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Detection of feline immunodeficiency proviral DNA in peripheral blood lymphocytes by the polymerase chain reaction

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ABSTRACT

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Feline immunodeficiency virus (FIV) proviral DNA was detected by the polymerase chain reaction method (PCR). PCR products were detected by gel electrophoresis and ethidium bromide staining. The P-10, P-15 and P-24 regions of the *gag* gene of FIV were chosen as the target sequences for amplification, and three primer pairs were prepared. The PCR products subjected to amplification with each primer pair were found to possess sites of digestion by a restriction enzyme, as hypothesized. They did not react with feline leukemia virus (FeLV)-infected or feline syncytium-forming virus (FeSFV)-infected cell-derived DNA, and specifically amplified FIV-infected cell-derived DNA. FIV proviral DNA was detected by the PCR method with either primer pair (one-step amplification: single PCR) in DNA derived from peripheral blood lymphocytes (PBL) from 7 of 12 FIV antibody-positive cats. When PCR products in each of the 12 cats were subjected to a second amplification using the same primer pair (two-step amplification: double PCR), FIV proviral DNA was detected in all of the cats. When PBL samples collected from three cats that were negative and three that were positive in the single PCR were cultured for a few weeks in the presence of interleukin 2, FIV proviral DNA was detected in all six cats by the single PCR method.

The results suggest that either the use of cultured PBL as the sample or the performance of the double PCR method enables simple and specific detection of FIV proviral DNA in PBL.

INTRODUCTION

Feline immunodeficiency virus (FIV) was isolated in 1986 from a feline leukemia virus (FeLV)-negative cat with chronic opportunistic infectons (Pedersen et al., 1987). FIV is a typical lentivirus morphologically resembling human and simian immunodeficiency viruses and showing similar pro-

tein structure, and the presence of reverse transcriptase. The nucleotide sequence of FIV has been determined, and some reports have shown a relationship between the genomic organization and that of other lentiviruses (Olmsted et al., 1989a,b; Talbott et al., 1989). When host cells are infected with FIV, viral RNA undergoes reverse transcription to DNA, which is mediated by reverse transcriptase, and is integrated into host cell DNA as a provirus, as with other lentiviruses. In this pattern of infection, FIV persists in the host cell DNA as a provirus.

FIV infections are diagnosed by demonstrating anti-FIV antibodies by the indirect fluorescent antibody technique (IFA) or enzyme-linked immunosorbent assay (ELISA) (Yamamoto et al., 1989; Grindem et al., 1989; Bennett et al., 1989; Belford et al., 1989; Ishida et al., 1989). In the case of kittens born of antibody-positive cats, however, diagnosis by serological techniques is difficult, and for diagnosis it is necessary to demonstrate the FIV antigen. A person with human immunodeficiency virus (HIV) infection is positive for virus antigen, but some studies have shown the absence of conversion of the antibody in some cases (Imagawa et al., 1989; Pezzella et al., 1989). In the case of FIV infection as well, the virus is isolated from antibody-negative cats (Hopper et al., 1988). The FIV antigen has been demonstrated by isolation of viruses from peripheral blood lymphocytes (PBL) (Yamamoto et al., 1988), but it will take a long time for this procedure to be evaluated. A simpler method must be developed.

In recent years the polymerase chain reaction (PCR) has been used to detect a trace of DNA, and it is increasingly used in the wide field of virology including HIV (Ou et al., 1988; Bel'ak et al., 1989; Albert and Fenyö, 1990; Garson et al., 1990; Verbeek and Tijssen, 1990; Ballagi-Pord'any et al., 1990). An attempt to establish a system for detection of FIV proviral DNA by PCR is described in this report.

MATERIALS AND METHODS

Viruses and cell cultures

The specificity of PCR was tested by using FIV, FeLV, and feline syncytium-forming virus (FeSFV). The FIV used was the Petaluma strain (Pedersen et al., 1987) which persistently infects Crandell feline kidney (CrFK) cells. FeSFV was inoculated onto CrFK cells for propagation. The FIV and FeSFV were received from Dr. J.K. Yamamoto of the University of California, Davis. The FeLV used was in the from of FL-74 cells persistently infected with FeLV (Theilen et al., 1969).

The CrFK and FL-74 cells were incubated in Eagle's minimum essential medium and RPMI-1640 medium, respectively, to each of which 10% FCS, 100 U penicillin per ml and 100 μg of streptomycin per ml were added.

