimpanzees, mandrills, greater spot nosed monkeys, mona monkeys, western red colobus etc. (Calvignac-Spencer et al. 2012; Mahieux et al. 1998; Peeters et al. 2014; Zheng et al. 2010)

Humans became most likely infected with simian retroviruses when they were exposed to infected blood, secretions or tissues through hunting and butchering primates as bushmeat. Recent reports showed ongoing transmission of simian retroviruses to humans in central Africa, i.e. SFV (simian foamy viruses) and new HTLV variants, closely related to viruses in co-habiting nonhuman primates (NHPs), have been observed in humans reporting primate hunting and butchering or who report severe bites from NHPs (Calattini et al. 2007; Filippone et al. 2015; Kazanji et al. 2015; Richard et al. 2016; Switzer et al. 2012; Wolfe et al. 2004; Wolfe et al. 2005; Zheng et al. 2010). Moreover, the description of HIV-1 group P in 2009 and HIV-2 group I in 2013 shows also that our knowledge on HIV diversity and possible cross-species transmissions is still incomplete (Ayouba et al. 2013; Plantier et al. 2009).

Cross-species transmission depends on the frequency of contacts, prevalence of infection in the animal host and subsequently on viral and host factors to establish efficient infection and disease. Our knowledge on exposure to simian retroviruses at the human/NHP interface is still largely incomplete and is only documented in limited regions across Africa (Aghokeng et al. 2010; Liegeois et al. 2012; Worobey et al. 2010). The Democratic Republic of Congo is the largest country in Sub-Saharan Africa, and among the approximately 80 million inhabitants, 60% live in rural areas. Given the enormous country size, and the diversity of ecosystems, at least 25 nonhuman primate species, often subdivided in many subspecies, are present in DRC (Groves, 2001). The absence of road infrastructure and difficult communications, people in DRC rely thus on bushmeat for subsistence in many areas. Given the importance of bushmeat in daily subsistence it is thus important to extend studies on pathogens at the human/animal interface. Our previous studies in NHP bushmeat were conducted in 3 sites in West-Central DRC and showed an overall SIV and STLV prevalence of almost 20% and 8% respectively, in NHP bushmeat (Ahuka-Mundeke et al. 2011; Ahuka-Mundeke et al. 2012). Here we study simian retroviruses in NHP bushmeat mainly in Northern and Eastern DRC.

Materials and Methods

Collection of nonhuman primate (NHP) samples

Samples from NHP bushmeat in DRC were collected between May 2012 and June 2015 as dried blood spots (DBS) in villages around Mbandaka (the Equateur Province), Goma and Walikale (North-Kivu Province) or as tissue samples in Kole (Oriental Kasai) (Figure 1).

Whole blood, present around injuries caused by hunting, was collected and spotted on Whatman 903 filter paper (GE-Healthcare, PA, USA). DBS were dried and subsequently stored into individual envelopes at ambient temperature. Tissue samples were collected in tubes with RNA-later and stored at ambient temperature in the field and at -20°C at the reference laboratory of INRB (National Institute for Biomedical Research) in Kinshasa. Samples were obtained from animals that died 6 to 78 hours prior to sampling. Samples were collected through a strategy specifically designed not to increase demand as previously described (Aghokeng et al. 2010; Ahuka-Mundeke et al. 2011). Species identification was determined in the field by visual inspection according to the Kingdon Field Guide to African Mammals (Kingdon, 1997) and the taxonomy described by Colin Groves (Groves 2001). The study was approved by the Ministries of Environment and Health and the National Ethics Committee.

