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# Markers of feline leukaemia virus infection or exposure in cats from a region of low seroprevalence

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Molecular techniques have demonstrated that cats may harbour feline leukaemia virus (FeLV) provirus in the absence of antigenaemia. Using quantitative real-time polymerase chain reaction (qPCR), p27 enzyme-linked immunosorbent assay (ELISA), anti-feline oncornavirus-associated cell-membrane-antigen (FOCMA) antibody testing and virus isolation (VI) we investigated three groups of cats. Among cats with cytopenias or lymphoma, 2/75 were transiently positive for provirus and anti-FOCMA antibodies were the only evidence of exposure in another. In 169 young, healthy cats, all tests were negative. In contrast, 3/4 cats from a closed household where FeLV was confirmed by isolation, had evidence of infection. Our results support a role for factors other than FeLV in the pathogenesis of cytopenias and lymphoma. There was no evidence of exposure in young cats. In regions of low prevalence, where the positive predictive value of antigen testing is low, qPCR may assist with diagnosis.

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Following the isolation of feline leukaemia virus (FeLV) from pet cats over 45 years ago, this gammaretrovirus became recognised as one of the most important pathogens of domestic cats and other Felidae worldwide.[1–3] Using diagnostic tests established to detect viral antigen (by enzyme-linked immunosorbent assay [ELISA] or immunofluorescence assay [IFA]) it became clear that up to 30% of exposed cats become persistently antigenaemic following exposure and have a significantly reduced life-expectancy with 83% mortality within 3.5 years.[4,5] The clinical consequences of FeLV are predominantly non-specific resulting from bone marrow disorders and immunosuppression, while around 25% of antigenaemic cats develop lymphomas or leukaemias.[6,7] Persistently antigenaemic cats were identified as the source of infection for susceptible cats and detection of antigen or virus formed the basis of test-and-removal programmes which, together with vaccination, have been successful in reducing the worldwide prevalence of FeLV.[8,9]

During the last decade the availability of molecular techniques to detect provirus (DNA) and free plasma virus (RNA) has enhanced our understanding of the pathogenesis of FeLV infection. It has become apparent that antigen detection is a relatively insensitive indicator of infection and that cats previously considered to have recovered using conventional techniques can harbour low levels of virus. Following experimental exposure most cats show evidence of persistent, transcriptionally active virus.[10–15] In the field, up to 10% of antigen-negative cats test positive for provirus in peripheral blood by polymerase chain reaction (PCR).[10,16–18] The absence of viral RNA in the saliva of 96% of provirus-positive/antigen-negative cats, the success of test-and-removal programs that relied on antigen detection to identify 'infected' cats and the results of attempted experimental

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transmission from latently infected cats,[4,17,19] suggest that regressive infection, characterised by absent antigenaemia and low, transient proviral load,[15] does not play a major role in natural transmission. The potential of FeLV to contribute to disease in antigen-negative cats requires clarification. While some epidemiological data demonstrate an increased risk of lymphoma and reduced survival in FeLV-exposed, antigen-negative cats compared with cats never exposed to the virus,[5,20,21] the results of immunohistochemical and molecular studies to date have yielded conflicting results.[22–28]

Cats in Eastern Australia are commonly diagnosed with problems that are potentially related to FeLV, such as anaemia and lymphoma, but FeLV antigenaemia is an uncommon finding.[29] ELISA-based, in-house antigen detection kits, despite their high specificity, have a low positive predictive value in this population where the prevalence of antigenaemia is similar to the incidence of false-positive results.[30] The aim of this study was to use a battery of serological and molecular techniques to look for evidence of infection with FeLV in cats from an area of low antigen prevalence. Cats at risk of infection by virtue of their clinical signs, cats with known exposure to persistently antigenaemic cats and a population of young, healthy cats undergoing routine procedures were tested. The derived information would more accurately describe the potential threat to Australian cats from FeLV, and inform testing and vaccination recommendations for cats in low-risk areas. This is of global importance as the prevalence of FeLV falls worldwide.

