

Development of Nested Polymerase Chain Reaction for Diagnosis of FeLV Infection in Cats

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ABSTRACT

The Nested Polymerase Chain Reaction test has been developed for a diagnosis of FeLV infection, by detecting proviral DNA of FeLV. In transient and latent infection cats, the freely circulating viral proteins present at high concentration in blood circulation within a few weeks to months. This latent infection is difficult to be detected by ELISA or ELISA application technique (Commercial test kit). The problem of latent infection status can be resolved by detection of proviral sequence which integrates in host genome. Nested PCR reaction was designed in two steps. First round, PCR produced 770 bp product and second round produced 601 bp product. The nested PCR method is more specific and sensitive than regular PCR method. Target gene of oligonucleotide primers is *gag* which could express very low rate of protein mutation. Therefore, the high mutation rates of retrovirus do not affect the specificity of this detection method.

Key words: nested polymerase chain reaction, FeLV, proviral DNA

INTRODUCTION

Feline leukemia virus (FeLV) is an exogenous retrovirus which is worldwide spread and affects domestic cat population. Three subgroups of FeLV; FeLV-A, FeLV-B, and FeLV-C were identified by detecting a variation of envelope proteins (env) (Rohn and Orerbaugh., 1997). The most common used method for diagnosis. FeLV infection can be unified by identification of viral p27 antigens which freely circulate in the plasma. For in-house use, immunochromatographic lateral flow test was developed. This test is practical for clinicians. Most of

commercial immunochromatographic lateral flow tests are in good agreement with ELISA test (Jarret *et al.*, 1991). FeLV p27 protein antigen may not be detected during latent FeLV infection. Some cats are able to eliminate the infection at this stage, when there is no viremia, however the viral genome has already integrated into feline genomic DNA. Latent period can persist for up to three years (Pacitti and Janett., 1985) and some latent infected cats can have a localized FeLV infection in other tissues such as spleen, lymph nodes, small intestine and mammary glands (Pacitti *et al.*, 1986; Hayes *et al.*, 1989).

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Molecular diagnosis methods like Polymerase Chain Reaction are becoming more advantageous than serological methods. The detection of proviral DNA in leukocytes from peripheral blood allows identification of the virus without the presence of antibodies or viremia. The use of two pairs of primers, outer and inner primers may increase the specificity and sensitivity of PCR (Kemp *et al.*, 1989).

In this study, we developed nested RT-PCR and nested PCR. These tests can detect both free RNA virus and proviral DNA that may be useful to indicate true status of the disease.

MATERIALS AND METHODS

RNA and DNA extraction

RNA was extracted from FeLV killed vaccine (Quantum, shcering-Plough) 100 μ l of vaccine was mixed with 500 μ l denaturing solution (4M guinidinium thiocyanate, 25mM sodium citrate pH 7, 0.5% N-lauroylsarcosine) to lyse cell membrane and protein components (Chomczynski, 1987) and then add 2M sodium acetate. Phenol-Chloroform extraction method was use for RNA purification. RNA was precipitated by isopropanol. RNA pellet was dissolved by distilled water and was immediately performed the RT-PCR (Sambrook and Russell, 2001).

Blood samples were collected from four infected cats confirmed by serological test (AGEN FeLV). Cats DNA and proviral DNA were extracted from 100 μ l of whole blood. Blood was mixed with 500 μ l denaturing solution (4M guinidinium thiocyanate, 25mM sodium citrate pH 7, 0.5% N-lauroylsarcosine), and was shaken for

5 to 10 minutes. Cell membrane and protein components were lysed with denaturing solution and then purified with phenol-chloroform extraction method. The DNA was precipitated by absolute ethanol. Purified DNA was kept in Tris-EDTA (TE) buffer (50 mM Tris, pH8.0, 100 mM EDTA) at -20°C until future analysis.

Primers for PCR

Outer and inner forward primers were based on the sequence of U3 region of long terminal repeat. Outer and inner reverse primers were complementary to the sequence of *gag* gene (Miysawa and Janett, 1997) (Table 1). Discrimination between endogenous FeLV-related sequences in cat genome and exogenous FeLV was done by using a unique U3 region as a target of PCR primers (Casey *et al.*, 1987; Berry *et al.*, 1988; McDougall *et al.*, 1994).

Nested RT-PCR detection

The 20 μ l RT-PCR reaction contained 1X buffer (0.2mM of each dNTP, 1.2 mM MgSO₄), 0.1 mM MgSO₄, 10 pmol of outer forward and reverse primers, 2 U of RT-*Taq* polymerase (Invitrogen® SuperScript™ III RT-*Taq*) and 12 μ l of extracted RNA in distilled water. PCR amplification was performed using thermal cycle (PTC 200, MJ Research, and USA). Reverse transcription and first round PCR were done in one step.

