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Title

Fecal shedding of infectious feline leukemia virus and its nucleic acids: a transmission potential

M. A. Gomes-Keller\textsuperscript{1*}, E. Gönczi\textsuperscript{1}, B. Grenacher\textsuperscript{2}, R. Tandon\textsuperscript{1}, R. Hoffman-Lehman\textsuperscript{1} and H. Lutz\textsuperscript{1}

\textsuperscript{1}Clinical Laboratory, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 260, 8057, Zurich, Switzerland

\textsuperscript{2}Institute of Veterinary Physiology, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 260, 8057, Zurich, Switzerland

* Corresponding author. Present mailing address: Flat 6, 99 Blackheath Park, SE3 0EU, London, UK. Phone: +44 20 3016 7658. E-mail: alice_gomes@yahoo.com

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Abstract

Although it is assumed that fecal shedding of feline leukemia virus (FeLV) constitutes a transmission potential, no study has been performed showing that feces of infected cats can be a source of infection. In this study, we investigated fecal viral shedding of FeLV and its role in viral pathogenesis with the goal to improve infection control. FeLV RNA and DNA levels were determined in rectal swabs of experimentally infected cats by real-time PCR, and the results were correlated with proviral and viral loads in whole blood and plasma, respectively, and plasma p27 levels. All antigenemic cats shed FeLV RNA and DNA in feces. To determine whether the viral RNA detected was infectious, virus isolation from feces was also performed. Infectious virus was isolated from feces of antigenemic cats, and these results perfectly correlated with the isolation of virus from plasma. Naïve cats exposed to these feces seroconverted, showing that infection through feces took place, but remained negative for the presence of FeLV provirus and p27 in blood, an outcome so far not described. Some of the organs collected after euthanasia were provirus positive at low copy numbers. From these results it is concluded that fecal shedding of FeLV plays a role in transmission, but it is probably of secondary importance in viral pathogenesis. Nevertheless, sharing of litter pans by susceptible and viremic cats could increase the environmental infectious pressure and appropriate measures should be taken to avoid unnecessary viral exposure.

Keywords

FeLV, feces, transmission, nucleic acids, virus isolation
Introduction

Feline leukemia virus (FeLV) is an enveloped, positive-sense, single-stranded RNA virus, member of the family Retroviridae, genus Gammaretrovirus. FeLV is one of the most common agents causing cancerous diseases, blood disorders and immunodeficiencies in cats worldwide.

It is assumed that FeLV initially infects the mucosae from the respiratory and gastrointestinal tracts of the head, and replicates subsequently in the tonsils and local lymph nodes (Rojko et al., 1979). The germinal centers from the lymph nodes and tonsils play an important role in this initial replication, functioning as an early reservoir for the virus (Racz et al., 1990). From these lymphoid tissues, infected lymphocytes, monocytes and probably mucosal dendritic cells (Langerhans cells) spread the virus to the bone marrow, thymus, spleen, and intestine, which favors the initiation of a systemic infection (Rohn and Overbaugh, 1999; Rojko et al., 1979).

Infectious FeLV is found in great numbers in saliva (Hoover et al., 1972), and transmission occurs mainly via the oronasal route, by mutual grooming and sharing of eating dishes and drinking bowls (Hoover and Mullins, 1991). Persistently infected, antigenemic cats shed large quantities of infectious virus in saliva and nasal secretions, and pose a great risk to susceptible cats (Hardy et al., 1973; Jarrett et al., 1973).

Although saliva is the most important vehicle for the transmission of FeLV, infectious virus has also been found in milk, urine, and rectal specimens (Hardy et al., 1976; Hinshaw and Blank, 1977). However, the importance of these clinical specimens in the horizontal transmission of the virus has been far less studied when compared to saliva.
Gastrointestinal dysfunction can be observed in cats persistently infected with FeLV, but the etiology of this FeLV-associated enteritis has not been clearly defined (Lutz et al., 1995; Reinacher, 1987, 1989). It was shown that p27 is expressed in intestinal epithelial cells not only of cats showing clinical signs of enteritis, but also in FeLV-positive cats without gastrointestinal disorders, suggesting that there is viral replication in those cells (Kipar et al., 2000). To our knowledge, although there are grounds for suspecting that FeLV can be shed in feces, no study has verified fecal viral shedding and its importance for the transmission of FeLV. In addition, no report has examined the correlation between FeLV nucleic acids detection and isolation of infectious FeLV in feces and blood.

Recently we demonstrated that the shedding of FeLV RNA in saliva is a consistent feature in viremic cats, and that latently infected cats (low proviral load, no antigenemia) sporadically shed viral RNA in saliva, but no virus could be detected by an infectivity assay (Gomes-Keller et al., 2006). To improve diagnosis and infection control, and to gain a better understanding on the importance of latently infected cats, it is essential that possible viral transmission routes are better investigated. In this study we aimed at investigating the possible role of feces in the transmission of the virus in cats harboring different proviral loads. We have applied noninvasive methods for the detection of FeLV. Rectal swabs from 24 experimentally infected cats were tested for the presence of FeLV RNA and DNA. In addition, the presence of infectious virus in the feces of these cats was also investigated and a transmission experiment was carried out to determine whether FeLV can be transmitted solely by contact of naïve cats with feces from persistently infected cats, harboring infectious virus.
Material and methods

Animals and study design

Two separate studies were performed with specific-pathogen-free (SPF) cats. In the first study, 24 animals were acquired from Charles River Laboratories (L’Arbresle, France), and were infected intraperitoneally with FeLV-A/Glasgow strain (kindly provided by Dr. O. Jarrett) at 17 to 19 weeks of age. We examined shedding of FeLV nucleic acids and infectious virus in the feces of these animals at week 98 post-infection, and correlated our findings with the presence of FeLV RNA, DNA, p27 and infectious virus in blood.

