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

Beverly A. Kidney, DVM, PhD; Deborah M. Haines, DVM, PhD; John A. Ellis, DVM, PhD; Micheline L. Burnham, BA; Marion L. Jackson, DVM, PhD

Objective—To evaluate a group of vaccine site-associated sarcomas (VSS) for the presence of feline foamy virus (FeFV) DNA, using polymerase chain reaction (PCR) methods.

Sample Population—50 formalin-fixed paraffin-embedded (FFPE) tissue blocks from VSS of cats.

Procedure—DNA was extracted from FFPE sections of each tumor, and regions of the *gag* and *pol* genes of FeFV were amplified by use of PCR methods, using 1 primer set for each region. Sensitivity of the method was compared between fresh and FFPE cells, using mouse kidney tissue that was injected with FeFV-infected cultured cells and using agarose-cell pellets.

Results—Feline foamy virus DNA was not detected in VSS tissues. Sensitivity of the method was 10 times greater in fresh versus FFPE mouse tissues. Sensitivity of the method in fresh FeFV-infected cultured cells versus FFPE agarose-cell pellets was equal when fixation was 24 or 48 hours and 10 times greater when fixation was 72 hours or 1 week.

Conclusions and Clinical Relevance—A PCR-based method can be successfully applied to FFPE tissues for FeFV DNA detection. Results suggest there is no direct FeFV involvement in the pathogenesis of VSS in cats. (*Am J Vet Res* 2002;63:60–63)

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] Feline **vaccine site-associated sarcomas (VSS)** have been linked to a variety of different vaccine types, including inactivated rabies virus vaccines, FeLV vaccines, and combination feline panleukopenia, rhinotracheitis, and calicivirus vaccines.[5-7] The low prevalence of VSS suggests that inflammation, necrosis, and healing may all play roles, but other factors in susceptible cats may be required for tumorigenesis. Retroviral infection represents 1 such factor that could be involved in the pathogenesis of feline VSS. Feline leukemia virus and feline immunodeficiency virus are retroviruses that have been investigated, but these viruses were not detected by use of immunohistochemical or **polymerase chain reaction (PCR)** methods in a large number of VSS.[8,9] **Feline foamy virus (FeFV)**, previously known as feline syncytium-forming virus, belongs to the retrovirus family. Foamy viruses are commonly isolated from cats; however, infection is usually not clinically apparent, and disease association remains obscure.[10] Although FeFV has not been considered to be oncogenic, it has been reported to cause malignant transformation of kidney cells in vitro.[11,12] Latent FeFV infection in lymphocytes and macrophages could be reactivated in a local inflammatory lesion containing these cells, and local replication of FeFV could predispose cells to insertional mutagenesis, altered expression of cellular genes, or altered expression of tumor suppressor genes. Our hypothesis is that FeFV is involved in the pathogenesis of VSS. Nonpathogenic or latent FeFV preexisting in host tissues could become oncogenic within the local environment created by vaccination. Alternatively, the virus could be introduced as a vaccine contaminant.

The objectives of the study reported here were to evaluate a group of VSS for the presence of FeFV DNA in **formalin-fixed paraffin-embedded (FFPE)** tissues, using PCR.

Received Mar 14, 2001.
Accepted Jun 7, 2001.
From the Departments of Veterinary Pathology (Kidney, Burnham, Jackson) and Veterinary Microbiology (Haines, Ellis), Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada S7N 5B4.
Supported by a grant from the Health Services Utilization and Research Commission, Saskatoon, SK, Canada.

Materials and Methods

Tissue specimens—The FFPE tissue blocks were obtained from feline biopsy specimens submitted during 1996 to a veterinary diagnostic laboratory.[a] The VSS tissues 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 histologic findings of inflammation and necrosis within each tumor.[13] Although detailed vaccination history was not available, all of the fibrosarcomas originated at locations commonly used for vaccine administration (interscapular, shoulder, thorax, thigh, hip, flank, lumbar).

