Comparison of endogenous feline leukemia virus RNA content in feline vaccine and nonvaccine site-associated sarcomas

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Objective—To determine whether feline vaccine site-associated sarcomas (VSS) contain a higher amount of endogenous FeLV (enFeLV) RNA, compared with feline nonvaccine site-associated sarcomas (non-VSS).

Sample Population—Formalin-fixed paraffin-embedded (FFPE) tissues from 50 VSS and 50 cutaneous non-VSS.

Procedure—RNA was extracted from FFPE sections of each tumor, and regions of the long terminal repeat (LTR) and envelope (*env*) gene of enFeLV were amplified by use of reverse transcriptase-polymerase chain reaction (RT-PCR). The density of each RT-PCR product band for enFeLV was compared with that of a constitutively expressed gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). An integrated density value (IDV) was determined by use of densitometry, and the IDV ratio for enFeLV to GAPDH was calculated for each enFeLV primer set.

Results—The median (interquartile range) of the IDV ratio for the enFeLV LTR primer set was 0.52 (0.26 to 1.17) for the VSS group and 0.84 (0.21 to 1.53) for the non-VSS group. The median (interquartile range) of the IDV ratio for the enFeLV *env* primer set was 0.60 (0.37 to 0.91) for the VSS group and 0.59 (0.36 to 1.09) for the non-VSS group.

Conclusions—Because the amount of enFeLV RNA within the LTR and *env* gene was not significantly different between the VSS and non-VSS groups, enFeLV replication or expression is unlikely to be involved in VSS development. (*Am J Vet Res* 2001;62:1990–1994)

It has been demonstrated that an association exists between vaccination and fibrosarcomas in cats.[1,2] Feline **vaccine site-associated sarcomas (VSS)** have been linked to a variety of different vaccine types, including inactivated rabies, FeLV, and feline panleukopenia-rhinotracheitis-calicivirus vaccines.[3-5]

The low prevalence of VSS (1 to 3.6/10,000 cats vaccinated)[6,7] suggests that congenital or acquired genetic factors within individual susceptible cats may act as initiators of oncogenesis. Vaccination could increase replication or expression of **endogenous FeLV (enFeLV)** within proliferating fibroblasts or inflammatory cells. Malignant transformation could then result from insertional mutagenesis, promoter/enhancer influence, or aberrant protein expression by enFeLV. Our hypothesis is that increased replication or expression of endogenous FeLV sequences contributes to the pathogenesis of VSS.

Cellular DNA from clinically normal domestic cats is known to contain sequences partially homologous to the genome of horizontally transmitted FeLV.[8] These sequences are present at a rate of 8 to 14 copies/cell, arranged as discrete genetic elements in a nontandem fashion.[9,10] Although infectious FeLV has not been induced in uninfected cats,[11] recombination between FeLV-A and enFeLV elements of the normal cat genome may give rise to FeLV-B isolates.[12]

Earlier studies[13,14] have revealed there is low expression of enFeLV genes in placenta, fetal lymphoid tissues, and some FeLV-negative lymphomas. A more recent study[15] indicated that a subset of defective enFeLV sequences is highly expressed in lymphoma cell lines, primary lymphoid organs (thymus, spleen, lymph nodes), and tissues with extensive lymphoid compartments (bone marrow, intestines) but not in other tissues such as muscle, brain, and kidney from healthy specific pathogen-free cats or in 2 feline fibroblast cell lines.

The purpose of the study reported here was to determine whether VSS contain an increased amount of enFeLV RNA, compared with **nonvaccine site-associated sarcomas (non-VSS)**. This was accomplished using **formalin-fixed paraffin-embedded (FFPE)** tissues and a semiquantitative **reverse transcriptase-polymerase chain reaction (RT-PCR)** analysis.

Materials and Methods

Case selection—Two groups of FFPE tissues were obtained from biopsy specimens obtained from cats and submitted during 1996 to a veterinary diagnostic laboratory.[a] The first group (VSS) consisted of 50 fibrosarcomas determined to be associated with administration of vaccine on the basis of historical and clinical findings consistent with an association with vaccination and histologic findings of inflammation and necrosis within each tumor.[16] The second group (non-VSS) consisted of 50 cutaneous fibrosarcomas that were from anatomic locations that could not possibly be vaccination sites such as the head, tail, and digits; inflammation and necrosis were not features of the non-VSS.

A third group of FFPE tissues was obtained at necropsy from 5 healthy adult cats that had been euthanatized. These served as control tissues.

The RT-PCR primers—A commercial laboratory synthesized the primer nucleotide sequences.[b] Primer sequences,

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genome positions, and expected length of PCR products were determined (**Appendix**).

