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Classification of lymphoma in cats and its relationship with the detection of feline leukemia virus proviral DNA[1]

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ABSTRACT.- Silva D.H.L., Ecco R., Pierezan F., Cassali G.D., Reis J.K.P., Gonçalves A.B.B., Bicalho J.M., Delarmelina E. & Leme F.O.P. 2022. **Classification of lymphoma in cats and its relationship with detection of feline leukemia virus proviral DNA.** *Pesquisa Veterinária Brasileira 42:e07021, 2022.* Departamento de Clínica e Cirurgia, Escola de Veterinária, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Belo Horizonte, MG 31270-901, Brazil. E-mail: fabiolapaesleme@vetufmg.edu.br

In this retrospective and prospective study, histopathological and immunohistochemical analyses of 62 cases of lymphomas in cats were performed to classify the anatomic forms and subtypes, according to the WHO guidelines, and correlate it to FeLV proviral DNA detected using PCR. The most common anatomical form was gastrointestinal (40.3%, 25/62), followed by multicentric (29%, 18/62), mediastinal (17.7%, 11/62) and extranodal (12,9%, 8/62). Among the lymphoma subtypes, diffuse large B-cell lymphoma (DLBCL) (30.6%, 19/62) was the most commonly diagnosed followed by peripheral T-cell lymphoma (PTCL) (29%, 18/62) and enteropathy associated T-cell lymphoma type 2 (14.5%, 9/62). DNA extraction from paraffin-embedded neoplastic tissue was obtained in 28 cases and FeLV proviral DNA was detected by PCR, in 23 of these. Of the cases presenting with FeLV proviral DNA, nine (32%) were of the multicentric form, five (22%) of the mediastinal and extranodal forms and four (17%) of the gastrointestinal form. The most frequent subtypes with FeLV proviral DNA, independent of the anatomical form, were DLBCL (39.1%, 9/23) and PTCL (34.7%, 8/23). The presence of the FeLV proviral DNA in 23 cats of this study, probably had association with the multicentric form of lymphoma and higher occurrence in the DLBCL and PTCL subtypes.

INDEX TERMS: Lymphoma, cats, feline leukemia vírus, proviral DNA, anatomical form, B-cells, T-cell, FeLV provirus.

RESUMO.- [**Classificação do linfoma em gatos e sua relação com a detecção do DNA pró-viral do vírus da leucemia felina.**] Neste estudo retrospectivo e prospectivo, análises histopatológicas e imuno-histoquímicas de 62 casos de linfomas em gatos foram realizadas para classificar as formas anatômicas o e subtipos do linfoma, de acordo com as diretrizes da OMS. Além disso, foi realizada a extração de DNA dos tumores incluídos na parafina para obtenção de DNA pró-viral do FeLV por PCR, e relacionada com os exames anteriores. A forma anatômica mais comum foi a gastrointestinal (40.3%, 25/62), seguida pela multicêntrica (29%, 18/62), mediastinal (17,7%, 11/62) e extranodal (12,9%, 8/62). Entre os subtipos de linfoma, o linfoma difuso de grandes células B (DLBCL) (30.6%, 19/62) foi o mais comumente diagnosticado, seguido por linfoma de células T periférico (PTCL) (29%, 18/62) e o linfoma de células T associado a enteropatia tipo 2 (14.5%, 9/62). A extração de DNA de tecido neoplásico emblocado em parafina foi obtida em 28 casos e o DNA pró-viral de FeLV foi detectado por PCR, em 23 deles. Dos casos com DNA pró-viral do FeLV,

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nove (32%) eram da forma multicêntrica, cinco (22%) das formas mediastinal e extranodal e quatro (17%) da forma gastrointestinal. Os subtipos mais frequentes com DNA pró-viral do FeLV, independente da forma anatômica, foram DLBCL (39.1%, 9/23) e PTCL (34.7%, 8/23). A presença do DNA pró-viral do FeLV em 23 gatos deste estudo, provavelmente teve associação com a forma multicêntrica do linfoma e maior ocorrência nos subtipos DLBCL e PTCL.

TERMOS DE INDEXAÇÃO: Linfoma, gatos, DNA pró-viral, vírus da leucemia felina, forma anatômica, células B, células T, provírus do FeLV.

INTRODUCTION

Lymphomas are among the most common feline malignancies representing more than 50% of all tumors in cats (Santagostino et al. 2015). The tumor cells develop from lymphoid cells of the immune system; thus, any tissue or organ can be affected in lymphoma (Wolfesberger et al. 2018). The anatomic forms of lymphoma in cats are multicentric (generalized lymphadenopathy), mediastinal (mediastinal lymphadenopathy), gastrointestinal and extranodal (renal, neural, ocular, and cutaneous) (Couto 2000).

Currently, the World Health Organization (WHO) classification, adapted from humans, is used for the diagnosis of lymphomas in domestic animals. It correlates each category of lymphoma to a cellular behavior and degree of malignancy (Valli et al. 2000, Vonderhaar & Morrison 2002, Valli 2007). Some publications relate some subtypes of lymphoma to the prognosis of the disease in cats (Moore et al. 2012, Santagostino et al. 2015, Wolfesberger et al. 2017, Musciano et al. 2020).

Retroviral infections are strongly associated with the development of feline lymphoma (Rojko et al. 1989), increasing its risk by approximately 60 times (Shelton et al. 1990, Rezanka et al. 1992). In the 1980s, approximately 70% of cats with lymphoma were serologically positive for feline leukemia virus (FeLV) (Rojko et al. 1989). FeLV infection has different stages, classified as progressive or regressive. In progressive infection, high viral replication occurs, a condition called "persistent viremia", diagnosed by ELISA and by PCR with detection of proviral DNA (Hofmann-Lehmann et al. 2007, Hartmann 2012). In regressive or latent infection, the molecular basis is the integration of a copy of the viral genome (provirus) into the chromosomal DNA of the host, with no active viral production. Thus, cats with regressive infection present negative results in all tests that detect the FeLV antigen; however, the proviral DNA can be detected using PCR. During cell division, proviral DNA is replicated and the genetic information is passed down to the daughter cells; however, proviral DNA is not translated into proteins and no infectious viral particles are produced. Therefore, PCR is potentially more sensitive in detecting FeLV proviral DNA at different stages of infection (Hofmann-Lehmann et al. 2007, Hartmann 2012, Jackson et al. 1996).

