

# Screening for simian foamy virus infection by using a combined antigen Western blot assay: evidence for a wide distribution among Old World primates and identification of four new divergent viruses

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## Abstract

Simian foamy viruses (SFVs) belong to a genetically and antigenically diverse class of retroviruses that naturally infect a wide range of nonhuman primates (NHPs) and can also be transmitted to humans occupationally exposed to NHPs. Current serologic detection of SFV infection requires separate Western blot (WB) testing by using two different SFV antigens [SFV<sub>AGM</sub> (African green monkey) and SFV<sub>CPZ</sub> (chimpanzee)]. However, this method is labor intensive and validation is limited to only small numbers of NHPs. To facilitate serologic SFV testing, we developed a WB assay that combines antigens from both SFV<sub>AGM</sub> and SFV<sub>CPZ</sub>. The combined-antigen WB (CA-WB) assay was validated with 145 serum samples from 129 NHPs (32 African and Asian species) and 16 humans, all with known SFV infection status determined by PCR. Concordant CA-WB results were obtained for all 145 PCR-positive or -negative primate and human specimens, giving the assay a 100% sensitivity and specificity. In addition, no reactivity was observed in sera from persons positive for human immunodeficiency virus or human T cell lymphotropic virus (HIV/HTLV) ( $n = 25$ ) or HIV/HTLV-negative U.S. blood donors ( $n = 100$ ). Using the CA-WB assay, we screened 360 sera from 43 Old World primate species and found an SFV prevalence of about 68% in both African and Asian primates. We also isolated SFV from the blood of four seropositive primates (*Allenopithecus nigroviridis*, *Trachypithecus francoisi*, *Hylobates pileatus*, and *H. leucogenys*) not previously known to be infected with SFV. Phylogenetic analysis of integrase sequences from these isolates confirmed that all four SFVs represent new, distinct, and highly divergent lineages. These results demonstrate the ability of the CA-WB assay to detect infection in a large number of NHP species, including previously uncharacterized infections with divergent SFVs.

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## Introduction

Foamy viruses (FVs), or *Spumaviruses*, are exogenous retroviruses present in many healthy mammals, including

monkeys and apes (Meiering and Linial, 2001). Among primates, FVs have been isolated from a variety of both Old and New World primates (Meiering and Linial, 2001). About 70–90% of captive-bred, adult nonhuman primates (NHPs) are infected with simian FVs (SFVs) (Meiering and Linial, 2001). However, this seroprevalence is based on screening of only a small number of primate species mostly representing animals used in biomedical research, such as

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rhesus macaques (MAC), chimpanzees (CPZ), orangutans (PON), baboons (BAB), and African green monkeys (AGM) (Meiering and Linial, 2001). While only limited information is available on the presence of SFV in captive NHPs, even less is known about the prevalence of SFV in wild-caught animals.

The SFV genome is organized similar to other complex simian retroviruses and consists of *gag*, polymerase (*pol*), and envelope (*env*) genes flanked by long terminal repeats (LTRs). In Western blot (WB) analysis, seroreactivity in SFV-infected primates is consistently detected to the Gag p68/71 precursor proteins and is thus considered to be a diagnostic marker of infection (Hahn et al., 1994; Schweizer et al., 1995). However, the Gag proteins from apes and monkeys share only about 60% amino acid identity and are only weakly cross-reactive in WB assays (Hahn et al., 1994; Herchenroder et al., 1994). Thus, serologic WB testing for SFV antibodies in monkeys and apes, as well as humans exposed to these primates, requires the use of two tests—one that contains antigen from an African green monkey (SFV<sub>AGM</sub>) and the other containing antigen from a chimpanzee (SFV<sub>CPZ</sub>), which would allow detection of antibodies to both the Old World monkey or the ape SFV variants, respectively (Schweizer et al., 1995). Other serologic methods such as immunofluorescence or radioimmunoprecipitation have also been used for the detection of SFV antibodies (Hahn et al., 1994; Khan et al., 1999). In addition to serologic testing, PCR testing for SFV sequences in peripheral blood lymphocytes (PBLs) by using generic integrase, *pol*, and LTR primers has also been used to detect the presence of SFV infection (Bieniasz et al., 1995; Broussard et al., 1997; Schweizer and Neumann-Haefelin, 1995).

Previous studies examining the genetic variability of SFVs suggested that these retroviruses have coevolved with their natural hosts (Blewett et al., 2000; Broussard et al., 1997; Heneine et al., 1998; Schweizer and Neumann-Haefelin, 1995). Thus, SFV sequences from baboons, AGMs, and mangabeys formed an African Old World monkey clade, while SFV sequences from Asian macaques were in a separate lineage. Similarly, SFV sequences from the great apes such as bonobos, chimpanzees, orangutans, and gorillas formed another distinct clade.

While SFVs are known to endemically infect a large number of NHPs, there is little evidence that humans are naturally infected with FVs. Surveys of a large number of humans from many different geographic locations and ethnic backgrounds have failed to show evidence of FV infection (Ali et al., 1996; Schweizer et al., 1995). Nonetheless, more recent investigations evaluating cross-species transmission of simian retroviruses to persons at risk for occupational exposures to NHPs reported transmission of SFVs to humans (Boneva et al., 2002; Brooks et al., 2002; Heneine et al., 1998; Sandstrom et al., 2000; Schweizer et al., 1995). To date, these studies have identified SFV infection in 2–3% of persons with occupational exposures to NHPs at research institutions or zoos. SFV in these primate workers was shown by phylogenetic analysis to have originated

from baboons ( $n = 4$ ), AGMs ( $n = 2$ ), chimpanzees ( $n = 6$ ), and macaques ( $n = 1$ ) (Brooks et al., 2002; Heneine et al., 1998; Sandstrom et al., 2000; Schweizer et al., 1995, 1997). Similar to the natural host, SFV-infected humans are apparently asymptomatic despite serologic evidence of a long persistent infection. However, longitudinal follow-up of a large number of infected persons may be necessary to fully assess any clinical outcomes associated with SFV infection.