Screening for cross reactive HIV/SIV antibodies

All DBS samples were screened with the recently described HIV/SIV multiplex microbead immunoassay (MIA), i.e. the Luminex® Multiple Analyte Profiling (xMAP) technology (Ahuka-Mundeke et al. 2011). Peptides covering the immunodominant region (IDR) of the gp41 transmembrane and/or V3-loop region from major SIV/HIV lineages were used: HIV-1, SIVcpz, SIVgor, HIV-2/SIVsmm, SIVmon, SIVmus, SIVgsn, SIVdeb, SIVrcm, SIVagm, SIVcol, SIVwrc, SIVsyk, SIVtal, SIVmnd, and SIVlho. Briefly, peptides were covalently coupled on carboxyl-functionalized fluorescent polystyrene beads (Luminex Inc., Austin, TX) using the Bio-Plex Amine Coupling Kit (BioRad Laboratories, Marnes la Coquette, France). Peptide-coupled microsphere preparations were counted and diluted to 4,000 beads/μL. Dilution and washing buffer consisted of PBS containing 0.75M NaCl, 1% (w/v) BSA (Sigma Aldrich, St Quentin Fallavier, France), 5% (v/v) fetal bovine serum (Gibco-Invitrogen, Cergy Pontoise, France) and 0.05% (v/v) Tween-20 (Sigma-Aldrich, St Quentin Fallavier, France). Ninety-six well flat-bottomed filter plates (Millipore, Tullagreen, Ireland) were pre-wet with 100μL assay buffer and subsequently 50 μL of bead mixture was added to each well. After aspiration of liquid with a vacuum manifold, wells were washed with 100 μL of assay buffer. Wells were then incubated with 100 μL of DBS eluate, obtained after overnight incubation of two 6 mm disks in 1 ml of hypertonic PBS. After washing, plates were incubated with biotinylated mouse anti-human IgG (BD Pharmingen, Le Pont de Claix, France) followed by washing, and incubation with streptavidin-R-phycoerythrin (Invitrogen/molecular Probes, Cergy Pontoise, France). After two final washes with reading buffer (PBS containing 1% (w/v) BSA), beads were resuspended in 125μL reading buffer/well and analyzed with BioPlex-200. The data were collected with the BioPlex Software Manager v5.0 (BioRad, Marnes La coquette, France). At least 100 events were read for each bead set and expressed as median fluorescence intensity (MFI) per 100 beads. The cut-off value was set at 200 MFI corresponding to a consensus value for all peptides and previously validated.

Extraction of nucleic acids and molecular confirmation of non human primate species

Depending of the material available, nucleic acids were extracted from one (50ul) or two (100ul) circles of DBS with the Nuclisens EasyMAG extraction Kit (Biomérieux, Craponne, France) using manufacturer's instructions, except for the incubation time of the viral lysis

step which was extended to 2 hours to increase DNA release or SMARTEExtract DNA kit (Eurogentec, Liege, Belgium). For tissue samples, DNA was extracted by using the Qiagen Blood and Tissue kit (Qiagen SAS, Courtaboeuf, France), following the manufacturer's recommendations, with minor modification, consisting in the extension of the incubation time of the viral lysis step to 3 hours to increase DNA release. In order to confirm the species identification in the field of the samples that were collected, a 450 bp mtDNA fragment of the 12S rRNA gene was amplified and sequenced using primers 12S-L1091 and 12S-H1478 (Van der Kuyl et al. 1995). PCR products were directly sequenced on an automated sequencer (ABI 3130XL, applied Biosystems, Courtaboeuf, France). Sequences were assembled using the software package Lasergene (DNASTAR, Inc, Madison, WI).

Characterization of SIVs in antibody positive samples

PCR analyses with universal HIV/SIV and SIV lineage-specific primers in *pol* (integrase region of the *pol* gene) or *env* (envelope gene) were done on all SIV antibody positive samples using previously described primers and conditions (Aghokeng et al. 2010, Ahuka-Mundeke et al. 2011). PCR products were purified on agarose gel (1%) and directly sequenced as described above.

Screening for STLV infection

All samples were tested for STLV infection by PCR, using universal and type-specific primers in *tax* gene (100-220bp), which previously identified a wide diversity of new STLV lineages in different nonhuman primate species (Van Damme et al. 1997). *tax* PCR positive samples were further characterized in 5'-LTR (386-689 bp) by amplifying a 450bp fragment with specific primers for the corresponding PTLV types as deduced from phylogenetic analyses of tax-PCR derived sequences. To that end, we used in the first round ENH280/PTLV1LTR5R1 and in the second round, Tatabox/5PLTR (Ahuka-Mundeke et al. 2012, Mahieux et al. 1997; Meertens et al. 2001). PCR products were directly sequenced using an automated sequencer as described above.

