New Strain of Simian Immunodeficiency Virus Identified in Wild-Born Chimpanzees from Central Africa

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Abstract

Studies of primate lentiviruses continue to provide information about the evolution of simian immunodeficiency viruses (SIVs) and the origin and emergence of HIV since chimpanzees in west–central Africa (Pan troglodytes troglodytes) were recognized as the reservoir of SIVcpzPtt viruses, which have been related phylogenetically to HIV-1. Using in-house peptide ELISAS to study SIV prevalence, we tested 104 wild-born captive chimpanzees from Gabon and Congo. We identified two new cases of SIVcpz infection in Gabon and characterized a new SIVcpz strain, SIVcpzPtt-Gab4. The complete sequence (9093 bp) was obtained by a PCR-based ‘genome walking’ approach to generate 17 overlapping fragments. Phylogenetic analyses of separated genes (gag, pol-vif and env-nef) showed that SIVcpzPtt-Gab4 is closely related to SIVcpzPtt-Gab1 and SIVcpzPtt-Gab2. No significant variation in viral load was observed during 3 years of follow-up, but a significantly lower CD4+ T cells count was found in infected than in uninfected chimpanzees (p<0.05). No clinical symptoms of SIV infection were observed in the SIV-positive chimpanzees. Further field studies with non-invasive methods are needed to determine the prevalence, geographic distribution, association species, and natural history of SIVcpz strains in the chimpanzee habitat in Gabon.

Citation: Souquière S, Makuwa M, Sallé B, Kazanji M (2012) New Strain of Simian Immunodeficiency Virus Identified in Wild-Born Chimpanzees from Central Africa. PLoS ONE 7(9): e44298. doi:10.1371/journal.pone.0044298

Editor: Zhiwei Chen, The University of Hong Kong, Hong Kong
Received April 18, 2012; Accepted August 1, 2012; Published September 12, 2012

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Funding: The CIRMF is funded by the Gabonese Government, Total-Gabon and the French Foreign Ministry. The funders had no role in study design, data collection or analysis, the decision to publish, or preparation of the manuscript. This does not alter the authors’ adherence to all the PLoS ONE policies on sharing data and materials.

Competing Interests: The authors received funding from a commercial source (Total-Gabon). However, this does not alter the authors’ adherence to all the PLoS ONE policies on sharing data and materials.
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Introduction

Simian immunodeficiency virus (SIV), a member of the Lentivirus genus (Retroviridae), has been isolated from various African nonhuman primates, including Cercopithecidae species and great apes (Pan troglodytes and Gorilla spp.) [1,2]. SIV has been clustered into six distinct lineages [3]. SIV from chimpanzees (SIVcpz) has been found to be genetically related to human immunodeficiency virus (HIV-1), with the same genomic organization [4], and the strong homology suggested that HIV-1 originated from chimpanzees [5]. This hypothesis was strengthened by the identification and characterization of two SIVcpz strains (SIVcpzPtt-Gab1 and SIVcpzPtt-Gab2) from captive wild-born chimpanzees in Gabon [6–9] and of one strain (SIVcpzPtt-US) in chimpanzees from an unknown central African country but kept in captivity in the USA [4]. Another strain (SIVcpz-Ant), which was not related to the HIV-1 group M (pandemic), N or O (nonpandemic), was characterized in a wild-born captured chimpanzee in Belgium [10].

Studies of hundreds of captive wild-born P. troglodytes troglodytes (P.t.t), revealed four SIVcpzPtt strains (SIVcpzPtt-Cam3, Cam5, Cam13 and Cam 155) in Cameroon [11–14]. These SIVcpz sequences clustered with SIVcpzPtt-US and SIVcpzPtt-Gab1. In 1998, identification of the HIV-1 group N strain which does not belong to HIV-1 group M or O, also in Cameroon, provided evidence of a close phylogenetic relation with SIVcpzPtt circulating in the same geographic area [11,12,15].

Analysis of faecal samples from wild-living gorillas (Gorilla gorilla) in Cameroon showed the presence of a new SIV lineage. These SIVgor viruses, forming a monophyletic lineage within the SIVcpzPtt group, suggested that SIVgor resulted from a chimpanzee-to-gorilla transmission [16]. Although there have been a few cases of SIV infection among western lowland gorillas (Gorilla gorilla gorilla), phylogenetic analyses of these SIVgor strains showed their close relation to human HIV-1 group O viruses [16–18]. Recently, a new RBF168 strain prototype of a lineage HIV-1 group P, closely related to SIVgor, was described in an old woman in Cameroon, indicating that gorillas, like chimpanzees, are probable sources of HIV-1 [17,19].

Chimpanzees are classified into four subspecies on the basis of differences in mitochondrial DNA sequences, with a characteristic geographic distribution: P. troglodytes verus (P.t.v) in west Africa, P. troglodytes ellioti (P.t.e) [20] (formerly termed P. t. vellerosus) in Nigeria and northern Cameroon, P. troglodytes troglodytes (P.t.t) in southern Cameroon, Congo and Gabon, and P. troglodytes schweinfurthii (P.t.s) in the Democratic Republic of the Congo and the countries of East Africa [21]. The only two subspecies of chimpanzees found to be infected are P.t.t and P.t.s [4]. Despite extensive testing, naturally
occurring lentiviruses have not been detected in West African chimpanzees (P.t.s or P.t.e), although one P.t.e (Cam 4) contracted SIV from a P.t.t in captivity [12,22,23]. The prevalence rate of SIVcpz varies considerably, ranging from 0 to 50% [22–24]. This finding, combined with the absence of SIVcpz in two of four subspecies, suggests that chimpanzee acquired the virus recently, before their differentiation into subspecies [25].

The origin of SIVcpz itself remains unclear [4,26]. Phylogenetic analyses of the SIVcpz-US strain and seven other SIV lineages revealed that the SIVcpz genome has a recombinant origin. SIVcpz clustered closely with SIVrcm from red-capped mangabeys (Cercopithecus torquatus) in the 5′ half of the genome, in Nef and in 3′LTR, and closely with SIV from several Cercopithecus species (C.nictitans, C. cephus, C. mona) in the env, tat, rev, and nef genes [26,27].

