Quantification of *Feline immunodeficiency virus* (FIV<sub>pco</sub>) in peripheral blood mononuclear cells, lymph nodes and plasma of naturally infected cougars

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Infection of domestic cats with *Feline immunodeficiency virus* (FIV) results in a fatal immunodeficiency disease, similar to *Human immunodeficiency virus* 1 (HIV-1) in humans. Elevated plasma viral loads in domestic cats are correlated to decreased survival time and disease progression. However, FIV is also maintained as an apathogenic infection in other members of the family Felidae including cougars, *Puma concolor* (FIV<sub>pco</sub>). It is not known whether the lack of disease in cougars is a result of diminished virus replication. A real-time PCR assay was developed to quantify both FIV<sub>pco</sub> proviral and plasma viral loads in naturally infected cougars. Proviral loads quantified from peripheral blood mononuclear cells (PBMC) ranged from 2 \( \times 10^3 \) to 6 \( \times 10^4 \) copies per 10⁶ cells. Plasma viral loads ranged from 2 \( \times 10^3 \) to 2 \( \times 10^6 \) RNA copies ml \(^{-1}\). These data indicate that FIV<sub>pco</sub> viral loads are comparable to viral loads observed in endemic and epidemic lentivirus infections. Thus, the lack of disease in cougars is not due to low levels of virus replication. Moreover, significant differences observed among cougar PBMC proviral loads correlated to viral lineage and cougar age (\( P = 0.014 \)), which suggests that separate life strategies exist within FIV<sub>pco</sub> lineages. This is the first study to demonstrate that an interaction of lentivirus lineage and host age significantly effect proviral loads.

**INTRODUCTION**

*Feline immunodeficiency virus* (FIV) is a lentivirus that infects members of the family Felidae worldwide. Although all FIV strains detected in wild and domestic cats form a monophyletic cluster in a phylogeny of lentiviruses, each feline species is infected with a distinct virus and infection results in disparate outcomes (Burkhard & Dean, 2003). FIV is maintained as an apathogenic infection in some members of the cat family such as lions (*Panthera leo*) and cougars (*Puma concolor*) (Biek et al., 2005; Brown et al., 1994; Carpenter & O’Brien, 1995; Olmsted et al., 1992; Packer et al., 1999). However, FIV infection in domestic cats results in a disease similar to that caused by *Human immunodeficiency virus* 1 (HIV-1) infection in humans that begins with an acute illness and progresses to immunodeficiency and ultimately death (Pedersen et al., 1987).

The amount of circulating virus is a strong prognostic indicator for disease progression in FIV and HIV-1 infections (Goto et al., 2002; Mellors et al., 1996). In both naturally and experimentally infected domestic cats, FIV replicates to high titres and elevated viral loads are associated with shorter survival time and progression to feline acquired immunodeficiency syndrome (AIDS) (Diehl et al., 1996; Goto et al., 2002). Similarly, high plasma virus loads are associated with disease progression in HIV-1 (Mellors et al., 1996).

African primates are also host to lentivirus infections (simian immunodeficiency virus, SIV) and, as is the case with endemic feline lentivirus infections, there is no evidence of disease (Beer et al., 1996; Broussard et al., 2001). However, plasma virus loads in African green monkeys (*Cercopithecus aethops*) and sooty mangabeys (*Cercocebus atys*) naturally infected with SIV<sub>agm</sub> and SIV<sub>sm</sub> respectively, are in the order of 10⁶ RNA copies ml \(^{-1}\) (Broussard et al., 2001; Chakrabarti, 2004). These data indicate that virus replication can be robust even in asymptomatic infections and thus high levels of circulating virus are not always associated with disease.
Currently, no viral load data have been determined for endemic FIV infections in wild felids. FIVpco infects free-ranging cougars in North and South America with infection prevalence averaging 30% (Carpenter et al., 1996), but reaching as high as 58% in some populations in western USA (Biek et al., 2003). This prevalence is remarkable because cougars are solitary carnivores with infrequent conspecific contacts. Intrahost viral diversity is less than 1% in infected cougars and the evolutionary rate of FIVpco has been estimated at 0.1–0.3% per site per year (Biek et al., 2003). This is an order of magnitude lower than the rate of SIV and HIV-1 could be due to increased virus replication resulting in a rapid accumulation of mutations and stronger selection on the virus population. Therefore, based on the lack of disease, the low intrahost viral diversity and low evolutionary rates, we hypothesized that FIVpco viral loads in infected cougars would be lower than in pathogenic FIV and HIV-1 infections or in endemic SIV infection in primates. We subsequently developed a real-time PCR assay for FIVpco and used the assay to determine the amount of cell-associated (proval DNA) and cell-free (viral RNA) virus present in a large set of naturally infected cougars.