## Materials and methods

Peripheral blood samples, obtained prospectively from three groups of cats, were tested for FeLV provirus, p27 antigen, and anti-feline oncornavirus-associated cell-membrane-antigen (FOCMA) antibodies. Informed consent was obtained and the study was approved by the University of Sydney's Animal Ethics Committee, N00/1-2009/1/4939.

### Group A (sick cats)

This group comprised 75 cats presented to the Valentine Charlton Cat Centre (VCCC), University of Sydney over a 7-month period (July 2007–January 2008). Cases were included if anaemia or another cytopenia was identified or if intermediate or high-grade lymphoma was diagnosed and there was residual sample after running a complete blood count. Data on signalment, environment (indoor only or outdoor access, single cat or multicat household) and feline immunodeficiency virus (FIV) vaccination status were recorded.

### Group B (in-contact cats)

These four cats were free-ranging from a multicat household and had been in-contact with a persistently antigenaemic cat during the previous 12 months. FeLV was isolated in culture from the antigenaemic cat, confirming its infection status and providing an isolate of Australian origin, FeLV Syd-1, for further study (unpublished data). The cats in group B had been in the household for 5 years or longer.

### Group C (young, healthy cats)

This group consisted of 169 healthy cats up to 1 year of age that were presented for routine veterinary procedures to one of three inner city veterinary clinics over a 12-month period (January–December 2009). Data on age, breed, sex, source and FIV vaccination status were recorded.

### Samples

Blood was collected into ethylenediamine tetra-acetic acid (EDTA) and was stored at 4°C for up to 7 days, centrifuged at 12,000 *g* for 2 min and the plasma was decanted. The cell pellet and plasma were stored at −20°C for up to 3 months or at −80°C for up to 3 years.

### FeLV quantitative real-time polymerase chain reaction (qPCR)

Cell pellets were thawed and mixed with an equal volume of sterile phosphate buffered saline (PBS). Polypropylene tubes (Matrix Storage Tubes, Thermo Fisher Scientific, New Hampshire, USA) were loaded with 200–400 µl of each sample and placed at −80°C. The plates were shipped on dry ice to the University of Bristol, UK. DNA extraction and qPCR were carried out as described previously using primers targeting the U3 region of the exogenous retroviral long terminal repeat (LTR).[16] Primers amplifying feline 28S rDNA were included to verify adequate DNA extraction. FeLV positive and negative controls were included as described previously.[16]

### Serology

FeLV p27 antigen and antibodies to FIV p15 and p24 were detected using a commercial in-clinic ELISA (Snap Combo, Idexx Laboratories, NSW, Australia) according to the manufacturer's instructions. Whole blood in EDTA was tested immediately or after storage at 4°C for up to 7 days. EDTA plasma samples were shipped on dry ice to the University of Glasgow for detection of antibodies to FOCMA, as described previously.[21]

### Virus isolation (VI)

Plasma from cats in group A that tested provirus positive ($n = 2$) and from all cats in group B ($n = 4$) was submitted for VI (Jarrett O et al, unpublished) using QN10 cells.

### Statistical analysis

Chi squared tests were used to compare group A with the entire VCCC hospital population to test for its representativeness with respect to sex and breed (crossbred versus purebred). Descriptive statistics were used to describe groups A and C.