The second study consisted of a transmission experiment. Eight cats were acquired from Liberty Research, Inc. (Waverly, NY), and entered the study at 12 weeks of age. For the transmission experiment, feces of 8 persistently infected cats were carefully collected and placed in the litter pan (size of 0.4 X 2.0 m) localized in the room that housed these 8 SPF cats. Feces were spread randomly on the surface of the litter pan. This procedure was performed twice daily, once in the morning and once in the afternoon, for a period of 26 weeks. Blood samples and rectal swabs were collected weekly until week 12, and at weeks 18 and 26. Blood samples were evaluated for the presence of FeLV-specific RNA and DNA sequences, anti-FeLV antibodies, and p27. Rectal swabs were used for the detection of FeLV-specific DNA and RNA.

In addition, 30 SPF cats acquired from Liberty Research, Inc., not infected by FeLV, were used as negative controls. These animals were acquired at the same time and at the same age as the animals from the first experiment, and were handled in the exact same manner. They were tested prior to the beginning of the experiment and shown to be negative for the presence of FeLV DNA and RNA sequences in
plasma and rectal swabs and p27 in plasma. Blood samples and rectal swabs from these negative controls were used to test the specificity of our extraction and detection methods.

All cited animals were kept under barrier conditions in large rooms furnished with running boards, climbing trees, ladders, hammocks and elevated sleeping places, under optimal ethological conditions at the Vetsuisse Faculty, Zurich, Switzerland. Animal handling protocols were reviewed and approved by the Swiss Federal Veterinary Office and followed strictly its guidelines.

**Sample collection and processing**

In study 1, to test whether cats shed FeLV-nucleic acids and infectious virus in their feces, blood samples and rectal swabs were collected from the 24 animals previously cited on the same day and under identical conditions. Blood samples were obtained by jugular venipuncture using evacuated tubes (Becton Dickinson, Plymouth, UK) containing K$_3$EDTA. For virus isolation, an additional blood sample was collected using evacuated tubes (Becton Dickinson, Plymouth, UK) containing lithium heparin. Rectal swabs were collected with the aid of commercially available cotton wool swabs, similar to Q-tips (Primella, Migros Genossenschafts-Bund, Switzerland). Swabs were inserted into the anus of the cats and rubbed gently. After collection, swabs were immediately placed into sterile 1.5-ml microcentrifuge tubes, the external tip was removed, and the microcentrifuge tubes were closed. Swabs were then processed as previously described (Gomes-Keller et al., 2006). Briefly, 200 µl of Hank’s balanced salt solution (without calcium chloride, magnesium chloride, or magnesium sulfate, GIBCO, Paisley, Scotland, UK) were pipetted into the tubes and briefly mixed by pulse-vortexing. Samples were incubated at 42°C for 10 min. The tubes were subsequently centrifuged at 8'000 X g for 1 minute to remove
drops from the inside of the lid. Swabs were inverted and samples were eluted by centrifugation at 8’000 X g for 1 min. Swabs were then discarded, and 140 µl of the whole eluate were used for extraction of total nucleic acids.

For study 2 (transmission experiment), 1 ml of blood was collected from 8 SPF cats using the closed collection system K$_3$EDTA S-Monovette (Sarstedt, Nümbrecht, Germany). Blood was collected weekly, starting from week 0 (no exposition to feces) until week 12, and at weeks 18 and 26.

For both experiments, plasma was obtained by centrifuging blood at 2300 X g, for 10 min. Blood and plasma samples were immediately frozen at -80°C until they were processed.

**Nucleic acid extractions**

One million blood leukocytes were used for DNA extraction using MagNA Pure LC DNA Isolation Kit I and MagNA Pure LC Instrument (Roche Diagnostics, Mannheim, Germany), according to the manufacturer’s recommendations. Leukocyte counts were determined by an electronic cell counter (Cell-Dyn 3500, Abbott, Baar, Switzerland), and the appropriate volume containing 10$^6$ cells were used for extraction. Total nucleic acids were extracted from 140 µl of fecal eluate or 140 µl of plasma using MagNA Pure LC Total Nucleic Acid Isolation Kit and MagNA Pure LC Instrument (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer. Each sample was eluted in a final volume of 60 µl of elution buffer. Extracted samples were stored at –80°C until analysis.

**Detection and quantification of FeLV-DNA and RNA**

FeLV DNA was detected by real-time PCR, and RNA by real-time RT-PCR (ABI 7700, Applied Biosystems, Foster City, USA). Five microliters of the eluate
obtained by nucleic acid extraction were used in each reaction. Primers, probe and assay conditions were as described (Tandon et al., 2005). The detection limit of the assays were determined using serial 10-fold dilutions of purified DNA and RNA from FEA/FeLV-A, -B, and -C spiked into a given sample (sample extracted either from blood, plasma or rectal specimens). The results obtained were compared to a standard curve. In the case of FeLV DNA detection in blood and rectal swabs, the lower limit of sensitivity was one proviral molecule per PCR reaction. For FeLV RNA detection, the RT-PCR procedure had a detection limit of 4.93 log10 copies/ml for plasma RNA and 3.16 log10 copies/RT-PCR reaction for fecal RNA, which cannot be compared directly as amounts of material in rectal swabs are not precisely quantitatable.

**Stability of total nucleic acids in rectal swabs**

Four rectal swabs each from five FeLV-infected cats were collected as mentioned above. Dry swabs were kept at room temperature, and processed and assayed as cited on days 0, 7, 21 and 42 post-collection for the evaluation of the stability of FeLV nucleic acids in rectal swabs.

