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# Competitive reverse transcription–polymerase chain reaction for quantitation of feline immunodeficiency virus

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**Abstract**

A competitive reverse transcription–polymerase chain reaction (RT-PCR) was developed to quantify RNA of feline immunodeficiency virus (FIV) in cats. The assay uses in vitro synthesized RNA derived from the *gag* region of the FIV genome as a competitive internal control. The synthesized RNA has a 22-base deletion with respect to the wild-type sequence. PCR products were quantitated by densitometric analysis of a digitalized image of the ethidium bromide stained gel. The non-radioactive method was evaluated in reconstruction experiments. RNA synthesis in FIV-infected feline thymocytes correlated well with the amount of viral p24 antigen produced. Viral RNA concentrations in the plasma of two cats experimentally infected with FIV strain UT113 were followed for 32 weeks; peak copy numbers ($2.3 \times 10^4$ and $1.3 \times 10^4$ per ml, respectively) were reached 11 weeks after subcutaneous injection of ten 50% cat infectious doses. With rising antibody titers against FIV-*gag* and FIV-*env* gene products, the amount of FIV RNA in plasma decreased. Nine asymptomatic cats that had been experimentally infected 3.5 to 4.5 years earlier had copy numbers between $5.6 \times 10^3$ and $4.3 \times 10^4$ per ml. This quantitative competitive RT-PCR will be useful to study the pathogenesis of the FIV infection, to evaluate the effectiveness of vaccines and to monitor antiviral and immunomodulating drugs.

*Keywords:* Feline immunodeficiency virus (FIV), RNA quantitation, Polymerase chain reaction (PCR)

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## 1. Introduction

After recovery from the primary infection, which can be accompanied by general and respiratory symptoms, feline immunodeficiency virus (FIV)-infected cats may remain asymptomatic for a long time. The progressive impairment of immune functions finally leads to immunodeficiency with opportunistic and secondary infections (Pedersen et al., 1987). The study of FIV is important as a feline pathogen, but also because the infection resemble human AIDS in its course and host response. The FIV model is used to study the pathogenesis of immune dysfunction, to develop vaccines and to test the efficacy of antiviral drugs (Jarrett et al., 1990; Siebelink et al., 1990; Egberink et al., 1992).

The infection is diagnosed routinely by demonstrating antibodies against FIV. In most serum samples viral antigens cannot be detected (Lombardi et al., 1994), and the dynamics of FIV replication in vivo are therefore difficult to study. Virus can be isolated from peripheral blood mononuclear cells (PBMCs) in all stages of the disease and quantified by titration in vitro (Meers et al., 1992). This applies to cell-associated virus; the amount of cell-free virus present e.g. in plasma cannot easily be determined by in vitro titration. Cocultivation of PBMC from specific pathogen free cats with plasma from FIV-infected cats leads to diminished cell viability and reduced infection rates (Matteucci et al., 1993).

Quantitation of FIV RNA using a reverse transcription–polymerase chain reaction (RT-PCR) should provide a reliable measure of the viral load in body fluids of the animals. So far, PCR studies of FIV have been limited to qualitative or semi-quantitative analyses of proviral DNA in PBMC (Philpott et al., 1992, Rimstad et al., 1992, Lawson et al., 1993). The present paper describes the development of a RT-PCR method using a competitive template that can be discriminated from that corresponding to wild-type RNA because of a 22-base deletion. Copy numbers of FIV RNA present in the sample can be calculated by regression analysis of data points collected around the molar equivalence point of amplified DNA bands derived from wild-type and competitive template.

