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Widely varying SIV prevalence rates in naturally infected primate species from Cameroon

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Abstract

Although it is now well established that a substantial proportion of wild-living primates in sub-Saharan Africa harbor SIV, no study to date has examined to what extent the various species are naturally infected. In this study, we first describe the development and validation of sensitive and specific SIV antibody detection assays representing all major known primate lentiviral lineages on a panel of 207 sera from 11 different primate species with known infection status. The newly developed assays were then used to determine SIV prevalence rates in nine primate species native to Cameroon. Analysis of 722 sera revealed widely varying prevalence rates, ranging from an apparent absence of SIV infection in crested mona (0/70), grey cheeked (0/36) and agile mangabeys (0/92), to prevalence rates of 3%, 4%, 11%, 27%, 39% and 52% for mustached (6/203), greater spot-nosed (8/193), northern talapoin (3/26), mantled guereza (14/52), De Brazza's (9/23) and mandrill (14/27) monkeys, respectively. The epidemiology of naturally occurring SIV infections is thus more complex than previously appreciated and the various non-human primate hosts seem to differ in their susceptibility to SIV infection. The newly developed assays should now permit to define with greater accuracy existing SIV reservoirs and associated human zoonotic risk.

Keywords: HIV; SIV; Prevalence; Non-human primates; Cameroon

Introduction

Simian immunodeficiency viruses (SIVs) comprise a diverse group of lentiviruses that infect a wide variety of non-human primate species in sub-Saharan Africa (Bailes et al., 2002; Peeters and Courgnaud, 2002). Although seemingly not pathogenic for their natural hosts, these viruses have gained public health attention since two of them have given rise to the human AIDS viruses, human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2). It is now well established that HIV-1 resulted from cross-species transmissions of SIVcpz naturally infecting chimpanzees (*Pan troglodytes troglodytes*) in west central Africa; similarly, HIV-2 resulted from multiple transmissions of SIVsmm infecting sooty mangabeys (*Cercocebus atys*) in west Africa (Corbet et al., 2000; Damond et al., 2001; Gao et al., 1992; Hahn et al., 2000; Yamaguchi et al., 2000). To date, serological evidence of SIV infection has been reported in 36 different primate species and partial or full-length viral sequences have been recovered from 29 of these (Bibollet-

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Ruche et al., 2004). Phylogenetic analysis of these sequences has revealed an extraordinary degree of genetic diversity, with different SIVs varying by as much as 60% of their protein sequences (Bibollet-Ruche et al., 2004); however, SIV diversity is generally species specific, that is SIVs cluster as monophyletic lineages according to their species of origin, which has been used to classify them by adding a three-letter code reflecting their common species name.

Although both SIVcpz and SIVsmm are known to have crossed the species barrier to humans on multiple occasions (Corbet et al., 2000; Damond et al., 2001; Gao et al., 1992, 1999; Yamaguchi et al., 2000), the transmission potential of the other primate lentiviruses remains unknown. In a recent survey of bushmeat markets in Cameroon, we found evidence suggesting that a substantial proportion of wild-living monkeys are SIV infected (Peeters et al., 2002). Among 788 monkeys, representing 16 different species, a considerable fraction (17%) was found to harbor antibodies that cross-reacted with HIV-1 and HIV-2 antigens in a confirmatory (recombinant protein and synthetic peptide based) immunoassay (INNO-LIA, Innogenetics, Ghent, Belgium). PCR analysis of a subset of these cross-reactive samples led to the discovery of new SIV strains not previously known to infect primates. This bushmeat market survey thus documented for the first time that humans who hunt and butcher primates are routinely exposed to a plethora of genetically divergent SIV strains. However, the exact prevalence of SIV infection in the 16 species under study could not be determined because of the unknown extent of antigenic cross-reactivity of the HIV-based test.

Given the extraordinary degree of primate lentivirus diversity, no assay based on a single HIV or SIV strain is likely to be sufficiently cross-reactive to reliably detect antibodies directed against all other strains. To develop SIV-specific antibody detection tests, several groups have examined linear or multiple antigenic peptides (MAPs) encompassing the V3-loop region and/or the immunodominant gp41 ectodomain of the SIV envelope glycoprotein as antibody capture antigens (Masciotra et al., 2000; Ndongmo et al., 2004; Simon et al., 2001). While these approaches have been useful in detecting strain-specific antibodies in selected primate and human reference sera, not all known SIV strains have thus far been targeted for peptide design and their utility for diagnosing SIV infection in wild primate populations representing different species remains to be tested (Ndongmo et al., 2004; Masciotra et al., 2000; Simon et al., 2001). Finally, confirmatory immunoblot assays for SIVs other than SIVcpz/HIV-1 and SIVsmm/HIV-2 have not been developed. Thus, sensitive and specific diagnostic tools suitable for systematic and comprehensive surveys of SIV infection in wild primate populations still need to be developed.

To begin to characterize the epidemiology of natural SIV infections, we expressed the gp41 ectodomain of 11 different SIV strains and used these recombinant proteins, along with corresponding V3 loop peptides, as ELISA antigens to screen 722 primate sera for antibodies directed against SIVs from the same (homologous) and other (heterologous) species. The results revealed a surprising array of seroprevalences, ranging from the apparent absence of SIV in some species to high level (50%) infection in others. Moreover, the overall number of SIV rgp41 ELISA-positive sera was considerably lower than that of INNO-LIA-positive sera, suggesting that for a subset of species, INNO-LIA had overestimated existing infection rates. These data thus indicate that not all naturally occurring SIV infections are common and wide spread, and that strain-specific screening and confirmatory assays will be required to determine the true extent of SIV infection in wild-living primate populations.

Results

Strain-specific ELISA-based assays for SIV antibody detection

To develop a comprehensive panel of SIV-specific serological assays, we selected antigens from 13 different strains which together represent all major known primate lentiviral lineages (Bibollet-Ruche et al., 2004). For 11 of these, we expressed the ectodomain of the transmembrane envelope glycoprotein (Figs. 1A and B) which is known to be highly immunogenic and has previously been used for SIV and HIV antibody detection (Allain et al., 1987; Georges-Courbot et al., 1998; Masciotra et al., 2000; Mauclere et al., 1997; Ndongmo et al., 2004; Saah et al., 1987; Simon et al., 2001). Recombinant proteins were generated as poly-histidine tagged fusion proteins and purified by metal ion affinity chromatography (Fig. 2). We also synthesized V3 loop peptides for 10 SIV strains, including SIVlho and SIVagm for which rgp41 proteins were not available (Fig. 1C; Table 1). Both rgp41 proteins and V3 loop peptides were evaluated as ELISA antigens using a panel of well-characterized reference sera from primates of known (PCR confirmed) infection status (Table 2).

To determine the cutoff values for the various peptide and rgp41 ELISAs, we examined the reactivity of sera from 144 uninfected monkeys (antibody and PCR negative) from 11 different species. For each test, the mean optical density (OD) plus five standard deviations was selected as the cut-off value, an approach deliberately intended to be conservative (Crowther, 2001). Fig. 3 depicts the results of these studies, with cut-off values indicated by red dots (vertical boxes with error bars represent the 25th and 75th percentiles of the negative serum reactivities for each antigen). The results revealed an overall lower background with peptide compared to rgp41 antigens.

The reactivity of the negative serum panel was then used to determine assay specificities. As shown in Tables 3a and 3b, none of the negative sera tested positive in the corresponding (homologous) rgp41 ELISA, and this was also true for the homologous peptide ELISAs except for one sooty mangabey serum that reacted weakly with the cognate SIVsmm V3 loop peptide. Thus, the overall specificity of homologous antibody detection was 100% for the rgp41 ELISAs, and 99% for the peptide ELISAs (Tables 3a and 3b). However, 3 of 144 negative sera reacted with low OD/cut-off ratios with

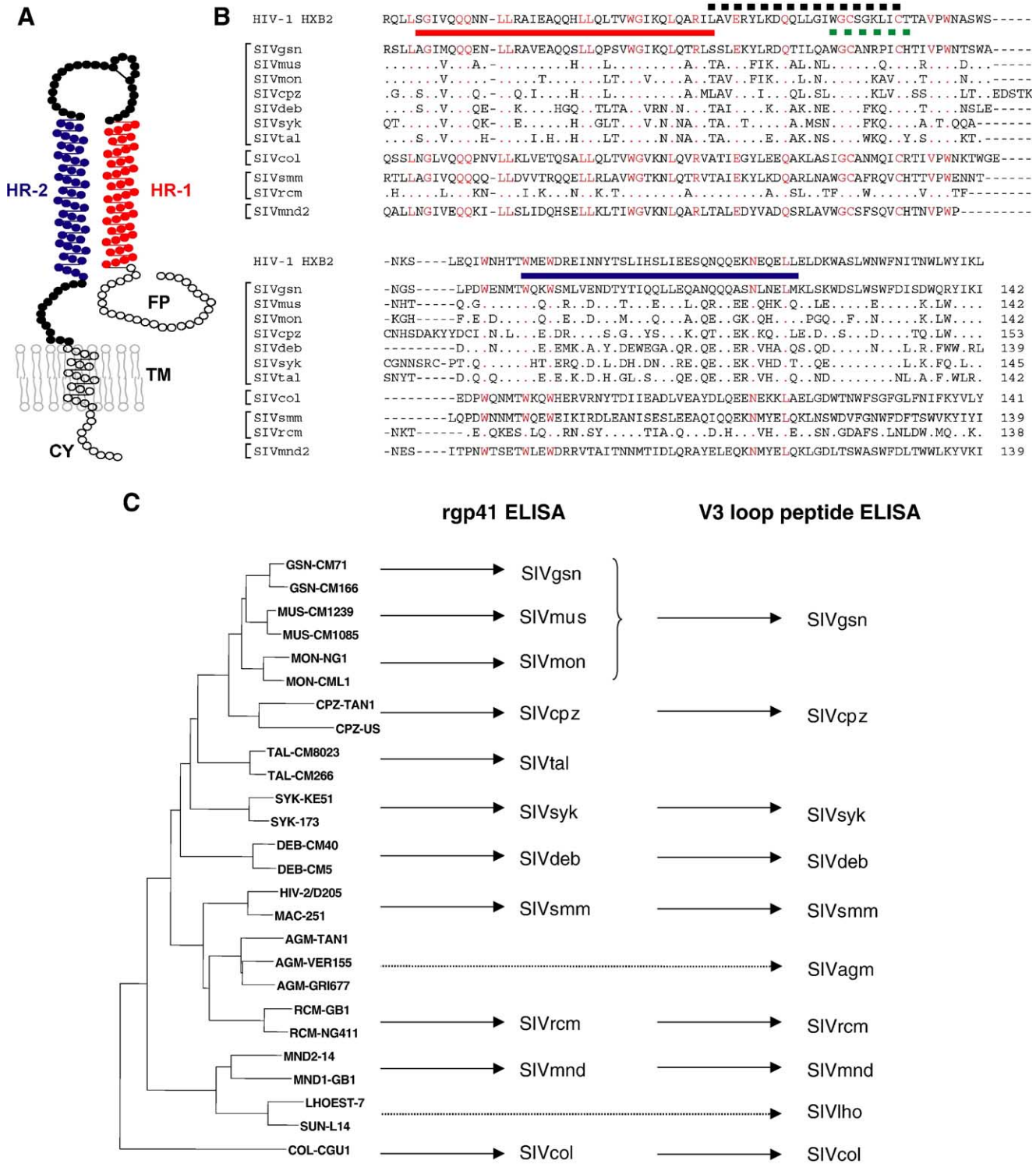

Fig. 1. (A) Schematic representation of the HIV/SIV transmembrane envelope glycoprotein (adapted from Desmezieres et al. (2005)). Amino acid residues included in the respective bacterially expressed gp41 fragments are shown as closed circles, with heptad repeat HR-1 and HR-2 domains highlighted in red and blue, respectively. Open circles represent rgp41 amino acid residues not present in the recombinant protein, including the fusion peptide (FP), the transmembrane domain (TM) and the intra-cytoplasmic region (CY). (B) Alignment of recombinant SIVrgp41 ectodomain sequences. The HIV-1 HXB2 amino acid sequence is shown as a reference, with the heptad repeat HR-1 and HR-2 domains underlined in red and blue, respectively. Amino acid residues conserved among all proteins are shown in red. SIV gp41 proteins are subdivided into four different groups (indicated by brackets) according to the phylogenetic relationships of their full-length Env protein sequences as shown in panel C (Bibollet-Ruche et al., 2004). Within groups, dots denote amino acid identity with the top sequence, while dashes were introduced to optimize the alignment. The length of the SIVrgp41 protein fragment is indicated for each strain. Black and green doted lines indicate the position of peptides used in previously reported ELISA assays as described in Ndongmo et al. (2004); Simon et al. (2001), respectively. (C) Phylogenetic relationship of primate lentiviruses based on Env protein sequences (as determined by the neighbor joining algorithm) (Saitou and Nei, 1987). SIV strains used to design recombinant gp41 proteins and/or V3 loop peptides are indicated.

