Full Genome Characterization of a New Simian Immune Deficiency Virus Lineage in a Naturally Infected *Cercopithecus ascanius whitesidei* in the Democratic Republic of Congo Reveals High Genetic Diversity Among Red-Tailed Monkeys in Central and Eastern Africa

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Abstract

Our knowledge on simian immune deficiency virus (SIV) diversity and evolution in the different nonhuman primate species is still incomplete. In this study, we report the full genome characterization of a new SIV from a red-tailed monkey (2013DRC-I8), from the *Cercopithecus ascanius whitesidei* subspecies, in the Democratic Republic of Congo (DRC). The new full-length genome is 9,926 bp long, and the genomic structure is similar to that of other SIVs with the absence of *vpx* and *vpu* genes. The new SIVasc-13DRC-I8 strain fell within the *Cercopithecus* specific SIV lineage. SIVasc-13DRC-I8 and previously reported SIVrtg from the *C.a. schmidt* subspecies in Uganda did not form a separate species-specific SIV lineage. These observations provide additional evidence for high genetic diversity and the complex evolution of SIVs in the *Cercopithecus* genus. More studies on a large number of monkeys from a wider geographic area are needed to understand SIV evolution.

Keywords: SIV, HIV, *Cercopithecus ascanius*, red-tailed monkey, Democratic Republic of Congo

TODAY, SIMIAN IMMUNE deficiency viruses (SIVs) have been described in numerous African nonhuman primate (NHP) species. Serological evidence has been shown in at least 45 different NHP species, and SIV infection was confirmed by viral sequence analysis in the majority.^{1,2} Despite the fact that each NHP species is generally infected with a species-specific SIV lineage, the overall genetic diversity among SIVs is extremely complex. There are many examples of coevolution between viruses and their hosts, but cross-species transmission of SIVs between primates living in the same habitats or in polyspecific associations followed or not by recombination between distant SIVs is not exceptional.^{1,2}

HIV/AIDS emerged from SIVs from central chimpanzees (*Pan troglodytes troglodytes*) and western lowland gorillas (*Gorilla gorilla gorilla*) from Cameroon that have been transmitted to humans on at least four occasions leading, respectively, to HIV-1 groups M and N and HIV-1 groups O and

P.^{3,4} SIVs infecting sooty mangabeys (*Cercocebus atys*) from West Africa crossed the species barrier on at least nine independent occasions leading to HIV-2 groups A to I.⁵ 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. Previous studies on NHP bushmeat in West and Central Africa showed ongoing exposure of humans to SIVs.⁶⁻⁹ These studies contributed also for a large amount to the identification and characterization of new SIV lineages in wild primate populations. However, with the exception of SIVs in chimpanzees and gorillas, our knowledge on SIV diversity and evolution is still incomplete because only a limited number of samples have been studied from a limited number of geographic regions.

Between 2009 and 2015, we analyzed more than 600 NHP bushmeat samples collected as dried blood spots (DBS) in six different localities in the Democratic Republic of Congo

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FIG. 1. Geographical distribution of the different *Cercocebus ascanius* subspecies in Central and Eastern Africa. The locations of SIV positive animals from this and previous studies are highlighted with black dots. Adapted from Gautier-Hion A, Colyn M, Gauthier JP. Histoire Naturelle Des Primates d'Afrique Centrale. Backhuys Publishers; Netherlands, UK: 1999. Li-breville Ecofac.

(DRC) or as tissue samples around Kole, the Oriental Kasai Province in south central DRC.^{7,10} Samples were obtained from 13 different monkey species, and red-tailed monkeys were widely present, representing 35% of the NHP bushmeat samples. During these previous surveys, a total of 234 DBS samples and 35 tissue samples were collected from this species. We previously reported that 50 (21.4%) of the 234 DBS samples had SIV/HIV cross-reactive antibodies when tested with the recently described HIV/SIV multiplex microbead immunoassay using the Luminex technology.^{7,11} More detailed analysis showed the presence of two subspecies, *Cercocebus ascanius schmidtii* in eastern DRC ($n=40$) and *C. ascanius whitesidei* in West and Central DRC ($n=229$). The 40 samples obtained from the *C.a. schmidtii* subspecies were negative, but infection rates among the *C.a. whitesidei* sub-

species were high and varied between 17% and 34% across the four sites.^{7,10} Unfortunately, due to low quantity of blood and low quality of samples collected as bushmeat, SIV infection could only be confirmed in four DBS samples and in a small region of the *pol* gene only.¹⁰ However, screening of the 36 tissue samples collected from *C. ascanius whitesidei* around Kole in Central DRC, with universal HIV/SIV primers in *pol* (integrase), was more successful, and new SIV sequences were obtained from 11 (30.2%) monkeys.⁷ Phylogenetic tree analysis of the partial *pol* sequences showed that they all fell in a single cluster different from SIVrtg recently described from the *C. ascanius schmidtii* subspecies from monkeys living in the Kibale national Park in Uganda, eastern Africa.^{10,11}