Endogenous FeLV—The PCR primers specific for enFeLV, designated enFeLV 1 and 2, were used to amplify a 217-**base pair (bp)** fragment of the U3 domain of the 3'- **long terminal repeat (LTR)**.[15] A second set of primers, designated enFeLV **envelope (*env*)** 4 and 2, was designed from the *env* nucleotide sequence of the enFeLV CFE-16 clone[17] and amplified a 96-bp fragment.

Glyceraldehyde-3-phosphate dehyrogenase—A sense primer was designed from the nucleotide sequence of rat GAPDH mRNA,[18] designated GAPDH3, whereas the antisense primer sequence, designated GAPDH2, was as published.[19] The 117-bp product was smaller than predicted by the previously published primer pair.[19]

RNA extraction—Sections (10 µm) of tissue were cut from each of the VSS and non-VSS tissue paraffin blocks and from lymph node (n = 5), skin (5), and kidney (2) from healthy feline tissues. Sections were placed into 1.5 ml Eppendorf tubes and deparaffinized by washing once with 1.2 ml of xylene and twice with absolute ethanol, both at room temperature (20 C). Each washing was followed by vortexing and then centrifugation at 13,000 × g for 5 minutes. After the last centrifugation step, the tubes were inverted on a paper towel to remove residual ethanol. Pellets were resuspended in 400 µl of digestion buffer 1 (500 µg/ml proteinase K, 50 m*M* Tris-HCl [pH 8.0], 5 m*M* EDTA [pH 8.0], and 0.5% sodium dodecyl sulfate), and digestion was performed as described.[20]

Ribonucleic acid extraction involved the following modifications of the procedure described by Chomczynski and Sacchi.[21] Following digestion, each sample was divided into 200-µl aliquots; then 200 µl of solution D (4*M* guanidium thiocyanate, 25 m*M* sodium citrate [pH 7.0], 0.1*M* 2 mercaptoethanol, 0.5% sarcosyl) and 200 µl of phenol:chloroform:isoamyl (24:24:1) were added to each tube. The final RNA preparation was dissolved in 50 µl of diethyl pyrocarbonate-treated water. The RNA concentration was measured by optical density.

DNase treatment—Ribonucleic acid was treated with DNase[e] to remove contaminating DNA.[22] The RNA samples were stored at –70 C until RT-PCR was performed.

Reverse transcriptase-PCR controls—Reverse transcriptase-PCR was performed on tissues from 5 healthy cats to determine whether enFeLV RNA was evident. Lymph node tissue from 1 of these cats was chosen as the positive control and internal standard to be included with each RT-PCR. Including a negative control that did not contain RT monitored contamination of RNA by DNA. A negative control containing no RNA was included in each RT-PCR to monitor contamination of RT-PCR reagents.

Reverse transcriptase-PCR—Reverse transcriptase-PCR was performed, using each of the 3 primer sets in the same run, on tumor RNA and on the normal lymph node RNA as an internal control. The cDNA obtained from the first step of the RT-PCR on each sample was divided equally among the 3 tubes containing the PCR reaction mix for each primer set. These precautions were important in this study to eliminate variability in efficiency of the RT step. The RT-PCR was performed on each tumor RNA sample once unless the GAPDH primers failed to yield product. Under these circumstances, the RT-PCR was repeated.

Reverse transcriptase-PCR was performed by use of a commercially available RNA PCR kit,[d] according to manufacturer's instructions. Random hexamers were used as the primer for cDNA synthesis. The final reaction volume for the PCR step was 50 µl, rather than 100 µl as in the kit instructions. The thermal cycler program was as follows: 95 C for 2 minutes, then 35 cycles of 95 C for 1 minute, 60 C for 1 minute, and 72 C for 7 minutes.

Electrophoresis and densitometry—A 10-µl aliquot of the RT-PCR product was electrophoresed[e] through a 2% agarose mini-gel according to standard procedure.[23] A 100-bp DNA size marker was included in each gel. The PCR products amplified by each primer pair for each sample were electrophoresed in the same gel to eliminate possible variability in staining intensity between agarose gels. The agarose gels were stained for 25 minutes with ethidium bromide, and the intensity of each RT-PCR band was determined by use of a documentation and analysis system,[f] providing a semiquantitative result referred to as **integrated density value (IDV)**. The IDV is a calculated value determined by the sum of all the pixel values after background correction.

