Quantification of Feline Immunodeficiency Virus (FIV) Proviral DNA in Peripheral Blood Mononuclear Cells of Cats Infected with Japanese Strains of FIV

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ABSTRACT. The polymerase chain reaction (PCR) method was applied for measurement of the proviral DNA copy number of feline immunodeficiency virus (FIV) in peripheral blood mononuclear cells (PBMC) of cats experimentally and naturally infected with FIV. In experimentally infected cats except one cat infected with the Petaluma strain, FIV-specific DNAs were efficiently amplified with the PCR method under the conditions used in this study. In the naturally FIV-infected cats, the specific DNAs were also amplified. We established a quantitative method for measurement of proviral DNA copy number in PBMC from cats infected with TM2-type of FIV strains, and found that the number was variable among the six cats examined, ranging from $10^{3.0}$ to $10^{7.7}$ copies per $10^{5}$ PBMCs. This method can be applicable to cats naturally infected with FIV of TM2-type. Proviral DNA quantitation developed here could be useful as an additional parameter to evaluate the relationships among the proviral load, immune response and development of the clinical symptoms, and to monitor efficacy of antiviral therapy in vivo.—key words: FIV, PCR, proviral DNA, quantification.


Feline immunodeficiency virus (FIV) was first isolated in 1986 from cats exhibiting an immunodeficiency-like syndrome [26]. The virus belongs to the lentivirus genus of the retrovirus family, which includes human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), visna virus, caprine arthritis encephalitis virus, equine infectious anemia virus and bovine immunodeficiency-like virus [23]. FIV is related by morphological and biological characteristics and genome organization to the other members of lentivirus [20, 21, 23]. Like other lentiviruses, FIV establishes persistent infection in cats [23]. Cats infected with FIV remain asymptomatic for several years, and some of them subsequently show clinical signs including fever, weight loss, anemia, gingivitis, stomatitis, chronic upper respiratory infection and diarrhea under natural conditions [10, 12, 37], which are similar to those in HIV infections in humans.

FIV infection in cats is indicated from the presence of antibody against FIV detected by indirect fluorescence antibody technique (IFA) [11, 38] or enzyme-linked immunosorbent assay (ELISA) [1, 6]. However, at early stages of the virus infection, it is known that antibody is not yet produced. Further it is impossible to quantify the amount of virus by IFA or ELISA. Moreover, it has been reported that FIV could be isolated from seronegative cats [7, 10, 35], suggesting that FIV can exist in vivo in a latent state without expressing any viral mRNAs or proteins. Therefore direct detection of proviral DNA is required.

The polymerase chain reaction (PCR), which has been used to detect specific nucleic acid of HIV [15, 25] and SIV [8], is also useful for detection of FIV proviral DNA in the infected cells [16, 22, 27]. For understanding the pathogenesis of FIV infection in the host, it is important to define the relationships among the viral load, immune response and development of the clinical symptoms. A quantitative analysis of the amount of proviral DNA in infected cats is therefore needed to evaluate the relationships, and to monitor the effect of antiviral treatment. In the studies on HIV and SIV, quantitative PCR has been achieved by either comparing the PBMC-derived signal to signal of externally amplified DNA of a standard dilution [4, 24, 28, 30, 31] or a limiting dilution of PBMC [2, 32]. On the other hand, in FIV, Lawson et al. [16] reported the use of the PCR method for quantification of FIV provirus using a limiting dilution of PBMC. In this study, we established a quantitative PCR assay for measurement of FIV proviral DNA copy number in PBMCs of cats infected with FIV.

MATERIALS AND METHODS

Cats: A total of 10 cats were used for quantitative PCR analysis. Three specific pathogen free (SPF) cats (Cats 103, 104 and 105) which had been experimentally inoculated with TM1, TM2 and Petaluma strains of FIV 6 years ago at the age of around 5-month-old [18], respectively, were used. One SPF cat (Cat 102) was used as a negative control [18]. In addition, four naturally infected cats positive for FIV antibody (Cats To-1, Tei-1, Tei-2 and Tei-3) and two cats negative for the antibody (Cats Tei-4 and Tei-5) were used. A 9-year-old cat To-1 was kept as a pet in Osaka area and was referred to a veterinarian for diagnosis. Five cats Tei-1 to Tei-5, of unknown ages, were kept at the Teikyo University. All of the ten cats were negative for FeLV antigen in sera.