Preparation of specimens for PCR

The cultured cells infected with FIV, FeSFV or FeLV and the feline PBL isolated from heparinized blood by the Ficoll-paque method were washed with PBS three times. The cell pellet (1×10^5) was suspended in 200 μl of PBS, mixed with 200 μl of lysis buffer [20 mM Tris–HCL (pH 7.4), 2 mM EDTA, 0.1% SDS, and 40 μg of proteinase K per ml], and incubated at 37°C for 90 min. The specimen was extracted twice with phenol–chloroform and once with chloroform to remove proteins from the lysed cells. After 3 M of sodium acetate and 99% ice-gold ethanol were added to the resultant, the mixture was held at −80°C for 10 min to induce DNA precipitation. DNA was pelleted in an Eppendorf centrifuge (15 000 rpm, 5 min), washed once in 70% ethanol, vacuum dried, and dissolved in 100 μl of TE buffer (10mM Tris–HCL, 1 mM EDTA, pH 8.0). The DNA level was determined at an OD of 260 nm, and 1 μg was used for PCR.

Primers for PCR

Three pairs of primer sets located at the P-10, P-15 and P-24 regions of the *gag* gene were synthesized on the basis of the nucleotide sequence of the Petaluma strain of FIV (Talbott et al., 1989). DNA oligonucleotide primers were synthesized in a Biosearch cyclone DNA synthesizer and purified by Sephadex G-25 (Pharmacia, Sweden) column chromatography. The nucleotide sequences of the primers and their locations in the FIV (Petaluma strain) genome are shown in Table 1.

DNA amplification

PCR was performed in 0.5 ml microfuge tubes in a total volume of 50 μl. A DNA sample (1 μg in 30 μl) was added to 5 μl of reaction mixture (100 mM Tris–hydrochloride (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin and

TABLE 1

Primers for the amplification of FIV genome

Primer designation	Primer sequence ('–3')	FIV Petalum strain		Target sequence length
		Region	Position	
P-10-1	GACAGACAATGTAGAGAAGT	*gag* p-10	1785-1804	
P-10-2	TTTCTCCTCCATTGGAGGTG	*gag* p-10	1945-1964	180 bp.
P-15-1	GTGATATACCAGAGACTTTA	*gag* p-15	778-797	
P-15-2	TTTACTGTTTGAATAGGATA	*gag* p-15	1029-1048	271 bp.
P-24-1	CTAGGAGGTGAGGAAGTTCA	*gag* p-24	1119-1138	
P-24-2	CTGCTTGTTGTTCTTGAGTT	*gag* p-24	1343-1362	244 bp.

10 μg of BSA), 5 μl of 10 mM deoxynucleotide mix (ATP, CTP, GTP and TTP), 5 μl of 1 μM primer mix, 1 μl of Taq polymerase (2 units/μl; New England Biolaboratories, USA) and 4 μl of distilled water (DH_2O), and mixed. DNA was amplified with a DNA Thermal Cycler (Atto Corp., Japan) after one drop of mineral oil was added to the mixture. The amplification involved incubation for 5 min at 94°C as the first denaturation, subsequent cycling which consisted of denaturation for 1 min at 94°C, primer annealing for 2 min at 55°C and synthesis for 2 min at 72°C, repeated 35 times, and a last incubation for 5 min at 72°C (one-step amplification: single PCR). Samples (10 μl) of the PCR products were analyzed by electrophoresis on an 8% polyacrylamide gel. Bands were visualized by ethidium bromide staining and photographed under a UV transilluminator at 312 nm. The double PCR (2-step amplification) involved a second amplification by the above-described methods with the same primers in 5 μl of PCR products.

Identification of the PCR products

Specificity of the PCR products was confirmed by digestion of the products with restriction endonucleases. Five microliters of the PCR products, 2 μl of tenfold concentrated enzyme-specific buffer, 1 μl of restriction endonuclease (4 U/μl) and 12 μl of DH_2O were mixed and incubated at 37°C for 2 h. Restriction endonuclease digests were analyzed by gel electrophoresis as described above.

Detection of antibodies against FIV

Antibodies against FIV were detected by indirect fluorescent antibody assay (IFA) using CrFK cells persistently infected with FIV as the antigen.

