

# Accuracy of polymerase chain reaction assays for diagnosis of feline immunodeficiency virus infection in cats

P. Cynda Crawford, DVM, PhD; Margaret R. Slater, DVM, PhD; Julie K. Levy, DVM, PhD, DACVIM

**Objective**—To determine the sensitivity, specificity, and overall diagnostic accuracy of polymerase chain reaction (PCR) assays offered by commercial diagnostic laboratories for diagnosis of FIV infection in cats.

**Design**—Prospective clinical trial.

**Animals**—124 cats.

**Procedure**—Blood was collected from cats that were neither infected with nor vaccinated against FIV, uninfected cats that were vaccinated with a licensed FIV vaccine, and cats experimentally and naturally infected with FIV representing subtypes A, B, and C. Coded blood samples were submitted to 3 laboratories in the United States and Canada offering PCR assays for diagnosis of FIV infection to veterinary practitioners. All laboratories tested fresh blood samples, and 1 laboratory also tested samples submitted as dried blood smears. The FIV infection status in all cats was confirmed by virus isolation. Sensitivity, specificity, and correct results were calculated for each PCR assay.

**Results**—Sensitivity ranged from 41% to 93%. Specificity ranged from 81% to 100% in unvaccinated cats and 44% to 95% in cats vaccinated against FIV. Correct results were obtained in 58% to 90% of 124 cats tested. All tests misidentified both uninfected and infected cats. False-positive results by all laboratories were higher in cats vaccinated against FIV than in unvaccinated cats, suggesting that vaccination interferes with the performance or interpretation of PCR assays used for diagnosis of FIV infection.

**Conclusions and Clinical Relevance**—PCR assays used for diagnosis of FIV infection presently marketed to veterinary practitioners in North America vary significantly in diagnostic accuracy and did not resolve the diagnostic dilemma resulting from vaccination of cats against FIV. (*J Am Vet Med Assoc* 2005;226:1503–1507)

Vaccination of cats against FIV with a whole-virus vaccine results in rapid and persistent production of antibodies that are indistinguishable from those

used for diagnosis of FIV infection.<sup>1,2</sup> As such, veterinary practitioners can no longer reliably determine the FIV infection status of cats that have positive antibody results in ELISA, western blot, or immunofluorescent antibody tests.<sup>1</sup> These cats may have antibodies attributable to vaccination against FIV, infection with FIV, or both. The inability of present serologic tests to distinguish between antibodies induced by infection or vaccination has created a diagnostic dilemma for FIV.

The polymerase chain reaction (PCR) assay has been promoted as a potential solution for confirming the true FIV status of cats.<sup>3,5</sup> Polymerase chain reaction assays exponentially amplify specific viral DNA sequences until they are present at detectable concentrations. Because diagnostic PCR assays can detect as few as 1 to 10 copies of viral DNA in a given sample, they are much more sensitive than many other testing methodologies.<sup>6</sup> However, the high sensitivity of PCR assays may lead to false-positive results if minute amounts of DNA contamination occur during collection, storage, or processing of samples.<sup>6</sup>

Like other lentiviruses, FIV has a high intrinsic mutation rate in the envelope (*env*) and capsid (*gag*) genes, which has led to the evolution of several distinct genetic subtypes. Sequence divergence in the *env* and *gag* genes ranges as much as 26% both within a subtype and between subtypes.<sup>3,7</sup> There are 5 well-characterized subtypes of FIV (A to E) based on genetic divergence in the *env* and *gag* genes.<sup>3</sup> The most common subtypes found in FIV-infected cats in the United States and Canada are subtypes A, B, and C.<sup>5</sup> Recently, a sixth subtype (TX) has been described in cats from Texas.<sup>8</sup> Additional subtypes may emerge as more strains of FIV are fully characterized.