Polymerase chain reaction primers—Sequences for the primers (**Table 1**) FUV 2662s and FUV 3065a that are published primers for the *gag/pol* overlap region[14] and the primers designed for our study, FeFV *gag* F and R, were synthesized by a commercial laboratory.[b] The primers FUV 2662s/3065a and FeFV *gag* F/R were expected to amplify a 404-**base pair (bp)** and a 122-bp fragment of FeFV, respectively.

Preparation of specimens for PCR—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 manufacturer's instructions. Concentration of extracted DNA was determined, using a 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.

Primer set FUV2662s and 3065a—A PCR mixture was

Table 1—Feline foamy virus (FeFV) polymerase chain reaction primer nucleotide sequences and genome coordinates

Primers	Nucleotide sequence (5'-3')	Coordinates*
FUV *gag/pol* 2662s	ACCTCCTCGTGGAAGTGG	2662–2679
FUV *gag/pol* 3065a	TTGCTGCCTAACAGGTTCTTCTCC	3042–3065
FeFV *gag* F	ACCACCAGGACCAAACCC	2734–2751
FeFV *gag* R	CTCCTCCTCTGGGATTGCC	2837–2855

*Complete nucleotide sequence of FeFV.[16]

prepared, using commercially available reagents. The final concentration of each reagent in the reaction mixture was as follows: 20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 µ*M* of each **deoxynucleoside triphosphate (dNTP)**, 1 µ*M* of each primer, 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. Amplification was accomplished with a thermal cycler, using the following program: 94 C for 5 minutes, then 35 cycles of 94 C for 1 minute, 50 C for 2 minutes, and 72 C for 2 minutes, followed by 72 C for 5 minutes.

Primer set FeFV gag F and R—The final concentration of reagents in the PCR mix was the same as for the FUV 2662s and 3065a primer set, except that 1 m*M* $MgCl_2$ and 0.5 µ*M* of each primer were used. Amplification was accomplished with a thermal cycler, using the following program: 94 C for 5 minutes, then 35 cycles of 94 C for 1 minute, 57 C for 2 minutes, and 72 C for 2 minutes, followed by 72 C for 5 minutes.

A 10-µl aliquot of PCR product was separated by electrophoresis[e] of the product through a 2% agarose mini-gel in accordance with a standard procedure.[15] A 100-bp DNA marker was included in each gel. The agarose gels were stained with ethidium bromide and photographed under ultraviolet transillumination,[f] and the image was captured by use of a documentation and analysis system.[g]

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]),[h] were used in a PCR. The final concentration of each reagent in the PCR reaction mix was as follows: 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, 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. Amplification was accomplished with a thermal cycler using the following program: 94 C for 3 minutes, then 30 cycles of 94 C for 1 minute, 52 C for 1 minute, and 72 C for 2 minutes.

Controls for PCR—Positive control DNA was prepared from cultured **felis catus whole fetus-4 (fcwf-4)** cells infected with FeFV.[i] The DNA was extracted from approximately 5×10^6 cultured cells, using a genomic DNA isolation reagent in accordance with manufacturer's instructions.[j] Extracted DNA was diluted to concentrations of 43 ng/µl and 4.3 ng/µl, and 2 µl of diluted genomic DNA was used in each PCR.

Negative control DNA was prepared from cultured fcwf-4 cells that were not infected with FeFV. Deoxyribonucleic acid was prepared in the same manner as for the infected cells. Two microliters of genomic DNA was used in each PCR. A second negative control sample that did not contain DNA was included in each PCR to monitor contamination of PCR reagents.

Sensitivity determination—Serial dilutions of the positive control DNA were prepared to determine the lower limit of FeFV detection, using both primer sets.

