Naturally occurring feline leukemia virus subgroup A and B infections in urban domestic cats

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A nested-PCR (n-PCR) was used to detect feline leukemia virus (FeLV) proviral DNA in blood samples from 464 sick and 608 healthy domestic cats (Felis catus) selected by convenience, and a significantly high prevalence of FeLV infection was observed. n-PCR results revealed the presence of FeLV proviral DNA in 47.2 % of sick cats and 47.4 % of healthy cats. Phylogenetic analysis revealed that FeLV samples from healthy or sick cats were grouped into separate clades. We determined FeLV subgroups by an n-PCR based on the envelope (env) gene. The partial env gene of FeLV Minas Gerais (MG) samples were compared to various exogenous FeLV isolates and endogenous (enFeLV) provirus from the same region. FeLV-B MG samples were more similar to endogenous sequences and to natural FeLV-B isolates than to either FeLV-A or FeLV-C. The results revealed the circulation of FeLV-B in large populations of urban domestic cats in Brazil.

Feline leukemia virus (FeLV) is an exogenous retrovirus, belonging to the genus Gammaretrovirus, which infects domestic and sporadically wild cats (Daniels et al., 1999; Arjona et al., 2007). By comparison of the envelope (env) sequence variation it has been possible to identify four FeLV subgroups: FeLV-A, -B, -C, and -T, each of which uses distinct cellular receptors to initiate infection of the host cell (Boomer et al., 1997; Tailor et al., 1999; Anderson et al., 2001; Mendoza et al., 2006; Cheng et al., 2007). FeLV has been associated with fatal neoplasia, degenerative diseases of the haematopoietic system and immunodeficiency (Chandhasin et al., 2004; Hofmann-Lehmann et al., 2006; Collado et al., 2007).

Traditionally, FeLV infections are diagnosed by virus isolation (VI), immunofluorescent antibody tests (IFA) or ELISA for the detection of soluble antigens (usually the p27 core protein) in whole blood, serum or plasma (Levy et al., 2008). However, FeLV infections in cats with a weakened immune response are not reliably detected by serological methods (Gomes-Keller et al., 2006). Recently, molecular methods such as PCR assays have been described for the use of detection of FeLV provirus DNA in peripheral blood mononuclear cells (PBMC), allowing virus detection independently of antibodies presence or viraemia, and enabling detection in latently infected cats (Hofmann-Lehmann et al., 2001; Torres et al., 2005; Tandon et al., 2008). Moreover, a highly sensitive nested-PCR (n-PCR) assay was developed and it is able to distinguish between endogenous and exogenous FeLV by using inner and outer pairs of primers based on the U3 long terminal repeat (LTR) region of FeLV provirus (Miyazawa & Jarrett, 1997).

Here, we investigated the occurrence of FeLV proviral DNA in healthy and symptomatic domestic cats with clinical suspicion of FeLV infection, from Belo Horizonte, Minas Gerais (MG), Brazil. We performed a cross-sectional study carried out over a 70 month period, from March 2002 to January 2008. Samples were obtained by convenience and included 608 healthy cats brought to a veterinary practice for vaccination or routine check up and 464 sick...
cats showing clinical signs commonly related to FeLV infection, including gingivitis, stomatitis, anaemia, apathy, weight loss, conjunctivitis, cachexia, cystitis or anorexia. Cats were clinically examined and owners completed a questionnaire indicating age, gender, breed, lifestyle, place of domicile and number of cats in the household. Veterinarians were requested to take EDTA-blood samples (1.0 ml) from the cats regardless of their clinical status. Written consent was obtained from each cat owner, and ethical guidelines were always followed.

Statistical analyses were performed using the $\chi^2$ or Fisher’s exact tests using the GraphPad InStat version 3.05 for Windows (GraphPad Software). Sample prevalence estimates were calculated and reported as the per cent of cats with a positive test result (number of cats classified as positive divided by the total number of cats tested). In addition, 95% confidence intervals (CIs) were determined using exact tables (Thrushfield, 1995). For all analyses, $P$-values <0.05 were considered significant. Table 1 shows the epidemiological data of the study population.

Upon collection, blood samples were identified, forwarded to the laboratory and processed on receipt. PBMCs were lysed by brief exposure of cell pellets to buffered ammonium chloride (0.155 M) as described. Cells were repeatedly washed in PBS and the final pellet was suspended in 0.1 M Tris-EDTA (Toth et al., 1992). Nucleic acids were extracted from PBMCs using sodium iodide as lysis buffer and silica particles according to published protocols (Boom et al., 1990). DNA concentration was determined by a GeneQuant spectrophotometer (Pharmacia).