The association between the presence of provirus or latent FeLV infection and the development of lymphomas is more complex and less clear. It is generally accepted that lymphoma induction by FeLV is associated with expression of virus antigen in the tumors, at least in their early stages (Rezanka et al. 1992, Weiss et al. 2010). The infection by FeLV with integration of FeLV provirus into the host cell genome induced tumors are caused in part by somatically acquired insertional mutagenesis (Forman et al. 2009). If the retrovirus has been integrated near a certain cellular gene, protooncogene, transcription of the gene can be upregulated by the promoter and enhancer function of the retroviral long terminal repeat (LTR). On the other hand, if it has been integrated inside the gene, in a tumor suppressor gene, the transcript can be altered or disrupted. And then, the cell acquires growth advantage (Fujino et al. 2008). Other mechanism in the formation of lymphoma is the U3-LTR region of FeLV activates NFκB-dependent gene expression. NFκB is intimately associated with growth factors that activates of cells proliferation (Abujamra et al. 2006).

Some studies have associated FeLV proviral DNA with lymphomas and relate them to the immunophenotype of the tumor (Jackson et al. 1996, Gabor et al. 2001) or have associated the predominance of some lymphoma subtypes with the FeLV antigen (Weiss et al. 2010, Santagostino et al. 2015, Leite-Filho et al. 2020).

Another study related the presence of proviral DNA and immunolabelling of the FeLV antigen in the tumors to the histological classification of lymphomas subtypes, according to the WHO classification, using a cohort study carried out in an area with low virus incidence (Weiss et al. 2010). In Brazil, epidemiological studies using serological tests to detect the FeLV p27 antigen in domestic cats identified populations with 31% of seropositive cats in the state of Rio Grande do Sul (Costa et al. 2017), 22.26% in the state of Santa Catarina (Biezus et al. 2019) and 11.52% in the state of Rio de Janeiro (Almeida et al. 2012).However, studies that used PCR as a diagnostic method for the detection of proviral DNA showed a higher prevalence of FeLV infection in the state of Minas Gerais, with a variation of 47.5-49.6% of positive cats (Coelho et al. 2011, Victor et al. 2020). Factors contributing to the high frequency of FeLV infection in Brazil include scarcity of vaccination, lack of detection, constant access to the outside and the male gender (Almeida et al. 2012, Biezus et al. 2019). In Brazil, only a few studies have established the relationship between antigens using immunohistochemistry, anatomical classification or histological aspects of lymphoma based on WHO guidelines (Leite-Filho et al. 2020); others have not pointed the subtypes (Cristo et al. 2019).

The objective of this study was to characterize the anatomical forms and subtypes of lymphoma in cats in Brazil and relate their forms to the molecular detection of FeLV proviral DNA.

MATERIALS AND METHODS

Cases of lymphoma in domestic cats from 2005 to 2018 were reviewed and included in this study. Of the 62 cases, 47 were derived from necropsies and 15 were biopsies from the small intestine, seven of which were obtained by endoscopy. All samples were recovered from the collection of the pathology sector of the "Escola de Veterinária" (Veterinary School) at "Universidade Federal de Minas Gerais" (UFMG). The inclusion criterion was cases with a confirmed diagnosis of lymphoma in viable samples to perform all analyses. Cases with severe autolysis were excluded, except for two cases of mild autolysis.

Classification of the anatomical forms was made according to Couto (2001) as follows: 1) multicentric lymphomas that were characterized by generalized lymphadenopathy with the involvement of the liver, spleen, and bone marrow; 2) mediastinal lymphomas that occurred at mediastinal location with enlarged lymph nodes; 3) gastrointestinal lymphomas that included focal, multifocal or

diffuse neoplastic infiltration of the gastrointestinal system with or without intra-abdominal enlarged lymph nodes; and 4) extranodal lymphomas, which were characterized by the involvement of any other organ or tissue (per example, renal, neural, ocular, and cutaneous).

Tissues were sectioned at 3μm thickness and subjected to routine staining [hematoxylin and eosin (HE)] and immunohistochemistry (IHC) according to the ABC streptavidin-biotin-peroxidase methodology (DAKO, California, USA).

Samples were labelled by incubating with an anti-CD3 antibody (CD3-12 clone, polyclonal, 1:300, University of California, Davis/CA, USA) and an anti-CD79a antibody (HM47/A9 clone, monoclonal, 1:500, DBS, Pleasanton/CA, USA), overnight inside a humid chamber at 8^0C. After 16 h of incubation, the slides were incubated with a secondary antibody for 30 min. All reagents were used according to the manufacturer's instructions (Novolink Kit – Leica).

Colorimetric development was performed using diaminobenzidine (DAB; Novolink Kit Leica) for 3 (CD3) or 2 min (CD79) (diluted 50μl of DAB to 1ml of diluent). Counter-staining was performed using hematoxylin. A lymph node from a healthy dog was used as control. Slides were examined using a 40× standard microscope objective, distribution and percentage of the stained cells were evaluated, and the predominant cell type was determined. All cases were classified according to the criteria established by the WHO (Vezzali et al. 2010, Valli et al. 2011, 2013, Wolfesberger et al. 2017, 2018).