## Results

### Group A (sick cats)

Group A comprised 75 sick cats of which 40 (53%) were desexed males and 35 (47%) were desexed females. Forty-eight cats (64%) were crossbred and 27 (36%) were purebred, encompassing 14 different breeds. Group A was not significantly different from the entire VCCC hospital population with regard to sex ($P = 0.5505$) and breed ($P = 0.2477$). The mean age was 11.5 years (standard deviation [SD] 5.1, range 1−21 years). Environmental history was available for 66 cats; 19 (29%) were housed indoors while 47 (71%) had outdoor access. Of 59 cats for which information was recorded, 21 (36%) were from single cat households and 38 (64%) from multicat households. Ten cats (13%) had intermediate or high-grade lymphoma described as renal ($n = 4$, one with concurrent spinal involvement), nasopharyngeal ($n = 2$), multicentric ($n = 2$), mediastinal ($n = 1$) and involving a solitary lymph node ($n = 1$, also FIV seropositive). Anaemia, present in 52 cases (69%), was non-regenerative in 36 (69%) and regenerative in 16 (31%). Twenty-one cats (28%) had other cytopenias (lymphopenia $n = 17$, neutropenia $n = 3$, pancytopenia $n = 1$, panleucopenia $n = 1$, thrombocytopenia $n = 1$). Some cats had >1 inclusion criterion concurrently. None of 75 cats in group A tested positive for FeLV antigen (Table 1). Eight of 75 cats were positive for FIV antibodies. One of these cats had been vaccinated against FIV (Fel-O-Vax FIV; Boehringer Ingelheim) and this cat tested seronegative prior to vaccination. Two cats were positive for FeLV provirus with threshold cycle (CT) values of 39.6 and 38.8. These cats were negative for FIV and anti-FOCMA antibodies. Repeat samples were available 14 and 19 weeks, respectively, after the first sample. FeLV VI carried out on these samples was negative as was repeat testing for FeLV provirus, p27 antigen, anti-FOCMA antibodies and FIV antibodies. Clinical data for cats with evidence of FeLV exposure are summarised in Table 2. A single cat in group A was positive for anti-FOCMA antibodies. This cat, a 12-year-old female domestic shorthair, was negative for FeLV provirus and p27 antigen but seropositive for FIV. The cat presented with splenomegaly, immune-mediated haemolytic anaemia and cyclic neutropenia. PCR testing for the feline haemoplasmas *Mycoplasma haemofelis*, '*Candidatus M. haemominutum*' and '*Candidatus M. turicensis*' was negative. Following splenectomy, all signs resolved and the cat was clinically well 2 years later. Splenic histopathology demonstrated extramedullary haematopoiesis and no evidence of neoplasia.

### Group B (in-contact cats)

The results for group B are summarised in Table 3. One cat tested positive for FeLV provirus, p27 antigen by ELISA and infectious virus by VI, one was positive for FeLV provirus and p27 antigen and a third tested positive for FeLV provirus only. All cats were negative for anti-FOCMA and FIV antibodies.

### Group C (young healthy cats)

Group C comprised 169 young, healthy, desexed cats of which 77 (46%) were males and 92 (54%) were females. One hundred and sixty-four cats (97%) were crossbred and five (3%) were purebred. The mean age was 3 months (SD 1.45, range 2−12 months). Cats had been acquired from rescue societies ($n = 148$, 88%), breeders ($n = 5$, 3%), a pet shop ($n = 1$, 0.6%) or other sources ($n = 15$, 9%). No cat was known to have been vaccinated for FIV. All tested negative for FeLV provirus, p27 antigen, anti-FOCMA antibody and FIV antibodies.

## Discussion

Among sick cats, those with peripheral cytopenia/s and/or lymphoma were selected for investigation because bone marrow suppression, lymphopenia and lymphoma are associated with FeLV infection.[6,31] FeLV infection in this group (A) was rare: none of the cats tested positive for p27 antigen and all but two

**Table 1**. Summary of results for group A (sick cats).

| Number of cats | p27 antigen | FeLV provirus qPCR | Anti-FOCMA antibody | FIV antibody |
|---|---|---|---|---|
| 65 | Negative | Negative | Negative | Negative |
| 7* | Negative | Negative | Negative | **Positive** |
| 1† | Negative | **Detected: CT 39.6** | Negative | Negative |
| 1† | Negative | **Detected: CT 38.8** | Negative | Negative |
| 1 | Negative | Negative | **Positive** | **Positive** |

CT = cycle threshold.
*One of these cats has been vaccinated against FIV.
†Negative on repeat testing on all tests, including VI, 4−5 months later.

