Feline Leukemia Virus Detection by Immunohistochemistry and Polymerase Chain Reaction in Formalin-fixed, Paraffin-embedded Tumor Tissue from Cats with Lymphosarcoma

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ABSTRACT

The prevalence of feline leukemia virus (FeLV) antigen and DNA was assessed in formalin-fixed, paraffin-embedded tumor tissues from 70 cats with lymphosarcoma (LSA). Tissue sections were tested for FeLV gp70 antigen using avidin-biotin complex (ABC) immunohistochemistry (IHC); DNA was extracted and purified from the same tissue blocks for polymerase chain reaction (PCR) amplification of a 166 base pair region of the FeLV long terminal repeat (LTR). Results were related to antemortem FeLV enzyme-linked immunosorbent assay (ELISA) for serum p27 antigen, anatomic site of LSA, and patient age. Viral DNA was detected by PCR in 80% of cases and viral antigen by IHC in 57% of cases. Seventeen cases were PCR-positive and IHC-negative; one case was PCR-negative and IHC-positive. Clinical records included FeLV ELISA results for 30 of 70 cats. All 19 ELISA-positive cats were positive by PCR and IHC; of the 11 ELISA-negative cats that were negative by IHC, seven were positive by PCR. When evaluated according to anatomic site, FeLV DNA and antigen were detected less frequently in intestinal LSAs than in multicentric and mediastinal tumors. Lymphosarcoma tissues from cats <7 yr were several fold more likely to be positive for FeLV antigen by IHC than were tumors from cats ≥7 yr. However, there was no significant difference in PCR detection of FeLV provirus between LSAs from cats <7 yr and those ≥7 yr. Immunohistochemical detection of FeLV antigen in formalin-fixed, paraffin-embedded tissues agreed with antemortem FeLV ELISA results, however, FeLV DNA could be detected by PCR in some LSA tissues with no demonstrable FeLV antigen, particularly in cats ≥7 yr. These provirus-positive, antigen-negative cases may represent infection with latent or replication-defective FeLV.

RÉSUMÉ

Nous avons déterminé la prévalence de l'antigène et de l'ADN du virus de la leucémie féline (VLFe) dans des lymphosarcomes (LSA) préservés dans la formaline et enrobés dans la paraffine qui provenaient de 70 chats. Nous avons examiné des sections de tissu pour la présence de l'antigène gp70 du VLFe en employant la méthode immunohistochimique (IHC) du complexe avidine-biotine (ABC); nous avons procédé à l'amplification en chaîne par polymérase (ACP) d'une région de 166 paires de bases située dans la longue répétition terminale du VLFe à partir de l'ADN extrait et purifié des mêmes blocs de tissus. Nous avons mis en relation ces résultats avec ceux du test immunoenzymatique ELISA effectué chez les patients vivants pour la détection de l'antigène p27, ainsi qu'avec le site anatomique des LSA et finalement avec l'âge des patients. L'ADN viral a été détecté dans 80 % des cas par ACP et l'antigène viral a été détecté dans 57 % des cas par IHC. Dix-sept cas se sont avérés positifs par ACP et négatifs par IHC; un cas a été négatif par ACP et positif par IHC. Les dossiers cliniques de 30 des 70 chats incluaient les résultats du test ELISA. Les 19 chats positifs par ELISA étaient tous positifs par ACP et IHC; des 11 chats négatifs par ELISA, qui étaient négatifs par IHC, sept étaient positifs par ACP. Quant au siège des tumeurs, l'ADN et l'antigène viraux étaient détectés moins fréquemment dans les lymphosarcomes intestinaux que dans les tumeurs pluricentriques et médiastinales. Les lymphosarcomes des chats âgés de moins de sept ans étaient beaucoup plus souvent positifs par IHC pour l'antigène viral que ne l'étaient les tumeurs des chats âgés sept ans et plus. Par contre, il n'y avait pas de différence significative en ce qui a trait à la détection du provirus du VLFe par ACP entre les LSAs des chats de moins de sept ans et ceux de sept ans et plus. La détection immunohistochimique et l'antigène du VLFe dans les tissus formulés et enrobés dans la paraffine étaient en accord avec les résultats obtenus par le test ELISA chez les patients vivants. Cependant l'ADN viral pouvait être détecté par ACP dans certains lymphosarcomes sans que l'antigène du VLFe n'ait été

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This study was supported by funds from the Companion Animal Health Fund of the Western College of Veterinary Medicine, SmithKline Beecham Animal Health of Toronto, and the Dean's Medical Research Council Fund at the Western College of Veterinary Medicine. Personal support for Dr. Jackson was from a fellowship awarded by the Medical Research Council, Ottawa.

