Detection and genetic characterization of feline retroviruses in domestic cats with clinical signs and hematological alterations associated with retroviral infection

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Abstract

Feline leukemia (FeLV) and immunodeficiency (FIV) viruses are globally distributed retroviruses that infect domestic cats and cause various syndromes, which can lead to death. The aim of our study was to detect and genotyping feline retroviruses in Mexican domestic cats. We used PCR assays to identify proviral DNA and viral RNA in 50 domestic cats with different clinical signs and hematological alterations. Endogenous FeLV (enFeLV) was identified in the genomic DNA samples from all cats in the study, and we detected enFeLV transcripts of LTR region in 48 individuals. Exogenous FeLV (exFeLV) was found in 13 cats. Furthermore, we detected FIV proviral DNA in ten cats. The enFeLV sequences were shown to be the most variable, while the exFeLV sequences were highly conserved and related to previously described subgroup A sequences. Additionally, FIV *gag* gene sequences revealed the presence of subtype B in the infected cats.

Full Text

The viruses that cause feline leukemia and immunodeficiency are globally distributed retroviruses that infect domestic cats and cause different syndromes that can lead to the death of infected animals [1]. Genetically, retroviruses are made up of three main genes: *gag*, which codes for structural proteins (matrix, capsid, and nucleocapsid), *pol*, which codes for enzymes that participate in viral replication (protease, integrase, and reverse transcriptase), and *env*, which codes for envelope proteins (transmembrane and surface). The retrovirus genome is flanked by non-translatable regulatory sequences called long terminal repeats (LTRs) [2]. Additionally, the feline immunodeficiency virus (FIV) genome contains three accessory genes (*vif*, *ORF A*, and *rev*) that are related to viral infectivity and replication [3].

Feline leukemia virus (FeLV) can be categorized as either endogenous or exogenous depending on how it is transmitted. Exogenous FeLV (exFeLV) is transmitted horizontally as an infectious agent. Endogenous FeLV (enFeLV) is part of the genome of species in the *Felis* genus, including domestic cats (*Felis silvestris catus*), and it is passed from parents to offspring [4].

Genomically intact, full-length enFeLV sequences have been detected in the genome of domestic cats [4]. While enFeLV have not been shown to directly induce disease presentation in their host, they are relevant to FeLV biology. Namely, enFeLV are expressed in various tissues and they are involved in the generation of new exogenous variants through recombination, which increases their pathogenicity [5–7].

Nucleotide sequences in enFeLV and exFeLV are about 86% similar [2]. However, differences found in the LTR genetic region allow for differential PCR amplification of either endogenous or exogenous FeLV sequences [8, 9].

Taxonomically, exFeLV is placed in the order *Ortervirales*, family *Retroviridae*, subfamily *Orthoretrovirinae*, genus *Gammaretrovirus* [10]. Based on its genetic and functional relationships, exFeLV is divided into

subgroups [11]. The main subgroups described to date are A, B, C, D and T, which differ in their degree of pathogenicity and therefore in the course of disease infection and presentation [12].

FeLV subgroup A (FeLV-A) is the most frequently described and least pathogenic type [11, 13]. FeLV-B arises through recombination of FeLV-A with enFeLV within the host, primarily in the *env* gene [14, 15]. FeLV-B is mainly associated with development of leukemia and lymphoma [16]. FeLV-C develops from *de novo* mutations in the FeLV-A *env* gene, and it has been associated with the development of aplastic anemia [17]. FeLV-D originated from recombination of FeLV *gag-pol* genetic regions with the domestic cat endogenous retrovirus (ERV-DC) *env* gene. This subgroup was detected in cats that developed hematopoietic tumors, including lymphoma and leukemia [18]. FeLV-T is a variant of FeLV-A that has insertions and deletions in the *env* gene. It presents T-lymphocyte tropism and induces an immunosuppressive disorder described as feline acquired immunodeficiency syndrome (FeLV-FAIDS) [19, 20].