The presence of zoonotic SFV infections in humans has increased the importance of defining the public health implications of these infections. These studies require an expanded surveillance to identify SFV-infected persons and therefore will need improved diagnostic assays. These assays should be less laborious by not requiring multiple testing with different SFV antigens, and they should be validated on a wide range of SFV variants.

In this study, we have developed a WB assay that combines antigens from SFV<sub>AGM</sub> and SFV<sub>CPZ</sub> into a single test. The combined-antigen WB (CA-WB) assay was validated with serum samples from primates representing 32 African and Asian primate species and 16 humans with known SFV infection. We also examined the seroprevalence of SFV in a large number of captive-bred and wild-born African and Asian NHP species. In addition, we report the isolation and identification of SFVs from four primate species not previously known to be infected with SFV and show phylogenetic evidence that each represents a new, distinct lineage.

## Results

### *Validation of the CA-WB assay*

Concordant CA-WB test results were obtained with serum samples from all 94 PCR-positive and 35 PCR-negative primates representing 23 African and 9 Asian monkeys and apes (Table 1). Seroreactivity to the combined SFV<sub>AGM</sub> and SFV<sub>CPZ</sub> Gag doublet proteins ranged from weak to strong positive for samples from the PCR-positive NHPs. Compared to other NHPs, the weakest seropositive reactivity was observed for sera from PCR-positive orangutans (Fig. 1). Concordant CA-WB results were also observed for all 11 PCR-positive and 5 PCR-negative human specimens. The sensitivity, specificity, predictive value positive, and predictive value negative measures of the CA-WB assay were determined to be 100% for these specimens. None of the 25 serum samples from humans infected with human immunodeficiency virus type 1 or 2 (HIV-1 or -2) or human T cell lymphotropic virus type 1 or 2 (HTLV-1 or -2) showed cross-reactivity to the diagnostic SFV Gag proteins. Similarly, negative CA-WB results were also seen with all 100 U.S. human blood donor sera.

### *Prevalence of antibodies to SFV in captive-bred and wild-born primates*

With the observed high sensitivity and specificity of the CA-WB assay, we proceeded to determine the seropreva-

Table 1

Validation of the combined simian foamy virus antigen Western blot assay (CA-WB) using sera from 32 nonhuman primate species (129 different animals) with known infection status determined by PCR analysis

Genus	Species	Common name	Number	CA-WB+/PCR+	CA-WB-/PCR-
<i>Cercopithecus</i>	<i>neglectus</i>	De Brazza's monkey	3	3/3	
	<i>lhoesti</i>	L'Hoest's monkey	1	1/1	
	<i>erythrotis</i>	Red-eared guenon	1		1/1
	<i>diana</i>	Diana monkey	1	1/1	
	<i>mitis albogularis</i>	Sykes's monkey	3	3/3	
<i>Chlorocebus</i>	<i>pygerythrus</i>	Vervet monkey	6	5/5	1/1
<i>Mandrillus</i>	<i>sphinx</i>	Mandrill	17	8/8	9/9
	<i>leucophaeus</i>	Drill	7	1/1	6/6
<i>Cercocebus</i>	<i>atys</i>	Sooty mangabey	2	2/2	
	<i>torquatus</i>	Red-capped mangabey	4	4/4	
	<i>chrysogaster</i>	Golden-bellied mangabey	4		4/4
<i>Lophocebus</i>	<i>aerrimus</i>	Black-crested mangabey	4		4/4
	<i>albigena</i>	Gray-cheeked mangabey	3	3/3	
<i>Papio</i>	<i>papio</i>	Guinea baboon	12	12/12	
	<i>hamadryas</i>	Hamadryas baboon	1	1/1	
	<i>cynocephalus</i>	Yellow baboon	1	1/1	
	<i>anubis</i>	Olive baboon	5	5/5	
<i>Theropithecus</i>	<i>gelada</i>	Gelada baboon	3	3/3	
<i>Macaca</i>	<i>silenus</i>	Lion-tailed macaque	2	2/2	
	<i>fuscata</i>	Japanese macaque	5	4/4	1/1
	<i>arctoides</i>	Stump-tailed macaque	4	4/4	
	<i>sylvanus</i>	Barbary macaque	10	6/6	4/4
	<i>Colobus</i>	<i>guereza</i>	Mantled guereza	5	5/5
<i>Pygathrix</i>	<i>nemaus</i>	Red-shanked Douc langur	1	1/1	
<i>Pan</i>	<i>trogodytes troglodytes</i>	Central African chimpanzee	3	3/3	
	<i>trogodytes schweinfurthii</i>	East African chimpanzee	3	3/3	
	<i>paniscus</i>	Bonobo	2	2/2	
	<i>Gorilla</i>	<i>gorilla</i>	Western lowland gorilla	6	6/6
<i>Hylobates</i>	<i>lar</i>	White-handed gibbon	2		2/2
	<i>leucogenys</i>	White-cheeked gibbon	2		2/2
<i>Pongo</i>	<i>pygmaeus</i>	Bornean orangutan	2	2/2	
	<i>abelii</i>	Sumatran orangutan	4	3/3	1/1
	Totals		129	94/94 (100%)	35/35 (100%)

lence of SFVs in a large number of captive-bred and wild-born NHPs. Of the 286 captive-bred NHPs tested, 211 (73.8%) were found to be seropositive, while 75 (26.2%) were seronegative (Table 2). There was no significant difference between the SFV seroprevalence rates among captive-bred NHPs from Africa (73.5%) and Asia (74.2%). These results show that SFV is present in a variety of NHPs, including those not typically used in biomedical research, such as langurs, gibbons, Allen's swamp monkeys, colobus monkeys, many guenons, and Barbary macaques. However, many serum samples from captive-bred red-eared guenons ( $n = 2$ ), black-crested mangabeys ( $n = 3$ ), Angolan colobus monkeys ( $n = 4$ ), drills ( $n = 11$ ), and siamangs ( $n = 4$ ) were found to be seronegative (Table 2a and 2b).

