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DIFFERENTIATION BETWEEN FELINE INTESTINAL T-CELL LYMPHOMA FROM INFLAMMATORY BOWEL DISEASE BY POLYMERASE CHAIN REACTION FOR ANTIGEN RECEPTOR REARRANGEMENI (PARR)

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Abstract

Intestinal lymphoma is a very common malignancy in cats, classified in low-grade intestinal T-cell lymphoma and high-grade intestinal T-cell lymphoma. Even the exact cause of the intestinal lymphoma remains uncertain, there may be a connection between persistent intestinal inflammation, such as inflammatory bowel disease (IBD), and the development of this tumor. Thus, differentiation between inflammation and low-grade lymphoma is always a challenge. This study included 22 cats with digestive syndrome featured by recurrent vomiting and diarrhea which have been also unresponsive to treatment. Full thickness intestinal biopsies from living animals and tissue samples from dead animals have been considered for routine cytopathological and histopathological diagnosis. Polymerase chain reaction for antigen receptor rearrangement (PARR) for T-cell CD3 region of the TCRy chain was used to differentiate lymphoma from non-lymphoma lesions. Cytological and histological findings have been represented by a residual heterogeneous population consisting of neutrophils, eosinophils and small mature lymphocyte, to which is added a dominant contingent of small- to medium-sized or large lymphocytes. Mesenteric lymph nodes contain characteristic cells which were consistent for incipient malignant lymphoid proliferation. PARR test discriminated 11 cases of T-cell lymphoma showing strong performance for discrimination of lymphoma from IBD.

Key words: *intestinal bowel disease, lymphoma, PARR.*

ABBREVIATIONS

EATL II- Feline enteropathy-associated T-cell lymphoma, type II
FFPE- formalin-fixed and paraffin-embedded
FNA- fine needle aspiration
HGITL- high grade intestinal T-cell lymphoma
IHC- immunohistochemistry
IgG1- immunoglobulin G1
IgG2- Immunoglobuline G2
IgH- immunoglobulin heavy chain
IgM- immunoglobulin M
LGITL- low-grade intestinal T-cell lymphoma
LPE- lymphoplasmacytic enteritis
PARR- polymerase chain reaction (PCR) to assess antigen receptor gene rearrangements
PBS- phosphate buffered saline
QC- quality check
TCL- T-cell lymphoma
TCR -T cell receptor
TRB- T cell receptor betta
TRD- T cell receptor delta
PCR- Polymerase chain reaction
V(D)J gene- genetic diversity joining

INTRODUCTION

The gastro-intestinal tract is the most frequently affected by neoplastic and inflammatory process in cats with an 80 percent of malignancy (Andrews, 2016). Gastric tumors in cats are highly common and varied in embryologic origin, including leiomyosarcomas, lymphomas, adenocarcinomas, intestinal mast cell tumors, gastro-intestinal stromal tumors (GISTs), plasma cell tumors, leiomyomas, adenomatous polyps, and adenomas (Kehl, 2022; Moulton, 2009; Peter 2005; Roth, 1990; Valli, 2000).
Lymphoma remains the single most frequent type of cancer experienced by cats as well as the most common feline gastric neoplasm (Richter, 2003).
Intestinal lymphoma is classified in low-grade intestinal T-cell lymphoma (LGITL) and high-grade intestinal T-cell lymphoma (HGITL), including an un- controlled proliferation of lymphocytes (Barko, 2023; Mc Lear, 2003; Kiupel, 2011).

Throughout maturation, lymphocytes generate specific antigens receptors by re-modelling the V(D)J sections of T-cell and B-cell receptor genes (TCR ɣ [T-cell receptor gamma gene] and immunoglobulin heavy- chain gene [IGH]), thus making them polyclonal at these genetic loci (Hardy, 1981; Van Dongen, 2003).

In T-cell lymphoproliferative disorders, the neoplastic T-cell population shares the same TCR rearrangement pattern and serves as a marker for monoclonality (Burnet, 1976; Richter, 2003). Lymphomas, on the other hand, are caused by the clonal expansion of an individual progenitor cell, and it has a monoclonal receptor loci (Burnet, 1976; Mazur, 1983; Mosli, 2014). The lymphocyte receptors are oligoclonal or monoclonal in low grade lymphomas in cats (Andrews, 2016; Cheroutre, 2004; Kiselow, 2008).

Another cause of lymphoma in cats is the viral infection, such as the feline leukemia virus infection (FeLV), which causes lymphoid and myeloid tumors in domestic cats (Marsilio, 2023; Maunder, 2016; Moore, 2012; Vail, 1998).