The PCR assay depends on precise matching between the genetic sequences of the virus and the primer sequences selected for detection of the genome. Primers are often selected on the basis of *env* and *gag* genetic sequences of a few well-characterized FIV strains. How well these *env* and *gag* primers detect the wide variety of genetically divergent FIV strains found in nature is unknown, but false-negative results ranging from 10% to 100% have been reported.<sup>5,7,9,10</sup>

Several reference laboratories offer unlicensed PCR assays for diagnostic purposes, but virtually nothing is known about the sensitivity, specificity, and overall performance of these assays. Accurate diagnosis of FIV is important for both uninfected and infected cats. Failure to identify infected cats may lead to inadvertent exposure and transmission of FIV to uninfected cats. Misdiagnosis of FIV in uninfected cats may lead to inappropriate euthanasia. This is especially a problem in cats for which complete medical histories are not

From the Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL 32610 (Crawford, Levy); and the Department of Veterinary Anatomy and Public Health, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4458 (Slater).

Supported by a grant from the George Sydney and Phyllis Redman Miller Trust in cooperation with the Winn Feline Foundation. IDEXX Laboratories Inc contributed testing reagents and services. The authors thank Drs. Christian Leutenegger, Susan Little, Leslie Sinclair, Ellen Collisson, and Pamela Berlinski for providing blood samples from cats infected with FIV and Marc Salute, Karen Scott, and Alex Trapp for technical assistance.

Address correspondence to Dr. Levy.

available, such as those in animal shelters or cats adopted as strays. The purpose of the study reported here was to determine the sensitivity, specificity, and overall diagnostic accuracy of PCR assays offered by commercial diagnostic laboratories to veterinary practitioners in the United States and Canada for diagnosis of FIV infection in cats.

## Materials and Methods

**Cats**—The study design was a prospective clinical trial involving 124 cats. Sensitivity and specificity of PCR assays used for diagnosis of FIV infection were determined with 42 **specific pathogen-free (SPF)** cats neither infected with nor vaccinated against FIV, 41 SPF cats not infected with but vaccinated against FIV, 19 SPF cats experimentally infected with FIV, and 22 client-owned cats with natural FIV infection. Mean  $\pm$  SD age of vaccinated cats was  $0.5 \pm 0.4$  years (range, 8 weeks to 1.1 years) at the time the first dose of vaccine against FIV was administered. The experimentally infected cats included 9 with FIV subtype A (4 NCSU<sub>1</sub>, 4 PPR, and 1 Stroker), 4 with FIV subtype B (Mt Airy), and 6 with FIV subtype C (PGammer). The client-owned cats with natural FIV infection were recruited from Florida, Maryland, and Ontario. The FIV subtypes were known for only 6 of those cats and included 3 with subtype A, 2 with subtype B, and 1 with subtype C.

The SPF cats were housed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The study was approved by the University of Florida Institutional Animal Care and Use Committee and the Clinical Research Review Committee.

**Vaccination against FIV**—The licensed FIV vaccine<sup>a</sup> used in this study is a dual subtype vaccine containing inactivated subtype A and subtype D viruses, cells in which the virus was propagated, and adjuvant. The vaccine was administered SC in the right hind limb every 2 weeks for a total of 3 doses as recommended by the manufacturer. Blood samples from vaccinated cats were collected  $5.0 \pm 1.5$  months (range, 3.5 to 7.2 months) after the first vaccine dose.

**Blood collection**—Cats were briefly anesthetized for blood collection with isoflurane administered by face mask. Blood (21 mL) was collected from the jugular vein into potassium EDTA tubes from each cat. Separate tubes were collected for each laboratory participating in the study to avoid the risk of contamination associated with preparation of multiple aliquots from 1 sample. Because 1 laboratory solicits submission of blood smears as a convenient alternative to collection of blood samples, 2 blood smears were made on glass slides for each cat by withdrawing 0.5 mL of blood from 1 of the EDTA tubes and allowing 0.25 mL of blood to air dry on each slide. The dried slides were packaged in cardboard carriers. Each blood tube and slide carrier was enclosed in an individual plastic bag to further reduce risk of leakage and contamination during shipping. All samples were coded so that laboratory personnel were unaware of the FIV status of the cats. Samples were shipped overnight with ice packs so that they arrived at the diagnostic laboratories within 24 hours of collection.

To determine whether the vaccine viruses could be detected by the PCR assays, each laboratory was asked to assay the vaccine itself. In addition, the vaccine was diluted 1:5 into a blood sample from an uninfected, unvaccinated cat and was coded and submitted to the laboratories along with the other blood samples.