Submitted December 23, 1992.

démontré, particulièrement chez les chats âgés de sept ans et plus. Ces cas positifs pour le provirus et négatifs pour l'antigène viral pourraient représenter des infections par des virus latents ou encore par des virus défectueux en ce qui a trait à la réplication. (*Traduit par Dr Daniel Martineau*)

INTRODUCTION

Feline leukemia virus (FeLV) is an exogenous retrovirus causing several diseases in domestic cats, including tumors of most hemopoietic cells, aplastic anemia, myeloproliferative disorders, and immunosuppression (1–19).

Lymphosarcoma (LSA) represents 30% of all tumors in cats and about 70% of feline LSAs contain FeLV antigen or infectious virus (12,18, 20,21). Cats with LSA in which FeLV is not detected are usually ≥7 yr and often have the intestinal form of the tumor (12,16,22).

Immunohistochemistry (IHC), particularly immunofluorescence, has been used extensively for viral diagnosis, including FeLV (23). Traditionally this technique has been applied to detect viral antigens in fresh or frozen tissues, but now enzyme-based IHC includes detection of antigens in formalin-fixed, paraffin-embedded tissues (24). The ability to detect viral antigens in fixed tissues is advantageous for ease of sample submission, improved morphological detail of tissues, and in combination with immunoenzyme detection systems, production of a permanent stain visible with ordinary light microscopy. However, while IHC is sensitive for identification of viral antigens in productive infections, latent viral infections are not discovered.

The polymerase chain reaction (PCR) is a molecular technique which can be applied to the amplification of viral DNA (25). The PCR has been used to amplify a region of the FeLV long terminal repeat (LTR) so that sequence variation in the LTRs of FeLV proviruses from T-cell LSAs could be investigated (26). Other researchers have compared enhancer duplication within the FeLV LTR in cells from FeLV-infected cats with neoplastic and nonneoplastic disease, by PCR amplification of the enhancer region followed by nucleotide sequencing (27). There are no reports of application of the PCR in a clinical setting for the diagnosis of FeLV infection, nor are there reports of PCR application to formalin-fixed, paraffin-embedded tissues for FeLV detection. The PCR is potentially more sensitive than IHC in detecting FeLV infection when virus load is low, the infection is latent, or a replication-defective virus is present. The purpose of this study was to apply these two techniques, PCR and enzyme-based IHC, to formalin-fixed, paraffin-embedded tissues for detection of FeLV in cats with LSA. The basic objective was to determine if FeLV DNA is associated with antigen-negative feline LSA. The prevalence of FeLV DNA and antigen in tumor tissue was related to patient age, anatomic tumor site, and antemortem enzyme-linked immunosorbent assay (ELISA) for serum FeLV antigen.

MATERIALS AND METHODS

CASE SELECTION

Seventy feline LSA cases diagnosed according to histopathological criteria (28), were retrieved from the pathology records at the Western College of Veterinary Medicine. The tumors were grouped by anatomic site as follows: multicentric, mediastinal, intestinal, and miscellaneous. Lymphocytic leukemia was included in the multicentric category. One to four paraffin blocks containing tumor tissue from various sites were used from each case. Medical records were reviewed to tabulate patient data, clinical history, and antemortem ELISA results.

IMMUNOHISTOCHEMISTRY

The avidin-biotin complex (ABC) method for IHC staining of formalin-fixed, paraffin-embedded tissues has been previously described (29). The following modifications were made for FeLV antigen detection. Nonspecific adherence of proteins to tissues was blocked by flooding sections with phosphate buffered saline supplemented with 4.0% serum from normal rabbits, the species in which the secondary antiserum was raised. Goat anti-FeLV gp70 primary antiserum (National Cancer Institute, Bethesda, Maryland) was applied to tissues followed by a biotinylated secondary antiserum, rabbit antigoat immunoglobulin (Vector Laboratories, Burlingame, California) and an ABC peroxidase detection system (Vectastain Elite ABC, Vector Laboratories). Primary antiserum was applied to duplicate serial sections of each tissue, diluted 1:4000 and 1:8000. The secondary antiserum was applied diluted 1:200. Serial sections of each tissue were tested with similar dilutions of an irrelevant goat serum substituted for anti-FeLV antiserum.