P.t.t in central Africa were thus recognized as a reservoir of SIVcpzPtt viruses, which have been transmitted at least twice to humans, resulting in infections with HIV-1 groups M and N [5,23,25]. Interestingly, the HIV-1 group N is closely related to SIVcpzPtt-EK505 from Dja forest (south-central Cameroon) and HIV-1 group M to SIVcpzPtt-MB/LB from the south-eastern corner of Cameroon [22,23]. The origin of emergences of HIV-1 group O and P remains unclear but was undoubtedly in the same area.

It was reported recently that chimpanzees can develop AIDS after natural infection with SIVcpz. Most of the documented cases have been found in P.t.t subspecies in Gombe National Park in Tanzania [28], resulting in a decline in the chimpanzee population in the area with the highest SIVcpz prevalence [24]. Recently, however, a case of natural SIVcpz infection in P.t.t, with clinical progression to AIDS-like disease, was reported [14].

Since 1994 a large survey of SIV prevalence in non-human primates in Gabon has been undertaken with synthetic peptide-based ELISA containing all known primate lentivirus lineages [29]. We recently found two new cases of SIVcpz in a wild-born orphan chimpanzee in Gabon and in a wild-born chimpanzee in Equatorial Guinea that had been seized as pets in Libreville, Gabon. We present here the characterization of a new SIVcpz strain, SIVcpzPtt-Gab4, isolated from one of these chimpanzees. Additionally, to assess the degree of pathogenicity of natural SIVcpz infection in P.t.t, its clinical and immunological features were investigated.

Results

Serologic Survey and SIV Strains

We screened 104 wild-born chimpanzees in two central African countries, Gabon and Congo, for the presence of SIV antibodies (Table 1). Two sera, one from a chimpanzee (Gab3) in Haut-Ogooué Province and the second from a chimpanzee (Gab4) in Estuaire Province, reacted positively to HIV-1/SIVcpz-specific peptides in a specific peptide-based ELISA (see Methods). Gab3 was only 1 month old when it arrived, in February 2000, at the Primatology Centre (CIRMF), and it died 2 months later of unknown causes. Gab4 was seized with two other chimpanzees from their owner in Libreville in October 2006 for transfer to La Mouila Park (Bakoumba, southern Gabon). Because of its serological status, Gab4 was not introduced into the sanctuary and is now at the CIRMF Primatology Centre. Neither Gab3 nor Gab4 had hepatitis B or C or STLV infection.

Gab4 showed the highest reactivity with SIVcpzPtt-Gab1 and HIV-1 group N-specific peptides derived from and mapping to the env-V3 region and HIV-1 group N-, SIVcpzPtt-Gab1- and SIVcpzPtt-Gab2-specific peptides derived from and mapping to the env-gp114 peptides. The plasma of Gab3 at 1 month of age showed greater reactivity to HIV-1 group M V3 peptide than to HIV-1 group N and SIVcpzPtt-Gab1 peptides and to the peptides mimicking gp41, the highest optical densities were found for HIV-1 group N and SIVcpzPtt-Gab1. Plasma sampled at 2 months showed greater reactivity to SIVcpzPtt-Gab1 V3 peptide and HIV-1 group N gp41 peptide. At 3 months, only weak reactivity was found to HIV-1 group N and SIVcpzPtt-Gab1 gp41 peptides. Analysis of the western blot profile revealed decreasing HIV-1 cross-reactivity with plasma from chimpanzee Gab3. High reactivity was detected on band C, corresponding to sampling at 1 month of age. Subsequent samples corresponding to bands D and E (Gab3 aged 2 and 3 months, respectively) clearly show progressive disappearance of antibodies. The virus could not be isolated or characterized, and western blot analyses suggested passive transmission of maternal SIV antibodies.

Conversely, strong HIV-1 cross-reactivity was observed with plasma from chimpanzee Gab 4, particularly with the HIV-1 gp160 and p24 proteins, whereas the cross-reactivity with the remaining HIV-1 antigens was weaker (Figure 1). The SIVcpzPtt-Gab4 virus was successfully isolated and the complete genome amplified and characterized.

Identification of Chimpanzee Subspecies

To identify the origin and subspecies of our positive animals, the mtDNA fragment spanning the hypervariable D-loop region was characterized phylogenetically. The sequences obtained were compared with known sequences retrieved from the GenBank. The two new SIV-positive chimpanzees from Gabon (accession numbers, GQ915583 and GQ915584) were identified as members of a P.t.t subspecies interspersed among chimpanzee strains from Gabon, Cameroon and other central African countries (data not shown).

Organization of the SIVcpz-Gab4 Genome

The complete SIVcpzPtt-Gab4 sequence was obtained with a PCR-based ‘genome walking’ approach to generate 17 overlapping fragments. The genome was determined to be 9093 bp long. It is characterized by the presence of three retroviral structural genes (gag, pol and env) and the regulatory genes (tine, vif, rev, tat, vpr and nef), including vpu. Complete open reading frames were found for all genes (Figure 2). The full genome sequences were analyzed

Table 1. Numbers of samples collected from wild-born chimpanzees by geographic origin, sex and SIV status.

| Country | Province | No. tested | Male | Female | SIV+ |
|---------|----------|------------|------|--------|------|
| Gabon   | Estuaire | 4          | 2    | 2      | 1    |
|         | Haut-Ogooué | 30       | 13   | 17     | 1*   |
|         | Moyen-Ogooué | 6        | 4    | 2      | 0    |
|         | Nyanga    | 2          | 1    | 1      | 0    |
|         | Ogooué-Ivindo | 5       | 0    | 5      | 0    |
|         | Ogooué-Lolo | 5        | 3    | 2      | 0    |
|         | Woleu-Ntem | 5          | 3    | 2      | 0    |
|         | Unknown   | 10         |      |        | 0    |
| Congo   | Konkouati | 37         | 16   | 21     | 0    |
| Total   |           | 104        | 42   | 52     | 2    |

*Juvenile that died at 3 months.

doi:10.1371/journal.pone.0044298.t001
gene by gene in MEGA and compared with SIVcpz sequences, in particular those of SIVcpzPtt-Gab1 and Gab2.