Although there was a broad range of seropositivity in both the captive-bred and the wild-born primates, the overall SFV seroprevalence was lower in animals born in the wild (73.8 vs 44.6%, respectively) (Table 3). In addition, when seroprevalence rates were compared directly between identical species of wild-born and captive-bred monkeys, a higher rate was observed in captive-bred guenons (2/7 or

28.6%) than in similar wild-born guenons (0/20). However, a lower seroprevalence was not seen in all captive-bred NHPs. For example, the prevalence of SFV antibodies among both captive-bred and wild-born red-capped mangabeys was very similar (62.5 and 61.5%, respectively). When the serology results for all 43 species of captive-bred and wild-born Old World primates are combined, there is an overall SFV seroprevalence of about 68% in both Asian and African NHPs.

CA-WB testing of one specimen from a wild-born Bornean orangutan showed indeterminate results (1/20 or 5.0%) (data not shown). However, this sample was found to be seropositive upon repeat WB testing using an antigen derived from an orangutan (SFV<sub>PON</sub>) (data not shown). These results may indicate a reduced cross-reactivity of SFV<sub>PON</sub> antibodies to the SFV<sub>AGM</sub> and SFV<sub>CPZ</sub> antigens likely due to a high degree of antigenic variability. DNA specimens were not available on the Bornean orangutans for PCR analysis. However, one serum sample from a PCR- and CA-WB-negative Sumatran orangutan remained seronegative upon retesting with the autologous SFV<sub>PON</sub> antigen.

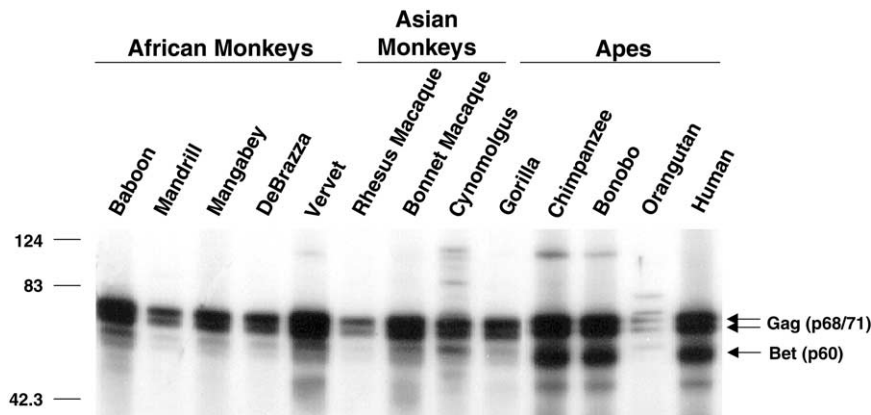


Fig. 1. Detection of antibodies to simian foamy virus (SFV) in representative nonhuman primates and in an occupationally exposed human by using the combined antigen [SFV<sub>AGM</sub> (African green monkey) and SFV<sub>CPZ</sub> (chimpanzee)] Western blot assay. Lanes 1–5 are sera from African monkeys; lanes 6–8 are sera from Asian monkeys; lanes 9–12 are sera from African and Asian apes; and lane 13 is from a human infected with SFV originating from a chimpanzee. Seroreactivity was observed to the diagnostic Gag doublet protein (p68/71) in all specimens and to the Bet protein (p60) in the chimpanzee specimens. The molecular weight protein marker sizes are indicated on the left.

#### Isolation of SFVs and phylogenetic analysis of novel integrase sequences

From the serosurvey of captive-bred NHPs, we identified several primate genera not previously known to be SFV-infected. Thus, to confirm the observed seropositivity, PCR amplification of integrase sequences with generic primers was attempted on PBL DNA lysates available from seropositive Allen's swamp monkeys (*Allenopithecus nigroviridis*), two species of langurs (*Pygathrix nemaus* and *Trachypithecus francoisi*), and two gibbon species (*Hylobates pileatus* and *H. leucogenys*). All 18 samples tested PCR-negative (Table 4), although the quality of each PBL DNA sample was confirmed by PCR amplification of  $\beta$ -actin sequences (data not shown). Cryopreserved PBL samples were available from one each of the Allen's swamp monkeys, François' langurs, and gibbons for virus isolation. The presence of SFV in the cocultures was confirmed by the presence of typical FV cytopathic effect, the detection of reverse transcriptase (RT) activity in the culture supernatants, and PCR amplification of SFV integrase sequences from the cultured cells. The positive PCR results found in cultured cells but not in uncultured PBLs reflect the reduced sensitivity of the generic PCR assay, which is likely due to sequence variability in the primer regions and which can be overcome in samples containing high copy numbers of SFV DNA.

By the formation of four distinct lineages, phylogenetic analysis confirmed that the integrase sequences obtained from the cell-culture isolates are novel and highly divergent from all available SFV sequences (Fig. 2). As expected, the *Hylobates* sequences formed two closely related yet distinct lineages nearest the integrase sequences from another ape, the chimpanzee. The *Trachypithecus* integrase sequence formed a separate lineage between the apes and Asian macaques, while the *Allenopithecus* lineage clustered between the *Cercocebus* and *Macaca* sequences. Identical tree

topologies were also obtained with both the maximum likelihood and the minimum evolution methods, providing further evidence of the genetic relationships of these unique SFV lineages (data not shown). The nucleotide diversity between the new lineages and known SFV integrase sequences ranged from 20 to 30%, with the highest percentage divergence seen with the gibbon and langur SFVs.