There are two main hypotheses on the origin of variation of tumor cells: different sub-clones originate from different tissue stem cells and have their own transformation pattern (polyclonal concept), or different clones develop from the initial clone due to genetic or epigenetic changes during evolution (monoclonal concept) (Marusyk, 2010).

Monoclonal rearrangements reveal limited intra-tumoral heterogeneity in their beginning stages, which rises with tumor size, in comparison with polyclonal population which has substantial intra-tumoral heterogeneity initially, but become more homogeneous as they grow due to clonal growth (Ibragimova, 2017). In polyclonal rearrangements, the formation of a dominant clone proceeds afterwards followed by a decrease in clonal diversity due to the replacement of minor clones. This clone divergence and the generation of dominant clones with minor clones' substitution may occur concurrently in a tumor (Janiszewska, 2015; McLear, 2003).

Polyclonal lymphocyte population's origin refers to cells that have undergone several modifications in their early stages, which may remain or be removed, representing intra-tumoral heterogeneity. The establishment of a dominant clone occurs later, being followed by a decrease in clonal diversity (Kreso, 2013).

Molecular analysis of monoclonal and polyclonal rearrangements of lymphocytes cells is an important key to diagnose the intestinal lymphoma, because morphologically, the intestinal tumors frequently include a polymorphous cell groups with different sizes (Ibragimova, 2017).

PARR has been created for the diagnosis of lymphoid neoplasia, being characterized by clonal proliferation of tumor cells with a rearranged immunoglobulin heavy chain (IgH) or T cell receptor gamma (TCR ɣ) gene in B-cell and T-cell lymphoid malignancies. It represents a major component of the diagnostic algorithm having the interest to differentiate feline inflammatory bowel disease from intestinal lymphoma, amplifying the T-cell receptor γ, but not having the role to determine the phenotype of lymphocytes involved in the processes (Andrews,2016; Jeffrey, 1993; Roth, 1990). PARR is an efficient and extremely sensitive approach (Holmberg, 1976; Van Dongen, 2003). The lymphocyte lineage determines antigen receptor loci rearrangement, which follows a precise sequence. T cells rearrange the T-cell receptor delta (TCR δ) locus first, followed by the TCR ɣ locus (Montañés, 2019; Valli,1981).

Routine microscopical investigation is a challenge, because of coexistence between lymphoma and inflammatory process or because of the progression of chronic inflammatory enteropathy to low-grade intestinal T-cell lymphoma (Moore, 2012).

MATERIALS AND METHODS

This study has considered 22 cats (Table 1) of different breeds and ages. The age of individual ranged between 9 and 14 years, all cats presenting digestive syndromes featured by recurrent vomiting and diarrhea (n=18). The patients presented also weight loss (n=19), icterus (n=2), pales mucous membranes (n=13), abdominal distension (n=6), and peripheral lymph nodes hypertrophy (n=12). Another clinical change observed as polyphagia was noted (n=3).

