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Applying a combination of semi-nested PCR and immunohistochemistry (IHC), the presence of exogenous feline leukemia virus infection was studied in 302 necropsied cats with various disorders. 9% showed the classical outcome of persistent productive FeLV infection which was represented by FeLV antigen expression in different organs. 152 cats (50%) harboured exogenous FeLV-specific proviral sequences in the bone marrow but did not express viral antigen. These cats were considered as horizontally but non-productively infected.

Statistical evaluation showed a significant association of non-productive horizontal FeLV infection with a variety of parameters. Non-productively infected cats were statistically significantly older and more often originated from animal shelters than cats without exogenous FeLV infection. Furthermore, some pathological disorders like anemia, panleukopenia, and purulent inflammation showed significant association with non-productive FeLV infection. No significant association was found with lymphosarcoma, known for a long time to be induced by productive FeLV infection.

The classical fatal outcome of FeLV infection is persistent viremia after horizontal transmission resulting in a permanent infection of, among others, bone marrow cells. However, most cats are able to terminate productive FeLV infection after an initial replicative phase in the bone marrow (Hoover and Mullins, 1991; Rojko et al., 1979). Latent FeLV infection is defined by the presence of provirus in the genome without virus protein production (Post and Warren, 1980; Rojko et al., 1982; Pacitti and Jarrett, 1985). Systemic latent infections were first described by Post and Warren (1980). Virus production was reactivated after corticosteroid treatment of previously virus-negative cats. It was shown by different authors that a high percentage of cats initially harboured reactivatable provirus after transient viremia which decreased permanently over time (Pacitti and Jarrett, 1985; Pedersen et al., 1984; Madewell and Jarrett, 1983). Some authors claimed latent FeLV infection as the cause for the presence of antibodies against feline oncornavirus-associated cell membrane antigen (FOCMA).
in cats (Swenson et al., 1990; Rojko et al., 1982; Rice and Olsen, 1981). Myelomonocytic bone marrow cells, T-lymphocytes and macrophages were identified as target cells for latent infections (Rojko and Olsen, 1984).

For detection of non-productive infection with molecular techniques, exogenously acquired provirus must be demonstrated in the host genome in the absence of viral protein production. U3 sequences, located in the long terminal repeat, are the only parts of the proviral genome showing major differences to endogenous FeLV-related sequences. These endogenous multiple integrated parts of the feline genome are highly homologous but replication deficient DNA sequences and represent phylogenetic remnants of former retroviral infections, which are passed on via the germ cell line (Kumar et al., 1989; Berry et al., 1988; Visscher et al., 1988).

Whether non-productive FeLV infection has any influence on the health status of cats remains doubtful up to now. Typical diseases reported in association with productive FeLV infection are, e.g., bone marrow suppression syndromes like anemia, neutropenia or thrombocytopenia, different forms of lymphosarcoma, and opportunistic infections as a consequence of FeLV-induced immunosuppression. Furthermore, immunopathologic phenomena like glomerulonephritis, iridocyclitis or polyarthritis are reported in exogenous FeLV infections, as are some rare but specifically defined conditions like panleukopenia-like syndrome, FeLV-associated enteritis, dermatosis, osteoporosis and exostosis (Favrot et al., 2005; Lutz et al., 1995; Hoover and Mullins, 1991; Reinacher, 1987; Cotter et al., 1975; Essex et al., 1975).

Conditions described until now in latent or supposedly latent FeLV infection and on its impact on the health status of domestic cats.

Table 1

| Reference                | Disease/condition                  |
|--------------------------|-------------------------------------|
| Pacitti and Jarrett (1985) | Pyometra                            |
| Swenson et al. (1990)     | Higher incidence of disease, Upper respiratory tract diseases |
| Swenson et al. (1987)     | Neutropenia                         |
| Jackson et al. (1996)     | Lymphosarcoma/leukemia              |
| Jackson et al. (1993)     | Higher mortality rate               |
| Francis et al. (1980)     | Variety of diseases/conditions      |
| Francis et al. (1981)     | Chronic gingivitis                  |
| Hardy et al. (1980)       |                                     |
| Tobey et al. (1994)       |                                     |
| McClelland et al. (1980)  |                                     |
| Pedersen et al. (1984)    |                                     |
| Uthmann et al. (1996)     |                                     |

2. Materials and methods

2.1. Animals and tissue processing

302 cats were routinely necropsied at the Department of Veterinary Pathology, Justus-Liebig-University Giessen, Germany. Tissues for histologic and immunohistologic examination were fixed in 10% formalin and embedded in paraffin. Furthermore, femoral bone marrow was collected for DNA extraction, embedded in Tissue Tek® O.C.T. Compound (Sakura Finetek Europe B. V., Zoeterwoude, The Netherlands), immediately frozen and stored at −72 °C. If required for diagnostic purposes, appropriate tissue specimens were transferred for microbiological, parasitological or viral examination.

