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ORIGINAL ARTICLE

Feline leukemia virus outbreak in the critically endangered Iberian lynx (*Lynx pardinus*): high-throughput sequencing of envelope variable region A and experimental transmission

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Abstract The Iberian lynx is the most endangered felid species. During winter/spring 2006/7, a feline leukemia virus (FeLV) outbreak of unexpected virulence killed about 2/3 of the infected Iberian lynxes. All FeLV-positive animals were co-infected with feline hemoplasmas. To further characterize the Iberian lynx FeLV strain and evaluate its potential virulence, the FeLV envelope gene variable region A (VRA) mutant spectrum was analyzed using the Roche 454 sequencing technology, and an *in vivo* transmission study of lynx blood to specified-pathogen-free cats was performed. VRA mutations indicated weak apolipoprotein B mRNA editing enzyme and catalytic polypeptide-like cytidine deaminase (APOBEC) restriction of FeLV replication, and variants characteristic of aggressive FeLV strains, such as FeLV-C or FeLV-A/61C, were not detected. Cats exposed to FeLV/*Candidatus* Mycoplasma haemominutum-positive lynx blood did not show a particularly severe outcome of infection. The results underscore the special susceptibility of Iberian lynxes to infectious diseases.

Introduction

The Iberian lynx (*Lynx pardinus*) is the most endangered felid in the world [34]. Not more than 200-250 animals remain, confined to two isolated populations in southern Spain: Doñana and Sierra Morena [20]. The genetic diversity in the Iberian lynx is very low, especially in the Doñana population [37] and may contribute to render the species particularly susceptible to pathogens [57]. In winter/spring 2006/7, an outbreak of feline leukemia virus (FeLV, a gammaretrovirus occurring naturally in domestic cats and some small felids worldwide [33, 43]) killed 7 out of 11 viremic animals [56].

Four subtypes of FeLV are known [2]: while FeLV-A is present in all FeLV-infected cats, FeLV-B arises by recombination between FeLV-A and an endogenous FeLV-related sequence, and FeLV-C and FeLV-T evolve through mutation of FeLV-A. The latter two cause pure red cell aplasia and a fatal immunodeficiency syndrome, respectively. The virus found in the Iberian lynxes is closely related to FeLV-A: the envelope gene from the first lynx found positive at the beginning of the epidemic shows 98.1% and 97.0% identity to FeLV-A/61E (GenBank accession M18247) and FeLV-A/Rickard (GenBank accession AF052723) reference strains, respectively, and 99.7-100% identity to FeLV blood and tissue viral DNA consensus sequences of the affected Iberian lynxes eight months later [9, 56]. The consensus sequences did not show the presence of highly pathogenic FeLV-A or FeLV-C variants [56], and FeLV-B cannot exist in Iberian lynxes

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because of the absence of endogenous FeLV-related sequences in their genome [56].

Four different outcomes of FeLV infection are known in domestic cats: abortive (no virus detected in blood after exposure, with or without bone marrow infection [54]), regressive, progressive, and the rare focal or atypical infection (no viremia, virus replication only in defined tissues) [31]. The majority of domestic cats exposed to FeLV develop regressive infection (with or without transient viremia) characterized by the presence of integrated viral DNA in the blood [8], low viral RNA and DNA loads, reduction or absence of antigenemia [31], and production of neutralizing antibodies [21], but the virus can occasionally be reactivated, e.g., through immunosuppression [73]. Progressively infected cats shed the virus continuously and can live for several years without developing disease symptoms, usually succumbing to subsequent FeLV-associated diseases [17]. The course of infection is influenced by both host and viral factors, and co-infections [42, 63, 66], e.g., resistance to persistent viremia, increase with the age of the host [32]. The severity of the disease in the Iberian lynx was rather unexpected, since in privately owned, persistently viremic FeLV-infected domestic cats, a six-month survival rate of about 90% and a mean survival time in the field of about one year has been reported [24]. In addition, the rare FeLV infections in Iberian lynx detected before 2006 did not lead to severe disease [48, 56, 71]. Studies in several populations of wildcats from Europe and the Middle East have shown a relatively high prevalence of FeLV antigenemia [22, 43, 57, 62], and at least one other FeLV outbreak has been described in the Florida puma [14]. However, the latter did not have as dramatic of consequences as the one seen in the Iberian lynx in 2007. Another hallmark of the FeLV outbreak was the clear association with infection by *Mycoplasma haemofelis* (Mhf) and '*Candidatus* Mycoplasma turicensis' (CMt) and the absence of FeLV-positive lynxes free of hemoplasma infection. No association with FeLV infection in the Iberian lynx was found for the other known feline hemoplasma, '*Candidatus* Mycoplasma haemominutum'(CMhm) [56]. In recent studies in domestic cats, no association between FeLV and hemoplasma infection was observed [41, 53, 75, 83, 87], but FeLV and hemoplasma infection are associated in European wildcats (*Felis sylvestris*) [88]. Thus, the association of FeLV and hemoplasma in the Iberian lynx may be linked either to host genetics (i.e., a general immune system dysfunction, which may support the assumption that a retrovirus-positive status alone is insufficient to induce hemoplasma disease and that retrovirus-induced immunosuppression may be required for the manifestation of hemoplasma-induced anemia [87]) or to a peculiar characteristic of the infecting virus strain that may have rendered the host more susceptible to specific pathogens.

Several management actions were implemented to avoid the spread of FeLV: progressively infected lynxes were removed from the field, non-infected animals were vaccinated against FeLV, and grown wild and domestic cats were removed in order to reduce inter-specific contacts with lynxes [13, 46]. So far, these measures have been successful: since 2007, the only new case had been from a mother-to-cub transmission in late 2009 (Guillermo López, personal communication). Nevertheless, the potential pathogenicity of the FeLV strain carried by the Iberian lynx cannot be neglected—not only because of the damage caused to the lynx population but also because retransmission from lynxes to domestic cats of a particularly virulent FeLV strain may have profound consequences, as seen for cross-species transmission in other species [13, 28, 39, 61, 65, 84, 89]. Thus, the aim of this study was to assess the disease-inducing potential of the lynx's FeLV. First, one of the regions of the envelope gene variable region A (VRA) of FeLV that is characteristic of extremely pathogenic FeLV variants, i.e., FeLV-C [7, 76] or FeLV-A/61C [63], was analyzed by next-generation sequencing. Second, specific-pathogen-free (SPF) cats were inoculated with either blood from a FeLV-viremic/CMhm- infected lynx, with CMhm-infected blood from a FeLV-free lynx mixed with the FeLV-A/Glasgow-1 strain or with the FeLV-A/Glasgow-1 strain alone, and the course of infection was followed for 20 weeks post-infection (p.i.).