The weight of the cats included in this study was between 3,2 kg and 5,6 kg.
Abdominal ultra-sound has been used for all patients. Aditionally, blood complementary tests, ELISA for FeLV and FIV diagnosis, serum cobalamin test, cytopathological and histopathological exams, PCR for antigen receptor rearrangement (PARR), DNA extraction, genomic analysis, immunohistochemistry for CD3 T-cell expression, and clonality assessments have been applied.
The abdominal ultrasound examination was performed in all 22 cases, and the standardized images were realized with a linear transducer with high frequency (15 MHz).
Standard necropsy has been done in dead or euthanized animals (n=7) followed by gross examination and by tissue sampling for microscopical investigations. The mesenteric lymph-nodes and intestinal segments were sampled and fixed in 10% neutral buffered formalin for 48 hours. Sections of 4 μm thickness were stained with Hematoxylin and Eosin (HE stain).
Cytological examination was significant in association with histological examinations, distinguishing the lymphoma from inflammatory process. Fine needle aspiration and full thickness intestinal biopsies have been sampled from living animals (n=12) of intestine (duodenal, ileal or jejunal mucosa) and mesenteric lymph nodes. Surgical resection of the jejunum and mass with 5 cm margins and an end-to-end anastomosis were performed (n=3). After smear preparation, the slides were air-dried and dyed with Diff-Quick staining protocol.
Histopathological investigations have been used for both categories of samples (intestinal segments and mesenteric lymph nodes), as well as for the intestinal loops removed by enterectomy, using a routine protocol of staining and examination, with hematoxylin and eosin staining for histological sections imbedded in paraffin. Following necropsy, surgical resections, the intestinal and mesenteric lymph-nodes components were frozen at -20°C in small containers with formal saline CH_2O:NaCl (10:0.9)%, before realizing PARR, histopathological and immunohistochemistry examinations, and before the treatment administration.
PARR test was used for the definitive diagnosis of T-cell lymphoma, with the interest to differentiate lymphoma from non-lymphoma lesions, in all of 22 patients, in concordance with primer sets, and TCR γ.
The interest to use the two methods in PARR evaluation was to develop a classification algorithm to distinguish proteomic signatures of lymphocytic-plasmacytic inflammatory lesions in intestinal lymphoma in cats. Therefore, PARR was based on the distinction of monoclonal lymphomas from polyclonal benign or reactive tissues (Andrews, 2016; Rychlick, 2007).
The examination of ePARR on FFPE samples was realized before and after applying DNA QC method, the FFPE method being significantly faster and more efficient, using the technique analysis performed with microcapillary electrophoresis (Pareek, 2011).
For FFPE method in PARR analyze, the samples of 4 μm thickness were dissected and prepared in 10% neutral buffered formalin, being maintained 70 hours. The final samples were placed in pure toluene, and after in a mixed solution with 50% toluene and 50% paraffin for one hour. In PARR evaluation, the flow cytometry pellets method was used for all 22 cases, to analyze and differentiate the tumoral cells and the smears passing through one or more lasers while suspended in PBS solution, utilizing cell sorter that purified and identified the lymphocyte population which are tumoral, and after, it neutralized these lymphocytes into isotonic saline liquid containing 10% bovine serum. The final preparation with suspended cells was combined with 20 μL of antibody solution, allowing quick quantitative and qualitative expression of lymphocyte population.

CD3 immunophenotyping analysis

The methodology of immunostaining was used to identify the immune-phenotype T, the morphology of T-cells, and to make the difference between this immune-phenotype (T) and from B-cells, this test being applied in all 22 cases. To perform immunophenotyping of CD3 expression, on tissues simples, 5 μm sections from FFPE preparation tissues simples were cut and immune-stained with antibodies that recognize the CD3 antigen stain T cells.

Clonality assessment

The examination of ePARR on FFPE samples was realized before and after applying DNA QC method. Every tissue sample was homogenized, and DNA was extracted following the manufacturer's instructions of QIAamp DNA Mini Kits. T cell clonality was determined by PCR in all 22 cases. Each sample with a polyclonal or oligoclonal population of T cells was tested for B cell clonality using PCR.

To confirm the specificity and reliability (monoclonality, oligoclonality, polyclonality) of the all 22 samples, the PCR reactions from all of these were visualized applying electrophoresis method analysis, and the primer sets tests were used. The PCR products have been separated according to melting rather than the differences between N sequences at the V-J joining, resulting in a very accurate genetic imprint for each TCR-V rearranged allele. Genomic DNA was obtained using the DNA FFPE kit in conformity with the manufacturer's instructions. The quality, quantity, and efficiency of extracted DNA were determined using the extraction of genomic DNA from the lymph-node biopsy samples, frozen pellet cohort, Diff-Quik stained FNA imprints, according to the DNA mini Kit instructions.

The primers from PCR reactions have been analyzed with DNA protocol, after DNA amplification using PARR primers in parallel. The primers from 1 to 10 have been optimized, and fluorescently marked using an identification for alignment to the feline IGH and TRG genomic parts.

To assess sample DNA quality, control primer sets Cμ and γ-actin were applied.

Table 1. Sampling methods in the 22 patients

Breed	Number	Gender	Sampling methods
European Cats	7	Sterilized females	Intestinal biopsies
	3	Sterilized males	Intestinal biopsies
Siberian Cats	2	Sterilized females	Intestinal biopsies
European Cats	3	Sterilized males	Surgical resection
European Cats	5	Sterilized males	Necropsy
	2	Sterilized females	Necropsy

RESULTS

The integration of clinical signs, complementary blood exams, abdominal ultrasound examination, gross evaluations, cytological and histopathological investigations, PARR test, Immunohistochemistry, and clonality assessment data were necessary at multiple stages to distinguish tumoral process from the inflamematory process, being important to minimizing misdiagnoses.

