Evaluation of formalin-fixed paraffin-embedded tissues obtained from vaccine site-associated sarcomas of cats for DNA of feline immunodeficiency virus

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Objective—To evaluate the use of a polymerase chain reaction (PCR) method for detection of feline immunodeficiency virus (FIV) DNA, using formalin-fixed paraffin-embedded (FFPE) tissues, and to use this method to evaluate tissues obtained from vaccine site-associated sarcomas (VSS) of cats for FIV DNA.

Sample Population—50 FFPE tissue blocks from VSS of cats and 50 FFPE tissue blocks from cutaneous non-vaccine site-associated fibrosarcomas (non-VSS) of cats.

Procedure—DNA was extracted from FFPE sections of each tumor and regions of the *gag* gene of FIV were amplified by a PCR, using 3 sets of primers. Sensitivity of the method was compared between frozen and FFPE tissues, using splenic tissue obtained from a cat that had been experimentally infected with FIV.

Results—We did not detect FIV DNA in VSS or non-VSS tissues. Sensitivity of the PCR method was identical for frozen or FFPE tissues.

Conclusions and Clinical Relevance—It is possible to detect FIV DNA in FFPE tissues by use of a PCR. We did not find evidence to support direct FIV involvement in the pathogenesis of VSS in cats. (*Am J Vet Res* 2000;61:1037–1041)

An epidemiologic association between site of vaccination and fibrosarcoma development in cats was first documented in the early 1990s.[1,2] Estimated prevalence of injection-site sarcomas is reportedly 1 to 3.6 tumors/10,000 cats vaccinated.[3,4] Although SC administration of inactivated rabies vaccine was initially implicated in the development of **vaccine site-associated sarcomas (VSS)** in cats, administration of rabies vaccine (IM), inactivated FeLV vaccine (SC), and inactivated feline panleukopenia virus-rhinotracheitis virus-calicivirus vaccine (SC) subsequently have been linked to development of these tumors.[5-7] Similarly, although aluminum adjuvants were incriminated in the pathogenesis of VSS in early studies,[1] vaccines containing nonaluminum adjuvants and vaccines without adjuvants also have been associated with VSS.[4,5]

The low prevalence of VSS suggests that inflammation, necrosis, and healing all may play roles, but other factors in susceptible cats may be required for tumorigenesis. One such factor, FeLV infection, has been investigated, but the virus was not detected by use of immunohistochemical or **polymerase chain reaction (PCR)** methods in a large number of VSS.[8] **Feline immunodeficiency virus (FIV)**, which belongs to the lentivirus genus of the retrovirus family, also is worthy of investigation as a possible factor in the development of VSS. Although cats with VSS have not reportedly had detectable FIV antibody,[6] FIV infection is not completely ruled out on the basis of that result.[9] Latent FIV infection in lymphocytes and macrophages could be reactivated in a local inflammatory lesion containing these cells. Proteins of feline herpesvirus 1[10] or other vaccine-derived proteins could potentially transactivate FIV regulatory sequences in vivo, resulting in viral reactivation or increased replication. Local replication of FIV could predispose cells to insertional mutagenesis, altered expression of cellular genes, or altered expression of tumor suppressor genes, without necessarily eliciting a systemic antibody response. Objectives of the study reported here were to evaluate the use of a PCR method for detection of FIV DNA, using **formalin-fixed paraffin-embedded (FFPE)** tissues, and to evaluate VSS and non-VSS of cats for FIV DNA.

Materials and Methods

Tissue specimens—Two groups of FFPE tissue blocks were obtained from all routine biopsy specimens obtained from cats and submitted during 1996 to a veterinary diagnostic laboratory.[a] The first group (ie, 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 between vaccination and histopathologic findings of inflammation and necrosis within each tumor.[11] The second group (ie, non-VSS) consisted of 50 cutaneous fibrosarcomas that were not associated with administration of vaccine. Tumors were included in this group only when it was indicated in the medical history that the anatomic location of the tumor was not consistent with that of a vaccination site (ie, head, tail, and digits). The FIV antibody status of the tumor-bearing cats in both groups was not known.

Polymerase chain reaction primers—Primer sequences used in fresh feline tissues[9,12-14] were synthesized by a commercial laboratory.[b] Primer sequences, FIV genome positions, and expected length of PCR products were determined (**Appendix**).