Detection of antibodies against FIV: Antibodies against
FIV were detected by IFA using MYA-1 cells [18] infected with FIV and by using a feline immunodeficiency virus antibody test kit (CITE Combo®, IDEXX, Maine, U.S.A.). ELISA titer was also determined by the kit.

Serum protein: Levels of γ-globulin and albumin/globulin (A/G) ratio in sera of individual cats were measured by electrophoresis using cellulose acetate membrane. These measurements were entrusted to Koto Biken Medical Laboratory (Tokyo, Japan).

Isolation of cells and DNA preparation: PBMCs were isolated from heparinized fresh blood by Ficoll-Paque (Pharmacia, Upsala, Sweden) gradient centrifugation and were washed with phosphate-buffered saline. DNAs were extracted from these PBMCs by QIAamp Blood Kit (QIAGEN, California, U.S.A.) according to the instructions of the manufacturer.

CD4/CD8 ratio: The CD4 and CD8 subsets of T lymphocytes and their relative frequencies were examined by a flow cytometry analytical system (CytoACE, Japan Spectroscopic Co., Tokyo, Japan) as described elsewhere [19].

Primers for PCR: The primers were constructed on the basis of the nucleotide sequence of the TM2 strain [17]. Most of the sequences of the primers were conserved between the TM2 and Petaluma strains [34]. The nucleotide sequences of the primers and their locations in the TM2 strain genome are shown in Table 1.

PCR: PCR was carried out by the method of Saiki et al. [29] in 0.1 ml microfuge tubes in a total volume of 30 μl. One μg of PBMC DNA was added to the PCR reaction mixture containing 0.1 μM each primer, 200 μM each of dATP, dCTP, dGTP and dTTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin, and 1.5 U of AmpliTaq® DNA polymerase (Perkin Elmer, Connecticut, U.S.A.). All reaction samples were overlaid with mineral oil (Sigma Chemical, Missouri, U.S.A.). DNA was amplified with a DNA Thermal Cycler (Perkin Elmer, Connecticut, U.S.A.) by 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 2 min), and extension (72°C, 2 min).

Hybridization and construction of standard curves: After amplification, 5 μl of the PCR products were electrophoresed on a 2% agarose gel in TBE buffer (0.09 M Tris-borate plus 2 mM EDTA). Then, the gels were soaked in HCl for 5 min, washed twice with H2O, denatured in 0.5 M NaOH plus 0.6 M NaCl for 30 min, washed again twice with H2O, neutralized in 1 M Tris-HCl (pH 7.0) plus 0.6 M NaCl for 30 min, followed by the transfer to a nylon membrane (Biodyne®, Pall BioSupport, New York, U.S.A.). The DNA was immobilized on the membrane by baking at 80°C for 2 hrs. The membrane was briefly rinsed with 2×SSC (1×SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Prehybridization was carried out in hybridization solution containing 50% formamide, 0.6 M NaCl, 0.2 M Tris-HCl (pH 8.0), 0.02 M EDTA and 0.5% sodium dodecyl sulfate (SDS) at 37°C for 1 hr. For detection of PCR products, a 153-base pair (bp) Sac I-Bal I fragment of TM2 strain (nucleotides 515–667) was used as a probe. The probe was labeled with [32P]dCTP using a Nick Translation Kit (Boehringer Mannheim, Mannheim, Germany). Hybridization was carried out at 42°C overnight in the same hybridization solution with [32P]dCTP-labeled probe and 100 μg/ml of denatured salmon sperm DNA. After hybridization, the membrane was washed in washing buffer containing 0.2×SSC, 0.1% SDS and 1 mM EDTA three times for 30 min at 42°C. The radiolabeled signal was analyzed using a Bio Imaging Analyzer, BAS 2000 (Fuji Photo Film, Tokyo, Japan), and then exposed to X-ray film (Eastman Kodak, New York, U.S.A.) between two intensifying screens, Lightning Plus (Du Pont, Massachusetts, U.S.A.). For the standard curve, serial ten-fold dilutions ranging from 10^6 to 10^5 copies of an infectious molecular clone of TM2 strain, pTM219 [21], were mixed with 1 μg of carrier DNA from 1×10^6 uninfected MYA-1 cells, and subjected to the PCR and hybridization analysis as described above.