Culture of PBL

Feline PBL (1×10^6/ml) isolated by the Ficoll-paque method were incubated at 37°C for 3 d in RPMI 1640 medium to which 5 μg of concanavalin A per ml and 10% FCS were added. Later the medium was changed to RPMI 1640 medium to which 100 units of human recombinant interleukin 2 (IL-2) per ml (Boehringer Mannheim, Germany) and 10% FCS were added. The cultured cells were diluted 3- to 4-fold every 3 or 4 d for passage. DNA was extracted by the above methods from the cultured cells after 2–3 weeks of culturing and used for PCR.

Cats

Twelve adult cats positive for FIV antibody and two adult cats negative for the antibody were used. Three of the FIV antibody-positive cats, KF 120, 177 and 281, had been infected spontaneously. Eight cats, KF 118, 140, 141, 159, 276, 289, 300 and 304, had received intravenous or subcutaneous inoculation of 2 ml of blood from FIV antibody positive cats. Cat KF 165 was experimen-

tally infected with the Petaluma strain. These cats had been inoculated with FIV 4 to 16 months previously. They were positive for FIV antibody, but clinically healthy.

RESULTS

Detection of FIV proviral DNA from FIV-, FeSFV- or FeLV-infected cell cultures by PCR

In the PCR method DNA from FIV-, FeSFV- or FeLV-infected cell cultures was combined with each primer pair. As shown in Fig. 1, bands of amplified DNA were detected at positions (primer P-15, 271 bp; primer P-24, 244 bp; primer P-10, 180 bp) which were consistent with those for theoretical values for DNA from only the FIV-infected cell cultures.

Restriction endonuclease digestion of PCR products

We determined, by restriction endonuclease digestion of the PCR products, whether the bands of DNA detected with each primer would amplify the target sequences as they did theoretically. The products detected with primer P-10 had the sequences cleaved into two fragments, 120 bp, and 60 bp, by Pst I (Fig. 2a). Similarly, the products detected with P-15 had the sequences cleaved into two fragments, 164 bp and 107 bp, by Pst I, and those detected with P-24 had the sequences cleaved similarly into two fragments, 150 bp and 94 bp, by Mbo I (Fig. 2b, 2c). In practice, the products of each primer were mixed with each restriction enzyme and incubated at 37°C for 2 h. As was hypothesized, they were cleaved at one site (Fig. 2a, 2b, 2c).

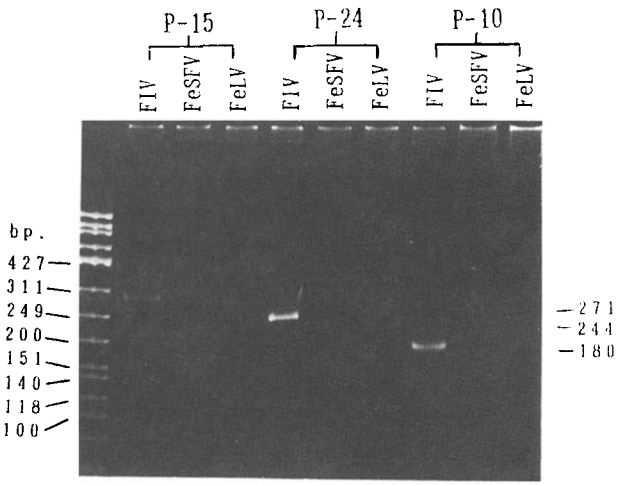

Fig. 1. Detection of FIV proviral DNA from FIV-, FeSFV- or FeLV-infected cell cultures by PCR.