SAMPLES FOR THE POLYMERASE CHAIN REACTION

Samples were prepared for PCR as previously described (25). From the same paraffin blocks used for IHC, 10 μm sections were cut to yield a minimum of 1 cm^2 of tissue. Sections were deparaffinized by extraction twice with xylene, then rinsed once with 95% ethanol, centrifuged, the liquid decanted, and the remaining ethanol evaporated under vacuum. Samples were resuspended in 200 μL of digestion buffer [50 mM Tris (pH 8.5), 1 mM EDTA, 0.5% Tween 20] containing 200 μg/mL of Proteinase K (Gibco BRL, Life Technologies, Inc., Gaithersburg, Maryland), and incubated at 37°C overnight. Samples were then extracted twice with phenol-chloroform-isoamyl (25:24:1), once with chloroform-isoamyl (24:1), and precipitated with ethanol according to standard technique (30). The dried samples were resuspended in 50 μL of HPLC grade water (BDH Inc., Toronto, Ontario). A 1–10 μL sample volume was used as template for the PCR.

POLYMERASE CHAIN REACTION

Primers targeting a 166 base pair (bp) segment of the FeLV U3 LTR region (26,27) were synthesized (University of British Columbia, Oligonucleotide Synthesis Laboratory, Department of Biochemistry, Vancouver, British Columbia). The primer sequences were as follows:

5′-TTACTCAAGTATGTTCCCATG-3′ (sense) and 5′-CTGGGGAGCC-TGGAGACTGCT-3′ (anti-sense). A computation was performed at the National Center for Biotechnology Information (NCBI) using the experimental GENINFO BLAST Network Service to assess degree of homology between these primers and other reported sequences. The BLAST service accesses GenBank, GenBank Update, EMBL Data Library, EMBL Update, Vector subset of GenBank, Kabat Sequences of Nucleic Acid of Immunological Interest Release, Eukaryotic Promoter Database Release 34, and Database of Expressed Sequence Tags Release 1.1. Reaction mixtures for the PCR consisted of 1× PCR Buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1.5 mM of $MgCl_2$, 200 μM of each deoxynucleoside triphosphate (dNTP), 1.25 units of Taq DNA polymerase (GeneAmp PCR Core Reagents, Perkin Elmer Cetus, Norwalk, Connecticut), 50 pmol of each primer, 1 to 10 μL of DNA template, and HPLC grade water (BDH Inc.) to a volume of 50 μL. The reaction mixture was overlaid with 50 μL of liquid paraffin. The PCR was carried out in a DNA Thermal Cycler (Perkin Elmer Cetus) as follows: initial denaturation of 3 min at 94°C followed by 35 cycles of: denaturation — 1 min at 94°C, primer annealing — 1 min at 52°C, and primer extension — 2 min at 72°C. Samples were cooled to 4°C or frozen at −20°C until further analysis.

A 10 μL aliquot of the aqueous PCR product was mixed with 3 μL of stop buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and electrophoresed through a 2% agarose minigel (Ultra Pure Agarose Electrophoresis Grade, Gibco BRL, Life Technologies, Inc.) in 0.5× TBE buffer (0.045 M Tris-borate, 0.001 M EDTA, pH 8.0) at 80–100 volts for 30-60 min. Either a 100 bp or 123 bp DNA size marker (Gibco BRL, Life Technologies, Inc.) was included in each gel. Gels were stained with ethidium bromide (Sigma Chemical Company, St. Louis, Missouri) for 15 min, and photographed (Polaroid film 667, Polaroid Corporation, Cambridge, Massachusetts) under UV transillumination (Foto UV 300 DNA Transilluminator, Fotodyne, Inc. New Berlin, Wisconsin). Sensitivity of the PCR had been determined using serially diluted cloned FeLV [FeLV clone 61E (1,31), a gift from Dr. J. Overbaugh, School of Medicine, Department of Microbiology, University of Washington, Seattle, Washington]. When evaluated by visualization of the ethidium bromide stained agarose gel, the lower limit of detection for cloned virus in a background of both fresh feline genomic DNA and DNA derived from paraffin-embedded, formalin-fixed feline tissue was between 3.2 fg and 160 fg. Generally, Southern hybridization enabled detection of PCR products to one dilution beyond that which could be visualized on agarose gels.