Phylogenetic analyses

Newly derived nucleotide sequences were compared to previously published HIV/SIV or HTLV/STLV reference sequences. Sequences were aligned using MEGA6 (Tamura et al. 2013), and where necessary, minor manual adjustments were performed. Sites that could not be aligned unambiguously or that contained a gap in any sequence were excluded from the analyses. Appropriate models of evolution were selected for each data set using MEGA 6 software and maximum likelihood phylogenies were reconstructed using phyML (Guindon et al. 2010) with 100 bootstrap replicates. For STLV sequences, the TrN93 models with a gamma distribution across sites was used for ML analyses. For SIV, the analyses were performed using discrete gamma distribution and GTR model.

Testing for cross reactive HTLV antibodies

A subset of samples confirmed by PCR and sequence analysis were retested with a commercially available confirmatory test, INNOLIA HTLV 1/2 (Innogenetics, Ghent, Belgium), a line immunoassay which discriminates between HTLV-1 and HTLV-2 cross-

reactive antibodies as previously described (Liegeois et al., 2008). This test configuration includes HTLV-1 and HTLV-2 recombinant proteins and synthetic peptides that are applied as discrete lines on a nylon strip. The antigenicity exhibited by these proteins and peptides is either common to HTLV-1 and HTLV-2 or specific to one of these two viruses allowing confirmation and discrimination in a single assay. Two Gag (p19 and p24) and two Env (gp46 and gp21) bands are included as non-type-specific antigens, which are used to confirm the presence of antibodies against HTLV-1 and HTLV-2. The type-specific antigens for HTLV-1 (Gag p19 and Env gp46) and HTLV-2 (Env gp46) are then used to differentiate between HTLV-1 and HTLV-2 infections. In addition to these HTLV antigens, control lines are present on each strip: one sample addition line (3+) containing anti-human immunoglobulin (Ig) and two test performance lines (1+ and +/-) containing human IgG. All assays were performed and interpreted according to the manufacturer's instructions.

Nucleotide sequence accession numbers

The new sequences have been deposited in Genbank under the following accession numbers: KX506859-KX506905

Results

NHP species collected at the different localities

DBS samples were obtained from 278 NHPs in rural villages around Mbandaka (n=136), Goma (n= 100) and Walikale (n=42), and tissue samples were collected from 36 animals around Kole (Fig.1). Sequence analysis of the 12S rRNA gene revealed that for almost 60% of the samples the species identification in the field was not correct and showed presence of 10 different NHP species: Allen's swamp monkeys (*Allenopithecus nigroviridis*) (n=44), red tailed guenons (*Cercopithecus ascanius*) (n=81) representing two subspecies, *C. a. whitesidei* in Mbandaka and Kole and *C. a. schmidtii* in eastern DRC, de Brazza monkeys (*Cercopithecus neglectus*) (n=18), greater spot nosed monkeys (*Cercopithecus nictitans*) (n=9), Wolf's monkeys (*Cercopithecus mona wolffi*) (n=36), Hamlyn's monkeys (*Cercopithecus hamlyni*) (n=5), l'hoest monkeys (*Cercopithecus l'hoesti*) (n=36), blue monkeys (*Cercopithecus mitis*) (n=45), agile mangabeys (*Cercocebus agilis*) (n=3), and one mantled guereza (*Colobus guereza*). The species distribution differs according to the sampling sites and reflects NHP distribution according to the biogeographic areas in DRC, for example l'hoest and blue monkeys are only seen in eastern DRC or Allen's swamp monkeys only in northern DRC.

Prevalence and genetic diversity of Simian Immunodeficiency Viruses (SIV)

SIV infection was documented in four species, antibodies were detected in DBS samples collected in the area from Mbandaka and viral RNA was detected in tissue samples from Kole. The overall SIV prevalence in Mbandaka was 10.3% and varied from 4.5% to 22.0% among the infected species. The highest SIV prevalences were seen in de Brazza monkeys (22%) and red tailed guenons (17%). All the NHP species from the two sites in eastern DRC were SIV negative, including, all the samples from the red tailed guenon subspecies (Table 1).

