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Comparison of four test kits for feline leukemia virus antigen

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Summary: *The sensitivity and specificity of 4 commercial FeLV ELISA kits, using blood, were compared with results of virus isolation from blood and immunofluorescent antibody (IFA) testing on blood. Significant differences were not found among the 4 ELISA kits. Marked decrease in sensitivity of the ELISA kits was detected when virus isolation was used as the standard of positivity rather than the IFA test. Virus isolation was a more sensitive indicator of early infection, with marked discrepancy among results obtained by virus isolation, ELISA, and the IFA test. Results became progressively more concordant as infection became fully established. Cats FeLV-positive by virus isolation alone were more likely to eliminate viremia. All cats FeLV-positive by IFA testing remained persistently viremic. Virus isolation, ELISA, and IFA testing appear to differ in their prognostic value. The use of blood rather than serum for the ELISA resulted in several discordant results. Six cats were FeLV-positive by ELISA when blood was tested but were FeLV-negative when serum was tested. Positive ELISA results were obtained for 4 of these cats when serum was tested, using extended incubation to increase sensitivity. It is possible that blood may actually be more sensitive than serum for use of the ELISA method.*

Detection of FeLV infection by use of an ELISA on serum is a sensitive, accurate, and simple procedure. Results of ELISA on serum were correlated (95%) to immunofluorescent antibody (IFA) detection of FeLV[1] on blood. Discrepancy usually is attributed to increased sensitivity of ELISA technology and to the ability of ELISA to detect infection prior to bone marrow involvement. False-negative IFA test results can be obtained when antigen concentration is low, when FeLV infection is sequestered outside the bone marrow, or when too few leukocytes are available to evaluate. False-positive ELISA results are attributable to improper washing technique or to anti-mouse immunoglobulin activity in the sample.[2]

In a recent study, 7 commercially available ELISA kits were compared using serum from known IFA-positive cats and FeLV-negative specific-pathogen-free (SPF) cats.[3] Since that report was published, several new or modified ELISA kits have been introduced on the market. To our knowledge, the sensitivity of FeLV ELISA kits, using a standard of virus isolation, has not yet been evaluated.

Many veterinarians use blood rather than serum when performing ELISA. Testing of blood may result in more false-positive reactions than does testing of serum.[4] False-positive results are attributed to the difficulty of adequate washing.[4] Serum samples were used in the previous study comparing ELISA kits.[3] To the authors' knowledge, direct comparison of specificity between use of blood and serum for ELISA kits has not been previously reported.

The objective of the study reported here was to compare the sensitivity and specificity of 4 commercially available FeLV ELISA kits, using blood, with IFA and virus isolation methods.

Materials and Methods

Commercially available ELISA kits—Four FeLV

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Supported by IDEXX Corporation, Portland, ME. Test kits were generously provided by their respective manufacturers.

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ELISA kits, designated A, B, C, D,[a-d] were compared. Kits were obtained directly from the manufacturers and were stored at 5 C. Kits were used according to manufacturers' directions. All samples were tested by a single technician. For a given sample, change in color greater than that of the control was considered a positive result.

Cats—All samples tested were obtained from 47 SPF kittens involved in an FeLV vaccine trial.[5] Thirty-six kittens were vaccinated for FeLV; 11 remained as nonvaccinated controls. All kittens were exposed to FeLV by housing 12 FeLV-positive carrier cats, not manifesting clinical signs of disease, with test cats for a period of 31 weeks.

Samples—Samples were obtained from all 47 cats every 2 weeks for a period of 18 weeks. Initial samples were drawn during the second week of exposure to carrier cats. At each sample collection time, blood was evaluated for FeLV by IFA testing, virus isolation, and serum ELISA.[e] Samples for blood ELISA testing were collected in EDTA-containing tubes and shipped on cold packs via overnight express to the University of Tennessee. All samples were tested within 24 hours of collection.

Virus isolation and IFA testing—Blood (collected in EDTA) smears were treated with a polyclonal bovine FeLV-specific antibody followed by addition of fluoroscein-labeled affinity-purified goat antibody to bovine IgG (H + L).[f] Slides were read, using a fluorescent microscope set at 400× magnification.

