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Detecting regressive feline leukemia infections and feline immunodeficiency coinfections in cats with clinical signs and hematological alterations related to retroviral infection

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Abstract

The infections caused by feline leukemia (FeLV) and feline immunodeficiency (FIV) viruses, are relevant in Feline Medicine due to the severe complications of the disease and related pathologies in domestic cats. This study describes clinical findings related to FeLV and FIV, regressive FeLV infections and identification of prevalent FeLV genotypes in domestic cats from Mérida, Yucatán, México. Hundred domestic cats with clinical manifestations of diseases associated with feline retrovirus infection in veterinary centers in Mérida, were submitted to a general physical examination, venipuncture to collect blood, and a quantitative hemogram. Detection of antigen (FeLV) and antibody (FIV) was used to estimate infection frequency. The percentage of regressive FeLV infections was determined by PCR of an *env* gene segment. Some FeLV amplified products were sequenced with the Sanger method and used to construct a phylogenetic tree. The predominant FeLV and FIV clinical infection findings were gingivitis, gingivostomatitis, periodontal disease and anemia. We found a 10% infection frequency for FeLV by antigen detection, and 17% for FIV by antibodies detection. PCR frequency detection for FeLV was 58%, and 52% were regressive infections. The phylogenetic analysis identified sequences associated with FeLV-A, and endogenous or recombinant FeLV that had not previously been identified in México. The frequency of infection by both retroviruses was higher in Mérida, Yucatán, than those described in previous studies. Cats with FeLV predominantly had regressive infections, but the role that endogenous or recombinant retroviruses play in disease development remains unknown.

Keywords: FeLV; FIV; PCR; Regressive infection; Antigens; Antibodies.

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Introduction

Feline leukemia and feline immunodeficiency viruses (FeLV and FIV, respectively) belong to the family *Retroviridae*, and to the Gammaretrovirus and Lentivirus genus respectively.[1-4] Their viral structure is composed of an envelope, and icosahedral nucleocapsid, a positive sense RNA strand, and enzymes such as reverse transcriptase. Retroviruses are characterized by inserting their DNA into the host genome (provirus) and can remain latent for a long time.[5]

FeLV and FIV have three main genes: *gag*, *pol*, and *env*, that respectively encode nucleocapsid, capsid and matrix proteins, enzymes that participate in replication, and envelope proteins. At the 3′ and 5′ ends, they have two terminal untranslated regulatory regions (LTRs).[2, 6] FeLV can recombine with endogenous sequences and forming new pathogenic subtypes.[7] The main subtypes described are FeLV-B, C, T, D and TG35, and they have been associated with the development of lymphomas, leukemia, red cell aplasia, and immunosuppression, among others. FeLV produces two types of infections: progressive (active bone marrow infection with viremia) and regressive (latency in bone marrow without viremia).[1, 8] On the other hand, FIV induces an acquired immunodeficiency syndrome (AIDS) by reducing the number and function of cd4+ T-lymphocytes.[9]

Antigen (FeLV) and antibody (FIV) detection using commercial tests based on lateral flow immunochromatography (LFI) is the most widely used in veterinary clinics and hospitals.[10, 11] FeLV diagnosis using this methodology is uncapable of identifying regressive infections.[12] On the other hand, antibody detection is considered adequate for FIV infection identification because the host does not eliminate the infection.[13] Nowadays, a 76% FeLV prevalence has been described for cats from central Mexico, while 7.5% FeLV and 2.5% FIV prevalences have been reported in Mérida, Yucatan.[14] No previous studies have assessed descriptions of clinical findings, molecular diagnoses and FeLV genetic characterization in infected cats from Mérida. The aim of our study was to describe FeLV and FIV infection clinical findings, estimating their frequency through antigen, antibody and proviral DNA detection, and to identify prevalent FeLV genotypes in domestic cats from Mérida, Yucatán, Mexico.

Materials and methods

Study animals

The study was cross-sectional from July to September 2019, and included 100 domestic cats under treatment at different veterinary centers (Figure 1) in Mérida, Yucatán, Mexico (latitude North 20° 58′ 01″; longitude West 89° 37′ 28″). Study inclusion criteria were: cats of 6 months of age and up, without recent vaccination against FeLV and presenting any clinical signs of infection related to feline retrovirus. Written informed consent was obtained from the legal guardians or owners of each cat described in this study.