Materials and methods

Ultra-deep sequencing of circulating viruses and proviruses from the Iberian lynxes

Plasma viral RNA and blood viral DNA from three Iberian lynxes were analyzed. The samples originated from the first lynx that had been tested positive for FeLV in December 2006 (lynx "Roman") and probably started the epidemic; from a lynx ("Uda") that also died in 2007; and from a lynx ("Coca") sampled in 2009 that was infected in 2007, yet survived up to now despite progressive FeLV infection. Blood samples from the lynx "Coca" were subsequently used for the transmission study. Reverse transcription of viral RNA was performed on 5 µl of plasma-extracted total nucleic acid (TNA) using an ABI high-capacity cDNA kit (Applied Biosystems, Rotkreuz, Switzerland) at a final concentration of 600 nM each of primers 8197R and 8262R (Table 1) in a final volume of 25 µl according to the manufacturer's instructions with incubation times of 25°C for 10 min, 37°C for 60 min and 70°C for 10 min. Samples were stored on ice until further processing. PCR generation

Table 1 Primers used. Italic bold underlined: tag-key, which allows differentiation of the samples after sequencing; the sequence on the key 5' side is 454-sequencer-specific, the sequence on the key 3' side is FeLV-specific. n.a. = not applicable

Name	5'-3' sequence	Length	Product length (bp)	Target/reference
FeLV subtyping primers				
FeA-F RB59	CAATGTAAAACACGGGGCAC	20	1071	FeLV-A subtype [16, 55]
FeA-R RB17	TAGTGATATTGGTTCTCTTCG	21		
FeB-F RB53	ACAACGGGAGCTAGTG	16	857	FeLV-B subtype [55, 77]
FeB-R RB17	TAGTGATATTGGTTCTCTTCG	21		
FeC-F RB58	AGATCTTGGGCACGTTATTCC	21	1754	FeLV-C subtype [77]
FeC-R RB47	TTGTGAAATGGCATTGCTGC	20		
Primers for reverse transcription				
8197R	GAAGGTCGAACCCTGGTCAACT	22	n.a.	FeLV LTR [56]
8262R	TTATAGCAGAAAGCGCGCG	19		
Next-generation multiple sequencing primers				
NGSA-CTA-F	GCCTCCCTCGCGCCATCAG***cta***CATGTWGACTTATGTGACCTAGTGGG	48	794	Reference (GenBank EU293182)
NGSB-CTA-R	GCCTTGCCAGCCCGCTCAG***cta***GGTCGAGAAACCAGGCAGA	41		
NGSA-ATC-F	GCCTCCCTCGCGCCATCAG***atc***CATGTWGACTTATGTGACCTAGTGGG	48	794	"Roman" blood virus
NGSB-ATC-R	GCCTTGCCAGCCCGCTCAG***atc***GGTCGAGAAACCAGGCAGA	41		
NGSA-ATA-F	GCCTCCCTCGCGCCATCAG***ata***CATGTWGACTTATGTGACCTAGTGGG	48	794	"Roman" blood provirus
NGSB-ATA-R	GCCTTGCCAGCCCGCTCAG***ata***GGTCGAGAAACCAGGCAGA	41		
NGSA-ACA-F	GCCTCCCTCGCGCCATCAG***aca***CATGTWGACTTATGTGACCTAGTGGG	48	794	"Uda" blood virus
NGSB-ACA-R	GCCTTGCCAGCCCGCTCAG***aca***GGTCGAGAAACCAGGCAGA	41		
NGSA-AGA-F	GCCTCCCTCGCGCCATCAG***aga***CATGTWGACTTATGTGACCTAGTGGG	48	794	"Uda" blood provirus
NGSB-AGA-R	GCCTTGCCAGCCCGCTCAG***aga***GGTCGAGAAACCAGGCAGA	41		
NGSA-TCA-F	GCCTCCCTCGCGCCATCAG***tca***CATGTWGACTTATGTGACCTAGTGGG	48	794	"Coca" blood virus
NGSB-TCA-R	GCCTTGCCAGCCCGCTCAG***tca***GGTCGAGAAACCAGGCAGA	41		
NGSA-TGA-F	GCCTCCCTCGCGCCATCAG***tga***CATGTWGACTTATGTGACCTAGTGGG	48	794	"Coca" blood provirus
NGSB-TGA-R	GCCTTGCCAGCCCGCTCAG***tga***GGTCGAGAAACCAGGCAGA	41		

of an 800-bp amplicon was performed on a Biometra gradient cycler (Biometra GmBH, Goettingen, Germany) using 2 µl amplified cDNA (for virus sequencing) or of blood TNA (for provirus sequencing), and forward and reverse primers tagged for multiplexing (Table 1) at a final concentration of 500 nM each with 0.4 µl 10 mM dNTP, 4 µl 5x GC buffer, and 0.5 µl Phusion Hot Start Taq Polymerase (2 U/µl, Finnzymes, Keilaranta, Finnlad) in a

final volume of 20 µl. Cycling conditions were as follows: an initial denaturation of 30 sec at 98°C followed by 40 cycles of 98°C for 10 sec, 64°C for 30 sec and 72°C for 20 sec with a final extension step of 72°C for 2 min. Successful amplification was verified by gel electrophoresis on a 2% TAE agarose gel, and the specific band was excised and purified using a GenElute PCR Clean-Up Kit (Sigma-Aldrich). The product was eluted with 30 µl water, and 5 µl thereof was visualized again by gel electrophoresis. The concentration of the purified PCR products was measured using a Quant-iT®PicoGreen dsDNA Reagent Kit (Invitrogen) on a Rotorgene 6000 (Corbett Life Sciences, Brisbane, Australia) according to the manufacturer's instructions. For next-generation sequencing, 100 ng of DNA was prepared after normalizing the samples to 10 ng/µl and pooling them together. Multiplex sequencing was performed at the Functional Genomics Center Zurich (Zurich, Switzerland) on a Roche 454 Genome Sequencer (Roche, Basel, Switzerland) using a GS FLX Standard Amplicon Kit (Roche) according to the manufacturer's instructions. The amplicons were sequenced starting from the forward primers. The resulting sequences were quality- and length-filtered (minimum read quality >20 for each read, length within the 95% confidence interval around the mean [26], i.e., 261-283 bases) and sorted according to their tags using the Galaxy pipeline at Penn State University, USA (http://galaxy.psu.edu/). The workflow used is available online (http://main.g2.bx.psu.edu/u/vcattori/w/fastq-filter-on-iberian-lynxes-454-dataset). Variant genotype analysis was performed using the SNP caller built in Geneious Software (version 5.0, Biomatters Ltd, Auckland, New Zealand). Variants were called if their frequency exceeded the expected 95% confidence interval for the binomial distribution of the variants detected while sequencing a reference plasmid that carried the same sequence as that of FeLV from the Iberian lynx (GenBank accession EU293182). Sequencing reads are deposited in the NCBI sequence read database (Accession number SRA020778.1) and can also be retrieved in Sanger FASTQ from http://main.g2.bx.psu.edu/u/vcattori/h/public.