Comparison of sensitivity of PCR for mouse kidney tissue—A suspension was prepared that contained approximately 5×10^6 FeFV-infected fcwf-4 cultured cells in 100 µl of **phosphate-buffered saline (PBS)** solution. This preparation was injected into a laboratory mouse kidney that had been previously frozen. Four 25-µl aliquots were injected into different locations of the kidney to establish even distribution of the cultured cells within the kidney tissue. Four 25-mg sections of kidney tissue were removed immediately, and extraction of DNA was performed on each section in accordance with manufacturer's instructions.[c] The remaining kidney tissue was placed in neutral-buffered 10% formalin. Following 24 and 48 hours of fixation, portions of the tissue were sectioned, and a paraffin block was prepared for each fixation period. Four separate DNA extractions were performed on sections cut from each paraffin block, as described for the VSS tumor tissues. A DNA extract pool of the fresh, 24-hour, and 48-hour FFPE extracts was prepared by combining the 4 extracts obtained for each period. Polymerase chain reaction was performed on 10-fold dilutions of each of the 3 extract pools, using the FeFV *gag* F and R primer set.

Comparison of sensitivity of PCR for agarose pellets—This experiment was designed to achieve even distribution of FeFV-infected cells, because uneven distribution of FeFV-infected cells within the mouse kidney tissue was considered a potential limitation. Comparison of sensitivity was also performed by preparing 150-µl aliquots of a suspension containing 5×10^5 FeFV infected Crandell feline kidney cells in PBS solution. Deoxyribonucleic acid was extracted from 2 of the aliquots, using the procedure described earlier. The remaining 8 aliquots were placed into ELISA plate wells and combined with an equal volume of low melting point 2% agarose and allowed to solidify. All of the agarose-cell pellets were placed in neutral-buffered 10% formalin. Two pellets were fixed for each of the following periods: 24, 48, and 72 hours and 1 week. Following fixation, each agarose-cell pellet was sectioned, and a paraffin block was prepared for each pellet. Deoxyribonucleic acid was extracted from sections cut from each paraffin block, and DNA was pooled for each fixation time. Polymerase chain reaction was performed on 10-fold dilutions of each extract pool, using the FeFV *gag* F and R primer set.

Nucleotide sequence of PCR products—Product bands that resulted from amplification of FeFV sequences from infected cultured cells using both primer sets were purified by use of a gel extraction kit[k] in accordance with manufacturer's instructions. Purified PCR products were submitted to another laboratory[l] for automated nucleotide sequencing. Sequence data from FeFV positive control samples were compared with the complete nucleotide sequences of the FUV[16] and F17[17] strains of FeFV.

Results

We did not detect FeFV DNA in the 50 VSS tissues with the PCR technique, using either of the 2 primer pairs. A single 404-bp product was amplified from the FeFV positive control with each PCR, using the FUV 2662s and 3065a primer pair. Similarly, a 122-bp product was amplified from the positive control with each PCR, using the FeFV *gag* F and R primer pair. The negative control and the reaction mixture that did not contain DNA were negative for each PCR.

Using the feline p53 PCR primers to assess amplifiable DNA, a single band was evident at the expected bp location in all of the tumor tissues except 1. The DNA extraction procedure was repeated on that tissue,