The SIVcpzPtt-Gab4 long terminal repeat comprised one binding site for NF-kB and two SP1 sites, the polyadenylation signal and TAATA box. No mutation was observed in gag p6 sequences in the PT/SAP domain of SIVcpzPtt-Gab4 after comparison with all known SIVcpz/HIV-1 strains. The importance of the mutation at position 30 (Met-to-Arg) of the gag p17 protein during interspecies transmission of SIVcpz to humans has been demonstrated [30,31]. The gag protein of SIVcpzGab4 has a methionine at position 30, and this is characteristic of all SIVcpzPtt, indicating that they belong to the same phylogenetic group.

Moreover, instead of the YPSL motif found in SIVcpzPtt-Gab2 strain, SIVcpzPtt-Gab4 shared an LTSL motif, as did all the SIVcpzPtt/HIV-1 strains. There was also no mutation in the YMDD motif in SIVcpzPtt-Gab4 pol sequences.

The vpr gene is made up of 80 amino acids and shows wide variation, like all SIVcpz strains. Nevertheless, we found the DSQGNE motif in the cytoplasmic domain of all SIVcpzPtt. This highly conserved residue is involved in down-regulation of CD4 expression and in degradation of the BST-2 host restriction factor [32,33,34].

In the env gene, the extracellular envelope domain (gp120) of SIVcpzPtt-Gab4 contained V1, V2, V3, V4, and V5 regions, a CD4 binding site, and the cleavage site for the transmembrane glycoprotein in the gp41 domain. The V1 region was longer than V2 because of the deletion of 15 amino acids in V2. Moreover, detailed inspection of the amino acid sequence revealed the presence of 18 highly conserved cysteine residues, common to the three SIVcpzPtt-Gab strains, which are involved in the formation of disulfide bonds and play an important role in the structure and function of gp120 (Figure 3).

The functional domains of the SIVcpzPtt-Gab4 rev gene were conserved; one is rich in arginine (amino acids 35–50) and the other is rich in leucine (amino acids 73–83), as described for SIVcpzPtt-Gab1 and SIVcpzPtt-Gab2 [9].

The nef gene contains 207 amino acids and groups of specific motifs with putative functional relevance. The consensus site of N-myristoyltransferase (MGXXXCA) is present, as in all SIVcpzPtt and HIV-1 strains [35]. Similarly, the site rich in proline PXXP representing the SH3 binding site is present [36,37]. The EXXXLL165 motif, which is involved in down-regulation of CD4, is also conserved, as in other HIV-1 and SIVcpz strains [38].

Phylogenetic Relation between the SIVcpz-Gab4 and Other Primate Lentiviruses

We examined the phylogenetic relation between the SIVcpzPtt-Gab4 strain and other primate lentiviruses and then the relation with SIVcpzPtt-Gab1 and SIVcpzPtt-Gab2 strains, by diversity plot analyses of concatenated nucleotide sequences. Pairwise sequence distances were plotted for windows of 450 nucleotides, which were moved in steps of 20 nucleotides along the alignment. SIVcpzPtt-Gab4 clustered closely with SIVern (red capped mangabey, Cercocebus torquatus torquatus) in pol and closely in env with SIVgsn (greater spot-nosed monkey, Cercocebus nictitans), did other SIVcpz strains (data not shown). As seen in Figure 4, the SIVcpzPtt-Gab strains showed the greatest homology of sequences across the genome, but the homology varied within genes. The results show crossing of diversity plots, indicating the presence of recombination events characteristic of mosaic genomes.

Phylogenetic Relations of SIVcpzPtt-Gab4

To estimate the phylogenetic relations between the new SIVcpzPtt-Gab4 strain and other SIVcpz strains, we constructed and analyzed the maximum likelihood phylogenetic trees from the gag, pol–vif and env–nef amino acid sequences (Figure 5). The position of the SIVcpzPtt-Gab4 strain varied: in the gag tree (Figure 5A), it belonged to the SIVcpzPtt-Gab1, SIVcpzPtt-Cam13 and SIVcpzPtt-Gab2 group, supported by a strong (96%) bootstrap value; in the pol–vif tree (Figure 5B), it was an outlier to the entire clade, which was composed of other SIVcpzPtt and HIV-1 groups M and N (bootstrap value, 100); and in the env–nef tree (Figure 5C), it was within the group of strains that includes SIVcpz/HIV-1 group M and HIV-1 group N, but with no strong relation to any of the strains present (bootstrap value, 72).

Comparison of the predicted protein sequences encoded by the gag, pol–vif and env–nef genes revealed that SIVcpzPtt-Gab4 is more closely related to SIVcpzPtt-Gab1 and SIVcpzPtt-Gab2 in the
Virus Isolation, Monitoring of Viral Replication, and Quantification of SIVcpz RNA

No SIV was detected after culture of PBMCs from chimpanzee Gab3. Real-time PCR for detection of the plasma viral load and the proviral load were negative. SIV was, however, isolated after T-cell depletion from PBMCs of chimpanzee Gab4, on day 11 of in vitro culture, confirming SIV infection in this chimpanzee. p24 antigen and RT activity were detected in supernatants collected between days 3 and 17, with a peak at day 11. p24 antigen was also detected in the plasma of this infected animal. The plasma viral load of this chimpanzee on arrival at the CIRMF was $9.2 \times 10^3$ RNA copies/ml, and no significant difference in viral load was found during 3 years of follow-up (Table 3).

Follow up of Hematologic and Immunologic Parameters in SIV-infected Chimpanzee Gab4

We evaluated the hematologic and immunologic effects of SIV infection in chimpanzee Gab4 and compared them with those of 16 uninfected chimpanzees housed at the CIRMF (Table 3). Of the basic hematologic markers, only the number of white blood cells was significantly lower than that in uninfected animals ($p<0.05$). Analysis of T-cell subsets showed a significantly low percentage and absolute number of CD4+ T cells ($p<0.05$). No significant difference was found in CD8+ T cells, and no difference was found in the distribution of naive or memory cells or in the proliferation and activation of CD4+ and CD8+ T cells.