In this study we report the full genome characterization of an SIV from one red-tailed monkey (2013DRC-I8), from the *C.a.*

FIG. 2. Similarity plots of nucleotide sequences illustrating the extent of genetic diversity between the new SIVasc-13DRC-I8 strain and other SIV lineages. The y-axis indicates the % similarity between the query sequence (SIVasc-13DRC-I8) and the other sequences. Similarity analysis was performed on the nucleotide alignment using the SIMPLOT package version 3.5.1¹⁵ using a sliding window of 400 nucleotides (nt) moved in steps of 30 nt. Only SIV lineages of interest are retained in the figure for clarity.

whitesidei subspecies, sampled in 2013 around Kolo in the DRC (Fig. 1). DNA was extracted from 10 mg of liver by using the Qiagen Blood and Tissue Kit (Qiagen SAS, Courtaboeuf, France), following the manufacturer's recommendations. Only the incubation time of the viral lysis step was slightly modified to increase DNA release and was extended to 3 h. The full genome sequence of the new SIVasc-13DRC-I8 strain was obtained by amplifying and sequencing eight overlapping polymerase chain reaction (PCR) fragments as previously described.^{12,13} Primers used to obtain full-length SIV sequence are provided as supplementary information (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/aid). PCR amplifications were performed using the Taq DNA Polymerase Core Kit (MP Biomedicals, Irvine, CA) or Expand Long Template PCR system (Roche Diagnostics, Indianapolis, IN) for long fragments, following manufacturers' instructions. Each amplification reaction included a

manual hot start at 94°C for 3 min, followed by 35–45 cycles with a denaturation step at 92°C for 20 s, an annealing temperature set according to the primers' melting temperatures, and a variable extension time depending on the size of the expected fragment (1 min/kb). PCR products were purified on agarose gel (1%) and directly sequenced using cycle sequencing and dye terminator methodologies (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq FS DNA polymerase) on an automated sequencer (ABI 3130XL; Applied Biosystems, Courtaboeuf, France). The full-length genome sequence was reconstructed by assembling overlapping sequences into contiguous ones using SeqMan Pro, version 11.2.1 software (DNASTAR, Madison, WI).

The new full-length genome is 9,926 bp long, and the genomic structure is similar to that of other SIVs, that is, presence of the structural genes (*gag*, *pol*, and *env*) and accessory genes (*vif*, *vpr*, *tat*, *rev*, and *nef*), but with the absence of *vpx* and *vpu*

FIG. 3. Phylogenetic relationships of the new SIVasc-13DRC-I8 strain to previously characterized SIVs in *gag* (1,115 bp) (A), *pol* (2,780 bp) (B), and *env* (1,440 bp) (C) genes. The trees show SIVasc-13DRC-I8 and other SIVs from the *Cercopithecus* specific SIV lineage. Trees were inferred by ML methods using PhyML.¹⁶ Asterisks on branches represent bootstrap values >80% from 1,000 pseudoreplicates. Scale bars are in substitutions per site. SIVasc and SIVrtg from *C. ascanius* species are highlighted in gray, and the new SIVasc-13DRC-I8 is highlighted with gray box. ML, maximum likelihood.

genes. Nucleotide and protein sequences were aligned using the online version of MAFFT (<http://mafft.cbrc.jp/alignment/server>). Alignments were subsequently edited manually with MEGA, and sites that could not be unambiguously aligned were excluded.¹⁴ The nucleotide and predicted protein sequences encoded by SIVasc-13DRC-I8 were compared to SIVrtg from red-tailed monkeys from Uganda and representatives of all known HIV/SIV lineages. SIVasc-13DRC-I8 fell within the *Cercopithecus* specific SIV lineage. Similarity plot analysis was performed with SimPlot version 3.5.1¹⁵ and did not reveal recombination with other SIV lineages. The analysis showed also that SIVasc-DRC-I8 was only distantly related to SIVmus-1,-2,-3, SIVgsn, SIVmon, and SIVrtg infecting mustached, greater spot-nosed, mona, and red-tailed guenons from Uganda, respectively (Fig. 2).