Six sections (4-um thick) of each of the 62 paraffin-embedded neoplastic tissues (corresponding to 62 cases) were stored in microtubes and DNA extractions were performed with the commercial Quick-DNA/RNA formalin-fixed paraffin-embedded (FFPE) Kit (Zymo Research), following the manufacturer's recommendations. Of the 62 cases from which DNA was extracted, viable DNA was obtained only in 28 cases and was used for performing semi-nested PCR. Proviral DNA was detected by amplifying the U3 region using semi-nested PCR with actin-β PCR as endogenous control (Souza et al. 2018) and standardized using the primers described by Suntz et al. (2010) (Table 1). DNA from the leukocyte layer of centrifugated blood of an FeLV-infected domestic cat was used as a positive control.

External reaction was composed of 2μl of DNA, 0.36pmol/μL of FW-1 and Rev primers, 0.20mM of dNTP (LGC Biotecnologia, Brazil); 1.5mM of MgCl2 (Promega, USA), 5μl of 5X Green GoTaq Flexi Buffer (Promega, USA), 0.06U/μL of Taq Gotaq DNA polymerase (Promega, USA) and sterile DEPC treated water to finalize 25μl. Reaction was performed in a thermal cycler Veriti™ 384 wells (Applied Biosystems, USA) with initial denaturation at 95°C (2 min), followed by 35 cycles of denaturation at 92°C (60 s), annealing at 54°C (30 s) and extension at 72°C (30 s). Final extension was performed at 72°C for 5 min.

Internal reaction involved 2μl of the amplified product of the external reaction, 0.36pmol/μL of FW-2 and Rev primers, 0.20mM of dNTP (LGC Biotecnologia, Brazil), 1.5mM of MgCl2 (Promega, USA), 5μL of 5X Green GoTaq Flexi Buffer (Promega, USA), 0.06U/μL of Gotaq DNA polymerase (Promega, USA) and sterile DEPC treated water to finalize the volume at 25μL. Reaction was performed as described before. Visualization of the amplified products was performed using 1.5% (w/v) agarose gel electrophoresis with ethidium bromide (1.5 mg/mL) using a 100 bp ladder (Ludwig Biotec).

Nucleotide sequencing of four of the amplified products was performed using the Sanger method. PCR Positive samples of each category had their bands extracted from the agarose gel using the GenElute™ Gel Extraction Kit (Sigma-Aldrich) following the protocol established by the manufacturer. Similarity of the sequences obtained was compared to those available in the Genbank using the NCBI BLAST program[5].

U3 partial sequences from FeLV Brazilian isolates 2, 4, 6 and 8 were aligned with the whole genome sequences from the GenBank (MH116005, MF681672, MF681669, MF681666, MF681665, MF681664, MF681668, MF681667, MH116004, MF681670, NC_001940, AF052723, KP728112, MF681671, MT1295, LC462187, AB060732, AB672612), using MAFFT.v7.471 L-INS-i with 1000 interactions (Katoh & Standley 2013). Sequences from the extremities were manually trimmed with AliViewV1.2.6 (Larsson 2014), resulting in a final alignment of 139. The phylogeny was estimated using MrBayesV3.2.7a (Ronquist et al. 2012), with 10 million MCMC replicates, 25% burnin, and the K80 substitution model, which was selected using Modeltest-NG.V.0.1.6, based on BIC (Darriba et al. 2020). Tree image was generated with ITOL online (Letunic & Bork 2007).

RESULTS

Data regarding sex, breed, and age were available for 48 cats. Of these, 56.2% (27/48) were female and 43.7% (21/48) were male, and 87.5% (42/48) of the cats had no defined breed; 8.3% (4/48) were Siamese and 4.1% (2/48) were Persian. The ages of these cats ranged from 10 months to 14 years (μ=8.70, σ=3.73). Cats lived in the metropolitan area of Belo Horizonte City and the majority were domestic pets.

Gastrointestinal was the most frequent anatomical form of lymphoma in 40% (25/62) of cats, followed by multicentric (29%, 18/62), mediastinal (17.7%, 11/62), renal (8%, 5/62), cutaneous (3.2%, 2/62) and cardiac (1.6%, 1/62). T-cell lymphomas were identified in 60% and B-cell lymphomas in 37% of the samples. It was not possible to determine the immunophenotype by immunohistochemistry in 3% of cases.

According to the World Health Organization classification, nine different lymphoma subtypes were identified (Table 2). The most frequently diagnosed subtypes were diffuse large B-cell lymphoma (DLBCL) (30.6%, 19/62) (Fig.1, 2, 3, and 4) and peripheral T-cell lymphoma (PTCL) (25.8%, 16/62) (Fig.5 and 6). DLBCL-related subtypes, such as T-cell rich B-cell lymphomas and thymic B-LCL, accounted for 3.2% (2/62) and 1.6% (1/62) cases, respectively. EATL2 (Fig.7 and 8) and EATL1 subtypes accounted for 14.5% (9/62) and 8% (5/62) of the cases, respectively. Four small T-cell lymphocytic lymphomas (6.4%), one B-cell lymphoplasmacytic lymphoma (1.6%), and one anaplastic large cell lymphoma (1.6%) were identified (Fig.9). In the case of anaplastic large cell lymphoma, immunohistochemistry was performed, but there was no labeling, which could be due to the autolysis of cells

[5] BLAST. Available at <https://blast.ncbi.nlm.nih.gov> Accessed on Jul. 25, 2020.

Table 1. Description of primers and size of PCR products for the FeLV provirus genes

Gene	Initiators (5'3')	Product size	Reference
U3 LTR -FeLV	FW-1: TTACTCAAGTATGTTCCCATG Rev: AGGTCGAACTCTGTCAACT FW-2: CTTGAGGCCAAGAACAGTTA	185 bp (external) 110 bp (internal)	Suntz et al. 2010

or longer fixation time of tissue in the formalin. In one case of T-cell lymphoma as well as in one case of renal lymphoma, it was not possible to perform the subtype classification, due to autolysis of cells, although proviral DNA was detected.

