Canine lymphoma, epidemiology and diagnosis

SUMMARY OF THE PhD THESIS

PhD student Roxana Cora (Popa)

Scientific coordinator Prof. univ. dr. Cornel Cătoi
INTRODUCTION

Lymphoma is a malignant type of tumor originated from lymphoreticular cells. This type of neoplasia was defined as solid tumor of the immune system (MORRISON, 2004; PETROV et al., 2009). In dogs, lymphomas represent between 7% and 24% of all types of tumors and approximately 83% of malignant neoplasias of hematopoietic system (KÄSE, 1981; MOULTON and HARVEY, 1990; VAIL et al., 2013). The dogs most predisposed to lymphoma are the average age and old ones, with a higher incidence of this type of tumor in dogs between 5.5 and 9 years old (BABA and CÁTOI, 2007). Recent studies show that females have a lower risk to develop lymphoma in comparison to males (VILLAMIL et al., 2010; VAIL et al., 2013). A higher predisposition to lymphoma was identified in some dog breeds as: Boxer, Chow Chow, German Shepherd, Saint Bernard, Scottish Terrier, Basset Hound, Airedale Terrier, English Bulldog, Beagle, Golden Retriever, Poodle (VONDERHAAR and MORRISON, 2002; MORRISON, 2004). Canine lymphoma has a multifactorial etiology and most of it is still unknown (VAIL et al., 2013). Among the possible causes of this type of neoplasm are: herbicides, as (2,4-D) phenoxyacetic acid, low frequency magnetic fields, toxic and radioactive waste, cigarette smoke, some genetic disorders, and some viruses (HAYES et al., 1991; HAHN et al., 1994; REIF et al., 1995; MORRISON, 2004; MARCONATO et al., 2009; PASTOR et al., 2009; MILMAN et al., 2011). Canine lymphomas are classified according to anatomical localization, histologic criteria and immunophenotype characteristics. Approximately 80-84% of the dogs with lymphoma develop the multicentric form, which is characterized by generalized peripheral lymphadenopathy (MADEWELL and THESEN, 1987; VAIL et al., 2010; VAIL et al., 2013). World Health Organization has conceived a system for clinical staging of lymphomas, considering the extension of lesions and the absence or presence of clinical symptoms (VAIL, 2003). To diagnose this type of tumor several exams may be performed: imagistics, cytologic, histologic, immunohistochemistry and molecular (SÖZMEN et al., 2005; KINNS and MAI, 2007; VAIL et al., 2010; VAIL et al., 2013; VALLI et al., 2017). The prognosis for dogs diagnosed with lymphoma is variable and it depends on several factors that influence the response to the therapy. For example it was confirmed that patients with T-cell lymphoma had a shorter remission period and a lower survival rate, than the ones with immunophenotype B lymphoma (RUSLANDER et al., 1997). Considering the fact that canine lymphoma is a systemic disease it necessarily a systemic treatment (chemotherapy). Most of the combined chemotherapy protocols used for dogs therapy were developed in the past 15-20 years (MacEWEN et al., 1977; VAIL, 2011).
THE STRUCTURE OF THE THESIS

In Romanian veterinary literature there is few of incomplete data regarding canine lymphoma. So, taking into consideration the fact that among this specie is a high incidence tumor and most of the times the diagnosis is established by an easy cytological exam, we chose to perform a more documented study for this type of neoplasia.

The thesis entitled "Canine lymphoma, epidemiology and diagnosis" contains 144 pages and it is written according to the rules suggested and it is structured in 2 parts.

The first part, the review of the literature, is structured in 5 chapters and it contains 26 pages. In this part of the thesis I have synthesized the general knowledge regarding epidemiological aspects of canine lymphoma, etiology of this group of neoplasms, anatomical and histological classification, diagnosis, prognosis and the treatments use for this type of neoplasm.

In the second part that contains 74 pages and it is also structured in 5 chapters I have detailed the personal research performed between 2013 – 2017. Every chapter is structured in subchapters that show the aim and the objectives, materials and methods that have been used, the results and discussions regarding the novelty of them, compared to other studies that have been performed and the partial conclusions for each study. The results of the research were illustrated in 36 figures and syntheized in 11 tables. The end of the thesis is represented by the references that have been cited (286 titles).