**Spiking control experiment**

To test for the presence of nonspecific inhibitors in fecal specimens, a spiking control experiment was performed. The experiment consisted of combining aliquots of pooled fecal swab eluates from SPF, non-infected cats, and supernatant of infected QN10S cells (virus stock), to reach an end volume of 200 μl. The following volumes of virus stock were used: 0 (negative control), 15, 40, 60, 80, 100, 140, 160, and 200 μl. The same procedure was performed using Hank's balanced salt solution (without calcium chloride, magnesium chloride, or magnesium sulfate, GIBCO,
Paisley, Scotland, UK), in place of fecal swab eluate. Combined samples were used for total nucleic acid isolation using the MagNA Pure LC total nucleic acid isolation kit and MagNA Pure LC instrument (Roche Diagnostics), as recommended by the manufacturer and tested for the presence of RNA by real-time RT-PCR, as described (Tandon et al., 2005).

**ELISA for the detection of FeLV p27 in plasma and cell supernatant**

FeLV p27 antigen was detected by a sandwich ELISA, as previously described (Lutz et al., 1983). Results are represented as percentages of absorbance in comparison to a positive control, FL-74 feline lymphoblastoid cell culture supernatant (Cockerell et al., 1976), which was considered to be 100%. In this study, p27 values above 5% were considered to be positive. Cats positive for p27 in plasma were considered to be antigenemic.

**Measurement of anti-FeLV IgG in plasma**

We detected anti-FeLV IgG in plasma, using ELISA wells coated with 200 ng/well of gradient-purified FL-74 FeLV under conditions previously described (Lutz et al., 1980). Samples were analyzed in duplicate and the absorbances were read using a microplate reader. For study 1, the results were calculated as the ratio of the mean optical density (OD) in comparison to the mean OD of a serum pool of the same 24 cats before inoculation with FeLV. In the case of study 2, the transmission study, ratios were calculated using the individual ODs obtained pre- and post-exposition to feces containing infectious FeLV. Ratios below 1.00 were considered negative for the presence of FeLV-specific antibodies. In addition, for the transmission experiment, Western blotting analysis (WB) was performed as described previously (Lutz et al., 1988), using 1 μg of gradient-purified FL-74 per
nitrocellulose strip. We analyzed samples from weeks 0 (before experiment begin) and 18.

**In vitro virus isolation from rectal swabs and plasma**

Virus was isolated by cell culture assay from rectal swabs and plasma specimens from cats from study 1 as described by Jarrett and Ganiere (Jarrett and Ganiere, 1996), with few modifications (Gomes-Keller et al., 2006). Rectal swabs were obtained from 21 experimentally infected cats as described above (same 24 cats cited above; 3 animals were not sampled), but swabs were immediately placed into a 1.5 ml-microcentrifuge tube containing 500 μl of cell culture medium. Swabs were processed essentially as described (Gomes-Keller et al., 2006). The supernatant was filtrated into a sterile 1.5 ml-microcentrifuge tube using a syringe-driven filter unit (Millex-HV filter unit, 0.45 μm, Millipore Corporation, Bedford, MA). Three hundred and fifty microliters of the filtrate were used for the infection of QN10S cells. Alternatively, 200 μl of lithium heparin plasma were used for inoculation of QN10S cells. Two hours after inoculation, the supernatant was replaced by fresh medium. Development of cytopathic effects (CPE) was monitored daily. Supernatants were tested on days 0, 10 and 18 post-inoculation by the detection of p27 in supernatant using ELISA, and by RT-PCR using total nucleic acids extracted from 200 μl of cell supernatant as described above. Medium was replaced by 50% once a week.

To test whether infectious virus was found in feces present in the litter pan of FeLV persistently infected cats, we randomly collected 1 g of 20 different fecal samples, being 10 collected in the morning and 10 collected in the afternoon. Fecal samples were resuspended in cell culture medium, and incubated for 10 min at RT. Samples were then centrifuged at 8000 X g for 1 min, and supernatant was filtrated.
as described above. Three hundred and fifty microliters of the filtrate were used for
the infection of QN10S cells.

**Detection of FeLV-DNA and virus isolation from tissues of cats in the
transmission experiment**

Cats used in the transmission study (study 2) were euthanized in week 26, and tissues samples from 27 organs were collected within 30 min post-mortem. Samples were immediately snap-frozen in liquid nitrogen. Approximately 10 to 20 mg of tissue were used for the extraction of DNA (DNeasy Blood and Tissue Kit, QIAGEN, Hombrechtikon, Switzerland), following manufacturer’s instructions. FeLV DNA was detected by real-time PCR as described above, using 800 ng of total DNA per reaction. For every eight cat samples, one water sample was co-extracted and tested by real-time PCR as negative control for extraction. In addition, four water samples were included in each 96-well real-time PCR plate to detect any possible contamination due to pipetting and processing.

In addition, we collected under sterile conditions samples from mesenteric lymph node, urinary bladder, lung, thymus, spleen, and duodenum for virus isolation. Tissue samples were processed and co-cultured with QN10S cells, in an attempt to isolate small quantities of FeLV. Assay conditions were as described (Jarrett and Ganiere, 1996), but tissue samples were cocultured with QN10S cells for 10 days, instead of being removed after an initial incubation of approximately 2 h. Supernatants of the cocultures (200 µl) collected at day 10 post-inoculation were used for total nucleic acid isolation (MagNA Pure LC Total Nucleic Acid Isolation Kit, Roche Diagnostics) and samples were tested for the presence of FeLV RNA as cited above.
**Statistical analysis**

The software StatView (Version 5. SAS Institute Inc., Cary, NC) was used to determine whether a correlation between different parameters characterizing FeLV infection and FeLV-RNA and DNA in feces existed (Spearman correlation analysis), to access the stability of total nucleic acids in rectal swabs (Kruskal-Wallis analysis), to analyze the results from virus isolation and to compare data in the spiking experiment (Mann-Whitney U test). All statistical analyses were two-tailed and P-values < 0.05 were considered as significant. Distributions were tested for normality by the Kolmogorov-Smirnov test using GraphPad Prism (version 3.00 for Windows, GraphPad Software, San Diego California USA).

