

**Detection of Feline Leukemia Virus in Bone Marrow
Using Polymerase Chain Reaction**

Erin Leigh Stimson

Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science

In

Veterinary Medical Sciences

Gregory C. Troy, Chair

Thomas E. Toth

S. Dru Forrester

March 31, 2000

Blacksburg, Virginia

Keywords: Feline Leukemia Virus, Polymerase Chain Reaction, Latent Infection,
Bone Marrow

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Latent feline leukemia virus (FeLV) infections, in which proviral DNA is integrated into host DNA, but not actively translated, are thought to be associated with many diseases. Bone marrow is the suspected site of the majority of latent infections. The purpose of the study was to determine if polymerase chain reaction (PCR) could be used to detect FeLV proviral DNA in bone marrow and potentially provide a method of detecting latent infections. Blood and bone marrow samples from fifty cats and bone marrow from one fetus were collected, including eleven laboratory cats, twenty-three cats and one fetus from random sources, and sixteen cats with diseases associated with FeLV. Serum and ELISA, blood and bone marrow immunofluorescent antibody test (IFA) and blood and bone marrow PCR were performed on each cat, and IFA and PCR were performed on bone marrow of the fetus. Forty-one cats were FeLV negative. Five cats and one fetus were persistently infected with FeLV. Four cats had discordant test results. Two were ELISA positive with other tests negative, one was bone marrow IFA negative with other tests positive, and one was bone marrow IFA positive with other tests negative. No cats were positive only on bone marrow PCR. These results indicate that PCR can detect FeLV proviral DNA in bone marrow, but no cats in this study harbored FeLV proviral DNA only in the bone marrow. Not all cats with FeLV-associated diseases are persistently or latently infected with FeLV.

Acknowledgments

I would like to acknowledge the assistance given by many individuals and groups in the completion of this project.

To Hanover County SPCA, Virginia Beach SPCA, Dr. Britt Culver, Dr. Gary Frenier, and VTH clinicians for their assistance in identifying cases for this study.

To Ms. Taryn Brandt, Lab Specialist, Virginia-Maryland Regional College of Veterinary Medicine and Dr. Michele Reimer, Internist, for their assistance in collection and storage of blood and bone marrow samples.

To Mr. Lee Weigt, Manager, Virginia Tech DNA Sequencing Facility, for his help in teaching PCR technology, and providing DNA sequencing services.

To Dr. Francois Elvinger, Associate Professor, Department of Large Animal Clinical Sciences, Virginia-Maryland Regional College of Veterinary Medicine, for assisting with statistical analysis of test results.

To Dr. Ian Herring, Assistant Professor, Department of Small Animal Clinical Sciences, Virginia-Maryland Regional College of Veterinary Medicine, for help in project design.

To the Virginia Veterinary Medical Association Veterinary Memorial Fund and the Office of Research and Graduate Studies at the Virginia-Maryland Regional College of Veterinary Medicine for providing funds for this project.

Table of Contents

I.	Introduction.....	1
II.	Literature Review.....	2
III.	Material and Methods.....	14
IV.	Results.....	19
V.	Discussion.....	22
VI.	References.....	26
VII.	Legend.....	31
VIII.	Figure 1.....	32
IX.	Figure 2.....	33
X.	Figure 3.....	34
XI.	Table 1.....	35
XII.	Table 2.....	36
XIII.	Table 3.....	38
XIV.	Table 4.....	39
XV.	Vita.....	40

Introduction

Feline leukemia virus (FeLV) is a retrovirus reported to infect 2-3% of the feline population, and is responsible for more feline diseases than any other infectious agent.^{1,2} FeLV has been shown to significantly decrease life expectancy of infected cats.³ Latent FeLV infections, in which provirus is integrated in myelomonocytic progenitor cells, are suspected to be associated with diseases such as lymphoma, leukemia, and cytopenias.^{1,4,5}

The current method for detection of latent FeLV infection involves bone marrow culture, primarily used in research settings. Use of polymerase chain reaction (PCR) to detect FeLV proviral DNA in bone marrow, the suspected site of the majority of latent infections, has not been reported. However, PCR is used in both human and veterinary medicine to detect latency with other viral infections.⁶⁻¹⁵ Specific viral infections PCR is used to detect latency include equine herpes virus-4 (EHV-4), feline herpes virus-1 (FHV-1), human herpesvirus 6 (HHV-6), canine herpes virus (CHV), Epstein-Barr virus, and herpes simplex virus.⁷⁻¹⁵ If PCR can detect untranscribed FeLV proviral DNA in bone marrow, this may provide a simple and sensitive method of detecting latent FeLV infections.

The purpose of this study was to determine if PCR can detect FeLV proviral DNA in feline bone marrow, and to determine if latent infections could be identified. Additionally, comparison of results of bone marrow PCR with standard FeLV tests (peripheral blood enzyme-linked immunosorbent assay (ELISA), immunofluorescent antibody (IFA), and PCR and bone marrow IFA) was performed.

Literature Review

Feline Leukemia Virus Structure and Forms

FeLV is a single-stranded RNA retrovirus of the family retroviridae, belonging to the subfamily oncovirinae. The structure of the virus includes an internal core composed of 60s and 70s single-stranded RNA genome, reverse transcriptase, and core proteins including p10, p12, and p15c. The genome is composed of the following genes: long terminal repeat (LTR), group-associated proteins (gag), polymerase (pol)-enveloped (env), and LTR.¹⁶ The LTR sequences have a regulatory function, controlling the expression of other viral genes.¹⁷ Included in the LTR is the U3 region, which functions as a transcriptional enhancer.^{17,18} The gag gene encodes for the internal structural proteins p15c, p12, p10, and p27. The pol specifies virion reverse transcriptase enzymes, which are responsible for synthesis of DNA from an RNA template. The env sequences encode for transmembrane p15e protein and surface unit glycoprotein (gp) 70 components of the FeLV envelope.¹⁶ The core is surrounded by a hexagonal capsid composed of repeated protein units, p27. An inner spherical coat and outer spherical envelope are present. Spikes of p15E are present and play a role in immunosuppression of the infected host. Disulfide bonds bind the p15E spikes to gp70 knobs. These structures allow attachment to specific gp70 receptors on feline cells, allowing cellular infection with the virus.