Viral titers

Tissue culture infectious doses 50 ($TCID_{50}$) were calculated using five-fold serial dilutions of the viruses. For each virus dilution tested, six wells of a 24-well plate, each with 10,000 QN10 cells, were incubated for 2 hours at 37°C with 250 µl virus diluted in cell culture medium [36]. The inoculum was washed away and the cells cultured further in 400 µl medium for 8 days with addition of 400 µl medium after 4 days. The presence of infectious virus was determined by p27 ELISA as described [49], and $TCID_{50}$ was calculated using the Karber formula [38].

In vivo transmission study: SPF cats

Sixteen 8-week-old SPF male domestic kittens (Liberty Research Inc., Waverly, NY, USA) were included in this study. The cats were divided into four groups of four cats each and housed in separate rooms under barrier conditions. All SPF cats were examined clinically prior to the study, and their SPF status was verified as described previously [60]. Identical methods were used to screen the lynxes designated as blood donors for the *in* vivo transmission study. EDTA serum was negative for FeLV p27 antigen and for antibodies against FCoV, FCV, FHV_1, FPV, and FIV [29, 51, 70]. The cats and the potential donor lynxes were blood typed using a commercial gel column technique (ID-Gel Test Feline A+B Typing; DiaMed AG, Cressier sur Morat, Switzerland).

Experimental design and infection

Cats of group "Iberian lynx - CMhm" (IbL-CMhm, cats LM1-LM4) received blood from a FeLV- and CMhm-positive Iberian lynx, a two-year old male called "Coca", which survived the outbreak in 2007 and belonged to the free-ranging Coto del Rey subpopulation in the Doñana area before being removed from the field. Each cat of group IbL-CMhm received 1 ml of EDTA blood in 20% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Buchs, Switzerland). The blood was stored at room temperature during shipment from Spain to Switzerland (24 h), preserved in 20% DMSO after receipt and stored in liquid nitrogen until use. The blood was positive for FeLV (p27 60% of the laboratory control, 9.8 x 10^7 copies virus/ml, a titer of approximately 2,000 focus-forming units (FFU)/ml as estimated by $TCID_{50}$ comparison with the Glasgow-1 stock virus used for the other groups) and for CMhm (6.9 x 10^5 copies/ml). Cats of the group "FeLV-A/Glasgow-1-CMhm" (G1-CMhm, cats GM1-GM4) were inoculated with blood from an FeLV-negative, CMhm-positive lynx together with a prototype FeLV-A virus strain (FeLV-A/Glasgow-1 [35]). Each cat received 1 ml of EDTA blood in 20% DMSO. The blood was collected from a free-ranging female Iberian lynx called "Esperanza" captured as cub from the Doñana area and held in captivity since, shipped at the same time as the group IbL-CMhm blood and handled identically. Esperanza's blood was positive by PCR for CMhm only (3.7 x 10^5 copies/ml). In addition, the cats received 0.2 ml FeLV-A/Glasgow-1 strain (500,000 FFU, 1.35 x 10^{10} viral RNA copies/ml inoculum) [35]. The two inocula were injected intraperitoneally with separate syringes. Cats of group "Felv-A/Glasgow1" (G1, cats G1-G4) served as an FeLV-A/Glasgow-1-only control group. They received 0.2 ml FeLV-A/Glasgow-1 strain (500,000 FFU) in 20% DMSO and 0.6 ml RPMI medium (Invitrogen AG, Basel, Switzerland), and cats C1-C4 (control group) served

as uninfected age-matched negative controls. They were inoculated with 1 ml RPMI medium (Invitrogen AG) in 20% DMSO. All cats were inoculated intraperitoneally at the age of 12 weeks. All inocula were thawed at 37°C and stored on ice until use. The cats were monitored daily for signs of illness after virus inoculation; body temperature and weight were recorded at each sampling day. To monitor for cryptic infections transmitted via lynx blood to the SPF cats, pathogen screening was performed again at week 16 p.i. as described above.

Sample collection and processing

Blood samples were collected under light anesthesia (3 mg/kg S-Ketamin (Keta-S®; Dr. E. Graeub AG, Bern, Switzerland), 5 µg/kg Medetomidin (Dorbene®; Dr. E. Graeub AG), and 0.2 mg/kg Butorphanol (Morphasol-4®; Dr. E. Graeub AG), administrated intramuscularly) at the time point of infection (week 0) and at weeks 1 to 6, 8, 10, 13, 16, and 20 p.i. Plasma was recovered from EDTA-anticoagulated blood after centrifugation for 10 min at 1700 g. Plasma and whole blood samples were immediately frozen at −80°C until use, with the exception of the hematology samples, which were processed at once. Hemograms were performed using a Sysmex XT-2000iV (Sysmex Corporation, Kobe, Japan) [85]. Packed cell volume (PCV) values between 22% and 39% and leucocytes between 4,600/µl and 12,800/µl (5% and 95% quantiles) were considered to be within the reference range. Biochemical test were performed on an automated analyzer (Cobas Integra 800, Roche Diagnostics, Rotkreuz, Switzerland). The infected cats were euthanized at week 20 p.i.

Detection and quantification of FeLV blood viral DNA and plasma viral RNA and CMhm loads by real-time PCR and RT-PCR

TNA was extracted from a volume of EDTA-anticoagulated blood containing 10^6 leucocytes or from a maximum volume of 100 µl using a MagNA Pure LC Total Nucleid Acid Isolation Kit (Roche diagnostics AG, Rotkreuz, Switzerland). Cell copy numbers, FeLV proviral DNA quantities and CMhm loads were determined by real-time PCR as described [79, 86, 87]. For detection of FeLV viral RNA in plasma, TNA was extracted from 200 µl EDTA plasma using a MagNA Pure LC Total Nucleic Isolation Kit (Roche) and quantified by real-time RT-PCR as described [79].

Detection of FeLV p27 antigen and FeLV antibodies by ELISA and western blot

FeLV viremia was determined by p27 double antibody sandwich ELISA as described [50]. Results were calculated as the percentage of a positive control (culture supernatant of FL-74 feline lymphoblastoid cell line permanently infected with FeLV), which was assayed with every plate. Values above 4% were considered to be positive. In agreement with the European Pharmacopoeia (2005), a cat was designated progressively infected when FeLV p27 antigen was positive for three consecutive weeks or on five occasions between weeks 3 and 15 p.i. Anti-FeLV whole virus antibodies were determined by ELISA using 100 ng of gradient-purified FL-74 FeLV and a plasma dilution of 1:100 as described [49]. Values above 25% of a SPF-serum pool were considered to be positive. In addition, FeLV antibodies were detected by western blot at weeks 0 and 20 p.i. as described [52], using serum obtained from a pool of immune cats and a mixture of monoclonal antibodies against p27, p15E and gp70 as a positive control [54].