Integrated density value ratios—Glyceraldehyde-3-phosphate dehydrogenase is constitutively expressed[19] and was chosen as an internal control within each sample, to identify variation in the degree of RNA degradation among samples. The ratio of the IDV obtained using enFeLV 1 and 2 primers to the IDV using the GAPDH primers (enFeLV 1 and 2 IDV/GAPDH IDV) was calculated for each tumor tissue and for the internal standard lymph node tissue for each RT-PCR run. Similarly, the ratio of the IDV using enFeLV *env* 4 and 2 primers to the IDV using the GAPDH primers (enfeLV *env* 4 and 2 IDV/GAPDH IDV) was calculated for each tissue. Each agarose gel was photographed under UV transillumination.[g]

Internal lymph node standard variation—Reverse transcriptase-PCR was performed in each run, using each primer set for the same RNA sample obtained from healthy feline lymph node. Mean (± SD) IDV ratios were calculated for samples from the lymph node. The SD assessed "between run" variability on an identical RNA sample.

Nucleotide sequencing of RT-PCR amplified products—The enFeLV and GAPDH RT-PCR products from normal lymph node were purified, using a gel extraction kit[h] in accordance with manufacturer's instructions. Purified RT-PCR products were submitted to another laboratory for automated sequencing.[i] Sequence data obtained using enFeLV 1 and 2 primer set and enFeLV *env* 4 and 2 primer set were compared with the nucleotide sequence of CFE-6 clone and enFeLV CFE-16 clone, respectively.[17] Sequence data obtained using the GAPDH 3 and 2 primer set was compared with the rat GAPDH mRNA sequence[18] and a feline GAPDH sequence.[24]

Data analysis—A median and interquartile range were calculated from the IDV ratio for each set of enFeLV primers for the VSS and non-VSS. The VSS and non-VSS IDV ratio values for each enFeLV primer set were compared by use of the Mann-Whitney test.[j] Means (± SD) were calculated from the IDV ratio values for each RT-PCR for the internal standard lymph node tissue.

Results

Examination of ethidium-bromide stained agarose gels of RT-PCR products typically revealed a single discrete band at the expected bp location using each of the 3 primer sets. None of the negative control samples revealed any bands. Invalid results were obtained with both enFeLV primer sets for 1 VSS and 1 non-VSS.

The RT-PCR results from the healthy feline tissues also revealed a single discrete band at the expected bp location for each of the 3 primer sets (**Fig 1**). The ranges of the IDV ratios for the healthy feline tissues

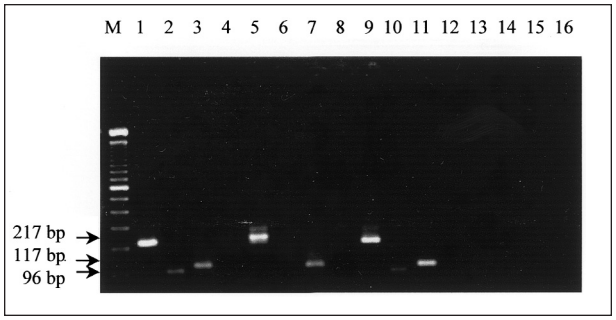

Figure 1—Ethidium bromide-stained agarose gel of reverse transcriptase-polymerase chain reaction (RT-PCR) products amplified from RNA extracted from healthy feline tissues. Lanes are as follows: M = 100-base pair (bp) DNA ladder; 1 to 4 = lymph node; 5 to 8 = kidney; 9 to 12 = skin. The 217-bp band (lanes 1, 5, and 9) represents RT-PCR product obtained using the endogenous FeLV (enFeLV) 1 and 2 primers; the 96-bp band (lanes 2, 6, and 10) represents the RT-PCR product obtained using the enFeLV *env* 4 and 2 primers; the 117-bp band (lanes 3, 7, and 11) represents the RT-PCR product obtained using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 3 and 2 primers. Lanes 4, 8, 12, and 16 are controls containing no reverse transcriptase enzyme. Lanes 13, 14, and 15 are negative controls containing no RNA for each primer set, enFeLV 1 and 2, enFeLV *env* 4 and 2, and GAPDH 3 and 2, respectively.

