

Development of the immunofluorescent antibody test for detection of feline leukemia virus infection in cats

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Summary: *Studies of the immunodetection of various microorganisms by various assay systems indicated that the most specific and sensitive assays are immunofluorescence, radioimmunoassay, and immunoblot analysis (western blot), followed by sensitive but less specific ELISA and agglutination assays and, finally, by even less sensitive but very specific virus isolation and double immunodiffusion techniques. The first test for the clinical detection of FeLV infection in pet cats was the immunofluorescent antibody (IFA) test, which was introduced in 1972. The FeLV test is used for detection for FeLV infection and not as a test for leukemia or any other feline disease. The IFA test was compared with an immunodiffusion (ID) test and with tissue culture isolation (TCI) of the virus in 26 cats to establish a standard for FeLV tests. Excellent correlation was observed between the IFA and the ID tests (100%)*

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and between the IFA and ID tests, compared with TCI (96.2%). From these studies, it is clear that the IFA test is more accurate and more practical, and that results can be obtained faster than can those for the ID or TCI tests for FeLV.

The FeLV, the feline immunodeficiency virus (FIV), and the feline syncytium-forming virus (FeSFV) are members of the Retrovirinae family of RNA-containing viruses.^{1,2} The FeLV is in the subfamily Oncoretrovirinae, FIV is a member of the Lentiretrovirinae subfamily, and FeSFV is a member of the Spumaretrovirinae subfamily. Distinctions between these subfamilies of retroviruses that are of importance to veterinarians are^{3,4}: FeLV infection is diagnosed by detection of viral antigens in leukocytes, plasma, serum, or saliva, whereas FIV and FeSFV are detected by the finding of antiviral antibody, which coexists and signals persistent FIV and FeSFV infections; FeLV-infected cats are dangerous to other cats that live in the same household, whereas FIV-infected cats are more dangerous to free-roaming cats that fight with them than they are to cats that live peacefully with them in the same household; FeLV is mainly transmitted among cats by licking and mutual grooming, whereas FIV and FeSFV are mainly transmitted by biting; FeLV in-

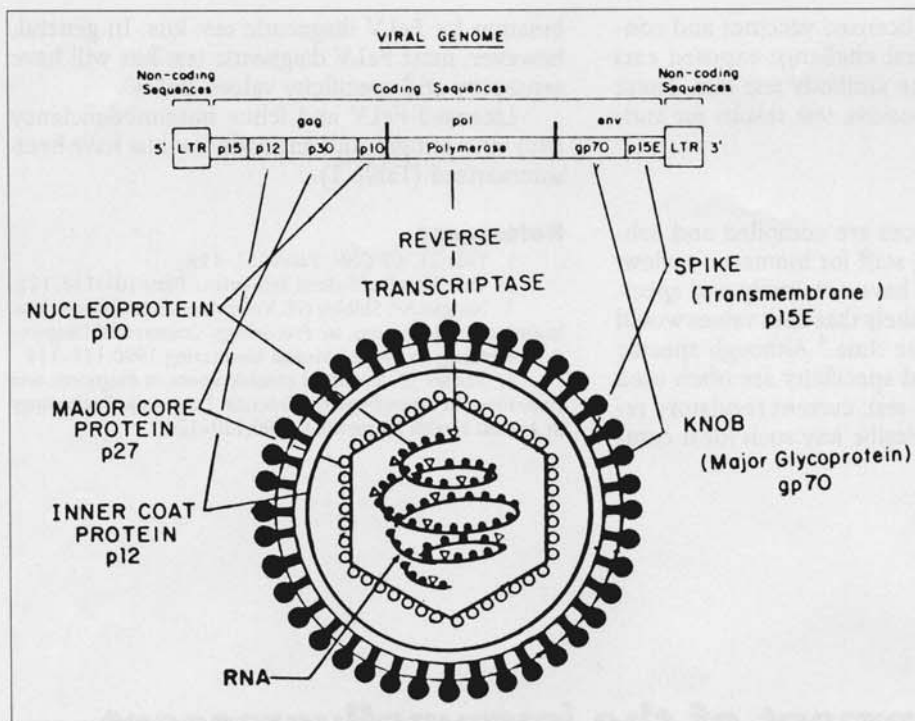


Figure 1—Antigens of the feline leukemia virus and their location in the virus. See Table 1 for additional information.