The anatomical distribution of the lymphomas and their histological classifications are shown in Table 3. In gastrointestinal forms, the most common subtypes were DLCBL (9/25) and EATL2 (9/25). Of the DLCBL subtype, two were in the stomach and seven in the intestine. All EATL2 subtypes were in the small intestine. For the multicentric form, PTCL was the most common subtype (8/18), followed by DLCBL (6/18). For the mediastinal form, the most common subtype was PTCL (7/11), followed by T-SLL (2/11). The extranodal form was observed at different sites, and the kidney was the most affected organ in this form, with PTCL (2/5) being the most common subtype.

DNA was obtained from paraffin-embedded tissue samples from 28 cases. Of these, 23 were FeLV positive corresponding to 82% of the sample size (Table 4). Considering anatomical forms, 39% of samples that had FeLV DNA detected were multicentric, 22% were mediastinal and extranodal forms and 17% were gastrointestinal. When only the samples of each anatomical form of the lymphoma were assessed, the FeLV proviral DNA was detected in 90% (9/10) of the multicentric, 83.3% (5/6) of the mediastinal, 100% (5/5) of the extranodal and 57.1% (4/7) of the gastrointestinal form. Regarding cell origin, 43% were T-cell lymphoma and 47% were B-cell lymphoma.

The most frequent detection of FeLV proviral DNA was that of DLBCL (32.1%, 9/28 of the cases that had viable DNA extracted) and PTCL (28.5%, 8/28 of the cases that had viable DNA extracted). T-cell rich B-cell lymphoma, thymic large B-cell lymphoma, and T-SLL subtypes had the same frequency (3.5%, 1/28) of the cases that had viable DNA extracted. One case of T-type lymphoma could not be classified due to autolysis and was classified as undefined.

Phylogenetic Bayesian analysis showed that FeLV Brazilian isolates 2, 4, 6 and 8 formed a separate clade closely related to isolates MH116004, MF681670 and KP728112 (Fig.10). Brazilian isolates had 98% to 99% similarity to the Feline leukemia virus strain Glasgow-1, which was recorded in GenBank (KP728112.1).

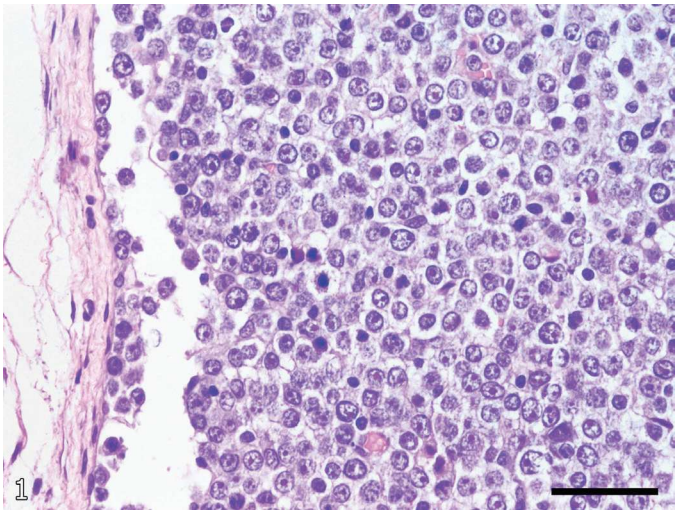

Fig.1. Cat, lymph node. Multicentric lymphoma: diffuse large B-cell lymphoma (DLBCL). Cell nuclei are large (more than two times the size of a red blood cell) and round (some nuclei have indentations) with a prominent, single, and central nucleolus (immunoblast). HE, obj.20x. Bar = 100µm.

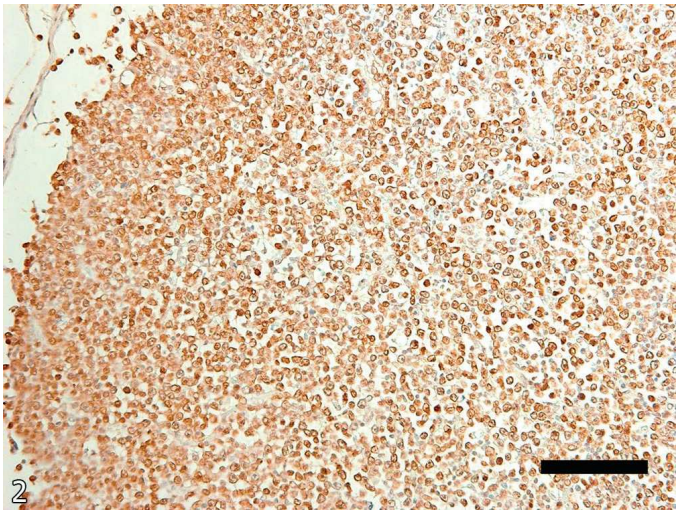

Fig.2. Cat, lymph node. Multicentric lymphoma: diffuse large B-cell lymphoma (DLBCL). Positive for cytoplasmic immunolabeling for the CD79 antibody of neoplastic B-cells. IHC, obj.20x. Bar = 100µm.

Table 2. Feline lymphomas classified according to the WHO classification

Abbreviation	Subtype	N	%
DLBCL	Diffuse large B-cell lymphoma	19	30.6%
T-cell rich B-LCL	T-cell rich B-large cell lymphoma	2	3.2%
Thymic B-LCL	Thymic B-large cell lymphoma	1	1.6%
PTCL	Peripheral T-cell lymphoma	18	29%
EATL1	Enteropathy-associated T-cell lymphoma 1	5	8%
EATL2	Enteropathy-associated T-cell lymphoma 2	9	14.5%
T-SLL	small T-cell lymphocytic lymphomas	4	6.4%
LPL	B-cell lymphoplasmacytic lymphoma	1	1.6%
ALCL	Anaplastic large cell lymphoma	1	1.6%
Undefined		2	3.2%
TOTAL		62	100%

DISCUSSION AND CONCLUSION

Cat lymphomas containing FeLV proviral DNA were frequent in the present study. According to other Brazilian studies, there are geographical regions considered endemic for this virus (Cristo et al. 2019, Leite-Filho et al. 2020). As described by Victor et al. (2020), the Brazilian FeLV proviral DNA obtained in our study were also closely related to the Glasgow1 (GenBank- KP728112.1) reference strain. These data support the predominance of this strain infecting cats in the same endemic study area.