METHODS

Study population and cougar samples. Peripheral blood mononuclear cells (PBMC), lymph nodes (LN) and plasma samples used in this study were obtained from free-ranging cougars from four Rocky Mountain populations determined previously to be infected with FIVpco. Genomic DNA samples from PBMC were obtained from 39 infected cougars. Genomic DNA samples from LN were obtained from a group of 10 hunter-killed animals submitted to the Montana Department of Fish, Wildlife and Parks. Plasma samples were also obtained from 32 infected cougars. Genomic DNA was extracted from samples as described previously (Biek et al., 2003).

Phylogenetic analysis. A 779 bp fragment of proviral env was amplified from serial dilutions of PBMC or LN DNA from all infected cougars evaluated in this study in order to determine lineage affiliation. PCR products were cloned and sequenced as described previously (Biek et al., 2003). Alignments were conducted in Lasergene (version 5.5) from DNASTAR using the CLUSTAL W algorithm. A maximum-likelihood (ML) tree was created in PAUP* (4.10b, Swofford, 2002) using a GTR + I + G model as described in MODELTEST (Posada & Crandall, 1998). PLV1695 (AY307116) was used as an outgroup (Biek et al., 2003). One hundred bootstrap iterations were performed. One env sequence for each cougar has been submitted to GenBank.

Sequence analysis of real-time FIVpco amplicon. A 690 bp fragment near the 3’ end of env was amplified by nested PCR from DNA derived from PBMC of 14 infected cougars, which included representatives of each viral lineage to determine the sequence variation in the FIVpco amplicon primer site. The oligonucleotides used for the first round were Co7990F (5’-ATGCAAGTTATGAGTGTAG-3’) and Co8958R (5’-TATTCAACGCGTTCGACTT-3’). The oligonucleotides used for the second round were Co3LTRF (5’-AAGCCCTATGTGTTCTCTAG-3’) and Co8859R (5’-CATTCCTTCAGCTGACCATG-3’). The conditions for the first round of PCR were as follows: 3 min at 94°C followed by 35 cycles of 94°C for 30 s, 48°C for 30 s, 71°C for 30 s and followed by 5 min extension at 71°C. The conditions for the second round of PCR were as follows: 3 min at 94°C followed by 35 cycles of 94°C for 30 s, 51°C for 30 s, 71°C for 45 s and followed by 5 min extension at 71°C. PCR products were cloned into the pDrive plasmid (Qiagen) and sequenced. The viral sequence from lineage four was an exact match to both the forward and reverse primers. The sequences from lineage one and three had the same single mismatch in the reverse primer. Sequences from lineage two had a single mismatch in the forward primer and those from lineage five had single mismatches in both the forward and reverse primers.

Plasma viral RNA preparation. The total volume of plasma or serum available from each cougar, which ranged from 100 µl to 65.5 ml per infected cougar, was centrifuged for 1-5 h at 100 000 g. The viral pellet was resuspended in 140 µl PBS containing Ca2+ and Mg2+ and incubated for 1 h with DNase. RNA was purified using the QIAamp viral RNA mini kit (Qiagen) and eluted in 30 µl DEPC-treated water. Where plasma volumes were greater than 2 ml, RNA was eluted in 60 µl DEPC-treated water. Samples were stored at −80°C until used for cDNA synthesis.