To implement a nomenclature system that more easily differentiates among unique SFVs, especially those obtained from the same genus, we propose using a three-letter code that consists of the first letter of the primate genus from which the SFVs were isolated with the first two letters of either the species or, if known, the subspecies name. Thus, the isolates from *A. nigroviridis*, *T. francoisi*, *H. pileatus*, and *H. leucogenys* are provisionally named SFV<sub>ANI</sub>, SFV<sub>TFR</sub>, SFV<sub>HPI</sub>, and SFV<sub>HLE</sub>, respectively. For completion, the name or code given to the animal could then be added to the three-letter primate designation. For example, SFV<sub>ANI</sub> isolated from a swamp monkey with the animal identification code 598422 would be named SFV<sub>ANI</sub>598422. This system will require approval by viral taxonomy specialty groups.

#### Discussion

Cross-species transmission of retroviruses from NHPs has been linked to the origin of the HIV pandemic, with HIV-1 and HIV-2 believed to be the result of zoonotic transfer of simian immunodeficiency viruses (SIVs) from chimpanzees and sooty mangabeys, respectively (Hahn et al., 2000). Similarly, phylogenetic evidence suggests that the HTLV-1 subtypes may have arisen from multiple introductions of the simian T cell lymphotropic virus type 1 (STLV-1) into ancient human populations (Slattery et al., 1999). The renewed investigations of the spread and clinical outcome of SFV infections among humans require the avail-

Table 2a  
Seroprevalence of simian foamy virus in 29 species of captive-bred African monkeys and apes

Genus	Species	Common name	Number seropositive <sup>a</sup>	Rate (%)
<i>Cercopithecus</i>	<i>neglectus</i>	De Brazza's monkey	4/5	80.0
	<i>cephus</i>	Mustached guenon	3/6	50.0
	<i>erythrois</i>	Red-eared guenon	0/2	0.0
	<i>diana</i>	Diana monkey	2/2	100.0
	<i>albogularis</i>	Syke's monkey	7/7	100.0
	<i>campbelli</i>	Campbell's guenon	15/16	93.8
	<i>nictitans</i>	Spot-nosed guenon	1/4	25.0
	<i>solatus</i>	Sun-tailed guenon	1/1	100.0
	<i>pogonias</i>	Crowned guenon	1/1	100.0
	<i>pogonias X mona</i> <sup>b</sup>	Hybrid	1/1	100.0
<i>Chlorocebus</i>	<i>pygerythrus</i>	Vervet monkey	2/2	100.0
	<i>tantalus</i>	Tantalus monkey	5/5	100.0
	<i>sabaeus</i>	African green monkey	4/8	50.0
<i>Allenopithecus</i>	<i>nigroviridis</i>	Allen's swamp monkey	2/2	100.0
<i>Miopithecus</i>	<i>talapoin</i>	Dwarf guenon	13/15	86.6
<i>Mandrillus</i>	<i>sphinx</i>	Mandrill	11/13	84.6
	<i>leucophaeus</i>	Drill	0/11	0.0
<i>Cercocebus</i>	<i>atys</i>	Sooty mangabey	5/5	100.0
	<i>torquatus</i>	Red-capped mangabey	5/8	62.5
	<i>chrysogaster</i>	Golden-bellied mangabey	2/3	66.6
<i>Lophocebus</i>	<i>aterrimus</i>	Black-crested mangabey	0/3	0.0
	<i>albigena</i>	Gray-cheeked mangabey	4/4	100.0
<i>Papio</i>	<i>hamadryas</i>	Hamadryas baboon	4/4	100.0
	<i>anubis</i>	Olive baboon	4/4	100.0
<i>Theropithecus</i>	<i>gelada</i>	Gelada baboon	13/16	81.3
<i>Macaca</i>	<i>sylvanus</i>	Barbary macaque	3/3	100.0
<i>Colobus</i>	<i>guereza</i>	Mantled guereza	4/4	100.0
	<i>angolensis</i>	Angolan colobus	0/4	0.0
<i>Pan</i>	<i>trogodytes</i>	Common chimpanzee	13/15	86.6
<i>Gorilla</i>	<i>gorilla</i>	Western lowland gorilla	4/7	57.1
		Totals	133/181	73.5

<sup>a</sup> Number of different animals.

<sup>b</sup> Crowned guenon and mona monkey hybrid; not included in species total.

ability of improved serologic assays capable of detecting SFV antibodies in a broad spectrum of Old World monkeys and apes. Thus, we developed and validated a serologic assay that combines two divergent antigens from SFV<sub>AGM</sub> and SFV<sub>CPZ</sub> in a single test.

The 100% sensitivity and specificity observed among samples from 32 primate species confirms the ability of this assay to detect divergent SFVs. These data support the use of the CA-WB assay in assessing risks of SFV transmission to persons exposed to a wide variety of NHPs and in determining the incidence and seroprevalence of SFV infection in captive and feral NHPs. We also show an absence of reactivity with human sera from persons infected with HIV or HTLV, confirming a lack of cross-reactivity with this assay. By requiring only a single test for SFV antibody detection, the new CA-WB assay will facilitate the screening of larger populations.

Previous studies of the prevalence of SFV infection in NHPs have been limited to small numbers and types of animals housed at research centers, such as baboons, macaques, chimpanzees, AGMs, and a few New World monkeys and prosimians (Blewett et al., 2000; Meiering and Linial, 2001). However, exposures to a larger variety of

NHPs and their simian retroviruses may occur in persons who work in zoological gardens and individuals who live in close contact with monkeys, such as bush-meat hunters or pet owners (Peeters et al., 2002). Nonetheless, little is known about the prevalence of SFV infection in monkeys and apes found in these settings.