Biological cloning of FeLV isolates has shown three distinct viral subgroups, designated A, B, and C.¹⁷ Type A is spread contagiously between cats, and types B and C are formed from recombination and mutation of type A FeLV and endogenous FeLV sequences.^{17,19} Endogenous FeLV sequences are found in healthy non FeLV-infected domestic cats and their close relatives, and are partially homologous to exogenous, horizontally transmitted FeLV. Endogenous FeLV sequences were likely acquired by domestic cats via transspecies infection with a rodent virus. Overall, there is approximately 85% sequence homology between the genomes of the three FeLV subgroups. All strains of FeLV isolated from infected cats contain FeLV A with 50% of

cats having only this subgroup. Forty-nine percent of infected cats have both FeLV A and B subgroups, with the remaining 1% of cats infected with combinations of either FeLV A, B, and C, or FeLV A and C.¹ Subgroup specificity appears to regulate the type of disease occurrence. FeLV A is slow to cause disease by itself, and may account for its high prevalence in nature. FeLV subgroup A is reported to be responsible for the majority of latent infections. If FeLV A proviral LTR inserts next to DNA carrying endogenous retroviruses, recombination can occur and FeLV B or C can be generated.^{1,20} These mutational events are uncommon. However, because viremic cats produce considerable amounts of FeLV A ($10^5 - 10^6$ infectious units/ml plasma) for years, this increases the chance that FeLV A will integrate into a sensitive region of DNA.¹ Emergence of FeLV B or FeLV C in bone marrow cells, lymphocytes, and plasma may also indicate onset of fatal hemolympathic disease. Cats experimentally infected with FeLV C develop fatal erythroid aplasia, thymic atrophy, and lymphoid depletion within 1 to 2 months.^{21,22} It is likely lymphogenesis proceeds after insertion of the provirus in the area of a cellular oncogene.¹⁶

Viral Infection

Cellular infection begins with viral attachment to gp70 receptors on the host cell. After viral entry, the envelope and outer coat are shed into the cell cytoplasm. Viral reverse transcriptase activity results in a complementary DNA (cDNA) copy of FeLV RNA. The cDNA strand serves as a template for second-strand DNA synthesis by the host's cellular-dependent DNA polymerase, resulting in a double-stranded (ds) DNA copy. The dsDNA circularizes and is integrated covalently into the feline cellular chromosomes. Integrated dsDNA is referred to as FeLV DNA provirus. Integration can only occur following DNA synthesis, and therefore dividing cells are particularly susceptible to FeLV infection.¹

Cats generally acquire the virus through oronasal contact with an infected cat, although FeLV can be transmitted transplacentally, or through milk.^{17,23,24} The virus

enters the caudal oropharynx, and local lymphoid infection occurs (phase one).¹⁷ Phase two occurs 2 to 12 days after exposure, when a mononuclear cell-associated viremia is present.²⁵ Phase three follows, with systemic lymphoid infection and phase four occurs when FeLV replication occurs in hemolymphatic and intestinal epithelial cells. Phase five is characterized by a marrow-origin viremia in which large amounts of virus are produced. The final phase, six, occurs with widespread replication of the virus in glandular tissue and epithelial cells allowing excretion of the virus into saliva, tears, and urine.²⁵

Approximately 70% of cats exposed to the virus are resistant to persistent infection.¹ Resistant cats eliminate replicating FeLV from their tissues in 4 to 6 weeks after exposure through development of virus-neutralizing antibodies and cell-mediated immune response. In minimally exposed cats, FeLV infection is restricted to phase one. The majority of remaining resistant cats eliminate replicating FeLV from their tissues during phase four. Occasionally, cats having a high viral burden or slower immune response can have a transient marrow-origin viremia before resistance occurs.¹⁷ Cats eliminating all infected cells are said to have extinguished FeLV infection. Experimentally, resistant cats extinguish FeLV infection by 16 to 88 weeks after exposure, and it is estimated that 30 to 60% of cats acquire a latent infection after extinguishing viremia.^{4,26,27} It has been noted that over several months time, the proportion of cats with detectable latent infections declines, possibly indicating that clonal extinction of infected myelomonocytic cells in bone marrow is occurring.^{26,27}

Latent Infections

Latency may be a phase in extinguishing the virus, in which the FeLV-infected cells in the bone marrow and lymph nodes are the last to be eliminated. In a small percentage of cats, the latent state persists for weeks to months or longer.^{26,27} Cats with persistent latent infections are suspected to be at risk for developing FeLV-related disease later in life.^{1,4,5} Approximately 2 to 6% of cats that ostensibly recover from acute FeLV

infection or feline sarcoma virus/FeLV infection die of FeLV-associated diseases.¹ Cats immunosuppressed with corticosteroids or through stressful conditions, can have a recrudescence of viremia.^{4,26} Reactivated virus is just as likely to cause lymphoma as acute viral infection.^{1,26}

The site of latent infection appears to reside predominantly in bone marrow, in myelomonocytic precursor cells, marrow stromal fibroblasts, and lymphoid cells.^{4,28} There appears to be a low level of gp70 expression on the membrane of infected marrow stromal fibroblast (FB) precursor cells. Therefore, these cells can escape immune surveillance and theoretically provide a reservoir of virus during active or latent FeLV infection. It is suspected that multiple marrow stromal cell types serve as reservoirs of latent virus.²⁸ Marrow FBs play an important role in the regulation of hematopoiesis within the marrow microenvironment. Therefore, stromal cell infection could play a role in the pathogenesis of FeLV-induced leukemia, or contribute to cytopenias if growth factor production or stromal cell viability is decreased.²⁸ It has been shown that FeLV-infection of long-term marrow culture (LTMC) stromal cells results in the alteration of the proliferative activity of adherent progenitors, and this correlates with down modulation of soluble progenitor growth-inhibitory activity production.²⁹ A high frequency of marrow stromal fibroblasts and macrophages are infected with FeLV in vivo, therefore it is likely that these mechanisms play a role in FeLV-associated hematologic diseases.²⁹ It is possible that spontaneous reactivation from latency occurs frequently, and the immune response acts to eliminate cells expressing viral antigens.¹⁷ Alternatively, the immune response may be directly responsible for maintaining the latent state by modulating virus expression.¹⁷ It has been shown that the complement system plays a role in the containment of latent feline leukemia virus. In a study using latently infected FeLV cats, an increase in viral antigen in marrow myelomonocytic cells and in circulating immune complexes was noted after complement depletion was achieved with treatment using cobra venom factor.³⁰ Viral excretion from the oropharynx is not present in latent infections, but transmission of virus from latently infected queens to their kittens

can occur either by reactivated virus from maternal leukocytes in the placenta or by virus shedding into milk.^{4,26,27}

FeLV-Related Diseases

Numerous diseases are associated with FeLV infection, including lymphoid and myeloid neoplasia.¹⁶ In reports from 10-15 years ago, approximately 70% of cats with lymphoma are positive for FeLV, and nearly 100% of cats with myeloproliferative neoplasia are FeLV positive by IFA. Prevalence is likely lower at this time due to the FeLV testing and eradication of cats, and due to FeLV vaccination. Anemia also occurs, and can be aplastic, hypoplastic, or hemolytic. Hemolytic anemia can be caused by either immune-system alterations with generation of autoantibodies, or secondary to *Hemobartonella* infection. Other FeLV associated diseases include infertility, neonatal death, and neuropathies. Hyperesthesia, lumbar pain, incontinence, paresis, and unilateral mydriasis are reported neurologic manifestations. Immune-complex disease due to virus specific immunoglobulins complexing with FeLV antigens may cause glomerulonephritis and occasionally polyarthritis.³ Rarely reported syndromes include panleukopenia, multiple cartilagenous exostoses, osteochondromatosis, and cutaneous keratin horns.¹ It is also speculated that the virus predisposes cats to develop a number of these diseases via immunosuppression. Viremia can induce neutropenia and leukopenia. Neutrophils of persistently infected cats have been shown to have deficient bacterial phagocytosis. Lymphocytes have decreased response to interleukin-2. The p15E spikes on the virus interfere with normal host cell-surface proteins, leading to pneumonia, pyothorax, enteritis, gingivitis, stomatitis, and dermatitis.¹ Increased susceptibility to toxoplasmosis, fungal infections, feline infectious peritonitis (FIP) virus, and upper respiratory viruses are also seen.