Sample collection

Three milliliters of blood were obtained from each cat either by jugular or cephalic venipuncture, using a sterile plastic syringe (21 × 1 1/2″; BD, USA) or introcan catheter (22G; B Braun, Germany), and stored in tubes with anticoagulant (EDTA).

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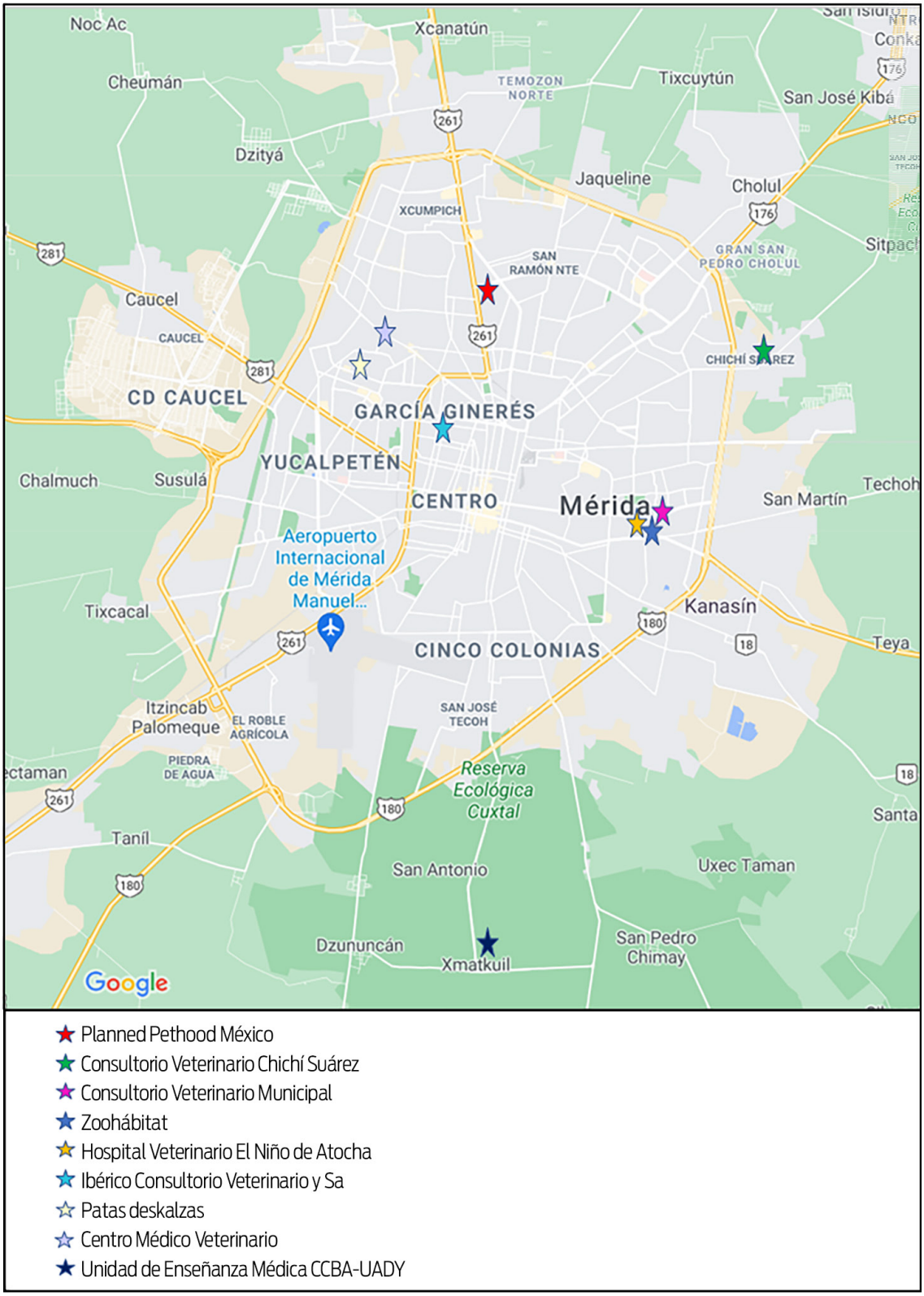

Fig. 1: Location of the veterinary clinics in the city of Mérida, Yucatán, Mexico, that participated in the study.