FeLV subtyping

TNA (2 µl) extracted from blood collected from all cats at week 20 p.i. (with the exception of GM1 and LM1, which were sacrificed for humane reasons at weeks 10 and 13 p.i., respectively) and from the FeLV viremic lynx used for the transmission experiment, was analyzed by conventional PCR for the presence of FeLV-A, -B and -C subtypes using previously described primers (Table 1) [16, 55, 56, 77]. Each reaction contained a final concentration of 500 nM of each primer with 0.4 µl 10 mM dNTP, 4 µl 5x Phusion HF Buffer, and 0.2 µl Phusion Taq Polymerase (2 U/µl, Finnzymes, Keilaranta, Finnlad) in a final volume of 20 µl. Target sequences were amplified on a Biometra gradient cycler (Labrepco, Horsham PA, USA) under the following conditions: initial denaturation for 2 min at 98°C followed by 40 cycles of 98°C for 20 sec, 52°C (FeLV-C) or 64°C (FeLV-A/-B) for 30 sec and 72°C for 80 sec with a final elongation step of 72°C for 10 min. PCR products were visualized by agarose gel electrophoresis.

Statistics

Due to the limited number of animals per group, we abstained from using a parametric approach and used a robust test instead. Longitudinal effects on FeLV viral, FeLV proviral, and antibody loads of the different groups were compared to each other using the area under the curve (AUC) and Mann-Whitney tests. Longitudinal effects on CMhm on the time until the bacterial peak were determined using the Mann-Whitney test. Outcomes between groups were compared using Fisher's exact test. P-values <0.05 were considered statistically significant. Analyses were conducted using the R-package (version 10.0, The R Foundation for Statistical Computing, Vienna, Austria) and

GraphPad software (version 5.00, GraphPad Software Inc. La Jolla, CA, USA).

Results

Characterization of circulating viruses

The FeLV viral RNA and proviral DNA envelope gene VRA from the three lynxes "Roman", "Uda" and "Coca" were analyzed (Fig. 1). A total of 3299 reads from a plasmid reference were analyzed for 454 sequencing error correction. The error rate was of 2.55% with a transition/transversion rate of 1.94, and the majority of errors were G-to-A and C-to-T transitions (16.8 and 14.4%, respectively, of the sequencing errors). After error correction, the sequences from the Iberian lynxes were 87.3 to 97.1% identical to the reference sequence over 238 bases (positions 150-387 of GenBank EU293182, Table 2). Mapping of the variants was possible for mutations that occurred in >0.2% of the viral/proviral sequences. No deletions or variants characteristic of the highly pathogenic FeLV-C [7, 76] or FeLV-A/61C [63] were found in the VRA (Fig. 1). Of the 25 sites of variation identified, 15 were due to a G-to-A substitution. Ten of the 25 sites found were conserved in the 2007 and 2009 sequences (Fig. 1 and Table 2). Twelve of the 15 G-to-A substitutions caused a nonsynonymous amino acid substitution, while only three nonsynonymous substitutions occurred in the other 10 variant sites. Nonsynonymous substitutions were mainly tryptophan to stop codon and glycine to arginine (Fig. 1 and Table 2). Overall, around 85% of the G-to-A substitutions identified caused a nonsynonymous substitution (Table 3). Substitutions other than G to A only caused nonsynonymous substitutions in a maximum of 40% of the sequences (Table 3). Furthermore, The fifteen G->A sites of the lynx FeLV variant spectrum are embedded in 8 different trinucleotide contexts (Table 2), three G->A sites (positions 10, 161 and 176) are present in all six samples analyzed, and three (74, 157, 173) in five out of six samples (Fig. 1 and Table 2).

Characteristics of Iberian lynx blood used as inoculum

The lynx blood that was used for the transmission experiment was PCR/RT-PCR negative for all tested feline

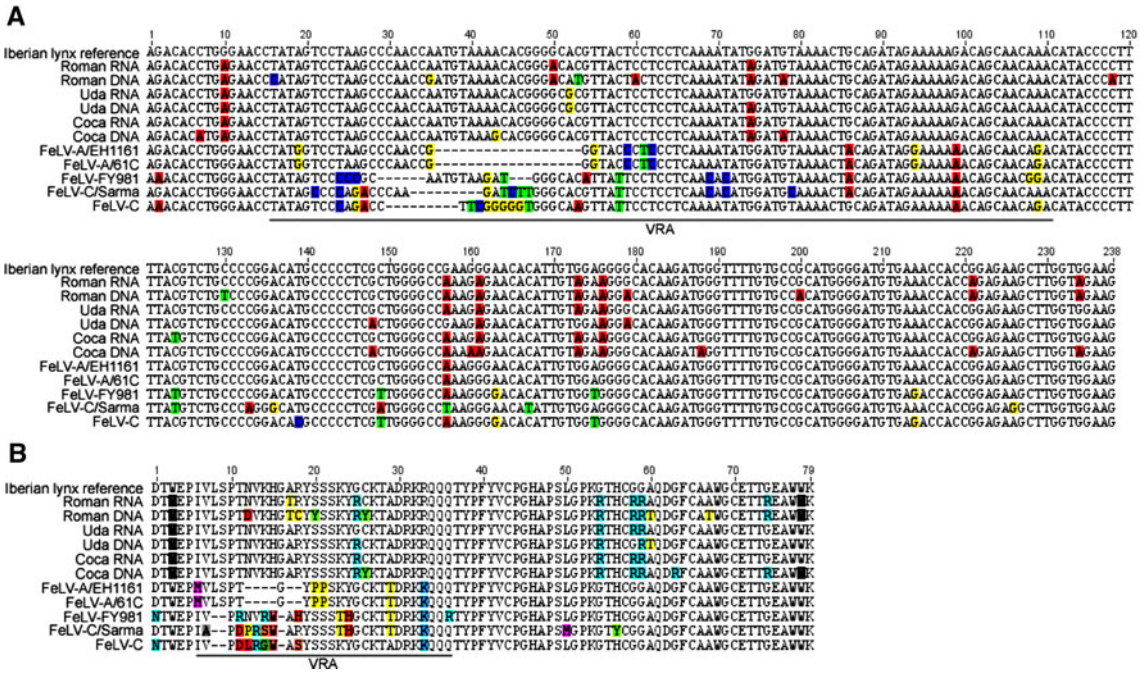

Fig. 1 FeLV sequence variants in the Iberian lynxes. Variations appearing at a frequency >0.2% and comparison with variations occurring in highly pathogenic FeLV strains are depicted. Differences from the Iberian lynx reference sequence are highlighted, with each nucleotide (**A**) and amino acid (**B**) shown with a different color. FeLV envelope VRA is indicated by a horizontal bar. A) DNA sequence comparison. FeLV-A/EH1161 and FeLV-A/61C are involved in feline AIDS pathogenesis [63]; FeLV-FY981 represents the transition between FeLV-A and FeLV-C subtypes [7, 76]. The samples "Roman", "Coca" and "Uda" originated from the first lynx that tested positive for FeLV in December 2006 ("Roman") and probably started the epidemic; from a lynx ("Uda") that also died in 2007; and from a lynx ("Coca") sampled in 2009 that was infected in 2007, yet survived up to now despite progressive FeLV infection. The GenBank accession numbers of the sequences are as follows: EU293182 (lynx reference), M87886 (EH1161), M18246 (61C), FJ436991 (FY981), M14331 and M89998 (FeLV-C). B) Nonsynonymous substitutions deriving from mutations in the DNA sequences from **A**)