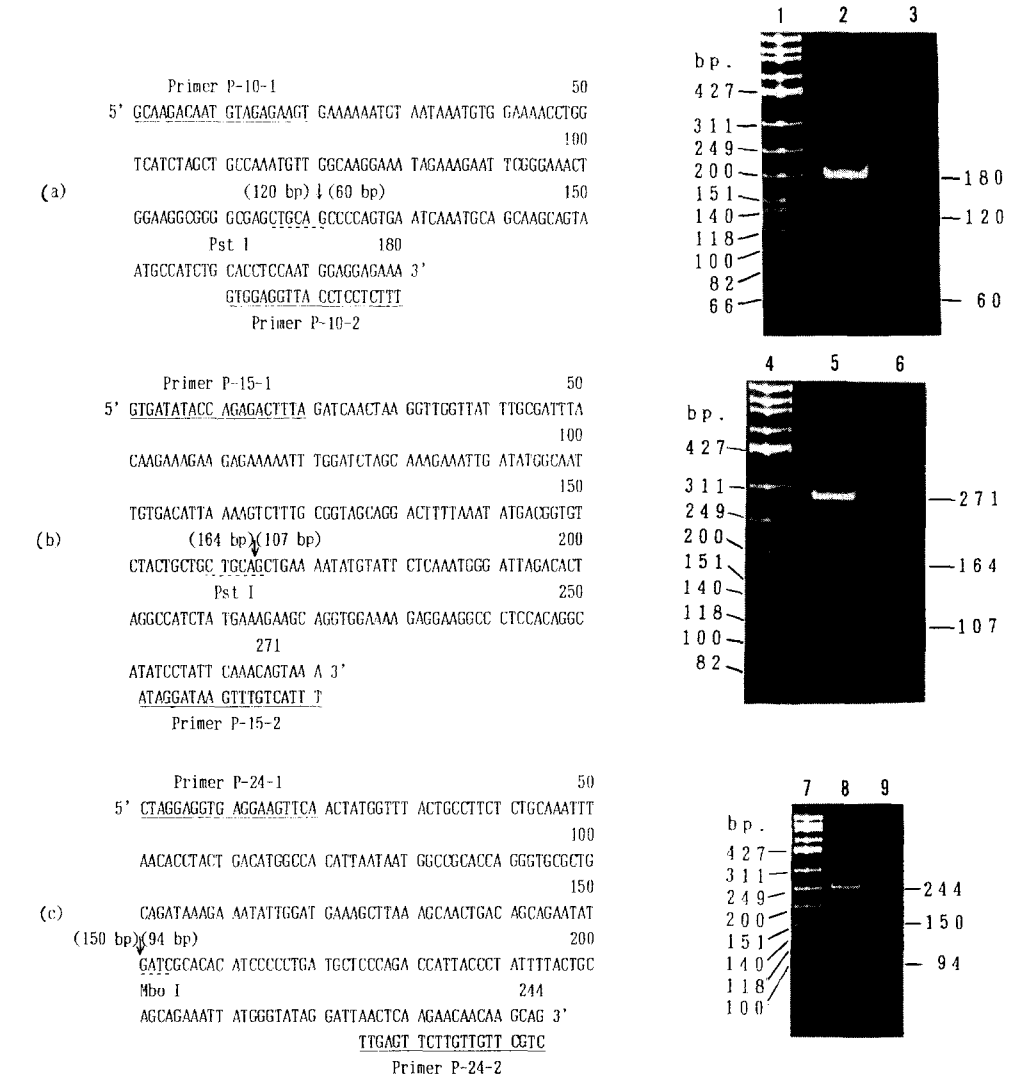

Fig. 2. Restriction endonuclease digestion of PCR products. (a) 1, Marker (Hinf I digest of $\phi\times174$) 2, P-10 product; 3, Pst I digest of P-10 product. (b) 4, Marker (Hinf I digest of $\phi\times174$) 5, P-15 product; 6, Pst I digest of P-15 product. (c) 7, Marker (Hinf I digest of $\phi\times174$) 8, P-24 product; 9, Mbo I digest of P-24 product.

Detection of FIV proviral DNA in feline PBL by PCR

Detection of FIV proviral DNA was performed in PBL from 12 FIV antibody-positive cats and two FIV antibody-negative cats. It was detected with a primer pair by the one-step PCR method (single PCR) in 7 of the 12 antibody-positive cats (Table 2). In contrast, no FIV proviral DNA was detected with any primer pair in five cats (Kf 120, 140, 289, 300 and 304). Subse-

TABLE 2

Detection of FIV proviral DNA in feline PBL by PCR

Cat No.	Single PCR			Double PCR			FIV antibody
	Primer			Primer			
	P-10	P-15	P-24	P-10	P-15	P-24	
KF 118	–	+	+	+	+	+	+
KF 141	+	+	+	+	+	+	+
KF 159	+	+	+	+	+	+	+
KF 165	+	+	+	+	+	+	+
KF 177	–	–	+	+	–	+	+
KF 276	–	+	–	+	+	+	+
KF 281	+	+	+	+	+	+	+
KF 120	–	–	–	+	–	–	+
KF 140	–	–	–	+	+	–	+
KF 289	–	–	–	–	+	–	+
KF 300	–	–	–	+	+	–	+
KF 304	–	–	–	+	+	+	+
KF 613	–	–	–	–	–	–	–
KF 614	–	–	–	–	–	–	–

TABLE 3

Detection of FIV proviral DNA in cultured feline PBL by PCR

Cat No.	Single PCR			Double PCR		
	Primer			Primer		
	P-10	P-15	P-24	P-10	P-15	P-24
KF 118	–	+	+	–	+	+
KF 159	ND	+	ND	ND	+	ND
KF 177	–	+	+	+	+	+
KF 289	–	+	+	+	+	+
KF 300	–	+	+	+	+	+
KF 304	–	+	+	+	+	+

quently, 5 μl of the products from the single PCR was subjected to another PCR by using the same primer pair (double PCR). FIV proviral DNA was then detected in the PBL even from the cats in which it was not detected by the single PCR (Table 2). No FIV proviral DNA was detected in the PBL from FIV antibody-negative cats.