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Peripheral blood leukocytes (PBL) were separated from 2ml of each blood sample using centrifugation at 3000 rpm for 15 minutes at 25°C. pbls were stored at -70°C in cryovials (Corning, USA) until use. The remaining 1ml of whole blood was used to perform quantitative hemograms (KontrolLab BC 7000 Plus Desego, Mexico) and lateral flow immunochromatography tests (LFI). These tests convey a FeLV sensitivity of 92.9% and specificity of 96.5%, and an FIV sensitivity of 93.8% and specificity of 93.4% (Witness, Zoetis. USA) when following the manufacturer's instructions.

PCR

We used previously described PCR amplification conditions and primers,[1] that generate a 508 bp amplicon of the FeLV *env* region. Lastly, we purified 10 PCR positive samples selected for greater intensity in amplification, and DNA quantified by spectrophotometry, using a NanoDropLite 2000 (Thermo Scientific, Waltham, MA. USA) with the FavorPrep™ GEL/PCR Purification Kit (Farvorgen, Ping-Tung, Taiwan). These were sent to the Biotechnology and Prototypes laboratory of the FES-Iztacala, UNAM for sequencing by the Sanger method.

Phylogenetic FeLV analysis

We researched the phylogenetic history of FeLV sequences using Maximun Likelihood inference and Kimura 2-parameter model.[15] A discrete Gamma distribution was used to model evolutionary rate differences between sites. This analysis involved 40 nucleotide sequences and included 1st + 2nd + 3rd + Noncoding codon positions. Evolutionary analyses were conducted in MEGA X.[16] The statistical confidence of the topology of the phylogenetic tree was secured with bootstrap values of 100 repetitions. Nodes with bootstrap values above 70 were considered significant. We included partial FeLV sequences (both exogenous and endogenous) from domestic and wild cats infected in different countries that are available in GenBank (Table 1).

Statistical analysis

Statistics were run in Windows Excel. We divided the number of positive cases by the total number of cats in the study to calculate the frequency obtained by LFI and PCR.

Results

Clinical and hematological findings in cats infected with FeLV or FIV

The clinical signs identified in 90% of the FeLV antigen positive cats (n= 10) included gingivitis, gingivostomatitis or periodontitis, and dental tartar or bacterial plaque (Figure 2). All cats had palpable lymph nodes and half had upper respiratory disease. In the hemogram, 70% presented thrombocytopenia and 50% had anemia (Table 2).

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Table 1. Accession numbers for endogenous and exogenous FeLV sequences available in GenBank, and used for the construction of the phylogenetic tree

Accession number	FeLV type	Reference
KY751005	Endogenous	Liu and Sun[40]
LC196053.1	Endogenous	Takeuchi and Nishigaki[39]
LC198317.1	Endogenous	Takeuchi and Nishigaki[39]
AY364319.1	Endogenous	Roca et al.[37]
AY364318.1	Endogenous	Roca et al.[37]
KY751001	Endogenous	Liu and Sun[40]
LC196054.1	Endogenous	Takeuchi and Nishigaki[39]
JF957363.2	Endogenous	Stewart et al.[38]
KR030130.1	Endogenous	Ramírez et al.[1]
KR030110.1	Endogenous	Ramírez et al.[1]
KR030120.1	Endogenous	Ramírez et al.[1]
KR030093	Endogenous	Ramírez et al.[1]
KR030098.1	Endogenous	Ramírez et al.[1]
KR030134.1	Endogenous	Ramírez et al.[1]
M18247.1	Exogenous	Donahue et al.[44]
AF403716.1	Exogenous	Anderson et al.[45]
KR349469.1	Exogenous	Filoni et al.[46]
AF052723.1	Exogenous	Chen and Roy-Burman[47]
V01172.1	Exogenous	Elder and Mullins[48]
KP728112.1	Exogenous	Helfer-Hungerbuehler et al.[49]
FJ436991.1	Exogenous	Shalev et al.[50]
M14331.1	Exogenous	Riedel et al.[51]
J03448.1	Exogenous	Nicolaisen-Strouss et al.[52]
MF681664.1	Exogenous	Chiu et al.[53]
AB635483.1	Exogenous	Watanabe et al.[54]
MF681669.1	Exogenous	Chiu et al.[53]
MG020273.1	Exogenous	Chiu et al.[53]
EU189490.1	Exogenous	Brown[55]
MF681672.1	Exogenous	Chiu et al.[53]

In the case of cats that were PCR FeLV positive (n= 58; Figure 3), 68.96% presented gingivitis, gingivostomatitis or periodontitis, bacterial plaque or dental tartar, 94.82% had palpable lymph nodes, and 48.27% had upper respiratory tract signs. Other prominent signs were 39.65% with pale mucous membranes, 32.75% with poor body condition, 34.48% with dermatological lesions, and 32.75% had ectoparasites. In the hemogram, 43.10% presented thrombocytopenia or lymphocytosis, 41.37% anemia, and 37.93% leukocytosis (Table 2).