Table 2 Variant frequency in the Iberian lynx FeLV after filtering of Roche 454 genome sequencer data. CID (%): percentage of sequencing reads identical to the reference sequence. Nucleotide: nucleotide substitution from -> to base. For G->A substitutions, the trinucleotide is given, with the central G being substituted. Position: position of the substitution in the reference sequence (see Fig. 1). Freq (%): frequency (%) of sequences showing the point variation at the position given. Substitution: amino acid substitution due to the mutation, -: synonymous substitution

Sample	454 reads	CID (%)	Nucleotide	Position	Freq (%)	Substitution
Roman RNA	5969	92.3	GGG	10	0.6	W-Stop
			GGC	50	0.2	A->T
			TGG	74	1.0	G->R
			CGA	157	1.7	–
			GGG	161	1.4	G->R
			TGG	173	1.2	G->R
			AGG	176	0.9	G->R
			CGG	221	0.2	G->R
			TGG	234	0.5	W->Stop
Roman DNA	759	87.3	GGG	10	0.7	W->Stop
			T->C	16	0.3	–
			A->G	35	0.3	N->D
			GGC	50	0.4	A->T
			C->T	53	0.3	R->C
			C->A	60	0.3	S->Y
			TGG	74	1.4	G->R
			TGT	78	0.3	C->Y
			C->A	118	0.3	–
			C->T	130	0.7	–
			CGA	157	1.9	–
			GGG	161	2.1	G->R
			TGG	173	1.4	G->R
			AGG	176	0.7	G->R
			GGC	179	0.9	A->T
			CGC	200	0.4	A->T
			CGG	221	0.3	G->R
			TGG	234	0.3	W->Stop
Uda RNA	4222	97.5	GGG	10	0.2	W->Stop
			A->G	52	0.2	–
			CGA	157	0.3	–
			GGG	161	0.6	G->R
			TGG	173	0.6	G->R
			AGG	176	0.6	G->R
Uda DNA	684	95.9	GGG	10	0.3	W->Stop
			A->G	52	0.4	–
			TGG	74	0.3	G->R
			CGC	148	0.4	–
			GGG	161	1.0	G->R
			AGG	176	1.0	G->R
			GGC	179	0.6	A->T
Coca RNA	6165	96.1	GGG	10	0.5	W->Stop
			G->A	74	0.2	G->R
			C->T	124	0.2	–
			CGA	157	0.5	–
			GGG	161	1.1	G->R

Table 2 continued

Sample	454 reads	CID (%)	Nucleotide	Position	Freq (%)	Substitution
			TGG	173	1.0	G->R
			AGG	176	0.5	G->R
Coca DNA	6382	92.1	C->A	7	0.3	–
			GGG	10	0.2	W->Stop
			A->G	43	0.3	–
			TGG	74	1.0	G->R
			TGT	78	0.4	C->Y
			CGC	148	0.4	–
			CGA	157	0.4	–
			AGG	160	0.6	–
			GGG	161	1.5	G->R
			TGG	173	0.5	G->R
			AGG	176	1.2	G->R
			TGG	188	0.2	G->R
			CGG	221	0.4	G->R
			TGG	234	0.4	W->Stop

pathogens with the exception of FeLV and CMhm, as described above. Inadvertently, the sample from the lynx used for group IbL-CMhm was serologically positive for antibodies to the feline calicivirus (immunofluorescence titer of 1:320). No antibodies against the other tested unwanted pathogens were detected. Both lynxes were of blood type A, as were all of the SPF cats in the present study.

Outcome of the FeLV transmission study

All cats of groups IbL-CMhm, G1-CMhm and G1 became infected with FeLV. Cats of the control group stayed FeLV-negative. According to the results obtained from p27 ELISA, the four cats of groups G1-CMhm and G1 and two cats of group IbL-CMhm (LM2, LM1) developed a progressive FeLV infection. The other two cats of group IbL-CMhm (LM3-LM4) were regressively infected (Fig. 2A). All cats of groups IbL-CMhm, G1-CMhm and G1 became FeLV provirus and viral RNA positive (Fig. 2B and C). There was no statistical difference in the outcomes (regressive vs progressive) among the three different groups, and this was independent of the FeLV strain used (lynx vs FeLV-A/Glasgow-1) and the absence or presence of CMhm co-inoculation. Differences were found between viral RNA loads of group G1 vs IbL-CMhm (p=0.0286) and G1-CMhm vs IbL-Cmhm (p=0.0286), and proviral loads of group G1 vs IbL-CMhm (p=0.0286). There was no statistical difference in the p27 values among the three groups. FeLV subtyping revealed that FeLV-B was present in one cat (R1) of group G1 at euthanasia 5 months p.i. The other cats of groups IbL-CMhm, G1-CMhm and G1 were positive for the FeLV-A subtype only. All cats were negative for FeLV-C.

Seroconversion to FeLV was found in all exposed cats, but not in cats of the control group. The FeLV-infected cats turned positive starting at weeks 1 to 2 p.i. Three cats of group IbL-CMhm (LM-2-LM4) showed a high humoral immune response against FeLV until the end of the experiment, while only two out of the remaining seven progressively infected cats (G3-G4, group G1) were still positive for FeLV whole-virus antibodies at the end of the experiment (Fig. 2D). Antibody levels to FeLV varied significantly over time until week 13 p.i. between the groups G1 and IbL-CMhm (p=0.0286), with group IbL-CMhm having the highest levels. This was also reflected by western blot results (Fig. 3): serum samples from 4 out of 12 FeLV-infected cats were western blot positive at week 20 p.i. (Fig. 3), and this included three cats of the group IbL-CMhm (LM2, LM3, LM4) and one cat of the group G1-CMhm (GM1). The two regressively infected cats, LM3 and LM4, were positive for p58, p27 and p15E, cat P2 was weakly positive for p58 and strongly positive for p15E, and cat GM1 was weakly positive for p15E.

Outcome of CMhm infection

All cats were hemoplasma PCR negative prior to inoculation. Seven of eight CMhm-inoculated cats became CMhm PCR-positive in the blood: three cats of group IbL-CMhm and all four cats of group G1-CMhm. One cat (LM1) of group IbL-CMhm was never positive during the whole experiment. While all four cats of group G1-CMhm and one cat of group IbL-CMhm became positive by week 1 p.i., the remaining two cats of the group IbL-CMhm turned positive at week 3 and 6 p.i., respectively (Fig. 4). No significant difference in

Table 3 Type and number of variations and nonsynonymous substitution frequency. Rows: base in the reference. Columns: substitution in the variant. (): % of derived nonsysnonymous substitutions

	A	C	G	T
Roman RNA				
A				
C				
G	460 (88)			
T				
Roman DNA				
A			2 (100)	
C	4 (50)			7 (29)
G	82 (83)			
T		2 (0)		
Uda RNA				
A			8 (0)	
C				
G	97 (83)			
T				
Uda DNA				
A			3 (0)	
C				
G	25 (88)			
T				
Coca RNA				
A				
C				12 (0)
G	234 (87)			
T				
Coca DNA				
A			19 (0)	
C	19 (0)			
G	460 (81)			
T				

copy numbers was found between groups after week 6 p.i. Nevertheless, the overall course of CMhm infection was significantly different between the two groups (time-to-peak $p = 0.0498$), where group IbL-CMhm showed the slowest progression. Clinical signs of CMhm infection were absent in the recipient cats. Anemia was observed in one cat (LM1, group IbL-CMhm) in which the PCV dropped from 25% to 18% before euthanasia at week 13 p.i., well below the reference range for the age of the cat [1]. However, this cat was never positive for CMhm in blood.