Table 1—Feline leukemia virus proteins

Viral gene	Protein	Protein 2-letter designation	Function or enzyme activity or location in virus
Internal core antigens			
gag 5'	p15	MA	Matrix protein
	p12	??	Unknown
	p27	CA	Capsid protein
	3'	p10	NC
pol 5'		p14†	PR
	p80	RT	Reverse transcriptase
3'	p46†	IN	Integration protein
Envelope antigens			
env 5'	gp70	SU	Surface protein
3'	p15E	TM	Transmembrane protein

*Unknown function-no name given yet; †Assumed from analogous murine leukemia virus protein, not yet identified for FeLV.

duces disease rapidly, whereas FIV induces disease only after prolonged periods, and FeSFV has not been shown to induce any disease.

In the 1950s and 1960s, clusters of cases of lymphosarcoma in cats were observed by veterinarians, which indicated that FeLV may be transmitted contagiously among cats.⁵ Until that time, all known retroviruses were thought to be transmitted only by hereditary means. However, the prevalence and mode of transmission of FeLV in the general pet cat population could not be studied until a rapid, sensitive, and accurate means of detecting the virus was developed that did not require large amounts of tissue. The most direct way to study the virus seemed to be by developing an immunodetection assay.⁶

To develop a specific FeLV antigen immunodetection test, the viral antigens had to be isolated and characterized. Such studies were done, and results indicated that 2 main types of FeLV antigens exist; (Fig 1; Table 1): viral envelope antigens, gp70 and p15E, and internal viral core antigens, p15, p12, p27, and p10.⁷⁻¹¹ The envelope antigens are mainly type specific (i.e., they are specific for 1 particular type or strain, of FeLV). This type specificity leads to the distinction of 3 subgroups of the virus: FeLV-A, -B, and -C.¹² In contrast to the envelope antigens, the FeLV internal core antigens are identical for all subgroups of FeLV and are termed group-specific (gs) antigens.⁷⁻⁹ Results of early studies indicated that FeLV-infected cats did not produce high titer of antibodies against the virus, which indicated that antibody tests were not possible.⁹ Because cats do not produce high antibody titers to the FeLV internal gs antigens,¹⁰ detection of these antigens in infected cells or as soluble antigens in body fluids in cats depends on use of antisera prepared in other species.⁷⁻⁹

In 1969, a specific rabbit anti-FeLV gs antigen serum with high titer was produced,^{8,9} thus enabling development of immunologic techniques for detection of the virus. Using this serum, the first test to be developed for detection of FeLV in feline tissues was the double immunodiffusion (ID) test.⁹ Shortly thereafter, a complement-fixation test for detection of FeLV was developed using this same rabbit serum, but the test proved difficult to perform and was not practical.¹² In general, the ID technique is able to resolve complex antigenic mixtures and has excellent specificity. The rabbit anti-FeLV gs serum was used in the ID test, and ini-

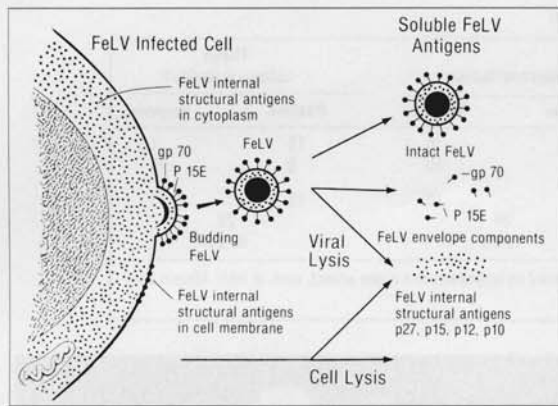


Figure 2—Feline leukemia virus antigens produced by infected cells. Excess viral antigens are produced in infected cells and can be detected in the cells by the use of immunofluorescent antibody (IFA) test or as free soluble antigens in the plasma by use of the ELISA.

tial studies of the epidemiologic features of FeLV and of some of the FeLV-induced diseases were begun.^{8,9} However, it was not until the immunofluorescent antibody (IFA) test was developed that the true extent of the incidence of FeLV and the diseases caused by the virus were determined in pet cats.