To determine the potential for detection of the vaccine viruses in blood samples from recently vaccinated cats, a single dose of the vaccine was administered to 4 SPF cats. Blood

samples were collected as previously described immediately prior to vaccination, then daily for 6 days, then every 3 days for a total of 24 days after vaccination. The samples were coded and submitted to the laboratories as for the other blood samples.

**PCR laboratories**—Three laboratories in the United States and Canada that routinely offer PCR assays for diagnosis of FIV infection agreed to participate in the study. Each laboratory provided basic information about their proprietary PCR reagents and assay.

Laboratory 1<sup>b</sup> (PCR1 assay) extracted genomic DNA from WBCs harvested from 4.5 mL of blood by use of a commercial kit. A real-time PCR assay was used to assess the quality and quantity of the extracted genomic DNA by amplification of a housekeeping gene.<sup>11</sup> For detection of FIV sequences in the genomic DNA, a real-time PCR assay was used to detect the *gag* gene for subtypes A, B, C, D, and TX.<sup>6,12,13,c</sup> Results were reported as positive or negative.

Laboratory 2<sup>d</sup> (PCR2 assay) isolated genomic DNA from WBCs in 0.25 mL of blood by use of a commercial reagent. The genomic DNA was subjected to conventional PCR assay by use of proprietary primer sequences for both the *env* and *gag* gene for the first round of amplification, followed by a second round of amplification using internal (nested) primers as described.<sup>14</sup> The PCR assay products were resolved by agarose gel electrophoresis and the outcome reported as positive or negative based on the presence or absence of a band of the expected size.

Laboratory 3<sup>e</sup> (PCR3 assay) isolated genomic DNA from 75  $\mu$ L of blood by use of a commercial kit. In addition, this laboratory rehydrated the dried blood smears with 200  $\mu$ L of saline (0.9% NaCl) solution and used 75  $\mu$ L of the rehydrated sample for DNA isolation (PCR4 assay). The genomic DNA samples were subjected to conventional PCR amplification by use of a mixture of 2 sets of primers for the *gag* gene as previously described.<sup>9</sup> The PCR assay products were resolved by agarose gel electrophoresis and the outcome reported as positive or negative based on the presence or absence of a band of the expected size for either primer set. Samples that were negative after the first round of amplification were subjected to a second round of amplification with the same primers.

**FIV culture**—The FIV status of each cat was verified by culture of peripheral blood mononuclear cells for virus as previously described.<sup>15</sup> Briefly, mononuclear cells were isolated by discontinuous density gradient centrifugation of blood. The CD8<sup>+</sup> T cells, which have been found to inhibit FIV replication in culture,<sup>15-17</sup> were removed from the mononuclear cells by immunomagnetic separation by use of monoclonal anti-feline CD8 antibody conjugated to phycoerythrin,<sup>f</sup> anti-phycoerythrin microbeads,<sup>8</sup> and magnetic separation columns<sup>h</sup> as described.<sup>15</sup> The remaining mononuclear cells were cocultured with a feline CD4<sup>+</sup> T-cell line permissive for FIV replication.<sup>15</sup> All cultures were performed in triplicate. Culture supernatants were collected at 7, 14, and 21 days and tested for FIV p24 antigen by ELISA.<sup>i</sup> Cats were considered as being infected with FIV if the culture supernatant was positive for FIV antigen at any of the 3 time points tested.

**Statistical analyses**—Data analysis was performed by use of a commercial software program.<sup>j</sup> Data with normal distribution were described as mean  $\pm$  SD and range. Data that were not normally distributed were summarized as median and range. Sensitivity of the PCR assays for FIV infection was calculated as the percentage of cats infected with FIV correctly identified as positive by the assay. The effects of viral subtype and of experimental versus natural infection on sensitivity were tested by use of  $\chi^2$  analysis. Specificity was cal-

culated as the percentage of cats not infected with FIV correctly identified as negative by the assay. Ninety-five percent confidence intervals were used to determine the precision of the sensitivity or specificity estimate. Assays with sensitivity or specificity outside the 95% confidence interval of another assay were considered significantly different at values of  $P < 0.05$ . The overall accuracy of each PCR assay was defined as the number of correct results for all 124 cats.