**Results**

**Animal contact with infected feces in the transmission study**

Before beginning the transmission study (study 2), we checked for the presence of infectious virus in the feces of 8 FeLV persistently infected cats. We randomly collected 20 fecal samples (10 in the morning and 10 in the afternoon) and individually tested them for the presence of FeLV RNA at the day of collection. Regardless the time of collection, all samples tested positive for the presence of FeLV RNA. Samples from morning had in average a cycle threshold (Ct) value (± SD) of 22.73 (± 1.87), while samples collected in the afternoon had a Ct value of 23.99 (± 0.78). There was no statistically significant difference between samples collected in the morning and in the afternoon. Fecal samples were also used for viral isolation to test for the presence of infectious virus, considering that FeLV looses relatively rapidly its infectiousness in the environment (Francis et al., 1979). We were able to detect infectious virus in all samples collected, except one from the morning.
pool, as shown by the presence of FeLV RNA in the supernatant of QN10S cells, detected 10 days after inoculation. There was no statistically significant difference between samples collected in the morning and in the afternoon, regarding the Ct values found by the real-time RT-PCR analysis of FeLV RNA in the supernatants of positive samples. Of note, the amount of virus isolated from feces exposed to the environment was relatively small in comparison to freshly collected feces (see results below for virus isolation). Based on these results, we conclude that infectious virus is commonly found in the feces of persistently infected cats and the virus remains infectious, a prerequisite for its use in the transmission experiment, even though at a lower load.

For the transmission study (study 2), feces from the same 8 FeLV persistently infected cats cited above were spread over the litter pan in a room housing 8 naïve, SPF cats. The procedure was performed daily in the morning and in the afternoon, for a period of 26 weeks. We observed that, after the spread of the feces, naïve cats entered in contact with the infected material as they immediately covered the deposited feces with litter. In this manner, we could assure that naïve cats were exposed twice daily to infectious virus present in the feces of persistently infected cats.

**Stability of total nucleic acids in rectal swabs**

The stability of total nucleic acids over time was monitored in rectal swabs collected from experimentally FeLV infected cats known to be shedding virus in the feces by cell culture assay. Rectal swabs were stored at room temperature, and individually tested for the presence and the amount of FeLV RNA and DNA on days 0, 7, 21 and 42 post-collection (Figure 1). We did not observe any statistically significant difference among different time points with reference to FeLV RNA
(Kruskal-Wallis, $P = 0.9719$) or DNA Ct values (Kruskal-Wallis, $P = 0.7089$). These results indicate that FeLV RNA and DNA present in feces are stable without refrigeration for up to 42 days post-collection. Considering the Ct values obtained, our results highly suggest that viral RNA is the predominant viral nucleic acid species in these clinical samples.

**FeLV-DNA and RNA detection in blood and rectal swabs by real-time PCR**

We subsequently investigated the presence of RNA and DNA in blood specimens and rectal swabs of experimentally FeLV infected cats from study 1. We were able to confirm infection of all 24 cats by detecting provirus in whole blood by real-time PCR (Table 1). FeLV RNA was detected in plasma of 22 (91.5%) of the 24 animals by RT-PCR. The levels of FeLV RNA and DNA in rectal swabs were quantitated by RT-PCR and PCR, respectively. Twenty-two (91.5%) of the 24 cats tested positive for both fecal RNA and DNA. Cats exhibiting an elevated proviral load were likely to have a higher plasma viral burden (Spearman’s correlation coefficient $r_S = 0.850$, $P < 0.0001$), higher viral RNA burden in rectal swabs (Spearman’s correlation coefficient $r_S = 0.626$, $P = 0.0027$), and higher viral DNA levels in rectal swabs (Spearman’s correlation coefficient $r_S = 0.612$, $P = 0.0033$) than cats harboring a low proviral load (Table 2). In addition, cats showing high plasma viral load had higher levels of RNA (Spearman’s correlation coefficient $r_S = 0.551$, $P = 0.0082$) and DNA (Spearman’s correlation coefficient $r_S = 0.664$, $P = 0.0014$) in rectal swabs (Table 2). Rectal swabs obtained from 30 control SPF cats tested negative for the presence of both FeLV RNA and DNA (data not shown). To ensure that negative RT-PCR results were not due to the presence of inhibitors in the feces, which could affect the RNA extraction and detection procedures, we spiked pooled rectal swab eluates with a cell-free virus stock, using different volumes. The results were
compared to HBSS spiked with the same virus stock and identical volumes. As shown in Figure 2, there was no statistically significant difference between Ct values obtained from eluates of rectal swabs or HBSS spiked with virus stock at any volume tested. This implies that negative results obtained for rectal swabs were not due to the presence of elements, which might inhibit the efficient RNA extraction and amplification, but rather due to the absence of FeLV RNA or too low amounts, not detectable by our assay.

Cats from the study 2 (transmission study) did not become provirus positive in any week tested (data not shown). Considering that our assay is able to detect one copy per PCR reaction, we believe that these cats did not harbor FeLV in cells from whole blood or the amount of FeLV present was below our detection limit. Similarly, we were also not able to detect RNA in plasma of these cats at any occasion tested.

Detection of p27 in plasma

Plasma samples were also assayed for the presence of p27 using a sandwich ELISA. In study 1, the antigen p27 was detected in plasma of 22 (91.5%) of the 24 cats tested, indicating an antigenemic state. There was a positive correlation between levels of p27 in plasma and RNA (Spearman’s correlation coefficient $r_s = 0.662$, $P = 0.0015$), and DNA (Spearman’s correlation coefficient $r_s = 0.534$, $P = 0.0105$, Table 2) from rectal swabs. On the other hand, there was no correlation between proviral load or FeLV RNA in plasma, and levels of plasma p27. Latently infected cats (cats 24 and 68), with no detectable levels of p27 and plasma viral RNA, tested negative for the presence of both RNA and DNA in rectal swabs.