RESULTS OF RESEARCH

In the second part of this thesis I have studied the canine lymphomas that were diagnosed at the Discipline of Pathology, Necropsy and Forensic Veterinary Medicine from the Faculty of Veterinary Medicine, Cluj-Napoca (Romania) and we have established the following aims:

- realization of an epidemiological study regarding canine lymphoma for a 12 years period
- investigation of some cases of lymphoma regarding macroscopic, cytological and histological aspects
- evaluation of this type of neoplasm using modern ways of diagnosis as immunohistochemistry and molecular exams (PCR-PARR)
classification of canine lymphomas according to the system implemented by the World Health Organization

- evaluation of some prediction markers as mitotic index, Ki67 cell proliferation index, nuclear size (large diameter, perimeter and area) and clinical stage.

**Chapter 7**, entitled "Canine lymphoma – epidemiology and morphology" aimed to carry out an epidemiological study over a 12-year period and the evaluation from the macroscopic and histological point of view the main lesions encountered in diagnosed lymphomas. 139 cases of canine lymphoma with different anatomical forms, males and females of different breeds, aged between 1 and 15 years were analyzed for this study. Thus, these cases were diagnosed either histopathologically (in the case of biopsies and corpses) or cytological (in the case of aspirate). Epidemiological data were obtained from the records of the discipline of Pathology, Necropsy and Veterinary Forensic Medicine at the Faculty of Veterinary Medicine, Cluj-Napoca. During the necropsy, the main lesions specific to lymphoma were highlighted, and 10% (pH 7) formol samples from the modified tissues were then collected and fixed for 24 hours. The tissues were then processed to perform the histopathological examination, being included in paraffin and stained by the usual Hematoxilin-Eosin technique. Following macroscopic and histopathological examination, we noticed that lymphoma in the dog is a very aggressive tumor with a high degree of metastasis, affecting various organs in the thoracic and abdominal cavity, but also other tissues and organs. The lymph nodes, spleen, tonsils, liver, bile, pancreas, intestine, kidneys, lungs, myocardium, skin, adrenal glands, encephalus, bone marrow and blood are some of the organs and tissues most affected by this neoplasm. In the 12-year study period (2005-2016), lymphoma had an incidence of 8.03% of all tumor pathology in the dog, so it can be considered one of the most important haematopoietic neoplasms in this species. Approximately 80% of the canine lymphomas were represented by the multicentric form and the rest were classified in descending order as follows: cutaneous (7.91%), digestive (6.47%), mediastinal (5.04%) and extranodal (3.6%). Most of the dogs with multicentric lymphoma were diagnosed with stage III (39.25%), stage IV (43%) and stage V (17.75%), and most of them were classified in clinical substation b (65.42%), because they have presented clinical symptoms. We noticed a slightly higher incidence of males (55%) compared to females (45%), and in terms of dog age, the most affected were individuals in the 6-9 year age group, with an average age of about 8 years. Among the breeds most likely to develop this tumor are: mixed-breeds (21.05%), Rottweiler (15.79%), German shepherd (8.27%), Boxer (4.51%) and Pekingese (4.51%).

**Chapter 8**, entitled "Differential cytological diagnosis in canine lymphoma", includes the cytological evaluation of fine-needle aspirate samples obtained from dogs diagnosed with different types of lymphadenopathy, including multicentric lymphoma.
but also from various round skin tumors (lymphoma, plasmocytoma, mastocytoma and histiocytoma). In this chapter I have presented some of the most important cytological features that differentiate canine lymphomas from other limfonodal and cutaneous lesions. For this study 75 lymphoid cytological samples and different skin tumors were collected from dogs of different breeds, males and females aged between 7 months and 14 years. Cytological preparations obtained by fine needle aspiration technique were then stained by Dia Quick Panoptic and Wright methods. Following the interpretation of cytological samples from lymph node aspirates, these were classified as follows: lymphoma, multicentric form 33.34%, lymphonodal reaction 18.67, purulent lymphadenitis 5.33%, lymph node reaction associated with eosinophilic lymphadenitis 1.33%, various metastatic tumors in lymph nodes 10.67%. The round cells skin tumors were: 1.33% cutaneous lymphoma, 1.33% cutaneous plasmocytoma, 9.33% cutaneous histiocytoma and 18.67% cutaneous mastocytoma. Fine needle aspiration has been shown to be a simple, early and rapid diagnostic procedure, which is extremely useful for differentiating round skin tumors and different lymphadenopathy in the dog, relying on the cytological characteristics specific to each type of lesion in part.