In order to detect FeLV-integrated provirus DNA in PBMCs from domestic cats an n-PCR assay was performed by using primers and amplification conditions as described previously, to amplify the U3 LTR region and gag gene (Miyazawa & Jarrett, 1997). For all n-PCR assays, plasmids containing the full-length p61E FeLV-A, pFRB FeLV-B and pFSC FeLV-C proviruses (kindly donated by Dr Sajal K. Ghosh, Cancer Research Center, Boston University School of Medicine, Boston, USA) were used as positive controls. The negative control consisted of a blank with all the reagents but no DNA. Furthermore, to confirm the presence of amplifiable DNA a specific PCR to amplify the feline glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene of provirus was obtained in 507 cats (47.5–95% CI=0.474–0.476). Each genomic DNA sample was subjected to another n-PCR to determine FeLV subgroups, as discussed below.

Of the 608 clinically healthy cats 47.4% were n-PCR positive, which was not significant when compared to the prevalence of infection in sick cats (47.2%). We did not find a significant relationship between health status and the presence of FeLV provirus in this population. The point prevalence rate was around 38.6% in cats from 1 year of age onward and remained at a high level of 56.1 and 52.7% through 9 years of age, after which it dropped to around 15.9%. The prevalence in purebred cats (43.2%) was not statistically divergent from that found in non-pedigree cats (48.1%). The number of cats with FeLV provirus was influenced by environmental status, as the prevalence of infection was significantly higher in indoor cats with outdoor access ($P<0.05$) when compared with sheltered cats. Moreover, the number of FeLV-positive cats increased as a function of the number of animals kept in the same household ($P<0.05$). Our data support the fact that FeLV is highly infectious and housemates of FeLV-positive cats are at higher risk of infection. These results are in agreement with previous findings that in households where the virus is enzootic, the prevalence is commonly 30–40% (Jarrett, 1999).

To determine the subgroup of the FeLV MG samples, the provirus env gene from the 34 randomly selected FeLV-infected cats were amplified by n-PCR using a pair of outer primers that recognize sequences in the pol gene upstream of the env gene start codon and sequences in the U3 region of the 3’LTR that are conserved among exogenous FeVs. One microlitre of product from the first round of amplification was used in the second round of PCR with subgroup specific primers as follows: RB59 and RB17 (specific for FeLV-A) that generate a 1072 bp amplicon (Chen et al., 1998), RB53 and RB17 (specific for FeLV-B) generating a 866 bp product (Pan et al., 2005) and RB58 and RB47 (specific for FeLV-C) that yield a 1755 bp amplicon (Mathes et al., 1994). We did not test samples using FeLV-T-specific primers. n-PCR products indicated that 15 MG samples were positive for FeLV-A, 19 MG samples were simultaneously positive for FeLV-A and FeLV-B and none were positive for FeLV-C. The sensitivity of detection of FeLV subgroups by n-PCR was determined using different amounts of cloned FeLV-A, FeLV-B and FeLV-C proviral DNA. The first round of amplification was performed with 1 µl (100 ng µl$^{-1}$) DNA template. The products of PCR were quantified by spectrophotometry and then 10-fold serial dilutions 100 ng µl$^{-1}$–1.2 fg µl$^{-1}$ were performed. The sensitivity of the n-PCR was determined by testing 1 µl of each dilution. The limits of detection by n-PCR for FeLV subgroups A, B and C were 12.8 fg (data not
shown). The specificity of this n-PCR assay was confirmed by nucleotide sequencing of the \textit{env} gene from three FeLV-A MG samples and five FeLV-B MG samples (out of the 34 pre-selected ones, as discussed above).

The U3 LTR-\textit{gag} and \textit{env} genes of the FeLV MG samples were cloned and sequenced in both directions using standard methods. Briefly, gel-purified amplicons were cloned into the pGEM-T vector (Promega), according to the manufacturer’s instructions. Samples were sequenced with M13 forward and reverse primers by using ET Dynamic Terminator kits (GE Healthcare). Sequencing was carried out in a MegaBACE 1000/Automated 96 Capillary DNA sequencer (Amersham Pharmacia Biotech). Nucleotide sequences were assembled using the CAP3 sequence assembling program (Huang & Madan, 1999) and aligned for subsequent phylogenetic analysis by CLUSTAL W (version 1.6 EMBL) (Thompson \textit{et al.}, 1994). The phylogenetic reconstruction was implemented in the MEGA software package, version 3.1 (Kumar \textit{et al.}, 2004) using three different methods [neighbour joining (NJ), maximum parsimony (MP) and minimum evolution (ME)]. The derived phylogenetic trees were topologically equivalent, irrespective of the method employed. The dataset was resampled 1000 times to generate bootstrap values.