Table 1—Integrated density value (IDV) ratios obtained for healthy feline tissues, using each endogenous feline leukemia virus (enFeLV) primer set, compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by use of reverse transcriptase polymerase chain reaction (RT-PCR)

	IDV Ratio ranges	
Tissue	enFeLV 1 and 2/GAPDH	enFeLV *env* 4 and 2/GAPDH
Skin (n = 5)	0.2–1.04	0–0.9
Lymph node (n = 5)	1.49–2.88	0.81–1.81
Kidney (n = 2)	1.48–2.47	0.4–1.74

using each enFeLV primer set are presented (**Table 1**). The mean (± SD) IDV ratio obtained for the healthy feline lymph node standard was 1.35 (± 0.32) using the enFeLV 1 and 2 primer set and 0.99 (± 0.26) using the enFeLV *env* 4 and 2 primer set. All IDV ratio values (n = 23) obtained for the lymph node standard using the same RNA sample were within 2 SD of the mean using the enFeLV 1 and 2 primer set, and all except 1 were within 2 SD of the mean using the enFeLV *env* 4 and 2 primer set; the 1 value that fell outside this range was within 3 SD of the mean. Therefore, 98% of the results for the lymph node standard were within 2 SD of the mean, and 100% of the results were within 3 SD of the mean.

The medians (interquartile range) of the IDV ratios calculated for the tumor groups using each enFeLV primer set are presented (**Table 2**). Results of the 2-sample Mann-Whitney test, in which the IDV ratios of the VSS and non-VSS groups were compared, yielded *P* values of 0.35 and 0.77 for the LTR and *env* enFeLV primers, respectively. Therefore, the IDV ratios of the VSS and non-VSS groups were not significantly different.

Nucleotide sequencing of the enFeLV 217 bp LTR and the 96 bp *env* regions of normal lymph node revealed 97% and 99% identity to the published enFeLV sequences, respectively. The nucleotide sequence of the 117 bp GAPDH segment amplified from normal lymph node was 94% homologous to the rat sequence and 100% homologous to the feline sequence.

Table 2—Integrated density value (IDV) ratio medians (and interquartile range) obtained for the vaccine site-associated sarcomas (VSS) and the non-VSS tumor groups, using each enFeLV primer set, compared with GAPDH by use of RT-PCR

	Median IDV ratios (interquartile range)	
Tumor group	enFeLV 1 and 2/GAPDH	enFeLV *env* 4 and 2/GAPDH
VSS (n = 50)	0.52 (0.26–1.17)	0.60 (0.37–0.91)
Non-VSS (n = 50)	0.84 (0.21–1.53)	0.59 (0.36–1.09)
P value*	0.35	0.77

*Obtained using the 2-sample Mann-Whitney test comparing median IDV ratios of VSS and non-VSS groups.

Discussion

Use of RT-PCR is common for the assessment of gene expression; however, this method is usually applied to RNA extracted from fresh or frozen tissues. The use of FFPE tissues in RT-PCR reactions has limitations, particularly because of the degradation of RNA; only short segments of RNA (usually between 100 and 200 bp) can be amplified.[25] However, a major advantage of using FFPE tissues is the availability of larger numbers of archival tissues. In our study, use of RT-PCR allowed for successful amplification of 2 different regions of the enFeLV genome. The LTR primer set (enFeLV 1 and 2) was specific for enFeLV,[15] whereas the *env* primer set (enFeLV *env* 4 and 2) would also detect exogenous FeLV-B. The quantitation of RNA from FFPE tissues presents specific problems that cannot be resolved with a quantitative competitive analysis because of the variable amount of RNA degradation that occurs in these samples.[25] Competitive RT-PCR is used for the quantitation of absolute values of target RNA and usually depends on the use of an external standard that closely imitates the target RNA species. Integrity of RNA within samples is a source of variability that is typically not controlled for in competitive RT-PCR assays.[26] Because comparison of values obtained using quantitative competitive analysis between samples would most likely be inaccurate because of the variability of the amount of intact RNA between samples, we chose to use a semiquantitative method that involved comparison of the amount of enFeLV RNA to the amount of GAPDH RNA in the same sample. Amplification of the GAPDH mRNA from the same RNA preparation used for target amplification provided an endogenous internal standard. The use of an internal sequence such as GAPDH to which the target is compared relies on the assumption that the amount of degradation is comparable for any mRNA within a given sample.[25] The same cDNA sample was used for each of the 3 PCR for each sample, thereby eliminating variability in the amplification efficiency during the reverse transcriptase step. The nucleotide sequences obtained from purified RT-PCR product using both sets of enFeLV primers and GAPDH primers were highly homologous to sequences reported in previous studies,[17,18,24] which indicates that

the correct region was amplified. We used RNA extracted from a healthy feline FFPE lymph node as an internal standard in each RT-PCR. Most (98%) of the enFeLV/GAPDH IDV ratios for the internal lymph node standard were within 2 SD of the mean, and the remaining result was within 3 SD. These represent acceptable control limits[27] and ensure validity of the test results. Inclusion of a sample lacking the reverse transcriptase enzyme as a negative control confirmed that PCR products were attributable to RNA, not DNA, amplification.

