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SHORT COMMUNICATION

The kinetics of feline leukaemia virus shedding in experimentally infected cats are associated with infection outcome

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Abstract

Feline leukaemia virus (FeLV) infection in felids results mainly from oronasal exposure to infectious saliva and nasal secretions, but the potential for viral transmission through faeces and urine has not been completely characterized. In order to assess and compare potential FeLV transmission routes, we determined the viral kinetics in plasma, saliva, faeces and urine during early experimental FeLV infection (up to week 15 post exposure) in specific pathogen-free cats. In addition to monitoring p27 antigen levels measured by ELISA, we evaluated the presence of infectious particles by cell culture assays and quantified viral RNA loads by a quantitative real-time TaqMan polymerase chain reaction RNA load was associated with infection outcome (high load – progressive infection; low load – regressive infection) not only in plasma, but also in saliva, faeces and urine. Infectious virus was isolated from the saliva, faeces and urine of infected cats with progressive infection as early as three to six weeks post-infection,
but usually not in cats with regressive infection. In cats with progressive infection, therefore, not only saliva but also faeces and to some extent urine might represent potential FeLV transmission routes. These results should be taken into account when modelling FeLV-host interactions and assessing FeLV transmission risk. Moreover, during early FeLV infection, detection of viral RNA in saliva may be used as an indicator of recent virus exposure, even in cats without detectable antigenaemia/viraemia. To determine the clinically relevant outcome of FeLV infection in exposed cats, however, p27 antigen levels in the peripheral blood should be measured. (250 words)

Keywords: feline leukaemia virus; real-time TaqMan® reverse transcriptase polymerase chain reaction; pathogenesis; retrovirus; virus-host interaction; viral loads; virus kinetics; virus shedding.

Introduction

Feline leukaemia virus (FeLV) is a gammaretrovirus of felids, and is not only of interest for veterinary practice, but is also an important model for tumour and AIDS research (Miyazawa, 2002; Onions, 1985). FeLV-host relationships were reevaluated recently using sensitive real-time PCR methods and categorized with respect to proviral and plasma viral RNA load in the peripheral blood of infected cats, and also based on the presence or absence of virus in the blood and bone marrow as well as antigenaemia (Hofmann-Lehmann et al., 2008; Hofmann-Lehmann et al., 2006; Torres et al., 2005).

Five putative categories were suggested; designated abortive infection (no detectable provirus, viral RNA or antigen subsequent to viral exposure), regressive infection with undetectable antigenaemia, regressive infection with transient antigenaemia, latent
infection, and progressive infection characterized by persistent antigenaemia and positive virus isolation (Cattori et al., 2006; Hofmann-Lehmann et al., 2008; Hofmann-Lehmann et al., 2007; Torres et al., 2005). In the latter four categories, all cats were found to become positive for provirus and viral RNA, and the loads had a significant association with infection outcome (Hofmann-Lehmann et al., 2006).

The intra-host kinetics of FeLV were characterized 30 years ago using non-molecular techniques (Francis and Essex, 1977). The virus was found to initially target local lymphoid tissues, where it infects lymphocytes and monocytes, causing a primary cell-associated viraemia that expands to the lymphoid tissues of the whole body within approximately two weeks. Neutrophil and platelet precursors in the bone marrow and small intestine crypt cells are infected within three weeks. This leads to a second cell-associated viraemia, including infection of circulating neutrophils and platelets starting from weeks two through four post-infection and excretion in mucosal and gland tissues from weeks four through eight post-infection (Rojko et al., 1979). The more recent development of sensitive FeLV-specific quantitative PCR assays has allowed the determination of proviral and plasma viral RNA loads in FeLV exposed cats and, therefore, the study of cats with undetectable antigenaemia and viraemia (Hofmann-Lehmann et al., 2001; Tandon et al., 2005; Torres et al., 2005). Viral and proviral loads were proposed to be implicated in the outcome of FeLV infection very early, but not prior to week one post-FeLV exposure (Hofmann-Lehmann et al., 2001; Hofmann-Lehmann et al., 2006). Nonetheless, the relationship between plasma viral loads and the potential shedding routes for FeLV has not yet been investigated.

In mathematical models of the interactions between pathogens and the immune response of the host, the parameters for the epidemiological spread of disease can be estimated from the intra-host dynamics, e.g., the variation in viral load or the
mechanism of pathogenesis (Ganusov et al., 2002). Thus, knowledge of intra-host viral
load kinetics and potential transmission routes is essential to fully understand the
ejpizootiology and virulence evolution potential of FeLV in infected animals. In this
context, saliva (as the primary source), blood and faeces have been identified as FeLV
transmission routes (Francis and Essex, 1977; Gomes-Keller et al., 2006a), but the
shedding kinetics during the early phase of the infection and the role played by urine in
this context are unknown thus far. Therefore, the goals of the present study were 1) to
estimate the transmission potential of FeLV via urine from FeLV-infected cats by
measuring the FeLV loads and kinetics in this compartment, and 2) to compare it with
those of the saliva and faeces from these same cats, as well as with those in the
peripheral blood during the early phase of infection (first 15 weeks). We studied cats
with both regressive and progressive infection. FeLV loads were determined using
FeLV antigen detection, virus isolation to measure replicating virus and molecular
methods for sensitive detection of viral RNA.