The animals were sent as corpses from variable sources in Germany (owners, practitioners, private veterinary hospitals, university veterinary hospitals) for diagnostic purposes. They all were included in the study continuously and independently from history, breed, age, origin and diagnoses in the period from May 2002 to December 2003. Supplied information on animals and case histories often was poor and not always reliable. Especially information on previous serological FeLV test results and vaccination status was supplied only infrequently.

Immunohistologically FeLV-positive cats, which all were U3-positive (see below) as well, were regarded as productively FeLV-infected. Antigen-negative cats with detectable provirus (U3) in bone marrow samples are referred to as non-productively FeLV-infected, whereas antigen- and provirus-negative cats are classified as FeLV-negative.

2.2. Diagnosis of FeLV infection

2.2.1. Immunohistology

An indirect immunoperoxidase method was performed to diagnose productive FeLV infection. Mesenteric lymph node, small intestine, spleen, bone marrow and tumor tissue if present were incubated with anti-gp70 and anti-p27 mouse monoclonal antibodies as described earlier by Kovacevic et al. (1997). This selection of organs was examined immunohistologically so as not to miss productive but localized FeLV infection (Hayes et al., 1992). The spleen of a productively FeLV-infected cat served as positive control.

Deparaffinized sections were incubated first with methanol/0.5% H2O2 to block activity of endogenous peroxidase. After washing with TBS buffer, the sections were
pretreated with TUF® (Target Unmasking Fluid®, Kreatech Diagnostics, Amsterdam, The Netherlands). After a further washing step, unspecific protein bindings were blocked with TBS/20% porcine serum. The sections were then incubated for 18 h with a mixture of two mouse monoclonal antibodies (anti-FeLV p27 and anti-FeLV gp70 (Custom Monoclonals Int., Sacramento, CA, USA)) at 4 °C. Negative controls consisted of tissue sections known from previous studies (Kovacevic et al., 1997; Reinacher et al., 1995; Reinacher and Theilen, 1987) to originate from FeLV-negative cats, and of incubating all tissue sections with an unrelated antibody directed against chicken T-lymphocytes (Hirschberger, 1987). A peroxidase-labelled rabbit anti-mouse IgG (DAKO Diagnostika GmbH, Hamburg, Germany) was then applied for 30 min after a further washing step. Specific binding was visualized by incubation with 0.05% buffered dianaminobenzidine tetrahydrochloride containing 0.01% H₂O₂ in 0.1 M imidazole buffer pH 7.1. Finally the sections were washed, counterstained with Papanicolaou's hematoxyline (1:10 in tap water) and dehydrated. The tissues were evaluated qualitatively as immunohistologically positive or negative. Positive reaction was morphologically identical to the previously described pattern (Kovacevic et al., 1997; Reinacher, 1987).

2.2.2. DNA preparation
A variable number (15–25) of bone marrow cryostat sections of 10–20 μm, depending on the expected cell count and the volume of the marrow cavity, were fragmented by agitation in a 2 ml Eppendorf tube cooled in liquid nitrogen. Whole genomic DNA was prepared using the Puregene® DNA (from cells, tissue, body fluids and Gram-negative bacteria) purification kit (Genentra Systems Inc., Minneapolis, USA). When a high blood content of the bone marrow was obvious macroscopically, the DNA sample preparation started with lysis of erythrocytes and pelleting of hemoglobin using Puregene® RBC (red blood cell lysis) solution.

An aliquot of the DNA sample was diluted with the standard kit buffer solution to a final concentration of 125 μg/ml.