In FIV-seropositive cats (n = 17), 64.7% presented gingivitis, gingivostomatitis or periodontitis, 100% had dental tartar or bacterial plaque and palpable lymph nodes, 70.58% had upper respiratory tract signs, 52.94% had abdominal pain, 41.17% had ectoparasites present or poor body condition, and 35.29% had non-specific signs. In the hemogram, 58.82% showed anemia, 64.7% thrombocytopenia, and 35.29% leukocytosis or lymphocytosis (Table 2).

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Figure 2. Different clinical findings in retrovirus positive cats. A) Severe gingivitis and mucopurulent nasal discharge (FIV+ / FeLV+ PCR). B) Pale gums, dental tartar and bacterial plaque (antigen and PCR FeLV+). C) Periodontal disease (FIV+ / PCR FeLV+). D) Spontaneous abortion (antigen and PCR FeLV+). E) Anisocoria (antigen and PCR FeLV+); F) Anisocoria and vestibulitis (antigen and PCR FeLV+). G) Felicola spp. (FIV+ / PCR FeLV+). H) Otodectes spp. (PCR FeLV+). I) Bilateral uveitis (FIV+ / PCR FeLV+). J) Tick bite (FIV+ / PCR FeLV+). K) Severe cachexia and pain (PCR FeLV+). L) Bite (PCR FeLV+). M) Gingivitis, halitosis and ptyalism (PCR FeLV+). N) Canker sore and faucitis (FIV+ / PCR and antigen FeLV+).

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Table 2. Frequency of clinical and hematological findings in cats infected with FeLV and/or FIV

Finding	Relative frequency %		
	LFI FIV	LFI FeLV	PCR FeLV
n	17	10	58
Nonspecific signs (fever, anorexia, wasting)	35.29	40	27.58
Pale gums	29.41	40	39.58
Gingivitis, gingivostomatitis or periodontitis	64.70	90	68.96
Bacterial plaque or dental tartar	100	90	84.48
Cachexia or low body condition	41.17	40	32.75
Palpable lymph nodes	100	100	94.82
Upper respiratory signs	70.58	50	48.27
Ophtalmological signs	11.76	---	8.62
Neurological signs	---	20	5.17
Dermatological signs	35.29	---	34.48
Bite history	29.41	20	17.24
Gastrointestinal signs	---	10	6.89
Abdominal pain	52.94	40	34.48
Lower urinary tract signs	5.88	---	10.34
Ectoparasites	41.17	10	32.75
Abortion history	---	10	3.44
Anemia	58.82	50	41.37
Leukocytosis	35.29	40	37.93
Leukopenia	11.76	10	5.17
Lymphocytosis	35.29	30	43.10
Monocytosis	5.88	---	18.96
Granulocytosis	11.76	10	13.79
Thrombocytopenia	64.70	70	43.10

LFI = lateral flow immunochromatography; FeLV = feline leukemia; FIV = feline immunodeficiency