The basic background for the development of the FeLV IFA test was established in 1968 when FeLV was shown, by electron microscopy, to be present in circulating blood leukocytes and platelets of infected cats.^{13,14} Because the virus was present in leukocytes, we assumed that the internal gs viral antigens would also be found in the cytoplasm of these cells (Fig 2). This assumption proved to be correct and was the observation that led to the development of the IFA test for detection of FeLV antigens in blood leukocytes and platelets and in bone marrow cells of pet cats.^{15,16} Later, the IFA test was used to elucidate the pathogenesis of FeLV by following the spread of the virus into various tissues of experimentally infected cats.¹⁷⁻¹⁹ The adaption of the IFA test to blood smears made the test practical for large screening studies of pet cats and for experimental studies of the pathogenesis of the virus. The IFA test can be performed on blood smears because the FeLV antigens are stable for weeks at room temperature and can be detected in leukocytes long after all FeLV infectivity is lost.

Since its introduction, the IFA test has had a pivotal role in research directed toward understanding the biology of FeLV.^{15,16,21} It has been used in every major study to determine prevalence and contagious spread of the virus,^{15,16,20-28} pathogenesis of FeLV,¹⁷⁻¹⁹ control of the spread of FeLV among cats,^{20,21,29,30} and identification of all diseases induced by or associated with the virus.^{3,16,31-35} In most of these studies, it was the only test used by the investigators, largely because of its specificity, sensitivity, accuracy, and practicality. No other test has been so widely used to generate basic knowledge about FeLV. The IFA test is

considered to be the reference standard (ie, the test by which the accuracy of other tests for infection with a specific agent is judged) for FeLV infection.

Most laboratory tests performed in veterinary medicine reflect their use in patient management. The FeLV IFA test-and-removal program for control of the spread of the virus has been available and used effectively worldwide for the past 20 years.^{20,21,29,30} In this program all FeLV-infected cats are removed from the household or isolated from noninfected cats. This program has been successful in stopping the spread of FeLV in thousands of households. However, with increasing use of the less specific FeLV in-hospital ELISA, numerous false-positive FeLV test results have been obtained that have complicated the management of cats in some households.³⁶

The goal of this study was to develop a practical and accurate test for detection of FeLV infection in pet cats that only required a few drops of blood. We also determined that the most accurate FeLV test method was IFA, and that the FeLV antigens were stable for months in leukocytes of unfixed blood smears. These studies were performed before FeLV ELISA were introduced into veterinary medicine.

Materials and Methods

Clinical specimens—Sera, blood smears, and lymphosarcoma tissue extracts from 26 cats (16 FeLV-infected and 10 FeLV-noninfected) were tested for FeLV by IFA, ID, and tissue culture isolation (TCI), (Table 2). Subsequent comparative assays were performed between IFA and ID on 1,549 cats (Table 3) and between IFA and TCI tests on 348 cats (Table 4). Results of 274 of the 348 comparative IFA and TCI tests have been reported.³⁴ We also compared FeLV ELISA performed by practitioners with the IFA test. Part of this comparative study has been published,³⁴ and the completed study is reported in this issue.³⁵

Preparation of antisera against FeLV—High-titer rabbit antisera to FeLV antigens were prepared as described.^{8,9,15} Fifteen young adult female New Zealand White rabbits were inoculated SC 3 times, at 1-month intervals, with purified ether-disrupted FeLV emulsified in complete Freund adjuvant (initial inoculation) and in incomplete Freund adjuvant (subsequent boosters). Blood was obtained from the rabbits 1 month after the final booster and biweekly thereafter. All rabbit sera were absorbed 3 times with equal volumes of washed packed RBC and WBC from feline blood as required. One rabbit serum (from rabbit No. 3) was chosen for this study because of its high titer against FeLV antigens, together with its low anti-cat nonspecific reactivity. Proper absorption of the rabbit antiserum to remove all anti-cat nonspecific reactivity is of paramount importance for this test.