Preparation of DNA and RNA real-time PCR standards. A plasmid standard for myosin was constructed by amplifying a 220 bp fragment of cougar genomic DNA with primers designed to exon 19 of the cougar myosin gene. The primers were used were MyoF (5’-CAAGAACCTGGGCCCTGGATGAA-3’) and MyoR (5’-CTGCCATTTGCGGAGTCTG-3’). The conditions for PCR were as follows: 3 min at 94°C followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, 71°C for 50 s and followed by 5 min extension at 71°C. PCR product was cloned into the pCR4-Topo plasmid (Invitrogen). For each FIVpco DNA standard, a 690 bp fragment of the FIVpco genome near the 3’ end of env was amplified by PCR from cougar proviral DNA as described above. All plasmids were linearized and purified with the QIAquick PCR Purification kit (Qiagen). Plasmid concentration was determined by UV spectroscopy. All DNA plasmid standards were diluted in 10 mM Tris (pH 8.5) containing salmon sperm DNA (Sigma-Aldrich) as a carrier at a final concentration of 6 ng µl−1.

Virus from the supernatant of a co-culture of 3201 cells and PBMC of a naturally infected cougar, 5RF631, was used for the RNA standards. Viral RNA concentration was determined by UV spectroscopy to estimate copy number. All RNA standards were diluted in DEPC-treated water with carrier tRNA (Sigma) at a final concentration of 63 ng µl−1. The viral RNA standards and viral RNA obtained from the plasma of infected cougars contained equivalent carrier tRNA concentrations.

FIVpco real-time PCR quantification. TaqMan chemistry was used to quantify the number of cell equivalents in each proviral reaction. For the myosin reactions, the primers used were MyoTaqMF (5’-TGGCCCTGGATGAACTCTACT-3’) and MyoTaqMR (5’-GCACATTCTCTGCTGCTTCT-3’). The probe sequence used for this primer set was Myosprobe (5’-FAM-CAGATCCAAGCCCCCTGCTCAAGGCAGG-TAMRA-3’).

SYBR green chemistry was used for quantification of FIVpco from genomic DNA and plasma because sequence divergence among FIVpco lineages precluded designing a suitable probe. The primers used were ETaqF (5’-TGATCCCTGATGGCTCCACCAAC-3’) and ETaqR (5’-TCTCACTGTTGTCGACCT-3’). The amplification with this pair of oligonucleotides produced a fragment of 170 bp.

Reactions consisted of 25 µl 2X Universal Master Mix (Applied Biosystems) containing 100 mM KCl, 40 mM HCl/Tris, 1·6 mM dNTP, 50 U Taq µl−1, 6 mM MgCl2 and 5 µl genomic template, in a
50 μl total reaction volume. Each reaction for myosin amplification
contained 300 nM MyoTaqMF, 100 nM MyoTaqMR and 50 nM
Myoprobe. Each FIVpco proviral reaction contained 300 nM ETaqF,
300 nM ETaqR and 1:10,000 dilution of SYBR Green I gel stain
(BioWhitaker).

Myosin amplification was as follows: 95°C for 10 min followed by a
two-step PCR procedure consisting of 95°C for 15 s then 60°C for 1
min for 45 cycles. FIVpco amplification was similar except that the
annealing temperature was at 61°C for 1 min. Amplification, data
acquisition and analysis were performed using the iCycler real-time
PCR detection system (Bio-Rad). All FIVpco reactions were evaluated
by melt-curve analysis to confirm the size of the amplicon and lack of
primer-dimer formation. Genomic DNA from uninfected cougars did not
amplify with FIVpco-specific oligonucleotides.

Reverse transcription (RT) was carried out as a two-step procedure for
both the RNA standards and plasma samples. The reaction mixture,
30 μl total, contained 1 μl SuperScript III Reverse Transcriptase
(Invitrogen), 4 μl 5 × RT buffer, 1 nM ETaqR and 10 μl purified RNA.
The reaction was conducted at 50°C for 50 min and 85°C for 5 min.

Plasma viral RNA quantification was determined using 50 μl reactions
consisting of 25 μl 2 × Platinum SYBR Green qPCR SuperMix
(Invitrogen), 300 nM ETaqF and 300 nM ETaqR. FIVpco amplification
was as follows: 50°C for 2 min followed by 1 cycle of 95°C for 2 min
then a two-step PCR procedure consisting of 95°C for 15 s then 60°C
for 45 s for 45 cycles.