In order to confirm SIV infection and document SIV diversity, PCR was done on all SIV antibody positive samples. Proviral SIV DNA could only be amplified in *pol* (400bp) for one of the 14 positive samples. The low PCR efficiency is most likely due to small sample volumes, low proviral DNA load and DNA/RNA degradation due to the fact that animals died several days before sampling and/or long and sub-optimal storage at ambient temperature in the field. However we documented here for the first time SIV infection in Allen's swamp monkeys, and phylogenetic tree analysis reveals that SIVasm represents a potential new species-specific SIV lineage (Figure 2). PCR attempts were more successful on tissue samples, and SIV sequences were amplified in spleen tissue which is a lymphoid tissue and known to harbor high viral loads of SIV. New SIVasc sequences were obtained from 12 (34.2%) out of 35 red tailed guenons from Kole. No SIV was amplified in the spleen sample from the wolf's monkey. Phylogenetic tree analysis of the partial *pol* sequences, showed that they all fell in a single cluster, together with previously identified SIVasc sequences obtained on DBS samples from the *C. a. whitesidei* sub-species in that area (Ahuka-Mundeke et al. 2011). Interestingly, SIVrtg recently described in Uganda in the subspecies *C. ascanius schmidtii* from eastern Africa, formed a separate cluster (Lauck et al. 2014) with mean genetic distance of 0.330 (± 0.032).

Prevalence and genetic diversity of Simian T-cell Lymphotropic Viruses (STLV)

Previous studies, observed that the sensitivity of commercial available serological assays, which are developed for the detection of HTLV-1 and HTLV-2, was not sufficient to identify divergent PTLV strains, especially PTLV-3 and 4, in blood samples from nonhuman primates and humans (Ahuka-Mundeke et al. 2012, Richard et al. 2016). For example, in our previous report, we observed the following results among 105 samples from primate bushmeat that were tested in parallel with Elisa (Murex HTLV I/II), INNOLIA HTLV I/II confirmatory assay and universal and type specific tax PCR: 11 (8 STLV-1 and 3 STLV-3) were identified by PCR, 10 by INNOLIA (8 corresponding to an HTLV-1 profile and 3 with an undetermined HTLV profile) and only one with Elisa (unpublished data from samples described in Ahuka-Mundeke et al. 2012). Therefore, all samples were tested for the presence of STLV infection by PCR.

STLV infection was identified in 43 samples from five species, four of the six species from the area around Mbandaka, and three of the five species from eastern DRC (Table 2). The overall STLV prevalence in the three sites was 15.4%; in Goma and Mbandaka, 20% and 16.9% of NHP, respectively, were infected with STLV but all NHP from Walikale were negative. High levels of STLV infection were seen among Allen's swamp monkeys (40.9%) and red tailed guenons (16.2%), with higher infection rates in the subspecies from eastern DRC, 27.5% versus 4.8% in the subspecies in Mbandaka. Thirty percent of blue monkeys from Goma were infected with STLV, but those from Walikale were negative.

Phylogenetic analysis of the small *tax* region showed that all STLV infections were with STLV-1 and revealed a high genetic diversity. To further characterize the new STLVs from this study, we attempted to amplify a fragment of the LTR region for the STLV-1 positive samples. For 36 of the 43 *tax* positive samples, PCR attempts with LTR primers were successful and new partial STLV-1 LTR sequences were obtained as shown in the

phylogenetic tree analysis (Figure 3). The high genetic diversity was confirmed and 3 potential new subtypes were identified because the strains did not cluster with any of the known STLV-1 LTR sequences available in Genbank. The majority of the STLV-1 strains from Allen's swamp monkeys formed a separate cluster. However, two strains from Allen's swamp monkeys were subtype B, which predominates in west central Africa and this subtype has also been previously reported in captive Allen's swamp monkeys captured in DRC (Meertens et al. 2001). All STLV-1 sequences from blue monkeys and from the large majority of red tailed monkeys from eastern DRC formed also another new subtype. In our previous study we observed also a new subtype among Tsuapa red colobus monkeys in Kole, and in this study we identified strains from a red tailed monkey and an agile magabey from Mbandaka which fell also in this cluster.