We were not able to detect p27 at any occasion in cats from the transmission study (study 2, data not shown). Either these cats did not become antigenemic or the levels of p27 in the circulation were below the detection limit from our assay.
Detection of FeLV-specific IgG in plasma

The levels of FeLV-specific IgG were determined by ELISA in plasma. In study 1, the levels of FeLV-specific IgG in plasma of the 24 experimentally FeLV-infected cats showed a strong negative correlation with proviral load (Spearman’s correlation coefficient $r_S = -0.865$, $P < 0.0001$) and with plasma RNA load (Spearman’s correlation coefficient $r_S = -0.865$, $P < 0.0001$). In addition, we also observed a strong negative correlation between the antibody levels in plasma and the RNA load (Spearman’s correlation coefficient $r_S = -0.592$, $P = 0.0045$) and DNA load (Spearman’s correlation coefficient $r_S = -0.719$, $P = 0.0006$) in rectal swabs. The levels of viral-specific IgG showed no correlation with the levels of p27 in plasma (Spearman’s correlation coefficient $r_S = -0.332$, $P = 0.1114$).

To test for seroconversion by ELISA in the transmission study (study 2), samples collected at week 0 served as individual negative controls for samples collected at week 18 and 26. The mean OD obtained by testing samples from these two latter weeks was divided by the mean OD of the samples collected at week 0, providing a ratio. Ratios below 1.00 were considered as negative for the presence of specific antibodies against FeLV. Results were confirmed by WB analysis (only week 18). Results are presented in Table 3 and Figure 3. We observed seroconversion in 5 out 8 cats (62.5%), as shown by the ELISA and WB results. Interestingly, not all positive ratios obtained by ELISA proved to be due to specific reactions to FeLV proteins, as revealed in the WB analysis. We were not able to detect antibodies to FeLV proteins other than p12 and p15. However, the seroconversion observed took place clearly after exposition of susceptible cats to infected feces.
**Isolation of infectious FeLV from plasma and fecal specimens from experimentally infected cats**

QN10S cells were inoculated with either a filtrate obtained from rectal swabs or heparin-plasma from 21 FeLV experimentally infected cats of study 1 (same animals cited above; 3 animals were not sampled). We monitored development of CPE, and the results were confirmed by the detection of FeLV RNA extracted from QN10S cell supernatant by real-time RT-PCR, and by the detection of p27 present in the cell supernatant using ELISA on days 10 and 18 post-inoculation. FeLV was isolated from rectal swabs and plasma of 17 (81.0%) out of 21 experimentally infected cats (Figure 4). Samples testing positive for virus isolation by ELISA and RT-PCR consistently showed CPE (data not shown). Cats 24 and 68, which were negative for the presence of p27 in plasma and did not shed fecal FeLV nucleic acids, tested negative for the presence of infectious virus. In addition, we were not able to isolate virus from cats 18 and 53, which tested positive for the presence of both p27 in plasma and FeLV RNA and DNA in rectal swabs. Cats with a negative result in virus isolation had a significantly lower proviral load (Mann-Whitney U Test, P = 0.0023), plasma RNA load (Mann-Whitney U Test, P = 0.0023), RNA (Mann-Whitney U Test, P = 0.0023) and DNA loads in rectal swabs (Mann-Whitney U Test, P = 0.0023), and levels of p27 in plasma (Mann-Whitney U Test, P = 0.0031). In addition, cats testing negative for the presence of infectious virus in rectal swabs and in plasma showed significantly higher concentrations of IgG in plasma than cats testing positive for virus isolation (Mann-Whitney U Test, P = 0.0031). In 28.5% of the cats, detection of virus in positive cultures inoculated with rectal swabs eluate required a longer incubation time than that required for detection of virus in plasma cultures (Figure 4).
Presence of FeLV proviral sequences in tissue samples from cats positive only in serological assays

At week 26 of feces exposure, cats from the transmission study (study 2) were euthanized, and different tissue samples were collected and analyzed for the presence of FeLV DNA sequences. In cats J1 and J2, which did not seroconvert, we were not able to detect FeLV DNA in any tissue examined. However, although we were also not able to detect seroconversion in cat F1, we were able to amplify viral DNA sequences in rectum, parotid gland and aorta. In cats that seroconverted (G1, G2, G3, I1 and I2), we found FeLV DNA in different tissues, such as aorta, diaphragm and myocardial muscles, thymus, popliteal lymph node, rectum, urinary bladder, thyroid, mandibular gland and lungs. The copy numbers were in average very low (2.5 copies/reaction). We were not able to detect viral DNA in tissue samples from the following organs: bone marrow, spleen, liver, kidneys, brain (cortex), spinal cord, ischiadic nerve, mesenteric, submandibular and sternal lymph nodes, tonsils, duodenum, jejunum, ileum, colon, and thigh muscle. Water samples co-extracted together with cat tissue samples and water negative controls added to real-time PCR plate tested negative for FeLV-specific DNA.

We also attempted to isolate infectious FeLV from mesenteric lymph node, urinary bladder, lungs, thymus, spleen and duodenum. However, we were not successful in isolating virus from any of these tissues.

Discussion

To gain a better knowledge on the pathogenesis, routes of transmission and control measures for FeLV, viral shedding patterns in different body fluids and secretions must be comprehensively elucidated. Although it has been cited
extensively that feces might play a role in the transmission of FeLV, there are no
published studies on the fecal shedding of infectious virus and the importance of this
route for transmission of FeLV. In addition, no report is available, which
demonstrates the correlation between proviral and viral loads in blood, and FeLV
fecal shedding, in the form of either nucleic acids or infectious virus. With the advent
of real-time PCR and RT-PCR, an excellent diagnostic tool with high analytical
sensitivity and specificity, and fast turnaround time became available for the
detection of FeLV in different specimens.