2.2.3. gp70-PCR
Each DNA sample preparation was tested with a PCR amplifying an endogenous as well as an exogenous FeLV gp70 sequence before employing the diagnostic U3-PCR in order to allow for identification of potentially false negative results due to poor DNA quality. The feline genome harbours multiple copies of endogenous FeLV-related sequences (Benveniste and Todaro, 1974). Therefore, a positive PCR of endogenous gp70 sequences indicated PCR suitability of the sample. A gp70 consensus sequence of FeLV-A, -B and -C subtypes was used to design the gp70 primer pair (NCBI Accession-Nr. M18247, M12500, M14331, X00188, 6612–6918 (M18247)). The expected gp70 sequence includes two potential FeLV-B- and endogenous gp70-specific insertions, yielding 305 bp FeLV-A and -C or 341 bp FeLV-B and endogenous gp70 amplicons (primer sequences: TATACCGITCTAGATAIGACC (sense), CAACAGTCTITAGTTTGG (antisense)). Each primer was applied in a final concentration of 0.4 pmol/μl in the 25 μl reaction volume. The deoxyribonucleotide triphosphate concentration was 0.2 mM. The reaction mixture contained 0.75 U Taq polymerase (BioTherm™ DNA Polymerase, NatuTec GmbH, Frankfurt, Germany) and a buffer solution supplied by the manufacturer with a final 1.5 mM MgCl₂ concentration. 190 ng of the extracted bone marrow DNA served as sample. The first PCR cycle started with a 2 min denaturation step at 94 °C. All 35 cycles started with a melting step at 92 °C for 60 s followed by 60 s with an annealing temperature of 47.9 °C and elongation for 60 s at 72 °C. The final elongation lasted 300 s. PCR products were analyzed by agarose gel electrophoresis (2.5%) and ethidium bromide staining. All of the 302 immunohistologically positive or negative animals showed a gp70-specific band at 341 bp after PCR in the electrophoresis. Therefore, we could rule out false negative results in the U3-PCR due to PCR inhibitors or poor DNA quality (fragmentation by autolysis or treatment). The band represents endogenous gp70 sequences, but may also be exogenously acquired (Fulton et al., 1990; Berry et al., 1988). 16 of the immunohistologically positive cats additionally showed a band at 305 bp indicating exogenously acquired gp70 originating from the FeLV subtypes A or C (Fulton et al., 1990; Berry et al., 1988).

For confirmation of specificity both PCR amplicons, from FeLV-A/C- and from FeLV-B-infected feline embryo cell cultures, were further analyzed.

The two different PCR products were excised each from agarose gel and commercially sequenced (MWG-Biotech AG, Ebersberg, Germany). One amplicon was 341 bp long and specific for FeLV-B and endogenous FeLV-related sequences, the other one was 305 bp long and specific for FeLV-A as well as for FeLV-C.

2.2.4. U3-PCR (Figs. 1 and 2)
For provirus detection a DNA PCR specific for exogenous U3 was applied. The PCR system was a two step semi-nested PCR. For the outer PCR reaction a previously published primer, TTATCAAGTATGGTCATAG (sense) (Jackson et al., 1993), was combined with the primer AGGTCAACCTGGTGAAC (antisense) for amplification of a 185 bp segment of U3 LTR. Reference genome primer design was the published genome sequence of FeLV-B by Stewart et al. (1986) (NCBI accession number D12500, location on reference genome, forward primer 2107–2127, reverse primer 2290–2271). Primer concentration was 0.4 pmol/μl in a total of 25 μl. The deoxyribonucleotide triphosphate triphosphate concentration was 0.2 mM. The reaction mixture contained 0.75 U Taq polymerase (BioTherm™ DNA Polymerase, NatuTec GmbH, Frankfurt, Germany) and a buffer solution supplied by the manufacturer with a final concentration of 1.5 mM MgCl₂. 250 ng of the extracted bone marrow DNA served as sample.