Finally, INNOLIA HTLV/II antibody tests were performed on 12 STLV positive samples two were negative, two were HTLV indeterminate and the eight remaining had serological profiles corresponding to HTLV-1 infections, confirming thus that serological testing is not sensitive enough.

Discussion

In this study, we analyzed to what extent nonhuman primate bushmeat from northern and eastern DRC is infected with simian retroviruses in order to have a more global picture of human exposure to these viruses in DRC. The overall SIV and STLV prevalence in the 3 sites was 5% and 15% respectively. SIV infection was only identified in the north, in the area around Mbandaka, and we confirmed also high rates of SIV infection in tissue samples collected from red tailed guenons in Kole, central DRC. No SIV infection was identified in samples collected in the east although SIV infection has been previously documented in captive and wild l'hoest monkeys from DRC and Rwanda (Beer et al. 2000; Santiago et al. 2003).

Today, studies on retroviral infections in NHP bushmeat have been limited both in number of samples tested as well as on number of geographic regions that were sampled. Large scale studies have been conducted in Cameroon, and data are also available from Equatorial Guinea and Gabon (Aghokeng et al. 2010; Liegeois et al. 2012; Worobey et al. 2010). These studies showed that SIV and STLV prevalences varied per species but also within species according to geographic areas where animals were sampled. Heterogeneous SIV prevalence, was also observed in the large scale studies on thousands of faecal samples from wild chimpanzees and gorillas across central Africa (D'arc et al. 2015; Keele et al. 2006; Li et al. 2012; Liegeois et al. 2008 and 2012; Van Heuverswyn et al. 2007). This suggests that exposure and potential risk for cross-species transmissions is heterogeneous. Interestingly, the ancestors of the different HIV-1 groups, were identified in chimpanzee and gorilla communities with high SIV prevalences (Keele et al. 2006; D'arc et al. 2015). Similarly as observed in previous studies, SIV and STLV prevalences in DRC varied also per species and per geographic site, most likely as the result of isolation of certain NHP populations due to natural geographic barriers such as rivers, eg. the Congo river for red-tailed monkeys from eastern and central DRC, or more recent habitat modification. Figure 4A and 4B summarizes all the data available today on simian retroviruses in NHP bushmeat in DRC. Taken together

the data from this study and our previous report, the highest SIV prevalence was seen in west-central DRC (17% to 31%), followed by the North (10%). We did not observe SIV infection in eastern DRC, however, the number of samples and sites are limited and SIV infection has been previously documented in some of the NHP species but in other geographic areas of east Africa; i.e. captive and wild l'hoest monkeys from DRC and Rwanda (Beer et al. 2000; Santiago et al. 2003) or red tailed guenons from Uganda (Lauck et al. 2014). Absence of SIV infection in certain NHP species or sites can also be explained by low sampling numbers, limited number of sites, or low quality of samples obtained from diseased monkeys reducing the sensitivity of antibody detection and low quality of DNA for PCR testing. SIVs have also been described in greater spot nosed monkeys from Cameroon and Gabon, in mantled guereza's from Cameroon and Uganda, and in an agile monkey from Cameroon (Ahuka-Mundeke et al. 2010; Courgnaud et al. 2001; Courgnaud et al. 2002; Lauck et al. 2013; Liegeois et al. 2012). In our studies in DRC, the species with highest SIV prevalences were Tsuapa red colobus, the red tailed guenon subspecies from central DRC and De Brazza monkeys. The differences between and within different NHP species or subspecies in the different sites can be related to separation by important geographic barriers like major rivers, for exemple central DRC harbors many endemic species because of the geographic barriers constituted by the Congo, Ubangui and Kasai rivers.