Discussion

During routine HIV/SIV screening of non-human primates, we identified two chimpanzees harboring anti-SIVcpz antibodies. With the previously described and characterized SIVcpz-Gab strains [6,9], SIVcpzPtt-Gab4 represents the third SIVcpz strain identified and characterized in wild-born captive chimpanzees in Gabon, central Africa. The absence of virus in the newborn seropositive chimpanzee Gab3 and the progressive disappearance of cross-reactive HIV-1 antibodies strongly suggests passive transplacental transfer of antibodies from infected mothers, as observed in infant chimpanzees [1]. Interestingly, Gab3 and Gab4 showed different serological patterns. As we did not have the original strain from the mother of Gab3, we can only hypothesize that Gab3 and Gab4, which were from different geographic areas, are sufficiently different that they induce distinct serological profiles. In a large study on the natural prevalence of SIVcpz, antibodies detected in urine and feces also showed different profiles, linked to viral diversity and, to a lesser extent, to the sites at which the samples were collected [23].

Analysis of mtDNA (D-loop) sequences showed that our SIV-positive chimpanzee Gab4 belongs to the *P.t.t* subspecies. Once we had sequenced the entire SIVcpzPtt-Gab4 genome, we found that the new strain has the same genomic organization found in all HIV/SIVcpz [39].

We evaluated the sequence homologies of SIVcpzPtt-Gab4 with two previously characterized Gabonese SIVcpz strains by phylogenetic analyses of evolutionary trees constructed for the three main genes. As reported for SIVcpzPtt-Gab1 and SIVcpzPtt-Gab2 [7,9], SIVcpzPtt-Gab4 is phylogenetically related to the SIVcpzPtt/HIV-1 lineage rather than to SIVcpzPti, but its position varies. The strongest amino acid identities were observed with SIVcpzPtt-Gab2 and SIVcpzPtt-Gab1. Interestingly, despite the relatively
New SIV in Chimpanzees

**CPZGab4**

```
MRVREMKK-- ---LWSFWVL GLGFLALSIT SDSN-WWTV YLGVPVWKDA 50
.K.MKTRRRR WQPYC----I TMAIIP.C.K TE.QQ....Y....E. 50
.K.MKR.D RD WNS.SIITII TIIL.TPC. .EL-----Y....H. 50
```

**CPZGab2**

```
ETTLFCASDA KAYSTEAHNI WATQACVPID PNPQEQFVSKN VKENFNMWDN 100
T.P....AN T.LK.E.P. . . . . T.S.E.I..I .T.E.V. 100
DPV. . . . . . . . . . . . . . . . . . . . . S.O.S.K 100
```

**CPZGab1**

```
PMVDQMDQI SLWDSQSLKP CVKLTPCLVT LNCTNVTNS PTKKPPTTP 150
A.E...... T........... N....Q .T..S..G NE.NATNGN-- 150
N....H. . . . . . . . . . . . . . . . . . . . . Q.SKA.FSQ AKN------- 150
```

**CPZGab4**

```
TTSTVSTTI PLNDSIFEDM CNCTFNVTE LRGSSNNSY- -------------- 200
I.EK.GLQ.. R....T. . . . . . . . . . . . . . . . . . . . . . 200
LT NQTS.PPLE. K..S..T. . . . . . . . . . . . . . . . . . . . . 200
```

**CPZGab2**

```
-------- NSYRLIC NCTTATAITQA CPTKSFEPIP IYHCAPAGFA ILRNEENSQ 250
GT.N.TF. . . . . . . . L.K..DKDPY 250
```

**CPZGab1**

```
GNEN.T.I. . . . . . . . . . . . . . . . . . . . . . . . . 250
```

**CPZGab4**

```
EMGYCENVST VHCTHGIKPV VTTQILNGS ITQQ-IMIRS KN---ISSNS 300
GK.K.K. . . . . . . . T . . . . . . . . . . . . . . . . . . . . 300
GK.K.T. . . . . . . . LIL. . . . . . . . . . . . . . . . . . . . 300
```

**CPZGab4**

```
FNIIQVFNET IPIICIRPGN NTRGQIQLGP AMTFYNIENI VGNTKRAFCK 350
DU..LKRA . . . . . . . . . . . . . . . . . . . . . . . . . . . 350
DVWL.LV.A VSLL.N.H. . . . . . . . . . . . . . . . . . . . . 350
```

**CPZGab2**

```
VNGSQWNMK QNIQFRKAE IK---LNTV FNSSAGDPOE ITNPMWNCIG 400
L.TL.N.N.L NR.K.K.K.I.NS TTWHRG-DI .TKHP.... VV...F.G. 400
```

**CPZGab1**

```
I..TT.NRTV EEEVKKALAT SRNTAA.-I. L.RAS. . . . . . . . . . . 400
```

**CPZGab4**

```
EFFYVINHPF FTGN-KINTN ILLPKKIRQI VNSMRVCGKS IYAPSRIGNL 450
........ SR. I.C.SSDTSE Y.. . . . . . . . . . . . . . . . . . 450
SOGI .D..I..GI . . . . . . . S..R.... . . . . . . . . . . . 450
```

**CPZGab2**

```
SCSNTITGL LTDRGDPKNI NETETLRPG GDMKDWRSE LKYYKVVKIE 500
T...T.... .EQNQ----T GNNTEVYL.. R.... . . . . . . . . . . 500
```

**CPZGab1**

```
T........ S.VT.N SGNL.F.T. .N...... . . . . . . . . . . . . 500
```

**CPZGab4**

```
PLAVAPTKR RYTINMKEKR AKRAAF---- ------AAG STMGAADVTL 550
G. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 550
```

**CPZGab2**

```
LPAPLQPL QIKA . . . . . . . . . . . . . . . . . . . . . . . . 550
```

**CPZGab1**

```
TVQARNSSLG IVQQMNLLR AIEAQQHLLQ LSJVWGV-QLQ ARLLAVERYL 600
```

**CPZGab2**

```
................. T..K. . . . . . . . . . . . . . . . . . . . . 600
```

**CPZGab1**

```
............ K. . . . . . . . . . . . . . . . . . . . . . . . . . 600
```

**CPZGab4**

```
KDQQGILQMG CBGKACIYTN VPWNRNWNS SYDNEIWNNL TWNENDKQVS 650
```

**CPZGab2**

```
.V.A..... TV..ST .TS.NSN KS.Ed.S . .QQ..LE 650
```

**CPZGab1**

```
Q.............. T . . . . . . . . . . . . . . . . . . . . . . 650
```

**immunodominant TM domain**
close similarity, clustering of these SIVcpzPtt-Gab strains was supported by a strong 96% bootstrap value only for the gag tree.