The nucleotide sequences corresponding to the U3 LTR region and \textit{gag} gene of FeLV provirus reported in this paper have been deposited in GenBank. These sequences were compared to other sequences of \textit{gag} and \textit{env} genes from GenBank and used to perform phylogenetic analysis (Fig. 1). \textit{Gag} and \textit{Env} sequences obtained from the GenBank (to which our sequences were compared) include: FeLV subgroup A, FeLV-FAIDS (M18247), FRA (AF052723); FeLV subgroup B, FeLV-B (K01803), GM1 (D13922); and enFeLV provirus, A1_enFeLV (AY364318), A2_enFeLV (AY364319).

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|}
\hline
\textbf{Variable} & \textbf{n-PCRs*} & \textbf{P value†} \\
\hline
\textbf{Gender} & & \\
Male & 48.5 % (251/517) & 51.5 % (266/517) & 0.445 \\
Female & 46.1 % (256/555) & 53.9 % (299/555) & \\
\hline
\textbf{Age} & & \\
<1 year & 38.6 % (88/228) & 61.4 % (140/228) & 0.001 ‡ \\
1–3 years & 56.1 % (221/394) & 43.9 % (173/394) & \\
4–9 years & 52.7 % (181/343) & 47.3 % (162/343) & \\
>10 years & 15.9 % (17/107) & 84.1 % (90/107) & \\
\hline
\textbf{Health status} & & \\
Healthy & 47.4 % (288/608) & 52.6 % (320/608) & 1.000 \\
Sick & 47.2 % (219/464) & 52.8 % (245/464) & \\
\hline
\textbf{Breed} & & \\
Pedigree & 43.2 % (73/169) & 56.8 % (96/169) & 0.0857 \\
Non-pedigree & 48.1 % (434/903) & 51.9 % (469/903) & \\
\hline
\textbf{Lifestyle} & & \\
Shelter & 39.4 % (210/533) & 60.6 % (323/533) & 0.001 § \\
Indoor (outdoor access) & 64.3 % (36/56) & 35.7 % (20/56) & \\
Indoor & 61.4 % (62/101) & 39.6 % (39/101) & \\
Catteries & 47.6 % (30/63) & 52.4 % (33/63) & \\
Outdoor & 53.0 % (169/319) & 47.0 % (150/319) & \\
\hline
\textbf{No. cats in household} & & \\
1 cat & 36.8 % (84/228) & 63.2 % (144/228) & 0.004 ‡ \\
2–29 cats & 48.5 % (167/344) & 51.5 % (177/344) & \\
30–60 cats & 51.9 % (28/54) & 48.1 % (26/54) & \\
>60 cats & 51.1 % (228/446) & 48.9 % (218/446) & \\
\hline
\end{tabular}
\caption{Characterization of the sampled cat population}
\end{table}

*No. of positive or negative/no. of tested cats.
†§Statistically significant (P<0.05).
‡Yates corrected or Fisher’s exact result.

FeLV-A and -B in urban domestic cats
Murine retrovirus sequences (RMU94692 and Z11128) were used as outgroups to generate rooted phylogenetic trees. The analyses indicated that our sequences were highly similar (97–100 %) to sequences of FeLV-FAIDS, FRA and FeLV-B and have low percentage identity (89–90 %) with sequences of A1_enFeLV and A2_enFeLV. The results revealed that FeLV MG samples formed distinct subclusters. Interestingly, samples obtained from healthy cats were separated from those obtained from sick cats presenting FeLV-related clinical signs (Fig. 1a).

Nucleotide sequences from FeLV MG samples 843-MG, 887-MG, 914-MG, 918-MG, 922-MG and 1230-MG, which were grouped as a distinct cluster in the gag/U3 LTR phylogenetic tree (Fig. 1a), revealed a closer relationship with isolates ST/FeLV-B, GA/FeLV-B, Ric/FeLV-B and E5.14, and were classified as FeLV-B by env gene nucleotide sequence analyses. While the FeLV MG samples 328-MG, 1235-MG and 1286-MG revealed a closer relationship with the isolates F3A/3281, FRA, Glasgow-1 and FeLV-FAIDS and were included in the subgroup A (Fig. 1b). Importantly, all 19 MG samples classified as FeLV-B by n-PCR were obtained from animals presenting clinical signs of infection. However, the 15 MG samples classified as FeLV-A were obtained from cats with no clinical signs. All these MG samples showed a closer relationship when compared to each other using sequences from the gag gene (Fig. 1a).

