Non-invasive follow-up of simian immunodeficiency virus infection in wild-living non-habituated western lowland gorillas in Cameroon.

Running title: SIVgor evolution and transmission in wild gorillas

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Abstract

Simian immunodeficiency viruses infecting western lowland gorillas (SIVgor) are closely related to HIV-1 and are most likely the ancestors of HIV-1 groups O and P. Today limited data are available on genetic diversity, transmission, viral evolution, and pathogenicity of SIVgor in its natural host. Between 2004 and 2011, 961 putative gorilla fecal samples were collected at the Campo Ma'an National Park, Cameroon. Among them 16% cross-reacted with HIV-1 antibodies, corresponding to at least 34 infected gorillas. Combining host genotyping and field data, we identified four social groups composed of seven to fifteen individuals with SIV rates ranging from 13% to 29%. Eleven SIVgor infected gorillas were sampled multiple times, two most likely seroconverted during the study period, showing that SIVgor continues to spread. Phylogenetic analysis of partial env and pol sequences revealed cocirculation of closely related and divergent strains among gorillas from the same social group indicating SIVgor transmissions within and between groups. Parental links could be inferred for some gorillas infected with closely related strains suggesting vertical transmission, but horizontal transmission, by sexual or aggressive behavior, was also suspected. Intra-host molecular evolution in one gorilla over a 5-year period showed viral adaptation, characteristic of escape mutants, i.e. V1V2 loop elongation and increased number of glycosylation sites. Here, we show for the first time the feasibility of non-invasive monitoring of non-habituated gorillas to study SIVgor infection over-time both at the individual and population levels. This approach can also be applied more generally to study other pathogens in wildlife.
INTRODUCTION

Chimpanzees and gorillas are the only non-human primates known to harbor viruses closely related to HIV-1 (22, 42, 58). Phylogenetic analyses showed that gorillas acquired SIVgor from chimpanzees (52), and SIVcpz/SIVgor viruses have been transmitted to humans on at least four occasions leading to HIV-1 groups M, N, O, and P. West central African chimpanzees (Pan troglodytes troglodytes) infected with SIVcpzPtt in southern Cameroon are recognized as the reservoir of the ancestors of pandemic HIV-1 group M and of HIV-1 group N (22). SIVgor from western lowland gorillas (Gorilla gorilla gorilla) is closely related to the two other HIV-1 lineages, group O and group P (36, 58). Group O represents 1% of HIV-1 infections in West central Africa and group P has been recently described in two Cameroonian patients (41, 44, 54, 59). The direct ancestors of HIV-1 group O have not been identified yet, but the short genetic distance between HIV-1 group P and SIVgor suggests that a zoonotic transmission of SIV from gorillas to humans has occurred (36, 44).

SIVgor infection was reported for the first time in 2006, in fecal samples from three gorillas, living about 400 km apart in Cameroon (58). Between 2006 and 2008, a comprehensive survey was conducted to determine the geographic distribution and prevalence of SIVgor in wild gorilla populations in southern Cameroon (36). This study was conducted on fecal samples from more than 1200 gorillas from 21 locations, and showed that SIVgor infection is much less widespread than SIVcpzPtt infection in chimpanzees. SIVgor infection was found only at three sites, whereas SIVcpzPtt infection was identified at ten locations. Moreover, the overall SIV prevalence in gorillas was 1.6% (ranging from 0% to 4.6%), significantly lower than the average prevalence of 5.9% (ranging from 0% to 32%) obtained for chimpanzees. However, a closer look at the locations where the SIVgor ratio reached almost 5% showed that a quarter of the individuals belonging to selected social groups were infected with this virus.
Our knowledge on the consequences of SIV infection on the health of wild-living ape populations is limited to a few studies on chimpanzees, and today we have no information on the impact of SIVgor infection on gorillas. Only one long-term study, initiated more than ten years ago on a few habituated communities of East African chimpanzees (*P.t.schweinfurthii*) living in Gombe National Park, Tanzania, provided evidence that SIVcpzPts infection has a negative impact on the health, reproduction, and survival of chimpanzees in the wild, and can cause the decline of chimpanzee populations (21, 46). SIVcpzPtt infecting *P.troglodytes* can also lead to an AIDS-like disease in this subspecies, as it has been documented in a recent report of a naturally infected chimpanzee rescued in Cameroon (13). Since gorillas have acquired SIV only recently by cross-species transmission from chimpanzees (52), we can hypothesize that SIV infection may also have a negative health impact on lowland gorilla populations. However, there are no studies to date that have included *G.g.gorilla* habituation to humans and long-term health monitoring of these populations. Studies that characterize in more detail SIVgor infection in its natural host are highly needed, but are particularly challenging in light of the elusive nature of this species, its endangered status, and the constant threat of poaching and human disturbance documented (65). During our previous exploratory surveys, we identified thirteen SIVgor infected gorillas in a relatively small territory of the Campo Ma’an National Park in South-west Cameroon (36). We therefore decided to focus our efforts on the non-habituated gorilla groups living in this area and to determine the feasibility of long-term monitoring of SIV infection in these apes by collecting fecal samples over time and by genotyping the SIVgor positive samples and a subset of negative ones at selected microsatellite loci. This follow-up study allowed us not only to characterize new viral strains but also to document potential routes of viral transmission within and between gorilla groups. Furthermore, sequential sampling of the same infected individuals enabled us to document viral evolution and adaptation. Finally, we show for the first
time that it is possible to sample and re-sample the same gorillas in a non-invasive way and study their viral infection. In the future, information obtained from such long-term studies will be crucial to understand the evolution and pathogenicity of SIVgor, one of the precursors of HIV-1 infection.