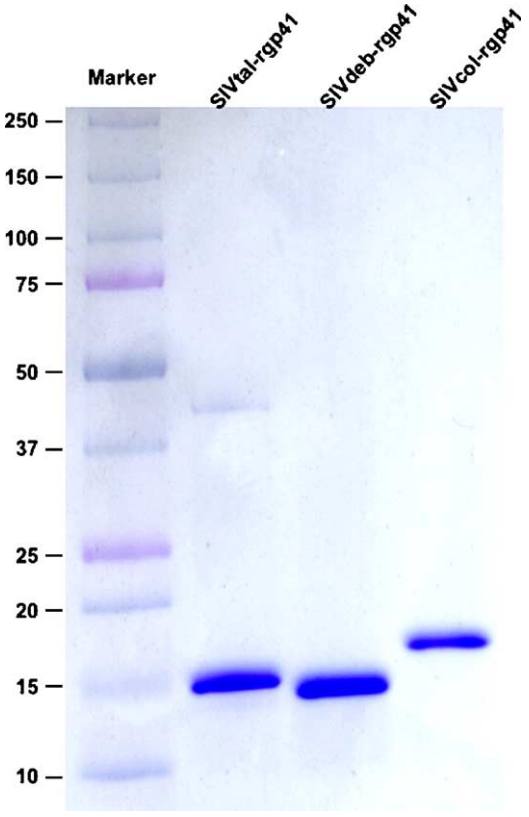

Fig. 2. Expression and purification of recombinant SIV gp41 proteins. The various SIV gp41 ectodomains were expressed as poly-histidine tagged fusion protein and purified by metal ion affinity chromatography. Purified aliquots of SIVtal-rgp41 (lane 2), SIVdeb-rgp41 (lane 3) and SIVcol-rgp41 (lane 4) were analyzed by SDS-PAGE electrophoresis, using a 4–20% linear gradient ready gel (BioRad) and stained using Coomassie brilliant blue G250. Protein markers of known molecular weight are shown in lane 1 (precision plus protein dual color standards, BioRad). Protein purity was estimated by using the ChemiDoc^{tm} XRS Image System (BioRad, Hercules, CA). All expressed purified recombinant SIVrgp41 proteins were greater than 85% pure.

heterologous rgp41 proteins, and six others reacted weakly with heterologous peptides (Tables 3a and 3b; 4a and 4b). Although such reactivities could be indicative of cross-species infection, all sera reacted only weakly (OD/cut-off ratios less than 1.65) and in each case only with a single heterologous antigen. Moreover, the same sera tested negative in the corresponding V3 loop or rgp41 ELISAs, strongly suggesting unspecific reactivities. Thus, the overall specificity of homologous and heterologous antibody detection was 98% for the rgp41 ELISAs, and 95% for the peptide ELISAs.

To determine the sensitivity of the various rgp41 and peptide ELISAs, a panel of positive reference sera from primates with PCR documented SIV infection was analyzed. As shown in Tables 3a and 3b, 62 of the 63 sera reacted strongly with the species-specific SIV gp41 proteins, indicating an overall sensitivity of 98%. The single false negative serum was from a known infected talapoin monkey. Although non-reactive in the homologous SIVtal rgp41 ELISA, this same sample scored positive in three other heterologous rgp41 ELISAs (SIVdeb, SIVsmm and SIVsyk) (Table 4a). Thus, taking both homologous and heterologous rgp41 reactivities into account, all antibody-positive sera were correctly identified. This was not the case for the synthetic peptide ELISAs (Table 3b). Overall, only 50 of 61 positive sera scored positive in the homologous peptide assays (82%), and this sensitivity did not improve when heterologous peptide reactivities were considered (Table 4b). Thus, while exhibiting similar specificities, the various peptide ELISAs were less sensitive at detecting homologous and heterologous antibodies than their rgp41 protein counterparts.

Extent of antigenic cross-reactivity between different SIV strains

To estimate how many strain-specific antibody detection tests would ultimately be required for comprehensive field testing, we examined the extent of antigenic cross-reactivity between the various (rgp41 and peptide) ELISAs and sera from different SIV-infected primate species. These data are summarized in Tables 4a and 4b, and in Fig. 4. Overall, the rgp41 ELISAs were considerably more cross-reactive than their peptide counterparts. For example, the SIVmon rgp41 ELISA detected all SIVmus, SIVgsn, SIVcpz, SIVmnd-2 and SIVsyk infections, and the SIVsmm rgp41 ELISA identified all SIVdeb, SIVtal and SIVsyk infections (Table 4a). By contrast, only SIVgsn and SIVcpz peptide ELISAs detected heterologous antibodies in an appreciable number of species, although most of these harbored closely related viruses (Table 4b; Fig. 1C); all other peptide ELISAs detected no, or only a negligible fraction of heterologous antibodies (Table 4b). Consistent with previous observations, these results indicate

Table 1
V3 peptide sequences representing different SIV lineages used in the ELISAs

SIV	SIV lineage	V3-sequence
SIVcpz-ant	HIV-1/SIVcpz	NRTVRNLQIGPGMTFYNVEIEATGDTRKAFC
SIVgsn-166	SIVgsn/mon/mus	GNKTIRNLQIGAGMTFYSQVIVGGNTRKAYC
SIVagm-consensus	SIVagm	GNKTVLPVTIMAGLVFHSQKYNTLLRQAWC
SIVsmm-consensus	HIV-2/SIVsmm	GNKTVVPITLMSGLVFHSQPINKRPRQAWC
SIVsyk	SIVsyk	GNESIKNIQLAAGYFLPVIQGKLKTGRDAKRAFC
SIVrcm	SIVrcm	SNRTVKGISLATGVFISLRVEKRPKGAWC
SIVmnd-1	SIVmnd	GNRSVVSTPSATGLLFYHGLEPGKNLKKGMC
SIVlho	SIVlho/sun	GNRSEVSTISSTGLLFYYGLEHGSRLRLAQC
SIVcol	SIVcol	GNSSHRNLNTANGAKFYYELIPYSKGIYGRC
SIVdeb	SIVdeb	GNKTYRAVHMATGLSFYTTFIPRLRIKRAHC

Table 2
Serum reference panel from SIV-infected and non-infected non human primate species

Species	Common name	SIV	SIV pos (*n*)	SIV neg (*n*)
Cercopithecus neglectus	De Brazza monkey	SIVdeb	6	3
Cercopithecus cephus	Mustached monkey	SIVmus	4	31
Cercopithecus nictitans	Greater spot nosed monkey	SIVgsn	6	47
Cercopithecus mona	Mona monkey	SIVmon	1	1
Cercopithecus albogularis	Sykes monkey	SIVsyk	14	35
Colobus guereza	Mantled guereza	SIVcol	9	9
Mandrillus sphinx	Mandrill	SIVmnd-2	7	4
Miopithecus ogouensis	Northern talapoin	SIVtal	2	6
Cercocebus atys	Sooty mangabey	SIVsmm	9	7
Cercocebus torquatus	Red capped mangabey	SIVrcm	2	0
Pan troglodytes	Chimpanzee	SIVcpz	3	1
Total			63	144

that antibodies directed against the V3 loop are more strain specific and thus more suitable to distinguish between different SIV infections than antibodies directed against the gp41 ectodomain (Masciotra et al., 2000; Ndongmo et al., 2004; Simon et al., 2001). Overall, the SIVdeb, SIVsmm, SIVmon and SIVtal rgp41 ELISAs were the most cross-reactive, detecting 80%, 74%, 72%, and 70% of all heterologous SIV infections, respectively. By contrast, SIVmus and SIVcpz rgp41 ELISAs were the least cross-reactive, identifying only about 30% of heterologous SIV infections. On a species level, sera from infected Sykes's monkeys reacted with the most, while sera from infected guereza colobus reacted with the least number of heterologous rgp41 proteins. Indeed, three sera from SIVcol-infected colobus monkeys reacted only with the homologous rgp41 ELISA. ELISA OD values were higher for homologous than heterologous reactivities (Fig. 4).

Although most rgp41 ELISAs were highly cross-reactive, the extent and direction of this cross-reactivity was variable. For example, the SIVdeb rgp41 ELISA identified all positive mandrill, Sykes's, talapoin, sooty mangabey and mustached monkey sera, but the reverse was only true for the SIVsmm rgp41 ELISA. SIVmnd2, SIVsyk, SIVtal and SIVmus rgp41 ELISAs identified only 2/6, 5/6, 4/6 and 0/6 of all positive De Brazza's monkey sera, respectively. Similarly, the SIVcol rgp41 ELISA detected all positive mustached and mona monkey sera, but only 2 of 6 positive greater spot nosed monkey sera; the remaining 4 went undetected despite the fact that greater spot nosed, mustached and mona monkeys all harbor genetically very closely related viruses (Fig. 1C). Finally, the SIVtal rgp41 ELISA identified only one positive talapoin serum, but detected 43 of 61 antibody-positive sera (70%) from other species. Thus, while strain-specific antibodies frequently cross-react with gp41 proteins from SIVs infecting other species, the magnitude and direction of this cross-reactivity is unpredictable and does not always correlate with the phylogenetic relatedness of the respective SIV strains.

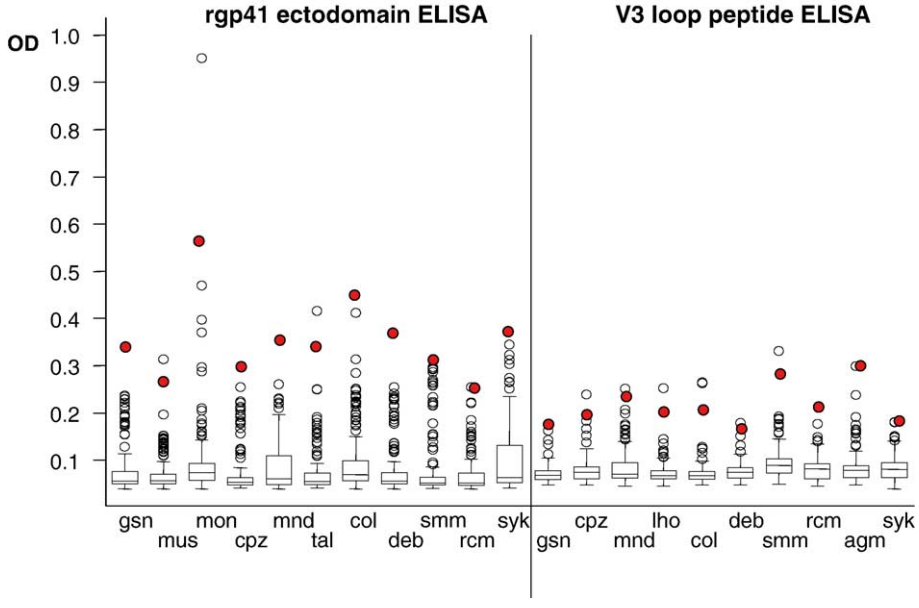

Fig. 3. Evaluation of recombinant protein (left panel) and synthetic peptide (right panel) based ELISAs (crude OD values) using sera from uninfected non-human primates ($n=144$). Vertical boxes with error bars represent the 25th [x_{25}] to 75th [x_{75}] percentiles (the so-called interquartile range, IQR) of seroreactivity ODs for each recombinant gp41 protein and peptide. The middle of the box represents the median or 50th percentile of the data, and the lines emerging from the box extend to the lower and upper adjacent values. The lower adjacent value is defined as the smallest data point greater than or equal to $x_{25}-1.5\times\text{IQR}$ and the upper adjacent value is defined as the largest data point less than or equal to $x_{75}+1.5\times\text{IQR}$. Observed points more extreme than the adjacent values are individually represented by open circles (Stata 7.0 software, Stata Corporation, College Station, Texas, USA). Red dots denote cut-off values calculated as the mean OD for each antigen plus 5 standard deviations (Crowther, 2001).