Crandall Feline Kidney (CRFK) cells were used for FeLV isolation from blood samples. The CRFK cells were plated at 20,000 cells/ml/well in 24-well culture plates. After overnight incubation at 37 C in a CO_2 incubator, 1 well/blood sample was inoculated with 5 μl of blood. After 1 to 2 hours' incubation (37 C, CO_2), the blood was removed and fresh minimal essential medium was added to cells. Plates were incubated for 7 days (37 C, CO_2). Cell fluids were then tested for p27 antigen activity, using a commercial ELISA kit.[c] Any OD value greater >0.100 was considered a positive result. Initially, positive ELISA results on all cell fluid samples were confirmed by IFA testing of the cultured cells. Because complete agreement was obtained between ELISA and the IFA test, subsequent samples were evaluated by ELISA only. Blood from FeLV-negative cats (negative controls) was tested with each sample.

[a]UNI-TEC FeLV Kit, lot No. RCO36B, Pitman-Moore, Inc, Mundelein, Ill.

[b]CITE/FeLV kit, lot No. 9640-04185G, IDEXX Corp, Portland, Me.

[c]Virachek, FeLV kit, lot No. CC056, Synbiotics Corp, San Diego, Calif.

[d]Diasystems FeLV-Flex II kit, lot No. 114007, Fermenta Animal Health, Omaha, Neb.

[e]Serum ELISA were performed at Fort Dodge Laboratories, Fort Dodge, Iowa, using kit C.

[f]Kirkegaard & Perry Laboratories Inc, Gaithersburg, Md.

Table 1—Comparison of four FeLV ELISA kits

Kit	Sensitivity-IFA* n = 15	Sensitivity-VI n = 25	Specificity n = 14	False positive results‡
A	100 (87 to 100)%	44 (26 to 63)%	100 (82 to 100)%	1/374
B	93 (80 to 100)%	60 (41 to 79)%	86 (68 to 100)%	8/374
C	100 (87 to 100)%	40 (21 to 59)%	100 (82 to 100)%	5/374
D	100 (87 to 100)%	56 (37 to 75)%	100 (82 to 100)%	7/374

*Immunofluorescent antibody (IFA)-positive, serum ELISA-positive, and virus isolation (VI)-positive cats used as positive standard. †VI-positive, positive, IFA negative cats used as positive standard. ‡False-positive results obtained from cats which had positive bone marrow culture after 23 weeks of exposure: kit B, 5/8; kit C, 2/5; kit D, 2/7.

Values in parentheses are confidence interval.

Calculation of sensitivity and specificity—Two calculations of sensitivity were done. The first determination of sensitivity of the blood ELISA kits was calculated by testing a population of cats that had positive results of virus isolation, serum ELISA, and IFA testing. Samples drawn during the second week of IFA positivity were tested using each kit. This was done to evaluate sensitivity during fully established infection.

The second determination of sensitivity of the blood ELISA kits was done early in the course of infection. The ELISA kits were evaluated, using samples from IFA-negative cats, on the first day that virus was isolated from blood.

Specificity of the test kits was determined by testing cats that had never become viremic on the basis of virus isolation, ELISA, or IFA results throughout the entire 18-week study. Samples tested were taken the last day of the study (week 18).

Sensitivity and specificity were calculated by use of a standard method[6] and were reported with 95% confidence interval (CI).[7]

Extended incubation of ELISA—Extended incubation of ELISA was performed on serum from 5 cats. A fifth FeLV[g] antigen test kit was used for these samples. Samples were tested according to the package insert, except for the incubation times, which were extended to 2.5 hours for these samples. A negative control, a positive control, and a positive control treated with a neutralizing reagent were tested concurrently with serum samples from test cats. Each test sample was assayed with diluent and again with neutralizing reagent. A positive result was defined by 3 criteria: OD 650 ≥0.1; OD value greater than that of the negative control; and reduction of the OD value by 50% after use of the neutralizing agent. All samples were tested in duplicate.