Evolutionary trees from *gag*, *pol*, and *env* nucleotide sequences were constructed to estimate the phylogeny of the new SIVasc-13DRC-I8 strain in relation to other primate lentiviruses in these three main genome domains. Phylogenies were inferred by both maximum likelihood (ML) and Bayesian methods using PhyML and MrBayes3.2 softwares, respectively.^{16,17} Both, Bayesian and ML phylogenies showed that SIVasc-13DRC-I8 and SIVrtg formed separate lineages within the *Cercopithecus* specific SIV lineage and fell within the cluster of SIVgsn/mus/mon and SIVtal sequences (Fig. 3). With the exception of *gag*, SIVrtg and SIVasc-13DRC-I8 were ancestral to SIVgsn/mon/mus in *pol* and *env*. In these latter regions, SIVasc-13DRC-I8 was ancestral to SIVgsn/mon/mus, and SIVrtg was ancestral to all. Overall, SIVasc-DRC-I8 and SIVrtg did not form a separate species-specific SIV lineage. Functional motifs in Env and Gag, characteristic to SIVs from the *Cercopithecus* specific SIV lineage, were also present in SIVasc-DRC, that is, 18 cysteine residues in the extracellular glycoprotein gp120, as

well as the PT/SAP and YPXL budding motifs in the Gag protein.¹⁸ In the envelope, the SIVgsn/mon/mus lineage is the ancestor of the SIVcpz/SIVgor lineage and they share a feature in the C1 domain of gp120 that is absent in other SIV lineages, that is, a 7-amino acid insertion.¹⁹ SIVasc-13DRC-I8 presents an insertion of only four amino acids, while the SIVrtg presented none (Supplementary Fig. S1). These different protein motifs indicate that, as observed with phylogenetic analyses, SIVasc-13DRC-I8 has an intermediate position between SIVrtg and SIVgsn/mon/mus lineages.

Red-tailed monkeys are widespread across Central and Eastern Africa, and five different subspecies have been reported, each with a different geographic distribution (Fig. 1). In this study we described for the first time the full-length SIV sequence in a red-tailed monkey from the *C. ascanius whitesidei* subspecies in the DRC. Overall our study confirms the high genetic diversity of SIVs in red-tailed monkeys previously observed on partial SIV *pol* sequences obtained from two subspecies in DRC and Uganda.^{7,11} The high genetic diversity is most likely due to the geographic distance (>1,000 km between Kileleshwa in DRC and Kibale in Uganda) and the fact that they are derived from two different subspecies which have been separated by geographic barriers, like the Congo river, for a long period of time. Moreover, the SIVrtg sequences have been obtained from monkeys living in the extreme east of the geographic range (Fig. 1). A partial SIV sequence, 1,800 bp in *pol* referred to as SIVasc-Qu, has also been obtained from a captive red-tailed monkey, from the *C.a.schmidti* subspecies, in a Zoo in the Netherlands, but the precise initial geographic origin in Africa of this sample was not reported.²⁰ We also obtained 1,800 bp sequences in *pol* for three additional samples from red-tailed monkeys from DRC, collected in the same area as SIVasc-2013DRC-I8. Phylogenetic tree analysis showed that SIVasc from DRC

FIG. 4. Phylogenetic relationships of the new SIVasc-13DRC-I8 strain and three other SIVasc strains from DRC, SIVrtg, and SIVasc-Qu to previously characterized SIVs in *pol* (1,800 bp). Trees were inferred by ML methods using PhyML.¹⁶ Asterisks on branches represent bootstrap values >80% from 1,000 pseudoreplicates. Scale bars are in substitutions per site. SIVasc and SIVrtg from *C. ascanius* species are highlighted in gray, and the new SIVasc-13DRC-I8 and other new SIVasc strains from DRC are highlighted with gray box.

and SIVrtg from Uganda each formed separate clusters and that SIVasc-Qu did not cluster with the other SIVs from red-tailed monkeys (Fig. 4). The genetic diversity of SIV in red-tailed monkeys is thus very high not only within a subspecies but also among the different subspecies. Like many other arboreal *Cercopithecus* species, red-tailed monkeys live also in polyspecific associations, and cross-species transmission followed by recombination can thus contribute to the SIV diversity in this species as is observed for SIVmus/gsn in mustached (*C. cephus*) and greater spot nosed (*C. nictitans*) monkeys in West Central Africa.¹⁹ Our study provides additional evidence for high genetic diversity and the complex evolution of SIVs in the *Cercopithecus* genus; only more studies on a large number of monkeys from a wider geographic area will allow to understand the evolution of SIVs.

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Sequence Data

The novel SIVasc nucleotide sequences were deposited in GenBank under the following accession nos. KY497574 (SIVasc-13DRC-I8), KY497571 (SIVasc-15DRC-AS4), KY497572 (SIVasc-13DRC-II1), and KY497573 (SIVasc-15DRC-OK3).

Author Disclosure Statement

No competing financial interests exist.

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