After an initial 2 min denaturation step, each of 35 cycles started with a melting step at 92 °C for 60 s followed by 30 s with an annealing temperature of 55 °C and elongation for 30 s at 72 °C. The final elongation lasted 90 s. PCR products were analyzed by agarose gel electrophoresis (2.5%) and ethidium bromide staining. A band of 185 bp was taken
as indicative for the presence of exogenously acquired provirus. For confirmation of specificity, the PCR product from the persistently FeLV-infected cell line FL 74-UCD-1 (Theilen et al., 1969) was sequenced after TOPO® TA cloning (pcDNA3.1/V5-His®TOPO® TA Cloning Kit, InvitrogenTM GmbH, Karlsruhe, Germany) into Escherichia coli (DH5αTM Subcloning EfficiencyTM Chemically Competent E. coli, InvitrogenTM GmbH, Karlsruhe, Germany). Isolated plasmid DNA contained a 185 base pair insert. BLAST query revealed a 93–100% sequence homology to 125 published sequences (Altschul et al., 1990), representing the exogenous U3 region of FeLV provirus. The PCR reaction products which showed no specific signal after this first outer PCR were diluted 1:1000 with sterilized water. A different forward primer, CTTGAGGCCAAGAACAGTTA (sense) (location on reference genome (M12500): 2187–2208), which anneals inside the product of the outer reaction was applied in combination with the reverse primer of the outer PCR to amplify a 110 bp region of U3. First, a 2 min denaturation step at 94°C was applied. Each of the following 30 cycles started with a melting step at 92°C for 60 s, followed by 30 s with an annealing temperature of 52.7°C and elongation for 30 s at 72°C. The final elongation lasted 150 s. PCR products were analyzed by agarose gel electrophoresis (3%) and ethidium bromide staining. A sample was categorized as provirus-positive if a band of 110 bp was detectable. The PCR product was also sequenced after routine TOPO® TA cloning (see above). Sequence annealing revealed the expected 110 bp FeLV U3-specific insert.

The semi-nested PCR testing procedure was repeated once if the sample was U3-negative in the first semi-nested run. Sensitivity of the PCR test was estimated by clone DNA dilution experiments. Plasmid DNA preparation (FL74, see above) with known initial concentration was serially diluted in sterilized water and gave reproducibly positive results down to approximately 100 U3 molecules. The amount of clone DNA molecules in the dilution steps was calculated on the basis of initial concentration and estimated molecular weight. In some PCR reactions sensitivity could be further lowered, hypothetically down to one U3 molecule.

2.3. Statistical evaluation

For statistical analysis, immunohistologically FeLV-positive cats were removed from the population. The remaining 276 cats were defined as continuous population. Provirus-positive, immunohistologically negative cats were classified as non-productively infected. FeLV-negative cats were defined as provirus- and antigen-negative. To obtain a diagnosis, clinical, necropsy, histopathological, microbiological and parasitological findings were analyzed. Rare and related diagnoses or conditions were grouped together. As statistical tests the Chi-square and Fisher’s exact tests (two-sided) were applied to demonstrate a significant association of a condition and non-productive FeLV infection. To test for significant association of age and FeLV status, the Wilcoxon–Mann–Whitney U-test was applied. A p ≤ 0.05 level of significance was chosen for each individual test. Two control populations were used for statistical comparison. The first control population comprised the cats that died of trauma and had no other pathological findings. We hypothesized that these cats did not suffer from conditions associated with non-productive FeLV infection. The complete continuous population was used as a second statistical control. Information on age, breed and origin of the animals was not available in every case. Origins were
Table 2
Diseases/conditions found in cats with productive FeLV infection (n = 26).

| Disease/condition                        | Total number |
|------------------------------------------|--------------|
| Lymphosarcoma                            | 15           |
| Meningioma                               | 1            |
| Bronchial adenoma                        | 1            |
| Mammary adenocarcinoma                   | 1            |
| Squamous cell carcinoma                  | 1            |
| Suppurative inflammation                 | 2            |
| Feline infectious peritonitis (FIP)       | 4            |
| End stage kidney                         | 1            |
| Renal cysts                              | 1            |
| Glomerulonephritis                       | 1            |
| Interstitial nephritis                   | 3            |
| Anemia                                   | 7            |
| Splenic hyperplasia                      | 3            |
| Phtisis bulbi                            | 1            |
| Pancreatic nodular hyperplasia           | 2            |
| Arteriosclerosis                         | 2            |
| Fatty liver                              | 5            |
| Pulmonary atelectasis                    | 4            |
| Follicular hyperplasia/follicular depletion | 1/2       |

Diseases/conditions were split in two categories. The first category comprised cats originating from animal shelters; the second category the privately owned cats. According to breed, the population was divided in a domestic short hair group, a Persian (pure and mixed) group, and a third group comprising all others.