**MATERIALS AND METHODS**

**Study site and sample collection**

The Campo-Ma’an National Park was created in 2000 and covers 2,640 km² of primary and mangrove forests in southwest Cameroon, spanning from the Atlantic coast to roughly 100 km inland (64). We conducted our follow-up study in an area of approximately 200 km² at the center of the Campo Ma’an National Park (CP), where previous surveys showed a relatively high rate of SIVgor infection in wild gorillas (36). In this follow-up study, the three previously described zones of collection (CP-GR, CP-OV, and CP-MV) were pooled together and were considered as a single site, because they are located at less than 10 km apart from each other and SIVgor positive individuals were identified in all three locations. A total of twelve sampling sessions were conducted between April 2004 and November 2011, including four previously reported from our prospective studies (36, 58). At each sampling session, three field teams collected fecal samples from gorillas around night nests, on the ground, feeding sites, or directly on track. At each collection, the GPS position and estimated time of fecal deposition were recorded. Fecal samples collected on the same day, at the same GPS location, and with the same estimated time of deposition were defined as a cluster of collected samples and were assumed to be from the same gorilla group, especially at nesting sites (1). Sequential numbers were assigned to these clusters of collected samples during the 7.5-year study period (cluster 01 to n). The species origin was inferred in the field according to cues like the type of nest, footprints, texture, and odor of the
samples. About 20 g of dung were collected in a 50 ml tube, containing 20 ml of RNAlater™ (Ambion, Austin, TX), and were kept at ambient temperature at base camp, for a maximum of three weeks, and then were stored at -80°C.

Detection of SIVgor antibodies in fecal samples from gorillas

All collected fecal samples were tested with the INNO-LIA™ HIV score confirmation test (Innogenetics, Ghent, Belgium) for the presence of HIV-1 cross-reactive antibodies. Briefly, 2 ml of fecal samples were incubated with 7 ml PBS-Tween-20 for 1 h at 60°C, centrifuged at 3,900 g for 10 min to clarify the solution, and dialyzed against PBS overnight at 4°C. One ml of these reconstituted extracts was then subjected to cross-reactive immunoblot analyses (58).

Nucleic acid extraction from ape fecal samples

Total nucleic acids were extracted from all antibody positive fecal samples using the NucliSens Magnetic Extraction kit (Biomérieux, Craponne, France), which utilizes magnetic silica particles (4). Briefly, 1.5 ml of sample were mixed with 7 ml of NucliSens Lysis buffer for 1 min and incubated at room temperature for 1-12 h before centrifugation at 3,900 g for 30 min (36). The supernatant was filtered through gauze and centrifuged at 3,900g for 5 min. The magnetic extraction procedure was then followed according to the manufacturer instructions to obtain a final elution volume of 50 µl fecal RNA. The QIAamp Stool DNA Mini kit (Qiagen, Valencia, CA) was used to extract fecal DNA for species confirmation and microsatellite analyses. Two ml of fecal sample were used to obtain a final elution volume of 100 µl of fecal DNA (22).

Species and sub-species determination
In a previous study, we showed that the species determination in the field matched 97.3% of the time with host mitochondrial DNA (mtDNA) sequence results (36). Nevertheless, for all seropositive fecal samples and a subset of SIV negative fecal samples, the species origin was confirmed by mtDNA analyses, as described previously (22, 55, 58). Briefly, a ~450 to 500 bp fragment spanning the hypervariable D-loop region was amplified using primers L15997 and H16498 and/or a 386 bp fragment spanning the 12S gene using primers 12S-L1091 and 12S-H1478. Phylogenetic analyses of these sequences allowed identification of gorilla samples. If both amplification strategies yielded no results, samples were considered degraded.

Identification of individuals

To identify each SIV positive individual and determine the number of SIVgor infected gorillas, microsatellite analyses were performed on all seropositive samples (36, 58). Furthermore, negative samples collected at the same location as SIV positive samples between April 2004 and November 2010 were also subjected to microsatellite analyses. Samples were genotyped at seven loci in two-multiplex PCRs (D18s536, D4s243, D10s676, D9s922, and D2s1326, D2s1333, D4s1627), and for gender determination, a region of the amelogenin gene that contains a deletion in the X, but not the Y chromosome (50), was amplified using the Taq DNA polymerase Core kit (MP Biomedical, Irvine, CA) with 2 to 10 µl fecal DNA. Homozygous loci were amplified from three to seven times to minimize allelic dropout (3, 35, 51), and when multiplex PCR reactions yielded poor results, fecal DNA was extracted again and a new set of PCRs was performed. We discarded all samples that did not provide successful results after five PCR attempts and two independent DNA extractions. All samples that displayed an incomplete allelic profile (less than four loci), a multiple peak profile for the same locus, or discordant results...
in subsequent analyses were also discarded from further analyses. The allele size data from successful amplifications were compared against each other and with previously genotyped individuals (36, 58).

We used Cervus v3.0 (20) and Genecap v3.1 (62) to assess allele frequency, expected heterozygoty, polymorphic information content (PIC), Hardy-Weinberg (HW) equilibrium, sibling identity, parentage analysis, and to identify SIV positive samples with matching genotypes (5, 19, 37). All genotypes mismatching at one locus were checked for data entry errors or allelic dropout possibilities (17). To guard for potential allelic dropout, we allowed an allelic mismatch at one locus, but only if it represented a missing allele (47). SIV positive matching samples were given a consensus ID number and genotype if their P(ID)\text{sib} value was inferior to 0.05, corresponding to at least 95% confidence that two matching samples originated from the same individual (assuming the individuals from the panel could be full-siblings) (63). Both P(ID)\text{HW} and P(ID)\text{sib} were calculated for each individual (14, 40, 60). P(ID)\text{HW} is the probability that two individuals drawn at random from a population will have the same genotype at multiple loci, whereas the P(ID)\text{sib} assumes that individuals are full-siblings. Here, the P(ID)\text{sib} will be more informative for samples with a possible full-sibling (genetically linked individuals) and for non-related gorillas from the study, the P(ID) to consider is between P(ID)\text{sib} and P(ID)\text{HW}. We assume that the loci we chose for these analyses are inherited independently (i.e. that they are unlinked). For parentage analysis, we first calculated allele frequencies, critical to run simulations and to assess confidence of parentage assignment. Cervus calculates the likelihood ratio which is the likelihood that the candidate parent is the true parent divided by the likelihood that the candidate parent is not the true parent. Cervus calculates this ratios for each candidate parent we selected taking into account possible typing errors. We estimated that we
collected and successfully genotyped half of the offsprings in every group and half of the candidate parents. The frequency of typing errors and the error rate in likelihood calculations were set at 0.01. The minimum number of typed loci was set at four out of seven and the proportion of loci typed was 0.83. Confidence in assignment is defined as the proportion of all candidate parents with LOD scores exceeding a given LOD score that are true parents. Simulations of parentage analysis reported that any candidate parent with a LOD score exceeding a critical value of 2.94 is assigned parentage with 95% confidence, and a LOD of 2 with a relaxed confidence of 80%.