## Results

Virus culture confirmed the FIV status of all 124 cats. Laboratory 1 (PCR1 assay) correctly identified 31 of the 41 FIV-infected cats (76% sensitivity; Table 1). All 3 FIV subtypes (A, B, and C) were found among the detected and undetected infections. There was no significant difference in the sensitivity when results were analyzed by viral subtype or by experimental versus natural infection. The PCR1 assay correctly identified 42 of 42 uninfected, unvaccinated cats (100% specificity) and 39 of 41 uninfected, vaccinated cats (95% specificity). Specificity was not significantly different between the 2 groups (unvaccinated vs vaccinated) of uninfected cats. Overall, the PCR1 assay correctly identified 112 of 124 cats tested (90% correct results; Figure 1).

Laboratory 2 (PCR2 assay) correctly identified 38 of 41 FIV-infected cats (93% sensitivity; Table 1). The PCR2 assay detected all 3 subtypes of FIV but also missed some cats with subtype A or B infection. There was no significant difference in the sensitivity when results were analyzed by viral subtype or by experimental versus natural infection. The PCR2 assay correctly identified 34 of 42 uninfected, unvaccinated cats (81% specificity). In contrast, only 27 of the 41 vaccinated cats were correctly identified as not having FIV (66% specificity). Thus, false-positive results were significantly more likely in vaccinated cats (34%), compared with unvaccinated cats (19%). Overall, the PCR2 assay correctly identified 99 of 124 cats tested (80% correct results; Figure 1).

Laboratory 3 correctly identified only 21 of 41 FIV-infected cats (51% sensitivity) when blood samples were used for analysis (PCR3 assay) and 17 of 41 FIV-infected cats (41% sensitivity) when blood smears were used (PCR4 assay; Table 1). All 3 subtypes of FIV (A, B, and C) were found among both the detected and missed infections, and there were no significant differences in the sensitivity when results were analyzed by viral subtype or by experimental versus natural infection. Both PCR3 and PCR4 assays correctly identified 34 of 42 uninfected, unvaccinated cats (81% specificity). However, only 18 of 41 vaccinated cats were correctly

reported as not having FIV by the PCR3 assay (44% specificity) and 21 of 41 by the PCR4 assay (51% specificity). Thus, false-positive results were significantly more likely in vaccinated cats (56% for PCR3 and 49% for PCR4 assays), compared with unvaccinated cats (19% for both PCR3 and PCR4 assays). Overall, the PCR3 assay correctly identified 73 of 124 cats tested (59% correct results), and the PCR4 assay correctly identified 72 of 124 cats (58% correct results; Figure 1).

In summary, the PCR2 assay was the most sensitive assay, followed by PCR1. The PCR1 assay was the most specific assay for both unvaccinated cats and cats vaccinated for FIV. Overall, the PCR1 assay was the most accurate, followed by PCR2.

Laboratory 3 used the same PCR assay for blood samples (PCR3) and blood smears (PCR4). As such, the results for each cat would be expected to match regardless of the sample format. However, discordant results between the assays used for blood samples and blood smears were reported for 24% of uninfected and unvaccinated cats, 61% of uninfected and vaccinated cats, and 34% of FIV-infected cats. For all 124 cats, 49 samples were discordant (40%), 27 samples matched but were inaccurate (22%), and 48 samples matched and were correct (39%).

To determine the effect of recent FIV vaccination on PCR testing, each laboratory performed its PCR