Table 3a
Sensitivity and specificity of the rgp41 SIV ELISA assays

Species	SIV	Homologous antibody detection				Homologous and heterologous antibody detection			
		N_{pos}/N_{tested}	Sensitivity	N_{pos}/N_{tested}	Specificity	N_{pos}/N_{tested}	Sensitivity	N_{pos}/N_{tested}	Specificity
C. neglectus	SIVdeb	6/6	100.0%	0/3	100.0%	52/63	82.5%	0/144	100.0%
C. cephus	SIVmus	4/4	100.0%	0/31	100.0%	23/63	36.5%	1/144	99.3%
C. nictitans	SIVgsn	6/6	100.0%	0/47	100.0%	35/63	55.6%	0/144	100.0%
C. mona	SIVmon	1/1	100.0%	0/1	100.0%	46/63	73.0%	1/144	99.3%
C. albogularis	SIVsyk	14/14	100.0%	0/35	100.0%	41/63	65.1%	0/144	100.0%
C. guereza	SIVcol	9/9	100.0%	0/9	100.0%	37/63	58.7%	0/144	100.0%
M. sphinx	SIVmnd-2	7/7	100.0%	0/4	100.0%	33/63	52.4%	0/144	100.0%
M. ogouensis	SIVtal	1/2	50.0%	0/6	100.0%	44/63	69.8%	1/144	99.3%
C. atys	SIVsmm	9/9	100.0%	0/7	100.0%	49/63	77.8%	0/144	100.0%
C. torquatus	SIVrcm	2/2	100.0%	NT	–	28/63	44.4%	0/144	100.0%
P. t. troglodytes	SIVcpz	3/3	100.0%	0/1	100.0%	21/63	33.3%	0/144	100.0%
Total		62/63	98.4% (91.5–100.0)[a]	0/144	100.0% (97.9–100.0)[a]	63/63	100.0% (95.4–100.0)[a]	3/144	97.9% (94.0–99.6)[a]

[a] 95% confidence intervals.

Prevalence of SIV infection in different primate species

Following assay validation, the newly developed ELISAs were employed to determine the prevalence of SIV infection in nine primate species native to Cameroon. Blood was collected from 589 additional monkeys sampled at various geographic sites, including bushmeat markets in and around the capital city of Yaounde, logging concessions in southeastern Cameroon, and villages in southwestern Cameroon. Thus, samples were collected over a wide range of locations representing different home ranges throughout southern Cameroon. All sera were subjected to homologous and heterologous rgp41 ELISA testing, while V3 peptide ELISAs were conducted in only selected cases. All sera were also screened by INNO-LIA to compare SIV strain specific with HIV cross-reactive antibody responses. Together with negative and positive reference sera from wild populations in Cameroon, INNO-LIA and rgp41 ELISA results were generated for a total of 722 primates.

Table 5 summarizes the proportion of SIV rgp41 ELISA-positive sera for members of six primate species for which an infecting SIV strain has been molecularly characterized (Bibollet-Ruche et al., 2004; Courgnaud et al., 2001, 2002; Souquiere et al., 2001) (F. Liegeois and M. Peeters, unpublished data). The results indicate a surprising range of naturally occurring SIV prevalences. Mandrills exhibited the highest infection rate, with over half of all animals harboring SIVmnd2-specific antibodies. De Brazza's and guereza colobus monkeys revealed intermediate seroprevalences, with 39% and 27% harboring strain-specific antibodies, respectively. By contrast, many fewer SIV seropositive talapoin (11%), greater spot nosed (3%) and mustached monkeys (4%) were identified, despite large numbers of available sera for the latter two species. All sera were tested using the entire panel of rgp41 ELISAs, with homologous assay results confirmed by heterologous assay results. For example, two talapoin monkeys initially misidentified as negative by the homologous SIVtal rgp41 ELISA scored positive in three heterologous rgp41 ELISAs (see below).

Table 3b
Sensitivity and specificity of the V3-peptide SIV ELISA assays

Species	SIV	Homologous antibody detection[a]				Homologous and heterologous antibody detection[b]			
		N_{pos}/N_{tested}	Sensitivity	N_{pos}/N_{tested}	Specificity	N_{pos}/N_{tested}	Sensitivity	N_{pos}/N_{tested}	Specificity
C. neglectus	SIVdeb	2/6	33.3%	0/3	100.0%	2/63	3.2%	1/144	99.3%
C. cephus	SIVgsn	4/4	100.0%	0/31	100.0%				
C. nictitans	SIVgsn	5/6	83.3%	0/47	100.0%	23/63	36.5%	1/144	99.3%
C. mona	SIVgsn	1/1	100.0%	0/1	100.0%				
C. albogularis	SIVsyk	14/14	100.0%	0/35	100.0%	16/63	25.4%	0/144	100.0%
C. guereza	SIVcol	8/9	88.9%	0/9	100.0%	8/63	12.7%	2/144	98.6%
M. sphinx	SIVmnd-2	7/7	100.0%	0/4	100.0%	8/63	12.7%	0/144	100.0%
C. atys	SIVsmm	6/9	66.7%	1/7	85.7%	6/63	9.5%	1/144	99.3%
C. torquatus	SIVrcm	1/2	50.0%	NT	–	3/63	4.8%	0/144	100.0%
P. troglodytes	SIVcpz	2/3	66.7%	0/1	100.0%	14/63	22.2%	1/144	99.3%
–	SIVlho[c]	NT	–	NT	–	1/63	1.6%	1/144	99.3%
–	SIVagm[c]	NT	–	NT	–	3/63	4.8%	0/144	100.0%
Total		50/61	82.0% (70.0–90.6)[d]	1/138	99.3% (96.0–100.0)[d]	51/63	81.0% (69.1–89.8)[d]	7/144	95.1% (90.2–98.0)[d]

[a] Samples from Northern talapoins (*M. ogouensis*) were excluded for the homologous detection due to absence of SIVtal peptide.
[b] All 207 sera from the reference panel were tested with all peptides.
[c] SIVlho and SIVagm were only validated for heterologous antibody detection due to absence of reference sera for the corresponding SIV infected primates.
[d] 95% confidence intervals.

Table 4a
Crossreactivity of SIV lineage-specific rgp41 ELISAs with sera from different primate species

Species	Primate sera	N_{tested}	SIVgsn ELISA N_{pos}/N_{tested}	SIVmus ELISA N_{pos}/N_{tested}	SIVmon ELISA N_{pos}/N_{tested}	SIVcpz ELISA N_{pos}/N_{tested}	SIVmnd-2 ELISA N_{pos}/N_{tested}	SIVtal ELISA N_{pos}/N_{tested}	SIVcol ELISA N_{pos}/N_{tested}	SIVdeb ELISA N_{pos}/N_{tested}	SIVrcm ELISA N_{pos}/N_{tested}	SIVsmm ELISA N_{pos}/N_{tested}	SIVsyk ELISA N_{pos}/N_{tested}
C. neglectus	SIVdeb antibody neg	3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
C. neglectus	**SIVdeb antibody pos**	**6**	**1/6**	**0/6**	**3/6**	**0/6**	**2/6**	**4/6**	**2/6**	**6/6**	**0/6**	**6/6**	**5/6**
C. guereza	SIVcol antibody neg	9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
C. guereza	**SIVcol antibody pos**	**9**	**1/9**	**5/9**	**3/9**	**1/9**	**0/9**	**1/9**	**9/9**	**2/9**	**0/9**	**0/9**	**0/9**
M. ogouensis	SIVtal antibody neg	6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
M. ogouensis	**SIVtal antibody pos**	**2**	**1/2**	**1/2**	**1/2**	**1/2**	**0/2**	**1/2**	**0/2**	**2/2**	**1/2**	**2/2**	**2/2**
M. sphinx	SIVmnd-2 antibody neg	4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
M. sphinx	**SIVmnd-2 antibody pos**	**7**	**6/7**	**2/7**	**6/6(1nt)[a]**	**1/7**	**7/7**	**5/7**	**7/7**	**7/7**	**5/7**	**7/7**	**5/7**
C. nictitans	SIVgsn antibody neg	47	0/47	1/47	1/47	0/47	0/47	0/47	0/47	0/47	0/47	0/47	0/47
C. nictitans	**SIVgsn antibody pos**	**6**	**6/6**	**5/6**	**6/6**	**4/6**	**1/6**	**3/6**	**2/6**	**4/6**	**0/6**	**3/6**	**4/6**
C. cephus	SIVmus antibody neg	31	0/31	0/31	0/31	0/31	0/31	0/31	0/31	0/31	0/31	0/31	0/31
C. cephus	**SIVmus antibody pos**	**4**	**4/4**	**4/4**	**4/4**	**3/4**	**0/4**	**2/4**	**4/4**	**4/4**	**1/4**	**4/4**	**1/4**
C. mona	SIVmon antibody neg	1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
C. mona	**SIVmon antibody pos**	**1**	**1/1**	**1/1**	**1/1**	**1/1**	**0/1**	**1/1**	**1/1**	**1/1**	**0/1**	**1/1**	**1/1**
P. troglodytes	SIVcpz antibody neg	1	0/1	0/1	0/1	0/1	0/1	1/1	0/1	0/1	0/1	0/1	0/1
P. troglodytes	**SIVcpz antibody pos**	**3**	**3/3**	**2/3**	**3/3**	**3/3**	**0/3**	**2/3**	**0/3**	**2/3**	**0/3**	**1/3**	**0/3**
C. atys	SIVsmm antibody neg	7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7
C. atys	**SIVsmm antibody pos**	**9**	**3/9**	**2/9**	**5/9**	**3/9**	**8/9**	**9/9**	**4/9**	**9/9**	**8/9**	**9/9**	**9/9**
C. torquatus	**SIVrcm antibody pos**	**2**	**0/2**	**0/2**	**0/2**	**0/2**	**1/2**	**2/2**	**0/2**	**1/2**	**2/2**	**2/2**	**0/2**
C. albogularis	SIVsyk antibody neg	35	0/35	0/35	0/35	0/35	0/35	0/35	0/35	0/35	0/35	0/35	0/35
C. albogularis	**SIVsyk antibody pos**	**14**	**9/14**	**1/14**	**14/14**	**4/14**	**14/14**	**14/14**	**8/14**	**14/14**	**11/14**	**14/14**	**14/14**

SIV-positive and -negative reference sera from different primate species (left panel) have been tested with the 11 different rgp41 ELISAs (right panel). Homologous Elisa's are highlighted by a frame.
[a] nt, not tested.