## 2. Materials and methods

### 2.1. Plasmids

A highly conserved region in the FIV *gag* gene was selected as target sequence, and two plasmids were constructed using standard cloning techniques (Sambrook et al., 1989). After PCR amplification of the *gag* gene of the Dutch isolate FIV-UT113 (EMBL data library accession no. X68019), a 508 basepair (bp) fragment ([*Apo*I/*Bam*HI] nucleotides 1164 and 1672; numbering as in FIV-Petaluma [Talbott et al., 1989]) was cloned into an *Eco*RI/*Bam*HI cut pSP73 vector (Promega, Leiden, The Netherlands) downstream from the SP6 promotor (plasmid pSP73-*gag*). A second plasmid, pSP73$\Delta$22-*gag*, was constructed containing a similar part of the FIV *gag* region cloned downstream from the T7 promoter of the pSP73 vector. This plasmid consists of sequences also present in the pSP73-*gag* plasmid, except for an internal 22

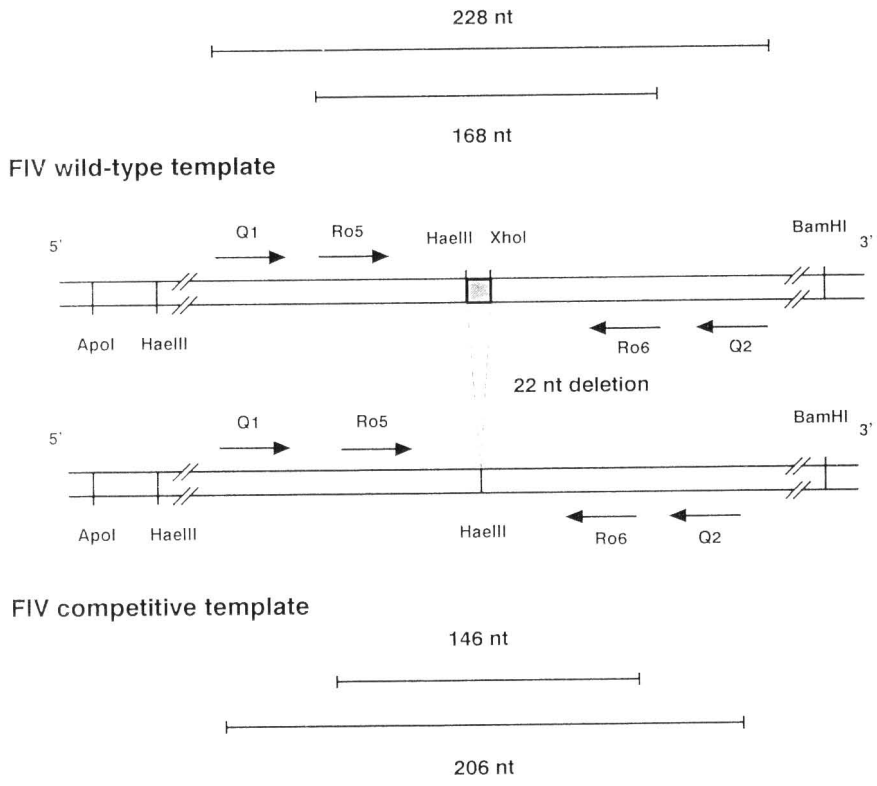


Fig. 1. Schematic diagram illustrating plasmid pSP73-*gag* (wild-type template) and plasmid pSP73Δ22-*gag* (competitive template), relative positions of primers and size of the PCR products.

bp deletion (*Hae*III/*Xho*I, nucleotides 1429 and 1451; Fig. 1). The construction was performed by inserting the *Bam*HI/*Xho*I *gag* fragment (nt 1672–1451) into pSP73 (*Bam*HI/*Xho*I cut) followed by inserting the *Hae*III *gag* fragment (nt 1201–1429) into the *Klenow* filled *Xho*I site. Hence, the pSP73Δ22-*gag* plasmid contained the FIV sequences from nucleotide 1201–1429 and 1451–1672.