FeLV infection is generally associated with hematological disorders, including cytopenias and neoplasms such as leukemia and lymphoma, which frequently lead to the death of infected cats [21]. Once a cat is infected with the virus, the infection can follow one of four different courses: abortive infection, progressive infection, regressive infection, and focal (atypical) infection [1].

FIV is taxonomically placed in the order *Ortervirales*, family *Retroviridae*, subfamily *Orthoretrovirinae*, genus *Lentivirus* [10], and infects animals in the Hyaenidae and Felidae families. FIV can cause an acquired immunodeficiency syndrome (AIDS) in domestic cats that begins with a latent phase. This is followed by decreased $CD4^+$ T-lymphocytes, which, in turn, lead to an increased risk of opportunistic infections, neurological diseases and tumors. However, FIV does not cause a severe clinical syndrome in most cats infected naturally. With proper care, infected cats can live for many years and die at an advanced age from causes unrelated to infection with this retrovirus [1].

FIV is classified into subtypes A – F, based on *env* genetic region divergence. Subtype A has been found in Australia, New Zealand, the United States, South Africa, and in northeastern Europe. Subtype B has been found in the United States, central and southern Europe, Brazil, and Japan. Subtype C has been identified in Canada, New Zealand, and Southeast Asia. Subtype D has been reported in Japan and Vietnam, subtype E in Argentina, and subtype F in the United States [22, 23].

It is important to consider feline retroviral genetic diversity, and to identify the viral types that are present and may be predominant in a particular region. These types of studies can generate information on the efficacy of diagnostic tools. In this study, we set out to detect feline retroviruses using PCR and RT-PCR from peripheral blood leukocytes and blood plasma. Our goal was to identify the retroviral genotypes present in Mexican domestic cats that had different clinical signs and hematological alterations.

The study included 50 cats of either sex that were at least six months old, which were patients either at private clinics or at the University Small Animal Hospital (Hospital de Pequeñas Especies of the FES-C, UNAM) in the State of Mexico. A convenience sampling was carried out in which animals that presented

clinical signs compatible with retroviral infection were included; mainly, oral disease, abscess, and hematological alterations; as well as cats that, according to their lifestyle, had risk factors associated with these infections, such as non-neutered animals, with access to the outside, in contact with infected animals, from animal shelters and/or rescued stray cats. Hemograms were performed on blood samples obtained by puncture of the radial or jugular vein in tubes with EDTA (BD Vacutainer® with EDTA K2, Mexico). Peripheral blood leukocytes (PBL) and blood plasma were obtained by centrifugation. These were then stored at -75°C until use.

Genomic DNA extraction from PBL was performed using the commercial FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen, Taiwan), and following the manufacturer's instructions. RNA was extracted from blood plasma with FavorPrep™ Viral Nucleic Acid Extraction Kit (Taiwan) and treated with enzyme ezDNAase™, Invitrogen™ (Thermo Scientific™, USA) to eliminate contaminating genomic DNA. Both DNA and RNA were quantified with a Nanodrop Lite™ spectrophotometer (Thermo Scientific™) and stored at -75°C until use.

We designed primers using the Primer3 Input program (v.0.4.0) with sequences available in GenBank that were also used for phylogenetic analysis. Primers were designed in the LTR region for endpoint PCR, in order to independently detect both exogenous and endogenous FeLV sequences. The enFeLV LTR FW and FeLV LTR RV primers amplified a 205 bp fragment of enFeLV. The exFeLV LTR FW and FeLV LTR RV primers amplified a 215 bp fragment of exFeLV (Table 1).

The reaction mix contained 1X buffer (Ampliqon, Odense M, Denmark), 1.5 mM $MgCl_2$ (Ampliqon), 200 µM dNTPs (Ampliqon), 500 nM of each primer, 5 U of Taq DNA polymerase (Ampliqon) and 0.5 µg of DNA per reaction, in a final volume of 25 µL. The amplification conditions included initial denaturation at 95°C for 5 min, followed by 35 cycles, each with three steps: denaturation at 95°C for 40 s, alignment at 62°C for exFeLV and 60°C for enFeLV, extension at 72°C for 13 s and a final extension at 72°C for 5 min.