In this study we demonstrated that FeLV nucleic acids are consistently found
in rectal swabs of antigenemic cats. Our results showed that the proviral load
strongly correlates with the viral load in rectal swabs and in plasma, as well as with
the proviral load in rectal swabs. Latently infected cats, however, do not appear to
shed either FeLV RNA or DNA in feces. These cats were positive only for the
presence of provirus in whole blood, and tested negative for the presence of viral
RNA and p27 in plasma.

We also observed that the FeLV-specific IgG levels strongly and negatively
correlate with the proviral load in whole blood, viral load in rectal swabs and in
plasma, and DNA load in rectal samples. Interestingly, no correlation between the
levels of p27 in plasma and antibody titers could be observed. These results are in
agreement with a previous study performed in this laboratory (Gomes-Keller et al.,
2006), suggesting that IgG plays an important role in viral containment. Animals
testing negative for virus isolation in feces tended to have higher plasma IgG levels
than those shedding infectious virus. We can only speculate that increased levels of
anti-FeLV specific antibodies may lead to an increase in the rates of viral removal
from the circulation, which would justify the strong negative correlations observed. Of
note, we did not evaluate in this study whether the FeLV-specific antibodies found
were present in the form of circulating immune complexes and whether they possessed neutralizing activity.

There are several studies reporting the detection of RNA and DNA in fecal specimens in viral infections causing gastrointestinal clinical signs for diagnostic purposes (Decaro et al., 2004; Pang et al., 2004; Phan et al., 2005). Cats persistently infected with FeLV can present viral-associated enteritis, characterized by histopathologic features in the small intestine similar to those observed in cats with feline panleukopenia (Reinacher, 1987). Of note, none of the 24 cats examined showed signs of gastrointestinal alterations, such as diarrhea, constipation or vomiting, suggesting that despite the involvement of the gastrointestinal tract, the FeLV-specific nucleic acids are consistently shed in feces of antigenemic and viremic cats without apparent clinical signs. It is known that tissues frequently infected in persistently infected cats include the gastric, intestinal and colonic epithelia (Rojko and Kociba, 1991), and villous stromal cells (Kästner, 1992). The FeLV RNA detected in rectal samples by our system could originate both from viral particles and from FeLV-infected cells originating from those tissues. In view of the fact that antigenemic cats shed also FeLV DNA, which had to be expected to originate from cellular material, at least part of the FeLV RNA detected most certainly originated from cells. However, the mean difference between Ct values from samples extracted from rectal swabs obtained by PCR (DNA measurement) and RT-PCR (RNA detection) was of 4.54 (SD = 2.30), and, therefore, FeLV DNA accounts for only 4.3% of the total signal obtained. Consequently, although total nucleic acids were extracted from fecal swabs, the signal obtained in the real-time RT-PCR originates mainly from viral RNA and not DNA. Of note, by performing a spiking experiment, we could exclude the presence of inhibitors affecting RNA extraction and amplification as a cause for a lack of a signal in the detection of the specific FeLV nucleic acid by real-
time RT-PCR. The PCR assay for detecting the presence of viral nucleic acids in rectal swabs of FeLV experimentally infected cats was shown to be of high diagnostic sensitivity and specificity, taking into consideration that we correctly diagnosed all antigenemic cats and we did not obtain a single false-positive result when testing fecal samples from 30 control SPF cats processed in an identical manner. These results are very similar to results obtained previously for the detection of FeLV nucleic acids in saliva (Gomes-Keller et al., 2006). We also observed that FeLV RNA and DNA are very stable at RT, considering that we were able to detect FeLV nucleic acids in rectal swabs stored for at least 42 days, even though very high concentrations of RNases appear to be present in fecal specimens. However, if we compare the collection of buccal swabs with the one of fecal swabs, it is comprehensible that saliva samples are easier to collect than rectal swabs. Nevertheless, the use of fecal swabs, rather than crude feces, due to the presence of large amounts of debris in the latter ones, remains an additional alternative as clinical sample that can be used for the diagnosis of FeLV. When this clinical specimen is used for diagnosis, it is important to keep in mind that cats with access to outdoors should necessarily be sampled by using rectal swabs, considering that feces from these animals are rarely available for collection. In addition, in a multiple cat household, collection of feces from a litter pan leads to cross-contamination with samples originating from different cats. Therefore, collection of individual rectal swabs avoids the risk of a false diagnosis.

Infectious virus was isolated from 17 (81%) fecal and plasma samples out of 21 tested. All 21 cats tested positive for the presence of provirus in whole blood, confirming infection with FeLV. Nineteen cats out of 21 (90.5%) tested positive for the presence of both FeLV RNA and DNA in rectal samples, as well as for the presence of viral RNA and p27 in plasma. Therefore, the presence of FeLV RNA in feces does
not absolutely reveal the production of infectious virions by intestinal and colonic epithelia. This is reflected by the fact that we were not able to isolate infectious virus from two cats, which tested positive for the presence of FeLV RNA in feces. However, the fact that virus was detected in rectal samples and concomitantly in plasma of 81% of the cats tested demonstrates that fecal shedding is a frequent feature of the infection. The reason why we were not able to isolate infectious virus in rectal swabs and plasma from two cats, which tested positive for the presence of FeLV RNA in both specimens, could be the presence of virus neutralization activity in these samples. Nevertheless, this possibility remains to be determined. This study confirms that antigenemia (presence of p27 in plasma) is a good marker for infection but a less reliable marker for viremia. We were not able to detect either FeLV RNA in plasma or isolate infectious virus from plasma from two cats testing positive for the presence of p27, indicating that antigenemia is not a synonym for viremia.