### 2.2. *In vitro transcription*

To generate known amounts of standard RNA, 2 μg linearized pSP73-*gag* DNA was transcribed in vitro for one hour at 37°C with 40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM NTPs, 50 μg/ml bovine serum albumin, 20 U RNA-Guard (Gibco BRL Life Technologies) and 10 U SP6 RNA polymerase (Pharmacia, The Netherlands). For plasmid pSP73Δ-*gag* the former reaction mixture was completed with 1 mM spermidine and the SP6 RNA polymerase was substituted by 10 U T7 RNA polymerase (Pharmacia, The Netherlands). After incubation for 15 min with RNAse-free DNAse (1 U per μg DNA), a phenol/chloroform extraction was performed, and RNA was pelleted after ethanol precipitation. The RNA was resuspended in TE (10 mM

Table 1
Sequence and location of FIV oligonucleotide primers

| Primer | Nucleotide sequence (5'-3') | Gene and location [a] |
|---|---|---|
| Q1 | GGATTAACTCAAGAACAGCAAGCAG | *gag* (1338-1362) |
| Q2 | GTTTTAAATATAGCTTAACTTCAGCTG | *gag* (1566-1540) |
| Ro5 | CAAGATTTGCACCAGCTAGGATGC | *gag* (1366-1389) |
| Ro6 | TGTTCTTGATCTATTTGGGC | *gag* (1534-1515) |

[a] Based on Talbott et al. (1989).

Tris–HCl pH 8.0, 1 mM EDTA) buffer. The quality of the RNA transcribed in vitro was evaluated after electrophoresis in a 1.6% agarose gel. Its amount was measured by comparison with known amounts of calf liver ribosomal RNA (Pharmacia, The Netherlands).

### 2.3. Oligonucleotide primers

The primers selected for reverse transcription and the nested PCR were derived from the nucleotide sequence of FIV-UT113. Nucleotide sequences of the sense and antisense primers used for reverse transcription and amplification are given in Table 1. Primers Q1 and Q2 generate a 228 bp product with plasmid pSP73-*gag* (wild-type) and a 206 bp product with plasmid pSP73$\Delta$22-*gag* (competitive template), respectively. The primers for the nested PCR Ro5 and Ro6 generate a 168 bp product with plasmid pSP73-*gag* and a 146 bp product with plasmid pSP73$\Delta$22-*gag*, respectively. Fig. 1 shows the relative primer positions and the PCR products derived from the wild-type and competitive templates.

The lower detection limit using this nested set of primers for amplification of FIV DNA was one proviral molecule, as determined by serial 10-fold dilutions of a plasmid containing the FIV-19k1 isolate (Siebelink et al., 1992) in 100 ng FIV-negative genomic DNA. The lower detection limit of RT-PCR was also one RNA template as determined by 10-fold serial dilutions of the in vitro synthesized RNA templates in 100 ng of calf liver t-RNA (Pharmacia, The Netherlands).

### 2.4. RNA isolation from plasma of infected cats

Blood samples in EDTA were centrifuged for 10 min at $3000 \times g$, the plasma was filtered (0.45 $\mu$m, MILLEX-HA, Millipore, France) and stored at $-70$°C in 150 $\mu$l aliquots until use. Nucleic acid was isolated from 150 $\mu$l plasma according to the method described by Boom et al. (1990). Briefly, plasma samples were lysed in a buffer containing 4.5 M guanidinium thiocyanate, 1.5% w/v Triton X-100, 100 mM Tris–HCl pH 6.4, and 20 mM EDTA pH 8.0. Nucleic acid was bound to 30 $\mu$l (1 mg/ml in 1 N HCl) size selected silicon dioxide particles (silica, Sigma Chemicals, USA) for 15 min at room temperature. Silica particles were washed twice in a buffer containing 5 M guanidinium thiocyanate, and 100 mM Tris–HCl pH 6.4, once with 70% ethanol and once with acetone. Nucleic acid was eluted from dried silica particles with 40 $\mu$l TE at 65°C for 10 min and stored at $-20$°C.