FIV detection was performed using nested PCR which amplified a *gag* gene fragment. The FIV *gag* FW1 and FIV *gag* RV1 primers amplified a 919 bp product in the first round, and then the FIV *gag* FW2 and FIV *gag* RV2 primers amplified a 458 bp product in the second round (Table 1). The reaction mix contained 1X buffer (Ampliqon, Odense M, Denmark), 2.5 mM $MgCl_2$ (Ampliqon), 200 µM dNTPs (Ampliqon), 500 nM of each primer, 5 U of Taq DNA polymerase (Ampliqon) and 0.7 µg of DNA per reaction, in a final volume of 25 µL. Amplification occurred with initial denaturation at 95°C for 5 min, followed by 40 cycles with three steps. First, denaturation at 95°C for 40 s, alignment at 57°C for the first reaction and 52°C for the second reaction, extension at 72°C for 56 s for the first reaction and for 30 s for the second reaction and a final extension at 72°C for 5 min.

For the detection of FeLV and FIV viral RNA, one-step RT-PCR was performed with a commercial kit (OneStep RT-PCR Kit, QIAGEN, USA), following the manufacturer's instructions, and using the primers previously described.

Following separation using horizontal electrophoresis in 1.5% agarose gel with ethidium bromide (0.5 µg / mL), final products were visualized in a UVP M-20E transilluminator (BioSurplus, San Diego, CA, USA). Positive amplicon purification was carried out with the commercial FavorPrep™ GEL/PCR Purification Kit (Favorgen, Taiwan), following the manufacturer's instructions.

Sample sequencing was carried out in the Biotechnology and Prototypes Unit of the National Autonomous University of Mexico, FES – Iztacala campus, using the Sanger method in an ABI 3130 × 1 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

All PBL genomic DNA samples were positive for enFeLV, and 48 enFeLV-positive animals were detected via RT-PCR of RNA extracted from blood plasma. Eleven cats that were only positive for enFeLV DNA and RNA presented clinical signs and/or blood count alterations consistent with retroviral infection. These included gingivitis, gingivostomatitis, lymphoma, lymphocytosis, lymphopenia, anemia, neutropenia and thrombocytopenia (Fig. 1).

Of the 50 cats evaluated, 13 tested positive for exFeLV infection by PCR. Nine of the 50 PBL samples were positive for exFeLV proviral DNA, while 13 were positive for exFeLV viral RNA in plasma. Cats that tested positive ranged in ages from six months to seven years old (2.65 years on average), five were female and eight were male. Among the exFeLV-positive animals, two showed no clinical signs, but three had gingivitis or gingivostomatitis, two had diarrhea and weight loss, and one had lymphoma. Likewise, there were cases of animals with bite injuries, vomiting and jaundice, one cat with pleural effusion and another with pulicosis (Fig. 1). Five of the exFeLV-positive animals did not have hemogram alterations, however three cats had non-regenerative anemia, two had lymphopenia, one had neutropenia, two presented eosinophilia, two neutrophilia and one had thrombocytopenia (Fig. 1).

Nested PCR detected FIV proviral DNA in 10 of the 50 study animals. These cats ranged from in age from 6 months to 15 years (7.05 years on average), five were female and five were male. Five animals presented depression and anorexia, there were two cases each of weight loss and diarrhea, and one case each with vomiting, gingivitis, and mammary adenocarcinoma. The hemograms of these FIV-positive animals showed the presence of neutrophilia in six cases, two cases with lymphocytosis, anisocytosis, neutropenia, monocytosis and eosinophilia (with one case each), and one of the animals presented a hemogram without alterations (Fig. 1). No sample tested positive for RT-PCR from RNA obtained from blood plasma.