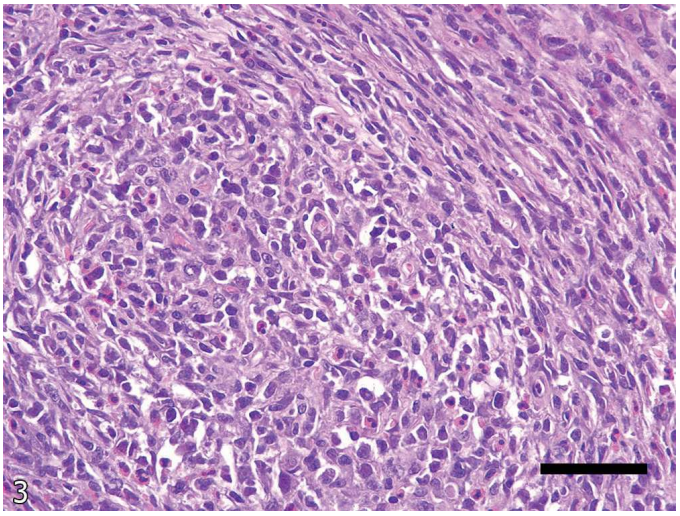

Fig.3. Cat, small intestine. Gastrointestinal lymphoma: anaplastic diffuse large B-cell lymphoma (anaplastic DLBCL) in which the neoplasm expands in all layers of the intestine (transmural). The cells are large (greater than two times the diameter of the red cell), pleomorphic, with elongated, round, oval nuclei (some edentate) and moderate to abundant cytoplasm. The nucleoli are evident and multiple. Some eosinophils are observed among neoplastic cells. HE, obj.40x. Bar = 50µm.

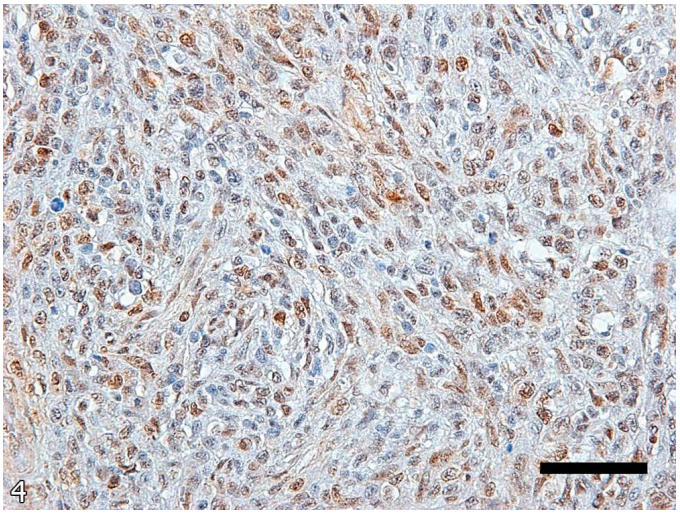

Fig.4. Cat, small intestine. Gastrointestinal lymphoma: anaplastic diffuse large B-cell lymphoma (anaplastic DLBCL). Positive for cytoplasmic immunolabeling for the CD79 antibody of neoplastic B-cells. IHC, obj.40x. Bar = 50µm.

PCR has been widely used as a standard test for the diagnosis of FeLV infection, because it's high sensitivity (Hofmann-Lehmann et al. 2001, Coelho et al. 2011, Chang-Fung-Martel et al. 2013, Victor et al. 2020). It has been proposed that the integration of FeLV proviral DNA into the cat's genome is sufficient for the transformation and development of lymphoma (Abujamra et al. 2006, Fujino et al. 2008). This hypothesis is supported by previous studies that associated the development of lymphoma with the detection of proviral DNA in tumors that were negative for the FeLV antigen by IHC (Jackson et al. 1996, Gabor et al. 2001, Weiss et al. 2010). Based on the aforementioned studies and data on the high FeLV infection rate in cats from the study area (Coelho et al. 2011, Victor et

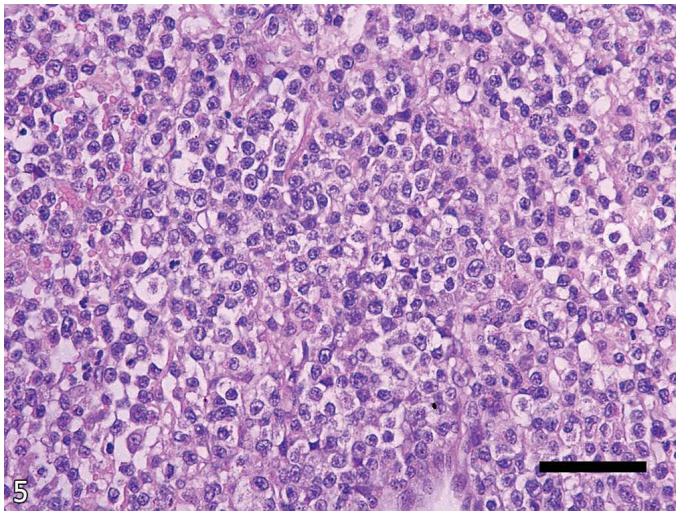

Fig.5. Cat, mediastinal lymph node. Multicentric lymphoma: peripheral T-cell lymphoma (PTCL). Dense and diffuse infiltration of neoplastic cells. The large cells are characterized by a round to oval nucleus, some with edentations. Chromatin is loose with single to multiple nucleoli evident. HE, obj.40x. Bar = 50µm.