A completed blood count test revealed an average approximative as: a-regenerative anemia (Htc = 20%) in 2/22 cats, regenerative anemia in 4/22 patients, hyperglobulinemia in 2/22 cats, neutrophilia 19.000/μL (reference ranges 10.000-30.000/μL), eosinophilia 2.270/μL (reference ranges 0.170-1.570/μL), monocytosis 10.270/μL (reference ranges 0.050-0.670/ μL), a lymphocytosis was revealed in 13/22 cats, and a thrombocytopenia in 4/22 cats. A serum biochemical profile revealed hypoalbuminemia (2.0 g/dl; reference ranges, 2.7-3.8 g/dl) due to chronical diarrhea, hyperglobulinemia (5.9 g/dl; reference ranges, 2.8-5.1 g/dl) all of these in 16/22 cats, and increased values of hepatic enzymes ALAT (220U/L, 10-130 U/L reference ranges) and PAL (440, 24-147 U/L reference ranges) and a hypercalcemia (7 mg/dL, 4.5-5.3 mg/dL reference ranges) in 6 of 22 patients. ELISA test for feline leukemia virus and feline immunodeficiency virus was tested in all of 22 patients and it presented negative results.

The echography examinations revealed in 10/22, thickening of the ileum and jejunal wall, and 12/22 reduction of luminal diameter of jejunum, 17/22 increased lymph nodes, 3/22 and gastric ulcerations, all of these being associated with clinical signs of gastro-intestinal disorders. In 2/22 patients, at the echography examination no modification at gastro-intestinal tract was observed.

Group I was represented by 11 of 22 cats diagnosed with IBD, whereas Group II is represented by 11 cats diagnosed with intestinal T-cell lymphoma. In Group II, 4 out of 11 patients were identified with HGITL, while 7 had been identified with LGITL.

Macroscopic alterations were observed during enterectomy surgery in duodenum, jejunum and ileonum in 5 cats from Group I and in another 7

from Group II, indicating a diffuse wall thickness of the intestinal tract, and an extended reddened fold in 3 out of Group I and in 7 out of Group II (LGITL) (Figure 2, Table 2).

The sizes of the jejunal lymph nodes were varied in cats diagnosed with LGITL (between 3.5 and 5.5 cm length, 0.3-4.8 cm length reference ranges and between 1-2.5 cm width, width reference ranges 0.2-1.0 cm width reference ranges), whereas the mesenteric lymph nodes were clearly enlarged (3 cm length, 0.1-2.0 cm length reference ranges, and 0.6 cm width, 0.1-1.0 cm width reference ranges) in 3 out of 4 cats with HGITL and in 3 out of 7 LGITL (Figure 3, Table 2).

During the necropsy, the evident lesions were severe with multifocal ulcerations of ileum (n=9 of Group II), of jejunum and duodenum (n=3), and nodular ulcerative masses and plaques were identified in duodenum and jejunum in 7 patients out 11 of Group II, having multifocal lesions aspects, spreading to mesenteric lymph-nodes, and determined multiple intestinal perforations (Figure 4, Table 2). The adjacent digestive mucosa reduced due to loss of mucosal folds. Among the 7 out of 11 patients diagnosed with LGITL, 5 had infiltration in the jejunum only, 3 had infiltration in the two areas of jejunum and the ileum, and 1 had infiltration just in the ileum.

In the necropsies, it was observed that in some cases of fibrinous peritonitis, the chronicity of the lesion with the formation of adhesions was found (n=3 HGITL).

Multiple malignant tumors were identified in the intestines (duodenum, jejunum), kidneys, urinary bladder, and lungs (n=4 HGITL), splenomegaly and hepatomegaly being remarked in lymphomas patients (Group II). In 4 out 11 lymphoma patients, the livers had a pale and a friable gross structure.

In HGITL cases, it was observed a necrotic tissue in jejunal wall, with an irregular surface, bowel ischemia in 3 out 4 cases. In 2 out of 4 HGITL patients, fusiform intramural jejunal and ileal lesions developed outward while the invaded muscle atrophied. All relevant described macroscopic lesions are illustrated in Table 2, including the localization of gastrointestinal segments.