FeLV Diagnostic Testing

Due to its availability and quick turnaround time, enzyme-linked immunosorbent assay (ELISA) is the most common testing method used for detecting FeLV. ELISA

detect soluble 27,000 dalton core protein (p27) antigen in whole blood, serum, plasma, solubilized tissue homogenates, saliva, or tears.^{5,31-35} Commercial ELISA kits have similar results, although test kits using saliva or tears are less accurate. Test kits use a mouse monoclonal anti-p27 to coat test wells. This antibody traps FeLV p27 present in the test fluid. A second mouse monoclonal antibody to FeLV p27 is applied that recognizes a different epitope and is conjugated to an indicator enzyme, often horseradish peroxidase. If FeLV p27 is trapped by the first monoclonal antibody, the trapped antigen will bind the second antibody with its conjugated peroxidase. Test wells are washed to remove unbound antibody, and a chromogenic peroxide substrate is added resulting in a color reaction that indicates the presence of FeLV antigen. Color development is proportional to the amount of virus specific antibody bound to the trapped p27 antigen solid phase. Negative ELISA results are predictive of a true disease-free state greater than 99% of the time.^{32,36} Positive ELISAs are not always diagnostic of viremia, nor are they correlated with the capacity for virus excretion and contagious transmission. Proper test protocol must be followed for accuracy, and positive tests should be confirmed with immunofluorescent antibody tests (IFA).

IFA tests detect presence of FeLV structural antigens in the cytoplasm of FeLV infected cells. The basis of this test is demonstration of excess FeLV core antigens present in any cell infected with replicating FeLV. Cells to be tested are smeared onto slides and fixed in acetone or methanol. Primary reagents for IFA are polyclonal antibodies to ether-disrupted FeLV prepared in rabbits or goats. Secondary reagents are fluorescent-conjugated antibodies to rabbit or goat IgG. A positive apple-green fluorescent reaction indicates the cell contains FeLV antigens, primarily core protein p27. In cats that are leukopenic, it may be difficult to detect leukocytes in the smear, and the smear may be inconclusive. In these cases, bone marrow smears or lymph node aspirates, which have higher concentrations of leukocytes, should be used. A positive IFA is diagnostic of persistent marrow-origin viremia. Performed correctly, 98% of IFA positive cats are also positive on virus isolation (VI).^{32,37-39} Rare discordancies between IFA and VI are likely due to early infection, prior to full infection of the marrow.

Virus isolation is the gold-standard for determining FeLV infection. VI is used to measure the number of infectious FeLV particles present in plasma, serum, saliva, urine, tissue fluids, and homogenates, or tissue culture medium. Assays generally are available through research laboratories. Standard VI assay takes 10 to 14 days to perform and requires a cell-culture laboratory.^{26,27} Virus isolation currently is the only proven method to detect latent FeLV bone marrow infections. In this assay, bone marrow mononuclear cells are harvested using density gradients, and are cultured for 7 to 30 days *in vitro* with glucocorticoids. If cells harboring latent FeLV are present, they are activated to release replicating FeLV into the supernatant. The supernatant can be analyzed for infectious FeLV using VI or ELISA.

Polymerase chain reaction (PCR) is a technique for amplifying specific sequences of DNA.⁴⁰ PCR has been used as a research tool for detection of FeLV DNA in peripheral blood, corneal tissue, and formalin-fixed, paraffin-embedded neoplastic tissue.^{5,41,42} Peripheral blood PCR results for FeLV DNA and FeLV ELISA tests are highly correlated.⁵ Results of PCR performed on 70 paraffin-embedded feline lymphomas revealed that more tumors were positive for FeLV using PCR than with immunohistochemistry techniques. These results suggest that latent or replication defective forms of virus may be involved in some feline tumors.⁴³

PCR is used in both human and veterinary medicine to detect latent viral infections.⁶⁻¹⁵ Specific viruses in which PCR has been useful to detect latency include equine herpes virus-4 (EHV-4), feline herpes virus-1 (FHV-1), human herpesvirus 6 (HHV-6), canine herpes virus (CHV), Epstein-Barr virus, and herpes simplex virus.⁷⁻¹⁵ For example, PCR performed on peripheral blood mononuclear cells has been used to differentiate between latent and actively replicating HHV-6.¹² Additionally, PCR has been performed on hemopoietic cells in human beings to determine that HHV-6 can latently infect early bone marrow progenitor cells.¹⁴ PCR is used in veterinary medicine to determine the sites of latent infections of CHV in dogs.¹¹ Bovine immunodeficiency-like virus was shown to have a latent state when PCR was used for viral detection,

indicating a higher rate of infection than previously determined using serology.¹⁵ Although serological assays for antibodies for many viruses have played a significant role in the identification of infected individuals, direct detection of viral pathogens through PCR is desirable. Factors that make serologic assays for retroviruses more difficult include transcriptional dormancy of the proviral genome, small number of infected cells in peripheral blood, small number of proviral copies per infected cell, existence of multiple related but distinct viral members, and presence of infected cell reservoirs that are not readily amenable to monitoring. PCR has proven to be an ideal procedure for detection of these viruses, as even one viral particle, and those in a latent, non-transcribing state can be detected.^{6,44}

PCR is named as it involves a polymerase, or extender. Products synthesized in each polymerase cycle serve as templates in the next, so the number of DNA copies doubles at every cycle, creating a chain reaction.⁴⁴ The first report of a specific DNA amplification using PCR was in 1985. Invented by Kary Mullis, it was originally applied to the amplification of human B-globulin DNA for the prenatal diagnosis of sickle-cell anemia.^{45,46} PCR can produce large amounts of specific DNA fragments from small amounts of a complex template.^{44,45} Basically, PCR involves combining a DNA sample with oligonucleotide primers, deoxynucleotide triphosphates, and thermostable Taq DNA polymerase in a suitable buffer. Repetitive heating and cooling of the mixture is performed until the desired degree of amplification has been achieved.⁴⁴

Specificity of PCR results from the use of oligonucleotides, or primers, which are designed to precisely anneal to a single DNA sequence. Two short sequences separated by a known sequence length are chosen as the primer. These short sequences are complementary to opposite strands of the sequence. Primers are generally between 18 to 28 nucleotides long, which is sufficient to ensure uniqueness of sequences if derived from nonrepetitive DNA, and avoid mispriming if a high annealing temperature is used. PCR can be performed either on DNA or RNA (following the production of complementary DNA using reverse transcriptase) for templates. Ability of primers to be pathogen

specific is critical for sensitive and specific amplifications and to allow detection of single pathogenic organisms or virus particles.⁴⁴

Polymerase chain reaction cycle includes three main steps: denaturation, annealing, and polymerization. The DNA sequence to be amplified by PCR is known as the template and usually is a fragment of double-stranded DNA. Taq polymerase, the DNA synthesizing enzyme most often used in PCR, requires DNA templates to be single-stranded before it is able to make a copy. Therefore, the template is denatured into two complementary single strands of DNA by heating and rapidly cooling the DNA before the reaction, an action which destroys the activity of most enzymes. In the annealing phase, Taq polymerase joins deoxyribonucleotides onto the 3' end of one DNA strand, called the primer, and is only able to carry out this activity when the primer is annealed in complementary fashion to the DNA template strand. In a population of denatured DNA strands, synthesis of new DNA can be made highly specific by supplying an excess of oligonucleotides (oligos). After denaturation, the reaction is quickly cooled, preventing immediate reannealing of long DNA strands. Due to their small size, oligos rapidly anneal to single strands of DNA at positions containing the specified template sequence. In these positions, they act as primers for Taq polymerase.