SOUTHERN TRANSFER AND HYBRIDIZATION

Specificity of the PCR for the FeLV U3 LTR was confirmed by Southern transfer and hybridization using a PCR synthesized digoxigenin-labeled probe directed to the 166 bp target. The template for the labeling reaction was a 1.1 kilobase (kb) *Eco*RI-*Hin*dIII fragment of pexU3, a plasmid containing two direct repeats of the 5′-end 249 bp of the FeLV-B-GA LTR (a gift from Dr. James I. Mullins, Stanford University School of Medicine, Stanford, California). This fragment was gel purified and then used as the source of FeLV DNA in the PCR labeling reaction. The PCR to generate labeled probe was modified as follows (32–34): the final reaction volume was doubled to 100 μL, the final concentration of the dTTP was reduced to 150 μM, and 50μM of 1 mM digoxigenin-11-2′-deoxyuridine-5′-triphosphate (Dig-11-dUTP) (Boehringer Mannheim Canada Ltd, Laval, Quebec) was added. Therefore, the labeled probe was 166 bp the same as the other FeLV PCR products except about every fourth dTTP was substituted with digoxigenin-labeled dUTP. A 10 μL aliquot of digoxigenin-labeled PCR product was used to probe one 100 cm^2 filter.

For hybridization, PCR products were transferred from agarose gels to nylon membranes (Gene Screen Plus Hybridization Transfer Membrane, Biotechnology Systems NEN Research Products, Dupont, Boston, Massachusetts) using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (BioRad, Richmond, California) according to the manufacturer's instructions (Trans-Blot SD DNA Blotting Kit Instruction Manual, BioRad). At the end of the procedure, membranes were cross-linked in a UV linker (UV Stratalinker 1800, Stratagene, La Jolla, California) at 1200 microjoules for 30 sec (autocrosslink mode). Hybridization and immunological detection of digoxigenin were done according to the manufacturer's instructions (DNA Labeling and Detection Kit Nonradioactive, Boehringer Mannheim Canada Ltd.).

CONTROLS FOR POLYMERASE CHAIN REACTION

Template DNA for positive controls was from cloned FeLV 61E initially, and later from cloned virus and/or paraffin-embedded, formalin-fixed tissues from clinical cases that consistently yielded intensely staining appropriate sized bands on agarose gel electrophoresis (AGE). Template DNA for negative controls was derived from uninfected cultured feline T cells (3201 cells, a gift from Dr. J. Overbaugh, School of Medicine, Department of Microbiology, University of Washington) and/or from blood, fresh or formalin-fixed bone marrow, or fresh or formalin-fixed tissue samples from cats with no hematological, clinical, or pathological evidence of FeLV-related disease. Reagent controls containing no DNA were also included in each PCR.

SEQUENCING OF AMPLIFIED PRODUCTS

Representative PCR products were sequenced using the AmpliTaq Cycle Sequencing Kit (Perkin Elmer Cetus) according to the manufacturer's instructions. The sequence of PCR products not hybridizing to the FeLV probe was analyzed for homology to reported sequences; this computation was performed at the NCBI using the experimental GENINFO BLAST Network Service.

INTERPRETATION OF POLYMERASE CHAIN REACTION RESULTS

Results from clinical cases were accepted and interpreted only when positive and negative controls yielded the expected results on AGE. An intensely staining 166 bp product on

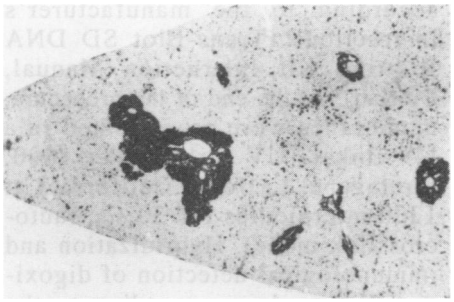

Fig. 1. Formalin-fixed, paraffin-embedded liver section from a cat with lymphosarcoma. The tissue has been stained for feline leukemia virus gp70 antigen; dark-staining foci of neoplastic lymphocytes are present within the tissue section. Avidin-biotin complex immunoperoxidase with hematoxylin counterstain.

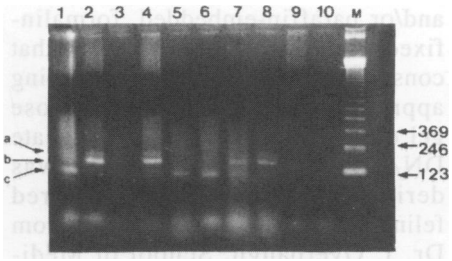

Fig. 2. Ethidium bromide-stained agarose gel of typical PCR products amplified from feline lymphosarcoma-derived DNA. Lane M is a 123 bp DNA ladder. The "b" arrow indicates the expected 166 bp product representing a region of the FeLV LTR. The "a" arrow indicates an apparent 210 bp product that also hybridized to the exogenous FeLV probe (see Fig. 3). The "c" arrow shows an approximate 100 bp product that did not hybridize to the FeLV probe (see Fig. 3). Lane 1 was interpreted as an equivocal result for the presence of FeLV product. Lanes 2,4,7 and 8 were considered positive for FeLV product. Lanes 3,5,6,9 and 10 were interpreted as negative for FeLV product. Southern hybridizations were routinely done when equivocal and negative results were obtained on agarose gel electrophoresis. Controls were included for each set of reactions but are not shown in this composite of PCR results.