3. Results

3.1. Productively FeLV-infected cats

In 26 of 302 cats, expression of viral antigen was demonstrated in all examined tissues. These cats were classified as productively FeLV-infected. All of these 26 cats were also positive for exogenous U3 in the PCR. 16 of these 26 cats showed an additional band at 305 bp, indicating exogenously acquired gp70 originating from the FeLV subtypes A or C. Diseases and conditions associated with productive FeLV infection are shown in Table 2.

3.2. Non-productively FeLV-infected and FeLV-negative cats

In the remaining 276 cats, no expression of the viral antigens gp70 or p27 could be detected. In 152 of the them exogenous U3 could be demonstrated in bone marrow by PCR. These were considered to be non-productively FeLV-infected. An overview of the FeLV status of the cats is shown in Table 3.

3.3. Statistical analysis

3.3.1. Age

Information about the age of 214 of the 276 antigen-negative cats was available. The mean age of these immunohistologically negative cats was 52 months (median: 24 months). 106 of these cats were up to 24 months old (group 1), 74 were between 24 and 120 months (group 2), and 34 cats were more than 120 months old (group 3). 45% of group 1, 64% of group 2 and 71% of group 3 were tested U3-positive by PCR. The youngest cat tested U3-positive was 1 month old, the oldest 20 years. The non-productively FeLV-infected cats (U3-positive (PCR), immunohistologically negative) had an age median of 24 months whereas FeLV-negative cats (U3-negative (PCR) and immunohistologically negative) had an age median of 12 months. The result of the Wilcoxon–Mann–Whitney U-test yielded a statistical significance (p = 0.026) of this age difference between immunohistologically negative, U3-positive (non-productively FeLV-infected) and immunohistologically negative, U3-negative cats (FeLV-negative).

3.3.2. Breed and sex

The evaluation of the different breeds yielded no statistically significant results. Of 227 cats breed information was available. Domestic short hair cats showed the highest percentage of non-productive FeLV infection (58%). For the different sex statuses, no statistically significant differences in the frequency of non-productive infections could be demonstrated either (p = 0.13).

3.3.3. Origin

The origin of 131 cats was known. 72% (n = 47) of cats originating from animal shelters harboured exogenous FeLV-specific U3 sequences in bone marrow DNA. This was significantly more frequent compared to the privately owned cats (p = 0.047). Median age differences between the two different origins and U3-statuses are shown in Fig. 3.

3.3.4. Association of non-productive FeLV infection with various disease conditions

Compared to the continuous population, non-viral infections (bacteria, fungi, parasites) showed a significant positive association with non-productive FeLV infection (p = 0.022); 70% of the 47 cats with such an infection were shown to be non-productively infected.

19 cats died of trauma and had no other pathological findings. Six of them were non-productively FeLV-infected. Some disease conditions were significantly (p ≤ 0.05) associated with the detection of exogenous U3-specific DNA when compared to this trauma population. They are presented in Table 4. For example, cats with anemia not associated with trauma, FIP, leukemia/lymphosarcoma, and feline panleukopenia were significantly more often non-productively FeLV-infected than the trauma population. Diseases and conditions with no significant association to non-productive infection are shown in Table 5. Age differences in the diagnosis groups are demonstrated in Table 6. For antigen-negative lymphosarcomas

Fig. 3. Median age differences, U3-status and origin of cats.
Table 3
FeLV status of necropsied cats.

| U3-PCR Immunohistology | + | − | No. tested cats |
|-------------------------|---|---|-----------------|
| +                       | 26 (9% productive infections) | 152 (50% non-productive infections) | 178 |
| −                       | 0 | 124 (41%; FeLV-negative) | 124 |
| No. tested cats         | 26 | 276 | 302 (100%) |

Table 4
Diseases and conditions significantly associated with non-productive FeLV infection when compared to trauma group (U3-prevalence 32%)a.