Our study is the first to our knowledge to document such an extensive distribution of SFV infection among NHP species. Our finding of an overall 73.8% SFV seroprevalence rate in 35 of 40 species of captive-bred African and Asian primates is significant and consistent with previous estimates on the high prevalence rates ( $\geq 70\%$ ) of SFV infections observed in small numbers of captive primates used in biomedical research (Meiering and Linial, 2001). In contrast, other simian retroviruses such as SIV and STLV have both a lower distribution and a lower seroprevalence (about 35%) among captive-bred and wild-born primates (Fultz, 1994). These data suggest that persons exposed to NHPs may be at higher risk for zoonotic infections with SFV than with SIV or STLV, and the data may also help explain the observed higher prevalence of SFV antibodies ( $\sim 3\%$ ) in occupationally exposed persons than either SIV (0.43%) or STLV (0%) (Heneine et al., 1998). These results

Table 2b  
Seroprevalence of simian foamy virus in 11 species of captive-bred Asian monkeys and apes

Genus	Species	Common name	Number seropositive <sup>a</sup>	Rate (%)
<i>Macaca</i>	<i>silenus</i>	Lion-tailed macaque	8/15	53.3
	<i>fuscata</i>	Japanese macaque	47/49	95.9
	<i>arctoides</i>	Stump-tailed macaque	2/2	100.0
	<i>mulatta</i>	Rhesus macaque	3/4	75.0
	<i>nigra</i>	Sulawesi-crested macaque	1/1	100.0
<i>Pygathrix</i>	<i>nemaeus</i>	Red-shanked Douc langur	4/5	80.0
<i>Trachypithecus</i>	<i>francoisi</i>	François's langur	5/9	55.5
<i>Hylobates</i>	<i>pileatus</i>	Pileated gibbon	4/9	44.4
	<i>leucogenys</i>	White-cheeked gibbon	3/6	50.0
	<i>syndactylus</i>	Siamang	0/4	0.0
<i>Pongo</i>	<i>pygmaeus</i>	Bornean orangutan	1/1	100.0
	Totals		78/105	74.2
	Combined totals <sup>b</sup>		211/286	73.8

<sup>a</sup> Number of different animals.

<sup>b</sup> Total for all captive-bred African and Asian primates in Tables 2a and 2b.

underscore the importance of work practices that prevent exposure to these viruses and highlight the need for increased surveillance and additional studies to define the clinical outcomes and transmissibility of these infections.

We also found a generally lower prevalence of SFV infection among wild-born primates (44.6%) than among captive-bred animals (73.8%). These observed differences in the prevalence of SFV among wild-born and captive-bred NHPs may be attributed to the limited number of wild-born NHPs tested or may reflect increased transmission in captive-bred animals. Interestingly, we also observed many SFV-seronegative primates from both Africa and Asia including captive-born red-eared guenons, black-crested mangabeys, Angolan colobus monkeys, and siamangs, suggesting that SFV in captive-bred NHPs may not be as ubiquitous as previously thought. However, these negative

findings and the observed lower SFV seroprevalence in wild-born primates may require confirmation by testing larger numbers of animals. The observed negative WB results in these animals may not be due to a lower assay sensitivity since SFV antibodies were detected in closely related primate species.

While this study provides evidence that the CA-WB assay is sensitive for the detection of antibodies to SFV variants in 32 different NHP species, our findings also show that the assay may not reliably detect antibodies to SFV in sera from some orangutans. A similar weak cross-reactivity of orangutan sera to SFV<sub>AGM</sub> and SFV<sub>CPZ</sub> has been observed by others (Hahn et al., 1994). However, we show that this limitation could be overcome by using the autologous SFV<sub>PON</sub> antigen. The lack of cross-reactivity between SFV<sub>PON</sub>-specific antibodies and the SFV<sub>AGM</sub> and SFV<sub>CPZ</sub>

Table 3  
Comparison of the seroprevalence of simian foamy virus (SFV) in eight species of wild-born and captive-bred primates

	Genus	Species	Common name	Number of wild-born seropositive (%)	Number of captive-bred seropositive (%) <sup>a</sup>
African	<i>Cercopithecus</i>	<i>mona</i>	Mona monkey	5/14 (35.7)	NA
		<i>nictitans</i>	Spot-nosed guenon	0/14	1/4 (25)
		<i>erythrotis</i>	Red-eared guenon	0/5	0/2
		<i>pogonias</i>	Crowned guenon	0/1	1/1 (100)
		<i>preussi</i>	Preuss's monkey	0/1	NA
		<i>sclateris</i>	Slater's guenon	0/6	NA
	<i>Cercocebus</i>	<i>torquatus</i>	Red-capped mangabey	8/13 (61.5)	5/8 (62.5)
Asian	<i>Pongo</i>	<i>pygmaeus</i>	Bornean orangutan	20/20 (100.0) <sup>b</sup>	1/1 (100.0)
		Totals <sup>c</sup>		28/53 (52.8)	8/16 (50.0)
		Combined total <sup>d</sup>		33/74 (44.6)	211/286 (73.8)

<sup>a</sup> NA, serum not available from this captive-bred species.

<sup>b</sup> Results are after repeat Western blot analysis of all orangutan sera with autologous SFV<sub>PON</sub> antigen (19 positive, 1 indeterminate with CA-WB testing). PON, orangutan.

<sup>c</sup> Seroprevalence in primates with sera from the same wild-born or captive-bred species.

<sup>d</sup> Seroprevalence in all wild-born or captive-bred primates.