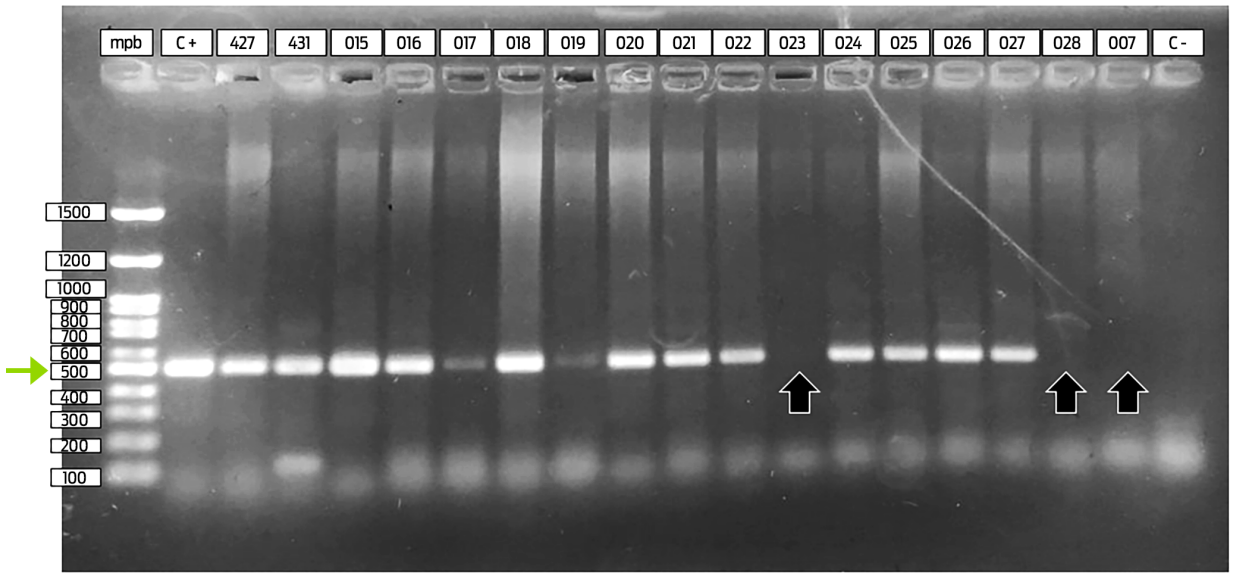

Figure 3. Agarose gel (1%) stained with ethidium bromide and some of the amplified 508 bp products from the FeLV *env* region. Lane 1: bp marker; lane 2: positive control; lanes 3-12 and 14-17: positive samples; lanes 13, 18 and 19, negative samples; lane 20: negative control.

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Table 3. Integration of positive (+) and negative (-) results obtained in diagnostic tests that detect antigen, antibodies, and proviral DNA in cats infected with retrovirus

Results in lateral flow immunochromatography and PCR							
Antigen	+FeLV	+ FeLV	+ FeLV	+ FeLV	- FeLV	- FeLV	- FeLV
Antibodies	+ FIV	- FIV	- FIV	+ FIV	+ FIV	- FIV	+ FIV
Proviral DNA	+ FeLV	+ FeLV	- FeLV	- FeLV	+ FeLV	+ FeLV	- FeLV
Absolute frecuency	(2/100)	(4/100)	(3/100)	(1/100)	(12/100)	(40/100)	(2/100)

FeLV = feline leukemia; FIV = feline immunodeficiency

Frequencies of feline retroviral infections

The commercial LFI test found 10% (10/100) of cats were positive for FeLV antigen, and 17% (17/100) tested positive for antibodies against FIV. In the case of the FeLV *env* PCR (Figure 3), 58% (58/100) of cats tested positive. Of these, 90% (52/58) had regressive FeLV infection, and 70% (40/58) of these did not have coinfection with FIV. In PCR positive cats, 26% (15/58) were coinfected, and only 3% (3/100) were only positive to LFI test to FIV (Table 3).

Characteristics of FeLV and/or FIV positive cats

Most of the FeLV antigen-positive cats were males, outdoor, uncastrated, or juvenile. Most FIV positive cats were males and outdoor cats; 52.94% were uncastrated and we identified more positive young animals than either adults or geriatric cats. Most of the PCR-positive cats were outdoor cats or juveniles, 56.89% were males, and the proportion was similar between castrated and whole.

Genotyping

The sequences obtained in this study are available from GenBank under accession numbers MW698945-MW698953. In the phylogenetic tree, we observed that one of the sequences (15G) was associated with FeLV-A and B sequences. The rest of the study sequences were associated with endogenous FeLV sequences from other countries, but not with endogenous FeLV sequences previously described in Mexico. These were associated in an independent clade (Figure 4).

