

Development of TaqMan® Real-time Reverse Transcription-Polymerase Chain Reaction for the Quantification of Feline Leukemia Virus Load

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ABSTRACT

Feline leukemia virus (FeLV), a gamma retrovirus of the domestic cat, is not only of veterinary interest, but is also an important model for the study of pathogenesis of tumors and AIDS in many animals. After initial infection, disease progression has been associated with cellular and humoral immune response and FeLV proviral load. Moreover, an earlier study showed that not only the proviral load, but also the plasma viral loads are important parameters, which are associated with progression of the disease. In this study, the TaqMan® real-time reverse transcription-polymerase chain reaction (RT-PCR) was developed for the quantification of FeLV loads. The assay was developed to amplify a 131 base pairs conserved domain within the unique region (U3) of a long terminal repeat (LTR). The detection limit of this assay was 8.3 copies of RNA standard template or 4.15 viruses per reaction that is equivalent to 1,778 viruses per ml of plasma or serum.

Key words: feline leukemia virus (FeLV), cat, TaqMan® real-time RT-PCR, quantification, viral loads

INTRODUCTION

Feline leukaemia virus (FeLV) was first described by Jarrett *et al.* (1964). It is a γ -retrovirus of domestic cats that contains a protein-coat core of encapsulated single-stranded RNA protected by an envelope, and is a member of the Oncornavirus subfamily of retroviruses (Hartman, 2006). The known main outcomes of the initial infection are: (i) a regressive infected cat, where viral replication can be stopped by an effective cell-mediated immune (CMI) response; (ii) a transiently infected cat, where viremia can be terminated within weeks; (iii) a latent non-productive infected cat, where bone marrow cells are infected after about three

weeks of viremia. (The cat is not able to completely eliminate the virus from its body, even if it terminates the viremia because the proviral DNA is present in the bone marrow stem cell); (iv) a persistent infected cat can develop FeLV-associated disease and persistent viremia, because it lacks specific humoral and cellular immunity (Hoover *et al.*, 1975; Rojko *et al.*, 1982; Hoover and Mullins, 1991; Flynn *et al.*, 2000, 2002).

Various methods have been used for the detection of an FeLV infection, such as immunofluorescence, enzyme-linked immunosorbent assay (ELISA) and virus isolation (Hardy *et al.*, 1973; Jarrett *et al.*, 1982; Hoover and Mullins, 1991; Flynn *et al.*, 2000, 2002). However, most assays

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(except viral isolation) detect FeLV p27 antigen in the serum or plasma (Lutz *et al.*, 1983).

More recently, TaqMan[®] real-time polymerase chain reaction (PCR) methods, using the 5'-3' nuclease activity of Taq DNA polymerases, have been developed for detection and quantification of FeLV proviral DNA (Holland *et al.*, 1991; Lyamichev *et al.*, 1993). This method has been used to study pathogenesis of FeLV (Hofmann-Lehmann *et al.*, 2001; Torres *et al.*, 2005). It offers several advantages, such as low DNA consumption, low risk of contamination, absolute quantification and rapid throughput of many samples (Holland *et al.*, 1991; Lee *et al.*, 1993; Lyamichev *et al.*, 1993; Heid *et al.*, 1996). Moreover, not only the proviral load, but also the plasma viral load has been shown to have a relationship with the pathogenesis of FeLV infection, and plasma viral loads have been related to the outcome of infection after exposure to FeLV (Tandon *et al.*, 2005). Thus, the aim of the present study was to establish a TaqMan[®] real-time reverse transcription-polymerase chain reaction (RT-PCR) assay to quantify the feline leukaemia virus load.

MATERIALS AND METHODS

Construction and production of RNA standards template

Whole blood of an FeLV-infected cat was used to extract genomic DNA using the phenol-

chloroform extraction method described by Sambrook and Russel (2001). An amount of 100 µl of whole blood from an FeLV-infected cat was mixed with 500 µl of D-solution (4M guanidium thiocyanate, 50mM Tris-HCl, 20mM EDTA, pH 8.0) and incubated at room temperature for 5 min. Subsequently, 200 µl each of phenol and chloroform were added, and vortexed and centrifuged at 13,000 rpm for 5 min. Total DNA was precipitated with 700 µl of absolute isopropanol and centrifuged at 13,000 rpm for 10 min. The DNA pellet was washed with 75% ethanol and then centrifuged at 13,000 rpm for 5 min. Finally, the DNA pellet was air-dried and resuspended in 30 µl of TE buffer (pH 8). The 468 base pairs of the unique region (U3) within a long terminal repeat, encompassing 131 base pairs long amplified by the designed TaqMan[®] RT-PCR system, were amplified using specific primers (Table 1) and cloned into the pGEM[®]-T Easy vector system (Promega). Positive colonies were selected by the blue/white colony screening method and the selected clones were confirmed by sequencing (Bioservice Unit, Bangkok, Thailand). The positive clones were linearized using the restriction enzyme *SacI* (Fermentus), purified (QIAquick[®] Gel Extraction Kit, Qiagen) and the concentration determined by spectrophotometer (Beckman, Amersham-Pharmacia Biotech, Otelfingen, Switzerland). Linearized plasmids were subjected to *in vitro*

Table 1 FeLV U3 LTR specific primers and TaqMan[®] probe used to produce standard templates and FeLV real-time RT-PCR assays (Tandon *et al.*, 2005).