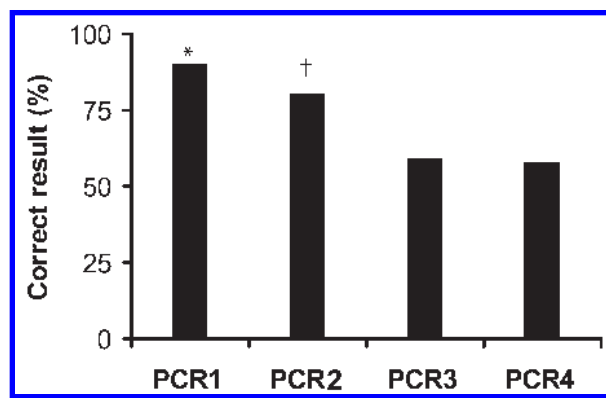


Figure 1—Overall diagnostic accuracy of polymerase chain reaction (PCR) assays from Laboratory 1 (PCR1), Laboratory 2 (PCR2), and Laboratory 3 (PCR3 and PCR4). The PCR assays were performed on blood samples (PCR1, PCR2, and PCR3) and blood smears (PCR4) from cats neither infected with nor vaccinated against FIV ( $n = 42$ ), cats not infected with but vaccinated against FIV (41), and cats infected with FIV (41). Overall diagnostic accuracy is defined as the number of correct results for the total population of cats ( $n = 124$ ) and is expressed as a percentage. \*Significantly ( $P < 0.05$ ) different from PCR2, PCR3, and PCR4. †Significantly ( $P < 0.05$ ) different from PCR1, PCR3, and PCR4.

Table 1—Sensitivity and specificity (95% confidence intervals) for 4 commercial polymerase chain reaction (PCR) assays used for detection of FIV infection in cats.

| Status of cats                  | No. of cats | PCR1                          | PCR2                        | PCR3                        | PCR4                        |
|---------------------------------|-------------|-------------------------------|-----------------------------|-----------------------------|-----------------------------|
| FIV-infected cats (sensitivity) | 41          | 76 <sup>a</sup><br>(60, 88)   | 93 <sup>b</sup><br>(80, 98) | 51 <sup>c</sup><br>(35, 67) | 41 <sup>c</sup><br>(26, 59) |
| Unvaccinated cats (specificity) | 42          | 100 <sup>a</sup><br>(92, 100) | 81 <sup>b</sup><br>(67, 91) | 81 <sup>b</sup><br>(66, 91) | 81 <sup>b</sup><br>(66, 91) |
| Vaccinated cats (specificity)   | 41          | 95 <sup>a</sup><br>(83, 99)   | 66 <sup>b</sup><br>(49, 80) | 44 <sup>c</sup><br>(26, 58) | 51 <sup>c</sup><br>(35, 67) |

<sup>a,b,c</sup> Within a row, values with different superscript letters are significantly different ( $P < 0.05$ ).



assay on the vaccine, blood samples spiked with the vaccine, and blood samples from recently vaccinated cats. Results of all PCR assays (PCR1, PCR2, and PCR3) were positive when the vaccine alone was assayed. Two of the assays (PCR1 and PCR3) also detected the vaccine virus in the blood sample spiked with the vaccine. The PCR1 assay reported negative results for all blood samples collected from 4 cats 1 to 24 days after a single vaccination. The PCR2 assay reported a single positive result in 1 cat 2 days after vaccination. The PCR3 assay reported positive results for several blood samples collected prior to vaccination and on days 1, 2, 3, 9, 12, and 15 after vaccination.

## Discussion

Performance of PCR diagnostic assays for detection of FIV infection presently marketed to veterinary practitioners in North America varied significantly among different laboratories. The PCR assays used by the 3 reference laboratories that participated in this study misidentified both infected and uninfected cats. Sensitivity (identification of truly infected cats) for FIV infection ranged from 41% to 93% (ie, 7 to 59 of every 100 FIV-infected cats tested would be incorrectly identified as not being infected with FIV, depending on the particular laboratory that was used). All PCR assays detected cats that were infected either experimentally or naturally with FIV subtypes A, B, and C, which are the predominant subtypes in North American cats. However, the PCR assays also missed cats infected with these subtypes. Sensitivity was not associated with the type of PCR assay (conventional vs real-time) that was used for detection of FIV infection or the primer specificity for *env* or *gag* gene sequences. For example, the real-time PCR assay used by Laboratory 1 (PCR1 assay) can reportedly detect as few as 1 to 10 copies of proviral DNA and uses reagents that identify *gag* gene sequences in 5 subtypes of FIV (A, B, C, D, and TX).<sup>6,12,c</sup> Despite the capacity for detection of only a few copies of viral DNA from a wide range of FIV subtypes, this assay failed to detect nearly 25% of cats infected with FIV.