Global analysis of the phylogenetic results showed high genetic diversity among the Gabonese SIVcpzPtt strains. As reported previously [13], SIVcpzPtt-Cam13 clustered more closely with SIVcpzPtt-Gab1 than with SIVcpzPtt-Gab2; in the present study, it clustered with the newly characterized SIVcpzPtt-Gab4 strain. This might be due partly to the origin of the two chimpanzees: Gab1 was from northern Gabon and Cam13 from a neighboring province in Cameroon, with no significant biogeographic barrier between the two. Conversely, the second SIV-positive chimpanzee, Gab2, was from eastern Gabon, isolated from the two others by the Ogooué and Ivindo rivers. Equatorial Guinea, the place of origin of Gab4, represents an intermediary geographic locality, with no hindrance to chimpanzee movement.

In contrast, the SIVcpz strains from Cameroon fell into the specific SIVcpzPtt-Cam cluster in the gag, pol, and env trees, as did the SIVcpzPtt-US strain from an unknown African country [5,13,22,23]. In this particular cluster, however, SIVcpzPtt-Cam strains differ according to their geographical origin and some appear to be particularly closer to HIV-1 group M or N [22,23]. These phylogenetic data strongly suggest that HIV-1 group M emerged from the south-eastern corner of Cameroon while HIV-1 group N appeared in south-central Cameroon [25].

Within a project for the epidemiological surveillance of primates in central Africa (Grant ROI AI44596) that began in 2000, we have systematically screened all samples taken from monkeys kept as pets representing 13 species of primate [47] including 104 chimpanzees. This type of sampling does not reflect the prevalence of SIVcpzPtt in Gabon, for which noninvasive studies involving the collection of faeces in Cameroon and Tanzania provide more convincing evidence [22-24,48]. Nevertheless, our study confirms

Figure 3. Predicted protein sequence of the env gene (gp120 and gp41) of SIVcpz-Gab4 in comparison with SIVcpz-Gab1 and SIVcpz-Gab2. Conserved cysteines are marked with asterisks, variable regions V1–V5 are indicated, and the CD4 binding site and immunodominant transmembrane domain are highlighted in grey.

doi:10.1371/journal.pone.0044298.g003

Figure 4. Diversity plot of SIVcpz-Gab4 with SIVcpz-Gab1 and SIVcpz-Gab2 sequences. Regions with uncertain alignment or sites with a gap in any sequence were excluded (8261 nucleotides after de-gapping). The nucleotide sequence difference is plotted for windows of 450 nucleotides and a 20-nucleotide step increment.

doi:10.1371/journal.pone.0044298.g004
the existence of SIV-infected chimpanzees circulating in northern Gabon and Equatorial Guinea (SIVcpz-Pt-Gab1, SIVcpz-Pt-Gab4), eastern Gabon (SIVcpz-Pt-Gab2), and southern Gabon (P.t.t Gab3). These geographic indications will be important for planning future non-invasive studies.

Few studies have been conducted of the pathogenesis of SIV in chimpanzees, for two main reasons. First, many SIV strains are known only from the available sequences and have not been isolated. Secondly, chimpanzees are endangered non-human primates and their use in experimental studies is prohibited. Only two P.t.s infected with SIVcpz-Pt-Ant have been monitored for several years [49]. We were able to study the chimpanzee Gab4 at the CIRMF where it is housed and could thus evaluate its immunological and virological parameters. No sign of disease associated with SIV has been identified during the 2 years of the survey. The level of viral replication in plasma was low, at about 4 log_{10}; however, the only other known viral loads are those of two chimpanzees, Cam 155 and Noah, which are 3.4–5.0 log_{10} [14,30]. Furthermore, the assumption that African primates infected with SIV have high viral loads is now in doubt, as several studies have shown wide variation among individuals of the same species, especially sooty mangabeys and African green monkeys [31,32]. It is therefore difficult to define a general viral load for all chimpanzees infected with SIVcpz.

No significant variation was found in the viral load of Gab4 during the study. The CD4/CD8 ratio also showed no significant variation, indicating that the moderate viral replication recorded in Gab4 did not impair its immune system. Only slight leukopenia and a lower level of CD4+ T cells were observed when compared with uninfected chimpanzees. Depletion of CD4+ T cells is not the only factor involved in disease progression, however, and chronic immune activation is a very strong predictor of pathogenic SIV infection [33,34]. SIVcpz-Pt-Gab4 infection was associated with neither T-cells proliferation nor T-cells activation. This correlates with the stable health of this animal and the absence of signs of immunodeficiency.

### Table 2. Protein sequence identities among SIVcpz/HIV-1 viruses.

| Strain compared with SIVcpz-Gab4 | Amino acid identity (%) |
|----------------------------------|-------------------------|
|                                  | Gag | Pol-vif | Env-nef |
| SIVcpz-Gab2                      | 82.4 | 72.0 | 62.5 |
| SIVcpz-Gab1                      | 81.3 | 78.3 | 67.0 |
| SIVcpz-Cam                       | 80.3 | 76.4 | 64.4 |
| HIV-1-N                          | 75.6 | 75.7 | 64.1 |
| HIV-1-M                          | 73.3 | 76.7 | 59.6 |
| HIV-1-O                          | 71.5 | 73.7 | 41.7 |
| SIVcpz-Tan                       | 61.7 | 64.2 | 36.5 |

doi:10.1371/journal.pone.0044298.t002

Gab4 is the oldest P.t.t infected with SIVcpz known in Gabon. All the animals were at least 2 years old when they were found to be seropositive, and Gab4 was 6 years old at that time. As it was not old enough to be sexually active, it was probably infected by maternal-fetal transmission, like the other chimpanzees [26]. By the end of our study (2008) this animal had been infected for 8 years.