### 2.5. Quantitative competitive RT-PCR

For quantitation of viral RNA in plasma, increasing known copy numbers of the competitive template were added to equal aliquots of the isolated (wild-type) RNA. The RNA mixture was reverse transcribed in a 20 μl volume for 45 min at 37°C using 100 ng of primer Q2, and 100 U Moloney murine leukaemia virus (M-MLV) RT (Gibco BRL, Life Technologies, USA) in 50 mM Tris–HCl pH 9.0, 3 mM $MgCl_2$, 75 mM KCl, 0.2 mM dNTP, and 20 U RNA-Guard. M-MLV RT was subsequently inactivated for 3 min at 94°C. Amplification was carried out in a total volume of 100 μl by adding a 80 μl reaction mix containing 2 U *Taq* polymerase (Promega, The Netherlands), 100 ng primer Q1, 2 mM $MgCl_2$, 40 mM KCl, 8 mM Tris–HCl pH 9.0, 0.1% Triton X-100 overlaid with 50 μl mineral oil. The PCR was carried out in a DNA thermal cycler (Perkin Elmer Cetus, Emeryville, CA). Amplification consisted of 1 min denaturation at 94°C, 1 min primer annealing at 55°C and 1 min extension at 72°C. After 35 cycles, 10 μl of the product was amplified using the same cycles in a 100 μl reaction mix containing 100 ng of the nested primers (Ro5, Ro6), 2 U *Taq* polymerase, 0.2 mM dNTP, 3.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl pH 9.0, 0.1% Triton X-100 overlaid with 50 μl mineral oil. Ten μl of the amplified products were subsequently separated on a 6% polyacrylamide gel. Gels were stained with ethidium bromide (1 μg/ml) and the Appligene CCD high-performance imaging system and software version 2.03 (B&L Systems, The Netherlands) were used to obtain a digitalized video image of the gel. The wild-type template was quantitated by densitometric analysis of the relative amounts of PCR products derived from wild-type and competitor using Molecular Dynamics Imagequant Software version 3.22 (B&L Systems, The Netherlands). Data points collected around the molar equivalence point of wild-type and competitor templates were used to calculate the FIV copy numbers by standard regression analysis (Piatak et al., 1993a).

### 2.6. Reconstruction experiments with known amounts of RNA (DNA)

In vitro RNA transcripts of plasmid pSP73-*gag* (wild-type) and plasmid pSP73Δ22-*gag* (competitive template) were used to compare the efficacy of competitive primer annealing and amplification. To 1250 wild-type templates, increasing amounts of competitive templates (250, 500, 1000, 2000, 4000) were added. The RNA mixture was reverse transcribed and amplified as described. Comparable titrations were done also with pSP73-*gag* and pSP73Δ22-*gag* plasmid DNA.

### 2.7. In vitro infection

In the presence of 2 μg/ml polybrene, $2 \times 10^6$ feline thymocytes were infected overnight with $10^3$ 50% tissue culture infectious doses ($TCID_{50}$) of FIV-UT113. The cells were washed three times to remove unbound virus and subsequently cultured at 37°C in 2 ml of RPMI 1640 containing 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 100 IU/ml human recombinant interleukin-2. Over a period of 12 days, 500 μl of the medium was replenished daily. An aliquot of 150 μl of the

culture supernatant was stored at −20°C to be tested for the presence of FIV p24 antigen using a capture ELISA, another 150 μl of the supernatant was treated with 2 μl DNAse (1 mg/ml) in the presence of 10 mM $MnCl_2$ for 1 h. Nucleic acids were isolated from 100 μl as described above.

### 2.8. Enzyme-linked immunosorbent assay (ELISA)

The FIV-*gag* p24 antigen capture ELISA developed in our laboratory makes use of two anti-p24 monoclonal antibodies (Egberink et al., 1992). The anti-FIV-*gag* and anti-FIV-*env* immune response was measured as described previously (Verschoor et al., 1993).