Discussion

In this study, we determined the frequency of FeLV and FIV infection in sick cats from Mérida, Yucatán for the first time using LFI, and the frequency of regressive FeLV infections via PCR. The most frequent clinical findings related to feline retrovirus infection and the characterization of the prevalent FeLV genotypes are also described for the first time. We found infection frequency estimated by LFI (10% for FeLV and 17% for FIV) was higher than previously reported in healthy cats from Mérida (7.5% for FeLV and 3.5% for FIV) by Ortega-Pacheco et al.[14] On the other hand, infection frequency was lower than that described in other studies of sick cats carried out in other regions such as Italy (18% FeLV and 24% FIV) by Bandecchi et al.,[17] Madrid, Spain (30.4% FeLV and 13.9% FIV) by Arjona et al.,[18] and Zagreb,

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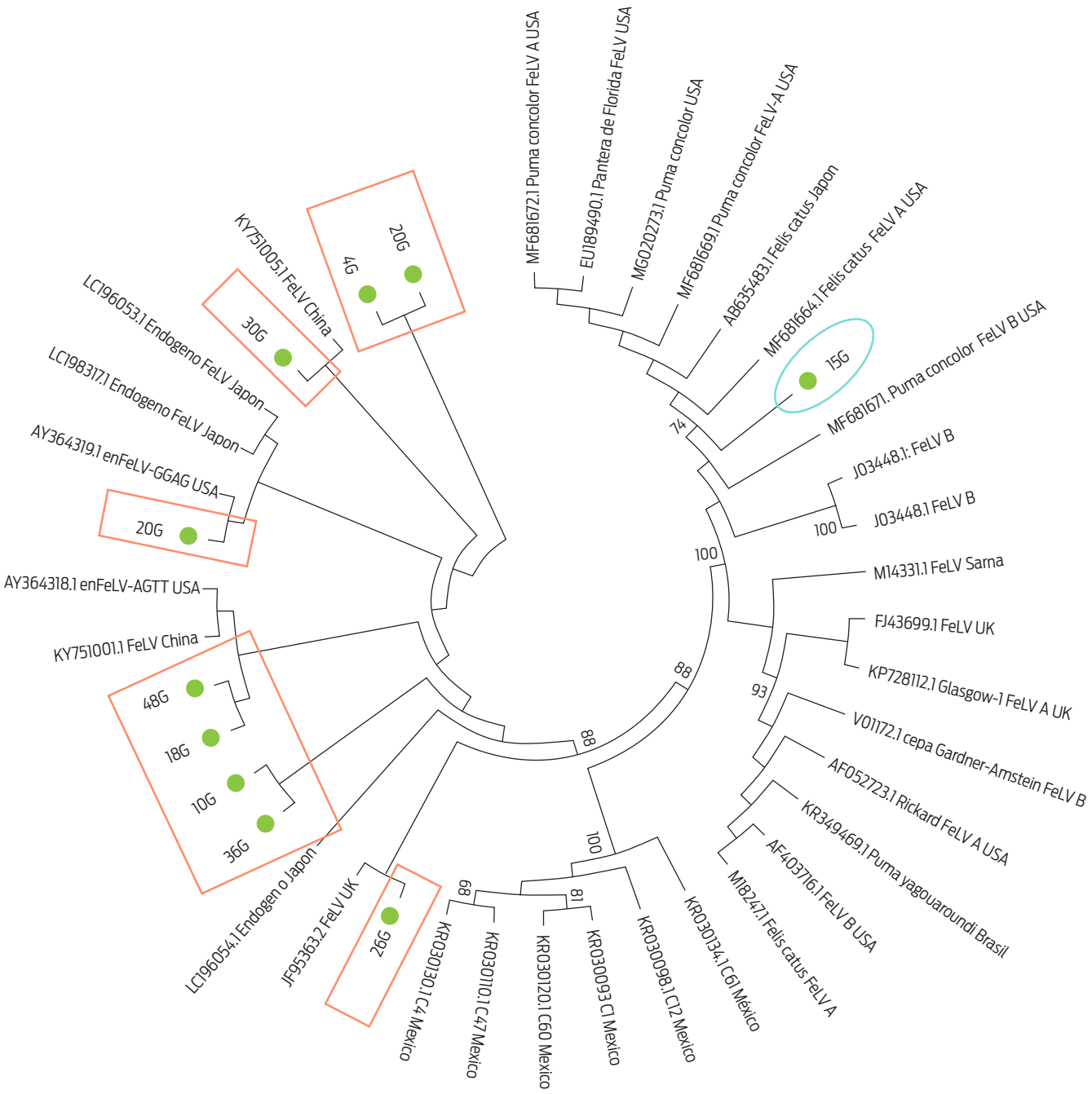

Figure 4. Phylogenetic tree constructed with the maximum likelihood method, including partial FeLV *env* gene sequences obtained from domestic cats in Mérida, Yucatán, Mexico, (●) and partial endogenous and exogenous FeLV subtype sequences available in GenBank. Red: endogenous associated sequences, purple: exogenous associated sequence.