Having shown that sera from multiple species harbor SIV-specific antibodies, we next examined which of the SIV ELISA-positive sera also reacted with HIV-1 and HIV-2 proteins in the INNO-LIA assay and which exhibited discordant results. This analysis identified widely varying results depending on the species analyzed. For De Brazza's monkeys, both the SIVdeb rgp41 ELISA and the INNO-LIA identified the same number of antibody-positive sera, indicating 100% concordance for the two tests. For mandrills, 14 of 27 sera were SIVmnd-2 rgp41 ELISA-positive, and 15 of 27 sera were INNO-LIA-positive. PCR analysis of the one discordant sample failed to detect SIVmnd-2 sequences, suggesting absence of infection. Thus, with the exception of one potentially unspecific INNO-LIA result, both tests yielded comparable results. However, this was not the case for guereza colobus monkeys. Only 8 of 14 SIVcol ELISA-positive sera were also INNO-LIA-positive. Two of the discordant sera were positive in the SIVcol V3 peptide ELISA, and two others were confirmed by PCR amplification of virus-specific sequences to be derived from SIVcol-infected monkeys (not shown). Thus, for guereza colobus, INNO-LIA reactivity clearly underestimated the true SIVcol infection rate. The opposite was observed for northern talapoin: only 1 of 26 sera tested SIVtal rgp41 ELISA-positive, while 3 were INNO-LIA-positive. These same 3 sera were also positive in heterologous SIVdeb, SIVsmm and SIVsyk ELISAs, and all 3 were confirmed by PCR to be derived from SIVtal-infected talapoin monkeys (Peeters et al., 2002), (F. Liegeois and M. Peeters, unpublished data). Thus, for northern talapoins, the INNO-LIA was more reliable than the lineage-specific rgp41 ELISA for detecting SIVtal infections.

By far, the most striking differences were observed for greater spot nosed and mustached monkeys. For mustached monkeys, only 6 of 203 sera were SIVmus rgp41 ELISA-positive, compared to 77 sera that were INNO-LIA-positive. Similarly, for greater spot nosed monkeys, only 8 of 193 sera were SIVgsn rgp41 ELISA-positive, compared to 33 that were INNO-LIA-positive, although all ELISA-positive sera were also INNO-LIA-positive. Further analysis indicated that all SIVgsn and SIVmus ELISA-positive sera also reacted with heterologous SIV rgp41 proteins (Table 5) and that the great majority was PCR-positive. By contrast, none of the discordant INNO-LIA positives reacted with the rgp41 proteins from SIVs infecting other species and none of a subset that was tested was PCR positive.

Table 4b
Crossreactivity of SIV lineage-specific V3 peptide ELISA assays with sera from different primate species

Species	Primate sera	N_{tested}	SIVgsn ELISA N_{pos}/N_{tested}	SIVcpz ELISA N_{pos}/N_{tested}	SIVmnd ELISA N_{pos}/N_{tested}	SIVlho ELISA N_{pos}/N_{tested}	SIVcol ELISA N_{pos}/N_{tested}	SIVdeb ELISA N_{pos}/N_{tested}	SIVrcm ELISA N_{pos}/N_{tested}	SIVsmm ELISA N_{pos}/N_{tested}	SIVagm ELISA N_{pos}/N_{tested}	SIVsyk ELISA N_{pos}/N_{tested}
C. neglectus	SIVdeb antibody neg	3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
C. neglectus	**SIVdeb antibody pos**	**6**	**0/6**	**0/6**	**0/6**	**0/6**	**0/6**	**2/6**	**0/6**	**0/6**	**0/6**	**0/6**
C. guereza	SIVcol antibody neg	9	1/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
C. guereza	**SIVcol antibody pos**	**9**	**0/9**	**2/9**	**0/9**	**1/9**	**8/9**	**0/9**	**0/9**	**0/9**	**0/9**	**0/9**
M. ogouensis	SIVtal antibody neg	6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
M. ogouensis	**SIVtal antibody pos**	**2**	**0/2**	**0/2**	**0/2**	**0/2**	**0/2**	**0/2**	**0/2**	**0/2**	**0/2**	**1/2**
M. sphinx	SIVmnd-2 antibody neg	4	0/4	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4	0/4
M. sphinx	**SIVmnd-2 antibody pos**	**7**	**0/7**	**0/7**	**7/7**	**0/7**	**0/7**	**0/7**	**2/7**	**0/7**	**0/7**	**1/7**
C. nictitans	SIVgsn antibody neg	47	0/47	1/47	0/47	0/47	1/47	0/47	0/47	0/47	0/47	0/47
C. nictitans	**SIVgsn antibody pos**	**6**	**5/6**	**5/6**	**1/6**	**0/6**	**0/6**	**0/6**	**0/6**	**0/6**	**0/6**	**0/6**
C. cephus	SIVmus antibody neg	31	0/31	0/31	0/31	1/31	0/31	1/31	0/31	0/31	0/31	0/31
C. cephus	**SIVmus antibody pos**	**4**	**4/4**	**4/4**	**0/4**	**0/4**	**0/4**	**0/4**	**0/4**	**0/4**	**0/4**	**0/4**
C. mona	SIVmon antibody neg	1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
C. mona	**SIVmon antibody pos**	**1**	**1/1**	**1/1**	**0/1**	**0/1**	**0/1**	**0/1**	**0/1**	**0/1**	**0/1**	**0/1**
P. troglodytes	SIVcpz antibody neg	1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
P. troglodytes	**SIVcpz antibody pos**	**3**	**2/3**	**2/3**	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**	**0/3**
C. atys	SIVsmm antibody neg	7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	1/7	0/7	0/7
C. atys	**SIVsmm antibody pos**	**9**	**2/7**	**0/9**	**0/9**	**0/9**	**0/9**	**0/9**	**0/9**	**6/9**	**3/9**	**0/9**
C. torquatus	**SIVrcm antibody pos**	**2**	**0/2**	**0/2**	**0/2**	**0/2**	**0/2**	**0/2**	**1/2**	**0/2**	**0/2**	**0/2**
C. albogularis	SIVsyk antibody neg	35	0/35	0/35	0/35	0/35	0/35	0/35	0/35	0/35	0/35	0/35
C. albogularis	**SIVsyk antibody pos**	**14**	**9/14**	**0/14**	**0/14**	**0/14**	**0/14**	**0/14**	**0/14**	**0/14**	**0/14**	**14/14**

SIV-positive and -negative reference sera from different primate species (left panel) have been tested with the 10 different V3- ELISAs (right panel). Homologous Elisa's are highlighted by a frame.

Confirmatory Western blot analysis

To examine whether INNO-LIA positive but SIV ELISA negative greater spot nosed and mustached monkeys lacked V3 loop and gp41 antibodies but harbored antibodies directed against other envelope and/or core protein epitopes, we developed a Western blot assay that utilized SIVgsn Env containing pseudovirions as immunoblot antigens. To generate these pseudovirions, we first modified the codon usage of the SIVgsn *env* sequence to resemble that of highly expressed human genes. Codon usage optimized genes are known to express large quantities of envelope glycoproteins, which are efficiently incorporated into virus particles when expressed in trans (Andre et al., 1998; Gao et al., 2003, 2005). The codon optimized SIVgsn env (gp160) gene was then cotransfected with an *env*-minus HIV-1 backbone vector (HIV-1ΔenvSG3) (Wei et al., 2003), which resulted in the production of SIVgsn Env containing pseudovirions. These were purified by centrifugation through a sucrose cushion, analyzed for p24 Gag content, and used as antigens on Western blot strips (100 ng of p24 per strip).

Fig. 5 depicts the Western blot profiles of three different groups of sera: (i) greater spot nosed and mustached monkey sera that were both SIV ELISA and INNO-LIA positive (left panel); (ii) greater spot nosed and mustached monkey sera that were INNO-LIA positive, but SIV ELISA negative (middle panel); (iii) greater spot nosed monkey sera that were both SIV ELISA and INNO-LIA negative (right panel). This analysis identified SIV-specific antibodies only in sera that were also SIV rgp41 ELISA positive: three greater spot nosed and three mustached monkey sera reacted strongly with the exterior portion (gp120) as well as the uncleaved gp160 precursor of the SIVgsn envelope glycoprotein (codon usage optimized envelope glycoproteins are only incompletely processed due to saturation of cellular proteases; (Andre et al., 1998;Gao et al., 2003, 2005)). Four of these same sera also recognized the transmembrane envelope glycoprotein (gp41) and three cross-reacted with the HIV-1 p24 Gag core protein. By contrast, all other sera including five that exhibited strong INNO-LIA reactivities with HIV-1 gp120 (S1079), gp41 (S43, S1267) and p17 Gag proteins (S1287, S1285) (not shown), failed to react with SIVgsn and HIV-1 proteins in the immunoblot assay. Thus, the discordant INNO-LIA positives represented unspecific reactivities.

Survey of primate species suspected to harbor SIV

Finally, we used the newly developed rgp41 ELISA to screen sera from 92 agile mangabeys (*Cercocebus agilis*), 70 crested mona monkeys (*Cercopithecus pogonias*) and 36 gray-cheeked mangabeys (*Lophocebus albigena*) for cross-reactive

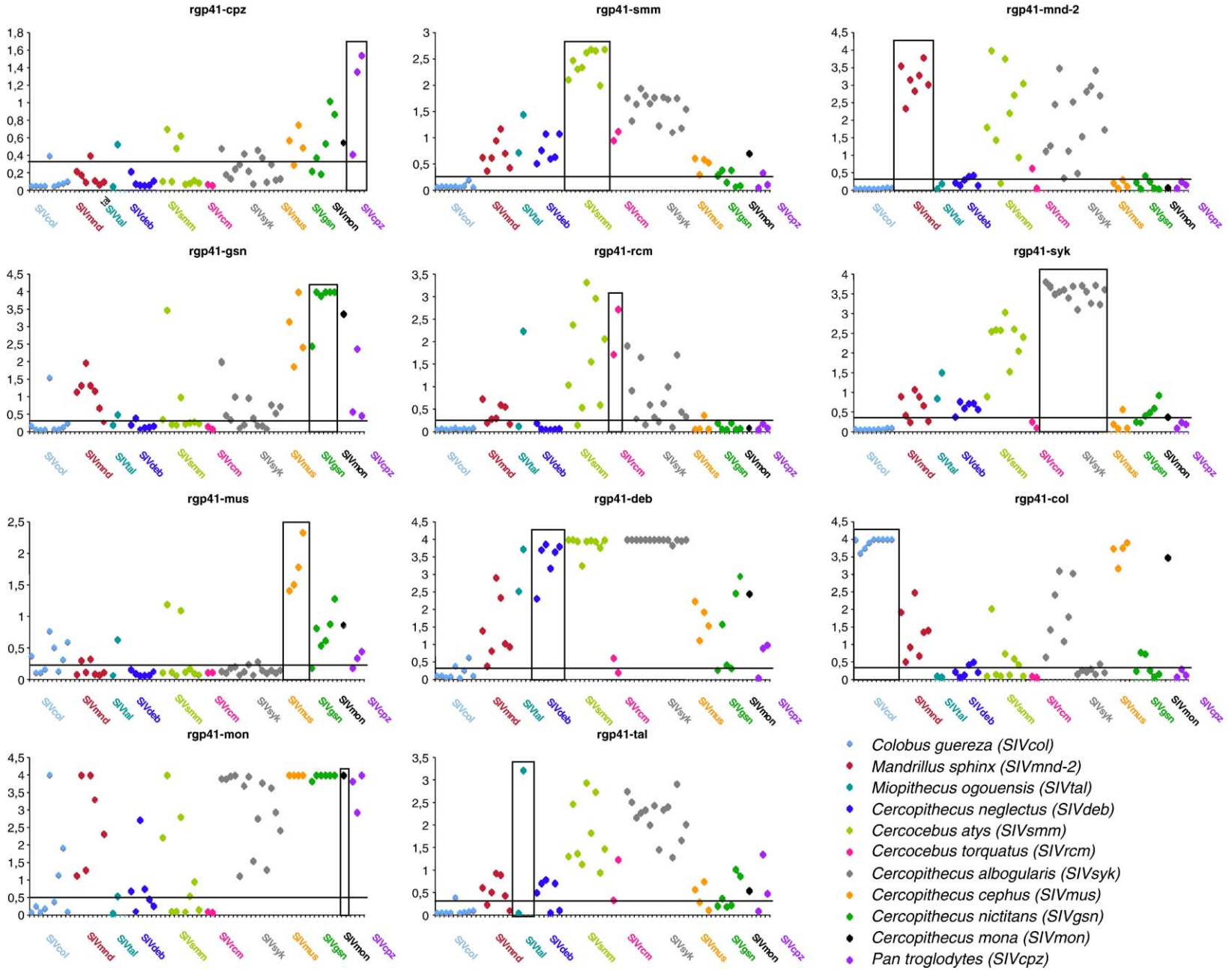

Fig. 4. ELISA cross-reactivity pattern of sera from primates with PCR confirmed SIV infection status. Crude OD values are shown on the *x* axis for eleven different rgp41 ELISA tests. Sera from different primate species are color-coded on the *y* axis. The cut-off for each test is indicated by a black horizontal line. For each ELISA, homologous antibody responses are shown in boxes.