Results

The sensitivity of the blood ELISA kits in 15 cats with fully established FeLV infection (positive results of virus isolation, serum ELISA, and IFA testing for at least 2 weeks) was determined (Table 1). Use of kits A, C, and D detected all IFA-positive cats (100% sensitivity). Use of kit B detected 14 of 15

[g]Petchek, IDEXX Corp, Portland, Me.

IFA-positive cats (sensitivity, 93%; 95% CI, 80 to 100%). Differences were not statistically significant.

The sensitivity of blood ELISA kits in 25 cats with early infection (negative results by IFA testing but positive results by virus isolation) also was determined (Table 1). Use of kit A detected 11 of 25 virus isolation-positive, IFA-negative cats for sensitivity of 44% (CI, 25 to 63%). Use of kit B correctly identified 15 of 25 cats for sensitivity of 60% (CI, 41 to 79%). Use of kit C detected 10 of 25 (sensitivity, 40%; CI, 21 to 59%). Use of kit D detected 14 of 25 (sensitivity, 56%; CI, 37 to 75%). Differences were not statistically significant.

The specificity of the test kits for blood from 14 cats negative for FeLV on the basis of results of virus isolation, serum ELISA, and IFA testing throughout the 18-week exposure period was calculated (Table 1). Kits A, C, and D had 100% specificity. Kit B had specificity of 85% (CI, 68 to 100%; 2 samples were incorrectly identified). Differences were not statistically significant.

A total of 374 blood samples were tested over the 18-week period. A false-positive result was defined as a positive result from a cat that had negative results of virus isolation, serum ELISA, and IFA testing. Cats that became FeLV-positive by results of any of the preceding methods within 2 weeks (before or after) of a false-positive result were excluded (ie, the kit may be detecting early or recovering infection). The rate of false-positive results on blood was as follows: kit A, 1 of 374 samples (0.3%); kits B and D, 8 of 374 samples (2.1%); kit C, 5 of 374 samples (1.3%).

The specificity of 2 kits (B and C), using blood, was compared with that obtained using serum. Serum ELISA had been performed using kit C every 2 weeks throughout the study. Using this kit, no false-positive results were obtained for serum from aviremic cats during the entire study, in contrast to 5 false positive results obtained using blood. The 8 samples (n = 6 cats) with false-positive results, using kit B on blood, were retested on stored serum, using kit B; results for all 8 samples were negative. Serum from 5 of the 6 cats for which discrepancy was found using kit B was available for further study. Extended incubation of the serum ELISA yielded positive results in 5 of the 5 cats retested. Neutralization of the serum samples by use of an antiserum to absorb p27 antigen resulted in >50% reduction in OD values in serum of all 5 cats retested. Virus was isolated from bone marrow cultures of 4 of the 6 cats with discrepant results, despite the fact that these cats had been aviremic on the basis of virus isolation, serum ELISA, and IFA testing throughout the entire study.

Significant differences were not observed in the abilities of the kits to detect early infection. Kits A through D detected FeLV-positive cats an average of 2.0, 2.5, 2.5, and 2.7 weeks, respectively, before IFA positivity was detected.

Discussion

Significant difference in sensitivity was not seen among the 4 blood ELISA kits, using IFA or virus isolation testing as the standard for positivity. Blood ELISA kits were similar in specificity (100%), with the exception of kit B (86%). The 2 false-positive results obtained using this kit later became negative results when serum was used. Differences in specificity among the kits were not significant.

Previously reported sensitivity and specificity of kits B (100%/100%), C (100%/100%), and D (95%/100%)[3] were compatible with our results, using IFA testing as the standard for sensitivity. Marked decreases were observed in sensitivity when virus isolation was used as the standard for FeLV infection. The decreased sensitivity of kit D reported when p27 antigen was added to fetal bovine serum[3] was not found in our study.