The absorbed rabbit No. 3 anti-FeLV serum was tested on mixtures of 50% FeLV-infected and

the leading edge of the blood smears where most of the leukocytes were expected to be located. Rabbit anti-FeLV serum, at dilution of 1:60, was placed in the reactive wells, and the slides were tested as previously described.^{15,16,20,21} Each FeLV test was performed with 2 positive control blood smears included.

COMPARISON OF THE FeLV TEST METHODS

Comparison of the IFA and ID tests—The ID test was the first test developed in our laboratory for detection of FeLV infection. We compared the ID and IFA methods by testing 1,549 cats, using both assays (Table 3). Unconcentrated sera were used for the ID test, and blood smears were used for the IFA test.

Comparison of the IFA and TCI tests—The IFA test was also compared with the ability to isolate FeLV in tissue culture from the blood of 348 cats (Table 4). Some of these data have been reported.³¹

Stability of FeLV Antigens in cat blood smears—To test the stability of the FeLV antigens in blood leukocytes, blood smears were prepared from 20 cats, then stored at room temperature and tested by IFA biweekly for up to 6 months.

Results

The relative sensitivities and specificities of the FeLV IFA, ID, and TCI immunodetection tests were compared to determine which test was the most accurate, and, thus, should be considered the reference standard (Table 2).

Specificity of FeLV antisera—Of the 15 rabbits immunized with FeLV, 5 produced high-titer very specific anti-FeLV sera. These rabbit sera required only minimal absorption with cat blood and were used for the immunodetection tests. Serum from rabbit No. 3 was chosen for this study because of its high titer against FeLV antigens, together with its low anti-cat nonspecific reactivity. The specificity against FeLV of the rabbit antiserum was confirmed when all IFA and ID reactivity against FeLV was abolished after absorption with purified ether-disrupted FeLV and lymph node powder from an FeLV-infected cat and tested against 50%/FeLV-infected/50% FeLV-noninfected FLF-3 mixture slides (IFA) and sera (ID) from 10 FeLV-infected cats. The reactivity against FeLV was not abolished after absorption with lymph node powder from an FeLV-noninfected cat. Ten of the rabbits produced good antibody titer against FeLV, but had interfering nonspecific reactivity to some cat proteins.

ID test—The rabbit anti-FeLV serum gave strong precipitin lines in the agar gel ID test against ether-disrupted purified FeLV. Lines of identity were seen between the purified FeLV and sera or tissue extracts from FeLV-infected cats (Fig 3). The ID test was able to resolve complex FeLV antigenic

mixtures in infected cat sera and tumor tissue extracts. Sixteen FeLV-infected and 10 FeLV-noninfected cats were compared by ID and IFA testing. A 100% correlation (26/26) existed between the IFA and ID test results (Table 2).

TCI of FeLV—There was 96.2% agreement (25/26) between the IFA test and TCI (Table 2). The FeLV was not isolated by TCI from 1 of the 16 IFA- and ID-positive cats. The TCI result in this case was most likely erroneous, because the TCI FeLV-negative cat had lymphosarcoma and the ID and IFA tests detected FeLV antigens in its tumor tissue.

IFA FeLV test—The IFA test for FeLV detects FeLV antigens as apple-green punctate fluorescent granules in the cytoplasm of neutrophils, eosinophils, lymphocytes, and platelets in blood smears of infected cats (Fig 4A and 4B). The FeLV antigens can also be detected in the cytoplasm of nucleated erythroid and myeloid cells in the bone marrow of infected cats (Fig 4C). Fluorescence is not seen in noninfected cat leukocytes, which appear red owing to the Evans blue counter stain (Fig 4D). Results in cats that are anemic or leukopenic may be difficult to interpret because of the low numbers or lack of leukocytes (Fig 4E). Blood smears that are too thick are also difficult to interpret because of lack of visible leukocytes or because of the nonspecific reaction attributable to trapping of the IFA reagents (Fig 4F).

Compared with the ID test and TCI, the IFA test was found to be the most accurate FeLV test, was the easiest test to perform, and required the least amount of time to complete.