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Preparation of specimens for the polymerase chain reaction—Sections of tumor tissue (10 µm) were cut from each paraffin block to yield a minimum of 1 cm^2 of tissue. A DNA extraction was performed, using a commercially available tissue kit[c] in accordance with the manufacturer's instructions, except final elution of DNA was performed with 50 µl of distilled water. Concentration of extracted DNA was determined, using a diode-array spectrophotometer[d] at a wavelength of 260 nm, and 2 µl of DNA (0.1 to 1.0 µg) was used as a template for the PCR.

Amplification of DNA—The PCR was performed in 0.5-ml thin-walled tubes, using each set of primers.

Primer sets FIV-1 and FIV-4—A PCR mixture was prepared, using commercially available core reagents and DNA polymerase.[e] The final concentration of each reagent in the reaction mixture was as follows: 10× PCR buffer II (10 m*M* tris HCl [pH 8.3], 50 m*M* KCl), 2.5 m*M* $MgCl_2$, 200 µ*M* of each **deoxynucleoside triphosphate (dNTP)**, 1 µ*M* of each primer (ie, FIV-1 and FIV-4), 0.625 units of *Taq* DNA polymerase, 2 µl of template DNA, and a sufficient amount of water to achieve a final reaction volume of 25 µl. Amplification of DNA was accomplished with a thermal cycler[f] and involved the following: 94 C for 10 minutes followed by 35 cycles (94 C for 1 minute, 55 C for 1 minute, and 72 C for 2 minutes).[14]

Primer sets P10 and P15—A PCR mixture was prepared, using *Taq* DNA polymerase, 10× PCR buffer, and $MgCl_2$.[g] The final concentration of each reagent in the reaction mixture was as follows: 10× PCR buffer (20 m*M* tris HCl [pH 8.4], 50 m*M* KCl), 1.5 m*M* $MgCl_2$, 200 µ*M* of each dNTP, 1 µ*M* of each primer (ie, P10-1 and -2 or P15-1 and -2), 1.25 units of *Taq* DNA polymerase, 2 µl of template DNA, and a sufficient amount of water to achieve a final reaction volume of 50 µl. A 1-step amplification (single PCR) was performed in the thermal cycler, using cycling variables previously described.[14]

A 10-µl aliquot of PCR product was separated, using electrophoresis[h] of the product through a 2% agarose minigel in accordance with a standard procedure.[15] A 100-**base pair (bp)** DNA marker[a] was included in each gel. The 2% agarose gels were stained with ethidium bromide and photographed under UV transillumination,[i] and the image was captured by use of a documentation-and-analysis system.[j]

Assessment of DNA—To ensure that there was sufficient amplifiable DNA for the PCR procedure, primers that amplify a 108-bp segment of exon 7 of the feline p53 tumor suppressor gene, (5′-GTCGGCTCTGAGTGTACCAC-3′ [sense] and 5′-GGAGTCTTCCAGGGTGATGA-3′ [antisense]),[k] were used in a PCR. Reagents and concentrations of reagents used in the PCR were identical to those used for the P10 and P15 FIV primer sets. Amplification was as follows: 94 C for 3 minutes followed by 30 cycles (94 C for 1 minute, 52 C for 1 minute, and 72 C for 2 minutes).

Controls for polymerase chain reaction—Positive-control DNA was prepared from a FIV-34TF10 plasmid,[l] which is a molecular clone containing a full-length insert from the FIV-Petaluma strain.[m] Plasmid DNA was purified, using a midiprep kit[n] in accordance with manufacturer's instructions. Extracted DNA was diluted to a concentration of 0.63 fg/µl, and 2 µl of the diluted FIV DNA was used in each PCR.

Negative-control DNA was prepared from FFPE tissue obtained from a healthy FIV-antibody-negative cat. A second negative-control sample that did not contain DNA was included in each PCR to monitor contamination of PCR reagents.

Sensitivity determination by use of the FIV molecular clone—Serial dilutions of the FIV-34TF10 clone were prepared to determine the lower limit of FIV detection, using each primer set. Two microliters of negative-control DNA was added to each reaction tube to provide a background of feline genomic DNA.

Comparison of sensitivity of the polymerase chain reaction—Frozen splenic tissue was obtained from a cat experimentally infected with FIV.[o] A small portion of tissue was routinely fixed in neutral-buffered 10% formalin for 12 hours and processed as a paraffin-embedded tissue block. The DNA was simultaneously extracted from frozen and FFPE tissue, using a tissue kit,[c] as described previously. Extracted DNA was serially diluted to determine the lower limit of FIV detection, using primer sets P10 and P15.