We got 19 enFeLV sequences, nine exFeLV sequences and ten FIV sequences from the amplicons obtained via PCR. We used the nucleotide sequences to construct two phylogenetic trees with Bayesian inference (MrBayes) using the Geneious 11.1.4 program. The first tree included endogenous and exogenous sequences from a fragment of the FeLV LTR (Fig. 2). Sequences obtained from a fragment of the FIV *gag* gene were included in the second tree (Fig. 3). In both trees, we considered sequences available in GenBank that included enFeLV genetic regions of interest AY364318.1, AY364319.1, AY364320.1, GU300829.1, GU300839.1, GU300855.1, GU300949.1, GU300970.1, GU300986.1, GU301018.1, GU301066.1, LC196053.1, LC196055.1, LC198317.1, MH325041.1, MH325043.1 and

MH325049.1. Similarly, with exFeLV regions: AB060732.3, AB672612.1, AB673426.1, AF052723.1, KP728112.1, M14331.1, M18247.1, M89997.1, MF681664.1, MF681665.1, MF681666.1, MF681667.1, MF681669.1, MF681670.1, MF681671.1 and X00188.1; and for FIV: AF474246.1, AY600517.1, AY679785.1, D37818.1, D37822.1, EU117992.1, GQ422125.1, KM880117.1, KM880121.1, M25381.1, M36968.1, M59418.1, MW142027.1, MW142038.1, MW142043.1, MW142046.1, MW142047.1, U11820.1 and Y13867.1. The sequences obtained in this study are available from GenBank under accession numbers MZ334429 - MZ334466.

Genetic distance analyses showed values ranging from 0.02–0.45 for the obtained enFeLV sequences, and values ranging from 0.02–0.46 with respect to previously described endogenous sequences. In the case of exFeLV sequences, the genetic distance was 0–0.03 between the sequences obtained in the study, and 0–0.13 with respect to previously described exogenous sequences.

Previous studies described the presence of these viruses in Mexico using FeLV antigen detection tests and FIV antibodies [24] and by detecting FeLV through PCR [25]. However, we detect and genetically characterize feline retroviruses (enFeLV, exFeLV and FIV) in domestic cats with different clinical signs and hematological alterations.

Our study used a pair of PCRs in the LTR region to differentiate endogenous and exogenous FeLV viruses, achieving the specific amplification of each virus. We corroborated this by analyzing the obtained nucleotide sequences, confirming that the LTR region is adequate for differential amplification of endogenous and exogenous FeLV sequences, which has been carried out mainly for real-time PCR assays [8, 9].

The enFeLV sequences present in the genome of species belonging to the genus *Felis* play an important role in the generation of exogenous FeLV variants that are more pathogenic [26]. Although recombinations that occur in the *env* gene are the most important and are the ones that allow the characterization of the FeLV subgroups [27], recombination has also been described in other genetic regions such as *gag* and *pol* [28, 29]. Endogenous sequence variability may impact the genetic variability of the exogenous types that result from recombination, and consequently on their level of pathogenicity, as has already been described in currently recognized subgroups [30, 31].

Eight of the enFeLV sequences obtained in our study were grouped with most of the previously described endogenous sequences [4]. However, in our phylogenetic tree the rest of them (n = 11) were grouped in an independent group close to exFeLV sequences, which had the highest values of genetic distance (data not shown). This could indicate recombinant sequences. The genetic distance values of enFeLV sequences showed that these can be highly variable. The lower genetic distance values were identified in previously reported domestic feline endogenous sequences [4, 32]. These data support that enFeLV sequences are genetically diverse [32–34]. In contrast, the exFeLV sequences obtained in this study showed smaller genetic distances (0-0.13), indicating that exogenous sequences are less variable. The shortest genetic distance was found in sequences from subgroup A that were previously described in the USA [35], and with which the sequences of our study were mainly associated. Exogenous sequences

belonging to subgroup A are the most conserved, even among isolates from different geographical areas, reaching similarity values of up to 98% in surface glycoprotein [2, 36].