Unfortunately, we could only confirm SIV infection by PCR and sequence analysis in a single DBS sample. Although SIV infection was suspected in captive animals (Lowenstine et al. 1986), this is the first time that SIV infection is confirmed by PCR in wild Allen's swamp monkeys, moreover partial sequence data suggest that they could be infected with a potential new species-specific SIV lineage. Only full-length sequence analysis will allow to identify unambiguously whether this virus represents a new lineage or an eventual recombinant of other SIV lineages. All attempts to obtain more sequence information of this virus remained unsuccessful. PCR attempts on tissue samples were more successful and a total of 12 new SIVasc sequences were obtained from red tailed guenons (*C. ascanius whitesidei*) collected around Kole. A high genetic diversity is seen in the SIVasc lineage. However, the SIVasc lineage was different from the SIVrtg strains isolated from the subspecies *C. ascanius schmidtii* living in east Africa. Similarly, only full-length genome sequences will allow understanding the evolutionary history of the new SIVasc viruses.

Overall, we showed that 15% of NHP bushmeat in our study is infected with STLV, which is higher than the 8% in our previous study in central DRC. As shown in Figure 4, STLV prevalences vary also by site and species. In sites where no STLV infection was identified, lower numbers of samples have generally been collected. Also, we found only STLV-1 infections and no STLV-3 infections, which represented 30% of the STLV infections in Kole (Ahuka-Mundeke et al. 2012). Today, STLV-2 infections have only been described in captive and wild bonobos (*Pan paniscus*), a species endemic to DRC (Ahuka-Mundeke et al. 2016; Giri et al. 1994; Vandamme et al. 1996). Further screening did not reveal STLV-2 in monkeys from DRC. Similarly, STLV-4 was recently identified in gorillas (LeBreton et al. 2014), but was not observed in NHP from our study. Prevalences varied also per species, ranging here from 5% in De Brazza and wolf's monkeys to 40% in Allen's swamp monkeys. In contrast to what we observed for SIV infection in the two red-tailed guenon subspecies, a high (27%) STLV-1 prevalence was observed in the eastern subspecies, versus 2.5% in the

other subspecies present around Mbandaka and Kole. No information was available on age of the monkeys from our study but in general SIV and STLV prevalences increase with age, as a result of sexual transmission and from aggressive behavior between juveniles and adults.

Although our sample size was small and covers only a limited number of areas in DRC, we observed a high variety of STLV variants and co-circulation of more than one variant in the same NHP species. For example, two different STLV variants co-circulate among Allen's swamp monkeys, STLV-1 subtype B and a potential new STLV-1 subtype. Some of the species in which we did not identify STLV infection, are known to be infected with STLV, ex greater spot nosed monkeys in Cameroon are infected with STLV-1 and STLV-3 (Liegeois et al. 2008, Sintasath et al. 2009), but less than 10 animals have been studied here for this species. Like in previous studies (Liegeois et al. 2012), we also observed SIV and STLV co-infection in four animals.

In summary, we found high prevalences of simian retroviruses and a broad diversity of SIV and STLV in NHPs from DRC and identified a new viral lineages. Our study illustrates clearly, even on a small sample size from a limited number of geographic areas, that our knowledge on the genetic diversity and geographic distribution of simian retroviruses is still limited and that humans continue to be exposed to relative high proportions of infected NHP bushmeat. Especially, red colobus and red tailed guenons are frequently hunted NHP and are highly infected. Moreover, simian foamy viruses from these species have been transmitted to humans in rural DRC (Switzer et al. 2012). Depending on viral and host factors, not all viruses can cross the species barrier but compared to the early 20th century, travelling between urban and rural areas has significantly increased (Locatelli et al. 2012). Viruses that are able to adapt and establish infection in a human host can now reach rapidly new areas with favorable conditions for epidemic spread. New epidemic outbreaks with diseases with a long incubation period can go unrecognized for a long period, especially because these SIV and STLV strains are not easily recognized by commercial screening assays, especially highly divergent strains. This is illustrated by the INNOLIA HTLVII antibody tests that we performed on a subset (n=12) of the STLV positive samples identified in this study and highlighted with a black circle in Figure 3. In addition, the weak health care structures will not timely recognize a new outbreak. Among the 13 documented cross-species transmissions of SIV to humans, one has lead to the current HIV/AIDS epidemic, it is thus important to continue to study whether other simian retroviruses crossed the species barrier, especially in human populations exposed to highly infected primates and in populations with risk behavior that is associated with high epidemic spread.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Sites where dried blood spots of nonhuman primates were collected are highlighted with circles on the map; in red for sites from this survey, in green for sites from our previous survey (Ahuka-Mundeke et al. 2011) and in green with red in sites included in both studies (DBS in the previous and tissue samples in this study)

Figure 2.