Table 4

Novel simian foamy viruses isolated from peripheral blood lymphocytes (PBLs) of African and Asian primates with discordant combined antigen Western blot (CA-WB) and integrase PCR results

Genus	Species	Common name	CA-WB+ <sup>a</sup>	PCR+ <sup>b</sup>	Culture <sup>c</sup>
African					
<i>Allenopithecus</i>	<i>nigroviridis</i>	Allen's swamp monkey	2/2	0/2	1/1
Asian					
<i>Pygathrix</i>	<i>nemaeus</i>	Red-shanked Douc langur	4/5	0/4	ND
<i>Trachypithecus</i>	<i>françoisi</i>	François' langur	5/9	0/5	1/1
<i>Hylobates</i>	<i>pileatus</i>	Pileated gibbon	4/9	0/4	1/1
	<i>leucogenys</i>	White-cheeked gibbon	3/6	0/3	1/1
	Totals		18/31	0/18	4/4

<sup>a</sup> Number of CA-WB-positive sera of the total tested.

<sup>b</sup> PCR analysis was done only on primates with positive CA-WB results.

<sup>c</sup> Coculture of PBLs from representative primates with canine thymocytes (Cf2Th); PBLs were not available from all animals. ND, not done.

antigens may be due to divergence in the highly conserved epitopes in the SFV<sub>PON</sub> Gag proteins. For example, the predicted SFV<sub>PON</sub> Gag sequence has only about 40–50% identity to the SFV<sub>AGM</sub> and SFV<sub>CPZ</sub> Gag proteins, respectively (McClure et al., 1994). In contrast, the Gag proteins from SFV<sub>MAC</sub>, SFV<sub>AGM</sub>, and SFV<sub>CPZ</sub> share at least 64% identity (Herchenroder et al., 1994). These results show that testing for antibodies to SFV<sub>PON</sub> in orangutans or in persons exposed to orangutans may require the inclusion of an SFV<sub>PON</sub> antigen in the serologic screening assay.

The new SFVs identified in the current study represent the first primate *Spumaviruses* described for Allen's swamp monkeys, gibbons, and langurs and demonstrate the broad genetic diversity of Old World primate FVs. Phylogenetic analysis of integrase sequences from the new SFV isolates is consistent with a coevolution with their primate hosts as previously suggested for other SFVs (Blewett et al., 2000; Broussard et al., 1997; Schweizer and Neumann-Haefelin, 1995; Heneine et al., 1998). Old World monkeys and apes are divided into four subfamilies, the Cercopithecines (cheek pouch monkeys), Colobines (leaf-eating monkeys), Hylobatids (lesser apes such as gibbons), and Hominids (orangutans, gorillas, chimpanzees, and bonobos) (Groves, 2001). Thus, as expected, the Hylobate SFVs cluster near the Hominid sequences; the *Allenopithecus* SFV sequence forms a separate lineage with SFVs from other Cercopithecines, and the SFV from a Colobine host, the *Trachypithecus*, forms another unique lineage between the Cercopithecine and Hominid subfamilies. The coevolution of SFVs in Old World monkeys and apes combined with wide distribution of distinct SFVs in the remaining primate taxonomic families, including New World monkeys and prosimians, suggests that ancestral SFVs were present in mammals before primate speciation. Thus, if primate divergence began at least 65 million years ago as previously estimated (Martin, 1993), then SFVs may be the oldest known exogenous simian retroviruses.

Our study also shows that generic PCR assays may not detect highly divergent SFVs. Thus, the CA-WB assay is a better diagnostic tool, and the study results further support the use of this method for the diagnosis and surveillance of

infection originating from divergent SFVs among both NHPs and humans. Our ability to isolate SFVs from these primates without detectable proviral sequences by PCR illustrates the utility of virus isolation and confirms the observed seropositivity in the CA-WB assay. Thus, our inability to PCR amplify SFV sequences from the seropositive *P. nemaeus* PBL DNA lysates may indicate that this species may harbor yet another highly divergent SFV.

In conclusion, we have described a highly sensitive and specific CA-WB assay for the detection of SFV antibodies from both naturally infected primates and accidentally infected humans. We have also shown that unlike other simian retroviruses, SFVs have both a wide distribution and a high seroprevalence among African and Asian NHPs. These results imply that persons who have frequent contact with NHPs may be at increased risk for cross-species SFV infection. The CA-WB assay should facilitate testing of larger human populations for evidence of SFV infection.

## Materials and methods

### *NHP specimens*

A total of 489 serum or plasma specimens were collected from 44 species of captive-bred African and Asian monkeys and apes and 7 species of wild-born Nigerian monkeys ( $n = 54$ ) and Bornean orangutans ( $n = 20$ ) as part of their initial or annual physicals (Tables 1–3). Some of these specimens ( $n = 286$ ) were archived serum samples available from NHPs residing at zoological gardens or research centers in North America. Also, for selected NHPs ( $n = 129$ ), fresh EDTA or sodium citrate treated blood specimens were obtained on an opportunistic basis in accordance with the animal care use committees at each institution. For PCR analysis, DNA or DNA lysates were prepared from the PBLs as described in previous studies (Broussard et al., 1997; Switzer et al., 1995). Primate taxonomic nomenclature used herein was as described by Groves (Groves, 2001).