Polymerization, or extension, is the final step of the PCR cycle in which the temperature of the reaction is adjusted to the optimum for Taq polymerase activity. During this step, the polymerase enzyme incorporates nucleotides into the nascent DNA strand, producing a complementary copy of the DNA template in the region specified by the annealed primer. The new temperature is above that at which annealing occurs, but does not lead to denaturation of the primer-template complex. This presumably occurs because the enzymes are already active at the annealing temperature, and significantly increase primer length during the annealing step, thus raising its denaturation temperature above that of the polymerization step. The mechanism of amplification involves first a polymerization step, in which a new DNA strand is synthesized onto each primer, giving a nucleotide sequence complementary to the original template. The polymerase enzyme will continue to extend this new strand for the duration of the polymerization step. The

same occurs on the other template strand, using the other oligo. Therefore, the original template has effectively been copied in this region, producing two DNA molecules which may be copied again in the next cycle. The DNA is again denatured in the first step of the second cycle. More primer anneals in the second step, both to the original template strands and to the new DNA strands produced in the first cycle. Polymerization proceeds as before, but in the latter case, synthesis of new DNA terminates when it reaches the 5' end of the template, represented by the primer of the first cycle. Thus, if the second oligo primes new DNA synthesis of the first oligo strand of cycle one, the complementary DNA strand terminates at the 5' end of oligo one, as there is no further template DNA beyond this point. The length of the new strand produced in this way corresponds to the distance between the two PCR primers. During the third cycle, the DNA strands of defined length synthesized in the second cycle are reprimed with the opposite primer in the third cycle. Polymerization leads to the synthesis of two copies of double-stranded DNA of the defined fragment size. The number of fragments is doubled during each PCR cycle, while the original template strands are copied only once. The ds DNA copies therefore become the major reaction product, accumulating logarithmically.

The three steps of the polymerase chain reaction are initiated by temperature changes. Temperatures chosen for each step are crucial to the specificity and efficiency of the chain reaction phenomenon, as are the times spent on each step and in adjusting temperatures between steps.⁴⁴ For denaturation, the reaction mix must initially be heated sufficiently to denature long stretches of double-stranded DNA. Degradation of DNA is accelerated at elevated temperatures, as is the breakdown of enzymes. A compromise must therefore be found between allowing enough time for the template DNA to denature and reducing the total amount of time spent at a high temperature, as even "heat stable" Taq polymerase loses activity at a rate proportional to temperature. Amplification reaction is not infinite. After a certain number of cycles, the desired amplification fragment gradually stops accumulating exponentially and enters a linear or stationary phase. This stage of the reaction is called the plateau stage.⁴⁴

Following denaturation, the DNA must be cooled rapidly to prevent reannealing of the original strands. The time spent at the predefined annealing temperature is somewhat of a tradeoff. If left too long, mispriming (annealing of oligos to the wrong sequences) or template reannealing may occur; if too short, insufficient primer annealing may occur.

In the last few years, advances in PCR have included modifications of the technique to enable amplification of DNA from archival material, making large data banks of paraffin-embedded material available for study.⁴³ DNA in paraffin-embedded sections tends to be more degraded than genomic DNA, and PCR of products much larger than 400 base pairs can be difficult. Inhibitors tend to be present in paraffin embedded sections. Amplification from paraffin-embedded tissue is less efficient than from blood or frozen tissue. To compensate for this, cycling parameters can be altered by increasing the number of cycles or increasing the time at each step in the cycle. Special digestion buffers are available for DNA extraction from paraffin sections fixed in Bouin's fixative, which until recently resulted in almost complete failure of PCR amplification.⁴³

The possibility of contamination during amplification is real, given the capacity of PCR to synthesize millions of DNA copies. Contamination of the sample reaction generally occurs with either products of a previous reaction (product carryover) or with material from an exogenous source.⁴⁴ In general, careful laboratory procedure, prealiquoting reagent, use of positive displacement pipettes, and physical separation of the reaction preparation from analysis of the reaction products are all precautions that reduce this risk. Carrying out only minimal numbers of PCR cycles required for analysis also minimizes the chance that a contaminating template will be amplified.⁴⁴ A panel of "blank" reactions with no template DNA is used to detect contamination.

Primer dimer is an amplification artifact often observed in the PCR product, especially when many cycles of amplification are performed on a sample containing very few initial copies of template. It is a double-stranded fragment whose length is very close to the sum of the 2 primers and appears to occur when one primer is extended by the

polymerase over the other primer. The result is an extremely efficient PCR template that can, if it occurs at an early cycle, easily overwhelm a reaction and become the predominant product.⁴⁴

Following PCR, products are separated by agarose gel electrophoresis. Ethidium bromide can be incorporated into the gel or applied in a subsequent step to bind DNA. The DNA-ethidium bromide complex will fluoresce under ultraviolet (UV) illumination, allowing visualization of PCR products. If a DNA marker of a known base pair size is included on the gel, it can be visually ascertained whether amplification of a sequence the size of the intended target has occurred. This is presumptive evidence that the DNA sequence in question was present in the original sample. Because there is a possibility of mispriming and production of products of a similar length, yet dissimilar sequence, confirmation of the identity of the reaction product is required. Evaluation of its nucleotide sequence is performed and then compared to the intended target sequence. If sufficient homology exists between target and product nucleotide sequence, this confirms the intended sequence was amplified. In the nucleotide sequence analysis of PCR products, sequences should be determined from multiple clones to distinguish misincorporated nucleotides from the faithful copies of the template sequence.

Detection of FeLV is complicated by the fact that the cellular DNA of healthy, uninfected cats contains nucleotide sequences related to, and with partial homology to, exogenous FeLV.⁴⁷ Complications can occur if the region selected for amplification by PCR is one where homology exists between the two. Major dissimilarities between endogenous FeLV-related sequences and exogenous are found within the U3 region of the LTR sequence.^{1,48,49} The U3 region is highly conserved among exogenous FeLV isolates, with an overall sequence conservation of at least 95% when multiple isolates from the United States and the United Kingdom were compared.⁴⁹ These properties make the U3 region an appropriate target for the PCR reaction. Oligonucleotide primers targeting sequences of variable base pair size within the U3 region have successfully identified FeLV proviral DNA in peripheral blood, corneal tissue, and formalin-fixed samples.^{5,41,43,50}

Materials and Methods

Sample Collection

Blood and bone marrow samples from fifty cats and bone marrow from one fetus were collected. Eleven of the cats were laboratory cats that were born and raised in a laboratory facility, tested FeLV negative as kittens, and housed together without contact with other cats for eight years. Thirteen cats were from a humane society. Ten cats and one fetus were brought to the Veterinary Teaching Hospital (VTH) (n = 9 cats, n = 1 fetus) or seen at another practice (n=1) as strays. Sixteen cats were evaluated at the VTH (n=15) or other practice (n = 1). These sixteen cats had diseases associated with FeLV-infection that were diagnosed by use of hematology, cytologic, and histopathologic evaluation, and laboratory information was assessed using VTH reference values. These diseases included granulocytic leukemia (n=3), lymphocytic leukemia (n=2), nonregenerative anemia (n=2), lymphoma (n=3), renal failure (n=1), pancytopenia (n=1), leukopenia (n=1), neutropenia (n=1), hemolytic anemia with thrombocytopenia (n=1), and thrombocytopenia with tetraparesis (n=1). Experimental design and procedures of this project were approved by the University Animal Care and Use Committee of Virginia Tech.