AGE, was recorded as a positive result for PCR detection of FeLV provirus. Southern hybridizations were done randomly on these cases. The PCR was repeated at least twice on cases yielding equivocal or negative results on AGE, using the original DNA extract as template. Southern hybridizations were done on PCR products from the second and

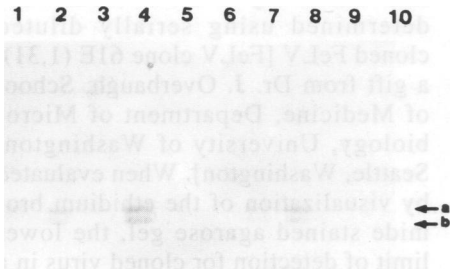

Fig. 3. Southern hybridization using a digoxigenin-labeled exogenous FeLV probe, of the PCR products shown in Fig. 2. The "a" and "b" arrows demonstrate hybridization of the probe to the 210 bp and 166 bp products, respectively. There was no hybridization of the exogenous FeLV probe to the approximate 100 bp product. Lanes 2,3,4,7 and 8 were interpreted as positive for FeLV, whereas lanes 5,6,9 and 10 were negative for FeLV. Lane 1 demonstrated an equivocal result and taken with other findings would be classified as negative for exogenous FeLV.

third amplifications of these samples. Results were recorded as positive, negative, or indeterminate. A positive result from those cases that had repeated PCR and Southern hybridizations, was defined as obtaining positive, negative, or equivocal 166 bp bands on AGE of one or both repeat PCRs, plus positive signals on both Southern hybridizations. An indeterminate result represented samples with equivocal or no evidence of the 166 bp FeLV product on AGE, and discordant results on the two hybridizations (one positive and one negative). A negative result was assigned to those cases with negative or equivocal bands on the two PCRs plus negative or equivocal bands on both Southern hybridizations.

STATISTICAL ANALYSIS

To assess the level of agreement between tests (PCR and IHC, PCR and ELISA, and IHC and ELISA) the kappa quotient was calculated. Kappa is a measure of agreement beyond that which might be expected due to chance. No agreement beyond chance yields a kappa of 0, and a kappa of 1 indicates perfect agreement. Moderate agreement is indicated by a kappa of at least 0.4–0.5 (35). Indeterminate results were not included in the calculation.

To assess the relationship between age group and PCR result, the Fisher exact test (36) was used. This test can be applied when cell frequencies in a 2×2 contingency table are too low to use the Chi-square test. The calculation was made by computer program (Epi Info USD Incorporated, Stone Mountain, Georgia), and indeterminate PCR results were not included. The relationship between age group and IHC result was determined using the Chi-square test (35,36). The critical Chi-square value for significance at the 5% level is 3.84 (35). When a significant relationship was found between test result and age group, the strength of the association was measured by calculating the odds ratio (35) from the 2×2 contingency table. Exact confidence limits for the odds ratio were calculated as described (37).

RESULTS

Immunohistochemical staining of tissue sections from 70 cases of feline LSA revealed FeLV gp70 antigen in 40 cases. Viral antigen was usually most apparent in neoplastic lymphoid cells, often within foci of cells infiltrating multiple organs and tissues (Fig. 1). There was no specific staining when sections were tested substituting an irrelevant serum for the FeLV antisera.

Although the search of various nucleotide databases using the BLAST Service detected homology between the primers and FeLV LTRs, the program identified no matches between the primers and other reported feline exogenous viruses or endogenous sequences.

In PCR testing, in addition to the expected 166 bp FeLV genome product, a band of lesser intensity representing DNA of about 210 bp was commonly seen (Fig. 2). Sometimes there was also a band of about 100 bp, particularly in those cases where no FeLV product band was present or where the FeLV band was faint (Fig. 2). The 166 and 210 bp bands hybridized to the FeLV U3 LTR probe in Southern hybridizations; the 100 bp band did not hybridize to the probe (Fig. 3). Occasionally samples yielded multiple larger sized bands in addition to the 166 and 210 bp products; these also hybridized to the

FeLV U3 LTR. Positive and negative controls yielded expected results in all cases for which PCR and Southern hybridization results were accepted and reported.