Table 2. Macroscopic features of IBD and gastrointestinal lymphoma

Diagnosisis	Localization	Gross lesions
IBD	Duodenum Jejunum	thickened wall reddened folds
LGITL	Jejunum-Duodenum	thickened wall, small exophytic growth
	Mesenteric lymphnodes	enlarged mesenteric lymp-node
HGITL	Jejunum-ileum	adenopathy, ulcerating nodules, fusiform intramural lesion, bowel ischemia
	Mesenteric lymph-nodes	adenopathy, necrotic tissue plaques

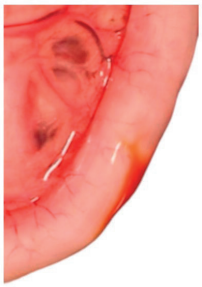

Figure 1. Enlarged intestinal segment

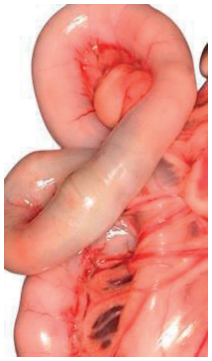

Figure 2. Enlarged small intestinal loops region in a 9 years old cat

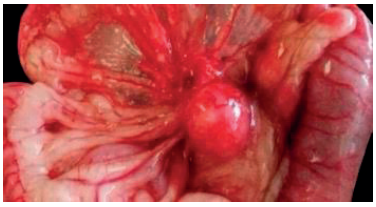

Figure 3. Enlarged mesenteric lymph node (central)

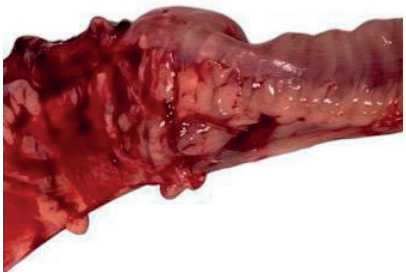

Figure 4. Massive enlarged and ulcerative jejunum mass

In all 22 cases, the cytologic diagnosis was in accordance with the gross and histopathologic examinations. Chronic enteritis was present in 11/22 cases, representing Group I, 6 patients of Group I were diagnosed with lymphocytic plasmacytic enteritis (LPE), while the rest of 5 cases of Group I had eosinophilic enteritis.

The grading system for the degree of inflammatory process was based on lymphocyte population sizes, presence of inflammatory cells, as follows, these being indicator for transition from inflammatory process to intestinal lymphoma lesions: mild inflammation had a few lymphocytes, the moderate inflammation which consisted in nests lymphocytes, and severe inflammation represented by the presence of small lymphocytes, in comparison with neutrophil size or with large lymphocytes (Table 3).

LPE was detected in 6/11 IBD cases and it was characterized by a moderate lymphocytic presence. Eosinophilic enteritis (EE), identified in 5/11 IBD cases, was characterized by a mild lymphocyte identification including eosinophils higher than 5 cells per 400x field. The mild inflammation comprised a few lymphocytes cells with numbered from 0 to 4 cells per 400x field, whereas the moderate inflammatory process consisted on a population of 5 to 20 lymphocytes per 400x field. The severe lesions included more than 20 lymphocytes per 400 × field. Low grade intestinal lymphoma was suspected in the sample which had homogenous and monomorphic population of small-medium-sized mature lymphocytes (Figure 5), an eccentric nucleus being noticed and representative in 7/11 cases, diagnosed with LGITL. The chromatin of the nucleus is less condensed that in the chromatin of mature lymphocytes. The nucleoli are indistinct or are missing. A moderately basophilic cytoplasm with several optically empty vacuoles, that extended to the nucleus regions were noticed. A small population of eosinophils was seen, and a few cells included fine azurophilic cytoplasmic granulations in small number, similar to chromaffin cells or potentially poorly granular mast cells (Figure 7). High-grade intestinal lymphoma was characterized by more than 30% homogenous cellular lymphocyte population. The population of medium to large round cells, well individualized, with a high nucleocytoplasmic ratio allowed lymphoid cells to be recognized, with spherical nucleus (Figure 6). This population is monomorphic, despite varied blast sizes and the presence of nucleolus, and the process was associated with neoplastic lesions, as identified in Figure 6. The lymphocyte's nuclei contained a fine stippled chromatin, with one or more small to medium-sized, spherical nucleoli, and a lightly reduced and basophilic cytoplasm, with azurophilic granulations, which indicated a raised fragility, and it was remarked at examination with magnification x1000.