Blood was collected via jugular venipuncture using a 3 ml syringe and 22 gauge needle, and was equally divided between an EDTA tube and a serum separator tube. Cats were either sedated with ketamine¹ (10 mg/kg body weight, intravenously (IV)) and diazepam² (0.2 mg/kg body weight, IV) or were placed under general anesthesia with thiopental³ (10 mg/kg body weight, IV) for induction and maintained with isoflurane⁴ in oxygen anesthesia for bone marrow collection. Bone marrow samples were obtained from the humerus using a 14 gauge bone marrow needle, or within three minutes of euthanasia using rongeurs to fracture the humerus, and the marrow carefully dissected. Bone marrow

¹ Vetamine, Schering Plough, Phoenix Scientific, Inc., St. Joseph, Missouri

² Valium, Elkins-Sinn, Cherry Hill, New Jersey

³ Pentothal, Abbot Laboratories, North Chicago, Illinois

⁴ Isovet, Mallinckrodt Veterinary, Mundelein, Illinois

was obtained in this manner from the fetus after euthanasia of the queen; blood could not be obtained from this fetus. Marrow samples were placed in EDTA tubes. Air-dried smears were made both from blood and marrow samples. EDTA tubes with samples were placed on ice within five minutes of being obtained. Thirty minutes after collection, serum separator tubes were centrifuged at 10,000 rpm for five minutes. Serum was separated and placed on ice. Blood, serum, and marrow samples were stored at -70° C within 15 minutes to 36 hours until analysis. Samples from two cats from referring veterinarians were obtained and stored in EDTA tubes using unknown methods prior to sending them to the VTH. Stray cats were euthanized via an overdose of intravenous barbiturates.

Enzyme-Linked Immunosorbent Assay and Immunofluorescent Antibody Test

A commercial ELISA test⁵ was performed on serum samples to detect the presence of free FeLV p27 antigen on 49 cats. The two cats from private practices were tested with unknown ELISA test kits. FeLV immunofluorescent antibody test for intracellular p27 was performed on the air-dried blood and bone marrow smears.⁶

DNA Extraction

Genomic deoxyribonucleic acid (DNA) was extracted from 200 µl aliquots of blood and bone marrow. DNA was extracted using standard extraction kits.⁷ Blood extraction protocols were used to extract DNA from blood samples while a tissue protocol was used to extract DNA from bone marrow and stored FL74 cell culture. FL74, a persistently FeLV-infected cell line, was used as a positive control. Confirmation of DNA was performed following extraction. Three microliters of the genomic DNA extraction was run on a low-melt 1.5% agarose gel with added ethidium bromide. A 100 bp DNA ladder was included on each gel as a size standard. Electrophoresis in 1X Tris-

⁵ Feline Leukemia Virus Antigen/Feline Immunodeficiency Virus Antibody Test Kit, IDEXX, Westbrook, Maine

⁶ National Veterinary Laboratory, Franklin Lakes, New Jersey

⁷ QIAgen tissue kit, QIAGEN Inc., Santa Clarita, California

Borate-EDTA (TBE) buffer was performed at 5V/cm for approximately 45 minutes. Following electrophoresis, the gel was visualized and photographed under UV illumination.

Primers

Oligonucleotide primers targeting a 495 base pair (bp) sequence in the FeLV U3 LTR region were designed and synthesized. The sequences were:

Forward primer: 5' – GCCAGCTACTGCAGTGGTGTTCAT - 3'

Reverse primer: 5' - CTGGGGGTTGGGGGTCTTTATCC – 3'

Oligonucleotide primers for a mitochondrial DNA copy of the 16s RNA gene were utilized as positive controls to test DNA integrity. The primer sequences were:

16s - AR: 5' – CGCCTGTTTATCAAAAACAT – 3'

16s - BR: 5' – CGCGTCTGAACTCAGATCACGT – 3'

Polymerase Chain Reaction

Ready-to-go PCR beads⁸ (0.2 ml) were used following the manufacturer's recommended protocol. For each reaction, a 1 µl sample of DNA template, 2 µl of each primer, and 20 µl of HPLC grade water were added to the PCR bead. Polymerase chain reaction was performed in a DNA thermal cycler.⁹ The PCR protocol included an initial denaturation step at 94° C, which was performed for 2 minutes. This was followed by 35 cycles of 20s denaturation at 94° C, 20s primer annealing at 60°C, and 45s extension at 72° C. A two minute final extension at 72° C was performed, and then samples were held at 4° C until further analysis. Samples that failed to amplify FeLV DNA at 60°C annealing temperature were repeated at an annealing temperature of 56° C, with other cycling parameters remaining constant to allow more nonspecific binding, and therefore decrease the chance of a false negative result. Two cats (cat 50 and 51) had bands

⁸ Amersham-Pharmacia Biotech, Piscataway, New Jersey

⁹ PCRExpress, Hybaid, Ashford, Middlesex

detected under UV illumination on both blood and bone marrow samples that were not of the expected base pair size. These samples were repeated using Q-Solution in PCR, which changes the melting behavior of DNA for systems not working well under standard conditions.

The integrity of genomic DNA was tested using the 16s primers. Samples which failed to amplify 16s DNA were repeated with 0.1X, 0.001X, 1X, 2X, and 5X DNA template concentrations. PCR analyses were run concurrently with a water template as a negative control.

Following PCR, 4 µl of each product was fractionated on a low-melt 1.5% agarose gel with added ethidium bromide. A 100 bp DNA ladder was included on each gel as a size standard. Electrophoresis in 1X TBE buffer was performed at 5V/cm for approximately 45 minutes. Following electrophoresis, the gel was visualized and photographed under UV illumination. If a weak positive was noted in the area of the expected size of the amplified FeLV product, a punch sample of the agarose gel containing the weak positive band was obtained. The gel was liquified at 62° C with 150 ul of HPLC grade water for ten minutes. A 1 µl sample of this was amplified at 60° C at 25 cycles, and fractionated on a 1.5% agarose gel as previously described. PCR test results were considered positive if a 495 base pair band was visualized on ethidium-bromide-agarose gel electrophoresis viewed with UV transillumination.

DNA Sequencing

Two randomly selected amplified bone marrow PCR products of the expected size (cats 12 and 36), and both blood and bone marrow PCR products of cat 51 were DNA sequenced.¹⁰ The PCR product was purified following the manufacturer's protocol for DNA purification.¹¹

¹⁰ DNA Sequencing Facility, Virginia Tech, Blacksburg, Virginia

¹¹ QIAquick PCR Purification Kit, QIAGEN Inc., Santa Clarita, California

Both strands of the purified DNA product from bone marrow of cat 36 were sequenced and aligned using Sequencher¹² by an automated fluorescent DNA sequencer¹³ using the Thermosequenase Dye-Terminator Cycle Sequencing Kit.¹⁴

Both strands of the purified DNA product from bone marrow of cat 12 and blood and bone marrow of cat 51 were sequenced using standard methods on an ABI 377 automated DNA sequencer.¹⁵

Statistical Analysis

Kappa statistics were calculated to determine agreement beyond that due to chance between the following tests: ELISA and IFA blood, ELISA and IFA bone marrow, ELISA and PCR blood, ELISA and PCR bone marrow, IFA blood and IFA bone marrow, IFA blood and PCR blood, IFA blood and PCR bone marrow, IFA marrow and PCR blood, IFA marrow and PCR marrow, and PCR blood and PCR marrow.⁵¹ Additionally, kappa statistics were calculated for the above listed tests for the clinical case cats alone.