Specificity (identification of truly uninfected cats) ranged from 81% to 100% when unvaccinated cats were tested. Laboratory 1 (PCR1 assay) correctly identified all uninfected, unvaccinated cats as not being infected with FIV; however, the other 2 laboratories (PCR2, PCR3, and PCR4 assays) identified 19% of the cats as being infected with FIV. In addition, these 2 laboratories reported significantly higher false-positive results for cats vaccinated against FIV than unvaccinated cats, such that 33 to 56 of every 100 vaccinated cats tested would be misidentified as being infected with FIV. In contrast, the specificity of the PCR assay used by laboratory 1 (PCR1 assay) was not significantly affected by the vaccination status of the cat.

A potential reason for the high rate of false-positive results in vaccinated cats is detection of virus administered in the vaccine. The PCR assays for all 3 laboratories detected FIV in the vaccine itself, but 1 laboratory (PCR2 assay) did not detect the vaccine virus added directly to whole blood. The most accurate PCR assay (PCR1) did not detect the vaccine virus in blood col-

lected from cats during the first 3 weeks after a single vaccination against FIV was administered. The least accurate PCR assay (PCR3) intermittently reported positive results for blood collected prior to vaccination and on days 1 to 15 after vaccination. These results, coupled with the fact that cats were tested 2 to 6 months after completion of vaccination, indicated that PCR detection of the vaccine virus was not a plausible explanation for the high rate of false-positive results in vaccinated cats.

Another possible explanation for the high rate of false-positive results in cats vaccinated for FIV may have been related to the type of PCR assay used by the laboratories. Vaccination did not significantly interfere with identification of uninfected cats by laboratory 1 (PCR1 assay). This laboratory uses a real-time PCR assay in which results are generated by a computer, thereby decreasing subjectivity inherent with human interpretation. Vaccination significantly interfered with identification of uninfected cats by laboratories 2 and 3. The conventional PCR assays used by these laboratories rely on visual assessment of agarose gels to diagnose FIV; thus, interpretation of results is influenced by human subjectivity. Although the blood samples were coded so that laboratory personnel would be unaware of the cats' FIV status, some laboratories pre-screen samples for FIV antibodies prior to performing PCR assays. If personnel are aware of serologic test results prior to viewing the gels, their subjective assessment could be biased. However, all laboratories denied performing any serologic screening tests before PCR assays were performed. Another possible explanation for decreased specificity observed by Laboratories 2 and 3 may have been the use of 2 rounds of PCR amplification, compared with use of only 1 round of real-time PCR by laboratory 1. Although multiple rounds of PCR amplification may decrease specificity overall, it should not have a greater effect on samples from vaccinated than from unvaccinated cats.

When results from all 124 cats were combined, the percentage of cats in which the FIV status was correctly identified by the PCR assays ranged from 58% to 90%. The PCR assay has been promoted as a solution to the diagnostic dilemma created by FIV vaccination because serologic tests can no longer accurately identify FIV-infected cats. However, PCR assays used for detection of FIV infection presently marketed to veterinary practitioners in North America vary significantly in diagnostic accuracy and do not resolve the diagnostic issues resulting from FIV vaccination against FIV in cats.

- 
- a. Fel-O-Vax FIV, Fort Dodge Animal Health, Fort Dodge, Iowa.
  - b. Lucy Whittier Molecular Core Facility, School of Veterinary Medicine, University of California, Davis, Calif.
  - c. Leutenegger CM, Lucy Whittier Molecular Core Facility, School of Veterinary Medicine, University of California, Davis, Calif: Personal communication, 2004.
  - d. Vita-Tech Laboratories, Buffalo, NY.
  - e. DyNagenics Veterinary Diagnostics Parasitology Research Lab LLC, Denver, Colo.
  - f. Mouse anti-feline CD8  $\alpha/\beta$  clone vpg9, Serotec Inc, Raleigh, NC.
  - g. Miltenyi Biotec Inc, Auburn, Calif.
  - h. MiniMACS, Miltenyi Biotec Inc, Auburn, Calif.
  - i. PetChek FIV Antigen Test, IDEXX Laboratories, Westbrook, Me.
  - j. Intercooled Stata 8, Stata Corp, College Station, Tex.
-