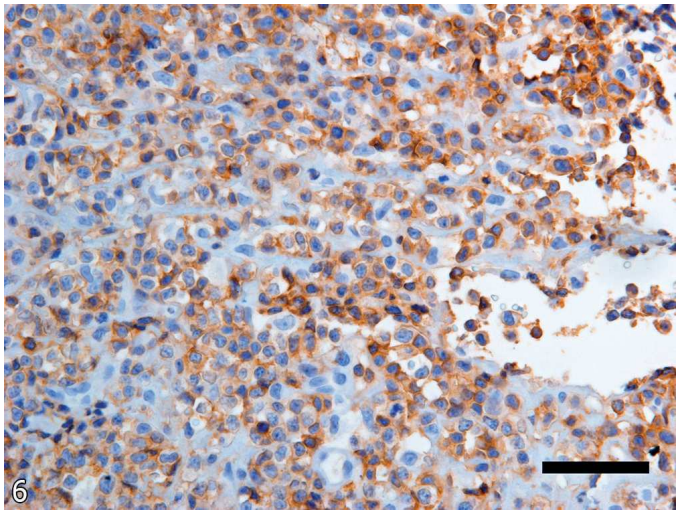

Fig.6. Cat, mediastinal lymph node. Multicentric lymphoma: peripheral T lymphoma (PTCL). Neoplastic T-cells are diffusely positive for cytoplasmic immunolabeling for the CD3 antibody. IHC, obj.40x. Bar = 50µm.

al. 2020), an association between lymphoma and FeLV proviral DNA was observed in cats in the present study.

In the current study, PCR was preferred to detect proviral FeLV DNA because it is potentially more sensitive than IHC in detecting FeLV infection. PCR, in addition to detecting the FeLV provirus in any of the stages of progressive (productive infections) and latent (without viral protein production) infection, allows the detection of a low viral load (Jackson et al. 1993). The IHC technique performed on lymphoma tissue is sensitive for the identification of viral antigens in productive infections (progressive), as performed by Leite-Filho et al. (2020). Thus, the ideal method for investigating and staging FeLV infection in lymphoma tissue is to perform IHC and PCR concurrently to identify the progressive and latent infections present in this neoplasm.

For extraction of high-quality DNA from tissue samples embedded in paraffin, fixation time is a determining factor, because formalin may cause crosslinking of cytosine nucleotides on either DNA strand. As a result, during PCR, the Taq-DNA polymerase fails to recognize cytosine and incorporates an adenine in the place of a guanosine, creating an artificial

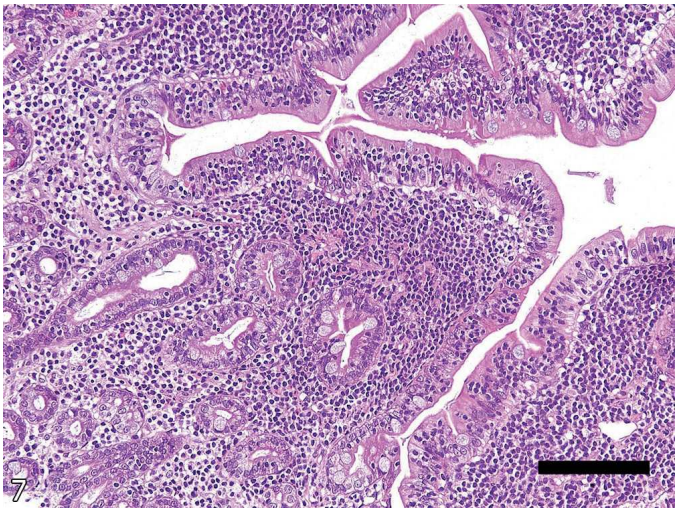

Fig.7. Cat, small intestine. Gastrointestinal lymphoma: enteropathy associated T-cell lymphoma type 2 (EATL2) infiltration of neoplastic lymphocytes in the lamina propria with evident nests and rare plaques of lymphocytes among the epithelial cells. The cells are small, and the nuclei are round and dense. HE, obj.20x. Bar = 100µm.

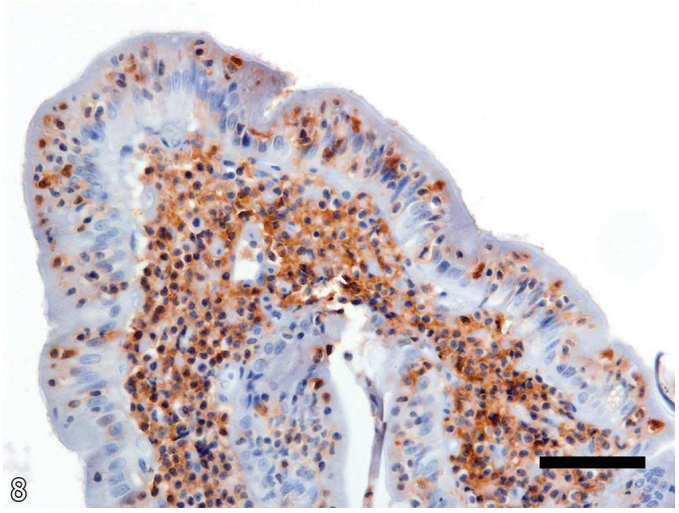

Fig.8. Cat, small intestine. Gastrointestinal lymphoma: enteropathy associated T-cell lymphoma type 2 (EATL2). Positive for cytoplasmic immunolabeling for CD3 T lymphocyte neoplastic cells. Immunohistochemistry significantly increases the ability to assess the epitheliotropism of neoplastic cells. IHC, obj.40x. Bar = 50µm.