COMPARISON OF FeLV TEST METHODS

Excellent correlation was evident between the IFA, ID, and TCI techniques, but the IFA test was the most accurate and thus, has come to be considered the reference standard FeLV test (Table 2). We further compared the FeLV ID test and TCI with the IFA test by testing more cats for FeLV by each method (Tables 3 and 4).

Comparison of the IFA and ID tests—Further comparisons between IFA and ID tests indicated overall agreement of 99.0%, 99.8% for FeLV-negative ID results and 96.2% for FeLV-positive ID results (Table 3). The ID test was not able to detect FeLV antigens in the sera of 13 of the 341 IFA-positive cats, which probably indicates the lower sensitivity of the ID technique. In support of this possibility, when sera from 3 of these 13 cats were concentrated threefold, 1 of them tested weakly positive by ID. The ID test sensitivity was below that of the IFA test, but most, (328/341) of the FeLV-infected cat sera were positive by ID and most (1,205/1,208) of the IFA-negative sera were negative by ID (Table 3). Compared with the IFA test, the sensitivity of the ID test was 96.2%, the specificity was 99.8%, the false-negative rate was 3.8%, and

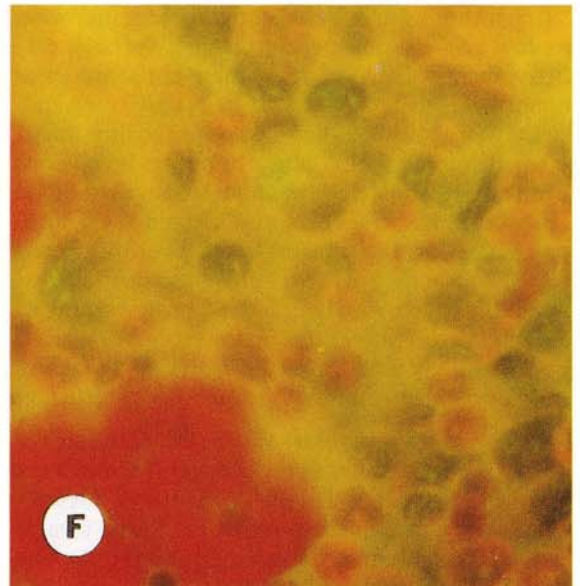
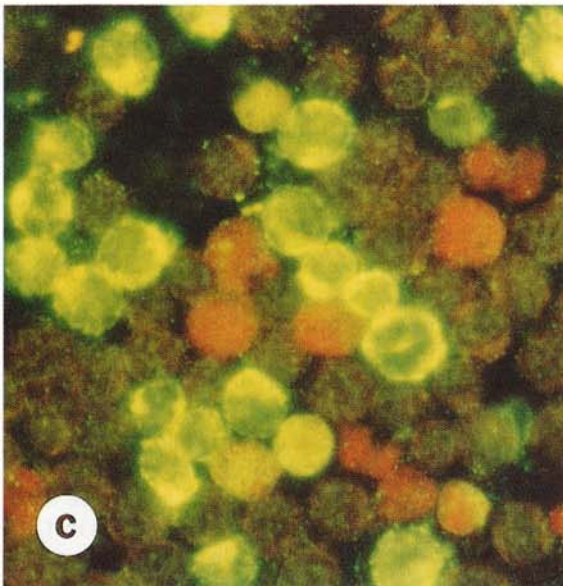
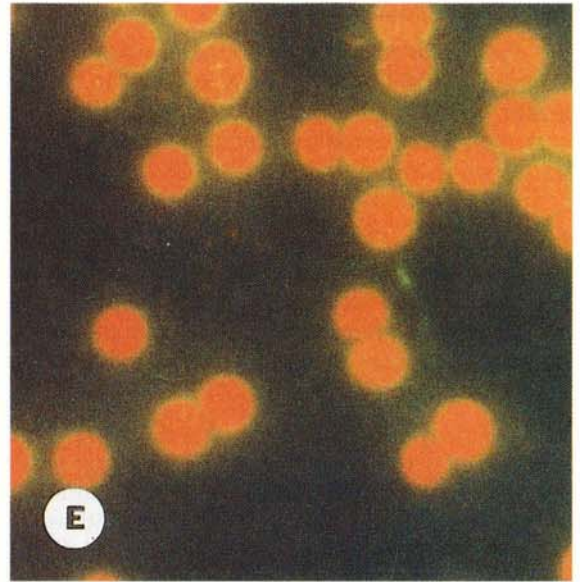
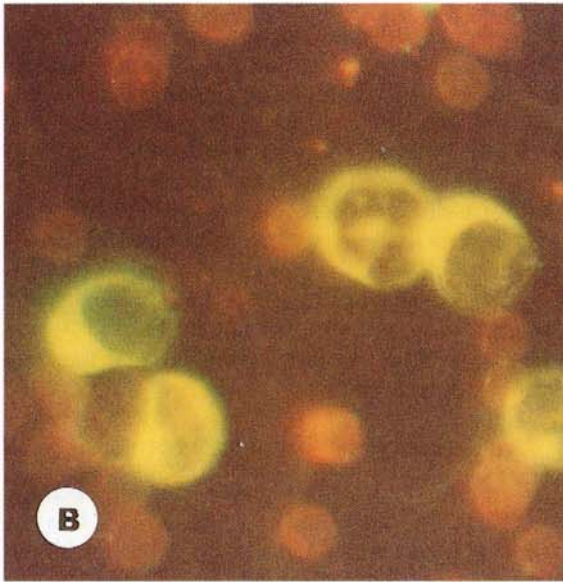
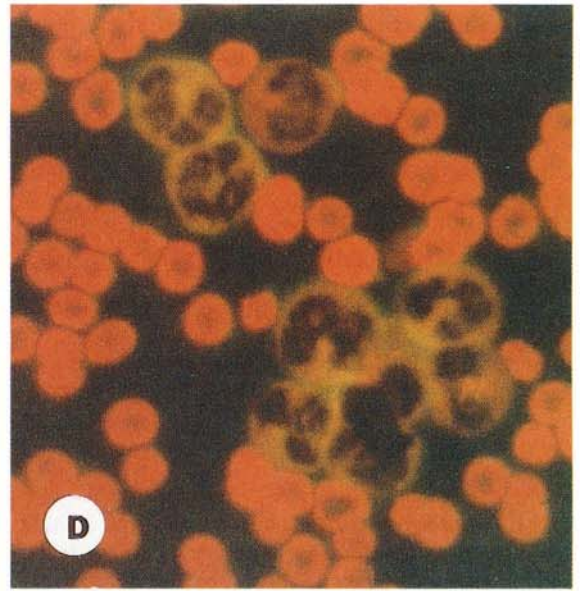
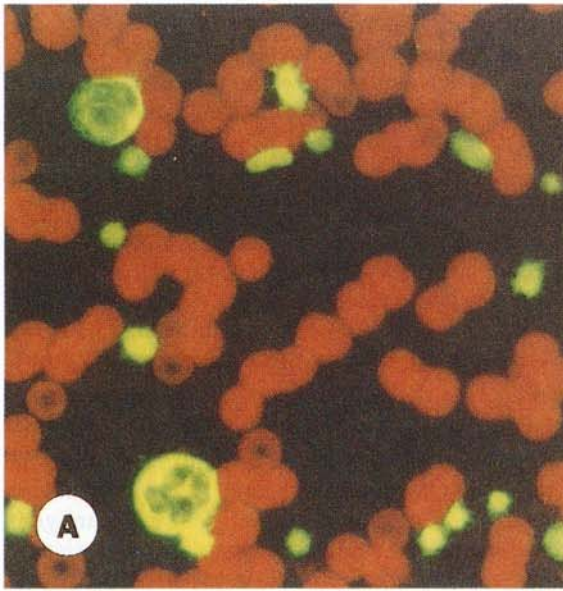


Table 5—Calculation of the sensitivity and specificity of the immunodiffusion (ID) test

	Immunofluorescent antibody (IFA) test results	
	Positive	Negative
ID test results Positive	a = 328	b = 3
Negative	c = 13	d = 1,205