This is the first study to show that feces from persistently infected cats harbor an infectious potential to susceptible cats. However, it appears that viral load present in feces exposed to environmental conditions is relatively low, leading to a novel outcome, in which cats only seroconvert, indicating infection, but virus replicates at very low levels, below the detection limit of very sensitive molecular assays. Based on this outcome, we can conclude from our results that the bone marrow was not successfully infected by FeLV, considering that cats tested always negative for the presence of viral DNA in whole blood. In addition, we could not detect FeLV in bone marrow samples from any of the 8 cats tested. The likelihood that all cats have been exposed to the virus is high, because we could observe that all cats equally entered in contact with feces harboring infectious virus. The level of antibodies remained low. This could be associated to either a limited initial viral replication (undetectable to our assay), or to a weak immune response directed to the viral particles present in the
feces. We were not able to test in this study whether the antibodies found possessed neutralizing activity. Interestingly, we were only able to detect seroconversion to p12 and p15 by WB analysis. In a previous experiment, we observed that antibodies to these two proteins appear very early after FeLV experimental infection (week 4 post-challenge) and last, in some individuals, up to week 98 (M. A. Gomes-Keller and H. Lutz, unpublished data). However, in this previous experiment, it appears that antibodies specific to p12 and p15 were not protective.

Although positive findings for seroconversion in a small population are difficult to generalize, our results suggest that infection with FeLV can occur by exposing susceptible cats to feces containing infectious virus, leading to solely development of a weak humoral response. However, virus was not completely eliminated, considering that we were able to find FeLV DNA sequences in a number of tissue samples from the euthanized cats. In an attempt to isolate infectious virus from mesenteric lymph node, urinary bladder, lungs, duodenum, spleen and thymus, we were not able to obtain a positive result. Tissues for virus isolation were collected at the same time as tissues for DNA isolation, and there were no means to know which samples were positive for the presence of DNA before sample collection. With the exception of thymus, we were not able to find DNA in any other tissue used for virus isolation. In addition, the number of DNA copies found in a single positive thymus sample was very low (1.21 copies/reaction). Thus, it appears that the presence of very low copy numbers of DNA in a certain tissue or its complete absence could be the reason for a negative result in vitro for virus isolation. In addition, our PCR assay amplifies both integrated and unintegrated FeLV DNA sequences. For viral replication, DNA must integrate before transcription can take place. The FeLV DNA sequences found in different tissues could originate from unintegrated DNA, justifying the negative result obtained. Low copy numbers could also be explained by...
contamination of some of the samples. However, contaminations are very unlikely as
all negative controls, which were routinely included during RNA and DNA extraction,
as well as during the real-time PCR testing, tested negative.

Conclusion

The present study raises some important points. First, the fact that FeLV RNA
and DNA, and infectious virus are consistently shed in feces of persistently viremic
cats, and that susceptible cats exposed to these feces seroconvert, demonstrates a
role of feces in the transmission of the virus. Although this route of transmission
appears to have a secondary importance, when compared to saliva, special care
should be taken when handling litter pans. However, to utterly evaluate the potential
for contact transmission via feces, the minimum dose required leading to infection of
susceptible cats, the duration and the titers of fecal shedding by persistently infected
cats remain to be determined. Second, the finding that FeLV nucleic acids and
infectious virus are shed in feces from persistently viremic cats, but not from latently
infected cats, i.e. cats testing positive only for the presence of provirus in whole
blood, but negative for p27 in plasma, provides a better understanding of the FeLV
epidemiology. This finding confirms that latently infected cats may not pose a direct
risk to susceptible cats at the time of proviral determination. Nevertheless, under
stressful conditions or coinfections, the infection may be reactivated, and latently
infected cats could become viremic. Finally, we were able to detect a novel outcome
of FeLV, which resulted to the exposition of susceptible cats to very low loads of the
virus. Whether this outcome has clinical importance or contributes to the
pathogenesis of FeLV remains to be defined. However, considering that these cats
tested negative for the presence of DNA and RNA in blood, and that the only signs
for an infection were a weak immune response and the presence of FeLV DNA in some organs, we believe that such infection state does not pose a risk to susceptible cats. Nevertheless, it would be interesting to induce such outcome in a larger population to further investigate these findings.

Acknowledgements

We thank Dr. O. Jarrett, who kindly provided the FeLV virus stock, and Dr. Marina Meli, Velia Fornera, Elisabeth Rogg, and Theres Meili for excellent assistance in this project. This work was supported by the Forschungskredit grant 55230201 from the University of Zurich. The laboratory work was performed at the Center for Clinical Studies from the Vetsuisse Faculty, University of Zurich, under the supervision of Dr. M. Meli. This study was conducted by M. A. Gomes-Keller in partial fulfillment of the requirements for a Ph.D. degree at the Vetsuisse Faculty, University of Zurich.

References


Figure captions

FIG. 1. Stability of FeLV RNA and DNA in fecal swabs stored at room temperature for up to 42 days. Results are expressed as the mean of cycle threshold (Ct) values from 5 experimentally infected cats and the respective standard deviations; RNA (□), DNA (■).

FIG. 2. Virus spiking control experiment. Supernatant of infected QN10S cell cultures (virus stock) that contained FeLV RNA were added into either pooled fecal swab eluates or HBSS before extraction and amplification of viral RNA. NC, negative control; PC, positive control. Results are expressed as cycle threshold (Ct) values from samples spiked with different volumes (µl) of virus stock (X axis); HBSS (□), pooled fecal swab eluates (■). As there were only two measurements, no standard deviation or error could be calculated.

