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 inactivated rabies vaccine (IM), inactivated FeLV vaccine (SC), and inactivated feline panleukopenia virus-rhinotracheitis virus-calcivirus vaccine (SC) subsequently have been linked to development of these tumors.5,6 Similarly, although aluminum adjuvants were incriminated in the pathogenesis of VSS in early studies,7 vaccines containing nonaluminum adjuvants and vaccines without adjuvants also have been associated with VSS.8,9 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.5 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, FIV infection is not completely ruled out on the basis of that result.5 Latent FIV infection in lymphocytes and macrophages could be reactivated in a local inflammatory lesion containing these cells. Proteins of feline herpesvirus 13-15 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. 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 in each tumor.9 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—Primers used in fresh feline tissues16-18 were synthesized by a commercial laboratory.19 Primer sequences, FIV genome positions, and expected length of PCR products were determined (Appendix).

Received Jun 24,1999.
Accepted Oct 19, 1999.

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Supported by a grant from the Health Services Utilization and Research Commission, SK, Canada.

Portions of this manuscript were presented at the 4th International Symposium on Predictive Oncology and Therapy, Nice, France, October 24–27, 1998.

The authors thank Dr. Sally Lester and Ian Shirley for technical assistance.
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 cm² of tissue. A DNA extraction was performed, using a commercially available tissue kit 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 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. The final concentration of each reagent in the reaction mixture was as follows: 10× PCR buffer II (10 mM tris HCl [pH 8.3], 50 mM KCl), 2.5 mM MgCl₂, 200 μM of each deoxynucleoside triphosphate (dNTP), 1 μM 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 and involved the following: 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).

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

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’-GTCCGCGCTTCAATGTGCTGCCAC-3’) [sense] and (5’-GGAGTGTTCACACGATGTA-3’) [antisense], 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, which is a molecular clone containing a full-length insert from the FIV-Petaluma strain. Plasmid DNA was purified, using a midiprep kit 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. 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, 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 in accordance with the manufacturer's instructions. Purified PCR products were submitted to another laboratory 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.
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 p33 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 p33 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 fg; 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 aspirates13 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; 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,23 and semi-nested PCR detects between 1 and 10 copies of FIV,23 the greatly increased risk of contamination with these procedures24 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 study20 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,23 investigators determined that the size of DNA fragments recovered from tissues treated in neutral-buffered 10% formalin was a less intense 300-bp band and a less intense 271-bp band.

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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.

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.

References


### Appendix

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5' to 3')</th>
<th>FIV Petaluma strain position</th>
<th>Length of target sequence (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIV-1</td>
<td>CTACGTCTCTGGAGGCTGAA</td>
<td>928–947</td>
<td>NA</td>
</tr>
<tr>
<td>FIV-4</td>
<td>CACCTCCTCAGCTGGTGC</td>
<td>1,374–1,393</td>
<td>465</td>
</tr>
<tr>
<td>P10-1</td>
<td>GCAAGACAATGTAGAGAAGT</td>
<td>1,785–1,804</td>
<td>NA</td>
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<tr>
<td>P10-2</td>
<td>TTTCCTCATTGGAGGTG</td>
<td>1,945–1,964</td>
<td>180</td>
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<tr>
<td>P15-1</td>
<td>GTGATATACCAGGCTTAA</td>
<td>778–797</td>
<td>NA</td>
</tr>
<tr>
<td>P15-2</td>
<td>TTTACTGTTTGAATGATT</td>
<td>1,029–1,048</td>
<td>271</td>
</tr>
</tbody>
</table>

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 23 µg/h).