**Amplification of partial SIVgor sequences in env and pol conserved regions from fecal RNA**

To amplify SIVgor fragments, RT-PCR reactions were performed from fecal RNA using SIVgor and SIVgor/SIVcpz/HIV-1 consensus primers in *env* (gp41 ectodomain, 315 or 440 bp) and *pol* (245 or 330 bp) as previously reported (36). To increase amplification success, a 200 bp fragment in gp41 was amplified with more specific primers designed from an alignment of available gp41 sequences previously obtained from SIVgor infecting gorillas in Campo Ma’an Park: (F1, 5’-GCT WGC YRT AGA AAG BTA YCT A-3’; R1, 5’-TCC AAT TCY AGY ARG GCY YTT TCA T-3’; F2, 5’-TCC TRG GAC TRT GGG GHT G-3’; and R2, 5’-TCA TTM TYH TCC TGY TGT CCY TGT GC-3’). The reverse transcription was carried out with 10 µl of fecal RNA and 40 pmol of R1 primer, using the Expand Reverse Transcriptase (Roche Diagnostics, Indianapolis, IN) or the Superscript Retro-transcriptase III (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions with minor adjustments as previously described (36). PCR reactions were then performed with the Expand Long Template PCR system (Roche Diagnostics,
Indianapolis, IN) or the Taq DNA polymerase Core kit (MP Biomedical, Irvine, CA, USA) following manufacturers’ instructions. Briefly, 10 μl of cDNA were used for first-round PCR amplifications with R1/F1 primers and 5 μl of the first-round reaction were used for the nested PCR with second round primers, F2/R2. Thermocycling conditions could slightly vary with annealing temperatures ranging from 45°C to 55°C, using a touch-down PCR strategy, and extension times were typically set at 1 min/kb. The resulting amplification products were purified using the Geneclean Turbo Kit (Qbiogene, Inc., Carlsbad, CA) and directly sequenced using an automated sequencer (3130xl Genetic Analyser, Applied Biosystems, Foster City, CA). If at least one sample of a given SIV positive individual yielded RT-PCR positive amplification, we considered that the serological status of this individual was confirmed by viral RNA amplification. We performed two to twelve independent RNA extractions for each SIV positive individual, followed by five to ten RT-PCR attempts for each RNA extract, with varying conditions and reagents, as described above.

**Amplification and molecular cloning of SIVgor gp120 V1V4 region**

To document the viral diversity and evolution in the envelope of SIVgor viruses, we studied the V1V4 region of gp120 (env) of the virus infecting gorilla ID1 over a five-year period. We amplified a fragment spanning the V1V4 env region (~1,000 bp) with specific primers from two samples: SIVgorID1-04CP684 infecting gorilla ID1 in 2004 and SIVgorID1-08CP3425 infecting the same animal in 2008. The primer sets were the following: 684envR1 (5’-CCT TGT AGG GGT ACA ATT CAC TC-3’) and 684/3425envF1 (5’-TGG GAT CAG AGC TTA AAG CC-3’) for the first round SIVgor-04CP684 V1V4 env PCR (~1,150 bp), 684envR2 (5’-TCA GCC AGG GGT GTA TCT CTT T-3’) and 684/3425envF2 (5’-AGC CTT GTG TAA AAC TAA CAG TC-3’) for the second round SIVgor-04CP684 V1V4 env PCR (~1,020 bp);
3425envR1 (5'-ATA GGC CTG AGC CTA CTC TCA T-3') and 684/3425envF1 for the first round SIVgor-08CP3425 V1V4 env PCR (~990 bp), 3425envR2 (5'-ACC TGT TTT AGG CGG CAT GGA A-3') and 684/3425envF2 for the second round SIVgor-08CP3425 V1V4 env PCR (~950 bp). The amplified and gel purified products were cloned with the pGEM-T system following the manufacturer’s instructions (pGEM-T Easy Vector System II, Promega, Madison, WI). We sequenced 20 and 24 SIV resulting clones for SIVgor-04CP684 and SIVgor-08CP3425, respectively, to analyze the viral envelope diversity and evolution.

**Phylogenetic and genetic diversity analyses of SIVgor sequences**

All new SIVgor nucleotide sequences were compared to SIVgor, SIVcpz, and HIV-1 reference sequences available in the database. MEGA5 (53) was used to align sequences and edit them where necessary. Ambiguously aligned sites were excluded from the analyses. We analyzed 220 nucleotides in the partial pol alignment, and 263 bp and 195 bp in gp41 alignments. We used a codon nucleotide alignment of 918 bp for the V1V4 env region. Phylogenies were inferred by maximum likelihood using PhyML (16) with 1,000 bootstrap replicates. According to Topali (34), the best evolution models were HKY85 with a gamma distribution across sites for the small pol and gp41 fragments and GTR+G for the V1V4 alignment. GenBank accession numbers for additional partial pol and gp41 sequences used in comparative analyses are: SIVgor: DJ3795 (FN554935), DJ4099 (FN554936, FN554958), DJ4112 (FN554957), BQ664 (AM296484, AM296488); SIVcpzPt: MB66 (DQ373063); SIVcpzPts: TAN1 (AF447763); HIV-1 group O: MVP5180 (L20571), ANT70 (L20587); HIV-1 group P: RBF168 (GQ328744), U14788 (HQ179987).
Viral diversity of SIVgor-04CP684 and SIVgor-08CP3425 V1V4 env region in 2004 and 2008, respectively, was determined by the mean pairwise nucleotide and amino acid distances between env clones at each time point using MEGA5 with the maximum composite likelihood method; the minimum, maximum, and standard deviation were also calculated. The cumulative number of non-synonymous and synonymous nucleotide substitutions (dN and dS) was estimated using SNAP (23). Amino acid sequence length and the number of putative N-linked glycosylation sites (PNGS) of SIVgor-04CP684 and SIVgor-08CP3425 VIV4 clones were calculated and the means at each time point were compared. Mann-Whitney test was used to assess statistical differences for viral diversity, sequence length, and PNGS between 2004 and 2008.