| Disease/condition | % U3-positive | p-Values |
|-------------------|---------------|----------|
| Follicular depletion (spleen) (n = 35) | 60 | 0.04607 |
| Cardiomyopathies (n = 16) | 69 | 0.02839 |
| Hydrothorax, -pericardium, -peritoneum, chylothorax (n = 7) | 78 | 0.04182 |
| Follicular hyperplasia (lymph node) (n = 17) | 71 | 0.01944 |
| Follicular depletion (lymph node) (n = 22) | 64 | 0.04058 |
| Otitis (n = 4) | 100 | 0.02372 |
| Parvovirus infection (n = 23) | 65 | 0.03 |
| Suppurative inflammationb (n = 50) | 58 | 0.04989 |
| Multiple foci of suppurative inflammation (n = 12) | 75 | 0.02901 |
| Epithelial tumorsc (n = 17) | 77 | 0.00707 |
| Lymphoplasmacellular interstitial nephritis (n = 33) | 67 | 0.01452 |
| Anemia (w/o trauma, FIP, panleukopenia, lymphosarcoma/leukemia) (n = 27) | 70 | 0.0093 |
| Intestinal worm infestation (n = 8) | 88 | 0.01275 |
| Enteritis (w/o panleukopenia) (n = 20) | 65 | 0.03688 |
| Infection with pathogenic bacteria (n = 37) | 65 | 0.01804 |

a No statistical significance when compared to continuous population.
b Suppurative inflammation (meningitis, otitis, endo-/myocarditis, dermatitis, cholangitis, keratitis, panophthalmitis, endometritis, osteomyelitis, arthritis, nephritis).
c Epithelial tumors (hepatocellular carcinoma (n = 3), intestinal/nasal adenocarcinoma (n = 1/1), pancreatic adenocarcinoma/adeno (n = 1/2), squamous cell carcinoma skin/gingiva (n = 1/2), bronchial adenocarcinoma/adeno (n = 4/1), thyroid adenoma (n = 1)).
d w/o = without.

Table 5
Diseases and conditions not significantly associated with non-productive FeLV infection when compared to trauma or continuous population (p > 0.05).

| Disease/condition | Total number | U3-positive |
|-------------------|--------------|-------------|
| Histoplasmosis | 1 | 1 |
| Fatty liver | 35 | 16 |
| Liver necrosis | 8 | 5 |
| Hepatocellular degeneration | 3 | 0 |
| Liver cysts | 2 | 0 |
| Liver fibrosis | 1 | 1 |
| Peliosis hepatitis | 1 | 1 |
| Hepatocytic dissociation | 4 | 2 |
| Pulmonary atelectasis | 3 | 3 |
| Hyperplasia of bronchial musculature | 2 | 1 |
| Lung sclerosis | 1 | 1 |
| Intestinal pneumonia | 2 | 1 |
| Renal cysts | 6 | 3 |
| End stage kidney | 16 | 9 |
| Renal glomerular sclerosis | 11 | 6 |
| Renal infarction | 3 | 0 |
| Non-suppurative encephalomyelitis | 4 | 3 |
| Adipose tissue necrosis | 1 | 1 |
| Atrophy of islet cells of pancreas | 1 | 1 |
| Chronic pancreatitis | 1 | 1 |
| Pancreatic necrosis | 2 | 0 |
| Nodular hyperplasia of pancreas | 1 | 0 |
| Accelerated thymus involution | 3 | 3 |
| Lymphocytic depletion thymus | 2 | 2 |
| Splenic hyperplasia | 20 | 11 |
| Splenic hemosiderosis | 2 | 0 |
| Streptotrichosis | 5 | 4 |
| Arterial wall calcification | 2 | 2 |
| Lipomatosis cordis | 1 | 1 |
| Non-suppurative myocarditis | 1 | 0 |
| Aortic thrombus | 5 | 3 |
| Thyreoiditis | 1 | 1 |
| Thyroid hyperplasia | 2 | 2 |
| Degenerative joint disease | 1 | 1 |
| Septicemia | 14 | 7 |
| Fibrosarcoma (incl. 1 meningioma) | 5 | 2 |
| Otis | 4 | 4 |
| Calicivirus infection | 6 | 5 |
| Anemia (all forms) | 49 | 28 |
| Feline Herpesvirus infection | 3 | 1 |
| Feline Enteric Coronavirus infection | 1 | 0 |
| Flea infestation | 3 | 2 |
| Lymphosarcoma | 3 | 4 |
| Haemangiosarcoma | 1 | 0 |
| FIP | 37 | 18 |
| Malformations | 13 | 6 |
| Dental calculi | 2 | 2 |
| Unclear cause of death | 26 | 14 |

and FIP, no significant association with non-productive FeLV infection could be demonstrated. 4 of 8 cats with lymphosarcoma and 18 of 37 cats with FIP were U3-positive.