Endogenization of enFeLV appears to have occurred after the initial divergence of the domestic cat lineage from the rest of the *Felidae* family [32], although more recent insertions of some enFeLV are recognized [4, 33]. While enFeLV is known to not generate infectious virions [37], enFeLV that retain functional open reading frames have been reported [4, 38].

In addition to detecting enFeLV in all the DNA samples from the cats in our study, enFeLV RNA was detected in 48 of the 50 cats tested, which may indicate a frequent transcription activity of the LTR region, as other studies have shown [6, 7]. The expression of enFeLV is associated with exFeLV infection [5–7] and the generation of exFeLV and enFeLV recombinants may take place in virions that have co-packaged transcripts of endogenous and exogenous origin [39].

On the other hand, we identified 11 cats that tested negative on all exFeLV and FIV tests but were positive for proviral DNA and enFeLV RNA transcripts. Additionally, these cats showed clinical signs and hemogram alterations that are compatible with feline retrovirus infection, such as gingivitis, gingivostomatitis, lymphoma, lymphocytosis, lymphopenia, anemia, neutropenia, and thrombocytopenia. This finding could be associated with recently integrated enFeLV retroviruses in the cat genome that may be capable of generating disease, however sequencing other genetic regions and verifying viral genome expression and replication would be necessary to confirm this. Another possibility relating to the cat population with clinical signs and hematological alterations not associated with exFeLV and FIV infection is a new exFeLV genetic variant that could not be detected with the PCR we used. The enFeLV sequences with the greatest diversity observed in the phylogenetic tree may be related to this new genetic variant (Fig. 2).

The clinical signs most frequently shown by cats at the time of sampling were depression and anorexia (18%), gingivitis or gingivostomatitis (12%), diarrhea (10%), emaciation (10%), pulicosis (8%) and vomiting (6%; Fig. 1). While most of these conditions can be associated with FeLV infection [40], the cases of gingivitis / gingivostomatitis are especially relevant because they are frequently observed in cats infected with FeLV or FIV. Infected cats are more susceptible to developing periodontal disease as a consequence of immune system deterioration and its consequent inability to respond to infections in general [41, 42].

Thirteen cats (26%) were exFeLV positive by PCR. Nine animals tested positive for both proviral DNA and viral RNA. These cats may be experiencing a progressive infection, since this course of FeLV infection is characterized by the presentation of persistent viremia, being possible to detect both viral DNA and RNA with PCR, and p27 antigen in blood [43]. However, in these cases it is recommended to follow up with antigen and/or viral RNA detection tests in which it is possible to obtain a negative result in the future, which would indicate a possible regressive infection [44]. In addition, progressive infections in cats are characterized by the development of diseases associated with the virus infection [1]. These include lymphoma and non-regenerative anemia, as well as gingivostomatitis and cytopenias that occurred in in

six of the nine FeLV proviral DNA and viral RNA positive cats. This could suggest a progressive course of infection [45, 46].

On the other hand, in regressive infection, viral replication is restricted by the animal's immune response either before or shortly after bone marrow infection, and they may or may not go through a state of viremia (transient viremia) [1]. Nevertheless, the infection can be reactivated during immunosuppression episodes and disease associated with the infection may occur [12]. We did not detect any animal only positive for proviral DNA, as occurs in regressive infections. In contrast, four of the animals were only positive to exFeLV using blood plasma RT-PCR. In these cats, viremia could be detected by RT-PCR, but the proviral form could not be detected. This may be due to different factors such as the quantity and quality of the DNA sample, low proviral load, or the sensitivity of the test used [47]. Real-time PCR tests have shown to be very sensitive in detecting FeLV at different stages of infection [8, 48].

Normal hemograms were the most frequent result found in cats that tested positive using any FeLV detection test. While FeLV infection is usually associated with a variety of hematological disorders [21, 49], the absence of hematological alterations has also been reported in FeLV positive animals [50]. This could be explained by these cats probably not having progressive type infections at the time of the study. It is important to note that two of these cats were positive for both proviral DNA and viral RNA, so it is possible that they will develop hematological alterations associated with the infection in the future [1].