Hematology results and clinical impact of the transmission

PCV values showed age-related fluctuation during the observation time but remained within the reference range. There was no significant difference in PCV and white blood cell counts between groups. Two cats had to be euthanized during the experiment. Cat GM1 (group G1-CMhm) developed a fatal panleukopenia at week 10 p.i., and cat LM1 (group IbL-CMhm) developed skin lesions on the back, head and neck at weeks 8 to 10 p.i. and, later on, an edema on the abdomen. At week 13 p.i., cat LM1 had to be euthanized for humane reasons. The PCV was 18%, with a low total protein of 32.8 g/l (reference: 64-80), albumin of 11.6 g/l (reference 30-40), increased lipase 558 U/L (reference 8-26) and amylase of 2,241 U/L (reference: 700-1,538), and 2,300 leukocytes/µl (reference range 4,600-12,800). Upon necropsy, in addition to the distinct edema, middle-grade acute pancreatitis and omentitis were found.

Post-infection pathogen screening

At week 16 p.i., a second pathogen screening was performed to test for cryptic infections transmitted via lynx blood. Indeed, cat LM2 (group IbL-CMhm) was found to be seropositive for FCV (titer 1:160) and FHV_1 (titer of 1:320). Conjunctiva and saliva swabs extracted and tested by RT-/PCR were negative at this time point. Retrospective testing of all earlier time points revealed that this cat was weakly positive for FHV_1 by real-time PCR analysis of blood at week 1 p.i. (crossing threshold of 36.9), when it was weakly seropositive (titer of 1:20) as well. This cat was seropositive for FHV_1 at weeks 10 and 13 p.i. (titer of 1:40 and 1:160), and was first positive for FCV at week 13 p.i. (titer of 1:80).

Discussion

The aim of this study was to further characterize the FeLV strain prevailing during a severe disease outbreak in Iberian lynxes in 2007 and to assess its pathogenic potential in domestic cats. For animal-welfare reasons, we had to minimize the number of animals used. Little is known about how hemotropic mycoplasma and FeLV interact, and dissecting the interactions between FeLV, hemotropic mycoplasma (which are clearly associated only in FeLV infection of the Iberian lynx [56]) and Iberian lynx blood in the domestic cat model would have required too large of a number of animals. Thus, far from being comprehensive, this analysis focused on three factors that, in our view, are important for the analysis of the pathogenic potential of the Iberian lynx FeLV. The first is the sequence analysis of FeLV envelope VRA, which was conducted using Roche 454 technology on plasma viral RNA and blood TNA samples. In addition, an *in vivo* transmission study was performed in which blood from an infected Iberian lynx

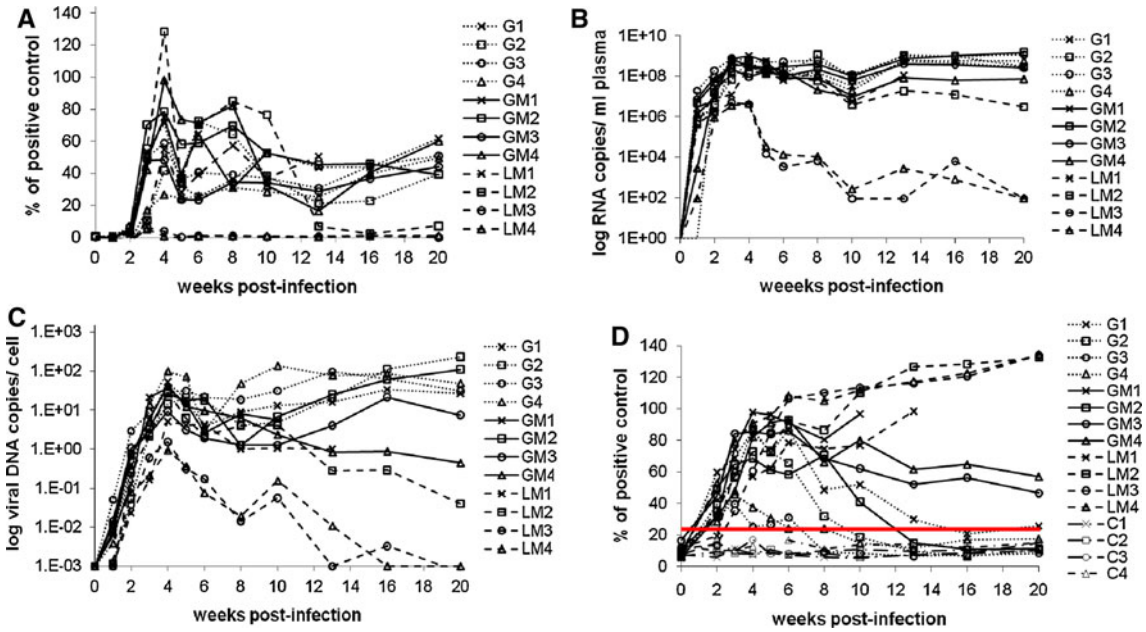

Fig. 2 FeLV antigen p27 (**A**), proviral (**B**), plasma viral RNA (**C**) and FeLV whole-virus (FL-74) antibodies (**D**) during the course of the experimental transmission study. The threshold for p27-positive results (**A**) is 4% of the positive control. Group IbL-CMhm: cats LM1-LM4. Group G1-CMhm: cats GM1-GM4. Group G1: cats G1-G4. Control group: cats C1-C4. Cats GM1 and LM1 were euthanized at weeks 10 and 13 p.i., respectively. The red line in **D** represents the threshold for positive results (25% of the positive control)