Nucleotide sequence of polymerase chain reaction products—Product bands that resulted from amplification of the FIV-34TF10 clone by use of primer sets FIV-1 and -4 and of FIV from experimentally infected splenic tissue by use of primer sets P10 and P15 were purified, using a gel extraction kit[p] in accordance with the manufacturer's instructions. Purified PCR products were submitted to another laboratory[q] for automated nucleotide sequencing. Sequence data from FIV positive-control samples were compared with the nucleotide sequence of the 34TF10 clone derived from the Petaluma strain of FIV.[16]

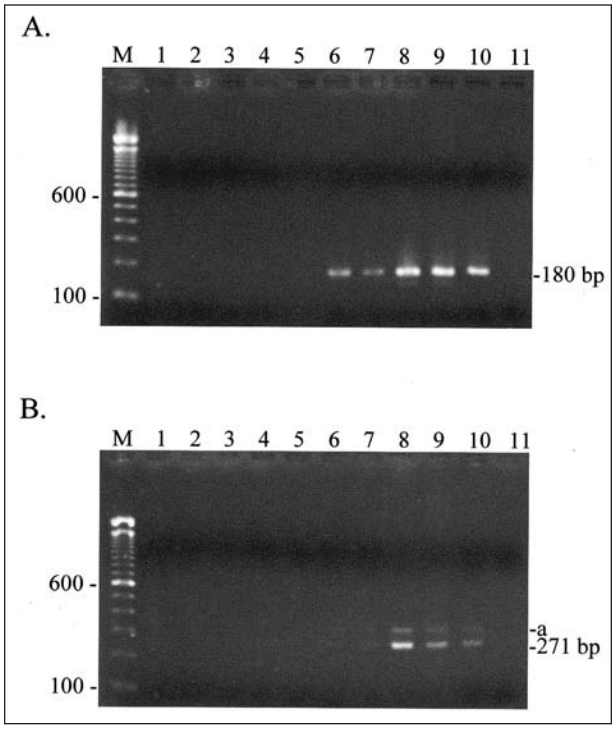

Figure 1—Ethidium bromide-stained agarose gels of polymerase chain reaction (PCR) products amplified from serially diluted frozen (FR) or formalin-fixed paraffin-embedded (FFPE) feline splenic tissue containing feline immunodeficiency virus (FIV) DNA, using primer P10 (A) or P15 (B). Lane M is a 100-base pair (bp) DNA ladder. Lane 1 is a negative-control sample derived from tissue lacking FIV DNA. Lanes 2, 4, 6, and 8 are FR. Lanes 3, 5, 7, and 9 are FFPE containing 0.114, 1.14, 11.4, and 114 ng of genomic DNA, respectively. Lane 10 is a positive-control sample, using cloned FIV DNA as template. Lane 11 is a negative-control sample that contained reagents but that did not contain DNA. Using primer P10, the 180-bp FIV product could be detected at a dilution of 11.4 ng of genomic DNA in FR (lane 6) and FFPE (lane 7) tissues, but lanes 1 to 5 and 11 yielded negative results. Using primer P15, the 271-bp FIV product could be detected at a dilution of 11.4 ng of genomic DNA in FR (lane 6, faint band) and FFPE (lane 7, faint band) tissues. The less intense band located at 300-bp (a) had the identical nucleotide sequence as the 271-bp band. Lanes 1 to 5 and 11 again yielded negative results.

Results

We did not detect FIV DNA in the 50 VSS or 50 non-VSS tissues with the PCR technique, using any of the 3 primer pairs. Using the feline p53 tumor suppressor gene PCR primers to assess amplifiable DNA, a single band was evident at the expected bp location in all of the tumor tissues, except for 1. The DNA extraction procedure was repeated on that tissue, and p53 product was evident on the second PCR of that tissue. The second extract was used as the template DNA in the FIV PCR.

The lower limit of detection of cloned FIV in a background of genomic DNA, using primer sets FIV-1 and -4, P10, and P15 was 0.063, 0.031, and 0.125 fg, respectively, corresponding to a sensitivity of approximately 5, 3, and 10 copies of FIV, respectively. Comparison of PCR by use of the primer sets P10 or P15 for FFPE and frozen tissue revealed that the lower limit of detection of FIV DNA in experimentally infected splenic tissue was identical (11.4 ng; **Fig 1**).