Phylogenetic relationships of the newly derived SIV sequences in *pol* to representatives of the other SIV lineages. The newly identified strains in this study are highlighted in red, sequences from our previous survey in DRC are in blue (Ahuka-Mundeke et al., 2011) and reference strains are in black. The trees were inferred from 391 bp nucleotides. The analyses were performed as described in methods. One hundred bootstrap replications were performed to assess confidence in topology, only those above 70% are shown with an asterisk. The scale bar represents 0.1 replacements per site. SIV lineages are generally referred to with a three letter code of the common name from the host species: SIVgsn/mus/mon, greater spot nosed, mustached and mona monkeys; SIVtal, talapoin monkeys; SIVasc, red tailed guenon subspecies (*C. ascanius schmidtii*) from Central DRC; SIVrtg, red tailed guenons from Uganda; SIVagm, african green monkeys; SIVasm, Allen's swamp monkeys; SIVmnd-2/drl, mandrills and drills from Cameroon and northern Gabon; SIVrcm, red cammped magabeys; SIVcpz/gor, chimpanzees and gorillas; SIVsmm, sooty mangabeys; SIVwrc, western red colobus; SIVolc, olive colobus; SIVlho/sun, l'Hoest and suntailed monkeys; SIVdeb, de Brazza monkeys; SIVsyk, Syke's monkeys; SIVcol, black and white colobus or mantled guereza's.

Figure 3. Phylogenetic relationships of the newly derived STLV sequences in LTR (373 bp) to representatives of the other HTLV-1/STLV-1 lineages. The analyses were performed as described in methods. One hundred bootstrap replications were performed to assess confidence in topology, asteriks at nodes are from bootstrap values above 70% of the replicates. Scale bar represents the number of nucleotide substitution per site. Sequences from this study are highlighted in red and those from our previous survey in DRC (Ahuka-Mundeke et al. 2012) in blue. Sequences derived from humans are in grey. Nonhuman primates from our studies are coded using the first letter of the genus followed by the first two letters of the species name: Ang, *Allenopithecus nigroviridis*; Cmi, *Cercopithecus mitis*; Cas, *Cercopithecus ascanius* (all samples from this study in red are from the *C.a.whitesidei* subspecies, in blue from the *C.a.schmidt* subspecies); Cag, *Cercocebus agilis*; Cwo, *Cercopithecus wolff*; Cne, *Cercopithecus neglectus*; and Pth, *Ptilocolobus tholloni*. INNOLIA HTLV-1/II antibody tests were performed 12 STLV positive samples highlighted with a black circle: Cas463 and Cas160 were negative; Ang496 and Ang502 were HTLV-1 indeterminate and the 8 remaining had serological profiles corresponding to HTLV-1 infections.

Figure 4.

Nonhuman primate species frequently sold at bushmeat markets in DRC and prevalence of SIV and STLV infection per sampling site (A) and per species (B). The data used for the figures are from this study and our previous surveys on SIV (Ahuka-Mundeke et al., 2011) and STLV (Ahuka-Mundeke et al., 2012) in DRC.

Table 1

The number and percentages of SIV antibody positive samples per species and number of samples confirmed by PCR and sequence analysis per species and per sites.