**Nucleotide sequence accession numbers.** All of the new SIVgor sequences are available at GenBank under accession numbers JQ924140 to JQ924164 for env V1V4 clones, JQ924165 to JQ924181 for env (gp41), and JQ924182 to JQ924187 for the pol fragment.
RESULTS

Detection of 34 SIV infected wild-living gorillas during a long-term non-invasive monitoring in the Campo Ma’an National Park

In two previous studies, we identified thirteen SIV infected gorillas in the center of the Campo Ma’an National Park in southwest Cameroon: two individuals were detected between April 2004 and March 2006 (58) and eleven in subsequent field surveys between June 2006 and April 2008 (36). Given the relatively high SIVgor rate previously observed in some gorilla groups, we increased our sampling efforts in this region. Since April 2004, a total of 1,001 putative gorilla fecal samples were collected during twelve independent missions. Gorilla dung was collected as clusters of samples at nest sites (50 clusters ranging from 2 to 28 fecal samples per cluster), at feeding sites or on track (67 clusters ranging from 2 to 20 fecal samples), and 46 individually isolated fecal samples were also collected. The host species was confirmed by mtDNA analyses on 437 (44%) out of 1,001 putative gorilla fecal samples. Fifteen samples were degraded and 25 were incorrectly assigned in the field (i.e. chimpanzee, monkey, African buffalo, deer, elephant). The results showed 94% concordance between field observations and host mtDNA analysis.

A total of 152 samples (16% of the remaining 961 putative gorilla fecal samples) cross-reacted with HIV-1 antibodies in serological analyses; 34 came from previous studies (36, 58) and 118 were obtained during the follow-up missions that started at the end of 2008. To ascertain how many individuals were infected with SIVgor, we ran host microsatellite analyses for seven selected loci. We also determined the sex of each SIV positive gorilla sample. Overall, the microsatellite loci used were polymorphic (mean polymorphic information content (PIC), 0.73), with a mean number of alleles per locus of 8.86, and an observed heterozygosity ranging from
0.76 to 0.9 (Table S1). For 131 out of 152 positive fecal samples usable genotypes were obtained, resulting in an 86% success rate for these SIV positive samples. We compared and combined the locus profiles of the 131 samples. Table 1 shows for each SIV infected gorilla, the consensus genetic profile and the matching probabilities, \( P(\text{ID})_{\text{HW}} \) and \( P(\text{ID})_{\text{sib}} \), including the thirteen previously reported individuals (58). We found that at least 34 SIV positive gorillas were circulating in the studied area.

**High SIVgor infection rates in gorilla groups**

To estimate infection rates in gorilla groups, we tried to assign individuals to social gorilla groups by combining microsatellite analysis and field data as previously described by Arandjelovic and colleagues (1). Overall, samples collected on the same day, at the same GPS location, and with the same time of deposition were assumed to belong to individuals from the same group. When two or more individuals from a same cluster of samples were consecutively found together, all the individuals from the different collections were considered to be from the same gorilla social group. In total, we performed microsatellite analysis on all SIV negative samples from 31 different clusters of collection in which SIV positive samples were identified. Complete genotypes were only obtained for all samples from nineteen clusters, while the remaining twelve included also degraded or mixed samples.

Our analyses showed that one cluster of three samples collected around one night nest, corresponded to a single SIVgor positive male, ID38, probably a solitary adult male. We identified four social groups with SIVgor positive gorillas: group A and C, already reported in 2007 (36), and group E and G, newly identified in this study (Figure 1). In addition, we identified three putative social groups, F1 to F3; groups F2 and F3 collected in 2009 and 2010, respectively,
each shared one individual with group F1 collected in 2006 (ID2 and ID36, respectively) (Figure 319). However, we considered these three groups as putative separate groups since they shared only one individual. Furthermore, given the three to four year gap of collection, the social structure of these groups may have changed.

As summarized in Figure 1, among the four identified social groups, three were detected on multiple occasions, up to seventeen times on different track or nest sites. The putative F1 to F3 social groups were each sampled twice during the same mission. Interestingly, group A was sampled in 2007, 2010, and on two occasions in 2011 with at least seven linked individuals (ID4+, ID5+, ID61, ID16, ID17, ID18, ID19), and was composed of a total of fifteen individuals between 2007 and 2010. Data on the number and linked individuals were not available for this group in 2011. Group E was detected in 2004 and 2008; two linked individuals (ID1+ and ID32+) and a total of seven individuals. The minimum number of gorillas for groups A, C, E, and G ranged from seven to fifteen individuals and the three putative social groups had between two and seven individuals. The ratio of SIV infected individuals in identified social groups ranged from 13% (group G with one female ID33 infected out of eight individuals sampled in this group) to 29% (group E with two infected individuals out of seven gorillas). These SIV rates were estimates based on the detected gorillas; we cannot exclude that some individuals were not sampled during our survey. Despite a possible sampling under-representation, we observed a trend suggesting that the ratio of infected versus the total number of females in a social group was generally higher than the ratio of infected versus total number of males (Figure 1).

Group C was detected only once during our study. However, one SIVgor positive individual from this group (ID10) was sampled again five years later (November 2011) at a
nesting site with SIVgor positive individuals (ID75, ID76, and ID77) not found in group C in
2006. Either the animal, ID10, moved to another group, or we did not sample all individuals from
this group in 2007 and in 2011. Based on the GPS information collected in the field, we could
determine the minimum home range of gorilla groups that were sampled at least twice. As shown
in Figure 2, we found that the different gorilla groups with SIVgor positive animals have
overlapping home ranges.