4. Discussion

The data presented here suggest that the non-productive FeLV infection is the most frequent outcome of FeLV infection. 50% of 302 cats harboured U3-specific exogenous FeLV sequences in bone marrow DNA. In comparison, only 9% of this cohort of cats was shown to be productively infected, as demonstrated by immunohistology. In 16 of the 26 productively infected cats an infection with FeLV subtype A and/or C could be demonstrated by PCR. An additional infection with FeLV subtype B cannot be ruled out in these cases because the applied gp70-PCR did not differentiate between endogenous and B-specific FeLV sequences. Conversely, the absence of the FeLV-A/C-specific band in the remaining 10 productively and in all non-productively infected cats is not indicative of FeLV-B infection. The presence of multiple copies of endogenous
FeLV sequences in the genome of the cat may have competitively inhibited sufficient amplification of few or single FeLV-A/C-specific gp70 sequences.

The prevalence of 9% productively FeLV-infected cats is considerably lower than in previous studies which identified 16% productive FeLV infection in necropsied cats between the years 1979–1991 at the same institution (Reinacher et al., 1995; Reinacher and Theilen, 1987). A possible cause for this decrease may be an increase in vaccination and control measures on the basis of FeLV in loco tests. FeLV vaccination was shown to reduce the rate of productive infections but not to prevent latent FeLV infection (Hofmann-Lehmann et al., 2006). With this background, the high prevalence of 50% non-productively infected cats is not unexpected in spite of the decrease of productive infection. A recent survey revealed a 10% prevalence of latently infected cats in a group of mostly healthy cats in Switzerland (Hofmann-Lehmann et al., 2001). Similar results were obtained by Arjona et al. (2006) in cats from Madrid, Spain, and by Pinches et al. (2006) in blood samples sent to the University of Bristol, UK. Diverging results could be explained epidemiologically: the prevalence of productive FeLV infection varies widely between different geographical regions and origins (Bandecchi et al., 2006; Dorny et al., 2002; Muirden, 2002; Arjona et al., 2000; Knotek et al., 1999; Malik et al., 1997). Furthermore, most cats in the mentioned studies were healthy, whereas in the present study cats died from different disease conditions, and often originated from animal shelters (36%). Differences may also be due to the applied methods of testing. Most studies did not use bone marrow DNA, but DNA from blood or lesional tissue as substrate for PCR reaction. After experimental infection, bone marrow myelomonocytic precursor cells and stromal bone marrow fibroblasts were shown to be able to harbour latent FeLV infection (Linenberger and Abkowitz, 1992; Rojko and Olsen, 1984). In the only other study that used bone marrow as test material, latent FeLV infection was not detected in cats with classical FeLV-associated conditions (Herring et al., 2001). This may be due to the likely lower sensitivity of the detection system in comparison to the highly sensitive semi-nested PCR applied in the present study.

19 cats died from trauma and had no other pathological findings. This subpopulation represented the group of otherwise healthy cats with a prevalence of 30% non-productive FeLV infections. In 1990, a study on anti-FOCMA antibodies demonstrated seropositivity in 33% of healthy aviremic cats and in 57% of aviremic cats with different diseases (Swenson et al., 1990). Schniewind et al. (1983) showed 28% anti-FOCMA seropositivity in a group of cats from Germany. The anti-FOCMA antibody prevalence in these former studies is similar to the prevalence of U3-positive cats in our study. This could support the hypothesis that latent FeLV infection is a cause for this immunological phenomenon (Swenson et al., 1990; Rojko et al., 1982).

The present study showed that the prevalence of productive FeLV infection decreases and the prevalence of non-productive infection increases with age. This result indicates that older cats are still susceptible to exogenous FeLV infection but can overcome the viremic (productive) phase more successfully. The data presented in this study also suggest that an effective immune surveillance does not prevent integration of proviral sequences into the host genome. The vaccination experiments of Hofmann-Lehmann et al. (2006) support this hypothesis. They demonstrated integrated provirus without detectable virus protein production after experimental infection of vaccinated cats. Furthermore, the recent results indicate that non-productive infection, as common outcome of prolonged exogenous FeLV exposure, has a relatively mild impact on the health status of immunocompetent animals. The latter interpretation is supported by the fact that 30% of the trauma cats were non-productively infected but did not show any other pathological changes. Host immune response could trigger evolution of low-replicative or non-replicative viral mutants during infection (Merezak et al., 2001; Linenberger and Abkowitz, 1992). New results