The nucleotide sequences of the 166 bp and apparent 210 bp bands were one and the same, and showed near homology to the sequence for FeLV 61E within this region (data not shown). The sequence of the 100 bp band showed complementarity to the FeLV target only for 13 bases within the sense primer, which was not chosen as the sequencing primer.

In total, 56 of 70 cases (80%) were positive for FeLV by PCR compared to 40 of 70 (57%) by IHC (Table I). Seventeen of the 56 cases that were positive by PCR, were negative by IHC; one of the seven cases that was negative by PCR, was positive by IHC. The seven cases that were indeterminate by PCR were negative by IHC. The kappa quotient to compare PCR and IHC test results, was 0.29 indicating a low level of agreement between the two tests.

In 30 of the 70 cases antemortem FeLV ELISAs had been performed. The 19 cases positive by ELISA were also positive by PCR and IHC (Table II). The 11 ELISA-negative cases were also negative by IHC, however, seven ELISA-negative cases were positive by PCR. The kappa quotient to compare PCR and ELISA results, was 0.29 indicating a low level of agreement between the two tests; kappa to compare IHC and ELISA results was 1 which indicates perfect agreement between these two tests.

Thirty-seven of the 42 cats (88%) <7 yr were positive by PCR, and 34 of these cats (81%) were positive by IHC (Table III). Eighteen of the 26 cats (69%) ≥7 yr were positive by PCR, whereas 5 of the 26 (19%) were positive by IHC. The Fisher exact calculation (p value = 0.23) indicated that there was no significant difference in PCR results between young and old groups of cats; LSA tissues from older cats (≥7 yr) were equally likely to be positive for FeLV DNA as were those from younger cats (<7 yr). However, IHC results were significantly different between young and old cats (Chi-square = 25.01, p<0.05; odds ratio = 17.85, 95% confidence interval = 4.51,76.37); LSA tissues from young cats (<7 yr) were more likely to be positive for FeLV antigen than were those from older cats (≥7 yr).

The distribution of PCR and IHC positive results according to anatomic site of LSA is shown (Table IV). The multicentric form was most common followed by the mediastinal and intestinal forms. Proportionately fewer intestinal LSAs were positive for FeLV by PCR and IHC than were the multicentric and mediastinal LSAs.

The anatomic locations of LSA in the 26 cats ≥7 yr were: multicentric — 17, mediastinal — 1, intestinal — 5, miscellaneous — 3. Three of the five cats with intestinal tumors were positive by PCR while one of the five was positive by IHC and this one was not positive by PCR. For the three cats <7 yr with intestinal LSA, two were positive for FeLV by PCR and IHC, and ELISAs were not done; the third was indeterminate on PCR and negative on IHC and ELISA.

TABLE I. FeLV detection by PCR[a] and IHC[b] in tumor tissues from 70 cats with lymphosarcoma

	IHC +	IHC −	Totals
PCR +	39	17	56/70 (80%)
PCR −	1	6	7/70 (10%)
PCR I[c]	-	7	7/70 (10%)
Totals	40/70 (57%)	30/70 (43%)	

[a]PCR = polymerase chain reaction
[b]IHC = immunohistochemistry
[c]I = indeterminate results
Kappa to assess agreement between PCR and IHC results = 0.29

TABLE II. FeLV detection by PCR[a], IHC[b], and ELISA[c] in tumor tissues (PCR and IHC) and serum (ELISA) from 70 cats with lymphosarcoma

		ELISA +	ELISA −	ELISA nd[d]
PCR + (n = 56)	IHC +	19	0	20
	IHC −	0	7	10
PCR − (n = 7)	IHC +	0	0	1
	IHC −	0	2	4
PCR I[e] (n = 7)	IHC +	0	0	0
	IHC −	0	2	5

[a]PCR = polymerase chain reaction
[b]IHC = immunohistochemistry
[c]ELISA = enzyme-linked immunosorbent assay
[d]nd = not done
[e]I = indeterminate results (for PCR)
Kappa to assess agreement between PCR and ELISA results = 0.29
Kappa to assess agreement between IHC and ELISA results = 1

TABLE III. FeLV detection by PCR[a] and IHC[b] according to age group in 70 cats with lymphosarcoma