Capture and re-capture of SIVgor negative and positive animals suggests ongoing
infection and transmission in the wild

In addition to some of the SIVgor infected gorillas from the above-mentioned groups,
more gorillas were sampled on two or more occasions. First, during a single survey, between one
and nine fecal samples from the same animal could be collected. More importantly, eleven of the
34 SIVgor positive gorillas were sampled on at least two independent field missions (Figure 3,
Table 2). Nine were collected and identified as positive during a first collection trip and were re-
sampled on subsequent field missions one and a half to five years later as summarized in Table 2.
Two other SIV positive gorillas were sampled in two independent field surveys, but SIV cross-
reactive antibodies were only detected in the follow-up sample; ID32 was SIV negative in April
2004 but SIV positive in December 2008 (almost five years later) and ID36 was SIV negative in
April 2006 but SIV positive in September 2009 (three and a half years later) (Figure 3, Table 2).
These negative serological observations with the INNO-LIA test were confirmed, for both
individuals, by Western-blot analyses (58) and importantly by SIVgor specific RT-PCR tests
(Table 1). Repeated PCR attempts on the antibody negative samples were negative, while the
virus could be amplified and sequenced from the antibody positive samples. These two gorillas
most likely seroconverted during the study period, indicating that SIVgor continues to spread in the Campo Ma’an National Park.

High genetic diversity of SIVgor strains circulating in gorillas from the Campo Ma’an National Park

We tried to amplify fragments of SIVgor in \textit{pol} and gp41 regions with SIVgor consensus and specific primers from all SIV seropositive samples. In total, we amplified and sequenced SIVgor fragments for 21 of the 34 identified SIVgor positive individuals (in bold in Table 1). For the remainders, SIV amplifications were unsuccessful, possibly as a result of viral nucleic acids degradation and/or because of the low viral shedding in feces. SIV amplification rates were variable and ranged from 0\% for antibody-positive gorillas sampled in November 2007 and April 2008 to 100\% in SIV positive gorillas collected in April 2004, April 2006, December 2008, and September 2009.

We compared phylogenetic relationships between new SIVgor viruses and previously characterized SIVgor, SIVcpz, and HIV-1 strains in \textit{pol} (alignment of 202 bp with 11 SIVgor strains) and in gp41 (alignments of 263 bp (without gaps) and 195 bp with 19 and 21 SIVgor strains, respectively). The phylogenetic tree of the 263bp gp41 fragment is shown in Figure 4A, and the phylogenetic tree analysis of the 195bp gp41 fragment and with the highest number of SIVgor strains is shown in Figure 4B. The phylogenetic tree from the partial \textit{pol} alignment is shown in Figure S1. The length of the fragment analyzed in Figure 4B was too short to obtain well supported clusters or to show any phylogeographic clustering, as previously reported for SIVgor strains from different locations in Cameroon (CP, DJ, BQ) and as observed in Figure 4A. Overall, we observed a high genetic diversity among SIVgor viruses infecting gorillas from the
Campo Ma’an National Park, with a maximum genetic distance of 0.403 in the gp41 fragment. Generally, viruses infecting individuals belonging to the same collection cluster or gorilla group were closely related with a low genetic distance (<0.05) as in the case for ID4 and ID5 from group A, ID9 and ID10 from group C, or ID1 and ID32 from group E (Figures 4A and 4B).

However, certain viruses obtained from animals from different groups were also closely related as observed for individuals from group E (ID1 and ID32) and G (ID33), or for group A (ID4 and ID5) and for some individuals from the putative groups F1 (ID36) and F2 (ID37) (supported with bootstrap values >80%) in Figure 4A). Similarly, we also observed divergent SIVgor strains co-circulating in the same collection clusters; for example, in the putative group F2 (Figure 4A), two females (ID2 and ID36) were infected with divergent SIVgor strains (genetic distance > 0.20) and each clustering in well separated clades supported with bootstrap values > 80% each. Also, in 2011, one male (ID35) and one female (ID73), sampled at the same nesting site (cluster 61), were infected with divergent strains (genetic distance > 0.20) (Figure 4B). Finally, ID10, infected with a strain closely related to SIVgor from ID9 collected at the same nesting site in 2007 (social group C), was re-sampled in 2011 at a new nesting site (cluster 64) together with the female ID76 infected with a more distantly related strain (Figure 4B).

**Suspected vertical and horizontal SIVgor transmissions within and between gorilla groups**

Combining phylogenetic analysis of the SIVgor strains and genotyping of the gorillas together with field data allowed us to trace potential transmission routes. The phylogenetic trees showed closely related viruses that suggested potential epidemiologic links between the infected animals. As mentioned above, some were derived from individuals from the same social group,
but they could also concern animals from different groups. We looked more in detail to the microsatellite analyses of the individuals infected with closely related viruses and performed statistical analyses to examine whether parentage relations existed. In the clade of viruses derived from ID4, ID5, ID36, ID38, ID73, and ID66 (Figure 4A (bootstrap >80%) and 4B), we observed that certain individuals shared one allele at each locus and a parent-offspring relation was inferred between ID4 and ID5 (both females; LOD=2.66, 80% confidence) and ID5 and ID73 (both females; LOD=3.12, 95% confidence). Similarly, in the clade of viruses derived from ID9, ID10, ID1, ID30, ID31, ID32 and ID33 (Figure 4A, bootstrap >80%), parent-offspring relations were seen between ID1 and ID32 (female and male, respectively; LOD=2.32, 80% confidence), ID1 and ID33 (both females; LOD=4.93, 95% confidence) and between ID10 and ID32 (female and male, respectively; LOD=2.98, 95% confidence). Thus, some of these linked viruses could be the results of vertical transmissions from mother to offspring. However, it is important to note that, given the fact that we do not have the age of the individuals, we cannot assess in which direction the parentage link is formed. Furthermore, it is possible that some represent other transmission routes, since closely related viruses were also seen among animals without parental links. The individuals with closely related strains and no parental link acquired their infection by horizontal transmission routes, which could be sexual or also a consequence of aggressive or grooming behavior. Sexual transmission could be suspected between male ID38 and female ID36, and male ID34 and female ID2 each infected with closely related viruses, because they were not displaying any significant level of parental linkage. Two male pairs (ID30/ID32 and ID34/ID70) without parental link and infected with closely related viruses belonged to different groups, suggesting horizontal transmission by blood contact during fights or sexual transmission from the same SIVgor infected female.
Viral adaptation of the SIVgor envelope variable loops over five years of natural infection in a non-habituated gorilla

To study SIVgor evolution over time in its natural host, we focused on the SIV positive individuals sampled in independent field trips (Table 2). Unfortunately, we could not characterize the virus infecting ID65 and we amplified an SIV for ID2, ID10, and ID37 at one time point only (Table 1), probably because of sample degradation. Samples from ID1, ID4, ID5, ID35, and ID66 were amplified from collections of different follow-up surveys (Table 1). The gp41 fragments of the SIVs infecting each of them one to five years apart were highly similar, as demonstrated by the phylogenetic analyses (Figure 4) and by the short genetic distance separating them in gp41 fragment (0.010 to 0.075). We did not observe any evidence of superinfection in these gorillas, but considering the low viral load in fecal samples, variants circulating at low concentration could have been undetected.