**Table 2**. Clinical data for cats in group A with evidence of FeLV exposure or infection.

| Number of cats | FeLV provirus qPCR | Anti-FOCMA antibody | Signalment | Clinical problems in FeLV-exposed cats | Outcome |
|---|---|---|---|---|---|
| 1 | **Detected: CT 39.6** | Negative | 9 years old FN DSH | Non-regenerative anaemia, cat fight abscess | Clinically well 28 months later |
| 1 | **Detected: CT 38.8** | Negative | 10 years old FN DSH | Regenerative anaemia, low-grade intestinal lymphoma | Euthanased 23 months later |
| 1 | Neg | **Positive** | 12 years old FN DSH | Splenomegaly, IMHA, neutropenia | Signs resolved after splenectomy. Clinically well 24 months later |

CT = cycle threshold.

tested negative for provirus. False negative qPCR results are unlikely as the internal control 28S rDNA demonstrated amplifiable DNA in the samples, and the sensitivity of this assay for FeLV provirus is around 92%.[16] The qPCR results are supported by negative p27 antigen test results as these tests have a high specificity and high negative predictive value in this population.[30] Two cats in group A were transiently positive for FeLV provirus with high CT values, consistent with low levels of provirus, but were negative on all other tests. Most likely these cats had been exposed to FeLV and developed regressive infections. One provirus positive cat presented with fight injuries documenting the potential for FeLV exposure, as fight wounds have been shown to be a risk factor for FeLV infection.[32] False-positive qPCR results are considered unlikely because this test, which targets the highly conserved U3 region of the exogenous retroviral LTR,[33] has been demonstrated to have excellent specificity at around 99%[16] and negative control qPCRs were negative throughout. The low prevalence of markers of infection in this group is unlikely to be explained by inadequate opportunities for potential exposure since the majority were from multicat households and had access outside. A higher prevalence of provirus positive cats might have been identified if a lower age had been included as a criterion for this category, since the highest prevalence of FeLV antigenaemia is found in cats less than 6 years of age.[34]

Three of four cats from a multicat household with known exposure to FeLV, confirmed by VI from a persistently antigenaemic cat, were positive on provirus testing. This is not surprising as FeLV spreads rapidly between cats in the same household.[4] The two cats with the lowest CT values were positive on p27 antigen testing, in line with previous findings where cats with higher proviral loads, and, therefore, lower CT values, are more likely to be antigenaemic.[11,14,16] One of these cats was also positive on VI. The CT values in antigenaemic cats here were higher than those typically reported in antigenaemic cats. For example, a previous study using the same qPCR test, found that only 9.4%

**Table 3**. Summary of results for group B (in-contacts).

| | Age (years) | Sex/ neuter status | Breed | FeLV p27 antigen | FeLV provirus qPCR | VI | Anti-FOCMA antibody | FIV antibody | Outcome |
|---|---|---|---|---|---|---|---|---|---|
| B1 | 14 | FN | DSH | **Positive** | **Detected: CT 26.5** | Negative | Negative | Negative | Euthanased 14 months from sampling, non-specific signs |
| B2 | 6.5 | FN | DSH | **Positive** | **Detected: CT 27.5** | **Positive** | Negative | Negative | Euthanased shortly after sampling, non-specific signs |
| B3 | 11 | FN | DSH | Negative | **Detected: CT 38** | Negative | Negative | Negative | Clinically well 3.5 years after sampling |
| B4 | 7.5 | FN | DSH | Negative | Negative | Negative | Negative | Negative | Clinically well 3.5 years after sampling |

of cats with CT values >25 were antigen and VI positive and 7.5% were antigen positive and VI negative. In that study, a CT value of <20 had a specificity of 100% for predicting circulating virus and values <25 maximised sensitivity with minimal loss of specificity.[16]

The presence of anti-FOCMA antibodies has been used to indicate exposure to FeLV.[4] Previously, these antibodies were detected in 13/16 provirus positive cats that were negative by VI and ELISA, whereas none of 16 provirus negative cats had anti-FOCMA antibodies.[16] A negative correlation between the humoral response to FeLV and provirus load has been reported,[10,35] and is supported by the work of Major et al,[36] who demonstrated anti-FOCMA antibodies in low level infection but not in cats with higher virus loads, suggesting that serological responses that might contain FeLV infection are not generated where there are high virus loads. Anti-FOCMA antibodies were not detected in cats with known exposure to FeLV (group B) or in provirus positive cats in group A. This is difficult to explain because the CT values obtained indicate relatively low provirus loads in these cats. An effect from virus replication in tissues other than the peripheral blood cannot be excluded. Anti-FOCMA antibodies were identified in a single cat from group A that was negative on both p27 antigen and provirus testing. This outcome, ie, seroconversion as the only indicator of a presumed abortive infection, has recently been described following low-dose oronasal exposure.[36] Alternative explanations for this result are polyclonal B cell stimulation secondary to concurrent FIV infection, or a false-positive anti-FOCMA result.