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Croatia (18.22% FeLV and 20.88% FIV) by Perharić et al.[4] In contrast, the frequency of feline retrovirus infection was higher than that identified in Japan (2.9% FeLV and 9.8% FIV) by Maruyama et al.,[19] and Canada (4.6% FeLV and 5.5% FIV) by Ravi et al.[20] Our findings were closer to those described in the United Kingdom (6.9% FeLV and 16.7% FIV) by Muirden.[21] Geographic region, cat traits, number of animals studied, and diagnostic tests used may influence the discrepancy between studies.[22]

Possible false negatives may bias the FIV frequency we detected, since the antibodies are detectable 2-6 weeks after infection.[2, 11] The host cannot eliminate FIV infection following exposure to the virus,[13] which represents a diagnostic advantage for serological tests. However, titers may be considerably decreased during the clinical phase of infection (AIDS), and lymphopenia in this phase would also make it difficult to detect proviral DNA by PCR.[2, 12]

Barely 2% of the population was exclusively positive to FIV, while 88.23% of cats positive for FIV were coinfected with FeLV. It is necessary to carry out more studies to elucidate whether coinfections favor any of the clinical presentations associated with feline retrovirus infection, as in our study where the presence of palpable lymph nodes, periodontitis, dental or bacterial tartar, gingivitis and thrombocytopenia were identified in more than 64% of cats.

These pathologies differed from those reported in cats from Argentina, where 42.9% of cats with FeLV and/or FIV had ulcers or gingivitis.[12] Persistent gingivostomatitis is common in both infections due to immunosuppression or immunodepression. FeLV causes direct cytopenias in bone marrow, while FIV decreases the number of TCD4+ lymphocytes.[5, 9, 13, 23] This favors changes in oral microbiota, facilitating colonization and chronic-antigenic stimulation by other microorganisms.[24] The finding of swollen lymph nodes cannot be solely attributed to retroviruses, since antigenic recognition of pathogens by dendritic cells and macrophages activate lymphocytes located in these organs. However, they can be tissue reservoirs for the active FIV replication in chronic stages.[9, 25]

Anemia (50%) in FeLV antigen-positive cats was similar to reports from Spain (50%) by Bandecchi et al.,[17] and Iran (55%) by Akhtardanesh et al.,[26] but thrombocytopenia (70%) was higher than a Brazilian report (42.2%) by da Costa et al.[27] Both findings are common in FeLV infection due to direct bone marrow cytopenias, but this virus more strongly affects erythroid precursors during hemoglobin synthesis, among other mechanisms such as leukemia, myelodysplasia, and erythroid aplasia.[5, 28, 29] However, automated blood counts overestimate thrombocytopenia prevalence fourfold, in addition to the fact that platelet aggregation is common in cats.[30] Therefore, it is important to include manual counts in the feline hemograms.

Unlike the report by Akhtardanesh et al.,[26] that identified 7.4% of cats with weight loss or anorexia, our study found a considerable percentage of cats with FIV presented ectoparasites, poor body condition or nonspecific signs (fever, decay and anorexia). Nonspecific signs can be attributed to FIV because they occur in the acute phase (6-8 weeks post-exposure),[2, 9] and antibodies are detectable before clinical signs appear, however, more studies are required. Despite the fact that FIV has a high degree of neurotropism,[31, 32] our study did not identify neurological damage in FIV-positive cats, possibly because a low percentage (up to 5.3%) has been described as manifesting nervous affection.[33] Our data on anemia (58.82%) and

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thrombocytopenia (64.70%) were higher than those described for cats in Brazil (32% anemia; 35% thrombocytopenia) by da Costa et al.,[27] but only 2% were exclusively positive for FIV. Anemia may be more associated with FeLV infection and/or poor body condition, and thrombocytopenia, as already mentioned, may be overestimated.