Table 5
SIV prevalence rates in wild primate populations in Cameroon

Species	SIV strain	HIV-1/2 INNO-LIA	SIV rgp41 ELISA		SIV prevalence[a] (95% CI)
		N_{pos}/N_{tested}	Homologous antibody detection N_{pos}/N_{tested}	Heterologous antibody detection N_{pos}/N_{tested}	
Cercopithecus neglectus	SIVdeb	9/23	9/23	9/23	39.1% (19.7–61.5)
Mandrillus sphinx	SIVmnd-2	15/27	14/27	13/27	51.8% (31.9–71.3)
Colobus guereza	SIVcol	8/52	14/52	8/52	26.9% (15.6–41.0)
Miopithecus ogouensis	SIVtal	3/26	1/26	3/26[b]	11.5% (2.4–30.2)
Cercopithecus cephus	SIVmus	77/203	6/203	6/203	2.9% (1.1–6.3)
Cercopithecus nictitans	SIVgsn	33/193	8/193	8/193	4.1% (1.8–8.0)
Cercopithecus pogonias	–[c]	19/70	NT	(1)/70[d]	0%[e] (0.0–4.2)
Cercocebus agilis	SIVagi[f]	27/92	NT	(1)/92[d]	0%[e] (0.0–3.2)
Lophocebus albigena	–[c]	20/36	NT	0/36	0% (0.0–8.0)

[a] SIV prevalence rates were calculated using the combined homologous and heterologous antibody reactivities as determined by the various SIV rgp41 ELISAs.
[b] Two *M. ogouensis* samples were identified as antibody positive by heterologous SIV rgp41 ELISAs and both were confirmed to be derived from SIVtal infected animals by PCR and sequence analysis.
[c] SIV infection has not yet been confirmed by amplification of SIV sequences.
[d] The single observed reactivities were most likely unspecific (see text for details).
[e] Confidence limits were calculated assuming zero positives among the sera tested.
[f] SIVrcm like partial *pol* sequences have been amplified from 2 captive agile mangabeys (see text for details).

antibodies. Sera from these three species have previously been observed to react with HIV-1 and HIV-2 antigens, suggesting they might harbor as yet unidentified SIV strains (Peeters et al., 2002). Moreover, SIVrcm-like viruses were identified in two captive agile mangabeys by PCR amplification of subgenomic integrase sequences (E. Nerrienet, C. Apetrei, Y. Foupouapouognigni, B. Ling, A. Luckay, L Chakrabarti, A. Ayouba, and P. Marx, Abstr. 14th Int. AIDS Conf., abstr. TuPeA4405, 2002). We thus examined whether our panel of strain-specific assays could uncover new SIV infections.

Consistent with previous results, we found 66 of the 198 sera to be INNO-LIA positive. However, using the entire set of rgp41 antigens, ELISA reactivity was only observed for two of the 198 sera. One from an agile mangabey scored positive in the SIVcol rgp41 ELISA, and the other from a crested mona reacted weakly in the SIVsyk rgp41 ELISA. In both instances, ELISA reactivities were limited to a single SIV rgp41 antigen, and all attempts to amplify SIV sequences from these two samples remained negative. Similarly, only 7 of the 198 sera scored weakly positive in the V3 peptide ELISAs. Again, none

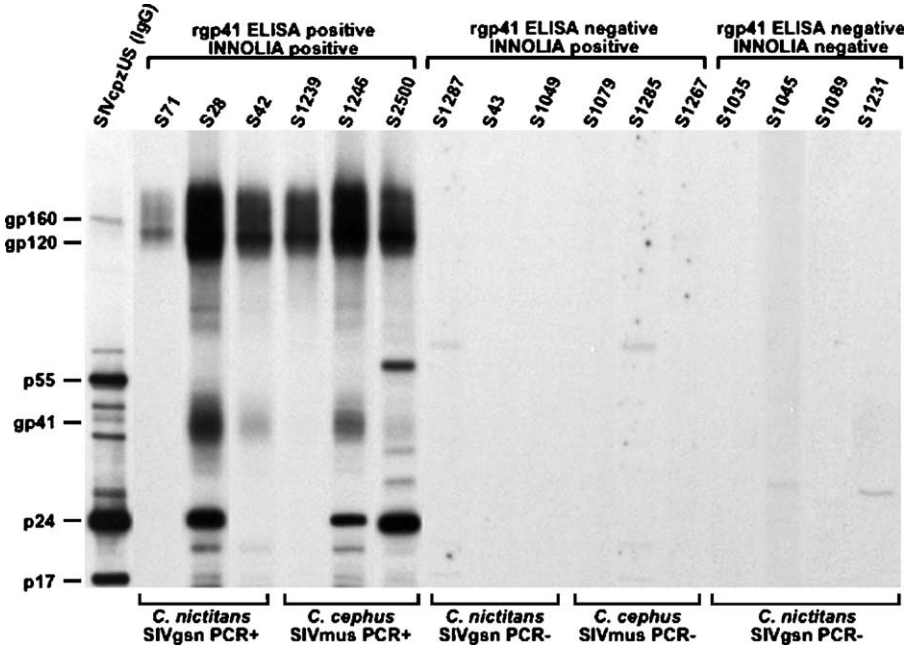

Fig. 5. SIVgsn-specific Western immunoblot. The Western blot reactivities of greater spot nosed (*C. nictitans*) and mustached (*C. cephus*) monkey sera (grouped by SIV PCR status as well as reactivity in SIVgsn and SIVmus rgp41 ELISA and INNO-LIA assays, respectively) with SIVgsn Env containing HIV-1 pseudovirions are shown. SIV-specific antibodies are detected only in sera that are both rgp41 ELISA and INNO-LIA positive. Pseudovirions were quantified by HIV-1 p24 antigen content; each strip contains 100 ng of p24 Gag protein. The positions of the SIVgsn gp160, gp120, and gp41 envelope glycoproteins, along with the HIV-1 backbone Gag proteins p55, p24, and p17, are shown on the left. Lane 1 depicts reactivities of a positive SIVcpz control (purified SIVcpzUS-specific IgG, 1 μg/ml).

of these was positive in the corresponding rgp41 ELISA and none was PCR positive. Finally, none of the 66 INNO-LIA-positive sera reacted with any of the SIV rgp41 ELISAs, and none contained amplifiable sequences. Taken together, the SIV-specific antibody detection assays failed to identify evidence of SIV infection in the crested mona, agile and grey-cheeked mangabey monkeys under study.

Discussion

Although it is now well established that a substantial proportion of wild-living primates in sub-Saharan Africa harbor SIV, no study to date has examined to what extent the various species are naturally infected, whether some species are the source of infection of others, and whether the viruses currently recognized represent the totality of SIV infection in nature. In this study, we describe the development and validation of a comprehensive panel of SIV-specific antibody detection tests and their use for SIV prevalence determinations in nine primate species. The results revealed an unexpectedly wide range of prevalence rates, with high levels of SIV infection in some species and rare or no infection in others. These data indicate that the epidemiology of naturally occurring SIV infections is more complex than previously appreciated and that the various non-human primate hosts differ in their susceptibility to SIV infection.

Assay development and screening strategies

Synthetic peptides have been used in the past to detect HIV- and SIV-specific antibodies in human and primate sera (Ayouba et al., 2000, 2001; Masciotra et al., 2000; Mauclere et al., 1997; Ndongmo et al., 2004; Simon et al., 2001). However, peptides cover only very short epitopes and are frequently not particularly sensitive as capture antigens unless their coating efficiency is increased by chemical modification. Moreover, antibodies directed against epitopes that differ in only few amino acids might not be detected. To generate a more robust set of antibody detection assays, we developed ELISA strategies that utilized both peptides and recombinant proteins as antibody capture antigens. For peptide design, we selected the V3 region of the SIV envelope because this domain is known to elicit antibodies that are suitable for distinguishing different SIV infections (Ndongmo et al., 2004; Simon et al., 2001). For the protein component, we expressed the entire ectodomain of the SIV transmembrane envelope domain because this region is highly immunogenic and comprises both linear and conformational epitopes due to partial refolding (Barin et al., 1985; Weissenhorn et al., 1997; Xu et al., 1991).

The sensitivity and specificity of the newly devised assays was determined using a comprehensive panel of well-characterized reference sera. These analyses showed that all but one of the rgp41 ELISAs detected homologous antibodies with 100% sensitivity and 100% specificity (Table 3a, 3b). Moreover, most of the rgp41 ELISAs were quite cross-reactive and thus provided corroborating evidence for homologous antibody results, although the extent and direction of cross-reactivity varied based on the particular test and primate species analyzed. The V3 loop peptide ELISAs were less sensitive, but capable of discriminating between the different SIV lineages. This panel of rgp41 and V3 peptide-based ELISAs thus represents a comprehensive and versatile set of SIV antibody screening assays.

Although the combination of SIVmon, SIVsmm and SIVcol rgp41 ELISAs was sufficient to correctly identify all SIV infections in the control panel, the number of positive reference sera for each of the 11 primate species is still rather limited (Table 2). Thus, future serological surveys of primate population should employ the entire panel of rgp41 ELISAs, especially for species suspected but not yet confirmed to harbor SIV. Based on current data, we would expect true positives to react with both homologous and heterologous rgp41 proteins as well as with selected V3 loop peptides, especially if the latter are modified to increase their sensitivity (Masciotra et al., 2000; Ndongmo et al., 2004, Simon et al., 2001). As additional SIV infections are identified and confirmed, it should be possible to identify patterns of seroreactivity that are highly predictive of infection, even in the absence of PCR confirmation. In this context, it will be important to improve the sensitivity of the current SIVtal rgp41 ELISA by expressing and testing additional SIVtal rgp41 ectodomains and by examining the genetic and antigenic diversity of the gp41 region among different SIVtal strains. Finally, inclusion of recombinant SIVlho, SIVmnd-1 and SIVagm gp41 proteins would ensure representation of all currently known SIV lineages in the rgp41 ELISA screening panel.

HIV cross-reactivity as a predictor of SIV infection

All major SIV lineages known to date were initially discovered because their primate hosts had antibodies that cross-reacted with HIV-1 and/or HIV-2 antigens (Beer et al., 1999; Bibollet-Ruche et al., 1997, 2004; Courgnaud et al., 2001, 2003a, 2003b; Emau et al., 1991; Gao et al., 1999; Georges-Courbot et al., 1998; Ohta et al., 1988; Osterhaus et al., 1999; Peeters et al., 2002; Souquiere et al., 2001; Takehisa et al., 2001). Since commercially available HIV screening assays contain only a limited number of antigens, most SIV studies have used Western blot type tests to screen for cross-reactive antibodies. One of these, the INNO-LIA HIV Confirmation test, has proven particularly useful in identifying genetically divergent SIV strains. This test, which contains recombinant HIV-1 and HIV-2 proteins and synthetic peptides coated as discrete lines on a nylon strip, correctly identified 7 of the 13 currently known major SIV lineages (Bibollet-Ruche et al., 2004; Courgnaud et al., 2001, 2002, 2003a, 2003b; Peeters et al., 2002). Given this performance record, we examined to what extent the INNO-LIA was also useful in determining SIV prevalence rates in different primate species.