### 2.9. Experimental FIV infection of cats

Two cats were infected subcutaneously with ten 50% cat infectious doses ($CID_{50}$) of FIV-UT113 grown in feline thymocytes. Blood samples were collected by jugular venipuncture after 1, 3, 5, 6, 11, 15, and 32 weeks of infection. Blood was centrifuged at $3000 \times g$ for 5 min, the plasma was withdrawn, filtered (0.45 μm, Millipore) and stored at −80°C.

### 2.10. $CD_4/CD_8$ T-cell analysis

Enumeration of the T lymphocyte subset was done by mixing $1 \times 10^5$ PBMC at 4°C with monoclonal antibodies directed against feline $CD_4$, $CD_8$, or $CD_3$ (Tompkins et al., 1990), followed by incubation with a fluorescein isothiocyanate conjugated goat anti-mouse IgG (Becton Dickinson, The Netherlands). Lymphocytes were enumerated by flow cytometry (FACScan) and analyzed using Research Software version 2.1 (Becton Dickinson, The Netherlands).

## 3. Results

### 3.1. Validation of the quantitative competitive RT-PCR

The RT-PCR of different amounts of competitive RNA templates in the presence of equal amounts of wild-type templates showed that they were reverse transcribed in vitro and amplified with the same efficiency as the wild-type templates (Fig. 2). The competitive template can therefore be used as an internal control in a quantitative PCR. The same result was observed at the DNA level, when plasmid molecules had been used in titration experiments (data not shown). A correction factor of 1.15 (168/146) was applied to compensate for the binding of a lesser amount of ethidium bromide per molar equivalent of the shorter fragment (Piatak et al., 1993a). Heteroduplexes consisting of equal molar amounts of both PCR products were observed predominantly at the equivalence point where the probability of formation is highest (Fig. 2).