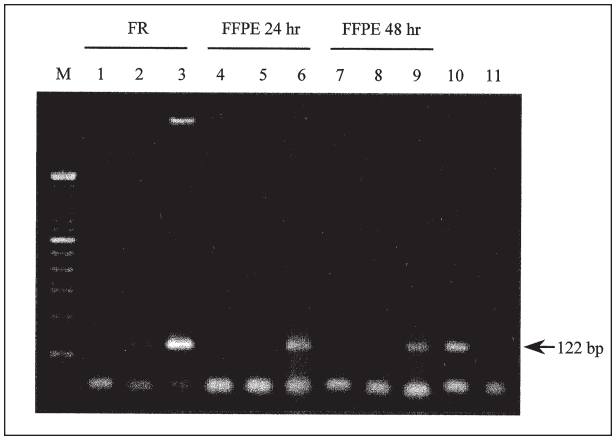

Figure 1—Ethidium bromide-stained agarose gel of polymerase chain reaction (PCR) products amplified, using primers FeFV *gag* F and R, from serially diluted fresh (FR) or formalin-fixed paraffin-embedded (FFPE) mouse kidney tissue injected with feline foamy virus (FeFV) infected cultured cells. Lane M is a 100-base pair (bp) DNA ladder. Lanes 1, 2, and 3 are from FR, lanes 4, 5, and 6 are from FFPE tissue fixed for 24 hours, and lanes 7, 8, and 9 are from FFPE tissue fixed for 48 hours. Product is not evident in lanes 1, 4, and 7, in which 0.01 µg of genomic DNA was used as template. Lanes 2, 5, and 8 represent PCR products in which 0.1 µg of genomic DNA was used as template. Lane 2 (FR) contains the 122-bp FeFV product, but lanes 5 and 8 (FFPE) do not. Lanes 3, 6, and 9 represent PCR products in which 1 µg of genomic DNA was used as template and demonstrate a positive result indicated by a 122-bp FeFV product. Lane 10 is a positive control sample containing 8.6 ng of DNA from cultured FeFV-infected cells. Lane 11 is a negative control sample containing reagents but no DNA.

and the p53 product was present on the second PCR of that tissue. The second extract was used as the template DNA in the FeFV PCR.

The lower limit of detection of FeFV in cultured cells, using primer sets FUV 2662 and 3065 and FeFV *gag* F and R, was 17.2 and 0.86 ng of genomic DNA, respectively. Using the primers FeFV *gag* F and R, the lower limit of detection of FeFV DNA in mouse kidney tissue was 0.1 µg for fresh tissue and 1 µg for FFPE tissue fixed for 24 or 48 hours (**Fig 1**). Using the same primers, the lower limit of detection of FeFV DNA in fresh cultured cells versus FFPE cells prepared in agarose was 1 ng (fresh cells), 1 ng (24 or 48 hours fixation), and 10 ng (72 hours or 1 week fixation).

The nucleotide sequence obtained from purified PCR product, using the primer pair FUV2662s and FUV 3065a to amplify a 404-bp fragment of the FeFV genome, revealed 98 and 96% homogeneity with the sequences published for the FUV and F17 strains, respectively. The nucleotide sequence obtained, using the primer pair FeFV *gag* F and R, revealed 98 and 99% homogeneity with the sequences published for the FUV and F17 strains of FeFV, respectively.

Discussion

Polymerase chain reaction techniques have been used to detect FeFV DNA in peripheral blood mononuclear cells.[10,18] In the study reported here, we documented the successful application of PCR for detection of FeFV in FFPE tissues. Analysis of our results, using mouse kidney tissue injected with FeFV infected cells, revealed a 10-fold greater sensitivity in fresh versus FFPE tissue that had been fixed for 24 or 48 hours. Because there is a possibility of uneven distribution of infected cells within the kidney tissue, we performed a similar experiment using infected cells in agarose pellets to ensure even distribution of cells. The sensitivity within fresh cells was equal to FFPE cells that had been fixed for 24 or 48 hours and 10-fold greater, compared with cells that had been fixed for 72 hours or 1 week.

The PCR primers used in our study were directed at the *gag* gene (FeFV gag F and R primer pair) and the *gag/pol* overlap region (FUV 2662a and 3065s primer pair). The highest degree of conservation among various FeFV field isolates was in the *gag* region (96% identity) and in the *pol* region (92% identity) of the viral genome.[10] We chose 2 sets of PCR primers to increase the likelihood of detecting FeFV field strains that may differ in nucleotide sequence. Nucleotide sequencing of FeFV infected cultured cells revealed a high degree of homology to published FeFV sequences. These minor differences in nucleotide sequence are consistent with a field strain that differs very slightly from the published sequences.