Materials and methods

Ten 18-week-old outbred, specific pathogen-free (SPF) domestic kittens (Liberty
Research Laboratory, Waverly, NY, USA) were infected intraperitoneally with $5 \times 10^5$
focus forming units of FeLV-A/Glasgow-1 (week 0). Six SPF cats of the same age and
origin served as negative controls. The cats were housed in two groups in separate
rooms in accordance with the Swiss federal research animal care regulations. Blood
samples were drawn weekly from week 0 to 15. Saliva specimens were collected in
triplicate with the aid of cotton wool swabs three times per week for the first four weeks
and weekly thereafter, and faecal specimens were collected in the same way at weeks
0 through 3 and weeks 6, 9, 12 and 15. Two swabs were placed in 1.5 ml
microcentrifuge tubes and stored at -80 °C until further processing. The third swab was
placed in a 1.5 ml microcentrifuge tube containing 500 µl RPMI medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Bioconcept, Allschwil, Switzerland), 2 mM L-glutamine (Invitrogen, Basel, Switzerland) and 400 IU/ml penicillin-streptomycin (Invitrogen). The swabs were inverted, and the tubes were centrifuged at 8,000Xg for 3 min. The swabs were then discarded, and the medium was stored at -80 °C for virus isolation. Sterile urine samples were collected by cystocentesis at weeks 3, 6, 9, 12 and 15.

Viral RNA in all samples was quantified by real-time TaqMan reverse transcriptase (RT)-PCR (Tandon et al., 2005); FeLV antigenaemia was measured by p27 sandwich ELISA (Lutz et al., 1983); and the presence of infectious FeLV was determined by virus isolation on QN10S cells (Jarrett and Ganiere, 1996) and testing of cell culture supernatants for the presence of replicating FeLV by p27 ELISA or RT-PCR (Gomes-Keller et al., 2006b). For p27 ELISA, results were calculated as the percentage of a positive control, which was assayed with every plate. Values above 4% were considered positive. In addition, virus neutralizing antibody titres were determined in plasma samples by focus reduction assays using FeLV-A/Glasgow-1 on QN10S cells (Jarrett and Ganiere, 1996).

For RT-PCR, nucleic acids were extracted from 140 µl of sterile EDTA-anticoagulated plasma (QIAamp Viral RNA Mini Kit (Qiagen, Hombrechtikon, Switzerland) or 200 µl of sterile urine (MagNA Pure® LC Total Nucleic Acid Isolation Kit, Roche, Basel, Switzerland). For faecal and saliva specimens, two swabs from each sample were used for nucleic acid extraction and ELISA as follows: 200 µl of HBSS (Hank's Balanced Salt Solution, without calcium chloride, magnesium chloride, and magnesium sulphate, GIBCO, Paisley, Scotland, UK) was pipetted into the tubes, which were vortexed, incubated at 42 °C for 10 min and centrifuged at 8,000Xg for 1 min. The swabs were
inverted, and the tubes centrifuged again at 8,000Xg for 1 min. The swabs were then discarded, and the tubes centrifuged again to pellet the cells and impurities. The eluates from duplicate samples were pooled, and the volume was adjusted to 400 µl with HBSS. An aliquot of 200µl was used for RNA extraction (MagNA Pure® LC Total Nucleic Acid Isolation Kit, Roche), and 200 µl were used for p27 ELISA after the addition of 50 µl of a solution of two monoclonal antibodies specific for p27 (Lutz et al., 1983) diluted 1:50 in 0.75 M sodium chloride, 5 mM EDTA disodium salt, 250 mM Tris-Base, 0.5% Tween20 and 0.5% bovine serum albumin (all reagents from Sigma, Buchs, Switzerland).

Statistical analysis of the data was performed using GraphPad Prism (version 3.0, GraphPad Software, San Diego, CA, USA).