To determine the extent of INNO-LIA cross-reactivity, we compared the relative proportions of INNO-LIA and SIV ELISA-positive sera among the 722 primates analyzed. The results revealed marked differences, depending on which species was analyzed. For De Brazza's monkeys, mandrills,

talapoins, Sykes's monkeys, and sooty mangabeys, SIV ELISA and INNO-LIA tests performed at comparable levels, with very similar numbers of SIV-infected monkeys correctly identified (Table 5) (F. Bibollet-Ruche and B. H. Hahn, unpublished data). For guereza colobus, INNO-LIA failed to identify 40% of infected animals, but this was not unexpected given the extent of genetic divergence of SIVcol from other SIVs in the envelope region (Fig. 1C) and the low level of antigenic cross-reactivity generally observed for positive guereza colobus sera (Table 4a) (Ndongmo et al., 2004). However, the markedly discordant results for greater spot nosed and mustached monkey sera came as a surprise (Table 5). To exclude the possibility that infected members of these two species lacked antibodies to the particular ELISA antigens used, we tested a subset for reactivity with SIVgsn Env containing HIV-1 pseudovirions (Fig. 5). This analysis failed to detect SIV-specific antibodies in ELISA negative sera, while SIVgsn envelope as well as the HIV-1 p24 Gag-reactive antibodies were readily identified in ELISA-positive sera (Fig. 5). Moreover, none of the discordant INNO-LIA-positive sera were PCR positive or reacted with heterologous rgp41 antigens. Based on these data, we conclude that the bulk of the observed INNO-LIA reactivities for greater spot nosed and mustached monkeys are unspecific, and that the same appears to be true for crested mona, agile and grey-cheeked mangabeys (Table 5). It remains to be determined whether the unspecific reactivities are directed against HIV gene products or contaminating proteins in the INNO-LIA test. Regardless of the outcome, it is clear that the INNO-LIA has overestimated SIV prevalence rates in a number of different primate species, and that HIV antigen-based assays cannot be used to measure SIV prevalences in primate populations or identify SIV infections in humans.

Widely varying prevalence rates in naturally infected primate species

The great majority of primate species known to harbor a primate lentivirus exhibit high levels of SIV infection. The most extensively studied species, sooty mangabeys and African green monkeys, exhibit prevalence rates that frequently exceed 50% in adult animals, and this has been documented both in captivity and in the wild (Apetrei et al., 2005; Bibollet-Ruche et al., 1997; Jolly et al., 1996; Phillips-Conroy et al., 1994). High levels of SIV infection have also been identified in wild-living mandrills, De Brazza's and Sykes's monkeys, although relatively fewer animals have been characterized (Bibollet-Ruche et al., 2004; Ellis et al., 2004; Souquiere et al., 2001; Takehisa et al., 2001). Based on these results, it is now generally assumed that all natural SIV infections are common and widespread. Our finding of only 3% and 4% SIV infection rates in mustached and greater spot nosed monkeys, respectively, indicates that this assumption is not correct.

The reasons for varying SIV infection rates in different primate species are not known, but are likely due to a combination of viral, host and/or environmental factors. For example, SIVgsn and SIVmus could represent SIVs that were acquired relatively more recently and are thus not yet fully adapted for efficient replication and spread in their new hosts. In this case, the low prevalence would be due to intrinsic viral properties. Alternatively, behavioral differences could somehow limit the extent of virus exposure of greater spot nosed and mustached monkeys relative to other species. However, it is known that SIVgsn and SIVmus are transmitted, albeit infrequently, among members of their respective primate species because their sequences form species-specific clusters in phylogenetic trees (Courgnaud et al., 2002, 2003a). In general, primates were sampled at many different sites throughout southern Cameroon and the few SIVgsn and SIVmus antibody positive samples that were identified, were derived from different geographic regions in Cameroon, excluding sampling bias as the reason for the different prevalence rates. Thus, while intrinsic viral properties or primate behavior may play a role, host genetic factors are likely the most important determinant of SIV transmission and persistence in wild primate populations. A number of intrinsic host defense mechanisms have recently been described (Bieniasz, 2004; Goff, 2004; Sheehy et al., 2002; Stremlau et al., 2004), and such restriction factors could greatly influence the relative susceptibility of primates to SIVs from their own as well as other species. Deciphering these host factors and their mechanisms of action will be important not only to understand the epidemiology and natural history of the various SIVs, but also to gauge their potential to cross-infect humans.

Finally, we used the newly developed antibody detection assays to screen members of three primate species, i.e., crested mona, agile and grey-cheeked mangabeys, which have been suspected but not yet confirmed to be naturally infected with SIV. Although a relative large number of sera scored INNO-LIA positive (see above), no evidence of infection was found using the SIV-specific assays. This result is particularly puzzling since two of 20 captive agile mangabeys have previously been reported to harbor SIVrcm-like viruses as determined by PCR amplification of a subgenomic (integrase) sequence (E. Nerrienet, C. Apetrei, Y. Foupouapouognigni, B. Ling, A. Luckay, L Chakrabarti, A. Ayouba, and P. Marx, Abstr. 14th Int. AIDS Conf., abstr. TuPeA4405, 2002). Our ELISA panel included both recombinant SIVrcm gp41 protein and V3 peptides; yet, none of the 92 agile mangabey sera reacted with these antigens, suggesting the absence of SIVrcm Env-reactive antibodies. It is possible that SIVagi is a recombinant containing SIVrcm-like sequences in its 5′ half and the sequences from a highly divergent SIV strain in its 3′ half. Alternatively, the prevalence of SIVagi in agile mangabeys may be exceedingly low. Additional studies are required to address these possibilities and to determine whether crested mona, agile and grey-cheeked mangabeys harbor divergent SIV(s) that are not detected by the current strain-specific assays.

Testing humans for SIV infection

Given the magnitude of existing SIV reservoirs, studies are needed to determine whether transmission of simian lentiviruses other than SIVcpz and SIVsmm have occurred in regions where SIV infection of non-human primates is most prevalent. In this

paper, we report diagnostic assays that are capable of recognizing a wide range of naturally occurring SIV infections in primates. These same assays should be applicable to screening human risk groups that might constitute carriers of such infections, such as individuals who are frequently exposed to blood or bodily secretions of infected monkeys. In this context, it is important to note that we have explored both screening and confirmatory antibody detection strategies. Specifically, we show that a codon usage optimized SIVgsn *env* gene can be used to generate pseudovirions which carry a significant amount of the SIVgsn envelope glycoprotein on their surface (Fig. 5). Although the SIVgsn Env containing pseudovirions did not mediate viral entry into CD4- and CCR5-positive human (JC53-BL) cells (not shown), they were readily produced and purified, and represented suitable Western blot immunogens (Fig. 5). Identical strategies can be used to generate SIV/HIV pseudovirions for all other naturally occurring SIV strains. In the absence of replication competent isolates which are unavailable for most SIV strains, this is the best strategy yet to document the presence of specific envelope (gp160, gp120 and gp41) antibodies in SIV rgp41 ELISA-reactive sera. Documentation of such antibodies in human sera would be highly suggestive of SIV infection even in the absence of PCR amplifiable viral sequences.

All SIV gp41 expression plasmids and the codon usage optimized SIVgsn *env* gene have been submitted to the National Institute of Health Research and Reagent Program, Bethesda, MD and are thus available to investigators interested in the epidemiology and natural history of natural SIV infections.

Materials and methods

Expression and purification of recombinant gp41 (rgp41) proteins from different SIV strains

A gene segment spanning the ectodomain of the transmembrane (gp41) envelope domain (Fig. 1A) corresponding to amino acids 31–168 of the HIV-1/HXB2 gp41 protein (GenBank Accession number K03455) was amplified from molecular clones of SIVcpzTAN1 (AF447763), SIVsmmCI2 (unpublished), SIVrcmGAB1 (AF382829), SIVgsn99CM166 (AF46 8659), SIVmon99CMCML1 (AY340701), SIVmus01CM1085 (AY340700), SIVmnd2-14CG (AF328295), SIVdebCM40 (AY523865), SIVtal00CM266 (unpublished), SIVsykKE51 (AY523867), and SIVcolCGU1 (AF301156) (Fig. 1B), and cloned into the bacterial expression vector pET-21d (Novagen, Madison, MI) in-frame with a C-terminal hexahistidine (6xHIS) tag. BL21-CodonPlus competent *E. coli* (Stratagene, La Jolla, CA) were transformed with the respective pET-SIVgp41 expression plasmids and grown overnight from a single clone. Aliquots of these cultures were then used to inoculate large-scale expression cultures. Expression of the 6xHIS-tagged SIV gp41 proteins was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (Fisher Scientific, Pittsburgh, PA) to the cultures (final concentration 1 mM) at an optical density (OD) of 0.6–0.8. After an additional incubation for 4 h at 37 °C, bacteria were harvested by low speed centrifugation at 4 °C, resuspended in 50 ml of phosphate-buffered saline (PBS, pH 7.2), disrupted by sonication and treated with rLysozyme (Novagen, Madison, MI) for 20 min at room temperature to release inclusion bodies (IB). These were then concentrated by centrifugation, treated with Benzonase Nuclease (90 U/ml) for 4 h at room temperature to digest bacterial nucleic acids, washed three times with B-Per II bacterial protein extraction reagent (1:20) (Pierce, Rockford, IL), and solubilized in 50 ml of denaturing buffer (PBS containing 8M urea and 0.1% SDS). After pelleting debris, lysate supernatant was passed through a column containing Ni-NTA Superflow resin (Qiagen, Valencia, CA). The 6xHIS-tagged gp41 proteins were subsequently eluted from the column with PBS containing 0.5M imidazole, 0.1% SDS, 8M urea and dialyzed against PBS containing decreasing concentration of urea (4M, 2M and no urea) and SDS (0.05%, 0.025% and 0.01%). Protein concentration was determined using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, CA), and the relative purity of 6xHIS-tagged rgp41 proteins was examined by SDS-PAGE electrophoresis, using 4–20% linear gradient ready gels (BioRad, Hercules, CA). Proteins were visualized by Coomassie brilliant blue G250 staining (Fig. 2) and their purity was estimated by using the ChemiDoc XRS Image System (BioRad, Hercules, CA). All expressed purified recombinant SIVrgp41 proteins were greater than 85% pure (Fig. 2).

Synthetic (V3 loop) peptides

Synthetic peptides corresponding to the V3 loop region of the extracellular envelope domain of ten different SIV strains or lineages were purchased from Neosystems (Strasbourg, France). Table 1 depicts the sequences of the different peptides and their corresponding SIV strains. Because the V3 amino acid sequences of SIVgsn, SIVmus and SIVmon were virtually identical (Courgnaud et al., 2003a), only the SIVgsn V3 loop peptide was synthesized. Similarly, the SIVmnd-2 V3 peptide was used as a representative of both SIVmnd-1 and SIVmnd-2 strains which are monophyletic in the envelope gene (Fig. 1C). Finally, consensus V3 loop sequences were synthesized for SIVagm and SIVsmm lineages (Table 1).

Recombinant gp41 ELISA

ELISA plates (Corning, Corning, N.Y.) were coated (100 µl/well) with recombinant gp41 (rgp41) protein (1 µg/ml of total protein in PBS, pH 7.2) and incubated at 4 °C overnight. Plates were blocked using StabilCoat Immunoassay Stabilizer (SurModics, Eden Prairie, MN) (100 µl/well) for 1 h at room temperature, dried at 37 °C for 2 h, and stored in a desiccator until use. To test primate sera for the presence of SIV-specific antibodies, plates were incubated with blocking buffer (PBS containing 0.5% Tween-20, 5% non-fat dry milk) for 1 h at 37 °C, and washed with PBST (PBS with 0.1% Tween-20). Serum or plasma samples were diluted 1:100 in blocking buffer, added to each well (100 µl), incubated for 1 h at 37 °C, and washed with PBST. Goat-anti-human IgG-HRP (Southern Biotech, Birmingham, AL) was diluted 1:20,000 in blocking buffer, added to each well (100 µl), and incubated for 1 h at 37 °C.