Name of oligonucleotide	Sequences(5'-3')	Length (bp)
Forward primer FeLV_standard_f ¹	CTA CCC CAA AAT TTA GCC AGC TAC T	25
Reverse primer FeLV_standard_r ¹	AAG ACC CCC GAA CTA GGT CTT C	22
Forward primer FeLV_U3-exo_f ²	AAC AGC AGA AGT TTC AAG GCC	21
Reverse primer FeLV_U3-exo_r ²	TTA TAG CAG AAA GCG CGC G	19
Probe FeLV_U3_probe	CCA GCA GTC TCC AGG CTC CCC A ³	22

¹ Primers used to construct the standard plasmid DNA

² Primers used in TaqMan[®] real-time reverse transcription- polymerase chain reaction (RT-PCR)

³ 5'-end labeled with reporter dye FAM; 3'-end labeled with the quencher dye TAMRA. The probe was designed to hybridize to the antisense strand.

transcription using an RNAMaxx™ high yield transcription kit (Stratagene). *In vitro* transcribed RNA was quantified using a spectrophotometer (Beckman, Amersham-Pharmacia Biotech, Otelfingen, Switzerland). An RNA standard was calculated and serially diluted ten times in PCR-grade water. The RNA was stored in 20 µl aliquots at -80°C until used.

Probe and primers

Primers and probes were identical to those previously described and are shown in Table 1.

TaqMan® fluorogenic real-time RT-PCR assay

RNA was reverse-transcribed, amplified and quantified using a Rotor-Gene™ 3000 real-time rotary analyzer (Corbett Life Science). Two microliters of virus RNA or RNA standard were amplified in a total reaction volume of 25 µl using the SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Invitrogen, Basel, Switzerland) with a final concentration of 2 µM of forward primer, 2 µM of reverse primer and 1 µM of fluorogenic probe. Firstly, the reverse transcription was performed for 5 min at 50°C and followed by a denaturation step of 95°C for 2 min and 50 cycles of 95°C for 15 s and 60°C for 30 s.

Specificity and sensitivity of the real-time RT-PCR assays

The specificity of the real-time RT-PCR assay was verified by sequencing of the PCR products. The sensitivity of the real-time RT-PCR assays was assessed at the end-point of the dilution experiments. A ten-fold serial dilution of 8.3×10^9 , 8.3×10^8 , 8.3×10^7 , 8.3×10^6 , 8.3×10^5 , 8.3×10^4 , 8.3×10^3 , 8.3×10^2 , 8.3×10^1 and 8.3×10^0 of the RNA standard was evaluated.

Amplification efficiency of the real-time RT-PCR assays

The amplification efficiencies were

performed in triplicate using serial ten-fold dilutions of purified RNA standard transcripts. The efficiencies of amplification were compared by assessing the slopes (s) of the regression line (threshold cycle (C_T) versus dilution) obtained by RT-PCR amplification of serial dilutions of either the RNA standard template or the viral RNA template of infected cats. The efficiencies of the assays for each sample can be considered equal if the difference in the slopes (Δs) was less than 0.1 (Gut *et al.*, 1999; Klein *et al.*, 1999).

Clinical samples

EDTA-treated blood (1ml) was collected from 30 FeLV-infected cats that tested positive by rapid test (ELISA, Simplify® FeLV, Australia) and nested-PCR. The samples were centrifuged at 4,000 rpm for 5 min and the plasma was collected. An amount of 140 µl plasma was extracted using a QIAamp® Viral RNA Mini Kit (Qiagen) and the RNA was stored at -80°C until used.

RESULTS AND DISCUSSION

Cloning of plasmid template

The 468 base pairs PCR products that contained the unique region (U3) within the long terminal repeat (LTR) were cloned into plasmid pGEM®-T Easy and were subjected to sequencing. The sequence (accession number FJ476268) showed 95-98% with other FeLV isolates (accession numbers EU189498, EU189496, EU189495, M12500 and D13922).