Results

Five out of ten FeLV challenged cats developed a progressive infection as judged by persistently positive p27 ELISA results, virus isolation from plasma and the absence of virus neutralizing activity. Five cats developed a regressive infection. One of the cats with a regressive infection showed transient antigenaemia, and four had undetectable antigenaemia (Table 1 and Fig. 1e). The latter five cats produced virus neutralizing antibodies starting at week 6 after virus exposure (Table 1). In cats with progressive infection, viral RNA was detectable before FeLV p27 antigen in all four investigated compartments (Fig. 1). In plasma and saliva, viral RNA was found as early as one week post-infection, while p27 ELISA was only positive starting at week three. In faeces, FeLV RNA and p27 appeared approximately one week later: RT-PCR was positive from week two on, and p27 became detectable in week four. For animal welfare reasons, urine samples were collected only every three weeks. In week three, FeLV RNA was readily detectable in urine. All ELISA results from urine samples were negative
throughout the observation period. In virus isolation, a similar sequence of appearance was observed in the four investigated compartments: in plasma, all cats with progressive infection were positive at week three (first time point investigated), while only one out of five animals tested positive in saliva. All saliva and faeces samples from cats with progressive infection were virus isolation positive at week six, while the majority of the urine samples became positive at week nine after virus exposure.

Interestingly, the cats with regressive infection were also found to be viral RNA positive, at least transiently, in all investigative compartments. In order to detect a potential association between the outcome of infection and viral RNA loads in saliva, plasma, faeces and urine, the cats were grouped according to their infection outcome and their loads were compared. The cats with progressive infection had significantly higher plasma viral RNA loads than the cats with regressive infection starting at week two post-infection (Mann-Whitney U-test: pMWU < 0.05). Faecal and saliva loads were higher from week three post-infection onward (both pMWU < 0.05), and urine loads were higher starting at week six post-infection (pMWU < 0.01) in the cats with progressive infection compared to cats with regressive infection. Virus isolation and p27 ELISA were negative in the saliva, faeces and urine of the cats with regressive infection, with one exception where the sample showed a weak signal.

Comparison of viral RNA loads among different compartments was only possible in samples collected quantitatively (e.g., plasma and urine samples). Plasma RNA loads were not significantly different from urine RNA loads in either the cats with progressive or the cats with regressive infection (Wilcoxon Signed Rank Test: pW > 0.05).

Nevertheless, a tendency was found in weeks 3, 6, 9 and 15 post-infection in the cats with progressive infection in that plasma viral RNA loads were somewhat higher than urine RNA loads (pW=0.0625).
Discussion

The best recognized natural route of FeLV infection is oronasal exposure of naïve cats to the saliva and nasal secretions of infected cats. The potential for transmission via other routes such as faeces or urine had been incompletely characterized prior to this study, and shedding kinetics had not been investigated. The data presented here assess and compare for the first time the viral kinetics of FeLV throughout early infection in four different compartments – plasma, saliva, faeces and urine – in cats with regressive and progressive infection outcomes. By using real-time RT-PCR for the quantification of viral RNA and cell culture techniques for the detection of infectious virus, we demonstrate that not only saliva and faeces, but also urine can bear viral RNA and infectious FeLV particles. These findings are important in light of inter-host modelling of FeLV infection: faeces and urine are additional potential transmission routes for FeLV and must be considered when modelling cat-to-cat FeLV transmission.

With one exception, infectious virus particles were only found in the plasma, saliva, faeces and urine of cats with progressive infection. This is in accordance with a previous study investigating mostly cats with progressive infection one year after experimental FeLV exposure (Gomes-Keller et al., 2006b), in which infectious particles were only found in saliva samples from cats with progressive FeLV infection. This is important with respect to the transmission potential of FeLV in infected cats: cats with progressive infection can shed infectious virus to the environment through their saliva, and can therefore infect other cats by mutual grooming and sharing of eating and drinking bowls (Francis and Essex, 1977). Our results demonstrate that faeces and, to a lesser extent, urine can also be considered to be potential routes of FeLV transmission. To our knowledge, this is the first study to demonstrate that infectious
virus is also shed via the urine of cats with progressive infection. In accordance with the saliva results, only cats with progressive infection, not those with regressive infection, were found to shed infectious particles in the urine and faeces. Thus, we suspect that FeLV might also be transmitted via contact with urine and sharing of litter boxes. So far, the infection risk via faeces and urine can only be estimated: while FeLV loads in faeces are relatively high, indicating a high potential for transmission, FeLV loads in urine were somewhat lower and might pose a lower risk of infection. However, further experiments (e.g., in vivo transmission) must be performed in order to confirm the potential of FeLV transmission through these two compartments.