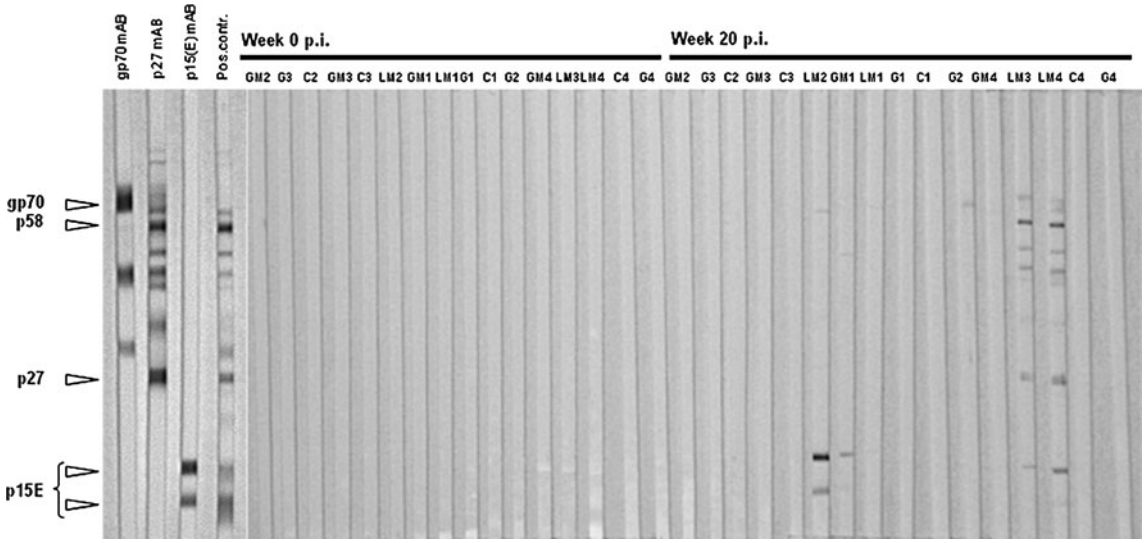

Fig. 3 FeLV western blot analysis of plasma samples collected at week 0 (prior to challenge) and week 20 p.i. from the 16 experimentally infected SPF cats (with the exception of cats GM1 and LM1, from which the last samples were collected at weeks 10 and 13 p.i., respectively. The positions of FeLV p15E (two bands), p27, p58 and gp70 are highlighted (open arrows). Positive controls: serum obtained and pooled from immune cats and a mixture of monoclonal antibodies against FeLV gp70, p27, and p15E (Gp70, p27, p15E mAB)

that was infected in 2007 and held in captivity ever since was transmitted to a group of SPF cats to assess extreme effects of the infection. Thirdly, the interaction between mycoplasma/Iberian lynx blood and FeLV was addressed in the well-tested FeLV-A/Glasgow-1 model using standard infectious viral doses.

Because the VRA of the FeLV envelope is one of the regions of the viral genome that contributes to the mutations characteristic of highly pathogenic FeLV strains (Fig. 1), the viral RNA and DNA sequence variation of this region was compared in lynxes that died in 2007, at the beginning of the FeLV epidemic, and in the

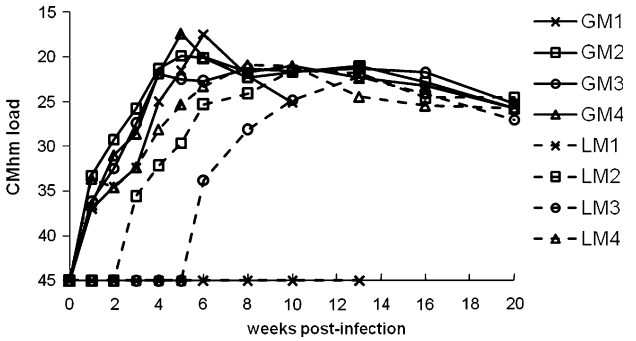

Fig. 4 Time course of CMhm loads in experimentally infected SPF cats. Group G1-CMhm (cats GM1-GM4) and group IbL-CMhm (cats LM1-LM4) loads are expressed as CMhm copies per ml blood. Cats GM1 and LM1 were euthanized at weeks 10 and 13 p.i., respectively

blood to be used for the transmission experiment. The remarkably high degree of identity found in both the consensus sequence and in the variants of one of the most changeable regions suggests that the overall characteristics of the virus may have remained the same over time, and the virus from 2009 was considered to be essentially the same as the one that caused the FeLV outbreak in 2007. This is the virus source that mostly resembles the original infecting strain, and its effect is more reliable than molecularly cloned and amplified viruses. Deletions or variations characteristic of highly pathogenic FeLV strains were absent from the mutant spectrum of the analyzed viral DNA and RNA sequence, suggesting that the FeLV-A strain infecting the Iberian lynxes is not, *per se*, extremely pathogenic, and other factors may have contributed to enhancing its effect on Iberian lynxes. However, since the determinants of pathogenicity of viruses such as FeLV-C or FeLV-A/61C are not well understood, variations in other regions may have contributed to the virulence of the virus. For RNA sequencing, cDNA was prepared from TNA extracted from plasma without DNase digestion. Therefore, a potential viral DNA contamination could have occurred and influenced our results. However, DNA contamination accounts for a maximum of 0.5% of the total as assessed by one-step real-time RT-PCR performed with or without reverse transcriptase (our own observations). As the minimum consensus identity is 83% (Table 2), this would lead to a mutant DNA contamination of less than 0.1%, which is not detectable under the analytical conditions used. In addition, some mutations found in the RNA were not detectable in the DNA sequences, and some mutations in DNA sequences were not present in the RNA, although their frequency would have resulted in detection if the sample had been highly contaminated. Together, the results do not suggest that DNA contamination influenced the RNA sequencing results.

Of note, only the G->A sites at position 78 (TGT->TAT) and 200 (CGC->CAC) are not embedded in a sequence context that may be compatible (although not proof of) with the antiviral action of APOBEC cytidine deaminases [12, 18, 44], and the six sites that are strongly conserved among the analyzed samples (positions 10, 74, 157, 161, 173 and 176) are within a strong feline APOBEC target sequence (AGG/GGG). Indeed, retrovirus restriction by feline APOBECs has been characterized for vif-deleted HIV and FIV [59, 78] and has been reported to be relatively nonspecific (first position of the GG/GC or GA dinucleotide [59]) or to show a preference for a purine before and/or after the substituted guanine [78]. However, most of the substitutions occurred alone within a single sequence, indicating a weak restriction activity. APOBEC restriction of FeLV replication was demonstrated *in vitro* for feline APOBEC3CH [59], and extensive APOBEC editing, although not associated with a clear effect on viral replication, has been shown *in vitro* and *in vivo* for Friend murine leukemia virus, a gammaretrovirus related to FeLV [67]. We therefore may expect more extensive editing of FeLV in felids than was observed in Iberian lynxes. The number of mutations caused by the action of APOBEC proteins on FeLV infection in felids other than Iberian lynxes is unknown, and therefore, no conclusion can be drawn about the role of APOBEC editing (e.g., driving virus evolution as proposed for HIV [40]) in FeLV infection in Iberian lynxes. Similar mutations have been detected in some thymic tumor clones from a cat experimentally infected with FeLV-A (GenBank accession FLU03204, FLU03207, FLU03219, FLU03208) and could potentially have been partly responsible for tumor development [72]. However, a direct involvement of such mutations (and therefore, indirectly, of APOBEC proteins) in tumorigenesis has not been demonstrated so far and does not seem to play a major role in the 2007 outbreak, since tumors were apparently not the cause of death of the Iberian lynxes [56]. The structure of the long terminal repeats (LTRs), which is known to influence the kinetics of FeLV replication, could have contributed to the pathogenicity of FeLV as well [4, 5, 11, 69]. However, LTR single genome amplification from blood and the LTR consensus sequence from kidney, liver and intestinal DNA from the lynx "Roman" did not reveal any tandem sequence duplication that could have caused an increased pathogenicity of the Iberian lynx FeLV, and the LTR sequences were 99% identical to that of the reference strain FeLV-A/61E (data not shown). Because all FeLV-infected Iberian lynxes were co-infected with feline hemotropic mycoplasmas [56], no transmission using blood containing only the lynx FeLV could be attempted. To control for any effect that feline hemoplasma (CMhm) from the lynx could have had on the experimental transmission (group IbL-CMhm), a second