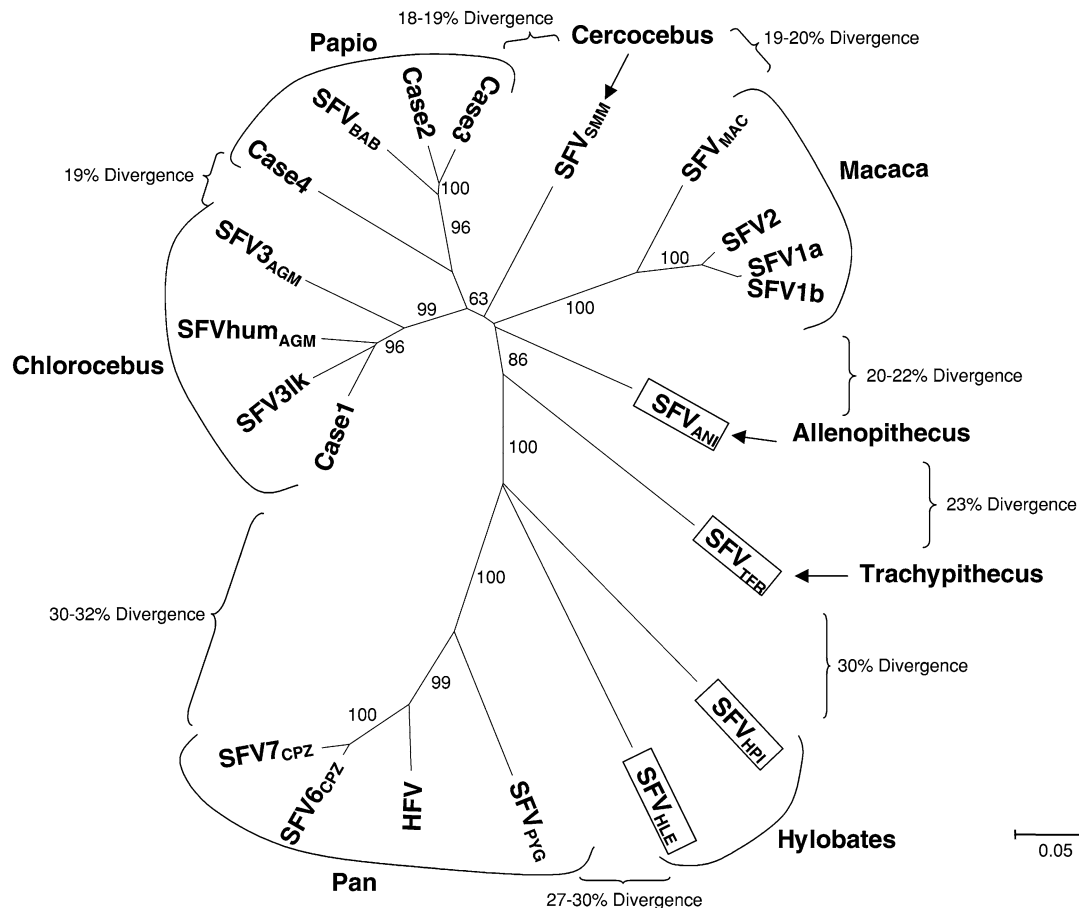


Fig. 2. Unrooted phylogenetic tree of new simian foamy virus (SFV) integrase sequences (boxed text) from peripheral blood lymphocyte coculture isolates from *Hylobates pileatus* (SFV<sub>HPI</sub>), *H. leucogenys* (SFV<sub>HLE</sub>), *Trachypithecus francoisi* (SFV<sub>TFR</sub>), and *Allenopithecus nigroviridis* (SFV<sub>ANI</sub>). Sequences used for comparison were available at GenBank. Case 1 and SFV<sub>hum-AGM</sub>, cases 2, 3, and 4, and the prototype HFV are from two SFV<sub>AGM</sub>-, three SFV<sub>BAB</sub>-, and one SFV<sub>CPZ</sub>-infected person(s), respectively (Achong et al., 1971; Heneine et al., 1998; Schweizer et al., 1997). SFV<sub>BAB</sub>, SFV<sub>3-AGM</sub> and SFV<sub>3Ik</sub>, SFV<sub>SMM</sub>, SFV<sub>MAC</sub> and SFV<sub>1a</sub> and 1b and SFV<sub>2</sub>, SFV<sub>PYG</sub>, and SFV<sub>6-CPZ</sub> and SFV<sub>7-CPZ</sub> are integrase sequences from a baboon, African green monkeys, sooty mangabey, macaques, bonobo, and chimpanzees, respectively. Distances were calculated by the Kimura two-parameter method, and phylogenetic relationships were inferred by the neighbor-joining method. The significance of the branching order was determined by 1000 bootstrap replicates. The values on the branches indicate frequencies of occurrence for 1000 trees and only values 60% or greater are shown. Branch lengths are drawn to scale. A similar tree topology was obtained by the maximum likelihood and minimum evolution methods (data not shown). Percentage nucleotide divergence of the new SFVs and their closest neighbors are shown.

### SFV CA-WB assay

To avoid using two separate WB assays to test for SFV antibodies, we combined antigens prepared individually from SFV<sub>AGM</sub>- or SFV<sub>CPZ</sub>-infected canine thymocyte cells (Cf2Th) into a single WB assay. Cf2Th cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine, penicillin-streptomycin, and fungizone (Gibco-BRL, Gaithersburg, MD). At 30–40% confluency the Cf2Th cells were infected with SFV<sub>AGM</sub> (ATCC VR-218), SFV<sub>CPZ</sub> (ATCC VR-632), or SFV<sub>PON</sub> (a kind gift of Dr. Paul B. Johnston). SFV-inoculated Cf2Th cells were monitored for characteristic cytopathic effects (CPE), and the cells were harvested by scraping when 40–50% CPE was observed. Uninfected Cf2Th cells were also maintained as negative virus antigen controls to

test for evidence of nonspecific cross-reactivity in some samples.

Cell pellets were washed twice in PBS (pH 7.4) and then lysed in detergent buffer containing 0.5 M Tris-Cl, pH 7.2, 10% SDS, and 1% glycerol. The lysates were boiled and sonicated for 5 min and then homogenized by passing them two or three times through a tuberculin syringe. The protein concentration of the cell lysates was determined with the Pierce protein kit (Rockford, IL). SFV<sub>AGM</sub>- and SFV<sub>CPZ</sub>-infected cell lysates (150  $\mu$ g of each) were mixed and denatured by boiling for 3 min in Laemmli buffer (Bio-Rad, Valencia, CA). SFV-mixed or -uninfected Cf2Th cell lysates were electrophoresed on 7.5% SDS-2D/prep Tris-glycine polyacrylamide gels (Bio-Rad) at 70 V for 2 h and then transferred to polyvinylidene difluoride membranes (Bio-Rad). The membrane filters were blocked overnight at 4°C in blocking buffer [5% nonfat dry milk in Tris borate