All standards, negative controls and samples were run in duplicate and
the mean value of the copy number was used to quantify both FIVpco
and myosin. The measurements of myosin and FIVpco-copy numbers
were accepted if the coefficients of variation (CV) were <20% for
myosin reactions and <35% for FIVpco reactions. FIVpco-copy number for provirus was divided by the number of cells assayed and reported
on the basis of 106 PBMC or LN cells. FIVpco-copy number for plasma virus was divided by the volume of plasma assayed and reported
as the number of viral RNA copies per millilitre of plasma.

Statistical analysis. The lower limits of detection for the proviral
and plasma viral load real-time PCR assays were set at 100 DNA
copies and 320 RNA copies per reaction to account for increased
variability in cycle number in quantifying low-copy numbers (see Results). Samples that amplified below the lower limit of detection
were confirmed by melt-curve analysis.

Proviral and plasma viral loads were determined from PBMC and
plasma that were above the lower limit of detection. The mean and
standard deviation of both proviral and plasma viral loads were calculated and 95% confidence intervals were then set for both
population means through the Student's t distribution. The lower limit
of both proviral and plasma viral loads was calculated. The minimum
number of cell equivalents and minimum volume of plasma per
reaction, which would generate viral loads within the 95% confidence
intervals, were determined to be 1.16 x 104 cells and 100 μl plasma per
reaction. Samples assayed that exceeded the calculated minimum
of cell equivalents or plasma volume, but had viral loads below the lower limit
of real-time detection, were down weighted with a factor of 1/10 to
account for increased variability in threshold-cycle numbers at low-
copy number. Proviral and plasma samples assayed below the calculated minimum of cell equivalents or plasma volume and samples
that did not reach threshold were excluded from the statistical analysis.

A weighted univariate analysis of covariance (ANCOVA) was used to
test if any significant differences existed among the log10 proviral and
plasma viral load means due to differences in age, gender and viral
lineage. Levene's test of equality of error variance was used to ensure
equal variance existed across the lineages. The proviral model was
created based on the ANCOVA of proviral load on age, which resulted in
separate slopes and intercepts for each lineage.

RESULTS

Experimental conditions of the FIVpco proviral and plasma viral load real-time PCR assays

A fragment of exon 19 of the cougar myosin gene was used to
determine the number of cell equivalents in samples to be
quantified for FIVpco provirus. Amplification of the myosin
gene producing a standard curve was based on Taqman chemistry and was linear over seven orders of magnitude.
The efficiencies (defined as 10(−1/slope)) of the plasmid and
genomic samples were 1.90 and 1.91, respectively, indi-
cating that plasmid and genomic DNA amplified with
equivalent efficiency in our assay (data not shown). The
number of cell equivalents determined for proviral
quantifications was established by calculating the mean of
two separate myosin quantifications of genomic cougar DNA. The inter-assay variation for myosin quantification
was determined by comparing the values obtained for
standard curves produced in four separate experiments.

Previous work established that mismatches within the real-
time primer sites do not enable accurate quantification
because of variable efficiencies in amplification (Klein
et al., 1999). Because the viral sequence diversity observed among
cougar lentiviruses is greater than the diversity observed
in FIV in domestic cats (Carpenter et al., 1996), we first
established the phylogenetic affiliation of all FIV pco samples
prior to quantification and then determined the effect of
nucleotide mismatches on FIVpco real-time amplification.

All samples clustered within five distinct viral lineages based on
a fragment of env (Fig. 1). Viral lineage associations were
consistent with those in ML trees of env and pol sequences
from 150 individual cougars (Biek et al., 2006). The
associations of lineage one and two were consistent with
previously published results (Biek et al., 2003).

We prepared separate FIVpco standards from sequences that
represented each viral lineage and investigated the effects
of these mismatches on amplification efficiency. Lineage
standards containing no mismatches or mismatches in
one or both primers were evaluated simultaneously by real-
time PCR. Standards from three lineages produced over-
lapping curves and were not significantly different based on
an F test for significant differences between lines (P = 0.114).