¹² GeneCodes, Ann Arbor, Michigan

¹³ ALFExpress II, Amersham-Pharmacia Biotech, Piscataway, New Jersey

¹⁴ Amersham-Pharmacia Biotech, Piscataway, New Jersey

¹⁵ PE Biosystems Big Dye Terminator chemistry, Foster City, California

Results

Blood and bone marrow samples from eleven laboratory cats, twenty-three random-source cats, one random-source fetus, and sixteen clinical cases were analyzed. DNA extracted from FL74 cells was consistently positive on PCR testing, and controls were consistently negative. A representative gel is shown in Figure 1.

DNA was detected from all DNA extractions from blood and bone marrow from the eleven laboratory cats. The eleven laboratory cats had negative FeLV ELISA, negative blood and bone marrow FeLV IFA, and negative blood and bone marrow FeLV PCR at 60° C and 56° C amplification (Table 1).

Results from 23 random-source cats and one fetus are summarized in Table 2. Intact DNA from blood and bone marrow extractions was detected from all random source cats and the fetus, except cat 13 which had degraded bone marrow DNA, seen as a smeared band when visualized under UV illumination. Eighteen of 23 random source cats (cats 17 through 34) had negative results on serum ELISA, blood and bone marrow IFA, and blood and bone marrow PCR (56° C and 60° C amplification). Three cats (cats 12, 14, and 15) were positive on ELISA, blood and bone marrow IFA, and blood and bone marrow PCR. Positive ELISA with negative blood and bone marrow IFA, and negative blood and bone marrow PCR (both 56° C and 60° C amplification) were noted in one cat (cat 16). One cat (cat 13) was positive on ELISA, blood and bone marrow IFA and blood PCR; DNA from the bone marrow was degraded and did not amplify. The fetus (cat 35) was positive on bone marrow IFA and bone marrow PCR.

Results of the 16 clinical cats are summarized in Table 3. DNA was detected from blood and bone marrow DNA extractions of all clinical case cats. Twelve of the sixteen clinical case cats (cats 38, 40 – 47, 49 – 51) were negative on serum ELISA, blood and bone marrow IFA, and blood and bone marrow PCR (both 56° C and 60° C amplification). PCR on both blood and bone marrow of cats 50 and 51 revealed bands of approximately 700 bp under UV illumination. Using Q-solution in PCR assays, cat 50

was negative on both blood and bone marrow PCR. Cat 51 had weak positive bands in the 495 bp region for both blood and bone marrow, however sequence analysis revealed that DNA was not FeLV. Therefore, cat 51 was considered negative on both blood and bone marrow PCR. One cat with lymphocytic leukemia (cat 36) was positive on ELISA, weak positive on blood IFA, and negative on bone marrow IFA, with positive blood and bone marrow PCR. One with renal failure (cat 37), was positive on ELISA, and blood and bone marrow IFA, and both blood and bone marrow PCR. One cat (cat 39) was negative on serum ELISA, negative on IFA blood, positive on IFA bone marrow, and negative on both blood and bone marrow PCR (both 56° C and 60° C amplification). One cat (cat 48) was weakly positive on serum ELISA, negative on blood and bone marrow IFA, and negative on blood and bone marrow PCR (both 56° C and 60° C amplification).

Polymerase chain reaction products of bone marrow from two of six randomly selected FeLV positive animals (cats 12 and 36) were sequenced. Comparison of each sequence with a previously published FeLV provirus 5' LTR-gag gene⁵² sequence revealed 97.5% homology.

Kappa values for all cats revealed perfect agreement between PCR blood and PCR bone marrow, IFA blood and PCR blood, and IFA blood and PCR bone marrow (k=1.0). Almost perfect agreement was noted between ELISA and IFA blood (k=0.84), ELISA and PCR blood (k=0.83), ELISA and PCR marrow (k=0.83), IFA bone marrow and PCR blood (k=0.81), IFA bone marrow and PCR bone marrow (k=0.81), and IFA blood and IFA bone marrow (k=0.81). Substantial agreement between ELISA and IFA bone marrow (k=0.69) was noted (Table 4).

Kappa values performed on results from the clinical case cats revealed perfect agreement with PCR blood and PCR bone marrow, IFA blood and PCR blood, and IFA blood and PCR bone marrow (k=1). Substantial agreement was present between ELISA and IFA blood (k=0.77), ELISA and PCR blood (k=0.76), and ELISA and PCR bone marrow (k=0.76). Moderate agreement was noted between, IFA blood and IFA bone marrow (k=0.47), IFA bone marrow and PCR blood (k=0.42), and IFA bone marrow and

PCR bone marrow ($k=0.42$). Fair agreement was noted between ELISA and IFA bone marrow ($k=0.29$). A scale for interpretation of kappa values can be found in Table 4. ⁵³

Discussion

Results indicate that PCR detects FeLV proviral DNA in both blood and bone marrow of infected cats. However, no latent infections (positive bone marrow PCR only) were detected. Although PCR techniques have not been previously evaluated for diagnosis of FeLV latency, PCR currently is used for this purpose in diagnosing latent infections in both human and veterinary medicine. Therefore, PCR would be expected to be useful for FeLV latency diagnosis.

The laboratory cats, which had tested FeLV negative as kittens, and had been housed together without contact with other cats for 8 years, were negative on all FeLV tests performed. These cats were not expected to have latent infections, as there had been no exposure to infected cats.

Of the random-source cats, animals 12, 14, and 15 were positive on all FeLV tests (ELISA, IFA blood and bone marrow, and PCR blood and bone marrow). This is the expected finding in persistently infected cats. Cat 13 had a degraded bone marrow sample, and DNA could not be amplified with the PCR method used in this study. This bone marrow sample was not placed on ice or refrigerated for 36-72 hours prior to long-term storage, accounting for degradation. It is possible that using a more sensitive PCR assay, such as nested PCR, would have resulted in DNA amplification. All FeLV tests performed on Cat 13 (ELISA, IFA blood and bone marrow, and PCR blood) were positive, consistent with persistent infection. Cat 35, the fetus of a persistently infected queen (cat 14), was positive on bone marrow IFA and PCR. The fetus had likely become persistently infected transplacentally, as genetic or vertical transmission has not been reported with FeLV infection. Cat 16 had discordant test results, with a positive ELISA and negative IFA and PCR on both blood and bone marrow. This likely represents a transient viremia. Other possible explanations include false positive ELISA results due to technician or test error. It is also possible, but unlikely, that both IFA and PCR tests were falsely negative. False negative IFA test results can be associated with technician error, leukopenia, or latency. PCR false negatives can occur with technician error or test error.