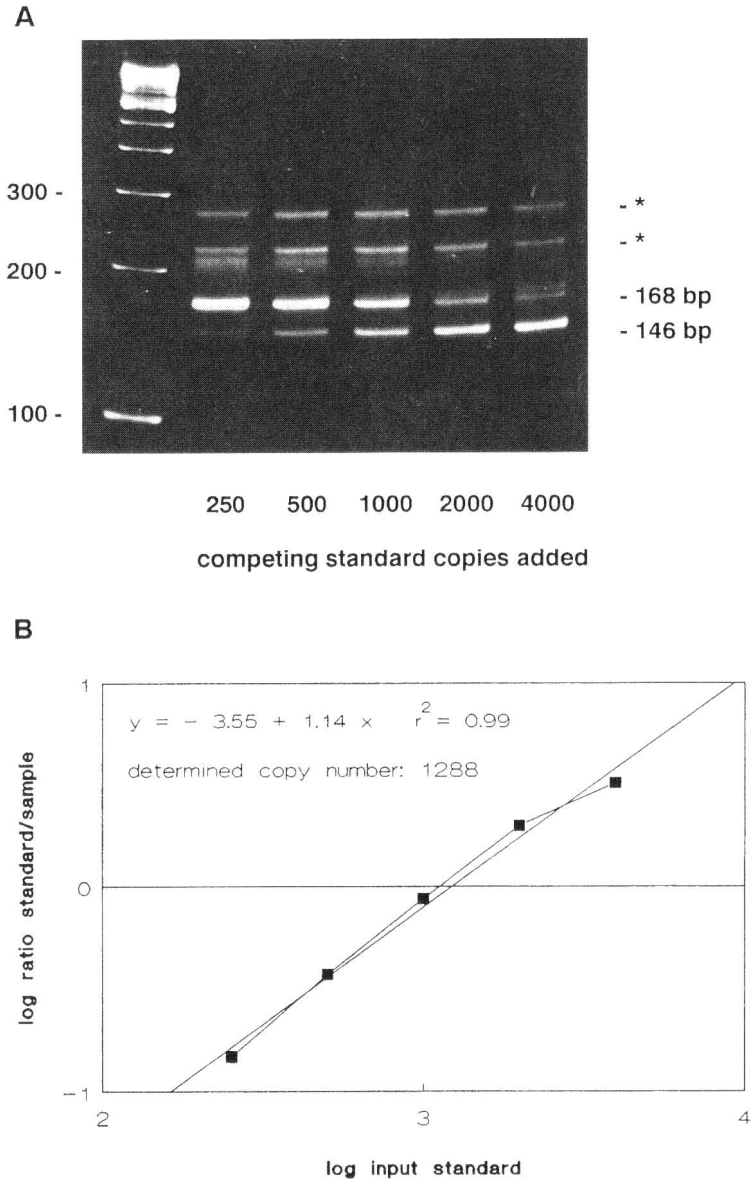


Fig. 2. Application of competitive RT-PCR to quantitate FIV target sequences. (A) Reconstruction experiment with in vitro transcripts from plasmid pSP73-*gag* containing full-length target sequence (168 bp product) and plasmid pSP73Δ-*gag* containing internally deleted competitive template (146 bp product). Electrophoretically resolved, ethidium bromide stained competitive PCR products of 1250 wild-type RNA templates (top) and increasing amounts of competitive templates (bottom). The first lane on the left consist of a 100 bp size standard (Gibco BRL, The Netherlands). * Heteroduplex formation was observed predominantly around the molar equivalence point of both templates. (B) Plot of quantitative fluorescence data (after correction for relative masses of product bands, 168/146) of the gel shown in (A) obtained by computer based video image analysis.

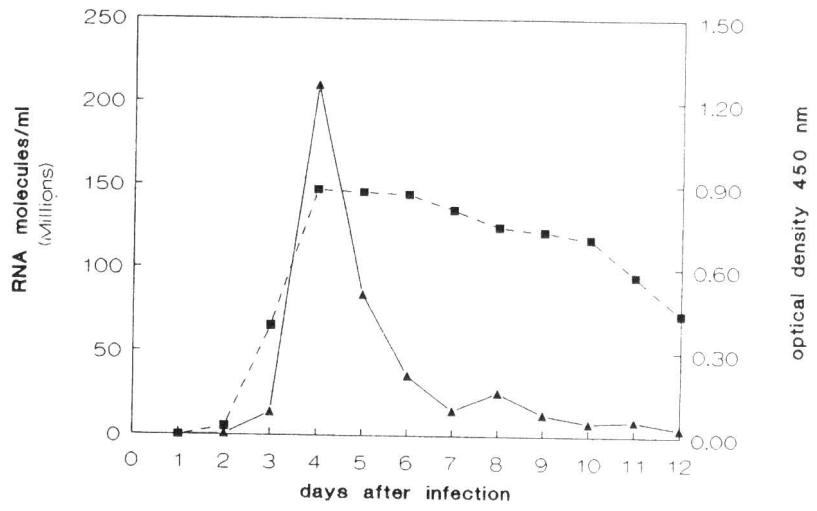


Fig. 3. Concentration of FIV RNA (▲) and FIV p24 (■) production after in vitro infection of $2 \times 10^6$ feline thymocytes measured by RT-PCR and ELISA, respectively.

### 3.2. Infection of cells in culture

The results of FIV RNA and P24 quantitation after infection of cultured feline thymocytes are illustrated in Fig. 3. Maximum yields of RNA released into the supernatant were measured four days after infection ($2.1 \times 10^8$ molecules/ml) which coincided with the peak of p24 production. One day later the RNA load had declined by about 50%, and to levels of $4.5 \times 10^6$ molecules/ml at day 12. Also p24 antigen production decreased, but more slowly than that of RNA.