Recently, immunopathological studies in communities of P.t.s in Tanzania showed that SIVcpz-Pt is also associated with progressive CD4+ T-cell loss, lymphatic tissue destruction and premature death. SIVcpz-Pt, correlated with high prevalence, has a substantial negative influence on the health, reproduction and lifespan of chimpanzees in the wild [28,55]. All these data suggest that SIVcpz is generally nonpathogenic but can induce pathogenic effects under certain circumstances. This is consistent with the idea of an increased susceptibility because of the recent introduction of SIVcpz into chimpanzees [56].

We have reported here the identification and characterization of a new SIVcpz-Pt strain, SIVcpz-Pt-Gab4, which is thus the third SIVcpz-Pt-Gab strain from a chimpanzee captured in the wild in Equatorial Guinea and then living in captivity in Gabon. It is interesting to note the strong genetic diversity that characterizes the three Gabonese SIVcpz strains. Up to now, no reliable clinical symptoms of SIV infection have been detected in the SIV-positive chimpanzee. Further field studies with non-invasive detection methods are needed to determine the prevalence, geographic distribution, species association and natural history of SIVcpz strains throughout the chimpanzee habitat in Gabon.

### Materials and Methods

#### Chimpanzee Collection at CIRMF

Within a project for the epidemiological surveillance of primates in central Africa (Grant ROI AI44596) that began in 2000, we have systematically screened all samples taken from monkeys kept as pets comprising 13 species of primates (Mouinga-Ondeme et al., 2012), including 104 chimpanzees. As shown in Table 1, the serologic survey involved 37 wild-born orphaned chimpanzees from the Habitat Ecologique et Liberte des Primates (HELP) Sanctuary in Conkouati-Douli National Park, Congo, from which plasma samples were collected during routine veterinary screening in 1992 and 1996 and 67 wild-born chimpanzees sampled in the wild throughout Gabon. All the animals were negative, except for Gab3 and Gab4, which were seropositive for SIV.

#### Ethics Statement

The animals were handled in accordance with standard national operating procedures in the CIRMF as well as in accordance with the United States National Institutes of Health guidelines for the Care and Use of Laboratory Animals. The
animal protocols and procedures were approved by the Gabonese ethics committee for animal experimentation at the CIRMF, and registered under No. CE08–010.

All work with animals was conducted according to the relevant national and international guidelines and in accordance with the recommendations of working group report chaired by Sir David Weatherall in December 2006 and Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. CIRMF collaborates with great apes sanctuaries to perform routine serological surveys of adopted orphans. In order to integrate animals safely, new orphans designated for a sanctuary or a releasing project are admitted to the CIRMF Primate Centre have examined animals and researchers in the Retrovirus Department have performed serological analyses for 20 years.

The housing conditions are in strict accordance with European Union guidelines for animal care (European Union Directive 86/609/EEC). The Primate Centre has spacious rooms equipped with branches, hammock, platforms and ball toys, which are changed regularly. Animal welfare ensure to prevent suffering in all work involving non-human primates: e.g. they are fed twice a day with various Gabonese fruits and with a “home-made” protein complement cake. Food enrichment and training (positive reinforcement) practised dayly to obtain cooperation for routine veterinarian examinations. Highly skilled staff spends 2 h a day with the non-human primates.

Each primate housed in the CIRMF Primate Centre has an annual health check under anesthesia (ketamine at 10 mg/kg body weight). The blood samples, taken for this study, were collected under strict health controls by Primate Centre veterinarians.

### Specimen Collection

Blood samples from SIV-positive chimpanzees were collected in EDTA K2 tubes under ketamine-HCl (10 mg/kg bw) and used for flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient centrifugation (Sigma-Aldrich), and plasma was centrifuged at 3000 x g for 10 min, dispensed into 1-ml aliquots and frozen at –80°C.

### Detection and Confirmation of SIV Antibodies

Plasma samples from all animals were first screened with the Determine HIV-1/2 rapid test (Alere Inc, San Diego, CA, USA). Positive samples were tested with the peptide-based primate lentivirus identification assay [29]. This indirect ELISA method is based on use of synthetic peptide antigens that map to the immunodominant gp41 region and the V3 region of HIV/SIVcpz reference strains: HIV-1 group M subtype A (consensus), HIV-1