 Although the variation between DNA plasmid standard
curves were not significantly different, the error associated
with the low-copy number standards increased with the
number of primer mismatches. Therefore, for proviral DNA quantification, a lineage-specific standard was used for samples of each lineage. The mean Ct CV (3.62%) and the mean absolute CV (38.52%) was calculated to determine FIVpco inter-assay variation (data not shown). The inter-assay variation of the FIVpco real-time assay was greater than the difference between different FIVpco-lineage standards. The mean absolute FIVpco inter-assay variation, obtained with SYBR green, of 38.52% is comparable to the variation described previously in real-time assays using Taqman probes, which has been reported to be greater than 35% (Damond et al., 2001; Desire et al., 2001). CV increased at the low-copy numbers, where the final standard, 6.45 × 10^1 copies, had the largest CV value (68.33%). Subsequently, we set the lower limit of detection at 100 DNA copies per reaction to account for the variability associated with the most dilute standard.

For plasma viral load quantification, a two-step real-time PCR assay was developed that was linear over six orders of magnitude from 3.2 × 10^8 to 3.2 × 10^2 copies. The lower limit of detection for this assay was set at 320 RNA copies per reaction to account for increased variation in threshold-cycle number with low-copy number standards. Only one RNA standard was amplified in the real-time RT assay because there were no significant differences between separate proviral standards in amplification and virus representative of all five lineages have not been isolated. The inter-assay variation for real-time RT-PCR was 3.92% for the mean Ct CV and 38.38% for the mean absolute CV (data not shown). This is similar to the inter-assay variation for the proviral quantification, suggesting that the RT step had minimal effects on assay reproducibility and is also comparable to variation reported for other real-time RT assays (Gibellini et al., 2004; Gueye et al., 2004).

**Proviral loads in naturally infected cougars**

Cougar samples quantified in this study were determined previously to be FIVpco-positive by nested PCR. Therefore,
the FIV<sub>pco</sub> real-time PCR assay was utilized only to quantify viral loads and was not used as a detection method. Thirty-nine cougar PBMC samples were quantified and 22 (56%) were within our level of detection (Table 1). Ten LN samples were also quantified and five were within the range of detection (50%). Samples that had less than 100 proviral copies, which we established as the lower limit of detection, were still valuable in our analysis. For example, FIV<sub>pco</sub>-copy number was below the limit of detection in four PBMC and two LN samples despite the fact that more than $1 \times 10^5$ cell

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*Indicates down weighted proviral or plasma viral loads.  
ND, Not determined.
equivalents were assayed, indicating that the proviral load was low in those animals. Therefore, 95% confidence intervals for the number of cells required for FIV pco detection was established (see Methods). Samples that were adequately assayed but below detection were down weighted to account for increased variability associated with threshold-cycle number. Eight PBMC samples were down weighted in the proviral analysis. Samples for which there were insufficient cell numbers or plasma volume for adequate sampling were omitted from the statistical analysis. Nine PBMC samples were omitted from the proviral analysis.

The mean proviral load per 10⁶ PBMC was 1·34 × 10⁴ and ranged from 2·9 × 10¹ to 6·72 × 10⁴ (Fig. 2). The lowest proviral loads were adequately assayed, however, the number of proviral copies quantified was below our limit of detection. Therefore, these samples were down weighted. The mean proviral load per 10⁶ LN cells was 1·51 × 10⁴ (range from 8·06 × 10³ to 2·51 × 10⁴). These data indicate that a mean of 1 in 75 circulating cells is infected with FIV pco and 1 in 66 cells in the LN is infected, assuming one proviral FIV pco molecule per cell.

We tested the hypothesis that mean proviral loads in PBMC were equal among gender, age and viral lineage using a weighted ANCOVA. These factors were considered because one or all may have a significant biological effect on the amount of virus in infected cougars. For example, the amount of virus in FIV-infected domestic cats differs significantly between different field isolates (Pedersen et al., 2001). The variation in proviral load was determined to be equal across viral lineages through Levene’s test of equality of error variance (P=0·239). Proviral loads were significantly different between lineages (P=0·029) and there was a significant interaction between viral lineage and cougar age, indicating that considered together these variables had an effect on proviral load (P=0·014). No correlation was observed between proviral load and gender (P=0·440).