### 3.3. Experimental FIV infection of cats

Upon experimental FIV infection virus was first isolated from PBMCs of cats #18 and #19 after three weeks. Plasma virus could be quantified after three and five weeks of infection, with peak RNA concentrations of $2.3 \times 10^4$ and $1.3 \times 10^4$ molecules/ml, respectively, at week 11 after infection. The amount of virus had decreased to $4.3 \times 10^3$ RNA molecules/ml and $4.7 \times 10^3$ RNA molecules/ml plasma, respectively, at 15 weeks after infection (Fig. 4). Antibodies against the *gag* and *env* gene products were detectable in cat #19 at seven weeks, in cat #18 at 11 weeks after infection. The antibody titers continued to rise while the viral RNA load in plasma had decreased. The peak concentration of FIV RNA of cat #18 exceeded that of #19, while it peak antibody titer was lower; cat #18 also had a slower immune response than #19.

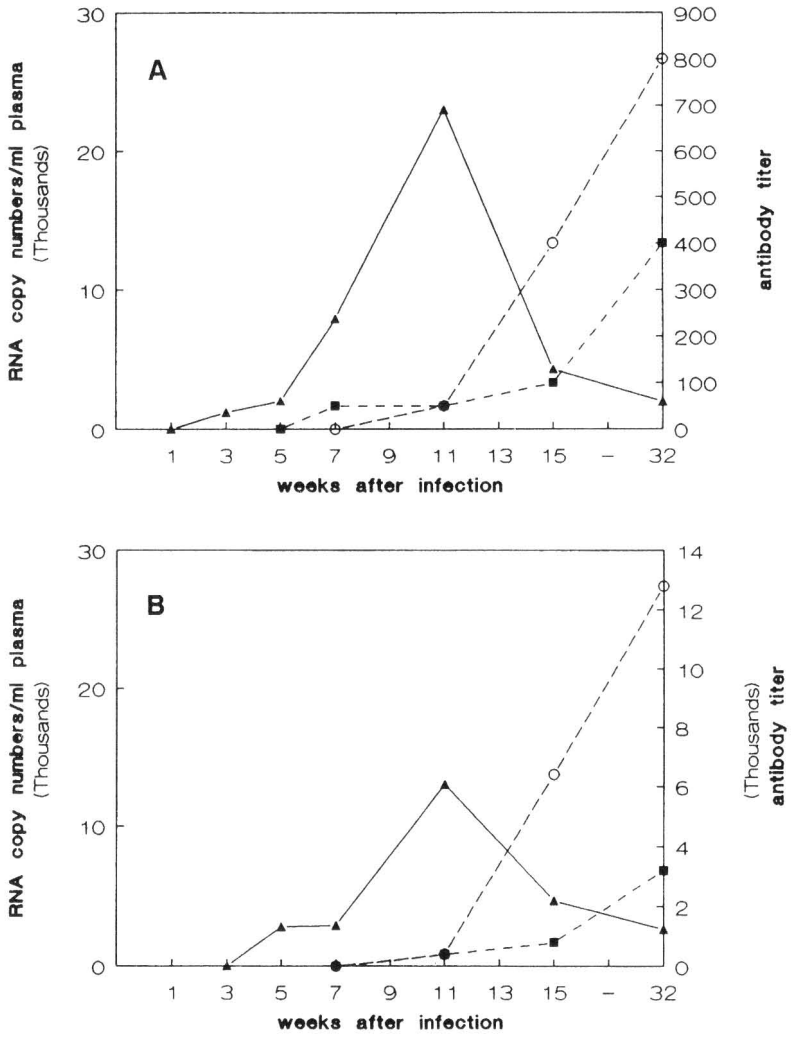


Fig. 4. Concentration of viral RNA in plasma and antibody titer after experimental FIV infection of cats #18 (A) and #19 (B). Plasma viraemia (▲) was determined by competitive RT-PCR; antibody titers against FIV-*gag* (■) and FIV-*env* (○) gene products were measured by end-point serum dilutions (Verschoor et al., 1993). Note the different antibody titer scale in (A) and (B).