Table 3. Cytological modifications in IBD, LGITL, HGITL

Diagnosis	Cytological modifications
IBD	LPE: -moderate lymphocyte infiltration; absence of eosinophils or mast cells EE: moderate lymphocyte infiltration; presence of eosinophils or mast cells
LGITL	-homogenous and monomorphic population -small matures lymphocytes -small-medium sized round and eccentric nucleus -less condensed chromatin - absent or distinct nucleoli -moderate basophilic cytoplasm
HGITL	-lymphocytic population -intermediate-large lymphocytes -medium-sized, round, irregular nuclei -multiple small-medium sized proeminant nucleoli -lightly to high basophilic cytoplasm

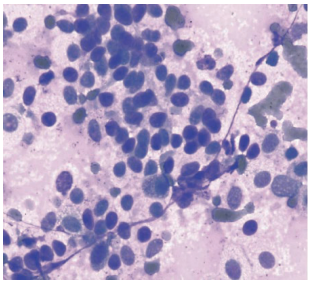

Figure 5. Small lymphocyte population (Diff-Quick staining, x1000)

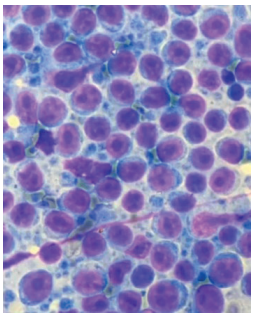

Figure 6. Population of granular lymphocytes, with anysokorya, giant nuclei, basophilic cytoplasm, and sometimes clear areas near the nucleus (May, Grünwald, Giemsa; X1000)

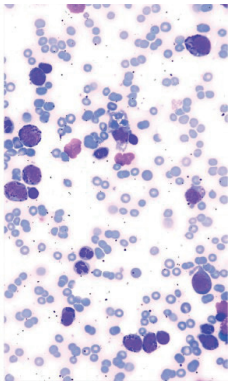

Figure 7. Ileum cells with azurophilic cytoplasmic granulations (Diff-Quick staining, x1000)

Histological examination of duodenal, jejunal, and ileal segments established a diagnosis of inflammatory bowel disease, low-grade or high-grade intestinal lymphomas, in correlation with cytologic and macroscopic analysis and findings for all patients. LPE was identified in the duodenal and jejunal samples, represented by non-neoplastic population of lymphocytes, being framed as mild in one cat, moderate in four cats, and severe in one cat. In the mild lesions of LPE, the histology structure of intestines was not modified. In the cases with moderate lesions, a marked and diffuse to severe focal agglomeration of lymphocytes was observed, with an unchanged histological structure of intestine. Eosinophilic enteritis was identified as with mild infiltration of eosinophils and dominant lymphocytic population. Some transmural eosinophilic infiltrates and muscle hypertrophy were detected in jejunum and ileum segments.

Low-grade intestinal lymphoma was marked by infiltration of small lymphoid T-cells population in the jejunal and ileal segments (Figure 8). In LGITL samples, the cellular group is represented by small T-cells groups arranged in plates, localized inside the villous epithelium. The regions revealed significant lymphocytic cryptis (6 out 7 LGITL) and neutrophilic cryptis in 5 out 7 LGITL patients. Villous atrophy was identified in all 7 LGITL patients. A discrete reactive follicular hyperplasia was noticed, and no invasion of the lymph-node parenchyma or peripheral tissue was observed.

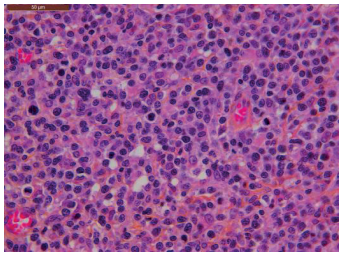

Figure 8. Duodeno-jejunal segment- Diffuse infiltrate with tumoral lymphocytes (Hematoxylin and Eosin staining, x300)

Specific to high-grade intestinal lymphoma were transmural lesions, which affected tunica muscularis along with severe perivascular lymphocytic infiltration, involving tunica muscularis and spreading into the serosa (Figure 10). High-grade intestinal lymphoma had determined the severe mucosal alterations, including blunt villi, and crypt effacement (Figure 10). In 3 out 4 HGITL patients, the lymphoid infiltrates comprised large lymphocytes, in the remaining case, the infiltrate was composed of small to intermediate-sized lymphocytes (Figure 9).