Age group (yr)	PCR +	IHC +
< 7 (n = 42)	37 (88%)	34 (81%)
≥ 7 (n = 26)	18 (69%)	5 (19%)
unknown (n = 2)	1 (50%)	1 (50%)

[a]PCR = polymerase chain reaction
[b]IHC = immunohistochemistry
Relationship of PCR result to age group, Fisher exact test, p = 0.23 (not significant)
Relationship of IHC result to age group, Chi-square = 25.01, p<0.05; odds ratio = 17.85, 95% confidence interval = 4.51,76.37

TABLE IV. FeLV detection by PCR[a] and IHC[b] according to anatomic site of lymphosarcoma in 70 cats

Anatomic site	PCR +	IHC +
Multicentric (n = 48)	39 (81%)	31 (65%)
Mediastinal (n = 11)	9 (82%)	6 (55%)
Intestinal (n = 8)	5 (63%)	3 (38%)
Miscellaneous (n = 3)	3 (100%)	0

[a]PCR = polymerase chain reaction
[b]IHC = immunohistochemistry

DISCUSSION

About 30% of cats with LSA are negative for FeLV by antigen detection and/or virus isolation; however, epidemiological studies suggest that exposure to FeLV is associated with the development of LSA in these cats (12,16,22,38–41). Some studies have attempted to further define this association. In one hybridization experiment however, no exogenous FeLV U3 sequences could be found in LSAs from six FeLV antigen-negative cats (4). Other research investigating the role of latency showed that FeLV could be reactivated from cultured bone marrow cells but not from blood or tumor cells in two cats with FeLV antigen-negative LSA (42). Although studies of FeLV infection suggest that latency may be a phase of recovery that is generally short-lived, the latent period may persist for months or years in some cats (43,44). These cats may be at increased risk for the development of virus-negative LSA as defined by absence of viral antigen and infectious virus. Explanations for the reported 30% of feline LSAs that are negative for infectious FeLV or viral antigen therefore include: the presence of a latent FeLV infection, as well as, the presence of a recombinant replication-defective virus, the presence of only a portion of the FeLV genome which also renders it replication defective, or, a "hit and run" phenomenon such that FeLV induces LSA but does not itself become integrated into the host cell genome (22).

In this study, 56 of the 70 cases (80%) were positive for FeLV using the PCR method. These included 17 of the 30 cases which were negative for FeLV by IHC. Seven of the 17 PCR-positive, IHC-negative cases had been tested by ELISA and were negative. These findings support the theory that some FeLV antigen-negative LSAs involve latent or replication-defective FeLV. Alternatively, the inherent amplification of target DNA in the PCR could facilitate detection of replication-competent FeLV present in low levels or intermittently producing viral protein, and therefore not identified by traditional methods.

Previous studies suggest that latent infections involve bone marrow cells and a minor subset of nodal lymphocytes but not necessarily tumor cells themselves (42). In this study, DNA from tumor tissue was tested for FeLV by PCR. Bone marrow contained tumor and was available for PCR in only a few cases. Therefore, if latent FeLV infection was being detected by PCR, tumor tissue was a source of the virus in this study.

Although PCR detected FeLV in cases that were antigen-negative by IHC and/or ELISA, there remained 20% of cases that were negative or indeterminate for FeLV by PCR. Possible explanations for this include: not all LSAs in cats are FeLV-related, the "hit and run" phenomenon may be occurring in a portion of LSAs, the FeLV infection was present/latent in tissues other than those tested (such as bone marrow), or FeLV DNA may have been undetectable by PCR due to interference, poor quality or otherwise inadequate template DNA.

The possible role of the feline immunodeficiency virus (FIV) in the LSA cases examined here, was not investigated. The relative risks of developing LSA are reported to be 5.6, 62.1, and 77.3 times greater in cats infected with FIV, FeLV, and FeLV with FIV, respectively, than in uninfected cats (45). Recently, a cat experimentally infected with FIV but FeLV antigen-negative developed LSA (46). The PCR for FeLV proviral detection was not applied in either of these two studies so that infection with replication-defective FeLV cannot be ruled out. To our knowledge, there is no conclusive evidence that FIV alone causes lymphosarcoma in cats. Nevertheless, since the presence of FIV antibody or DNA was not determined in this study, the possible role of FIV in the FeLV-negative LSAs is not known.