The difference between positive frequencies found by LFI and PCR in FeLV infection detection are due to PCR having greater sensitivity for detecting lymphocyte and monocyte proviral DNA, even during regressive infection, and not depending on the viremia phase.[1, 12, 34] Regressive infections are the most common and cannot be diagnosed with antigen-detection tests.[35] Up to 52% of the cats in this study had regressive infection, equivalent to 89.65% of the population of PCR-positive animals. Antigen detection considerably underestimates the frequency of FeLV infection in both sick and healthy cats. This would mean high numbers of false negatives with the LFI test, and for a long time in the life of the hosts, with chronic viral effects on bone marrow that can have fatal consequences on the immune and/or hematopoietic system. It is also possible that the lack of sensitivity is linked to the viral genetic subtype that is used for the design of the LFI tests available in Mexico. All of these tests are made in other countries and do not necessarily represent the infectious viral genotype present in Mexican cats.

The FeLV PCR frequency (58%) we found was lower than what had previously been reported in healthy cats from central Mexico (76%) by Ramírez et al.[1] This may be related to the fact that many cats that appear clinically healthy may be in a regressive phase.[10] Additionally, the PCR frequency we found was higher than others, such as a study in Argentina (11.82%) by Galdo-Novo et al.,[12] where they used a PCR to amplify the U3 region of the LTR. Their PCR was designed to only recognize the FeLV-A genotype with 100% sensitivity, but it did not detect endogenous FeLV sequences,[36] which it was where the majority was found in our study. The heterogenicity of the env gene makes it possible to differentiate endogenous and exogenous retroviruses, as it is the least conserved FeLV region and it's involved in recombination.[1, 6] The primers used to amplify the env gene in the study PCR maintain 70% identity with exogenous FeLV.[1] Thus, it is possible that the sequences identified in the study are related to recombinant FeLV and that the amplified region has a greater similarity with endogenous FeLV sequences.

One of the sequences obtained in the study was associated with reference FeLV-A and/or B sequences, while the rest of the sequences were associated with endogenous FeLV reported in other studies.[37-40] FeLV-A recombination with endogenous FeLV sequences generates various pathogenic subtypes.[1, 7, 41] Endogenous retroviruses are part of cat evolutionary history, and they are endogenous ancestral infections that cannot replicate or be pathogenic without FeLV-A .[42, 43] However, complete endogenous FeLVs have been identified that could complement each other and have replicative capacity.[42] To date, much remains unknown about the role of endogenous FeLV in the pathogenic process caused by exogenous FeLV, but they participate in recombination. The endogenous-like sequences found in the study were grouped in a different clade from endogenous sequences previously described in Mexico, and had greater affinity with sequences identified in China by Liu and Sun (2017),[40] Japan by Takeuchi and Nishigaki,[39] the United States by Roca et al.,[37] and the United Kingdom by Stewart et al.[38] This shows the need for studies that clarify the role of endogenous FeLV in the history of do-

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mestication or evolution, the development of tools to differentiate FeLV recombinants, and the possible involvement of endogenous FeLV in disease generation in cats from Mérida, Yucatán.

Conclusions

FeLV and FIV detection frequencies through LFI were higher than previously reported in healthy cats from Mérida, Yucatán. Regressive type infection was predominant in cats with FeLV. Coinfection with both feline retroviruses was more common than isolated infections. The most common clinical findings were gingivitis, periodontitis, and anemia. Phylogenetic analysis revealed endogenous FeLV or recombinant FeLV sequences not previously described in Mexico and their role in the disease process in domestic cats is unknown.

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Conflicts of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contributions

Conceptualization, M.C.C., M.E.B. and H.R.; methodology, M.C.C and G.E.A.; formal analysis, M.C.C., M.E.B., L.C., A. R. and H.R.; investigation, M.C.C., G.E.A., L. C., A. R. and M.E.B.; resources, H.R. and M.E.B.; writing original draft preparation, M.C.C., M.E.B., G.E.A. and H.R.; writing review and editing, all authors; supervision, H. R. and M.E.B.; funding acquisition, H. R. and M.E.B. All authors have read and agreed to the published version of the manuscript.

Ethical approval

The study was approved by Campus de Ciencias Biológicas y Agropecuarias, Universidad Autónoma de Yucatán (CCBA-UADY) Bioethics Committee (registration number CB-CCBA-M-2019-007).

Consent to participate

Informed consent (either verbal or written) was obtained from the owner or legal custodian of all animal (s) described in this work (either experimental or non-experimental animals) for the procedure(s) undertaken (either prospective or retrospective studies). No animals or humans are identifiable within this publication, and therefore additional informed consent for publication was not required.

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