The second most frequent result found in these animals (three cases) was non-regenerative anemia, followed by lymphopenia, neutrophilia, and eosinophilia, which occurred in two cases each. Neither neutrophilia nor eosinophilia can be associated in any direct way with FeLV presence. Cytopenias are the most frequently reported hematological disorders that characterize FeLV infection [21, 49–51]. These are attributed to viral infection of bone marrow hematopoietic and structural cells (stromal and fibroblasts), which alters the necessary microenvironment for hematopoiesis, and leads to myelosuppression [40].

FIV was detected in its proviral form using nested PCR. Phylogenetic analysis of obtained sequences revealed that they are grouped with sequences belonging to subtype B, which is one of the most frequently reported subtypes in other studies [22, 52–56]. We were unable to detect FIV positive cats using RT-PCR. In this case, the proviral form may have been the only detectable form because the virus was not replicating, or viral load was too low to be detected.

Depression and anorexia were the most frequent clinical signs found in FIV-positive cats. However, these signs are considered nonspecific, and thus cannot be attributed to infection with this virus. One female presented mammary adecarcinoma and neutropenia while another cat presented gingivostomatitis. Both of these conditions can be attributed to FIV infection given that the retrovirus predisposes hosts to various infections including those of the oral cavity [40, 57], and FIV infection is associated with a greater risk of neoplasm development, including mammary tissue neoplasms [58]. Cytopenias, including neutropenia, are frequent alterations found in FIV-positive cat blood counts [21, 49–51].

In summary, our results confirmed the presence of enFeLV, exFeLV-A and possibly recombinant viruses. This work also represents the first report describing FIV subtype B in naturally infected domestic cats in Mexico. The clinical characteristics of cats infected by feline retrovirus were the ones most frequently described in other studies. The joint study of feline retroviruses is important to better understand the role that enFeLV plays directly in disease presentation, as well as for the development of diagnostic tools that can provide global information on their efficiency, course of infection and genetic characterization of feline retroviruses.

Declarations

Ethical approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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Declaration of Conflict of Interest

The authors declare that they have no conflict of interest.

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Table

Table 1

Primers designed to detect feline retroviruses by PCR.

Primer	Sequence 5’ – 3’	Position [a]	TM	Amplicon size
enFeLV LTR FW	CCTCATTCTGGGAAATCGCC	8977–8996	60° C	205 bp
FeLV LTR RV	GCGGTCAAGTCTCAGCAAAG	9162–9181		
exFeLV LTR FW	CCAAGAACAGTTAAACCCCGG	8120–8140	62° C	215 bp
FeLV LTR RV	GCGGTCAAGTCTCAGCAAAG	8315–8334		
FIV gag FW1	GAGAAGGGAAYTTYAGATGGGC	719–740	57° C	919 bp
FIV gag RV1	GTACTTTCTGGCTTAAGRTGRC	1616–1637		
FIV gag FW2	ATGGGATTAGACACYAGRCC	964–983	52° C	458 bp
FIV gag RV2	CCTAATGCYTCRAGATACCA	1402–1421		

[a] Positions based on enFeLV-AGTT sequence (AY364318.1) for enFeLV, on Rickard strain sequence (NC_001940.1) for exFeLV and on Petaluma strain sequence (NC_001482.1) for FIV.