### *3.4. RNA load in chronically FIV-infected asymptomatic cats*

To determine the plasma RNA concentration in chronic FIV infection, nine cats were studied that had been experimentally infected with another Dutch isolate (FIV-UT48) 3.5–4.5 years earlier. They were clinically healthy and had low–normal haemoglobin ($7.02 \pm 0.36$ mM) and packed cell volume ($0.35 \pm 0.02$ l/l) levels as well as low–normal lymphocyte counts ($2.03 \pm 0.22$ $10^9$/l). The $CD^4/CD^8$ T-cell ratios varied be-

Table 2
Viral RNA copy numbers per ml plasma and $CD_4/CD_8$ T-cell ratios of nine experimentally FIV-infected asymptomatic cats

| Cat | Viral RNA copy numbers | $CD_4/CD_8$ ratio |
|---|---|---|
| #1 | $8.5 \times 10^3$ | 1.13 |
| #2 | $1.0 \times 10^4$ | 1.02 |
| #3 | $1.4 \times 10^4$ | 0.50 |
| #4 | $5.6 \times 10^3$ | 0.90 |
| #5 | $4.3 \times 10^4$ | 0.85 |
| #6 | $9.2 \times 10^3$ | 1.60 |
| #7 | $7.4 \times 10^3$ | 0.85 |
| #8 | $1.5 \times 10^4$ | 1.20 |
| #9 | $1.0 \times 10^4$ | 0.90 |

tween 0.5 and 1.6. As summarized in Table 2, the concentration of viral RNA in plasma ranged between $5.6 \times 10^3$ molecules/ml and $4.3 \times 10^4$ molecules/ml. No correlation was found between the concentration of viral RNA in plasma and the $CD^4/CD^8$ T-cell ratio.

## 4. Discussion

A competitive RT-PCR was developed to quantify FIV-specific RNA that may prove useful for evaluating vaccine efficacy, antiviral therapy and in the study of FIV pathogenesis. This non-radioactive method uses an in vitro synthesized RNA template as an internal control. The template has been derived from a conserved region of the FIV genome and differs from that of the wild-type by an introduced deletion of 22 bases. Reconstruction experiments showed that the competitive template is amplified with the same efficiency as that of the wild-type, their amplification taking place in the same reaction mixture and competing for the same primers and other reagents. Factor affecting amplification will therefore equally influence both templates. The ratio between competitive and wild-type template can be used to calculate the absolute number of wild-type molecules if the amount of added competitive template is known. To increase the accuracy of the calculation, different copy numbers of the competitive template are added to aliquots of the sample to be quantified.

Heteroduplexes around the molar equivalence point do not significantly affect the relative abundance of wild-type and competitive PCR products; they do not influence the results if data points at both sides of the molar equivalence points are included in regression analysis. We therefore did not quantitate heteroduplexes, attributing equal amounts to wild-type and competitor molecules (Araki et al., 1993).

Quantitation was validated by assaying FIV production in infected feline thymocyte cultures. The concentration of viral RNA produced was compared to the production of the viral protein p24, both components of viral particles. The kinetics of RNA production correspond to those of p24 production measured in the culture supernatant.

The slower decrease of p24 is most likely due to the higher stability of the protein as compared to that of RNA.

The competitive RT-PCR was used to follow the FIV RNA concentrations in the plasma of experimentally infected cats for 32 weeks; these peaked 11 weeks after infection and decreased as antiviral antibodies appeared. A similar pattern has been observed in the primary phase of HIV-1 infection albeit that the peak of HIV-1 RNA load was more pronounced (Clark et al., 1991; Daar et al., 1991; Mulders et al., 1994). However, the actual RNA peak in the two cats studied may have occurred between two consecutive bleedings.

The competitive RT-PCR was also used to determine RNA concentrations in cats with asymptomatic chronic infections; these varied between about $10^3$ and $10^5$ molecules/ml of plasma. Copy numbers in plasma samples from HIV-1 infected patients determined after the acute viraemic phase generally ranged between $10^4$ and $10^6$ per ml (Piatak et al., 1993b, c). This amount of virus equivalents is indicative of active replication of both lentiviruses during the asymptomatic stage and illustrates the validity of the FIV/cat infection model for the study of AIDS.

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