Species	Common name	SIV Antibody detection in DBS samples			PCR detection DBS antibody+ n/Ntested	SIV detection tissues by PCR* Kole n/Ntested (%)
		Mbandaka n/Ntested (%)	Goma n/Ntested (%)	Walikale n/Ntested (%)	Total n/Ntested (%)	
<i>Allenopithecus nigroviridis</i>	Allen's swamp monkey	2/44 (4.5%)	- [#]	-	2/44 (4.5%)	-
<i>Cercopithecus ascanius denti ascanius schmidti</i>	Red tailed monkey	7/41 (17.%)	-	-	7/41 (17.%)	12/35 (34.2%)
	-	0/33 (0%)	0/7 (0%)	0/40 (0%)	-	-
<i>Cercopithecus neglectus</i>	De Brazza monkey	4/18 (22.2%)	-	-	4/18 (22.2%)	-
<i>Cercopithecus nictitans</i>	Greater spot nosed monkey	0/9 (0%)	-	-	0/9 (0%)	-
<i>Cercopithecus wolffi</i>	Wolf's monkey	1/20 (5.0%)	0/7 (0.0%)	0/9 (0.0%)	1/36 (2.8%)	0/1
<i>Cercopithecus hamlyni</i>	Hamlyn's monkey	-	0/5 (0.0%)	-	0/5 (0.0%)	-
<i>Cercopithecus lhoesti</i>	I'Hoest monkey	-	0/30 (0.0%)	0/6 (0.0%)	0/36 (0.0%)	-
<i>Cercopithecus mitis</i>	Blue monkey	-	0/25 (0.0%)	0/20 (0.0%)	0/45 (0.0%)	-
<i>Cercocercus agilis</i>	Agile mangabey	0/3 (0.0%)	-	-	0/3 (0.0%)	-
<i>Colobus guereza</i>	Mantled guereza	0/1 (0.0%)	-	-	0/1 (0.0%)	-
Total		14/136 (10.3%)	0/100 (0.0%)	0/42 (0.0%)	14/278 (5.0%)	12/36 (33.3%)

[#] -; Not applicable because samples non available

* : only results from PCR in *pol* (integrase region of the *pol* gene) regions are shown. PCR attempts in *env* (envelope gene) were negative.

Number and percentages STLV positive samples per species and per sites detected by PCR in *tax* and number samples with additional sequences in LTR.

Table 2

Species	Common name	STLV detection in DBS samples				LTR sequence ^s n/Ntested	STLV detection tissues by PCR Kole n/Ntested (%)
		Mbandaka n/Ntested (%)	Goma n/Ntested (%)	Walikale n/Ntested (%)	Total n/Ntested (%)		
<i>Allenopithecus nigroviridis</i>	Allen's swamp monkey	18/44 (40.9%)	-	-	18/44 (40.9%)	15/18	-
<i>Cercopithecus ascanius denti ascanius schmidti</i>	Red-tailed monkey	2/41 (4.8%)	-	-	2/41 (4.8%)	1/2	1/35 (2.8%)
-	-	11/33 (33.3%)	0/7 (0.0%)	11/40 (27.5%)	10/11	-	-
<i>Cercopithecus neglectus</i>	De Brazza monkey	1/18 (5.5%)	-	-	1/18 (5.5%)	0/1	-
<i>Cercopithecus nictitans</i>	Greater spot nosed monkey	0/9 (0%)	-	-	0/9 (0%)	-	-
<i>Cercopithecus wolffi</i>	Wolf's monkey	1/20 (5.0%)	1/7 (14.3%)	0/9 (0.0%)	2/36 (5.5%)	1/2	0/1
<i>Cercopithecus hamlyni</i>	Hamlyni's monkey	-	0/5 (0.0%)	-	0/5 (0.0%)	-	-
<i>Cercopithecus lhoesti</i>	L'Hoest monkey	-	0/30 (0.0%)	0/6 (0.0%)	0/36 (0.0%)	-	-
<i>Cercopithecus mitis</i>	Blue monkey	-	8/25 (32.0%)	0/20 (0.0%)	8/45 (17.8%)	8/8	-
<i>Cercocebus agilis</i>	Agile mangabey	1/3 (33.3%)	-	-	1/3 (33.3%)	1/1	-
<i>Colobus guereza</i>	Mantled guereza	0/1 (0.0%)	-	-	0/1 (0.0%)	-	-
Total		23/136 (16.9%)	20/100 (20.0%)	0/42 (0.0%)	43/278 (15.4%)	36/43 (83.7%)	1/36 (2.7%)

-: Not applicable because samples non available;

* : LTR sequences were only attempted on tax positive samples.