Figures

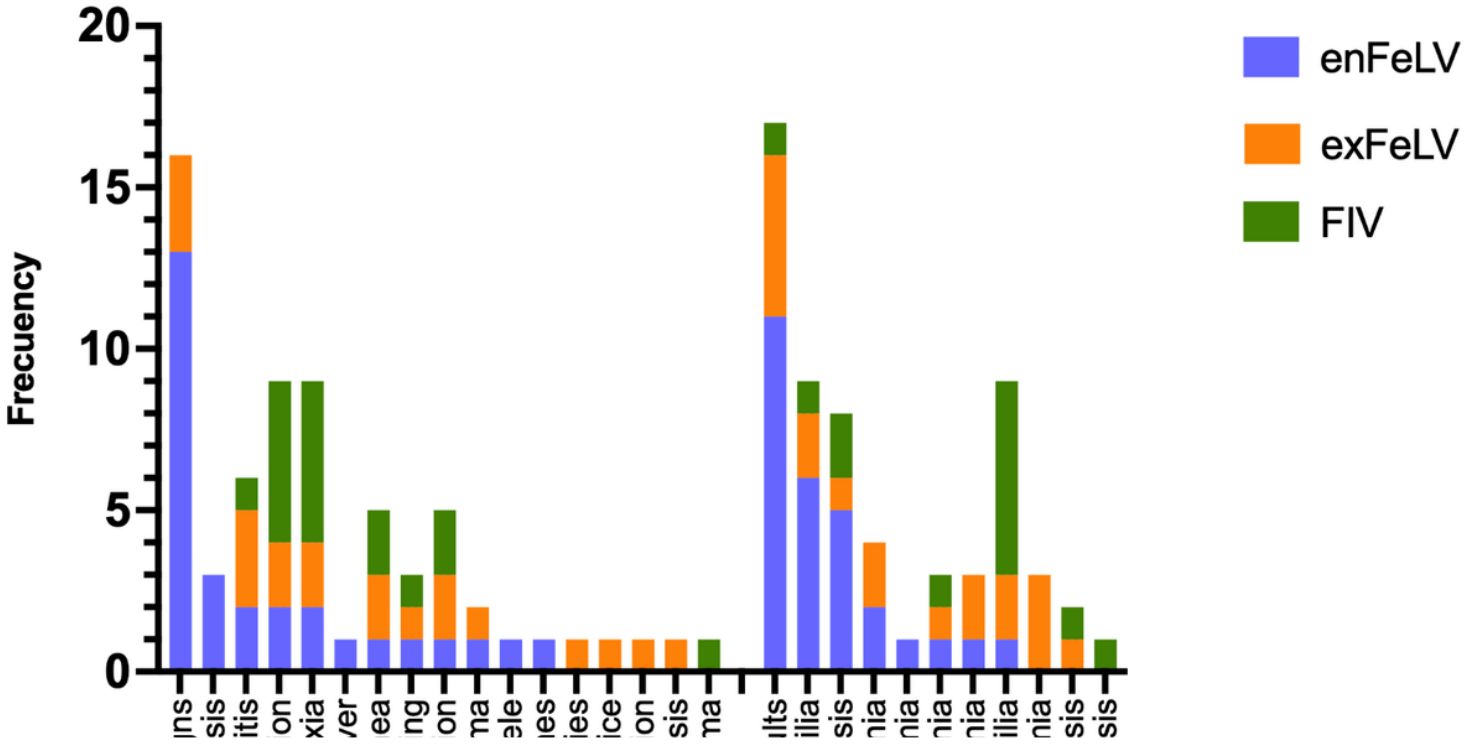

Figure 1

Clinical signs and hematological findings found in domestic cats that tested positive for feline retroviruses.

Figure 2

Phylogenetic tree constructed with partial enFeLV and exFeLV LTR region sequences, using Bayesian inference (MrBayes) in Geneious 11.1.4. The sequences obtained in this study are shown with an asterisk.

Figure 3

Phylogenetic tree constructed with partial FIV *gag* gene sequences, using Bayesian inference (MrBayes) in Geneious 11.1.4. We included sequences from different viral subtypes. Subtype A: GQ422125.1, KM880117.1, KM880121.1, M25381.1 and M36968.1. Subtype B: M59418.1, MW142027.1, MW142038.1, MW142043.1, MW142046.1, MW142047.1, U11820.1 and Y13867.1. Subtype C: AF474246.1 and AY600517.1. Subtype D: AY679785.1, D37818.1 and D37822.1. Subtype E: EU117992.1. The sequences obtained in this study are shown with an asterisk.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- enFeLVexFeLVandFIVsequences.txt