Let a = IFA/ID-positive; b = IFA-negative; ID-positive c = IFA-positive; ID-negative; and d = IFA/ID-negative results. Total tested = a + c + b + d = 1,549; overall agreement = 99.0%

Calculations:

$$\text{ID test sensitivity} = \frac{a}{a+c} \times 100 = \frac{328}{328+13} \times 100 = 96.2\%$$

$$\text{ID test specificity} = \frac{d}{b+d} \times 100 = \frac{1205}{3+1205} \times 100 = 99.8\%$$

$$\text{ID False-negative rate} = \frac{c}{a+c} \times 100 = \frac{13}{328+13} \times 100 = 3.8\%$$

$$\text{ID False-positive rate} = \frac{b}{b+d} \times 100 = \frac{3}{3+1205} \times 100 = 0.2\%$$

Table 6—Calculation of the sensitivity and specificity of tissue culture isolation (TCI) of FeLV

	IFA test results	
	Positive	Negative
TCI Results Positive	a = 173	b = 3
Negative	c = 3	d = 169

Let a = IFA/TCI-positive; b = IFA-negative; TCI-positive; c = IFA-negative; TCI-positive; and d = IFA/TCI-negative results. Total tested = a + c + b + d = 348; overall agreement = 98.3%

Calculations:

$$\text{TCI sensitivity} = \frac{a}{a+c} \times 100 = \frac{173}{173+3} \times 100 = 98.3\%$$

$$\text{TCI specificity} = \frac{d}{b+d} \times 100 = \frac{169}{3+169} \times 100 = 98.3\%$$

$$\text{TCI False-negative rate} = \frac{c}{a+c} \times 100 = \frac{3}{173+3} \times 100 = 1.7\%$$

$$\text{TCI false-positive rate} = \frac{b}{b+d} \times 100 = \frac{3}{3+169} \times 100 = 1.7\%$$

the false-positive rate was 0.2% (Table 5). However, in comparison with the IFA test, the ID test required 1,000-fold more rabbit anti-FeLV serum per test, which made use of this technique less practical. In addition, the test required 4 hours' time to plate the sera and 15 hours before the test could be read. The ID test for FeLV is very specific but less sensitive than the IFA test.

Comparison of the IFA test and TCI—Virus was isolated from the sera of 173 of the 176 (98.3%

concordance) IFA-positive cats, but not from 169 of the 172 IFA-negative cats (Table 4). The FeLV isolates were easy to detect in cultured cells by IFA, usually after only 1 week. Because the IFA test and TCI failed to detect FeLV in 3 cats in which the other test results were positive, it was not possible to determine which test was more sensitive. Thus, the sensitivity and specificity of the IFA test and TCI are identical, whereas the ID test for FeLV is less sensitive.

Calculations of the characteristics of the TCI technique (Table 6) indicate that sensitivity of TCI is 98.3%, specificity is 98.3%, false-negative rate is 1.7%, and false-positive rate is 1.7%. Analysis of these data indicate that the TCI technique is sensitive and specific.

Stability of FeLV antigens in cat blood smears—For all 20 FeLV-positive cats tested, FeLV antigens were detected in the leukocytes of blood smears that were stored for 30 days at room temperature. Although viral antigens were weaker in some blood smears after 30 days, most blood smears had detectable FeLV antigens for 60 to 90 days when stored at room temperature.

Discussion

Early observations by veterinarians suggested that FeLV was transmitted contagiously, but methods were not available at that time to test pet cats for FeLV. Thus, to study the biology of FeLV, it was necessary to develop tests for detection of the virus in cats. The FeLV was first studied by visualization of the virus, using electron microscopy, and by isolation of the virus in tissue culture.^{13,14} Both techniques were not practical for large epidemiologic studies. We then developed an ID test that could detect FeLV in tissue homogenates and concentrated serum. The need for tissues and large amounts of serum restricted use of this test. We then realized that if the virus grew in leukocytes in the bone marrow and blood, it should be possible to detect the viral antigens in blood leukocytes by the sensitive technique of immunofluorescence. We were able to develop the IFA test for FeLV, thus enabling performance of large epidemiologic studies of the prevalence of FeLV. In addition, the IFA test was used to elucidate naturally acquired diseases caused by this virus in pet cats.