The IDV ratios for healthy feline tissues used in our study indicated a detectable amount of enFeLV RNA in lymph node, kidney, and skin. Our results are in partial contrast to another study[15] in which expression of enFeLV genes was evaluated; results of that study indicated that enFeLV was expressed in primary lymphoid organs but not in other tissues including muscle, brain, and kidney nor in 2 feline fibroblast cell lines. Because RT-PCR is more sensitive than Northern immunoblot analysis,[28,29] this would most likely explain why detectable enFeLV RNA was found in skin and kidney tissue in our study.

Evaluation of the amount of enFeLV RNA in the VSS and non-VSS revealed no significant differences between the group median for each set of enFeLV primers. This indicates that the amount of enFeLV RNA within the LTR and *env* gene is not enhanced in sarcomas that are associated with vaccination, compared with fibrosarcomas that are not associated with vaccination. The amount of enFeLV RNA was variable among healthy feline tissues, the VSS, and the non-VSS, which is consistent with previous reports[12,30] indicating there is differential expression of enFeLV genes when introduced into susceptible cells. Transcriptional regulatory activity studies using isolated 5' LTR of enFeLV clones indicate that the basic characteristics of promotion and enhancement of transcription were retained in each LTR.[12,31] Also, the presence of enhancer functions residing in the enFeLV LTR lends support to the idea that enFeLV LTR may have the capability to influence the expression of cellular genes, which are in the same vicinity.[31]

The transcriptional activity of enFeLV LTRs is also variable because of the influence of strong transcription regulatory sequences in the 702-bp region of cellular DNA immediately upstream of the 5' boundary of the endogenous LTR.[12,31] The expression of endogenous FeLV sequences appears to be tightly regulated by proximal DNA sequences.

Replication of endogenous retroviral elements can result in movement of elements and, therefore, insertional activation or inactivation of genes.[32] Studies have revealed that members of endogenous retrovirus families are heterogeneous with respect to transcription and that subsets of a given family of endogenous retroviral elements are expressed in different tissues at different times. Long terminal repeat sequences have a key role in controlling expression of endogenous elements, and a correlation exists between specific LTR sequences and specific expression patterns.[32-35] Alternatively, other host factors may also control expression of endogenous retroviruses. The variability in expression of enFeLV among the tumor tissues may be related to the stage of differentiation of the cell. A common genetic lesion in cancer is reactivation of genes that are normally only active in fetal tissues.[11] This increased expression of certain genes may be related to the arrest of cells at an undifferentiated stage. In conclusion, the amount of enFeLV RNA from within the LTR and *env* gene is not increased in VSS as compared with non-VSS, indicating that enFeLV sequences are unlikely to be involved in the pathogenesis of VSS.

[a]Central Laboratory for Veterinarians Ltd, Langley, BC, Canada.
[b]Custom primers, Gibco BRL Life Technologies Inc, Burlington, ON, Canada.
[c]DNase I (RNase-free), Boehringer Mannheim Canada, Laval, QC, Canada.
[d]Gene Amp RNA PCR kit, Perkin Elmer Applied Biosystems, Mississauga, ON, Canada.
[e]Mupid-2 mini gel migration trough, Topogen Inc, Columbus, Ohio.
[f]Alpha Imager 2000 documentation and analysis system, Alpha Innotech Corp, San Leandro, Calif.
[g]FBTIV-816 transilluminator, Fisher Scientific Co, Edmonton, AB, Canada.
[h]QIAquick Gel extraction kit, Qiagen Inc, Mississauga, ON, Canada.
[i]DNA technologies unit, National Research Council of Canada, Plant Biotechnology Institute, Saskatoon, SK, Canada.
[j]Statistix for Windows, Analytical Software, Tallahassee, Fla.

Appendix

Reverse transcriptase-polymerase chain reaction primers for the amplification of endogenous feline leukemia virus (enFeLV) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Primer Name	Primer sequence 5' to 3'	Genome position	Target length (bp)
enFeLV 1	GACCCCCTGTCATAATATGC	2124–2143	NA
enFeLV 2	CCAGAATGAGGGGAACAAAC	2321–2340	217
enFeLV *env* 4	TGTAGTGGAGGTGGTTGGTG	560–579	NA
enFeLV *env* 2	GATCAAGGGGTTGCACCG	638–655	96
GAPDH 3	GCACCACCAACTGCTTAGCC	449–468	NA
GAPDH 2	GGCCATCCACAGTCTTCT	548–565	117

bp = No. of base pairs. NA = Not applicable.

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