The distribution of LSAs according to anatomic site was similar to previous reports when lymphocytic leukemia had been included with the multicentric form (12,22,45). In a study involving LSAs in 507 cats, 80.3% of the multicentric tumors, 77.0 % of the thymic (mediastinal) tumors, and 23.2% of the alimentary (intestinal) tumors were positive for FeLV antigens (22). The PCR results of the present study were similar for the multicentric tumors (81% PCR positive) and the mediastinal tumors (82% PCR positive), however, five of the eight intestinal tumors (63%) were FeLV-positive by PCR. The number of intestinal tumors available in the current study was small, however, the results suggest that previous findings that alimentary LSAs are usually FeLV negative (12,22,47,48), may relate more to the method of testing rather than the true presence or absence of the viral genome.

Lymphosarcoma in older cats is not usually associated with the presence of FeLV antigen or infectious virus (12,22,49). An interesting finding in the current study was that 18 of 26 (69%) cats ≥7 yr were FeLV-positive by PCR compared to 5 of 26 (19%) that were FeLV-positive by IHC. While there was no significant difference in PCR results according to age group, there was a significant difference in IHC results according to age group. Lymphosarcoma tissues from young cats (<7 yr) were several fold more likely to be FeLV-positive by IHC than were tumors from older cats (≥7 yr). In general, older cats have probably been infected with FeLV for a longer period and there may be more opportunity for the development of recombinant forms of FeLV. Immune responses may effectively eliminate replication-competent forms and inadvertently select for replication-defective strains of FeLV which are still capable of inducing transformation by insertional mutagenesis, transduction of cellular oncogenes, or some other unknown mechanism.

There was one PCR-negative, IHC-positive result in an older cat with an intestinal tumor. Since viral antigen was demonstrated within tumor cells, viral DNA would also be expected to be present locally within the tumor. Perhaps the DNA template was degraded or inadequate, or there was some chemical or physical interference prohibiting amplification. The simultaneous inclusion of a feline genomic positive control may have been of value to examine this discrepancy; however, the nonspecific 100 bp product was seen on AGE, indicating that amplifiable DNA was present.

The frequent finding of both 166 and approximate 210 bp PCR products that hybridized to the FeLV probe, is interesting. The region of the

FeLV LTR being amplified in the PCR contains several enhancer binding consensus sequences, and it has been reported that duplications of a 50–90 bp segment of the enhancer relates to leukemogenic potential in FeLV-related neoplastic disease (26,27,50). In this study, enhancer duplication was considered to explain the apparent 210 bp band, however, the sequence determination showed that the 166 and 210 bp bands represented the same DNA with no repeats. The difference in migration on AGE might relate to secondary structure formation in some of the product retarding movement through the gel. Heating the product after adding the stop solution and before loading the gel may remove any alterations in configuration that have occurred during cooling of the amplified product.

The 100 bp product that was commonly seen showed no homology to known sequences. This likely represents a feline genomic sequence present in some but not necessarily all cats, and with some homology to the primers. When ample target FeLV was present, the primers appeared to preferentially bind to the exogenous virus. The 100 bp product was most evident when little or no target virus was amplified.

Contamination, particularly from amplified PCR product, is a major problem that commonly occurs in laboratories performing PCR regularly (51–60). Occasionally in this study, even though there were no visible bands on stained agarose gels, signals were present on the Southern hybridizations of PCR products. For this reason, some samples were tested repeatedly by PCR and hybridization, and a system of interpreting results was devised (Materials and Methods). The weak, equivocal, and discordant results sometimes obtained on the repeated Southern hybridizations, may have been due to a low level of contamination which some report to be unavoidable during the various steps involved in the PCR (61).

The IHC and ELISA results agreed in those 30 cases for which FeLV ELISAs had been done, supporting the application of enzyme-based IHC for FeLV studies using paraffin-embedded, formalin-fixed specimens. The advantages of enzyme-based IHC over most antigen-detection methods include the ability to localize the FeLV antigen to specific tissues and to maintain a permanent record of the results simply by storing the stained slides.

Feline leukemia virus DNA was frequently amplified from tumor tissues of older cats that were IHC-negative, thus supporting the hypothesis that latent or replication-defective FeLV may be responsible for some cases of "virus-negative" LSA. An increased ability of the PCR to detect low levels of replicating virus compared to IHC, would not likely explain PCR-positive/IHC-negative results involving mainly a subgroup of tumors — those from older cats. More study is warranted in applying the PCR technology to help elucidate the pathogenesis of FeLV antigen-negative LSA in cats.

ACKNOWLEDGMENTS

We are grateful to Mr. Brian Chelack, Ms. Karen Martin, Mr. Ed Bueckert, and Ms. Barbara McCoy for their technical expertise and assistance, and we thank Dr. Paul Morley for his advice and assistance with the statistical analysis.

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