experimental group was introduced that received a prototype FeLV-A (FeLV-A/Glasgow-1) and the lynx CMhm (group G1-CMhm). For this purpose, FeLV-free CMhm-infected Iberian lynx blood was obtained as matched control CMhm. To further address possible influences of CMhm coinfection on the pathogenesis of FeLV infection, another control group of SPF cats was included and infected with the FeLV-A/Glasgow-1 strain alone (group G1). The high levels of viremia reached in the infected cats (2-3 orders of magnitude higher than HIV, for example [6, 45]) were typical for a FeLV-A infection [10, 30, 79] in all three groups. Due to the small group size, the outcome of FeLV infection in these three groups was not significantly different, although the IbL-CMhm group showed lower levels of FeLV viral RNA and DNA loads during the course of infection, and two of the cats from the same group had a regressive infection. Even if no direct comparison of experimental infection using FeLV-A/Glasgow1 is available, cats infected intravenously with 200 FFU of FeLV-A Rickard strain [68] at 8-10 weeks of age all developed a progressive infection, and cats injected intraperitoneally with 50,000 FFU FeLV-A/Glasgow-1 showed the same course of infection as cats injected with a 10x and 100x higher load [25]. Hence, the IbL-CMhm group showed a similar, if not better, response to the infection as the one that may be expected using the same amount of FeLV-A/Glasgow-1 strain.

One cat of Group G1-CMhm (GM1) was diagnosed with fatal panleukopenia at week 10 p.i., which can develop during FeLV infection as a result of FeLV replication in the bone marrow [33, 74]. We therefore assume that a potential contribution to increased pathogenicity of FeLV from intrinsic factors present in the Iberian lynx blood is not likely.

In addition, whole-virus antibody levels varied significantly between group G1 and IbL-CMhm, and western blot analysis indicated that three cats of group IbL-CMhm may have become regressively infected. This is in agreement with the fact that a much lower titer of the lynx FeLV was used for infection compared to the $\sim$250x higher FeLV-A prototype FeLV-A/Glasgow-1 dose used to infect cats of groups G1-CMhm and G1. The low dose may have contributed to the establishment of a lower number of progressive infections.

Although the cause of disease in cat LM1 is still unclear, biopsy of a skin lesion (neck) was positive for FeLV nucleic acid and FeLV antigens, as demonstrated by RT-PCR and immunohistology, respectively (data not shown). This may explain the sudden lesions, as ulcerative dermatitis is described for FeLV-infection [19]. Intestinal protein loss may have been responsible for the very low protein concentration and development of edema, a pattern associated with FeLV-related diseases such as intestinal lymphoma. Interestingly, although intestines, liver and kidneys were examined macroscopically and microscopically for the presence of lymphoma, no evidence of neoplasia was found. Thus, the etiology of the extremely low plasma protein concentration was not elucidated.

Cat P2 showed signs of contact with FHV_1 and FCV, implying that these two pathogens had been present in the lynx blood in quantities below the detection limit of the PCR assays. Nevertheless, these subclinical infections introduced through lynx blood inoculation apparently did not influence the outcome of FeLV infection, since this cat may have progressed towards a regressive infection according to our serological results.

We report here, to our knowledge, the first successful feline hemoplasma transmission after 24 h storage of the blood at room temperature followed by cryopreservation. The course of CMhm infection in cats of group IbL-CMhm was significantly different from that seen in group G1-CMhm, despite of the similar CMhm loads in the blood utilized for infection. A possible explanation may be a difference in CMhm viability. While real-time PCR assays provides reliable quantification of CMhm in blood samples [87], the true correlation between copy number and the number of living organisms would require quantitative culture, which is not yet possible [80]. A higher infectivity of the inocula and/or the use of intraperitoneal (obligatory because of the experimental infection with FeLV) rather than intravenous administration may explain the differences compared to previously described courses of CMhm infection, where the loads peaked in weeks 2 to 4 [81, 82]. Alternatively, the different infection course of CMhm may have been influenced by the outcome of FeLV infection itself, but this was not testable in the experimental setting that was used. Although a relationship between retroviruses (e.g., FeLV or FIV) and coinfection with hemotropic mycoplasmas has been postulated by different authors [23, 27, 42, 81], recent field studies have found an association for FIV, but not for FeLV infection [41, 53, 75, 83]. The results presented here support these findings and show that a retrovirus-positive status with CMhm coinfection does not necessarily result in a particular progression or outcome of disease, at least on a short term. However, it should be noted that these observations relate to the domestic cat model. Hemotropic mycoplasma and FeLV may interact specifically in the Iberian lynx to cause high pathogenicity, but this can only be tested in an *in vitro* system, which is unavailable so far. Thus, the low pathogenicity of the transmitted lynx FeLV strain in SPF cats underscores the importance of alterations in host-related factors that, together with environmental changes, may interact to affect the susceptibility of Iberian lynxes to disease [13, 64]. For example, the lack of endogenous FeLV-related sequences

[56] might also contribute to a higher pathogenicity of FeLV infection in the Iberian lynxes, since a protective role of endogenously expressed retroviruses has been postulated, e.g., for gammaretroviruses in mice [3, 15, 47, 58]. The lack of exposure to pathogens (e.g., FeLV, FIV, canine distemper virus) might have rendered the Iberian lynx vulnerable to infections with these pathogens and to outbreaks of the associated diseases [71]. Nevertheless, the most important factor may be the loss of genetic diversity as a consequence of inbreeding (especially in the Doñana region), which resulted in the apparent lack of acquired immunity and immunocompetence of the Iberian lynxes against infectious agents [37, 57, 64]. A primary immune deficiency of the lynxes may have made this species more vulnerable to infectious diseases — and to FeLV in particular — a fact that should be thoroughly investigated and that may pose a real threat to the future survival of the species.

In conclusion, the results obtained show that the FeLV strain responsible for the outbreak in Iberian lynxes in 2007 does not harbor potentially highly pathogenic variants — at least in the genomic regions we have investigated so far. In addition, it has remained stable in its genetic composition, may undergo partial APOBEC-mediated restriction and it is apparently not extremely pathogenic when inoculated into SPF domestic cats. Hence, we assume that there is no increased risk of transmission of the lynx FeLV from lynxes to cats and suggest that the severe outcome of the FeLV outbreak in 2007 was due rather to the particular susceptibility of the Iberian lynx to pathogens.

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