RESULTS

ELISA titer, serum protein and CD4/CD8 ratio: The ELISA titer of FIV antibody, concentration of γ-globulin and albumin/globulin (A/G) ratio in sera from cats used in

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence(5'-3')</th>
<th>Region</th>
<th>Location(1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pr-1</td>
<td>GAACTTCTGCCAGACCTGTTG(2)</td>
<td>downstream of LTR</td>
<td>436–455</td>
</tr>
<tr>
<td>KA-14</td>
<td>GTCTCTGTATATACACCTG</td>
<td>gag</td>
<td>790–780</td>
</tr>
<tr>
<td>KA-18</td>
<td>GGATCAAGGOGCTACATACG</td>
<td>gag</td>
<td>1085–1066</td>
</tr>
<tr>
<td>unigA</td>
<td>GGTGATACGAGACACCTTACG</td>
<td>gag</td>
<td>784–805</td>
</tr>
<tr>
<td>unigR</td>
<td>GGTGACGCTGCTCACAGCTG</td>
<td>gag</td>
<td>1415–1395</td>
</tr>
<tr>
<td>unipolF</td>
<td>TGGAGGATGCTCATAGATTAG</td>
<td>pol</td>
<td>2547–2569</td>
</tr>
<tr>
<td>unipolR</td>
<td>CCTGCTATTCTTTGCTAGTTATT</td>
<td>pol</td>
<td>3121–3099</td>
</tr>
<tr>
<td>HV3F</td>
<td>ATACCCAAAATGCTATGCTGAGGA</td>
<td>V3 in env</td>
<td>7322–7344</td>
</tr>
<tr>
<td>HV3R</td>
<td>TGAACCTCTCATGTCAGCAGA</td>
<td>V3 in env</td>
<td>7561–7544</td>
</tr>
<tr>
<td>HV3R</td>
<td>TCTGTTTCTTTTACACTTGTG(3)</td>
<td>V5 in env</td>
<td>8049–8027</td>
</tr>
</tbody>
</table>

a) Based on the TM2 strain [17].

b) Nucleotides underlined are different from those of the Petaluma strain.
c) Nucleotides written in small letters are different from those of the TM2 strain.
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this study are shown in Table 2. The ELISA titer of Cat 104 was the highest of the FIV-infected cats. The levels of γ-globulin in sera of all the seven FIV-infected cats were higher than those of control cats. Especially, the level of Cat To-1 was two-fold higher than those of control cats. A/G ratios of the infected cats were generally lower than those of control cats. CD4/CD8 ratios in three experimentally infected cats (Cats 103 to 105) were lower than that of the control cat (Cat 102) (Table 2).

Comparison of sensitivity with primer pairs: The sensitivities for amplifying specific DNA with each primer pair were compared under the conditions of PCR described in Materials and Methods (Fig. 1). The sequence and location of the primers used are listed in Table 1. The sensitivity was tested by using serial dilutions of a FIV molecular clone, pTM219, in a background of 1 μg (1×10⁶ cells) DNA from MYA-1 cells. With primer pair Pr1/KA-18, the limit of visible detectability by ethidium bromide staining was 10⁶ copies of pTM219 DNA in 1×10⁶ cells, whereas the detectabilities with primer pairs unigagF/unigagR, unipolF/unipolR, HV3F/HV3R and HV3F/HV5R were 10⁵, 10⁶, 10⁵ and 10⁷ copies of pTM219, respectively (Fig. 1). From these experiments, it was demonstrated that the primer pair Pr1/KA-18 was the most efficient for detection of FIV-specific DNA of the primer pairs tested. Thus, we used this pair for the first PCR throughout further experiments.

Comparison of amplification efficiencies using one and two rounds of PCR: After the first PCR, using primer pair Pr1/KA-18, the amplification products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. For the second PCR, 3 μl of the each product from the first PCR were added to the new reaction solution containing the primer pair Pr1/KA-14 and subjected to PCR amplification. In the second PCR, the limit of detectability increased from 10⁵ to 10⁶ copies. With Southern blot analysis using first PCR products, the quantitative application of the PCR method was found to be reliable and reproducible (Fig. 2A). Furthermore, as few as 1 copy per 1×10⁵ cells was detected in Southern blot analysis using second PCR products (data not shown).