Univariate analysis of covariance demonstrates whether differences between means are statistically significant but not how means differ. Therefore, to understand the influence of age and lineage on proviral loads, the linear regression from the ANCOVA of proviral load on age was conducted to model the change in lineage-specific proviral loads versus age, which resulted in separate lines for each lineage (r² value=0·549) (Fig. 3). Cougars infected with viruses from either lineage one or two have an increase in

![Fig. 2. FIV pco proviral and plasma viral load for each viral lineage. The mean values for each viral lineage are shown next to the horizontal lines. Closed shapes denote proviral or plasma viral loads that were within the detection limits established for this assay. Open shapes denote down weighted proviral or plasma viral loads.](image)

![Fig. 3. A weighted analysis of covariance of cougar PBMC proviral loads versus cougar age. The colours for each viral lineage correspond to the phylogeny displayed in Fig. 1. The change in lineage-specific proviral loads versus cougar age was based on an ANCOVA of cougar PBMC proviral loads with viral lineage and gender as factors and age as a covariate. Lineage-specific PBMC proviral loads were averaged across gender because gender was not a significant influence on PBMC proviral loads. The linear regressions of proviral loads from lineages one and two, indicated with A, were statistically different from those of lineages three, four and five, indicated with B (P<0·05). However, the differences observed between lineages three, four and five were not significant (P>0·24).](image)
PBMC proviral loads with age. In contrast, cougars infected with viruses from either lineage three, four or five exhibit a decrease in proviral loads with age. The differences between lineages three, four and five or between lineage one and two were not significant (P>0.24). However, the linear regression of proviral loads from lineages one and two were statistically different from those of lineages three, four and five (P<0.05) and this difference remained after random deletion of three proviral load values.

**Plasma viral load in naturally infected cougars**

Thirty-two plasma samples were quantified and in 21 (66%) of these samples FIVpco was detectable by our real-time assay (Table 1). The plasma viral loads ranged from 2.30 × 10^2 to 2.81 × 10^6 RNA copies ml^-1, with a mean of 5.69 × 10^5 (Fig. 2). Of the 11 plasma samples that were below our limit of detection, eight samples were not included in the statistical analysis because an insufficient volume of plasma was assayed and three samples were down weighted as described previously. The variance in plasma viral loads was determined to be equal across lineages through Levene’s test (P=0.086). Through a univariate ANCOVA no significant differences were observed between mean plasma viral loads for different genders, ages or lineages (P=0.958, 0.830 and 0.783, respectively). Additionally, we were able to quantify proviral and plasma viral loads from the same blood sample of 11 cougars in the study (Fig. 4). There was no correlation between proviral and plasma viral loads (r² value =0.133) from the same individual. Similarly, there was no correlation between FIVpco plasma viral loads and PBMC proviral loads within viral lineages (data not shown). These data demonstrate that the level of plasma viraemia is not correlated to the number of infected PBMC, age, gender or viral lineage.

[Fig. 4. Correlation of PBMC proviral loads versus plasma viral loads of infected cougars. Provirals and plasma viral loads were quantified from the same blood sample of 11 cougars. All samples had at least one viral load parameter that was within the detection threshold. Diamonds denote proviral and plasma viral loads within the detection threshold. Down weighted proviral loads are indicated with ‘x’ and ‘+’ denotes down weighted plasma viral loads.]

**DISCUSSION**

Free-ranging cougars are one of several feline species that harbour an endemic lentivirus infection without any apparent signs of disease (Biek et al., 2005; Brown et al., 1994; Carpenter & O’Brien, 1995; Olmsted et al., 1992; Packer et al., 1999). However, it was not known if the lack of disease observed in naturally infected cougars was a result of low-level virus replication. Consequently, FIVpco proviral and plasma viral loads were quantified from naturally infected cougars by real-time PCR. This study represents the most extensive analysis of proviral and plasma viral loads in natural, endemic lentivirus infections to date.