To study the diversity and evolution of the hypervariable region of the viral envelope over the course of infection, we amplified and sequenced 20 and 24 molecular clones of gp120 V1V4 env region (~1,000 bp) of the viruses infecting ID1 in April 2004 and in November 2008, SIVgor-04CP684 and SIVgor-08CP3425, respectively. The 20 clones of SIVgor-04CP684 V1V4 region were 100% similar along the 1,020 bp fragment. The 24 clones obtained from SIVgor-08CP3425 displayed very little variability with a nucleotide diversity of 0.005 (min 0, max 0.011, SD 0.001) and an amino acid diversity of 0.010 (min 0, max 0.027, SD 0.002). These observations reflected more the limit of the low viral load in fecal samples than the actual viral diversity of viruses infecting gorillas. The calculation of the dN/dS ratio did not show evidence of positive selection (dN/dS<0) but was not considered informative since the clones displayed low
or no genetic diversity as a consequence of the low viral load in fecal sample RNA extracts. The comparison of the SIVgorID1 V1V4 regions after almost five years of infection revealed an extensive elongation of 24 amino acids (from the first cysteine of V1 to the last cysteine of V4: 273 aa in 2004 vs 297 aa in 2008, p<0.0001) (Figure 5). This considerable amino acid elongation was mainly due to an 18 aa extension of the V1 loop. The number of PNGS in the V1V4 region significantly increased between 2004 and 2008, from 17 to 20 PNGS (p<0.0001). Actually, four additional PNGS in SIVgor-08CP3425 (2008) were located in V1 loop (+2), V2 loop (+1), and C3 region (+1), while one PNGS was lost in the V3 loop (-1) (Figure 5). This latter loop was relatively conserved over the years, except for the loss of one PNGS and a V3 crown switch, from a GPL to a GPM motif. In an attempt to deduce the phenotype of these two strains, we used the 11/25 rule (no K or R at positions 11 and 25) and the net charge rule designed for HIV viruses (the net charges in 2004 and 2008 were of two, under the threshold of five). Both methods suggest that the characterized viruses infecting ID1 in 2004 and 2008 were from the R5 phenotype, but further analyses are here needed.
Our goal was to study SIVgor infection over time in non-habituated wild-living gorillas in an area with a relatively high density of SIVgor infected animals. We were able to identify at least 34 SIVgor infected gorillas, characterize additional SIVgor strains, resample SIV positive and negative individuals over time, reconstitute gorilla groups using non-invasive methods among non-habituated animals, and obtained evidence for SIVgor transmissions within and between groups. Finally, we could document the genetic diversity of SIVgor in different gorilla groups and we found viral adaptation in the envelope of SIVgor over time in its natural host.

During the study period, we identified SIV antibodies in 16% of the gorilla samples, corresponding to at least 34 SIVgor positive gorillas living in an area of approximately 200 km² in the center of the Campo Ma’an Park. Similarly as for SIVcpzPtt and SIVcpzPts, SIVgor is also unevenly distributed, but the SIVgor prevalence at a site does not reach the 30% to 40% rates observed in certain central and eastern chimpanzee populations ((22); (57); (25) (26)). For other non-human primate species, prevalences can also vary according to species or geographic localities from 0% to >50% (reviewed in (29)). Importantly, eleven of the 34 gorillas were resampled at different time intervals allowing us to follow infected non-habituated gorillas non-invasively. We were also able to identify four social groups and three putative social groups with SIVgor infected animals by combining microsatellite analysis and field data. Thus, we confirmed, on a 7.5-year survey, the previously reported data from Arandjelovic et al. which showed effectiveness of non-invasive monitoring of wild gorilla groups during a 2-year period with frequent missions (1). During our 7.5-year study, SIVgor positive animals and social groups with SIVgor infected gorillas were detected on up to seventeen occasions (for group A), during the
same or independent missions. Between 2010 and 2011, follow-up missions were conducted at shorter time intervals and, as a consequence, resampling of individuals and groups increased accordingly. The identified social groups had overlapping home ranges and varied in size from two to fifteen individuals with different male/female sex ratios, which is typical of western lowland gorillas (1, 15, 45). We determined the SIV ratio (13 to 29%) in four non-habituated identified social groups. We were also able to identify a single adult male (ID38) infected with SIV, illustrating that migrating individuals infected with SIVgor can be responsible for the spread of the viruses in the area, when fighting for a dominant position or by other routes while transferring in a new gorilla social group (2, 31, 45). Despite previous studies, reporting that the use of RNAlater™ is not optimal for DNA analyses (38), we obtained a microsatellite amplification success rate comparable to previous studies on non-invasive samples showing that storage of fecal samples in RNAlater™ allows for combined studies on the host and its pathogens.

By combining the analyses of viral genetic diversity, the information on the social groups, and the genotypes of the individuals we could infer potential routes of transmission. In a previous study, we reported that SIVgor strains infecting individuals from two groups, identified at nesting sites, were highly similar (36). Here, we confirmed this pattern in two additional groups. However, in this new survey, divergent strains were also found to circulate within groups (three examples found). In fact, there are no limiting natural geographic barriers in the studied area and we found that different groups are circulating with overlapping ranges. These characteristics made possible both male and female transfers (11) and thus SIV strains circulation among various groups of gorillas.
The ratio of infected/total females in a social group was generally higher than the ratio of infected/total males. In gorilla groups, where several females and only one male are sexually active (24, 49), this observation could support the hypothesis that SIVgor transmission between individuals in a group mostly occurs via the horizontal route by sexual contact. Furthermore, we identified two pairs in which sexual transmission was highly suspected since the implicated males and females were not genetically linked but were infected with highly similar viruses. We can thus hypothesize that SIVgor transmission by sexual contact could also drive the epidemic in lowland gorillas. Rudicell and colleagues showed that the SIVcpzPts transmission probability is approximately 0.001 per coital act in Eastern chimpanzees, which is comparable with HIV-1 transmission probability in humans by the same route (46). Nevertheless, horizontal transmission could also occur via blood contact during grooming or aggressive behavior.