Random source cats 17 through 34 were negative on all FeLV tests performed. Due to the unknown history of these cats, recent infection and latent infection were considered possible. However, PCR did not detect proviral FeLV DNA, and therefore no evidence of latency existed in this group of cats.

The 16 clinical case cats had diseases that are associated with FeLV infection, and therefore persistent and latent FeLV infections were considered possible. Cats 38, 40 through 47, and 49 through 51 tested FeLV negative on ELISA, IFA blood and bone marrow, and PCR blood and bone marrow. Therefore, these cats were neither persistently nor latently infected. In these cases, FeLV infection does not appear to be related to the disease process. Cat 36, diagnosed with lymphocytic leukemia, which had a positive ELISA, weak positive blood IFA, negative bone marrow IFA, and was positive on both blood and bone marrow PCR, was likely persistently infected. It is unusual for IFA to be positive on blood and negative on bone marrow, as the marrow has a greater concentration of white blood cells. The discordancy in the IFA test results may have resulted from technician or test error. Cat 37, an animal with renal failure, was positive on all FeLV tests, and was therefore persistently infected with FeLV. Cat 39, an animal with granulocytic leukemia, also had discordant results. FeLV IFA bone marrow was positive, and all other tests were negative. The discordancy is likely due to false positive bone marrow IFA, as it is unlikely that all four other tests would be falsely negative. Cat 48, an animal with multicentric lymphoma, had a weak positive ELISA, and negative IFA and PCR results. Weak positive ELISA results generally represent a lower antigen burden. The discordancy noted in this case likely resulted from a transient viremia with low viral burden, or a false positive ELISA from technician or test error. However, the latter was considered more likely as PCR blood should be positive with a positive ELISA.

Using standard PCR methods, cats 50 and 51 had PCR products amplified from the blood and bone marrow that were larger than expected. Although these were technically negative results, these samples were further evaluated to confirm this was not in fact FeLV. One explanation for obtaining a larger PCR product than expected include

nonspecific primer annealing at different sites of DNA if the target sequence is not present. Though a PCR product in the approximate expected range of the 495 bp fragment was obtained from DNA from blood and bone marrow from cat 51 after additional techniques were performed, this cat was considered negative. The negative result could be explained by the following: technique modifications were needed to amplify the sequence and resulted in only a weak positive band, and the samples did not reveal FeLV proviral DNA upon sequencing.

Cats 12 and 36, that had PCR products of the expected weight, were chosen at random to be sequenced. Sequence analysis of these products revealed 97.5% homology to a previously published FeLV sequence. This high degree of homology revealed that these were true FeLV amplicons. Though not all positive PCR results were DNA sequenced, the remainder of positive PCR samples all had distinct bands of the expected weight on gel electrophoresis when run under standard conditions, and are assumed to have similar degrees of homology. Previous studies involving PCR of FeLV have not reported DNA sequence confirmation of any sample, and therefore the sequencing of two samples from the six positive animals was considered adequate for this study.

Kappa statistics revealed perfect agreement between test results for PCR blood and PCR bone marrow, IFA blood and PCR blood, and IFA blood and PCR bone marrow. All remaining kappa statistics for all cases revealed almost perfect or at least substantial agreement. Some degree of test result variation is expected between cases as the various tests use different methodologies and may detect FeLV infection at different stages, but overall a high agreement between tests was present. In the cases of clinically affected cats, kappa statistics did not reveal as strong agreement for some test comparisons. Therefore, in cats presenting with a high suspicion of FeLV-related disease, it appears that test result agreements may be lower. In this study, PCR results were similar to IFA blood results, and therefore PCR testing did not provide any additional information. To determine if these results on IFA and PCR are in fact similar, a larger number of clinical cases with FeLV-associated diseases should be evaluated.

The prevalence of FeLV latent infection is reported to diminish with time after exposure to the virus. Fifty-six percent of susceptible cats in a household in which a viremic cat was introduced had latent infections confirmed by virus isolation 9 months after exposure. This number diminished gradually, so by three years after exposure, only eight percent of cats were latently infected.^{26,54} It is likely that a smaller percentage of cats have latent infections which persist for a longer period of time, although an exact number is unknown. In the present study, no evidence of latency was detected in any group of cats, which is lower than expected compared to these previously reported experimental studies.^{26,54} This is also unusual as the diseased cats were highly suspected to have FeLV related conditions. A decrease in prevalence of FeLV infection has been noted from the Tufts Veterinary Diagnostic Laboratory. At this laboratory, a gradual decline has been noted in the number of positive ELISA test results, from 8% in 1989 to 4% in 1995.⁵⁵ Previous studies have anecdotally noted that fewer FeLV positive cats are affected by lymphoma than was noted 10-15 years ago. In a recent study of feline lymphoma, only 25% of lymphoma cases were associated with FeLV antigenemia, compared with the earlier published 60-70% rates.⁵⁶ Possible causes for this decrease in prevalence include routine testing of kittens with subsequent eradication, and the development of FeLV vaccinations.

Although FeLV latency is reportedly responsible for a variety of diseases, and it is suspected that bone marrow may be the primary site, it appears this is not true in all cases. The lack of evidence of latency in any group in this study indicates these cats were either not latently infected, the PCR results were falsely negative, or infection may be sequestered in areas other than the bone marrow. Further studies on a larger number of cats with high suspicion for naturally-occurring FeLV-related disease are needed to determine if these results can be verified.

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Figure Legend

Figure 1. Results of polymerase chain reaction of feline leukemia virus on ethidium-bromide stained gel. Expected size 495 bp. Lane1 – molecular weight marker, arrow indicates 500 bp; 2 – PCR product from FL74 (persistently infected FeLV cell culture) used as positive control; 3 – PCR product from negative control; 4- PCR product from blood of cat 12; 5 – PCR product from bone marrow of cat 12 6 – PCR product from blood of cat 10; 7 – PCR product from bone marrow of cat 35.

Figure 2. DNA sequence of PCR product from bone marrow of cat 12. This sequence was 97.5% homologous to a previously published FeLV LTR sequence.

Figure 3. DNA sequence of PCR product from bone marrow of cat 36. This sequence was 97.5% homologous to a previously published FeLV LTR sequence.



Figure 1 - Results of polymerase chain reaction of feline leukemia virus on ethidium-bromide stained gel. Expected size 495 bp. Lane1 – molecular weight marker, arrow indicates 500 bp; 2 – PCR product from FL74 (persistently infected FeLV cell culture) used as positive control; 3 – PCR product from negative control; 4- PCR product from blood of cat 12; 5 – PCR product from bone marrow of cat 12; 6 – PCR product from blood of cat 10; 7 – PCR product from bone marrow of cat 35.

TTCACAAGGCATGGAAAATTACTCAAGTATGTTCCCATGA
GATAACAAGGAAGTTAGCAGCTAAAACAGGATATCTGTGGT

TAAGCACCTGGGCCCCGGCTTGAAGCCAAGAACAGTTAAA
CTCCGGATATAGCTGAAACAGCAGAAGTTTCAAGGCCGCT

GCCAGCAGTCTCCAGGCTCCCCAGTTGACCAGAGTTCGAC
CTTCCGCCTCATTTAAACTCACCAATCCCCACGCCTCTCGC

TTCTGTACGCGCGCTTTCTGCTATAAAACGAACCATCAGC
CCCCAACGGGCGCGCAAGTCTTTGCTGAGACTTGACCGCC

CCGGGTACCCGTGTACGAATAAACCTCTTGCTGTTTGCATC
TGACTCGTGGTCTCGGTGTTCCGTGGGCACGGGGTCTCAT

CGCCGAGGAAGACCTAGTTCGGGGGTCTTTCATTTGGGGG
CTCGTCCG

Figure 2. DNA sequence of PCR product from bone marrow of cat 12. This sequence was 97.5% homologous to a previously published FeLV LTR sequence.