Detection of FIV proviral DNA in PBMC: To detect FIV-specific proviral DNAs in PBMCs from FIV-infected cats, we performed PCR analysis using primer pair Pr1/KA-18. In the first PCR, specific DNAs were amplified in PBMCs from cats experimentally infected with the TM1 and TM2 strains. In a cat infected with the Petaluma strain, however, specific DNA was not amplified (Fig. 2B). The same result was obtained using a second PCR employing Pr1/KA-14 followed by Southern blot analysis (Fig. 2B). These results indicate that the primer pair Pr1/KA-18 can be used to detect cats experimentally infected with the TM1 and TM2 strains of FIV but not the Petaluma strain. Further, a specific product was also amplified with primer pair Pr1/KA-18 in PBMCs from all naturally infected cats used. None of the three seronegative cats was PCR positive in these analysis (Fig. 2B).

Determination of FIV proviral DNA copy number in

![Fig. 1. Comparison of sensitivity with primer pairs. The numbers indicate copy number of pTM219 in 1×10⁶ MYA-1 cells. P, DNA of MYA-1 infected with pTM219 as a positive control. N, MYA-1 DNA as a negative control. Primer pair of Pr1/KA-18 was most sensitive for amplifying as few as 10⁵ copies of pTM219 per 1×10⁶ cells. With primer pairs of Pr1/KA-18 and unigagF/unigagR, amplifications of FIV non-specific DNA were observed. The arrows indicate FIV specific band.](image)

![Fig. 2. Second PCR and Southern blot analysis of first PCR products and detection of FIV proviral DNA in PBMC from cats. (A) The numbers indicate copy number of pTM219 in 1×10⁵ MYA-1 cells. (B) Cat samples amplified by PCR. Pos., DNA of MYA-1 infected with pTM219 as a positive control. 103, cat infected with the TM1 strain of FIV. 104, cat infected with the TM2. 105, cat infected with the Petaluma. 102, control cat. To-1, Tei-1, Tei-2 and Tei-3; cats infected naturally with wild type. Tei-4 and Tei-5; control cats. The arrows in (A) and (B) indicate FIV specific band.](image)
PBMC: To quantitate the number of FIV proviral DNA copies in the PBMCs from FIV-infected cats, pTM219 DNAs were serially diluted. Then, the signal intensities from the first-PCR-Southern blot analysis were analyzed by using a Bio Imaging Analyzer and used as a standard curve. As shown in Fig. 3, there was a linear relationship between the signal intensity and the amount of proviral DNA copy number in the range from $10^5$ to $10^6$ copies. Therefore, in that range, this standard curve of signal intensities could be employed to quantify the copy number of FIV proviral DNA in $1 \times 10^5$ PBMCs for unknown positive samples. By extrapolation of the standard curve, the copy numbers of FIV proviral DNAs in $1 \times 10^5$ PBMCs were $10^{4.0}$ and $10^{5.7}$ in experimentally infected cats and ranged from $10^{4.3}$ to $10^{5.2}$ in naturally infected cats (Table 2).

**DISCUSSION**

The purpose of the present study was to establish a method for the evaluation of proviral DNA copy number of FIV in vivo. First, we constructed several primers based on the sequence of the TM2 strain. Most of the sequences of the primers were conserved between the TM2 and Petaluma strains. Mismatch sequences of both strains were shown in Table 1. The sensitivities of PCR employing each primer pair were compared by using serial dilutions of a molecular clone of the TM2 strain, pTM219. As shown in Fig. 1, primer pair Pr-1/KA-18 was most sensitive and could detect as few as $1 \times 10^4$ copies of pTM219 per $1 \times 10^5$ cells by ethidium bromide staining. The apparent difference in proviral copy number between cats infected with different FIV strains might be caused by differences in nucleotide sequence between the TM2 and Petaluma strains, leading to differences in efficiency of amplification. A similar observation of amplification efficiency was reported by Momoi et al. [22].