Despite the use of 2 sets of primers, the PCR did not detect FeFV DNA in any of the VSS tumor tissues tested. Failure to detect FeFV DNA in the sarcomas most likely indicates that FeFV is not involved in the pathogenesis of these tumors. False-negative results for the PCR are also possible. The number of cells containing viral DNA may have been less than the detection limits of the PCR used. Alternatively, false-negative results could occur with mutation or deletion within the FeFV *gag* and *pol* regions such that the primers fail to recognize the gene sequence. We attempted to overcome this potential problem by using 2 primer sets that would bind to different sequences. Finally, FeFV DNA may have been undetectable in these tumors as a result of technical problems. There is a potential concern that formalin fixation may degrade DNA, rendering only short sequences detectable by use of PCR. Polymerase chain reaction products up to a range of 450 to 650 bp can be amplified from most FFPE sections.[19] We chose to design our second primer pair, FeFV gag F and R, to amplify a fragment of only 122 bp to avoid false-negative results because of DNA degradation. However, even the larger PCR product of 404 bp is still within acceptable size limits for formalin-fixed tissue. The length of time that the tissue remains in formalin prior to processing may affect PCR results. There was a substantial decline in efficiency of PCR amplification when tissue was fixed in neutral-buffered 10% formalin for 1 to 4 weeks, but little or no effect occurred at ≤ 48 hours fixation in 1 report.[20] The PCR sensitivity in our study was similar when tissue or cells were fixed for 24 or 48 hours and only slightly decreased when the cells were fixed for 72 hours or 1 week. Presumably, a false-negative result may be possible for any VSS tissue fixed for greater than 48 hours. However, we were able to detect p53 DNA in all of the VSS tissues in our study, ensuring that sequences of at least the size of the p53 amplicon (108 bp) could be amplified. Detection of amplifiable p53 DNA in our study also ensured that our DNA extraction system was functioning and ruled out the possibility of interfer-

ence of the PCR, particularly inhibition of the Taq DNA polymerase by contaminants in the DNA sample.

We did not document an association between the exogenous retrovirus FeFV and a unique group of sarcomas in cats. Lack of FeFV DNA within any of the sarcomas suggests that this virus is unlikely to be directly involved in the pathogenesis of these neoplasms.

[a]Lester S, Central Laboratory for Veterinarians Ltd, Langley, BC, Canada.

[b]Custom primers, Gibco BRL Life Technologies Inc, Burlington, ON, Canada.

[c]QIAamp tissue kit, Qiagen Inc, Mississauga, ON, Canada.

[d]Diode array spectrophotometer, 8452A, Hewlett-Packard (Canada) Ltd, Edmonton, AB, Canada.

[e]Pedersen N, University of California, Davis, Calif.

[f]DNAzol reagent, Gibco BRL Life Technologies Inc, Burlington, ON, Canada.

[g]Mupid-2 mini gel migration trough, Topogen Inc, Columbus, Ohio.

[h]FBTIV-816 Transilluminator, Fisher Scientific Co, Edmonton, AB, Canada.

[i]Alpha imager 2000 documentation and analysis system, Alpha Innotech Corp, San Leandro, Calif.

[j]Nambiar P. *Immunohistochemical and mutational analysis of the tumor suppressor gene p53 in feline vaccine-associated sarcomas*. MS thesis, Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, 1998.

[k]Concert rapid gel extraction system, Gibco BRL Life Technologies Inc, Burlington, ON, Canada.

[l]DNA Technologies Unit, National Research Council of Canada, Plant Biotechnology Institute, Saskatoon, SK, Canada.

References

1. Hendrick MJ, Goldschmidt MH, Shofer FS, et al. Postvaccinal sarcomas in the cat: epidemiology and electron probe microanalytical identification of aluminum. *Cancer Res* 1992;52:5391–5394.

2. Hendrick MJ, Goldschmidt MH. Do injection site reactions induce fibrosarcomas in cats (lett)? *J Am Vet Med Assoc* 1991;199:968.