After washing with PBST, 100 μl of 3,3′, 5,5′-Tetramethylbenzidine (TMB) Solution (Sigma, St. Louis, MO) was added and incubated for 20 min at room temperature. The reaction was stopped by adding 100 μl of 1N sulfuric acid, and OD values were measured at 450 nm against a reference wavelength of 630 nm in EX-800 ELISA reader (BIO-TEK Instruments, Inc., Winooski, VT).

Synthetic peptide ELISA

Polyvinylmicrotiter plates were coated with 0.25 μg per well of each peptide in a 0.05M bicarbonate buffer, pH 9.6, by incubation at 37 °C for 20 h. After washing with phosphate-buffer saline (PBS) containing 0.5% Tween 20, unoccupied sites were blocked with PBS containing 5% fetal calf serum, for 2 h at 37 °C followed by washing with PBS-Tween. Sera were diluted 1:100 in a hypertonic PBS solution (0.01M sodium phosphate buffer, pH 7.4, containing 0.75M NaCl, 10% fetal calf serum, and 0.5% Tween 20). After incubation for 30 min at room temperature, plates were washed and incubated with peroxidase-conjugated goat anti-human IgG for 30 min at room temperature. After washing, the reaction was developed with hydrogen peroxide-*o*-phenylendiamine for 15 min at room temperature in the dark. Reactions were terminated by adding 2N H_2SO_4, and optical densities (OD) were read at 492 nm.

Data analysis

Cut-off values for each peptide and rgp41 ELISA were determined by calculating the mean optical densities (ODs) for all antibody negative reference sera plus 5 standard deviations (Crowther, 2001). A sample with an OD/cut-off ratio of equal and/or greater than 1 was scored positive, and a OD/cut-off value of less than 1 was scored negative. For each peptide and rgp41 antigen, the sensitivity and specificity of detecting antibodies to the homologous virus were calculated. Sensitivity was calculated as the number of ELISA-reactive samples divided by the total number of SIV antibody-positive samples, multiplied by 100. Specificity was calculated as the number of ELISA negative samples, divided by the total number of SIV antibody negative samples, multiplied by 100. Confidence limits were determined given the assumption of binomial sampling (Stata 7.0 software, Stata Corporation, College Station, TX, USA).

Reference sera from primates of known infection status

A panel of well-characterized reference sera was selected from 207 primates in whom SIV infection was either confirmed or excluded by PCR amplification of viral sequences from peripheral blood mononuclear cell DNA (Table 2). The majority of these sera were chosen from the previous bushmeat survey (Peeters et al., 2002) and included sera from (infected/uninfected) De Brazza's (5/3), mustached (4/31), greater spot nosed (6/47), mona (1/1), mandrill (7/4), mantled guereza (8/9) and northern talapoin (2/6) monkeys. Additional sera from two infected red capped mangabeys, one De Brazza's monkey and one guereza colobus were identified more recently. Reference sera were also obtained from captive chimpanzees (3/1), captive sooty mangabeys (9/7) and wild-living Sykes's monkeys (14/35). The latter were collected from Lowland Sykes' monkeys (*C. a. kibonotensis*) in southern Kenya (close to Mombasa) and Highland Sykes's monkeys (*C. a. kolbi*) in central Kenya (in the vicinity of Nairobi), which were trapped by investigators of the Institute of Primate Research after obtaining approval by the Kenya Wildlife Service. Following blood drawing, all animals were released back into the wild. Blood samples were first screened for HIV-1/HIV-2 cross-reactive antibodies and subsequently subjected to PCR analysis using two sets of virus-specific primers targeting *pol* and gp41 genes (F. Bibollet-Ruche and B. H. Hahn, unpublished data).

Sera from primate bushmeat and pet monkeys

For prevalence determinations, 589 additional primates were studied from geographically diverse sites throughout southern Cameroon, including bushmeat markets in and around the capital city of Yaounde, logging concessions in southeastern Cameroon and villages in southwestern Cameroon. 265 of these were selected from the previous bushmeat survey (Peeters et al., 2002), while an additional 324 were obtained more recently. The selection criterion for all sera was that they were available in sufficient quantities for multiple serological analyses. As described previously, blood was collected from primate bushmeat by cardiac puncture and from pet monkeys by venipuncture after tranquilizing the animals with ketamine (Peeters et al., 2002); all samples were obtained with approval from the Cameroonian Ministry of Environment and Forestry. Plasma and cells were separated on site by Ficoll gradient centrifugation. All samples were screened for HIV-1 and HIV-2 cross-reactive antibodies using the INNO-LIA HIV Confirmatory assay (Innogenetics, Gent, Belgium); however, PCR analysis was not performed to confirm their respective infection status.

Codon usage optimization of the SIVgsn env gene

The codon usage of the SIVgsn99CM166 *env* sequence (AF468659) was inspected and transcribed manually to that of highly expressed human genes as described (Andre et al., 1998; Gao et al., 2003). A Kozak sequence (GCCGCCGCC) was added immediately prior to the first methionine codon (ATG) and the modified sequence was subdivided into three non-overlapping fragments, each of which was bounded by unique restriction enzyme sites to facilitate cloning following gene synthesis (fragment A: 1–873nt, *Xba*I–*Bsp*HI; fragment B: 866–1834nt, *Bsp*HI–*Sbf*I; fragment C: 1827–2667nt, *Sbf*I–*Bam*HI). None of these sequence modifications resulted in changes of the SIVgsn encoded Env glycoprotein sequence. The three fragments were purchased from a commercial vendor (DNA 2.0, Menlo Park, CA), sequence confirmed upon receipt, concatenated and cloned into the eukaryotic expression vector pCMV/R (kindly provided by Dr. Gary Nabel). The codon usage optimized CMV/R-SIVgsn *env* gene sequence is available under GenBank accession number AY995800.

SIVgsn-specific Western immunoblot

293T cells were co-transfected in 100 mm dishes ($n=20$) with the codon usage optimized SIVgsn *env* gene (pCMV/R-SIVgsnEnv; 10 μg) and an *env*-minus HIV-1 backbone vector (pSG3ΔEnv; 30 μg) (Decker et al., 2005; Wei et al., 2003) to generate HIV-1 pseudovirions containing the SIVgsn envelope glycoprotein (Effectene Transfection Reagent, Qiagen, Valencia, CA). Forty-eight hours post transfection, culture supernatants were harvested, passed through 0.22 μm filter (Millipore Express Plus Membrane filter; Millipore Corp., Bedford, MA), and ultracentrifuged twice through a 20% sucrose cushion (23,500×*g*; 2 h). Pseudovirion pellets were resuspended in 500 μl PBS and quantified by determining their HIV-1 p24 antigen content (Beckman Coulter, Fullerton, CA). Pseudovirions were lysed in Reducing Sample Buffer (Pierce, Rockford, IL) and run on a 4–15% linear gradient gel (BioRad, Hercules, CA). Following electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (BioRad, Hercules, CA), incubated with StabilCoat Immunoassay Stabilizer (SurModics, Eden Prairie, MN) for 1 h at room temperature, and dried. Individually cut membrane strips (4 mm width) were first incubated with blocking buffer (5% nonfat dry milk, 3% fetal bovine sera, 0.5% Tween-20 in PBS) for 1 h at room temperature, and then reacted over night at 4 °C with sera from greater spot-nosed and mustached monkeys (diluted 1:2,000 in PBS) which had been pre-incubated with 293T cell lysates (1 mg total protein per ml of serum dilution) at room temperature for 1 h to remove unspecific reactivities to cell derived antigens. Protein-bound antibody was probed with goat-anti-human IgG-HRP for 1 h at room temperature (1: 2,000; Southern Biotech, Birmingham, AL) and developed using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Piscataway, NJ) on Hyperfilm-ECL.

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References

Allain, J.P., Laurian, Y., Paul, D.A., Verroust, F., Leuther, M., Gazengel, C., Senn, D., Larrieu, M.J., Bosser, C., 1987. Long-term evaluation of HIV antigen and antibodies to p24 and gp41 in patients with hemophilia. Potential clinical importance. N. Engl. J. Med. 317, 1114–1121.

Andre, S., Seed, B., Eberle, J., Schraut, W., Bultmann, A., Haas, J., 1998. Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. J. Virol. 72, 1497–1503.

Apetrei, C., Metzger, M.J., Richardson, D., Ling, B., Telfer, P.T., Reed, P., Robertson, D.L., Marx, P.A., 2005. Detection and partial characterization of simian immunodeficiency virus SIVsm strains from bush meat samples from rural Sierra Leone. J. Virol. 79, 2631–2636.

Ayouba, A., Souquieres, S., Njinku, B., Martin, P.M., Muller-Trutwin, M.C., Roques, P., Barre-Sinoussi, F., Mauclere, P., Simon, F., Nerrienet, E., 2000. HIV-1 group N among HIV-1-seropositive individuals in Cameroon. AIDS 14, 2623–2625.

Ayouba, A., Mauclere, P., Martin, P.M., Cunin, P., Mfoupouendoun, J., Njinku, B., Souquieres, S., Simon, F., 2001. HIV-1 group O infection in Cameroon, 1986 to 1998. Emerg. Infect. Dis. 7, 466–467.

Bailes, E., Chaudhuri, R.R., Santiago, M.L., Bibollet-Ruche, F., Hahn, B., Sharp, P.M., 2002. The evolution of primate lentiviruses and the origins of AIDS, p. 65–96. In: Leitner, T.A. (Ed.), The Molecular Epidemiology of Human Viruses. Kluwer Academic Publishers, Boston, MA.

Barin, F., McLane, M.F., Allan, J.S., Lee, T.H., Groopman, J.E., Essex, M., 1985. Virus envelope protein of HTLV-III represents major target antigen for antibodies in AIDS patients. Science 228, 1094–1096.

Beer, B.E., Bailes, E., Goeken, R., Dapolito, G., Coulibaly, C., Norley, S.G., Kurth, R., Gautier, J.P., Gautier-Hion, A., Vallet, D., Sharp, P.M., Hirsch, V.M., 1999. Simian immunodeficiency virus (SIV) from sun-tailed monkeys (*Cercopithecus solatus*): evidence for host-dependent evolution of SIV within the *C. lhoesti* superspecies. J. Virol. 73, 7734–7744.

Bibollet-Ruche, F., Brengues, C., Galat-Luong, A., Galat, G., Pourrut, X., Vidal, N., Veas, F., Durand, J.P., Cuny, G., 1997. Genetic diversity of simian immunodeficiency viruses from West African green monkeys: evidence of multiple genotypes within populations from the same geographical locale. J. Virol. 71, 307–313.

Bibollet-Ruche, F., Bailes, E., Gao, F., Pourrut, X., Barlow, K.L., Clewley, J.P., Mwenda, J.M., Langat, D.K., Chege, G.K., McClure, H.M., Mpoudi-Ngole, E., Delaporte, E., Peeters, M., Shaw, G.M., Sharp, P.M., Hahn, B.H., 2004. New simian immunodeficiency virus infecting De Brazza's monkeys (*Cercopithecus neglectus*): evidence for a Cercopithecus monkey virus clade. J. Virol. 78, 7748–7762.

Bieniasz, P.D., 2004. Intrinsic immunity: a front-line defense against viral attack. Nat. Immunol. 5, 1109–1115.

Corbet, S., Muller-Trutwin, M.C., Versmisse, P., Delarue, S., Ayouba, A., Lewis, J., Brunak, S., Martin, P., Brun-Vezinet, F., Simon, F., Barre-Sinoussi, F., Mauclere, P., 2000. *env* sequences of simian immunodeficiency viruses from chimpanzees in Cameroon are strongly related to those of human immunodeficiency virus group N from the same geographic area. J. Virol. 74, 529–534.

Courgnaud, V., Pourrut, X., Bibollet-Ruche, F., Mpoudi-Ngole, E., Bourgeois, A., Delaporte, E., Peeters, M., 2001. Characterization of a novel simian immunodeficiency virus from guereza colobus monkeys (*Colobus guereza*) in Cameroon: a new lineage in the nonhuman primate lentivirus family. J. Virol. 75, 857–866.