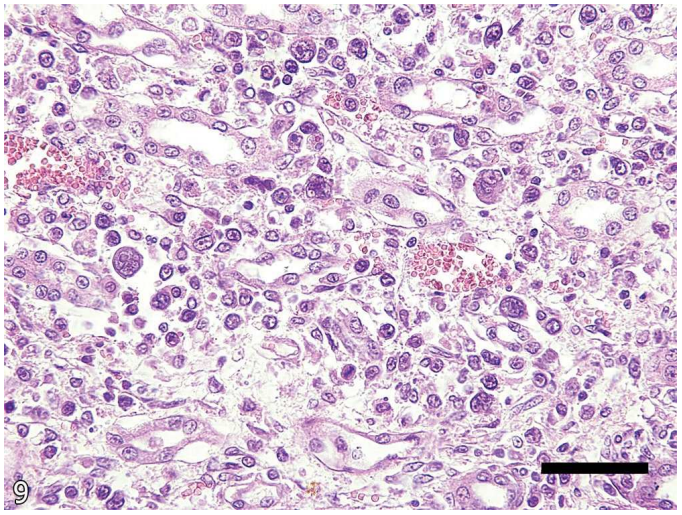

Fig.9. Cat, kidney. Extranodal lymphoma: anaplastic large cell lymphoma (ALCL). The cells are multivariate, large, with bizarre, multilobulated, multiple, edentulous nuclei and others with horseshoe shapes; the nucleolus is evident. HE, obj.40x. Bar = 50µm.

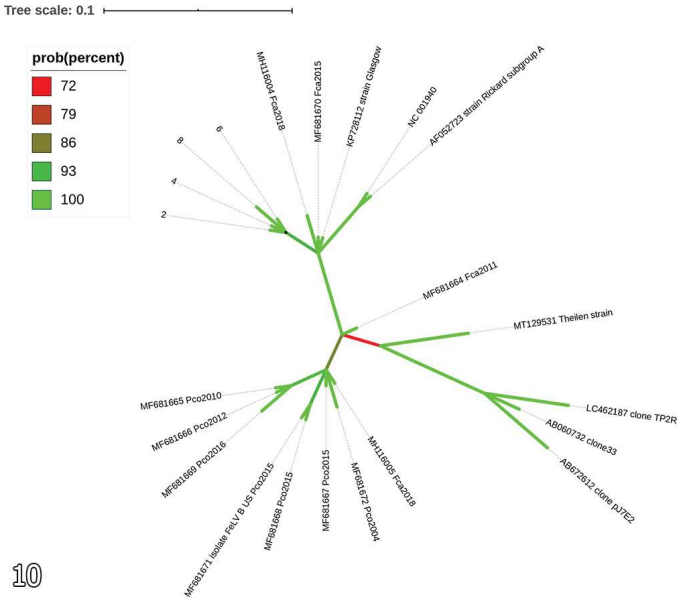

Fig.10. Bayesian phylogeny of the Brazilian FeLV isolates. Unrooted Bayesian phylogenetic tree based on the FeLV U3 partial sequence. In this image, branch support is represented in a scale from red to green, and the branch lengths are scaled based on sequence distances.

mutation between cytosine-thymine (C-T) or guanine-adenine (G-A) (Hamoud et al. 2007). Although this factor may have contributed to low positivity, considering all samples used in the present study, the high percentage of PCR positivity in FFPE tissue samples is similar to other studies in countries that were endemic for FeLV (Canada), with an occurrence of >70% positivity for proviral DNA (Jackson et al. 1993, 1996). The results of the present study indicate that FeLV infection, is highly common in cats with lymphoma in the metropolitan region of Belo Horizonte.

Regarding the anatomical forms of lymphoma in cats, in our study, a higher frequency of gastrointestinal, multicentric and mediastinal forms was observed. These results differ somewhat from those of other studies in Europe and the United States, where a lower incidence of multicentric and mediastinal forms of lymphoma has been reported (Meichner et al. 2012). Since the implementation of vaccination programs for the control of FeLV in these countries, gastrointestinal lymphoma has been reported to be the most commonly recognized anatomical form. This anatomic form is not frequently associated with FeLV infection (Leite-Filho et al. 2020). Other studies in Brazil have shown a higher frequency of multicentric (Cristo et al. 2019), gastrointestinal (Leite-Filho et al. 2020) and mediastinal forms (Cristo et al. 2019, Leite-Filho et al. 2020), similar to our findings.

FeLV proviral DNA was more frequent in multicentric and mediastinal lymphomas in this study. This result corroborates the findings of other studies (Rojko et al. 1989, Vail et al. 1998, Couto 2001, Cristo et al. 2019). In Brazil, high immunoreactivity to FeLV in mediastinal lymphomas has been described (Cristo et al. 2019, Leite-Filho et al. 2020), demonstrating the high association between FeLV infection and this type of lymphoma. Based on epidemiological data, this relationship between mediastinal lymphoma and FeLV was observed to be

Table 3. Anatomical distribution, histological classification and FeLV status of 62 feline lymphomas

Anatomical distribution *n* (%)	Histological classification	n	FeLV provirus DNA positive PCR
Gastrointestinal	DLBCL	9	2/2(100%)
25 (40%)	T-cell rich B-LCL	2	1/1(100%)
	EATL1	5	0/3 (0%)
	EATL2	9	1/1(100%)
Multicentric	DLBCL	6	4/4(100%)
18 (29.0%)	PTCL	8	2/2(100%)
	T-SLL	2	1/2(50%)
	ALCL	1	1/1(100%)
	T-cell lymphoma	1	1/1(100%)
Mediastinal	DLBCL	1	1/1(100%)
11 (17.7%)	PTCL	7	3/3(100%)
	T-SLL	2	0
	Thymic B-LCL	1	1/1(100%)
Extranodal	DLBCL	1	1/1(100%)
Renal	PTCL	2	2/2(100%)
5 (8%)	Renal lymphoma	1	0
	LPL	1	0
Cutaneous	DLBCL	1	0
2 (3.2%)	PTCL	1	1/1(100%)
Cardiac	DLBCL	1	1/1(100%)
1 (1.6%)			

Table 4. Lymphoma subtypes of the 28 cases in which it was possible to extract DNA

Lymphoma subtype	*n*	Samples with FeLV proviral DNA within each subtype
DLBCL	9 (32.1%)	9/9(100%)
T-cell rich B-LCL	1 (3.5%)	1/1 (100%)
Thymic B-LCL	1(3.5%)	1/1 (100%)
PTCL	8 (28.5%)	8/8 (100%)
EATL2	1 (3.5%)	1/1 (100%)
ALCL	1 (3.5%)	1/1 (100%)
T-SLL	1 (3.5%)	1/2 (50%)
Undefined	1 (3.5%)	1/1 (100%)
EATL1	0 (0%)	0/3 (0%)

common in several countries in the 1980s, when there was no vaccination and effective prevention against viral infection. Currently, the prevalence of FeLV antigenemia among cats with lymphoma was approximately 70% (Fabrizio et al. 2013).