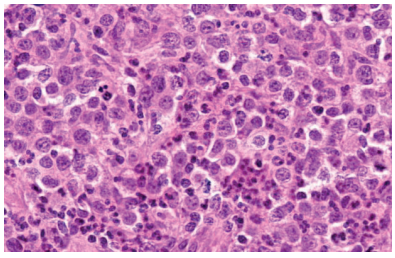

Figure 9. Infiltration with small-intermediate sized lymphocytes (Hematoxylin and Eosin staining, x300)

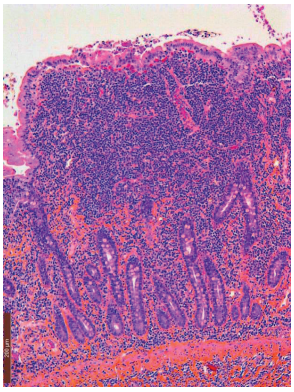

Figure 10. High grade intestinal invasive lymphoma-jejunal and ileal segments (Hematoxylin and Eosin staining, x200)

The PARR test was performed to identify T-cell lymphoma versus non-lymphoma, by monoclonal properties of T-cells population. Table 4 and Figure 11 summarized the PARR performance (sensitivity, specificity, and accuracy) in all 22 cases, in concordance with QC, and DNA extraction. Each DNA sample was amplified using various PARR primers simultaneously, however only QC (quality control) pass resulted in a high percentage of accuracy: in FFPE method, 100% ePARR negative in all 11 IBD cases, 100% ePARR positive in 11 lymphomas out 22. In Flow cytometry pellets method, the accuracy was calculated at 81% e-PARR negative in 9/11 IBD, and 90%-ePARR positive in 10/11 lymphomas. DNA for FFPE method was optim for PARR accuracy evaluation, having a high quality. The polymerase chain reaction for antigen receptor rearrangement (PARR) for the T-cell CD3 region of the TCRγ, utilized in all 22 cases, had as results 11 out 22, diagnosed with inflammatory bowel disease classified in Group I and 11 out 22 cases with intestinal lymphoma, comprised in Group II.

PCR test was performed in all 22 samples. In 11 out 22 samples, the PCR for B-cells identified polyclonal population, the presence of B-cells being significative. The final diagnosis was non-neoplastic lesions. For the rest of 11 cases, the PCR for the B-cells was negative, while the T-cells were dominant. Of 11 samples with monoclonal T-cell population, one sample had the dual clonality for T and B-cells, influenced by a polyclonal background, but the final diagnosis was given as T-cell intestinal lymphoma, being based on correlation with immunohistochemistry (CD3) and on primer sets peak's tests.

By DNA extraction, the values of each sample were ranged from 1 (degraded DNA) to 10 (intact DNA), the results from primer sets 1-10, in combination with Qc and Cμ revealed 11 out 22 samples scored with rang 10, represented by T-cell primer sets, being diagnosed with T-cell lymphoma. The rest of 11 revealed less than rang 3 of DNA input. The presence of B-cells was identified in 2 out 11 (non-lymphomas samples) graded with rang 3 and 2 of DNA. The recombination of genes identified that in 11 out 22 cases, the lymphoma processes provided between V and J genes.

The clonal primer sets improved the diagnosis in concordance with TRG genomic regions. TRGγ primers determined higher monoclonal peaks (1-10) in 11 out 22 cases, the final result being scored as lymphoma lesions. The polyclonal rearrangements had the patterns of Ig genes and TRGγ genes in 11 out 22 cases, being considered as non-lymphoma processes.

In the 11 lymphoma samples, the primer of sequences the both V and J primers for TRGγ genes, which influenced the sensibility and accuracy for this final diagnosis.

The monoclonal rearrangements were represented in 11 out 22 samples by a bigger size band in width with 1 or 2 evident sharp bands with the same size, in comparison with polyclonal populations, the test being realized with electropherograms method. The polyclonal samples had one or multiple small size in width bands with a normal distribution in electropherogram test, with clearer size regions, in the rest of 11 samples, non-lymphoma lesions. In one out 22 cases, the electrophoresis method assessment has shown a monoclonal sample with taller and bigger size band in width, having a few polyclonal peaks localized basal, with variable sizes, representing oligoclonality.

The primer sets from 1 to10 were analyzed with implemented QC method based on adequate input DNA, having a final result 10 out 22 T-cell specific primer sets, and 11 out 22 polyclonal primer sets, while 1 out 11 lymphomas presented T-cell primer sets on a polyclonal background, with 1 to 3 peaks.

The primer sets from 11-14 checked in concordance with γ-actin method revealed 11 T-cell intestinal lymphoma out 22 samples.
The CD3 exam matched with IgH and TCR γ, identifying the antigen region of T-cell receptor in 11 out 22 samples containing the T-cell receptor sequence. The lymphoma cells were positive for cytoplasmic CD3 (Figure 12), with medium sized atypical lymphocytes.