Positive results for 8 and 5 blood samples were obtained, using kits B and C, respectively; these samples were from cats that had been FeLV–negative by blood virus isolation, serum ELISA, and blood IFA testing throughout the entire study. All 13 samples had negative results when retested using the same kits on serum. On initial evaluation, these results were classified as false positive, most likely attributable to difficulty in complete washing. Further investigation of kit B suggested, however, that blood may be more sensitive than serum for FeLV ELISA testing. Of the 6 cats from which discrepant results were obtained, 4 had FeLV isolated from the bone marrow cell culture, indicating that these cats were truly infected. Increasing the sensitivity of kit B (on serum) by prolonging the incubation period yielded positive results that could be neutralized >50% by pretreatment with anti-FeLV antibody. It is possible that p27 may have an affinity for blood cells, and that using blood results in larger amounts of antigen in the assay. A portion of the false-positive results obtained from kit C(2/5) and D(2/7) were also from cats that had positive bone marrow culture after 31 weeks of exposure. Further study of a greater number of FeLV-exposed cats is required before firm conclusions can be drawn.

Poor agreement between virus isolation, serum ELISA, and IFA detection of p27 was seen early in the course of FeLV infection. The viremic status of the individual cats in this study was determined by virus isolation, serum ELISA, and IFA testing throughout the course of infection.[5] Virus isolation was documented to detect FeLV viremia approximately 2 to 6 weeks sooner than could ELISA and/or IFA testing. As viremia began to appear in cats during the eighth week of exposure, results of virus isolation, ELISA, and IFA testing agreed in only 6 of 18 (33%) virus isolation-positive cats. As the course of infection progressed, progressive concordance between virus isolation, ELISA, and IFA results became evident. Midway through the study after 14 weeks

of exposure, complete agreement was apparent among results of the 3 tests in 10 of 20 (50%) virus isolation–positive cats. Once infection became established during week 31, 95% agreement was evident among virus isolation, serum ELISA, and IFA results in the 19 virus isolation-positive cats. Discordant results were likely attributable to a combination of the differing sensitivities of the 3 methods, the probable low degree of viremia during early infection, and the localization of virus antigens outside the bone marrow during early viremia.

To the authors' knowledge, the marked increase in sensitivity of virus isolation over ELISA and IFA testing has not previously been reported. An earlier study indicated high degree of correlation between virus isolation and IFA results.[8] Virus could not be isolated from 30% of ELISA-positive cats in that study. Virus was isolated from all ELISA-positive cats in our study. The difference in the sensitivity of virus isolation in the previous study and our study may be related to the differing techniques used in isolating the virus. Those investigators[8] used plasma samples to infect clone–81 cells and feline embryo cells of the FEA strain; transformation of the cells was used to detect virus. In our study, blood was used to infect CRFK cells. An ELISA was used to detect p27 viral antigen in the supernatant. It is unlikely that the increased sensitivity of virus isolation by use of our method was attributable to nonspecific false-positive reactions. Negative-control CRFK cell cultures were used in each test. These controls were subjected to the same conditions and procedures as were test sample groups and were never found to express p27 antigen. Also, all prechallenge– and 2-week postchallenge-exposure samples collected from the 47 test cats had negative results of virus isolation. Finally, in the initial part of the study, the positive ELISA results for the virus isolation cultures were confirmed by IFA testing of infected CRFK cells. These results promote confidence in the specificity of the virus isolation technique used in this study.

Sixty percent (12/20) of cats initially positive for FeLV by virus isolation alone were able to eliminate viremia. The remaining 40% progressed to become ELISA positive, IFA positive, or both. Of the 11 cats, which at some point during the exposure period had positive results of virus isolation and ELISA but had negative results by IFA testing, only 4 (36%) were able to eliminate viremia; these findings were similar to those of the aforementioned study of ELISA-positive, IFA-negative cats.[8] The remaining 7 cats became IFA positive. Of the 18 cats that became positive by IFA testing, none were able to eliminate the viremia; all became persistently viremic, with complete agreement among virus isolation, ELISA, and IFA results.