We found infectious FeLV in saliva at the first time points investigated – three to six weeks after virus exposure. Thus, cats undergoing progressive infection may shed infectious virus via their saliva even at the earliest time point at which the routine diagnostic test for FeLV, detection of plasma p27, typically yields a positive result (Tandon et al., 2005). We speculate that prior to week three, saliva samples would have been negative for virus isolation. Four out of five samples collected from cats with progressive infection in week three were still negative. This assumption would be in accordance with results from Rojko and co-workers, who have demonstrated that the infection of mucosal and glandular epithelial tissues and, in turn, the excretion of FeLV usually starts approximately four weeks after infection, subsequent to bone marrow infection and secondary viraemia in neutrophils and platelets around week three (Rojko et al., 1979). The latter viraemia is presumably associated with the spread of the virus to other compartments. FeLV compartmental kinetics in the present study also followed this scheme: infectious virus appeared first in the plasma and subsequently in saliva, faeces and urine (following the granulocyte-monocyte phase). This was also evident when considering the association between infection outcome and viral RNA loads: in all compartments, cats with progressive infection had significantly higher loads than cats
with regressive infection. The loads started to differ at different time points; i.e., the difference was first found in plasma, and only later in saliva, faeces and urine. The use of a sensitive real-time RT-PCR assay allowed the assessment of FeLV shedding even in the urine of cats with regressive infection and undetectable antigenaemia/viraemia, which had not previously been possible using non-molecular assays. The concentration of RNA that could be found in urine, however, was somewhat lower than in plasma, indicating that a widespread infection of the kidney and urinary tract did not occur in the first four months post-infection.

During early infection, viral RNA was detectable in cats with progressive and regressive infection, not only in plasma as described previously (Hofmann-Lehmann et al., 2001; Hofmann-Lehmann et al., 2006), but also in saliva, and to some extent in faeces and urine. Since four out of the five cats with regressive infection had undetectable antigenaemia/viraemia during the investigated period up to week 15 after virus exposure, they would not have been categorized as FeLV-infected using non-molecular assays. Nonetheless, these four cats were clearly infected with FeLV: they were plasma viral RNA positive for weeks and developed virus-neutralizing antibodies. Thus, during the investigated period up to 15 weeks after FeLV exposure, saliva viral RNA did not seem to be a marker for antigenaemia/viraemia as had been demonstrated for long-term infected cats, i.e., cats infected with FeLV for one year (Gomes-Keller et al., 2006b). Therefore, p27 antigen detection performed on repeated occasions still appears to be the method of choice to determine the outcome of FeLV infection during the early phase of infection investigated here. The outcome of FeLV infection is of clinical importance since cats with progressive infection usually succumb to FeLV associated diseases (McClelland et al., 1980). Nevertheless, testing for viral RNA in saliva could provide insight into possible recent exposure to FeLV, especially in the absence of detectable p27 antigen in the peripheral blood.
In conclusion, these results indicate that urine, in addition to saliva and faeces, is an additional potential means for FeLV transmission. We also show that FeLV shedding via saliva, faeces and urine is subject to different kinetics during the early phase of infection. These parameters must be taken in account in epidemiological models and can be used to assess the risk that infected cats may pose to naïve cats. Moreover, these results demonstrate that during early FeLV infection, detection of viral RNA in saliva is a more sensitive marker of recent virus exposure than p27 detection in the peripheral blood. The latter, however, is still the diagnostic method of choice to determine the outcome of FeLV infection in exposed cats.

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**Figure legend**

**Figure 1.** Viral RNA (a-d) and p27 (e-g) loads in plasma (a,e), saliva (b,f), faeces (c,g) and urine (d,h) in experimentally FeLV-infected cats. Closed symbols and continuous lines represent data from cats with progressive infection (plasma p27-positive from onset to week 15); open symbols and broken lines indicate data from cats with regressive infection. ⋄=cat B8; ■=cat L2; ▲=cat M1; ●=cat Y3; ж=cat Y4; ○=cat A6; x=cat A7; Δ=cat J1; □=cat K5; ◊=cat Y2. Weeks post-infection are represented on the X-axes. Y-axes represent the log10 RNA copies/15 µl for plasma (a) and urine (d); log10 RNA copies/100 µl swab eluate for saliva (b) and faeces (c); or the percentage obtained by p27 ELISA in comparison to a positive control that was considered to be 100% (e-h).
Table 1. Virus isolation from different compartments and viral neutralising antibodies at weeks 3, 6, 9, 12 and 15 post-infection in experimentally infected SPF cats.

<table>
<thead>
<tr>
<th>Cat</th>
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<td>Virus isolation</td>
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<td>UR</td>
<td>PL</td>
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</table>

a B8, L2, M1, Y3, Y4: cats with progressive infection (plasma p27-positive from onset to week 15). A6, A7, K5, J1, Y2: cats with regressive infection.


c Virus isolation; open symbols: negative result, closed symbols: positive result. PL: plasma, SA: saliva, FE: faeces, UR: urine.
Low copy number: RT-PCR positive but with copy numbers lower than those of the last positive RNA standard controls. Virus replication in these samples is either confined to a few cells or is minimal.