Table 6
Age of non-productively FeLV-infected and FeLV-negative cats in diagnosis groups.

| Diagnosis (group) | Median (months) | FeLV-negative | Median (months) | FeLV-negative |
|-------------------|----------------|---------------|----------------|---------------|
|                   |               |               |               |               |
| Follicular depletion (spleen) | 77 | 49 | 63 | 24 |
| Follicular depletion (lymph node) | 63 | 39 | 9 | 9 |
| Follicular hyperplasia (lymph node) | 9 | 23 | 12 | 12 |
| Suppurative inflammation | 9 | 80 | 12 | 12 |
| Multiple foci of supp. inflammation | 104 | 27 | 12 | 12 |
| Parvovirus infection | 14 | 35 (n=2) | 114 | 35 (n=2) |
| Otitis | 2 | 108 | – | 108 |
| Non-viral infection | 51 | 9 | 18 | 6 |
| Epithelial tumor | 15 | 141 | 144 | 160 |
| Hydrothorax, hydropericardium, hydroperitonaeum, chylothorax | 4 | 42 | – | 15 |
| Lymphoplasmacellular interstitial nephritis | 27 | 99 | 82 | 90 |
| Anemia (w/o trauma, FIP, panleukopenia, lymphoma/leukemia) | 23 | 71 | 29 | 12 |
| Intestinal worm infestation | 6 | 38 | 36 (n=1) | 24 | 36 (n=1) |
| Enteritis (w/o panleukopenia) | 18 | 50 | 37 | 3 |
| Cardiomyopathy | 11 | 51 | 45 | 8 |
| Infection with pathogenic bacteria | 30 | 58 | 7 | 6 |
indicate that the majority of circulating viral DNA is transcriptionally active even in the absence of antigenemia shortly after experimental infection (Torres et al., 2008). Replication-defective proviruses could occur for example after provirus silencing through methylation or loss of essential viral sequences by recombination or other molecular mechanisms (Svoboda et al., 1977, 2000).

For the first time statistically significant associations between non-productive FeLV infection and different disease conditions could be demonstrated. Interestingly, not only classical FeLV-associated conditions like anemia or suppressive inflammatory processes, but also conditions not yet FeLV-associated, especially panleukopenia and cardiomyopathies, were significantly associated. For panleukopenia, an age-related statistical side effect is not relevant because panleukopenia is a disease of primarily young cats. The same holds true for the non-productively infected cats from animal shelters, which were younger than the non-productively infected cats from private households. Undoubtedly, cats in animal shelters are more often young and therefore their immune response might sometimes not yet be fully developed or can be impaired by stress, etc. The phenomenon of high prevalence of non-productive infection in this subpopulation could be explained by the high risk of getting into close contact with virus shedding cats in crowded facilities (high virus load in combination with a high population density). In contrast, association of epithelial tumors with non-productive FeLV infection could be biased by age. Previous studies have revealed possible pathogenetic mechanisms of latent FeLV infection. Depression of the specific and unspecific cellular immune response has been shown (Lafrado et al., 1989; Olsen et al., 1987). True latent infections are characterized by the possibility to reactivate virus replication. They must be differentiated from infections resulting in proviral DNA that is replication-defective or silently sequestered by host defense mechanisms (Torres et al., 2005). With the PCR method applied in our study, reactivatability of virus production to define true latent FeLV infection was not proven. The U3 region of the proviral long terminal repeat region (LTR) which is unique for exogenous FeLV infection was identified in the host genome. The LTRs are flanking the viral structural protein genes as 450 bp long DNA regions at both ends of the provirus genome. Formed during reverse transcription, the LTRs have a typical U3-R-U5 sequence structure containing non-coding regulatory sequences important for provirus integration as well as for transcription and replication of virus genes (Goff, 2001; Coffin, 1979). However, the LTR promoter and enhancer regions can also influence transcriptional activity of cell genes without virus protein production (Ghosh et al., 2000). Moreover, the integration of provirus or even proviral parts should be regarded as an infectious mutation that can change genome structure and function (Fujino et al., 2008; Jun et al., 2000). Definite pathomechanisms cannot be deduced from the presented results, as statistical associations are not to be interpreted as a causative linkage between the respective diagnosis and non-productive FeLV infection. The results, however, indicate that the presence of translationally silent proviral sequences can have a negative impact on the health status of affected cats.

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