buffer (TBS) with 0.1% Tween 20] and washed once for 5 min in wash buffer (1% Tween 20 in TBS) at room temperature. The proteins were incubated overnight at 4°C with sera diluted 1:50 in blocking buffer. The membranes were washed three times for 5–10 min each and incubated with a 1:6000 dilution of horseradish peroxidase conjugated protein A/G for 2 h at 4°C, followed by two 15-min washes. Reactivity to protein bands was visualized by chemiluminescence with the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ). Seroreactivity to both Gag p68 and p71 precursor proteins was interpreted as seropositive. Specimens without reactivity to either Gag protein were considered seronegative and those with reactivity to a single band in the 68- to 74-kDa molecular weight range with an absence of similar reactivity to antigen from uninfected Cf2Th cells were called seroindeterminate.

#### *Validation of the SFV CA-WB assay*

The CA-WB assay was validated with serum samples from 32 African and Asian NHP species ( $n = 129$ ) (Table 1) and 16 humans [SFV<sub>BAB</sub>-infected ( $n = 4$ ), SFV<sub>CPZ</sub>-infected ( $n = 6$ ), SFV<sub>AGM</sub>-infected ( $n = 1$ ), and SFV-uninfected ( $n = 5$ )]. The infection status of these NHPs and human samples was determined by PCR analysis using generic integrase primers as described below. Reactivity of human sera from 25 persons infected with HIV-1/2 or HTLV-1/2 and of HIV and HTLV-negative sera from 100 U.S. blood donors was also evaluated.

#### *SFV seroprevalence in a large number of captive-bred and wild-born NHPs*

To define the prevalence of SFV infection in captive-bred and wild-born NHPs and to assess the risks of occupational exposures to SFVs in these settings, we used the CA-WB assay to screen sera or plasma from 286 captive-bred African and Asian NHPs (40 species) (Table 2) in 25 North American zoos and sera from 74 wild-born Nigerian monkeys (7 species,  $n = 54$ ) and Bornean orangutans (1 species,  $n = 20$ ) (Table 3).

#### *Isolation of SFV from NHP PBLs by coculture*

Cocultivation of equal numbers of primate PBLs and Cf2Th was done by using culture conditions reported previously (Heneine et al., 1998). Cultures were monitored every 3–4 days for syncytial CPE typical of FV and for RT activity using the Amp-RT assay as performed elsewhere (Heneine et al., 1995). When at least 50% of each culture showed CPE, the cells were trypsinized and DNA lysates were prepared as described above. Isolates were confirmed as SFVs by PCR and sequence analysis using generic integrase primers as described below.

#### *PCR and DNA sequence analysis*

Amplification of SFV proviral sequences was performed using 25  $\mu$ l of either PBL or PBL coculture DNA lysates in a generic, nested PCR of the integrase region by using methods previously described (Heneine et al., 1998; Schweizer and Neumann-Haefelin, 1995). These generic primers, termed 1–4, have been previously shown to have a copy number sensitivity of 1–10 genomes per  $10^5$  cells and can detect divergent SFV sequences from both monkeys and apes (Schweizer and Neumann-Haefelin, 1995). Nested PCR products were electrophoresed in 1.8% agarose gels and visualized by ethidium bromide staining. For sequence analysis, PCR products were purified using the Qiaquick PCR purification kit (Qiagen Inc., Valencia, CA) and then sequenced in both directions using a Big Dye terminator cycle kit (PE Biosystems, Foster City, CA) and a 373 automated sequencer (PE Biosystems). Percentage nucleotide divergence was determined with the GAP program in the Wisconsin sequence analysis package on a UNIX workstation. Sequences were aligned using the Clustal W program (Thompson et al., 1994), and the alignments were imported into various programs in either the PAUP\* (beta version 5.0) or the MEGA (version 2.1) packages (Kumar et al., 2001; Rogers and Swofford, 1999).

Distance-based trees were generated by using the Kimura two-parameter model in conjunction with the Neighbor Joining and Minimum Evolution methods in the MEGA program. A total of 1000 bootstrap replicates were used to test the reliability of the final tree topology. Character-based tree-building methods were performed by using the maximum likelihood (ML) procedures included in the PAUP\* software (Rogers and Swofford, 1999). For ML analyses, the quartet puzzling method was used with the following parameters: quartets evaluated using the least-squares method with ML distances; 10,000 puzzling steps; all sites assumed to evolve at the same rate; empirical frequencies; transition/transversion ratio = 2; HKY (Hasegawa–Kishino–Yano) two-parameter model for unequal base frequencies; and molecular clock not enforced. The GenBank accession numbers for the four new SFV integrase sequences are AF516484–AF516487. GenBank accession numbers for the 17 sequences used for phylogenetic comparison are M74895 (SFV31k), X83293 (SFVhum<sub>AGM</sub>), X83291 (SFV3<sub>AGM</sub>), AF049077 (SFV<sub>AGM</sub>-infected human or case 1), AF049080 (SFV<sub>BAB</sub>-infected human or case 2), AF049084 (SFV<sub>BAB</sub>-infected human or case 3), AF049080 (SFV<sub>BAB</sub>-infected human or case 4), AF049081 (SFV<sub>BAB</sub>), AF049079 (SFV<sub>SMM</sub>), X83292 (SFV<sub>MAC</sub>), X83290 (SFV-2), X54482 (SFV-1a), X58484 (SFV-1b), AF049086 (SFV<sub>PYG</sub>), M54978/M38712 (HFV), X83296 (SFV-6<sub>CPZ</sub>), and X83296 (SFV-7<sub>CPZ</sub>), where SFV<sub>SMM</sub> and SFV<sub>PYG</sub> are from sooty mangabey monkey and pygmy chimpanzee (bonobo), respectively.

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