Primer pair Pr-1/KA-18 was most sensitive for amplification of pTM219 DNA, but it could not detect FIV specific DNA from PBMC of a cat infected with the Petaluma strain (Fig. 2B). The presence of genomic diversity among FIV strains has been noted [21]. The amino acid homologies between the TM2 and Petaluma strains in gag, pol and env regions are 90, 87 and 81%, respectively [17]. Sodora et al. [33] reported that FIV can be separated into three subtypes by env sequence, and the TM2 and Petaluma strains belong to different subtypes. Recently, we found the presence of four or more subtypes among FIV isolates by LTR and env sequences [36, Pecoraro, M. R. et al., unpublished data]. Further, Johnson et al. [13] reported that the Shizuoka strain isolated in Japan belongs to a different subtype from the TM2. Although Pr-1/KA-18 could not detect the specific DNA in MYA-1 cells infected with the Shizuoka strain (data not shown) and there are some different subtypes of FIV in Japan [Kakinuma, S. et al. and Nishimura, Y. et al., personal communications], the majority of Japanese isolates conceivably belong to the subtype similar to the TM2 strain [36]. It is further supported by the fact that Pr-1/KA-18 was able to detect FIV specific DNA from all naturally infected cats tested in this study. For accurate estimation of proviral DNA copy number in PBMCs from

![](image)

Fig. 3. Signal intensities of serial dilutions of pTM219. In the range from $10^5$ to $10^6$ copies of pTM219 per $1 \times 10^5$ PBMCs, a linear correlation between signal intensity and copy number was observed.

<table>
<thead>
<tr>
<th>Cat no.</th>
<th>Copy no.</th>
<th>Virus strain</th>
<th>FIA antibody titer</th>
<th>ELISA antibody titer</th>
<th>CD4/CD8 ratio</th>
<th>γ-Globulin (mg/ml)</th>
<th>A/G ratio</th>
<th>Clinical status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental infection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>103</td>
<td>$10^{4.0}$</td>
<td>TM1</td>
<td>+</td>
<td>5120</td>
<td>0.49</td>
<td>19.3</td>
<td>0.90</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>104</td>
<td>$10^{5.7}$</td>
<td>TM2</td>
<td>+</td>
<td>10240</td>
<td>1.02</td>
<td>23.4</td>
<td>0.81</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>105</td>
<td>–</td>
<td>Petaluma</td>
<td>+</td>
<td>1280</td>
<td>0.42</td>
<td>21.4</td>
<td>0.80</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>102</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>&lt;10</td>
<td>1.45</td>
<td>15.8</td>
<td>1.03</td>
<td>Control</td>
</tr>
<tr>
<td><strong>Natural infection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>To-1</td>
<td>$10^{5.2}$</td>
<td>wild</td>
<td>+</td>
<td>1280</td>
<td>ND*</td>
<td>42.0</td>
<td>0.40</td>
<td>Stomatitis, Rhinitis, Anemia, Lymphadenopathy</td>
</tr>
<tr>
<td>Tei-1</td>
<td>$10^{4.5}$</td>
<td>wild</td>
<td>+</td>
<td>1280</td>
<td>ND</td>
<td>26.5</td>
<td>0.71</td>
<td>Gingivitis</td>
</tr>
<tr>
<td>Tei-2</td>
<td>$10^{4.5}$</td>
<td>wild</td>
<td>+</td>
<td>640</td>
<td>ND</td>
<td>22.0</td>
<td>0.87</td>
<td>Stomatitis</td>
</tr>
<tr>
<td>Tei-3</td>
<td>$10^{4.6}$</td>
<td>wild</td>
<td>+</td>
<td>640</td>
<td>ND</td>
<td>27.1</td>
<td>0.72</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>Tei-4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>&lt;10</td>
<td>ND</td>
<td>15.4</td>
<td>1.00</td>
<td>Control</td>
</tr>
<tr>
<td>Tei-5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>&lt;10</td>
<td>ND</td>
<td>17.9</td>
<td>0.90</td>
<td>Control</td>
</tr>
</tbody>
</table>

*ND: Not done.
FIV-infected cats, we developed a quantitative PCR method using the strain specific primers. This method will provide a useful tool for understanding the pathogenesis of FIV infection in the host, and could be used to improve the evaluation of the relationships among the proviral load, immune response and development of the clinical symptoms, and to monitor efficacy of antiviral therapy in vivo, especially in asymptomatic carriers in which no other reliable marker of viral burden is available.

Finally, this study indicated no correlation between the FIV proviral DNA copy number in PBMCs and the stage of FIV infection. In HIV, many studies have exhibited a positive correlation between the amount of provirus-carrying cells and progression of the disease [3, 5, 9, 31, 32]. Therefore, in the studies on FIV, further attempt will be required to clarify the relationship between proviral DNA copy number and disease development using a large number of cats infected with FIV under natural conditions.

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REFERENCES