Besides from horizontal transmission, we reported cases of suspected vertical transmissions of SIVs in gorillas. Mother to infant viral transmission (MTIT) could occur in utero or by breastfeeding, since gorillas are weaned at approximately four years of age and therefore can be infected after birth (21, 66). Sexual transmission between related individuals (at first degree) is unlikely, as females and males leave their natal group at sexual maturity to avoid inbreeding (12, 24, 49). By deducting microsatellite matches and highly related viruses, we identified five putative vertical transmissions, but because we had no data on the age of apes, it was impossible to ascertain whether we were looking at father/daughter pairs or mother/son pairs, or to discriminate mother or daughters among linked female pairs. Interestingly, for a few of them, the presumed mother/infant pairs involved individuals from different groups, e.g. ID1 (group E) and ID33 (group G), which suggested that the infected infants did not die early of SIV infection and were able to emigrate from their natal groups. This behavior is common in healthy
weaned female and male western lowland gorillas and was reported in several studies (24, 45, 49). Mother to infant transmission (MTIT) in non-pathogenic SIV infection like SIVsm in sooty mangabeys or SIVagm African green monkeys is rarely seen (reviewed by (7)). In contrast, for SIVcpz which is pathogenic in its natural host, vertical transmissions are observed and infants born to SIVcpz positive mothers had a significantly higher mortality rate than others born to uninfected mothers (22). Besides, many naturally SIVcpz positive infected chimpanzees in captivity died as infants upon arrival in the sanctuaries (reviewed in (13)). Further studies are thus needed to confirm the putative vertical transmissions of SIVgor viruses and the survival rate of infected infants, this characteristic being highly informative for the impact of SIVgor on the structure and survival of the gorilla populations. More generally, it would be interesting to better understand if there is a link between SIV/HIV pathogenicity and MTIT.

Since we identified two possible cases of seroconversions during the time of survey, we show here that there are ongoing SIVgor transmissions in wild-living gorilla populations. Nevertheless, only a few samples were available to confirm these seroconversions and, even after PCR amplification controls, we cannot exclude false negative results mainly because we detected SIVgor antibodies in fecal samples by cross-reactivity with the INNO-LIA™ HIV-1/-2 assay. In fact, the assay sensitivity is not determined on SIVgor infection, since there is no captive SIV positive gorilla for comparison. Furthermore, the test was performed on fecal samples with lower sensitivities and which may also have varied antibody shedding over time.

SIVgor viral evolution within its natural host has never been characterized since no SIV naturally infected gorilla has been documented in captivity. Here, we characterized the virus infecting the positive non-habituated gorilla ID1 in April 2004 and in December 2008. The clones obtained from the V1V4 gp120 region (~1,000 bp) were very similar at a given point in time,
showing that the low viral load in fecal samples can be a drawback to non-invasive genetic diversity studies. However, we could compare the strains infecting the animal 4.7 years apart. We showed that the V1V4 region was significantly longer in 2008 comparing to 2004 (+24 aa), mainly due to a longer V1 loop. Furthermore, we could observe a significant increased number of PNGS in the V1V2 region over time. Like SIVcpz in naturally infected chimpanzees (13, 39) or SIVsmm in naturally infected sooty mangabeys (10), SIVgor V1V2 appears to be highly glycosylated. The increasing number of PNGS in the gp120 hypervariable loops of HIV-1 has been associated with the escape of viral mutants (33, 61). Besides, longer HIV-1 V1V2 loops (particularly V1) with increased number of PNGS in Env have been correlated with escape from HIV-1 specific neutralizing antibodies, both at the individual and at a population level (6, 56), possibly by the shielding of envelope epitopes from antibody recognition (8, 30, 43, 48). Furthermore, these V1V2 characteristics were recently associated with disease progression in humans (9). Whether they would also be associated with an AIDS-like disease progression in gorillas still has to be determined. In addition, a switch from a GPL to a GPM motif (found in HIV-1 group O) in the V3 crown was observed. To our knowledge, such a switch or its implication has not been reported in the literature yet, apart from a follow-up study of a SIVcpzPt infection in a naturally infected captive chimpanzee over five years (GPAM to GPGM switch) (13). The role of such mutation in the V3 crown and the biological properties of the corresponding viruses have to be further studied.

In conclusion, our study demonstrates the feasibility of monitoring SIV infection in wild-living non-habituated gorillas over a long period of time in a region of Cameroon where SIVgor prevalence is relatively high. We could not only identify new SIVgor strains during our survey, showing ongoing SIVgor transmissions in the wild, but we also obtained follow-up samples of
infected gorillas and their putative social group from which we could retrieve important information on SIVgor infection rate, routes of transmission, and viral evolution within its natural host. The possibility that SIVgor may be pathogenic for its natural host is of major concern, given the highly endangered status of western lowland gorillas, and can only be studied by non-invasive surveys implemented over a sufficiently long period of time allowing for the documentation of both new SIVgor infections and deaths of infected gorillas. It will also be important to compare SIVgor infection in gorilla versus SIVcpz infection in chimpanzee populations with different social structures which could have different consequences on modes and efficiency of viral transmission, viral evolution at the population level, pathogenicity, and survival of the populations in general. Many different pathogens can be detected in fecal samples, as reported in previous studies (18, 27, 28, 32); coupling long-term monitoring of SIVgor infection with the investigation of the presence of other viruses or parasites will provide us with a better picture on the role that SIV plays on the health and survival of wild gorillas, both at the individual and population level. Finally, non-invasive monitoring can also be applied more generally to study other pathogens in wildlife.
We thank the staff and the SIV team from PRESICA for logistical support in Cameroon; the Cameroonian Ministries of Health, Environment and Forestry, and Research for permission to collect samples in Cameroon. The study was supported by grants from National Institutes of Health (R01 AI50529), the Agence Nationale de Recherches sur le SIDA, France (ANRS 12125, ANRS 12182 and ANRS 12255) and the Institut de Recherche pour le Développement (IRD). Lucie Etienne is supported by a PhD grant from Sidaction and Fonds de dotation Pierre Bergé.
FIGURES LEGENDS

Figure 1. The inferred composition of gorilla groups of interest at Campo Ma’an field site over the 7.5-year study period, underlining the follow-up of SIVgor positive individuals and social groups.