RT-PCR cycle consisted of reverse transcription 45°C, 50 minutes followed by an initial 5-minute denaturation at 94°C, then 35 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 30 seconds, and extension

Table 1 Nested PCR specific primers targeting on U3 region and *gag*.

Primers	Region	Sequence (5' → 3')	Products(bp)
U3-F (1)	U3 region	ACA GCA GAA GTT TCA AGG CC	770
G-R (1)	<i>gag</i> gene	GAC CAG TGA TCA AGG GTG AG	
U3-F (2)	U3 region	GCT CCC CAG TTG ACC AGA GT	601
G-R (2)	<i>gag</i> gene	GCT TCG GTA CCA AAC CGA AA	

at 72°C for 60 seconds, and final extension at 72°C for 10 minutes. The inner primers were used in a nested PCR. The conditions of the second-round PCR cycles consisted of an initial 5-minute denaturation at 94°C, then 35 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, followed by final extension at 72°C for 10 minutes. PCR products were analyzed on 1.5% agarose gels in TAE buffer (0.04M Tris-acetate [pH8.5], 0.002 M EDTA).

Nested PCR detection

The 20 μ l PCR reaction contain 1X buffer (20 mM Tris-HCl; pH8.4, 50 mM KCl), 2 mM MgCl₂, 10 pmol of outer forward and reverse primers, 2 U of *Taq* DNA polymerase (Invitrogen™), and 2 ml of extracted DNA in TE buffer. The following conditions were used: After predenaturation 94°C for 45 seconds, 35 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for

60 seconds, followed by a final extension at 72°C for 10 minutes. The conditions of the seconds-round PCR were the same as mentioned above. PCR products were purified with a QIAquick® gel extraction kit (QIAGEN) and ligated into pGEM-T easy vector (Promega) for the DNA sequencing.

RESULTS

The results of electrophoresis by the nested RT-PCR and nested PCR were shown in Figure 1 and 2, respectively. The nested RT-PCR FeLV from killed vaccine and nested PCR from all infected samples produced bands of 601bp. The first round PCR products of infected blood samples still remained in the second round PCR (Figure 2). DNA sequence was analyzed from the sample which has originated from the infected blood sample. DNA sequence of *gag* gene isolated from infected cats in Thailand was 95 % identical to FeLV sequence reference (GenBank accession number AY745878) (Figure 3).

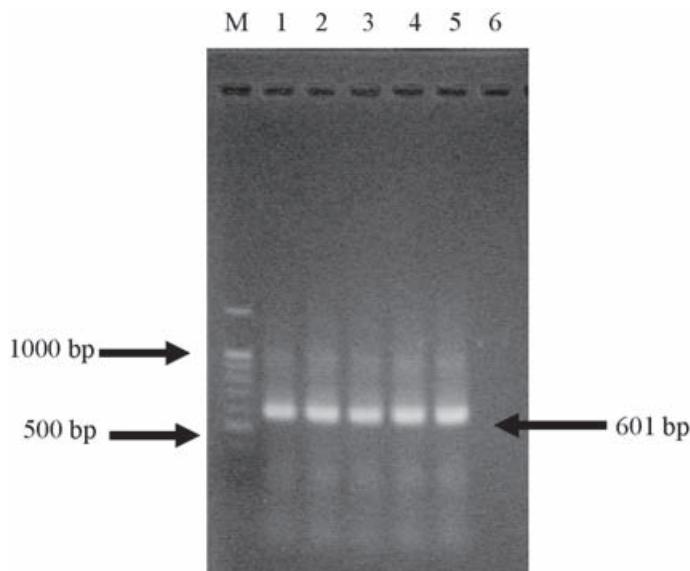


Figure 1 Agarose gel electrophoresis of the RT- PCR product from FeLV killed vaccine (nested RT-PCR). The 601 bp products were shown in lane 1 to 5. Lane M = 100-bp marker (Fermentas), Lane 6 = Negative control.

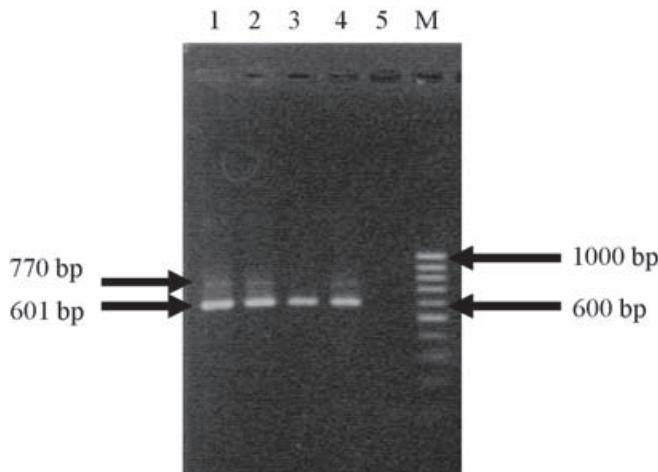


Figure 2 Agarose gel electrophoresis of PCR product from blood sample of FeLV-infected cats. The results of the first round PCR (770 bp) and nested PCR products (601 bp) are shown in lane 1 to 4. Lane M = 100-bp marker (Fermentas), Lane 5= Negative control.