3. Coyne MJ, Reeves NC, Rosen DK. Estimated prevalence of injection-site sarcomas in cats during 1992. *J Am Vet Med Assoc* 1997;210:249–251.

4. Kass PH, Barnes WG Jr, Spangler WL, et al. Epidemiologic evidence for a causal relation between vaccination and fibrosarcoma tumorigenesis in cats. *J Am Vet Med Assoc* 1993;203:396–405.

5. Hendrick MJ, Kass PH, McGill LD, et al. Postvaccinal sarcomas in cats. *J Natl Cancer Inst* 1994;86:341–343.

6. Hendrick MJ, Shofer FS, Goldschmidt MH, et al. Comparison of fibrosarcomas that developed at vaccination sites and at nonvaccination sites in cats: 239 cases (1991–1992). *J Am Vet Med Assoc* 1994;205:1425–1429.

7. Lester S, Clemett T, Burt A. Vaccine site-associated sarcomas in cats: clinical experience and a laboratory review (1982–1993). *J Am Anim Hosp Assoc* 1996;32:91–95.

8. Ellis JA, Jackson ML, Bartsch RC, et al. Use of immunohistochemistry and polymerase chain reaction for detection of oncornaviruses in formalin-fixed, paraffin-embedded fibrosarcomas from cats. *J Am Vet Med Assoc* 1996;209:767–771.

9. Kidney BA, Ellis JA, Haines DM, et al. Evaluation of formalin-fixed paraffin-embedded tissues obtained from vaccine site-associated sarcomas of cats for DNA of feline immunodeficiency virus. *Am J Vet Res* 2000;61:1037–1041.

10. Winkler IG, Flügel RM, Löchelt M, et al. Detection and molecular characterisation of feline foamy virus serotypes in naturally infected cats. *Virology* 1998;247:144–151.

11. McKissick GE, Lamont PH. Characteristics of a virus isolated from a feline fibrosarcoma. *J Virol* 1970;5:247–257.

12. Pedersen NC. Feline syncytium-forming virus infection. In: Holzworth J, ed. *Diseases of the cat: medicine and surgery*. Philadelphia: WB Saunders Co, 1987;268–272.

13. Hendrick MJ, Brooks JJ. Postvaccinal sarcomas in the cat: histology and immunohistochemistry. *Vet Pathol* 1994;31:126–129.

14. Winkler IG, Löchelt M, Levesque J, et al. A rapid streptavidin-capture ELISA specific for the detection of antibodies to feline foamy virus. *J Immunol Methods* 1997;207:69–77.

15. Sambrook J, Fritsch EF, Maniatis T. Gel electrophoresis of DNA. In: Sambrook J, Fritsch EF, Maniatis T, eds. *Molecular cloning. A laboratory manual*. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989;6.1–6.62.

16. Winkler I, Bodem J, Haas L, et al. Characterization of the genome of feline foamy virus and its proteins shows distinct features different from those of primate spumaviruses. *J Virol* 1997;71:6727–6741.

17. Helps CR, Harbour DA. Comparison of the complete sequence of feline spumavirus with those of the primate spumaviruses reveals a shorter *gag* gene. *J Gen Virol* 1997;78:2549–2564.

18. Winkler IG, Löchelt M, Flower RLP. Epidemiology of feline foamy virus and feline immunodeficiency virus infections in domestic and feral cats: a seroepidemiological study. *J Clin Microbiol* 1999;37:2848–2851.

19. Wright DK, Manos MM. Sample preparation from paraffin-embedded tissues. In: Innis MA, Gelfand DH, Sninsky JJ, et al, eds. *PCR protocols. A guide to methods and applications*. San Diego: Academic Press Inc, 1990;153–158.

20. Rogers BB, Alpert LC, Hine EAS, et al. Analysis of DNA in fresh and fixed tissue by the polymerase chain reaction. *Am J Pathol* 1990;136:541–548.