We compared the amino acid sequences inferred from the partial env gene from FeLV-A and FeLV-B MG samples to exogenous sequences representing the four FeLV subgroups (A, B, C and T), and with the endogenous FeLV provirus clone (CFE-6) present in the uninfected cat genome. The comparison revealed that the deduced amino acid sequences of FeLV-B MG samples were more similar to the group represented by exogenous FeLV-B virus and to the endogenous provirus than to either FeLV-A, FeLV-C or FeLV-T env genes. Furthermore, the FeLV-B samples contained a motif, a distinctive cluster of lysine residues

**Fig. 1.** Phylogenetic trees based on the partial U3 LTR-gag (a) and env (b) genes from FeLV MG samples. The trees were constructed by the MP method. Bootstrap values are given at the nodes of the tree and only values ≥70 are shown.
that has been previously described for natural FeLV isolates from subgroups B and C, and may have been derived from recombination episodes with endogenous FeLV-related elements (Mendoza et al., 2006). The amino acid sequence alignment also revealed that all FeLV-A MG samples share high sequence identity with the natural isolates of the horizontally transmissible subgroup A FeLV-FAIDS, FRA and Glasgow (Fig. 2).

In this study, the overall rate of FeLV proviral DNA found by n-PCR assays was 47.5%. This rate is one of the highest observed in similar investigations reported so far and supports the notion that FeLV is the most common infection of cats in the MG state, and perhaps in Brazil. Our results are in accordance with a recent survey performed in Spain, which found FeLV prevalence of 35.7% by using PCR (Arjona et al., 2007). Brazilian serosurveys conducted in São Paulo and Rio de Janeiro reported lower rates of infection, 12.5 and 20.3%, respectively (Hagiwara et al., 1997; Souza et al., 2002). The differences in prevalence can be explained by the fact that in our study an n-PCR assay was used to detect FeLV DNA provirus in PBMCs from domestic cats. The n-PCR can detect latently infected cats and, thus, FeLV prevalence is higher than the infection rate predicted by tests that detect soluble antigens. Nonetheless, some inconsistencies in the n-PCR method used have been pointed out in the past and serologically positive cats were eventually negative upon PCR analysis (Miyazawa & Jarrett, 1997). One possible solution to that would be the use of more sensitive molecular approaches like real-time PCR assays (Tandon et al., 2008). A second possible drawback of the employed methods is the use of convenience samples in order to build our study population. We have enrolled animals that were brought to veterinary clinics, which could partially justify the high FeLV prevalence found. However, more than half of this large population is made up of healthy animals brought in for vaccination or routine check up. Yet, the prevalence in this group was found to be equally high when compared to the prevalence among sick animals, and this fact minimizes the convenience sampling problem.

All FeLV-B-positive cats were apparently co-infected with FeLV-A. The subgroup FeLV-C proviral DNA was not detected. These results are in agreement with previous epidemiological studies showing that subgroup B viruses can be isolated from 30 to 60% of infected cats, but only in conjunction with FeLV-A, suggesting that horizontal transmission of FeLV-B alone occurs rarely (Neil et al., 1991; Phipps et al., 2000). FeLV-A is present in all natural infection and gives rise to the other subgroups by the env gene.
mutation, insertion or recombination events (Hoover & Mullins, 1991; Roy-Burman, 1995; Rohn et al., 1998).

In conclusion, the identification of FeLV subgroups in infected animals and provirus detection is important for a number of reasons: (i) clinical signs are not often indicative of infection and FeLV-infected animals are frequently asymptomatic; (ii) FeLV-B naturally originates from FeLV-A infected animals through recombination with enFeLV (Overbaugh et al., 1988; Hoover & Mullins, 1991; Roy-Burman, 1995; Rohn et al., 1998); (iii) identification of asymptomatic infected cats is important for prophylactic protection of non-infected animals once these latently infected animals may, at some point, turn into viraemic and infectious cats; (iv) FeLV infection is an important model for understanding pathogenesis of retroviral diseases. Identification of latently infected cats is essential in this respect.

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