Proviral loads reported previously in infected PBMC from African green monkeys and sooty mangabeys are in the order of 10^2–10^3 proviral copies per 10^6 cells, respectively (Beer et al., 1996; Broussard et al., 2001; Rey-Cuillé et al., 1998). The mean cougar PBMC proviral load was 1.34 × 10^4 proviral copies per 10^6 cells. Therefore, FIVpco proviral loads in infected PBMC are in order of magnitude higher than in PBMC from infected primates. The mean LN proviral load, which was determined from a separate group of infected cougars, was 1.51 × 10^4 proviral copies per 10^6 cells. Although the variation around LN proviral loads was markedly lower than in PBMC, both cougar PBMC and LN cells had similar mean proviral loads. Equivalent PBMC and LN proviral loads have been reported previously in a large cohort of long-term naturally infected African green monkeys (Beer et al., 1996). These data stand in contrast to other studies that have reported elevated proviral loads in lymphoid tissue in naturally infected primates and HIV-1-infected humans (Broussard et al., 2001; Fauci et al., 1996). Finally, plasma viral loads in naturally infected cougars ranged from 10^2 to 10^6 RNA copies ml^-1 and are comparable to viraemia levels reported previously in SIVsm and SIVsm infections (Broussard et al., 2001; Goldstein et al., 2000; Holzammer et al., 2001; Rey-Cuillé et al., 1998). These data clearly indicate that the absence of detectable disease in naturally infected cougars and primates is not a result of low-level virus replication.

In pathogenic lentivirus infections, such as HIV-1 or FIV in domestic cats, the amount of circulating virus is an accurate predictor of disease severity. In humans and domestic cats, plasma viral loads greater than 10^6 copies ml^-1 are correlated to disease progression and shorter survival time (Goto et al., 2002; Mellors et al., 1996). FIVpco-infected cougars maintain plasma viral loads that are greater than 10^5 copies ml^-1 during infection, but these animals remain asymptomatic. Furthermore, we could not detect any relationship between plasma viral loads in infected cougars and factors such as age, gender or viral lineage. Different rates of cell-free virus clearance and production have been reported in patients infected with HIV-1, but the lifespan of infected cells was not significantly different among patients (Perelson et al., 1996). The lack of correlation in plasma viral loads with age, gender or virus lineage may reflect the
transient nature of cell-free virus compared with the integrated provirus.

There was no correlation between the FIVpco and PBMC proviral loads in 11 infected cougars from which both plasma and blood were available. In fact, the animal with the highest plasma viral load (2.81 × 10^6) maintained a proviral load that was below the lower limit of detection (Fig. 4). These data suggest that circulating PBMC may not be the primary source of FIVpco particles in the blood. This is consistent with studies of HIV-1 infection, which established that the primary site of virus production is lymphoid tissue (Haase, 1999) and greater than 90% of HIV-1 plasma viraemia is maintained by a fraction of the CD4^+ T-cell population (Hufert et al., 1997).

The widespread distribution of FIVpco in North America and the extensive sequence divergence between FIVpco lineages indicate that FIVpco infection in free-ranging cougars is not a recent event (Carpenter et al., 1996). In addition, the lack of disease may be an outcome of coevolution between FIVpco and its cougar host (Carpenter & O’Brien, 1995). Based on our data, the low FIVpco intrahost viral diversity reported previously (Biek et al., 2003) cannot be attributed to low-level virus replication and may be a result of other factors including an absence of strong-positive selection on the virus, an increased fidelity of the FIVpco reverse transcriptase or longer virus generation time. Additionally, the high cell-associated and cell-free viral loads documented in infected cougars perhaps may be an effective mechanism by which FIVpco can sustain a high prevalence rate (30–58%) in a solitary species (Biek et al., 2003; Carpenter et al., 1996). Indeed, both FIV cell-associated and cell-free virus are able to cause infection in domestic cats (Burkhard et al., 1997).

Our results indicate that over half of the variability in PBMC proviral loads can be ascribed to viral lineage and cougar age (\(r^2 = 0.549\)). Although the number of PBMC samples quantified was moderate (n = 30), there was a strong correlation of PBMC proviral loads to viral lineage and cougar age (P = 0.014). Because differences in proviral loads among viral lineages were most pronounced in adult cougars (Fig. 3), changes in hormone levels associated with sexual maturation or activity may influence virus replication. Activation of viral transcription occurs in type B (mouse mammary tumour virus) and type C (murine leukaemia virus) retroviruses in response to adrenal steroids B (mouse mammary tumour virus) and type C (murine leukaemia virus) retroviruses in response to adrenal steroids B (mouse mammary tumour virus) and type C (murine leukaemia virus) retroviruses in response to adrenal steroids B (mouse mammary tumour virus) and type C (murine leukaemia virus) retroviruses in response to adrenal steroids.
Viral loads in FIV\textsubscript{pco}-infected cougars