The nucleotide sequence obtained from purified PCR product, using primer sets FIV-1 and -4 to amplify a 465-bp fragment of the FIV-34TF10 clone, revealed 100% homogeneity with the sequence published for the FIV Petaluma strain. The nucleotide sequence obtained from purified PCR product, using FIV primer set P10 to amplify a 180-bp fragment and FIV primer set P15 to amplify a 271-bp fragment of frozen splenic tissue from an experimentally infected cat, revealed 96 and 97% homogeneity, respectively, with that of the published FIV sequence. Using primer set P15, an additional 300-bp product was identified, but it was identical in sequence to the 271-bp product.

Discussion

Polymerase chain reaction techniques have been used to detect FIV DNA in fresh or frozen mononuclear cells,[9,13,14,17-24] frozen tissues,[12] fresh bone marrow,[9] and lymph node aspirates[13] of experimentally infected cats. In the study reported here, we documented the application of PCR for successful detection of FIV DNA in FFPE tissues. Analysis of our results revealed an identical amount detected, using primer set P10 or P15, between frozen and FFPE tissue from a cat experimentally infected with FIV. The 3 primer sets used for detection of FIV DNA in this study had similar sensitivities. The lowest amount of cloned FIV detected in a background of genomic DNA was estimated to correspond to 3 to 10 copies of FIV. This sensitivity is comparable to the sensitivity reported in other studies of FIV.[22,25,26]

The PCR primers used in our study were directed at the *gag* sequence of the FIV genome, which is the most highly conserved region of the virus.[27] In addition, we chose 3 sets of *gag* region primers to increase the likelihood of detecting FIV field strains that may differ in nucleotide sequence. Nucleotide sequencing results of frozen splenic tissue from an experimentally infected cat revealed 96 and 97% identity (using primer sets P10 and P15, respectively) with that published for the Petaluma FIV sequence. These minor differences in nucleotide sequence would be expected in a field strain distinct from Petaluma FIV and did not preclude detection by PCR.

Two PCR product bands were obtained when primer set P15 was used: the expected 271-bp band and a less intense 300-bp band. The nucleotide sequence of the less intense 300-bp band was identical to that of the expected 271-bp band. Therefore, the difference in agarose gel electrophoretic migration was considered to be attributable to secondary structure formation in a portion of the PCR product that resulted in slower movement through the gel.

Despite the use of 3 sets of primers, the PCR did not detect FIV DNA in any of the tumor tissues tested in the VSS and non-VSS groups. Failure to detect FIV DNA in the sarcomas most likely indicated that FIV was not involved in the pathogenesis of these tumors. A less direct role of immunosuppression attributable to FIV infection without virus within tumor tissue is not completely ruled out.

False-negative results for the PCR were also possible. The number of cells containing viral DNA may have been less than the detection limits of the PCR used. In a study[17] in which cats were experimentally infected with FIV, the proportion of mononuclear cells in the peripheral blood that contained provirus ranged from 0.001 to 1.4%. Cats that have a low percentage of FIV infected cells may not have a positive result when tested by PCR. Sensitivity of the PCR methods used in our study was between 3 and 10 copies of FIV; therefore, a positive result would only be missed if fewer than 3 to 10 viral copies were in the template DNA. Although nested PCR reportedly allows detection of a single copy of human immunodeficiency virus contained in a sample,[28] and semi-nested PCR detects between 1 and 10 copies of FIV,[26] the greatly increased risk of contamination with these procedures[28] precluded their use for the large number of samples in our study. A second explanation for false-negative results included deletion or mutation within the FIV *gag* region resulting in a failure of the primers to recognize the gene sequence. We attempted to overcome this potential problem by using 3 primer sets for distinct areas of the *gag* gene. Finally, FIV DNA may have been undetectable in these tumors as a result of technical problems. Formalin fixation may degrade DNA and prohibit amplification by PCR. However, FIV DNA from splenic tissue of an experimentally infected cat was readily amplified from both frozen and formalin-fixed tissues, thereby ensuring that formalin fixation does not preclude FIV DNA amplification. Although formalin-fixed tissues can be used for PCR amplification, the length of time that the tissue remains in formalin prior to processing may be important. It was reported in 1 study[29] that there was a substantial decline in efficiency of PCR amplification when tissue was fixed in buffered 10% formalin for 1 to 4 weeks but that little or no effect was evident for tissue fixed ≤ 48 hours. Although we were able to document an identical amount of detection between frozen and FFPE tissue, a 12-hour fixation period was used. There may have been false-negative results if we had allowed tumor tissues to remain in formalin for > 48 hours. Also, there is a potential concern that formalin fixation may degrade DNA, rendering only short sequences detectable by PCR. In 1 study,[30] investigators determined that the size of DNA fragments recovered from tissues treated in neutral-buffered 10% formalin was