| Variable | Time of follow-up | Uninfected chimpanzees (mean of 16±SD) |
|----------|------------------|----------------------------------------|
|          | At arrival | 6 months | 2 years |          |
| Age (years) | 6 | 6 | 8 | 8.8±2.9 |
| Sex | M | 11M, 5F |
| Plasma viral load (RNA copies/ml) | 4.5×10³ | 2.5×10³ | 1.9×10³ |
| White blood cells | 8.2 | 4.1 | 5.8* | 9.3±2.8 |
| Red blood cells | 6.0 | 4.7 | 5.8 | 5.3±0.9 |
| Platelets | 483 | 363 | 345 | 351±144 |
| % CD4⁺ T cells | 22.7 | 25.1 | 25.7* | 38.8±8.8 |
| CD4⁺ T cells (number/mm³) | 149 | 268 | 283* | 534±196 |
| % CD8⁺ T cells | 28.2 | 34.4 | 25.8 | 262±8.8 |
| CD8⁺ T cells (number/mm³) | 185 | 367 | 284.3 | 363±158 |
| CD4⁺ T cells/CD8⁺ T cells (ratio) | 0.8 | 0.7 | 1.0 | 1.7±0.8 |
| %HLA-DR in CD4⁺ T cells | 16.5 | 3.0 | 1.1 | 1.9±1.3 |
| %HLA-DR in CD8⁺ T cells | 28.8 | 12.3 | 4.8 | 6.1±0.3 |
| %K667 in CD4⁺ T cells | ND ND | ND | 4.0 | 2.4±1.0 |
| %K667 in CD8⁺ T cells | ND ND | ND | 4.3 | 2.1±1.3 |
| %CD25 in CD4⁺ T cells | 50.3 | 55.2 | 44.0 | 36.9±8.8 |
| %CD25 in CD8⁺ T cells | 3.4 | 2.7 | 2.9 | 4.4±2.6 |
| %CD28⁺CD95⁻ in CD4⁺ T cells (naives) | 55.0 | 52.2 | 69.0 | 53.4±17.8 |
| %CD28⁺CD95⁺ in CD4⁺ T cells (CM) | 44.5 | 46.5 | 29.0 | 41.8±13.9 |
| %CD28⁺/⁻CD95 in CD4⁺ T cells (EM) | 0.5 | 1.3 | 2.1 | 4.8±5.9 |
| %CD28⁺CD95⁺ in CD8⁺ T cells (naives) | 26.0 | 19.7 | 21.9 | 31.3±16.1 |
| %CD28⁺CD95⁻ in CD8⁺ T cells (CM) | 23.5 | 16.4 | 25.5 | 17.1±6.2 |
| %CD28⁺/⁻CD95 in CD8⁺ T cells (EM) | 50.5 | 64.0 | 52.7 | 53.4±15.2 |

CM, central memory; EM, effector memory.

*p<0.05 determined with Mann-Witney U test.

doi:10.1371/journal.pone.0044298.t003
group O (Ant-70), HIV-1 group N (YBF30), SIVcpzPtb-Gab1 (Gab1) and SIVcpzPtb-Gab2 (Gab2). All positive and equivocal samples were subjected to western blotting confirmation (New Lat Blot 1, BioRad, Marnes la Coquette, France).

Amplification of SIVcpz Viral RNA by RT-PCR

The complete SIVcpzPtb-Gab4 genome was amplified from RNA extracted from the plasma of the infected chimpanzee. PCR amplification was performed on a thermal cycler Perkin Elmer 9700. We first used degenerated primers to amplify a 330-bp fragment of pol (PoliS4 and PoliOR for the first round and Hpol4235-Hpol4358 for the second round) and a 550-bp fragment of env (gp40F1 and gp41R1 for the first round and gp46F2 and gp47R2 for the second round) with RT-PCR, as described previously [57–59]. The PCR products were directly sequenced. We then amplified the full-length provirus by the long-PCR procedure (GeneAmp XL kit Perkin Elmer, Norwalk, Connecticut, USA) with two sets of primers, LPBS15’–Hpol4538 and Hpol4235-Hpol4538 for the second round) and a 550-bp fragment of Ptt–Gab4 sequence was deposited in GenBank (accession number, GQ217539).

Virus Isolation and Viral Replication Monitoring

A portion of the PBMC was used for virus isolation. CD8 depletion was performed on 10 million lymphocytes with magnetic beads coupled to CD8 antibody, as recommended by the manufacturer (Dynabeads, Invitrogen Dynal, Ås, Oslo, Norway). After washing, the enriched CD4+ cells were suspended in RPMI 1640 growth medium (Cambrex Bioscience, Walkersville, Maryland, USA) supplemented with 20% heat-inactivated foetal bovine serum (Gibco BRL, Eragny, France), 1% penicillin–streptomycin mixture (Gibco BRL, Eragny, France), 1% L-glutamine 200 mmol/l (Gibco BRL, Eragny, France), and 20 U/ml human recombinant interleukin-2 (Roche Diagnostics, Manheim, Germany). The lymphocytes were stimulated with 3 μg/ml of the mitogen concanavalin-A (Sigma-Aldrich, Saint Quentin Fallavier, France) and incubated at 37°C in 5% CO2. To maintain the cells, 50% of the medium was changed twice a week. The second portion of PBMC was aliquoted in 10% DMSO (Sigma-Aldrich) in foetal bovine serum (Gibco-BRL) and frozen at −80°C.

Viral replication was monitored with a reverse transcriptase (RT) assay (Lenti-RT Kit, Cavidix Tech AB, Uppsala, Sweden) and by measuring p24 antigen (Genetic Systems HIV-1 Ag EIA, BioRad, Marnes-la-Coquette, France).

Phylogenetic Analyses

Pairwise alignments were performed for the nucleotides and deduced amino acids of separated SIVcpzPtb-Gab4 genes (gag, pol–vif and env–nef) with CLUSTAL W (1.7), which constructs neighbor-joining trees in a Kimura two-parameter model (transition/transversion ratio = 2) [64]. The SIVcpzPtb-Gab4 sequences were aligned with the corresponding sequences of representative SIVcpz and HIV-1 strains. The GenBank accession numbers were: HIV-1 MU455, M62320; HIV-1M HX32, K03453; HIV-1N YBF30, AJ006022; HIV-1N YBF106, AJ271370; HIV-1O AN70, L20587; HIV-1O MVP5180, L20571; SIVcpz-Gab1, X52154; SIVcpz-Gab2, AF382828; SIVcpz-Cam3, AF115393; SIVcpz-Cam5, AJ271369; SIVcpz-Cam13, AY169968; SIVcpz-US, AF103818; SIVcpz-TAN1, AF447763; SIVcpz-TAN2, DQ374657; SIVcpz-TAN3, DQ374658; SIVcpzANT, U42720; SIVcpz-MT145, DQ373066; SIVcpz-MB66, DQ373063; SIVcpz-LB7, DQ373064 and SIVcpz-EK505, DQ373065. The concatenated amino acid alignments were used for the phylogenetic analysis after exclusion of all sites that could not be aligned unambiguously or sites with a gap in any sequence and after removing the gag/pol and pol/vif overlaps from the C-terminus of the deduced gag and pol protein sequences. Trees were inferred by the Bayesian method implemented in MrBayes version 3.1 software (2005) [63] with the Jones, Taylor and Thornton model [65] and the R8rev model [66] of evolution and gamma distributed rates at sites, with one million generations and burn-in of 2.5%. Bayesian parameters were examined with the Tracer program (http://evolve.zoo.ox.ac.uk/software.html/id = tracer), and all estimated sample sizes were greater than 545.