Sensitivity of the real-time RT-PCR assay

The quantitative real-time RT-PCR was designed to amplify a 131 base pairs conserve fragment within the unique region (U3) of the long terminal repeat (LTR) of FeLV. The sensitivity was assessed by the end-point dilution experiment. The assay consistently detected 8.3 copies of the RNA standard template or 4.15 virus particles per reaction. By calculation, this assay can detect at

least 1,778 virus particles per ml of plasma or serum. However, the detection limit of a previous study, using the same primer and probe, was higher (Tandon *et al.*, 2005). Moreover, other studies that detected U3 LTR, but used a different set of primer and probe could detect FeLV at a lower level (Rohn and Overbaugh, 1995; Hofmann-Lehmann *et al.*, 2001). The reason for the different detection limits may have been the different use of primers and

probes, the concentration of RT-PCR mixtures, cycling conditions and the real-time PCR machine. If the real-time RT-PCR condition in this study were optimized, the sensitivity of the test might increase. The linear range of quantification of FeLV-virus RNA was evaluated using ten-fold serial dilutions of the standard RNA. Linearity was found between 8.3×10^9 copies and 8.3×10^0 copies of standard RNA (Figures 1 and 2). The

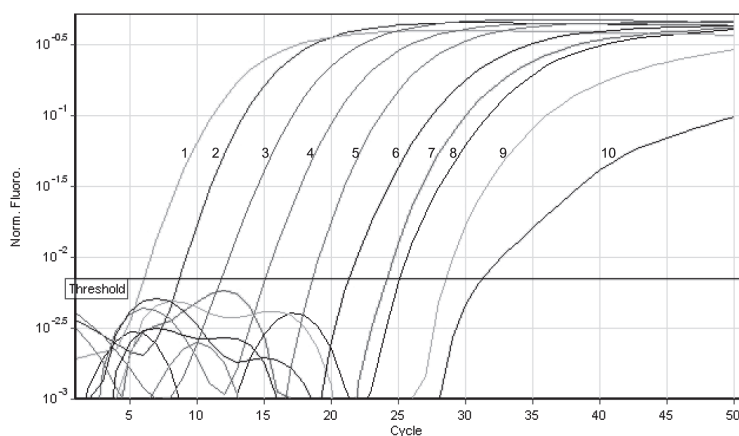


Figure 1 Amplification plot of RT-PCR assay (1= 8.3×10^9 copies, 2= 8.3×10^8 copies, 3= 8.3×10^7 copies, 4= 8.3×10^6 copies, 5= 8.3×10^5 copies, 6= 8.3×10^4 copies, 7= 8.3×10^3 copies, 8= 8.3×10^2 copies, 9= 8.3×10^1 copies and 10= 8.3×10^0 copies).

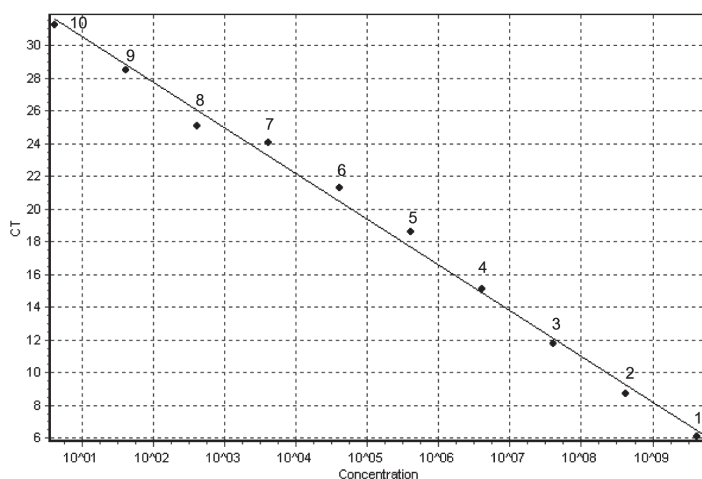


Figure 2 The measured threshold cycle vs ten-fold serial dilution of RNA standard template (1= 8.3×10^9 copies, 2= 8.3×10^8 copies, 3= 8.3×10^7 copies, 4= 8.3×10^6 copies, 5= 8.3×10^5 copies, 6= 8.3×10^4 copies, 7= 8.3×10^3 copies, 8= 8.3×10^2 copies, 9= 8.3×10^1 copies and 10= 8.3×10^0 copies).

amplification efficiency was 0.99 generated by Rotor-Gene™ 3000 analyzer software.

For clinical samples, the serum of naturally FeLV-infected cats was used, which tested positive to the rapid test and nested-PCR. All samples showed positive results and had an RNA load ranging from 2.2×10^4 to 3.60×10^9 copies per ml of plasma or serum. This may be the first quantitative RT-PCR assay to determine the FeLV virus load in Thailand. The FeLV viral RNA load has been shown to be correlated with disease progression and the outcome of infection (Saag *et al.*, 1996; Watson *et al.*, 1997; Tandon *et al.*, 2005). Thus, this quantitative RT-PCR assay may be a useful tool for monitoring disease progression and determining the responsiveness to certain drugs during treatment.

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