Table 4. PARR performances

Methods control primer/ Diagnostic	IBD	Lymphomas
DNA QC pass - FFPE	Sensitivity:100%- ePARR negative in 11/11 IBD Specificity: 100%- ePARR negative in 11/11 IBD Accuracy: 100%- ePARR negative in 11/11 IBD	Sensitivity:100%- ePARR positive in 11/11 lymphomas Specificity: 100%- ePARR positive in 11/11 lymphomas Accuracy: 100%- ePARR positive in 11/11 lymphomas
DNA QC pass- Flow cytometry pellets	Sensitivity: 90%- ePARR negative -in 10/11 IBD Specificity: 81%- ePARR negative -in 9/11 IBD Accuracy: 81%- ePARR negative in 9/11 IBD	Sensitivity: 90%- ePARR positive in 10/11 lymphomas Specificity: 81%- ePARR positive in 9/11 lymphomas Accuracy:90%- ePARR positive in 10/11 lymphomas

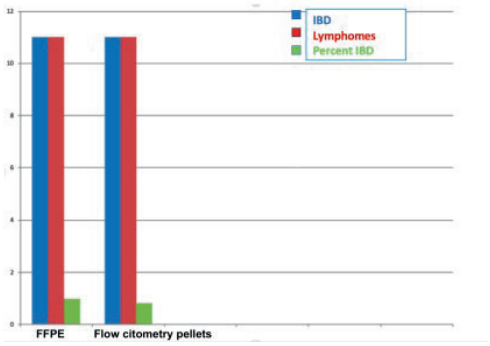

Figure 11. Accuracy of ePARR assay with FFPE and Flow cytometry methods

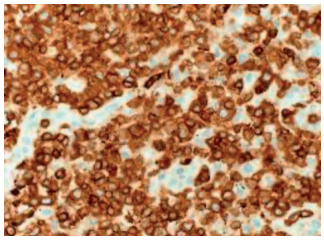

Figure 12. CD3 cytoplasmic positivity for T-cell lymphoma (x300)

DISCUSSIONS

The present study showed that the correlation between clinical, cytological, histological, macroscopically and complementary examinations as PARR test and clonality evaluation, in correlation estimate diagnosis, prognosis, and therapy in IBD and intestinal lymphoma in felines.
Previous studies have indicated that thickening of the small intestine in felines is a significant indicator for distinguishing inflammatory bowel disease and lymphoplasmacytic gastrointestinal inflammation from high-grade intestinal lymphoma (Freiche, 2021). In our study, the difference of thickness of different intestinal segments was significative and representative for IBD, LGITL and HGITL. Researches in the main pathogen causes related to IBD and feline intestinal lymphoma permit greater comprehension of the same illness in humans, showing diagnostic and prognostic indicators (Paulin, 2018).
Increased clonality analysis in human medicine developed from expanding the variety of targeted loci to include TRβ, TRδ, TRγ among others, to compensate for the reduced sensitivity of individual analyses. In veterinary medicine, assays for different objectives have not been used and described for the moment in many studies. The standardization of clonality testing across institutions was a significant advancement in human medicine. Current researches in veterinary medicine demonstrated that LPE processes are often polyclonal, while LGITL patients with percent bigger than 90 illustrated clonal or oligoclonal TCRγ gene rearrangement (Moore, P., F., 2005, Keller, 2016). In our study, the clonality analysis was used in all 22 patients, within 11/22 showing polyclonal rearrangement and the rest of 11/22 monoclonal rearrangement, but just one has shown oligoclonal arrangement.
Polymerase chain reaction for receptor antigen rearrangement is currently the only one technique that can be applied to FFPE tissue samples, and thus it is the most often conducted procedure for cats with enteritis.

CONCLUSIONS

The PARR test is an important method for a definitive diagnosis of monoclonal lymphomas in intestinal neoplasms in cats, versus polyclonal benign or reactive tissues and it revealed in 11/22 the presence of monoclonal rearrangement of the T-cell ɣ receptor gene.
A successful amplification of a control primer quality was observed in FFPE method, having 100% accuracy in diagnosis of lymphomas.
The jejunum and ileum were the most frequently affected regions in all cases of study.
The predominant intestinal cells in 11/22 cases are represented by T-cells, being compatible with Type II Enteropathy in cats.
This article reinforces the importance of PARR laboratory method to accurately diagnose the intestinal lymphoma.
The improvements in immunohistochemical and clonality examinations have increased our therapeutic and results assurance, after a closer diagnosis, as follows: 10/11 of Group I were completely healed after a variable period of treatment, and 3/11 of Group responded to chemotherapic treatment.

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