FIG. 3. Western blotting using gradient-purified FL-74 and sera from susceptible cats exposed to feces containing infectious FeLV. Strips 1 and 9, negative controls; strips 8 and 20, positive controls; strips 2 (cat G1), 4 (cat G2), 6 (cat G3), 10 (cat F1), 12 (cat I1), 14 (cat I2), 16 (cat J1) and 18 (cat J2), sera before exposure to infected feces (week 0); strips 3 (cat G1), 5 (cat G2), 7 (cat G3), 11 (cat F1), 13 (cat I1), 15 (cat I2), 17 (cat J1) and 19 (cat J2), sera after exposure to infected feces (week 18).

FIG. 4. FeLV virus Isolation from feces and plasma from experimentally infected cats at days 10 and 18 post-inoculation using QN10S cell cultures. Results are expressed as Ct value 45 (considered to be negative) minus Ct values measured obtained by real-time RT-PCR done in total nucleic acids extracted from QN10S cell culture
supernatant after inoculation; and as percentage obtained by p27 ELISA performed using cell culture supernatant as sample, in comparison to a positive control (FL74 feline lymphoblastoid cell culture supernatant), which was considered to be 100%. The four cats found to be negative in RT-PCR and p27 ELISA were identical.
Figure 1

Days post-collection

Ct-values (median, n = 5)
Figure 2
Figure 3
Figure 4

Feces

Plasma

Percentage

Difference in Ct values (45-Ct value)

ELISA
RT-PCR

ELISA
RT-PCR
### TABLE 1. Detection of FeLV DNA and RNA in rectal swabs, plasma and blood samples, and detection of p27 in plasma from FeLV infected cats collected one year after experimental infection

<table>
<thead>
<tr>
<th>Cat</th>
<th>Provirus</th>
<th>Plasma RNA</th>
<th>Fecal RNA</th>
<th>Fecal DNA</th>
<th>Plasma p27</th>
<th>Plasma IgG</th>
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<td>nd</td>
<td>nd</td>
<td>&lt; 5.00</td>
<td>2.931</td>
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nd, not detectable, that is below the lower detection limit of the assay (1 copy/PCR reaction for provirus in blood and for fecal DNA; 4.93 log10 copies/ml for plasma RNA; 3.16 log10 copies/reaction for fecal RNA).

\(^a\) copies (log10)/10^6 cells

\(^b\) copies (log10)/ml of plasma

\(^c\) copies (log10)/reaction

\(^d\) values are expressed in percentage in comparison to a positive control (FL74 feline lymphoblastoid cell culture supernatant), which was considered to be 100%.

\(^e\) values are expressed as ratios of the mean optical density (OD) in comparison to the mean OD of samples collected from the same cats before infection (n=24) and processed in an identical manner. Ratios ≤ 1 are considered negative for the presence of FeLV-specific antibodies.
TABLE 2. Correlation between different parameters characterizing FeLV infection and FeLV-RNA and DNA levels in rectal swabs

<table>
<thead>
<tr>
<th>Variable</th>
<th>Correlation with fecal viral load (log_{10} FeLV RNA copies/reaction)</th>
<th>Correlation with fecal viral load (log_{10} FeLV DNA copies/reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spearman's $r_s^a$</td>
<td>P value$^f$</td>
</tr>
<tr>
<td>Plasma FeLV RNA$^a$</td>
<td>0.626</td>
<td>0.0027</td>
</tr>
<tr>
<td>Provirus$^b$</td>
<td>0.551</td>
<td>0.0082</td>
</tr>
<tr>
<td>Plasma p27$^c$</td>
<td>0.662</td>
<td>0.0015</td>
</tr>
<tr>
<td>Plasma anti-FeLV IgG$^d$</td>
<td>-0.592</td>
<td>0.0045</td>
</tr>
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</table>

$^a$ Detection of FeLV RNA sequences by real-time RT-PCR in plasma samples;
$^b$ Detection of FeLV DNA sequences by real-time PCR in whole blood;
$^c$ Concentrations of p27 in plasma samples were measured by ELISA assay;
$^d$ Levels of anti-FeLV IgG in plasma samples were determined by ELISA assay;
$^e$ Spearman's $r_s$, Spearman's correlation coefficient;
$^f$ P-values $< 0.05$ were considered as statistically significant;
TABLE 3. Detectable anti-FeLV antibodies against gradient-purified whole FeLV and its proteins in cats exposed to feces from FeLV persistently infected cats

<table>
<thead>
<tr>
<th>Cat</th>
<th>ELISA$^a$</th>
<th>WB$^b$</th>
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<tbody>
<tr>
<td></td>
<td>Week 18</td>
<td>Week 26</td>
</tr>
<tr>
<td>F1</td>
<td>0.674</td>
<td>0.720</td>
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<tr>
<td>G1</td>
<td>2.682</td>
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<td>G2</td>
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<td>G3</td>
<td>1.235</td>
<td>1.426</td>
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<td>I1</td>
<td>1.593</td>
<td>1.537</td>
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<tr>
<td>I2</td>
<td>1.321</td>
<td>1.612</td>
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<tr>
<td>J1</td>
<td>1.216</td>
<td>1.162</td>
</tr>
<tr>
<td>J2</td>
<td>0.918</td>
<td>1.194</td>
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</table>

$^a$ Anti-FeLV IgG in plasma samples were determined by ELISA. Values are expressed as ratios of the mean optical density (OD) in comparison to the mean OD of samples collected from the same cat before FeLV exposition and processed in an identical manner. Ratios ≤ 1 are considered negative for the presence of FeLV-specific antibodies;

$^b$ Anti-FeLV IgG measured by WB analysis. Responses defined as (+) for a positive reaction, and (−) for a negative reaction to either p12 and/or p15. Responses to other FeLV proteins could not be detected in our assay. WB analysis was performed at week 18 of exposition; plasma samples collected at week 0 (no exposition to FeLV-infected feces) tested negative for the presence of anti-FeLV antibodies.