not significantly affected by fixation time, but the prolonged duration for fixation prohibited amplification of target sequences that were ≥ 989 bp. Our largest FIV product was 465 bp, which is still considered to be within the amplifiable range for formalin-fixed tissues. Detection of amplifiable p53 DNA in our study ensured that our DNA extraction system was functioning and ruled out the possibility of interference of the PCR, particularly inhibition of the *Taq* polymerase, by contaminants in the DNA sample, including ionic detergents, phenol, heparin, xylene cyanol, and bromphenol blue.[31]

We did not document an association between exogenous retrovirus, FIV, and a unique group of VSS in cats. Lack of FIV DNA within any of the sarcomas suggested that this lentivirus is unlikely to be directly involved in the pathogenesis of these neoplasms. These findings do not preclude the possibility that other genetic factors or oncogenic viruses may be involved in development of VSS in cats.

[a]Central Laboratory for Veterinarians Ltd, Langley, BC, Canada.
[b]Custom primers, Gibco BRL Life Technologies Inc, Burlington, ON, Canada.
[c]QIAmp Tissue Kit, Qiagen Inc, Mississauga, ON, Canada.
[d]Diode Array Spectrophotometer, 8452A, Hewlett-Packard (Canada) Ltd, Edmonton, AB, Canada.
[e]AmpliTaq Gold, Perkin Elmer Applied Biosystems, Mississauga, ON, Canada.
[f]PTC-200 Peltier Thermal Cycler, MJ Research Inc, Watertown, Mass.
[g]*Taq* DNA Polymerase, Gibco BRL Life Technologies Inc, Burlington, ON, Canada.
[h]Mupid-2 Mini Gel Migration Trough, Topogen Inc, Columbus, Ohio.
[i]FBTIV-816 Transilluminator, Fisher Scientific Co, Edmonton, AB, Canada.
[j]Alpha Imager 2000 Documentation and Analysis System, Alpha Innotech Corp, San Leandro, Calif.
[k]Nambiar P. MS thesis. Immunohistochemical and mutational analysis of the tumor suppressor gene P53 in feline vaccine-associated sarcomas. Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, 1998.
[l]Dr Robert Jacobs, Department of Pathology, Ontario Veterinary College, Guelph, ON, Canada.
[m]FIV-34TF10 provided by Dr. John Elder, Acquired Immunodeficiency Syndrome (AIDS) Research and Reference Reagent Program, Division of AIDS, National Institutes of Health, Ogden Bioservices Corporation, Rockville, Md.
[n]Wizard *Plus* Midipreps DNA Purification System, Promega, Madison, Wisc.
[o]Dr Edward A Hoover, Department of Pathology, College of Veterinary Medicine and Biomedical Sciences, Fort Collins, Colo.
[p]QIAquick Gel Extraction Kit, Qiagen Inc, Mississauga, ON, Canada.
[q]DNA Technologies Unit, National Research Council of Canada, Plant Biotechnology Institute, Saskatoon, SK, Canada.

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Appendix

Polymerase chain reaction primers used for the amplification of feline immunodeficiency virus (FIV) genome

Primer	Primer sequence (5′ to 3′)	FIV Petaluma strain position	Length of target sequence (bp)
FIV-1	CTACTGCTGCTGCAGCTGAA	928–947	NA
FIV-4	CACTGCATCCTAGCTGGTGC	1,374–1,393	465
P10-1	GCAAGACAATGTAGAGAAGT	1,785–1,804	NA
P10-2	TTTCTCCTCCATTGGAGGTG	1,945–1,964	180
P15-1	GTGATATACCAGAGACTTTA	778–797	NA
P15-2	TTTACTGTTTGAATAGGATA	1,029–1048	271

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

Correction: Comparison of pharmacokinetics of fentanyl after intravenous and transdermal administration in cats

In the Structured Abstract for "Comparison of pharmacokinetics of fentanyl after intravenous and transdermal administration in cats" (*AJVR*, Jun 2000, pp 672), the dosage of fentanyl indicated in the Procedure and the rate of transdermal delivery indicated in the Results are incorrect. Correct dosage for IV administration was 25 µg/cat (mean ± SD dosage, 7.19 ± 1.17 µg/kg of body weight), and correct rate of delivery was 8.48 ± 1.7 µg/h (< 36% of the theoretical 25 µg/h).