FeLV is a directly oncogenic retrovirus and persistent antigenaemia is associated with an increased risk of lymphoma.[37] A role for latent FeLV in lymphomagenesis has long been debated. While some studies have demonstrated FeLV provirus in formalin-fixed lymphoma or bone marrow tissue from up to 26% of antigen-negative cats,[22,23] others, using a variety of testing methods including PCR of blood, bone marrow and lymphoma tissue, have found that FeLV latency is rare in cats with cytopenias or lymphoma.[24–26,28] A recent study of over 300 cats undergoing necropsy, where the prevalence of antigen expression was 9%, reported provirus detection in 50% of cases.[27] A significant association with potential FeLV-related problems including anaemia and panleukopenia but, interestingly, not lymphoma, was demonstrated. We found no evidence of FeLV infection or exposure in 10 cats from group A that were diagnosed with intermediate or high-grade lymphoma. The association between FeLV and lymphoma varies with anatomic form, being strongest for mediastinal and spinal lymphoma and weaker for intestinal locations.[3] The results cannot be explained by low-risk anatomic location; there were no cases of intestinal lymphoma in group A, but there was one case each of mediastinal and spinal lymphoma. Although we did not test bone marrow samples, other studies have demonstrated general concordance between provirus detection in the periphery and the bone marrow.[10,24,26] Our findings support a role for factors other than FeLV in lymphomagenesis in antigen-negative cats. No conclusions can be drawn regarding other pathogenic potential for FeLV among cats with evidence of FeLV exposure on qPCR testing because of small numbers. Long-term follow-up studies are needed to determine the clinical significance of regressive FeLV infection.

The in-house ELISA for p27 antigen also detects FIV antibodies. The prevalence of FIV infection in group A, determined by serology in unvaccinated cats, was 9.3% which is similar to the proportion found in recent serosurveys of sick cats in Eastern Australia.[38,39] The FIV infected cats had been included because of non-regenerative anaemia ($n = 4$, one with concurrent lymphopenia), regenerative anaemia ($n = 2$, one with concurrent neutropenia) and lymphoma ($n = 1$). FIV infection is associated with a mild increased risk for lymphoma development, likely by indirect mechanisms in most cases,[40,41] and is more commonly associated than FeLV with lymphoma in Australian studies.[25,42,43]

There was no evidence of FeLV exposure in cats <1 year undergoing routine procedures. All were negative on p27 antigen testing, qPCR and anti-FOCMA testing. Most of these cats had been acquired from rescues societies. Vertical transmission of FIV is rare but it is perhaps surprising that maternally derived anti-FIV antibodies were not detected in this group since most cats were less than 4 months of age.[44]

In contrast to previous prevalence studies carried out in Australia, which have relied on serology, we have demonstrated definitively, by VI and qPCR, that FeLV is present in the Australian cat population. Even though evidence of exposure to FeLV among those cats not known to be in contact with FeLV-infected cats was low, routine FeLV testing is recommended for all cats because of the poor prognosis that accompanies infection, the absence of effective treatments and the potential for rapid spread between cats housed together. Although p27 antigen detection has the greatest clinical relevance and negative antigen tests are likely to be reliable, the risk of false-positive antigen tests in this background means that confirmation using IFA, VI or qPCR should always be sought.

While the benefits to an individual animal of vaccinating against FeLV in areas of low prevalence, a phenomenon of increasing worldwide importance, may be perceived as small, vaccination should be considered for any cat at risk of FeLV infection for reasons outlined above for FeLV testing. Additionally, given that commercial vaccines against FeLV are highly effective,[12,15] that the reservoir of FeLV infection is the infected cat and that, in contrast to the situation with FIV, cats most likely to be infected with FeLV are also those most likely to be presented for vaccination, eradication of FeLV should be considered as a possibility.[45]

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