Chapter 9, entitled "Classification of canine lymphomas according to the World Health Organization system", aims to classify cases of canine lymphoma diagnosed in the discipline of Pathology, Necropsy and Veterinary Forensic Medicine, according to the system proposed by the World Health Organization (WHO). To achieve this, we included 44 cases of canine lymphoma with different anatomical forms. Tissue samples were included in paraffin after the standard protocol and then cut and stained by the Hematoxylin-Eosin method. We also performed the immunohistochemical examination by the automated method for each case using the following antibodies: antiCD3 (clone LN10) specific for T lymphocytes and antiCD20 (clone MJ1), antiCD21 (clone 2G9), antiCD79a (clone 11E3) are specific for type B lymphocytes. Based on anatomic location, histopathological aspects and immunohistochemical examination results, the classification of the lymphomas studied was performed. Of the total lymphomas, 65.91% (29 cases) had phenotype B, 31.82% (14 cases) were diagnosed with immunophenotype T and 2.27% (1 case) were null phenotype (possibly originated in natural killer lymphocytes - NK), the results being similar to those obtained in other studies. Canine lymphomas with immunophenotype B predominated in spleen (100%), multicentric (80%), mediastinal (60%) and central nervous system (100%), while digestive lymphomas (100%) and most of the cutaneous lymphomas (55.55%) originated in type T lymphocytes. Lymphomas with null immunophenotype were only diagnosed in multicentric form, with a 4% average. After determining the anatomical type and the immunophenotype of each lymphoma, the lymphomas were classified according to the WHO system based on histopathological characteristics (nodular or diffuse tumor growth, nuclear dimension,
detailed nuclear morphology, mitotic index). Of all cases, large B-cell diffuse lymphomas (52.28%) prevailed, followed by unclear T-cell peripheral lymphoma (9.1%). The remaining cases were classified as follows: marginal zone lymphoma (6.82%), lymphoplasmocytic lymphoma (2.27%), plasmacytoma (4.54%), lymphoblastic T lymphoblastoma (4.54%), intestinal T cell lymphoma 6.82%), epitheliotrophic cutaneous lymphoma (6.82%) and non-epitheliotropic T-cells lymphoma (4.54%) and aggressive NK cell lymphoma (2.27%).

Chapter 10, entitled "Predictability assessment of some morphological markers in canine lymphoma", includes the evaluation of predictive markers such as the mitotic index, Ki67 expression, clinical stage and nuclear morphometry (large diameter, perimeter and area) in the diagnosis of canine lymphoma. The biological material consisted of 25 lymphoid tissue specimens harvested during necropsic examination in dogs with multicentric lymphoma. Depending on the extent of the tumor in different organs and tissues, the clinical stage (from I to V according to the WHO system) was established for each case of lymphoma. The tissue fragments were processed by standard paraffin technique, after which tissue sections were obtained which were stained by the Hematoxylin-Eosin technique and others were used to perform the immunohistochemical examination using the anti-Ki67 antibody (clone ab15580), by automatic method. The histopathological examination aimed at determining the mitotic index and assessing the nuclear morphometry. Evaluation of the Ki67 proliferation index was performed following the brown marking of various tumor cell nucleus intensities. The data obtained following the evaluation of the predictive markers were statistically processed using the Sperman correlation test. The multicentric lymphomas included in the study were staged as follows: stage III (12%), stage IV (36%) and stage V (52%). None of the dogs were diagnosed at the onset of the disease, in clinical stages I and II. In terms of mitosis, 80% of cases had an average mitotic index of less than or equal to 9. Approximately half of the lymphomas (48%) had an average Ki67 expression of between 41% and 60%. 32% of the lymphomas had mean Ki67 expressions ranging from 21% to 40%, and the remaining 20% had an average Ki67 greater than 60%. A 44% of lymphomas had nuclei with a mean diameter of less than 6 μm, an average perimeter of 18 to 32 μm and a mean area less than 30 μm². The remaining 56% had nuclei with a mean diameter between 6 and 11 μm, a medium perimeter of less than 18 μm and an average area of 30 to 65 μm, respectively. In the present study there was a moderate correlation between Ki67 expression and clinical stage of lymphomas, but we noticed the lack of correlation between nuclear morphometric parameters (large nuclear diameter, perimeter, area) and the Ki67 cell proliferation index. We also found a correlation between the mitotic index and the clinical status of the studied cases. Nuclear morphometric parameters had much higher values in lymphomas with increased mitotic index than those with...
low mitotic index. The predictive markers included in this study (Ki67 expression, clinical status, mitotic index, large nucleus diameter, nuclear area and perimeter) can be used successfully to characterize and determine the aggressiveness of canine lymphomas, being real use in formulating a prognosis and in making an optimal therapeutic decision.