Unlike monoclonal antibodies that are used in modern ELISA for FeLV and that react with only 1

Figure 4—The IFA test for detection of FeLV in blood leukocytes of cats.

- A—An IFA-positive blood smear from a cat with normal hematologic profile. The FeLV antigens are in neutrophils, lymphocytes, and platelets.
- B—An IFA-positive blood smear from a cat with a leukemic blood profile. The FeLV antigens are present in neutrophils, platelets, and neoplastic lymphoid cells.
- C—An IFA-positive bone marrow smear. The FeLV antigens are present in the cytoplasm of nucleated cells of the erythroid and myeloid series.
- D—An IFA-negative blood smear. Feline leukemia virus antigens are not present in neutrophils and lymphocytes.
- E—An IFA test on blood smears from a cat with a severe leukopenia. Leukocytes are not in the smear, and thus, evaluation is not possible.
- F—An IFA test on blood smears that are too thick. This causes no-evaluation result owing to nonspecific reaction.

FeLV antigen, the rabbit anti-FeLV serum used in these studies reacts with all FeLV proteins. This serum has proved to be highly specific and sensitive after proper absorption to remove anti-cat reactivity.

Correlation was excellent between the IFA and ID tests and TCI. In addition, because of the ease of use and short time needed to perform it, the IFA test is the most practical of these 3 tests for clinical use. The main limitations of this test are that it has to be performed by trained individuals, and fluorescent microscopes have to be used to read the tests. These factors preclude its use in veterinary hospitals and necessitate that veterinarians submit blood smears to specialized laboratories if they want the FeLV IFA test performed.

The first commercially available FeLV test was the IFA test, which was introduced in 1972.^{15,16} The IFA test for detection of FeLV in blood leukocytes is practical, because the viral antigens remain stable and can be detected immunologically in most blood smears stored unfixed at room temperature for as long as 3 months. The stability of the FeLV antigens in leukocytes allows blood smears to be mailed to laboratories in sufficient time so as not to degrade the antigens.

The FeLV antigens are detectable in the cytoplasm of leukocytes and in platelets in blood on ordinary smears (Fig 4). One report warned of nonspecific IFA staining of feline eosinophil granules, but this problem has not been associated with the antiserum and conjugates used in our study.⁴⁰ Detection of FeLV antigens in blood leukocytes indicates that the bone marrow, the main site for FeLV replication, is infected.^{15,18,19,34} Replication of FeLV in the bone marrow indicates that the cat is viremic, is shedding the virus in its saliva,^{15,16,18,19,34,41,42} and will most likely remain viremic for life.^{15,31} In this regard, between 90 and 97% of IFA-positive cats will remain infected for life^{16,34,37} and 83% of these cats will die of an FeLV-induced disease within 3.5 years.^{35,43} However, between 3 and 10% of viremic cats that are IFA-positive (blood leukocytes and bone marrow cells) can reject the virus and become FeLV-negative (by IFA) and immune to the virus.^{35,37}

The FeLV ELISA were introduced after the establishment of the IFA test as the reference standard, and comparative studies of these 2 test systems are reported elsewhere in this issue.³⁵ The biology and pathogenesis of FeLV infection have been elucidated by the IFA test. In contrast, the biology of ELISA-positive pet cats has not been extensively studied and the outcome of ELISA-positive but IFA-negative discordant cats is not known. However, the biology of ELISA true-positive cats should be identical to that of IFA-positive cats.

The management of ELISA-positive cats presents a considerable problem for veterinarians. There have been conflicting recommendations regarding the disposition and management of these cats. Some veterinarians recommend removal of

the cat from the multicat household, others recommend euthanasia, and still others recommend that these cats be retested by ELISA in 1 month. Many veterinarians, however, recommend immediate IFA confirmation of ELISA-positive test results before making any decision concerning management of the cats. We recommend that all FeLV ELISA-positive results be immediately confirmed by the IFA test. If the IFA test result is positive, the cat should be considered viremic and most likely infected for life.

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