The multicentric form of lymphoma is closely related to FeLV infection, in which the presence of proviral DNA has been described (Jackson et al. 1993, Weiss et al. 2010). Other studies with multicentric lymphomas have demonstrated high immunoreactivity for FeLV antigen (Cristo et al. 2019, Leite-Filho et al. 2020). The results of this study corroborate these findings, considering that 90% of multicentric lymphomas are positive for infection.

The extranodal form was positive for FeLV proviral DNA in more than 50% of cats. This result corroborates some studies that detected FeLV proviral DNA in 25- 42% of renal lymphomas (Vail et al. 1998, Gabor et al. 2001). The only skin lymphoma tested in our study was FeLV-positive. Despite divergent results in the literature, a high proportion of FeLV provirus in these lymphomas has already been reported (Weiss et al. 2010).

The gastrointestinal form had the lowest occurrence of FeLV provirus in the current study. The low ratio in this form is well documented in the literature, having little association with FeLV antigenemia (Barrs & Beatty 2012). However, when considering the detection of proviral DNA, no consensus has been reached yet with proviral DNA detected in different ratios (Gabor et al. 2001, Weiss et al. 2010, Russell et al. 2012). Including intestinal biopsy samples tended to increase the incidence of gastrointestinal lymphoma cases in this study. This may have interfered with the actual occurrence of this anatomical form in the cat population inhabiting the metropolitan region of Belo Horizonte. Therefore, the frequency of gastrointestinal lymphoma may be lower than reported in the present study.

The most common immunophenotype in the present study was T-cell (60%). Some authors have reported immunophenotype B as the most common (Gabor et al. 1999). Minimal difference was seen between the results related to the immunophenotype and the presence of the FeLV provirus. Association between phenotype and the presence of FeLV were described in a higher proportion for positive B-cell compared to T-cell lymphomas (Gabor et al. 2001). However, contrasting results have been described in other study, with a higher occurrence of the FeLV provirus detection in T lymphomas, followed by B and non-B or -T lymphomas (Weiss et al. 2010).

B-cell tumors were generally considered FeLV-negative (Hardy & Macewen 1989, Rojko et al. 1989, Rezanka et al. 1992). However, until the 1990s, there was no reactive B-cell marker, which compromised the most appropriate investigation of feline B-cell lymphoma (Jackson et al. 1996). *In vitro* research has shown that FeLV not only transforms mature T-cells, but also immature or prothymocyte cells, null cells and possibly auxiliary T-cells and monocytes. Later, a study associated feline lymphoma immunophenotype with more specific B-cell markers and the presence of FeLV in neoplastic cells (Rojko et al. 1989). Furthermore, another investigation identified the phenotype and the FeLV antigen by IHC, in addition to the FeLV provirus using PCR. These results indicated that B-cell lymphomas were as frequent as T-cell lymphomas when IHC for FeLV antigen and PCR for FeLV proviral DNA were performed (Jackson et al. 1996). In the present study there was no difference in the proportion of T and B-cell lymphomas positive for FeLV proviral DNA.

For subtype classification of lymphomas in cats, based on the WHO classification, the most common subtypes in our study were DLBCL and PTCL, followed by T-cell intestinal lymphomas. These results were similar to those of another study in Brazil (Leite-Filho et al. 2020) and differ from the findings reported by Wolfesberger et al. (2017), in which the PTCL subtype was more common, followed by DLBCL and T-cell intestinal lymphoma.

There is only one study associated with lymphoma subtypes and following the WHO classification, with detection of FeLV provirus and immunolabeling of FeLV viral antigen (Weiss et al. 2010). In that study, DLBCL and EATL subtypes had the highest proportion of proviral DNA occurrence. In our study, FeLV proviral DNA was detected in only one case of the EATL2 subtype. However, for the DLBCL subtype, the proportion of FeLV proviral DNA was higher.

The main limitation of this study was the lack of epidemiological data on the animals evaluated, such as the information about vaccination against FeLV and whether they had outdoors access. In addition, there is a lack of information on the clinical history of the disease and serological diagnosis with staging of the infection. Therefore, these factors limited an adequate epidemiological and clinical study to relate the findings of molecular analysis of the proviral FeLV DNA in the lymphoma tissues of these cats.

In this study, histopathology, immunohistochemistry, and PCR enabled the characterization of nine lymphoma subtypes, according to the WHO guidelines, and the identification of FeLV provirus. DLBCL was the most common subtype. Genetic material from the neoplastic tissues was not recovered for all cases; nevertheless, the rate of FeLV proviral positivity in association with the other analyses, enabled us to consider some important considerations. FeLV proviral DNA is highly associated with multicentric and mediastinal lymphomas and strongly suggests that FeLV infection contributes to the development of these anatomical forms. More extensive epidemiological studies are necessary to characterize the relationship between lymphomas and FeLV, and influence of the lymphoma subtype on the survival rate of animals. In addition, it is important to investigate other viruses, that are known or suspected to be involved in the development of lymphomas, such as FIV and feline gammaherpesvirus, and identify their role along with FeLV in the development of lymphoma.

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