It is evident that the 3 methods differ not only in their ability to detect early viremia but also in their ability to predict persistent infection. Most cats FeLV–positive by virus isolation alone are able to eliminate the infection. A positive IFA result in cats of this study was an excellent indicator of persistent viremia, as previously established by other investigators.[9]

The question remains as to the clinical relevance of detection of this early and often transient viremia. Most FeLV infections seen by veterinarians are fully established. Results of virus isolation, ELISA, and IFA testing would be expected to be in agreement. The increased sensitivity of virus isolation is not relevant in these cases, and the prognostic value of the IFA test becomes important. Increased sensitivity may be valuable, however, for detection of viremia early in the course of infection when screening cats prior to vaccination or introduction into a household. In addition, clinical relevance of even transient viremia, such as that detected only by virus isolation, is not known. Because FeLV is incorporated into host DNA, transformation of the host cell, with resulting tumorogenesis may be possible. Adequate host immunity may be able to suppress virus production and expression, leading to FeLV-negative status as detected by conventionally used diagnostic testing. Transient viremia could then theoretically be associated with FeLV-negative neoplasia.

Overall, significant difference was not seen among the blood FeLV ELISA kits in sensitivity and specificity. The sensitivity of all ELISA kits was markedly reduced, compared with virus isolation as a standard. A negative ELISA result may not rule out early or low degree of viremia. Virus isolation, ELISA, and IFA testing differed in their ability to detect early viremia. Results became progressively more concordant as infection became fully established. Excellent agreement was seen in cats with persistent viremia. Cats FeLV-positive by virus isolation alone were more likely to eliminate viremia, whereas cats positive by IFA testing were unable to eliminate viremia. The use of blood for ELISA testing is controversial. Previous work has suggested increased incidence of false-positive results using blood. Initial work in this study indicates that blood may actually be more sensitive than serum for use in detecting FeLV infection.

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Comparison and interpretation of diagnostic tests for feline immunodeficiency virus infection

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Summary: *Feline sera were submitted to the Cornell Feline Health Center (n = 497) or to the New York State Diagnostic Laboratory (n = 1,565) for feline immunodeficiency virus (FIV) testing. Some sera (n = 166) were submitted for confirmation of previous FIV-positive results; 151 of these sera had been tested at the referring veterinary practice or laboratory, using an in-house ELISA. Excluding the samples submitted for confirmation, a total of 173 samples (9.1%) were FIV-positive; 11.6% of the clinically ill or high-risk cats and 0.49% of the healthy, low risk cats were positive for FIV antibody.*

A commercially available ELISA for detection of antibody to FIV was evaluated in relation to the immunofluorescent antibody (IFA) test and the immunoblot assay. The ELISA was interpreted according to the manufacturer's instructions, with the ratio of sample optical density to positive control optical density (S/P) determining a positive or negative result. The ELISA results based on the S/P interpretation were compared with a kinetics-based (KELA) interpretation of the ELISA. The KELA values were reported as positive, negative, or equivocal.

Using the immunoblot as the standard, ELISA (S/P interpretation) had sensitivity of 0.93 and specificity of 0.98, whereas the IFA test had sensitivity of 0.95 and specificity of 0.98. However, the sensitivity and specificity of the ELISA (S/P interpretation) were markedly reduced for sample results falling in the KELA equivocal range, indicating that equivocal results were valid interpretations for some sera.

A high number (22.5%) of the samples submitted for confirmation of a positive result from use of the in-house ELISA were determined to be negative for FIV antibody. Operator error or incorrect interpretation of the in-house ELISA were thought to be the cause of most of these false-positive test results.

During the past quarter century, the quantity and quality of feline medicine practiced by veterinarians in the United States have markedly increased. As interest in domestic cats as household pets has grown, so has the demand for preventive and critical veterinary care for them. Diagnosis of many feline diseases has become increasingly dependent on the merging of clinical findings and results of appropriate diagnostic tests. However, many of these tests have at least some controversy associated with their use and interpretation. For example, a number of tests are available for detection of antibodies to feline infectious peritonitis (FIP) virus, yet the value of a positive result is severely limited because of high variability among the tests, cross reactivity between antibody to feline enteric coronavirus and that to other coronaviruses, and false-positive results caused by reactivity to bovine serum components used in cell culture and vaccine production.[1]

Diagnostic tests for feline immunodeficiency virus (FIV) do not have the complexities of FIP testing; however, certain problems must be recognized and either overcome or accepted as inevitable. All the commonly used diagnostic tests for FIV detect virus-specific antibody rather than the virus itself.

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