## References

1. Levy JK, Crawford PC, Slater MR. Effect of vaccination against feline immunodeficiency virus on results of serologic testing in cats. *J Am Vet Med Assoc* 2004;225:1558–1561.
2. MacDonald K, Levy JK, Tucker SJ, et al. Effects of passive transfer of immunity on results of diagnostic tests for antibodies against feline immunodeficiency virus in kittens born to vaccinated queens. *J Am Vet Med Assoc* 2004;225:1554–1557.
3. Uhl EW, Heaton-Jones TG, Pu R, et al. FIV vaccine development and its importance to veterinary and human medicine: a review. FIV vaccine 2002 update and review. *Vet Immunol Immunopathol* 2002;90:113–132.
4. Connell S. Manufacturer addresses concerns about FIV vaccine (lett). *J Am Vet Med Assoc* 2003;222:149.
5. Steinrigl A, Klein D. Phylogenetic analysis of feline immunodeficiency virus in Central Europe: a prerequisite for vaccination and molecular diagnostics. *J Gen Virol* 2003;84:1301–1307.
6. Leutenegger CM, Klein D, Hoffmann-Lehmann R, et al. Rapid feline immunodeficiency virus provirus quantitation by polymerase chain reaction using the TaqMan fluorogenic real-time detection system. *J Virol Methods* 1999;78:105–116.
7. Bachmann MH, Mathiason-Dubard C, Learn GH, et al. Genetic diversity of feline immunodeficiency virus: dual infection, recombination, and distinct evolutionary rates among envelope sequence clades. *J Virol* 1997;71:4241–4253.
8. Weaver EA, Collisson EW, Slater M, et al. Phylogenetic analyses of Texas isolates indicate an evolving subtype of the Clade B feline immunodeficiency virus. *J Virol* 2004;78:2158–2163.
9. Hohdatsu T, Yamada M, Okada M, et al. Detection of feline immunodeficiency proviral DNA in peripheral blood lymphocytes by the polymerase chain reaction. *Vet Microbiol* 1992;30:113–123.
10. Klein D, Janda P, Steinborn R, et al. Proviral load determination of different feline immunodeficiency virus isolates using real-time polymerase chain reaction: influence of mismatches on quantification. *Electrophoresis* 1999;20:291–299.
11. Leutenegger CM, Mislin CN, Sigrist B, et al. Quantitative real-time PCR for the measurement of feline cytokine mRNA. *Vet Immunol Immunopathol* 1999;71:291–305.
12. Pedersen NC, Leutenegger CM, Woo J, et al. Virulence differences between two field isolates of feline immunodeficiency virus (FIV-A Petaluma and FIV-C PGammar) in young adult specific pathogen free cats. *Vet Immunol Immunopathol* 2001;79:53–67.
13. Klein D, Leutenegger CM, Bahula C, et al. Influence of pre-assay and sequence variations on viral load determination by a multiplex real-time reverse transcriptase-polymerase chain reaction for feline immunodeficiency virus. *J Acquir Immune Defic Syndr* 2001;26:8–20.
14. Rimstad E, Ueland K. Detection of feline immunodeficiency virus by a nested polymerase chain reaction. *J Virol Methods* 1992;36:239–248.
15. Crawford PC, Papadi GP, Levy JK, et al. Tissue dynamics of CD8 lymphocytes that suppress virus replication in cats infected neonatally with feline immunodeficiency virus. *J Infect Dis* 2001;184:671–681.
16. Bucci JG, English RV, Jordan HL, et al. Mucosally transmitted feline immunodeficiency virus induces a CD8+ antiviral response that correlates with reduction of cell-associated virus. *J Infect Dis* 1998;177:18–25.
17. Hohdatsu T, Sasagawa T, Yamazaki A, et al. CD8+ T cells from feline immunodeficiency virus (FIV) infected cats suppress exogenous FIV replication of their peripheral blood mononuclear cells in vitro. *Arch Virol* 2002;147:1517–1529.