AY745878 Thai isolate	TTCTGCTATAAAACGAGCCATCAGCCCCAACGGCGCGCAAGTCTTGCTGGGACTTGA 120 TTCTGCTATAAAACGAGCCATCAGCCCCAACCGCGCGCAAGTCTTGCTGAGACTTGA 120 *****
AY745878 Thai isolate	CCGCCCCGGGTACCCGTGTACGAATAAACCTCTTGCTGATTGCATCTGACTCGTGGTCTC 180 CCGCCCCGGGTACCCGTGTATGAATAAACCTCTTGCTGCTGCATCTGACTCGTGGTCTC 180 *****
AY745878 Thai isolate	GGTGTCTCGTGGGCACGGGTCTCATCTCGAGGAAGACCCAGTCAGGGGTCTTCATT 240 GGTGTCTCGTGGGCACGGGTCTCATCGCAGGAAGACCTAGTCAGGGGTCTTCATT 240 *****
AY745878 Thai isolate	TGGGGGCTCGTCGGGATCGAGACCCCCAACCCCCCGGGACCCACCGACCCACCATCAGGAG 300 TGGGGGCTCGTCGGGATCGAGACCCCCAACCCCCCGGGACCCACCGACCCACCATCAGGAG 300 *****
AY745878 Thai isolate	GTAAGCTGGCCGGCACCATATCTGTTGCTTATATAAGTGTCTCTGTCATTGATCTG 360 GTAAGCTGGCCGGCACCATATCTGTTGCTTGTATGAATGTCTCTGTCATTGATCTG 360 *****
AY745878 Thai isolate	ATTTGGGCGGTGGGATCGTAGGAGCTGACGAGCTTCGACTTCGCCCCCACACCCCTGGA 420 ATTTGGGCGGTGGGAGCGAAGGAGCTGGCGAGCTTCGACTTCGCCCCCACACCCCTGGA 420 *****
AY745878 Thai isolate	AGACGTCCACGGGCCTGATGTCTGGAGCCTCTAGTGGGACAGCCATTGGGCTCATC 480 AGACGTCCACGGGTGTCGATGTCTGGAGCCACTAGTGGGACAGCCATTGGGCTCATC 480 *****
AY745878 Thai isolate	TGTTTGGGTCTCACCTGAATACAGGGTGTGATCGGAGACGAGGGAGCCGGACCCCTCAA 540 TGTTTGGGTCTCACCTGAATACAGGGTGTGATCGGAGACGAGGGAGCCGGACCCCTCAA 540 *****
AY745878 Thai isolate	AGTCTCTTCTGAGGTTCA 560 AGTCTCTTCTGAGGTTCA 560 *****

Figure 3 DNA sequencing from infected specimen aligns to *gag* gene (AY745878).

DISCUSSION

We performed nested PCR method to detect proviral DNA of exogenous FeLV in peripheral blood leukocytes (PBL) and nested RT-PCR to detect plasma viral RNA. Not only proviral DNA in PBL and plasma viral RNA that can be detected by this PCR tests, but also proviral DNA and viral RNA from tumor masses (Stile *et al.*, 1999; Gabor *et al.*, 2001), tissue biopsy (Herring *et al.*, 2001), and RNA virus shedding in excretion (Gomes-Keller *et al.*, 2006).

FeLV is discriminated in three subgroups; FeLV-A, FeLV-B, and FeLV-C. There are genetic variations among three subgroups. The primers in this method were designed based on FeLV-A sequence. Because the U3 region and *gag* gene are strongly conserved among three subgroups, the PCR methods in this study would detect the other subgroups as good as subgroup A. The sensitivity of the test should be confirmed by PCR reactions using the serial dilution of DNA and RNA standard template. Because of the limitation for preparing the standard template, we can not determine the detection limit of the test.

The study of pathogenesis of FeLV infection in cats is not only the important research in veterinary medical science, but also a well-acknowledged animal model for studying the pathogenesis of retroviral disease. The present PCR technique will be useful for the laboratory diagnosis of FeLV infection and for the research of FeLV pathogenesis. The detection of proviral DNA and viral RNA provides valuable information of the disease status in infected animals and the duration of virus shedding. Genetic variation of FeLV isolation in Thailand requires further investigation to identify FeLV-subgroup distribution in domestic cats in Thailand.

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