Species and Sub-species Determination (mtDNA-D loop)

To confirm the subspecies origin of the SIV-positive chimpanzees, a 341-bp region of the mtDNA genome (D-loop) was amplified, as previously described [61], with the primers L15997 5’-CACCATTAGCAGCCAAAGCT-3’ and H16498 5’- CCTGAAGTAGGAACCAG. AGTG-3’. The resulting PCR products were directly sequenced (Macrogen Inc., Kumchun-ku, Republic of Korea).

These new mtDNA sequences were compared with those found in the GenBank database originating from 25 chimpanzees from Gabon and 26 from Cameroon, the Congo, the Central African Republic and the Democratic People’s Republic of the Congo, all representatives of P. troglodytes troglodytes subspecies. Of the 25 mtDNA sequences from Gabon, 16 originated from pets or wild-born, orphaned chimpanzees sampled within the country, and nine were from wild chimpanzees in the Lope Reserve. The samples were obtained by noninvasive methods [62]. mtDNA sequences characterizing the remaining P. troglodytes subspecies (P. troglodytes schweinfurthii, verus, and ellioti) and P. paniscus were also included.

Sequences were aligned with CLUSTAL W (1.7); all ambiguous sites with a gap in any sequence were excluded. Phylogenetic trees were constructed by the Bayesian method with MrBayes version 3.1 software (2005) [63] and the GTR model for gamma distributed rates at sites and one million generations with a burn in of 2.5%. Bayesian parameters were examined with the Tracer program (http://evolve.zoo.ox.ac.uk/software.html/id = tracer); all the estimated sample sizes were greater than 220. The mtDNA chimpanzee sequences were deposited in GenBank (accession numbers, GQ915383 and GQ915384).

RNA and DNA Extraction

RNA was extracted from 150 μl plasma with a QiaAmp Viral RNA Mini kit (Qiagen) and eluted in 60 μl TE buffer as recommended by the manufacturer.

DNA was extracted from PBMCs with a QiaAmp DNA Mini kit (Qiagen) and eluted in 200 μl AE buffer.
Quantification of SIVcpz RNA

Quantification by real-time RT-PCR was performed with 5 μl extracted RNA with a Quantitect SYBR Green RT-PCR kit (Qiagen) in capillary tubes, by the LightCycler System (Roche Diagnostics).

Quantification was based on amplification of a 119-bp fragment located in the long-terminal-repeat region of the HIV-1 major group. We used the forward and reverse primers (AF) 5′-GCCCTCAATAAAGCTTGCCCTTGA-3′ (66-87) and (BR) 5′-GCGGCACTCTGCTAGAT-3′ and inactivated HIV virus (5×10³ RNA copies per ml) as the standard (Biocentric, Bandol, France). The primers were used at a final concentration of 1 μmol/l, and the final MgCl₂ concentration was 2.5 mmol/l. The amplification protocol for SIVcpz quantification consisted of reverse transcription (30 min at 50°C), followed by denaturation and activation of HotStart Tag DNA polymerase (15 min at 95°C) and cDNA amplification (45 cycles of denaturation for 15 s at 95°C, annealing for 15 s at 55°C, and elongation for 22 s at 72°C). The RNA copy number was determined by comparison with an external standard curve and was expressed as RNA copies per ml plasma. The detection limit of the SIVcpz quantification assay was 100 RNA copies per ml plasma.

Flow Cytometric Analysis of Cell-surface and Intracellular Marker Expression

In addition to GAb4, we selected 16 uninfected chimpanzees aged 5–10 years and analyzed whole blood samples by four-color flow cytometry with a standard procedure and a panel of monoclonal antibodies: anti-CD4-fluorescein isothiocyanate (FITC) (clone MT4-77), anti-CD4-phycoerythrin (PE) (clone L200), anti-CD3-allophycocyanin (clone SP34-2), anti-CD8-peridinin chlorophyll protein (clone SK1), anti-HLA DR-PE (clone L200), anti-CD4-fluorescein isothiocyanate (FITC) (clone MT4-77), anti-CD4-phycoerythrin (PE) (clone L200), anti-CD3-allophycocyanin (clone SP34-2), anti-CD8-peridinin chlorophyll protein (clone SK1), anti-HLA DR-PE (clone L200), anti-T cell receptor-phycoerythrin-Cy5 (clone 2H7), anti-CD14-phycocerythrin-Cy5 (clone 61D3), anti-CD11c-phycocerythrin-Cy5 (clone 6B4), anti-CD16-phycocerythrin-Cy5 (clone 6H12), anti-CD25-phycocerythrin-Cy5 (clone BY/6B3), anti-CD56-phycocerythrin-Cy5 (clone 6H12), anti-CD69-phycocerythrin-Cy5 (clone 10B8), anti-CD154-phycocerythrin-Cy5 (clone OKT15), and anti-CD107a-phycocerythrin-Cy5 (clone H4A3). Data were analyzed with FlowJo software v7.2 (Tree Star, Inc., Ashland, Oregon, USA).

Statistical Analysis

The Mann-Whitney U test was used to compare the results of flow cytometry. Significance was assumed at p<0.05. All analyses were performed with Statistica software v7.1. (StatSoft France, www.statsoft.fr).

Supporting Information

Table S1 Oligonucleotide primers used to amplify SIVcpz-Gab4 genome.

(DOC)

Acknowledgments

We thank Dr Olivier Bourry and Dr Nina Jaffre for technical help.

Author Contributions

Conceived and designed the experiments: SS MM MK. Performed the experiments: SS MM. Analyzed the data: SS MM MK. Contributed reagents/materials/analysis tools: BS. Wrote the paper: SS MM MK.

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