TTCACAAGGCATGGAAAATTACCCAAGTATGTTCCCATGAG
ATACAAGGAAGTTAAAGGCTAAAACAGGATATCTGTGGTTA

AGCACCTGGGCCCCGGCTTGAGGCCAAGAACAGTTAAACC
CCGGATATAGCTGAAACAGCAGAAGTTTCAAGGCCGCTGC

CAGCGGTCTCCAGGCTCCCCAGTTGACCAGAGTTCGACCTT
CCGCCTCATTAAACTAACCAATCCCCACGCCTCTCGCTTCT

GTACGCGCGCTTTCTGCTATAAAACGAGCCATCAGCCCCCA
ACGGGCGCGCAAGTCCTTGCTGAGACTTGACCGCCCCGGGT

ACCCGTGTACGAATAAACCTCTTGCCGATTGCATCTGACTCG
TGGTCTCGGTGTTCCGTGGGCGCGGGGTCTCATCACCGAGGA

AGACCTAGTTCGGGGGTCTTTCATTTGGGGGCTCGTCCG

Figure 3. DNA sequence of PCR product from bone marrow of cat 36. This sequence was 97.5% homologous to a previously published FeLV LTR sequence.

Table 1. Feline leukemia virus test results of eleven laboratory cats.

Cat No.	ELISA	IFA BLD	IFA BM	PCR BLD	PCR BM
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	-	-	-	-	-
6	-	-	-	-	-
7	-	-	-	-	-
8	-	-	-	-	-
9	-	-	-	-	-
10	-	-	-	-	-
11	-	-	-	-	-

ELISA = peripheral blood enzyme-linked immunosorbent assay, IFA BLD = peripheral blood immunofluorescent antibody, IFA BM = bone marrow immunofluorescent antibody, PCR BLD = blood polymerase chain reaction, PCR BM = bone marrow polymerase chain reaction, (-) = negative result.

Table 2. Feline leukemia virus test results of twenty-three random source cats (12 through 23).

Cat No.	ELISA	IFA BLD	IFA BM	PCR BLD	PCR BM
12	+	+	+	+	+
13	+	+	+	+	ND
14	+	+	+	+	+
15	+	+	+	+	+
16	+	-	-	-	-
17	-	-	-	-	-
18	-	-	-	-	-
19	-	-	-	-	-
20	-	-	-	-	-
21	-	-	-	-	-
22	-	-	-	-	-
23	-	-	-	-	-
24	-	-	-	-	-
25	-	-	-	-	-
26	-	-	-	-	-
27	-	-	-	-	-
28	-	-	-	-	-
29	-	-	-	-	-
30	-	-	-	-	-
31	-	-	-	-	-

Table 2. Continued

Cat No.	ELISA	IFA BLD	IFA BM	PCR BLD	PCR BM
32	-	-	-	-	-
33	-	-	-	-	-
34	-	-	-	-	-
35	N/A	N/A	+	N/A	+

ELISA = peripheral blood enzyme-linked immunosorbent assay, IFA BLD = peripheral blood immunofluorescent antibody, IFA BM = bone marrow immunofluorescent antibody, PCR BLD = blood polymerase chain reaction, PCR BM = bone marrow polymerase chain reaction, (-) = negative result, (+) = positive result. ND = not done, as DNA was degraded. N/A = test not performed.

Table 3. Feline leukemia virus test results and clinical diagnosis of sixteen clinical case cats (36 through 51).

Cat No.	ELISA	IFA BLD	IFA BM	PCR BLD	PCR BM	Diagnosis
36	+	+(w)	-	+	+	Lymphocytic leukemia
37	+	+	+	+	+	Renal failure
38	-	-	-	-	-	Pancytopenia
39	-	-	+	-	-	Granulocytic leukemia
40	-	-	-	-	-	Leukopenia
41	-	-	-	-	-	Lymphoblastic leukemia
42	-	-	-	-	-	Non-regenerative anemia
43	-	-	-	-	-	Alimentary lymphoma
44	-	-	-	-	-	Non-regenerative anemia
45	-	-	-	-	-	Hemolytic anemia, TCP
46	-	-	-	-	-	Hepatic/Splenic Lymphoma
47	-	-	-	-	-	TCP, Tetraparesis
48	+(w)	-	-	-	-	Multicentric lymphoma
49	-	-	-	-	-	Neutropenia
50	-	-	-	-	-	Granulocytic leukemia
51	-	-	-	-	-	Granulocytic leukemia

ELISA = peripheral blood enzyme-linked immunosorbent assay, IFA BLD = peripheral blood immunofluorescent antibody, IFA BM = bone marrow immunofluorescent antibody, PCR BLD = blood polymerase chain reaction, PCR BM = bone marrow polymerase chain reaction, (-) = negative result, (+) = positive result, +(w) = weak positive result, TCP = thrombocytopenia

Table 4. Kappa statistics values for test comparisons and interpretation for all cats and for clinical case cats.

FeLV Test Comparison	Kappa Value for All Cats (n=51)	Kappa Value for Clinical Cases (n=16)
IFA Blood and PCR Blood	1	1
IFA Blood and PCR Bone Marrow	1	1
PCR Blood and PCR Bone Marrow	1	1
ELISA and IFA Blood	0.84	0.77
ELISA and PCR Blood	0.83	0.76
ELISA and PCR Bone Marrow	0.83	0.76
IFA Blood and IFA Bone Marrow	0.81	0.47
IFA Bone Marrow and PCR Blood	0.81	0.42
IFA Bone Marrow and PCR Bone Marrow	0.81	0.42
ELISA and IFA Bone Marrow	0.69	0.29

ELISA = enzyme-linked immunosorbent assay, IFA = immunofluorescent antibody, PCR = polymerase chain reaction

Kappa Statistic Interpretation⁵³

Kappa Value	Strength of Agreement
0	No Better than chance
0.01 - 0.20	Slight
0.21 - 0.40	Fair
0.41 - 0.60	Moderate
0.61 - 0.80	Substantial
0.81 - 0.99	Almost Perfect
1	Perfect

Erin Leigh Stimson

Erin Leigh Stimson was born on August 17, 1969 in Hanover, New Hampshire. She attended high school in Gilford, New Hampshire prior to attending college at Duke University in Durham, North Carolina. She graduated in 1991 with a B.S. in Zoology. She worked for a year as a veterinary assistant at the Museum of Life and Science in Durham, N.C. following graduation. She attended North Carolina State University College of Veterinary Medicine, where she graduated as a Doctor of Veterinary Medicine in 1996.

Erin completed a small animal medicine and surgery internship at Virginia-Maryland Regional College of Veterinary Medicine in Blacksburg, VA in 1997. Following this, she began a residency in internal medicine also at Virginia-Maryland Regional College of Veterinary Medicine. She is currently board-eligible and will take the certifying examination in internal medicine in May, 2000.