**Chapter 11**, entitled "Diagnosis of canine lymphoma by detection the clonal rearrangement of the antigen receptor gene and the immunohistochemical examination", aimed to evaluate the immunohistochemical and molecular examination of cases of canine lymphoma. By using the molecular and polymerase chain reaction (PCR) technique, we wanted to highlight the rearrangement of the antigen receptor gene (PARR) and tumor cell cloning, and to determine the lymphoma phenotype in the dog. By the immunohistochemical examination (IHC) we established the immunophenotype of the same cases of lymphoma, and finally we correlated the results of the two diagnostic methods. In the present study, tissue samples were collected from 24 dogs diagnosed with various anatomical lymphoma forms, which were processed by the standard paraffin inclusion technique, after which histopathological stains were obtained by the Hematoxylin-Eosin method. For the PCR test, we included 2 tissue samples, considered negative controls, from a dog diagnosed with metastatic melanoma in the lymph node and from another one without alterations. The immunohistochemical test was performed by the automated method using the anti-CD3 antibody specific for T lymphocytes and antiCD20, antiCD21, antiCD79a antibodies for type B lymphocytes. For the PCR analysis in order to detect the rearrangement of the antigen receptor gene (PARR), the DNA was extracted from the 24 lymphomas and the 2 negative controls. The extraction was made from fresh tissue (7 cases of lymphoma and 2 negative controls) and from tissue included in paraffin (17 cases of lymphoma). For each sample, the CDR3 region of the antigen receptor genes: TCRγ for T-lymphocytes, major IgH and minor Ig lymphocytes for B lymphocytes was amplified. After the immunohistochemical examination, the lymphomas were subdivided according to the immunophenotype as follows: lymphomas origin in B lymphocytes (66.66%), immunophenotype T lymphomas (29.18%) and null phenotype (4.16%). Immunophenotype B lymphomas predominated in multicentric form (84.62%), mediastinal (60%) and central nervous system (100%). Most cutaneous lymphomas (66.66%) and those with gastrointestinal tract localization (100%) originated in T-cells lymphocytes. In the PCR test, the reaction to detect clonal rearrangement of the antigen receptor gene was positive in 100% of the lymphomas from which fresh tissue was analyzed, but the positivity was 41.18% in the canine lymphoma samples from which it was used for the extraction the paraffin tissue. Approximately one-third of all cases analyzed revealed clonal rearrangement of the immunoglobulin gene (29.17%), another third of the lymphomas
showed cross-clonal rearrangement (both for IgH and TCRγ) (29.17%), and the remaining cases of lymphoma were negative (41.66%). Approximately half of the lymphomas with immunophenotype B (43.75%) and those with null phenotype (100%) showed a positive correlation between the results of the IHC and PARR examinations. Cross-reactivity with PARR was identified in both T-type lymphomas (42.86%) and Immunophenotype B lymphomas (25%).
SELECTED REFERENCES