Detection of FIV proviral DNA in cultured feline PBL by PCR

PBL from three cats (KF 118, 159, 177) that were positive and from three cats (Kf 289, 300, 304) that were negative in the single PCR in the previous experiment were cultured in the presence of IL-2, and the cells were subjected to PCR after 2–3 weeks of culture. PBL from the three cats (KF 289, 300, 304) as well as the cats that were positive in the single PCR became positive in the single PCR (Table 3). When these products were subjected to the double PCR, all of them were positive for FIV proviral DNA except for the PCR at primer P-10 in cat KF 118 (Table 3).

DISCUSSION

FIV was isolated by in-vitro culture of PBL with susceptible cells, but it has taken a long time for evaluation of the procedure. Detection of antigen by ELISA using monoclonal antibody is a simpler method. When the blood levels of FIV antigen are considered, however, problems are predicted to arise regarding the sensitivity and specificity of detection. There are some reports of detection of provirus DNA by the PCR method in HIV infection, and its usefulness (Ou et al., 1988; Albert and Fenyö, 1990). Pedersen et al. (1990) reported cases in which PCR was used for FIV infection. They used two primer pairs in the *gag* region of FIV. Some strains of FIV, including the Petaluma strain, have been isolated (Pedersen et al., 1987; harbour et al., 1988; Miyazawa et al., 1989). In the present study, the P-10, P-15 and P-24 regions of the *gag* gene, which are believed to be conserved relatively well among FIV strains, were amplified by the PCR method, and proviral DNA was detected. The PCR products amplified with each primer pair were found to possess the sites of cleavage by a restriction enzyme at the same position as those of the target sequences, showing that the target sequence for the FIV gene was precisely amplified (Figs. 2a, 2b and 2c). It was further indicated that the PCR with each primer pair did not react with FeSFV- or FeLV-infected cells and that the PCR was specific to FIV (Fig. 1). The PCR system was applied to PBL from FIV antibody-positive cats. Proviral DNA was detected in the PBL from only 7 of 12 cats by the PCR whose single step consisted of 35 cycles (single PCR), an unsatisfactory result (Table 2). If the single PCR products from the cats in which proviral DNA was not detected are subjected to detection of DNA by Southern blot or dot-blot hybridization using radioisotopes, DNA detection will be more sensitive. Pedersen et al. (1990) also detected the PCR products by Southern blot hybridization using radiolabeled probes. However, these procedures are troublesome for routine PCR procedures. Albert and Fenyö (1990), and Garson et al. (1990) amplified human immunodeficiency viral sequences and hepatitis C viral sequences, respectively, by 2-step PCR, in which two primer pairs (an outer primer pair and an inner primer pair) were used. The PCR products yielded by the outer primer pair

were subjected to another amplification with the inner primer pair, and DNA was detected in the products by gel electrophoresis and ethidium bromide staining (PCR with nested primers or "nested" PCR). The 2-step amplification may increase the sensitivity of detection, and the use of nested primers may increase its specificity. The authors also conducted a second amplification of PCR products from PBL from 12 cats using the same primer pair (double PCR). As a result, FIV proviral DNA was detected in the PBL from all 12 cats (Table 2). We prepared the outer primer pair of the primer pair used in this study, and the system of "nested" PCR is now under investigation. The use of cultured PBL enabled detection of products even by single PCR (Table 3). This result may be due to the fact that the proportion of FIV-infected lymphocytes is increased by in vitro culture. It is not known at present what percentage of FIV proviral DNA is included in PBL. How the incidence of infection varies even among antibody-positive cats is also unknown. The use of the PCR technique should answer these questions. An inactivated FIV vaccine may be developed in the future. Then it will be increasingly necessary to distinguish the antibody induced by vaccination from that induced by infection. The detection of proviral DNA by PCR seems to be a useful method for diagnosis of FIV infection from the viewpoint of such a background factor.

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