For each studied group, collected individuals are shown with information on sex (F, female; M, male; ?, unknown) and SIV serology (seropositive gorillas appear in bold). The number of feces from each gorilla sampled at a given collection location and date are noted. Putative group F1 is linked with group F3 and group F2 by two independent gorillas, ID2 and ID36, respectively, highlighted by vertical arrows. These later three putative groups could possibly be a single one, but they were analyzed separately because of the lack of certainty. Cluster 64 collected in 2011 is linked with group C by ID10, and cluster 61 collected in 2011 is linked with putative group F2 by ID35. These two individuals could have emigrated from their original group, or clusters 64 and 61 are in fact groups C and F2, respectively. Data from the collections carried out in 2011 are shaded by dots since genotyping data were only available for positive samples.

Figure 2. Inferred minimum home ranges for gorilla groups of interest showing overlapping ranges.

From April 2004 to November 2011, an area of approximately 200 km² was covered during successive field missions in the Campo Ma’an National Park (CP). The scale bar represents 5 km. Inset, map of Cameroon, arrow indicates CP field site. Minimum home ranges inferred from collections sites (small circles) are delineated by plain line ellipses for identified social groups and for the three putative social groups (F1, F2, and F3) (Table 1, Figure 1). The positive individuals and the years of collection are given for each gorilla.
group. The hypothetical home ranges of the possible groups (Group C? ; Group F2? ; Group F??) with linked individuals are highlighted by dashed line ellipses and the corresponding linked IDs are noted (Figure 1).

Figure 3. SIVgor infected gorillas’ capture-recapture in Campo Ma’an study area.

From April 2004 to November 2011, an area of approximately 200 km² was covered during successive field missions in the Campo Ma’an National Park. The scale bar represents 5 km. The eleven SIVgor positive individuals that were captured and recaptured in at least two different collecting missions (Table 2) are plotted and identified by their ID number, and a positive sign when they were found SIV seropositive or a negative sign if they were firstly found SIV negative (ID32 and ID36).

Figure 4. Phylogenetic analyses of partial env (gp41, 263 bp (4A) and 195 bp (4B)) nucleotide sequences of SIVgor strains.

The trees were inferred by maximum likelihood phylogeny (PhyML) with previously characterized SIVcpzPtt/SIVgor/HIV-1 strains (in black). Stars represent the support values above 800 (4A) or 700 (4B) from 1,000 maximum likelihood bootstraps. The scale bar represents 0.1 substitutions per site. Sample number and collection date (i.e. 11-CP6466, sample CP6466 collected in 2011), individual identification (IDs), clusters (i.e. 20, 61, and 64, in grey), and social groups (A, C, G, E, and F1/2/3, in color) are noted and refer to Table 1. The color code for social groups is conserved through Figures 2 to 4 and S1.

Plain brackets highlight individuals from the same group/cluster infected with closely related strains, (very low genetic distance) whereas dash links stand for individuals from the same group/cluster infected with divergent strains.
Figure 5. Amino acid evolution of the SIVgorID1 Env hypervariable region between April 2004 (SIVgor-04CP684) and December 2008 (SIVgor-08CP3425).

The hypervariable loops V1, V2, V3, and V4, and C2 and C3 regions were analyzed. SIVgor-04CP684 sequence (the 20 clones found for SIVgor-04CP684 were 100% similar), indicated at the top (684 clones), was compared with the consensus of all clonal sequences from SIVgor-08CP3425 (3425 clones). The dots stand for gaps, dashes for the same amino acids as above. The putative N-linked glycosylation consensus motifs (NXT/S) are highlighted in grey, important cysteines in bold, and the V3 crown in dashed rectangular stressing the switch from GPL to GPM.
Table legends

Table 1. SIVgor positive gorillas detected in the Campo Ma’an National Park from April 2004 to November 2011.

For each gorilla, an ID number was given with collection information: collected cluster (01 to 64) or social group (A to H), collection details (t, collected on track; 3n, collected on three nests; n, collected on a nesting site but nests were not counted), collection date (mmyy), and number of collected samples (multiple samples could be collected for one individual; SIV sequences are available for samples in bold). The result of the cross-reactive serological test is given (+, positive in the INNO-LIA™ HIV-1/2 serological test; -, negative). Samples confirmed by SIV amplification and sequences are shown in the SIV RT-PCR part (pol, pol fragment amplification; gp41, env fragment amplification; other, other regions in the genome could be amplified and sequenced; nd, not done; FL, full-length (52). A consensus multilocus genotype profile of seven microsatellite loci is given for each gorilla with two allele sizes per locus (-, repeatedly negative) and the sex (F, female; M, male). The maximum P(ID)HW (significant, ** < 10^-6 ; * < 10^-3) and P(ID)Sib (significant, ** < 0.01 ; * < 0.05) are given for each individual and confirm the matches between samples. Eight seropositive samples from the April 2010 field trip that were too degraded to be assigned to an individual are not shown. Samples of poor quality (degraded RNA and/or DNA) appear in grey.
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**Genetic profile (consensus multilocus genotype)**

- **D9S922**
- **D2S1326**
- **D2S1333**
- **D4S1627**
- **Sex**
- **P(ID)**
- **Sib**

- **(1207 bp)**
- **(1207 bp)**
- **(1207 bp)**
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- **Sib**

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**References:**
- [JVI](http://jvi.asm.org)
Table 2. Capture and re-capture of eleven SIV positive non-habituated gorillas in Campo Ma'an Park.

For each SIV infected gorilla, the social group or collected cluster is indicated with its serology status. Details on the collections are given: number of collections in total, dates of collections, and concluded intervals. The location of sample collections are depicted in Figure 3.

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39


## Figure 1

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*Note: Table details are not transcribed here due to the complexity and nature of the data presented.*
Figure 2
Figure 5