Courgnaud, V., Salemi, M., Pourrut, X., Mpoudi-Ngole, E., Abela, B., Auzel, P., Bibollet-Ruche, F., Hahn, B., Vandamme, A.M., Delaporte, E., Peeters, M., 2002. Characterization of a novel simian immunodeficiency virus with a *vpu* gene from greater spot-nosed monkeys (*Cercopithecus nictitans*) provides new insights into simian/human immunodeficiency virus phylogeny. J. Virol. 76, 8298–8309.

Courgnaud, V., Abela, B., Pourrut, X., Mpoudi-Ngole, E., Loul, S., Delaporte, E., Peeters, M., 2003a. Identification of a new simian immunodeficiency virus lineage with a vpu gene present among different *Cercopithecus* monkeys (*C. mona*, *C. cephus*, and *C. nictitans*) from Cameroon. J. Virol. 77, 12523–12534.

Courgnaud, V., Formenty, P., Akoua-Koffi, C., Noe, R., Boesch, C., Delaporte, E., Peeters, M., 2003b. Partial molecular characterization of simian immunodeficiency viruses (SIV) from African colobids, SIVwrc from Western red colobus (*Piliocolobus badius*) and SIVolc from Olive colobus (*Procolobus verus*). J. Virol. 77, 744–748.

Crowther, J.R., 2001. The ELISA guidebook. In: Walker, J.M. (Ed.), Methods in Molecular Biology, vol. 149. Humana Press, Totowa, NJ.

Damond, F., Apetrei, C., Robertson, D.L., Souquiere, S., Lepretre, A., Matheron, S., Plantier, J.C., Brun-Vezinet, F., Simon, F., 2001. Variability of human immunodeficiency virus type 2 (HIV-2) infecting patients living in France. Virology 280, 19–30.

Decker, J.M., Bibollet-Ruche, F., Wei, X., Wang, S., Levy, D.N., Wang, W., Delaporte, E., Peeters, M., Derdeyn, C.A., Allen, S., Hunter, E., Saag, M.S., Hoxie, J.A., Hahn, H.B., Kwong, P.D., Robinson, E.J., Shaw, M.G., 2005. Antigenic conservation and immunogenicity of the HIV co-receptor binding site. Exp. J. Med. 201, 1407–1419.

Desmezieres, E., Gupta, N., Vassell, R., He, Y., Peden, K., Sirota, L., Yang, Z., Wingfield, P., Weiss, C.D., 2005. Human immunodeficiency virus (HIV) gp41 escape mutants: cross-resistance to peptide inhibitors of HIV fusion and altered receptor activation of gp120. J. Virol. 79, 4774–4781.

Ellis, B.R., Munene, E., Elliott, D., Robinson, J., Otsyula, M.G., Michael, S.F., 2004. Seroprevalence of simian immunodeficiency virus in wild and captive born Sykes' monkeys (*Cercopithecus mitis*) in Kenya. Retrovirology 1, 34.

Emau, P., McClure, H.M., Isahakia, M., Else, J.G., Fultz, P.N., 1991. Isolation from African Sykes' monkeys (*Cercopithecus mitis*) of a lentivirus related to human and simian immunodeficiency viruses. J. Virol. 65, 2135–2140.

Gao, F., Yue, L., White, A.T., Pappas, P.G., Barchue, J., Hanson, A.P., Greene, B.M., Sharp, P.M., Shaw, G.M., Hahn, B.H., 1992. Human infection by genetically diverse SIVSM-related HIV-2 in west Africa. Nature 358, 495–499.

Gao, F., Bailes, E., Robertson, D.L., Chen, Y., Rodenburg, C.M., Michael, S.F., Cummins, L.B., Arthur, L.O., Peeters, M., Shaw, G.M., Sharp, P.M., Hahn, B.H., 1999. Origin of HIV-1 in the chimpanzee (*Pan troglodytes troglodytes*). Nature 397, 436–441.

Gao, F., Li, Y., Decker, J.M., Peyerl, F.W., Bibollet-Ruche, F., Rodenburg, C.M., Chen, Y., Shaw, D.R., Allen, S., Musonda, R., Shaw, G.M., Zajac, A.J., Letvin, N., Hahn, B.H., 2003. Codon usage optimization of HIV type 1 subtype C gag, pol, env, and nef genes: in vitro expression and immune responses in DNA-vaccinated mice. AIDS Res. Hum. Retroviruses 19, 817–823.

Gao, F., Weaver, E.A., Lu, Z., Li, Y., Liao, H.X., Ma, B., Alam, S.M., Scearce, R.M., Sutherland, L.L., Yu, J.S., Decker, J.M., Shaw, G.M., Montefiori, D.C., Korber, B.T., Hahn, B.H., Haynes, B.F., 2005. Antigenicity and immunogenicity of a synthetic human immunodeficiency virus type 1 group M consensus envelope glycoprotein. J. Virol. 79, 1154–1163.

Georges-Courbot, M.C., Lu, C.Y., Makuwa, M., Telfer, P., Onanga, R., Dubreuil, G., Chen, Z., Smith, S.M., Georges, A., Gao, F., Hahn, B.H., Marx, P.A., 1998. Natural infection of a household pet red-capped mangabey (*Cercocebus torquatus torquatus*) with a new simian immunodeficiency virus. J. Virol. 72, 600–608.

Goff, S.P., 2004. Retrovirus restriction factors. Mol. Cell 16, 849–859.

Hahn, B.H., Shaw, G.M., De Cock, K.M., Sharp, P.M., 2000. AIDS as a zoonosis: scientific and public health implications. Science 287, 607–614.

Jolly, C., Phillips-Conroy, J.E., Turner, T.R., Broussard, S., Allan, J.S., 1996. SIVagm incidence over two decades in a natural population of Ethiopian grivet monkeys (*Cercopithecus aethiops aethiops*). J. Med. Primatol. 25, 78–83.

Masciotra, S., Rudolph, D.L., van der Groen, G., Yang, C., Lal, R.B., 2000. Serological detection of infection with diverse human and simian immunodeficiency viruses using consensus env peptides. Clin. Diagn. Lab. Immunol. 7, 706–709.

Mauclere, P., Damond, F., Apetrei, C., Loussert-Ajaka, I., Souquiere, S., Buzelay, L., Dalbon, P., Jolivet, M., Mony Lobe, M., Brun-Vezinet, F., Simon, F., Barin, F., 1997. Synthetic peptide ELISAs for detection of and discrimination between group M and group O HIV type 1 infection. AIDS Res. Hum. Retroviruses 13, 987–993.

Ndongmo, C.B., Switzer, W.M., Pau, C.P., Zeh, C., Schaefer, A., Pieniazek, D., Folks, T.M., Kalish, M.L., 2004. New multiple antigenic peptide-based enzyme immunoassay for detection of simian immunodeficiency virus infection in nonhuman primates and humans. J. Clin. Microbiol. 42, 5161–5169.

Ohta, Y., Masuda, T., Tsujimoto, H., Ishikawa, K., Kodama, T., Morikawa, S., Nakai, M., Honjo, S., Hayami, M., 1988. Isolation of simian immunodeficiency virus from African green monkeys and seroepidemiologic survey of the virus in various non-human primates. Int. J. Cancer 41, 115–122.

Osterhaus, A.D., Pedersen, N., van Amerongen, G., Frankenhuis, M.T., Marthas, M., Reay, E., Rose, T.M., Pamungkas, J., Bosch, M.L., 1999. Isolation and partial characterization of a lentivirus from talapoin monkeys (*Miopithecus talapoin*). Virology 260, 116–124.

Peeters, M., Courgnaud, V., 2002. Overview of primate lentiviruses and the evolution of non-human primates in Africa. In: Kuiken, C., Foley, B., Freed, E., Hahn, B., Korber, B., Marx, P., McCutchan, F., Mellors, J.W., Wolinsky, S. (Eds.), HIV Sequence Compedium. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM, p. 2–23.

Peeters, M., Courgnaud, V., Abela, B., Auzel, P., Pourrut, X., Bibollet-Ruche, F., Loul, S., Liegeois, F., Butel, C., Koulagna, D., Mpoudi-Ngole, E., Shaw, G.M., Hahn, B.H., Delaporte, E., 2002. Risk to human health from a plethora of simian immunodeficiency viruses in primate bushmeat. Emerging Infect. Dis. 8, 451–457.

Phillips-Conroy, J.E., Jolly, C.J., Petros, B., Allan, J.S., Desrosiers, R.C., 1994. Sexual transmission of SIVagm in wild grivet monkeys. J. Med. Primatol. 23, 1–7.

Saah, A.J., Farzadegan, H., Fox, R., Nishanian, P., Rinaldo Jr., C.R., Phair, J.P., Fahey, J.L., Lee, T.H., Polk, B.F., 1987. Detection of early antibodies in human immunodeficiency virus infection by enzyme-linked immunosorbent assay, Western blot, and radioimmunoprecipitation. J. Clin. Microbiol. 25, 1605–1610.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Sheehy, A.M., Gaddis, N.C., Choi, J.D., Malim, M.H., 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature 418, 646–650.

Simon, F., Souquiere, S., Damond, F., Kfutwah, A., Makuwa, M., Leroy, E., Rouquet, P., Berthier, L.J., Rigoulet, J., Lecu, A., Telfer, P.T., Pandrea, I., Plantier, J.C., Barre-Sinoussi, F., Roques, P., Muller-Trutwin, M.C., Apetrei, C., 2001. Synthetic peptide strategy for the detection of and discrimination among highly divergent primate lentiviruses. AIDS Res. Hum. Retrovir. 17, 937–952.

Souquiere, S., Bibollet-Ruche, F., Robertson, D.L., Makuwa, M., Apetrei, C., Onanga, R., Kornfeld, C., Plantier, J.C., Gao, F., Abernethy, K., White, L.J., Karesh, W., Telfer, P., Wickings, E.J., Mauclere, P., Marx, P.A., Barre-Sinoussi, F., Hahn, B.H., Muller-Trutwin, M.C., Simon, F., 2001. Wild *Mandrillus sphinx* are carriers of two types of lentivirus. J. Virol. 75, 7086–7096.

Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., Sodroski, J., 2004. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature 427, 848–853.

Takehisa, J., Harada, Y., Ndembi, N., Mboudjeka, I., Taniguchi, Y., Ngansop, C., Kuate, S., Zekeng, L., Ibuki, K., Shimada, T., Bikandou, B., Yamaguchi-Kabata, Y., Miura, T., Ikeda, M., Ichimura, H., Kaptue, L., Hayami, M., 2001. Natural infection of wild-born mandrills (Mandrillus sphinx) with two different types of simian immunodeficiency virus. AIDS Res. Hum. Retroviruses 17, 1143–1154.

Wei, X., Decker, J.M., Wang, S., Hui, H., Kappes, J.C., Wu, X., Salazar-Gonzalez, J.F., Salazar, M.G., Kilby, J.M., Saag, M.S., Komarova, N.L., Nowak, M.A., Hahn, B.H., Kwong, P.D., Shaw, G.M., 2003. Antibody neutralization and escape by HIV-1. Nature 422, 307–312.

Weissenhorn, W., Calder, L.J., Dessen, A., Laue, T., Skehel, J.J., Wiley, D.C., 1997. Assembly of a rod-shaped chimera of a trimeric GCN4 zipper and the HIV-1 gp41 ectodomain expressed in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 94, 6065–6069.

Xu, J.Y., Gorny, M.K., Palker, T., Karwowska, S., Zolla-Pazner, S., 1991. Epitope mapping of two immunodominant domains of gp41, the transmembrane protein of human immunodeficiency virus type 1, using ten human monoclonal antibodies. J. Virol. 65, 4832–4838.

Yamaguchi, J., Devare, S.G., Brennan, C.A., 2000. Identification of a new HIV-2 subtype based on phylogenetic analysis of full-length genomic sequence. AIDS Res. Hum. Retroviruses 16, 925–930.