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*Original article*

# High agreement of routine cytopathology and immunocytochemistry in canine lymphomas

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## Abstract

The aim of this study was to compare the concordance of immunophenotype established with routine cytopathology (slides stained with Giemsa solution) and immunocytochemistry according to CD3 and CD79 alpha immunoreactivity. The study was performed on cytological samples of 70 canine lymphomas subtyped on the basis of the updated Kiel classification system. Additionally, cytologic samples were examined immunocytochemically for the CD3 and CD79 alpha antigens presence and thus immunophenotype of neoplastic growth was confirmed. The cytopathological and immunocytochemical diagnoses were then compared; in order to measure the concordance between immunocytochemistry (IC) result and Giemsa stain result of the same sample the Cohen's kappa coefficient was calculated. On the basis of the results of immunocytochemistry of 70 cases of canine lymphoma examined 42 were recognized as B cell lymphoma and 28 as T cell lymphoma. Full accordance between the results of routine cytopathology and IC was obtained in 63 out of 70 examined dogs (90% of cases). It can be concluded that cytopathological examination of Giemsa stained smears is helpful in determining the lymphoma cells immunophenotype. Additionally, it seems that combination of routine cytopathology and immunocytochemistry in cases of canine lymphomas allows to obtain the precise diagnosis in 90% of cases, and allows to receive most important information that is necessary for planning of appropriate therapy and to determine prognosis. Finally, this routine procedure allowed to eliminate the need of collection of tissue samples during surgery or core-biopsy and thus time, cost and patient discomfort related to more complex and invasive medical procedures can be easily reduced.

**Key words:** canine lymphoma, cytology, immunocytochemistry, immunophenotype

## Introduction

Lymphomas are one of the most common malignancies recognised in dogs and a major clinical manifestation in these tumours is single, regional or systemic lymphadenomegaly (Foulner-Fleury et al. 2002,

Pastor et al. 2008, Sapieryński et al. 2010). Cytopathological examination of lymph node samples done in such cases allow to diagnose a lymphoma in majority of cases, and additionally also subtype the tumor into low-grade or high-grade lymphoma class (Teske and van Heerde 1996, Foulner-Fleury et al.

1997, Foulner-Fleury et al. 2002, Sozmen et al. 2005, Guija de Arespachochoga et al. 2007, Sapieryński 2010, Sapieryński et al. 2010). The assessment of the updated Kiel classification, in which the inclusion of cells morphology allows to establish the immunophenotype of neoplastic cells, provides very important information that is useful in treatment planning, its outcome and general prognosis. Unfortunately, in some cases in the establishment of the lymphoma cells immunophenotype additional methods of staining (i.e. histopathology and immunohistochemistry) of tissue samples are required, although as previous studies revealed it is possible to make immunocytochemical staining or flow cytometry of cytologic samples obtained by fine-needle biopsy according to CD3 and CD79alpha antigen presence (Fisher et al. 1995, Vail et al. 1997, Caniatti et al. 1999, Sozmen et al. 2005, Sapieryński 2010, Comazzi et al. 2011). It seems that immunophenotyping of lymphoma based on staining of cytologic samples collected via low invasive methods can be an excellent alternative to histopathologic examination of tissue samples that are usually obtained during general anesthesia. It seems also, that immunocytochemistry (IC) allows to avoid repeated biopsy in uncertain cases. Although, numerous studies focusing on cytological classification of canine lymphoma exist, particular analysis of efficacy of routine cytopathology (Giemsa stain) in immunophenotyping of canine lymphoma cells is lacking. The aim of this study was to compare the concordance of immunophenotype established basing on routine cytopathology (slides stained with Giemsa solution) and immunocytochemistry for CD3 and CD79 alpha antigens.

## Materials and Methods

The study was performed on cytological samples collected from 70 dogs, with recognised lymphoma that were submitted to Department of Pathology and Veterinary Diagnostic, Faculty of Veterinary Medicine, Warsaw University of Life Sciences (SGGW).

Samples for cytological examination were obtained by fine-needle aspiration or fine-needle nonaspiration biopsy of peripheral lymph nodes, body cavities lymph nodes (mediastinal, abdominal), affected internal organs (liver, spleen), and pathologic masses in serosal cavities. From pathological masses, internal organs affected (liver, spleen) and enlarged lymph nodes detected within body cavities at least five smears were made from every case. In cases of peripheral lymphadenomegaly at least two enlarged lymph nodes were examined and at least four samples from each examined lymph node were taken. For routine

examination in every case at least 3 smears of aspirates were dried, fixed in 70% methanol, stained with Giemsa stain and examined by light microscope. Collected smears were examined by light microscopy according to main types of cells present and final cytopathologic diagnosis was established. Diagnosis of lymphoma was established according to the updated Kiel classification (Foulner-Fleury et al. 1997, Sozmen et al. 2005), assuming that at least 80% of the cells present on slides are blastic cells (excluding small cell lymphomas in which characteristic cell morphology was observed in at least 90% of cells). Based on the same updated Kiel classification system subtype of lymphoma with suggestion of immunophenotype (B cell lymphoma or T cell lymphoma) was established (Sozmen et al. 2005). In cases when cytology did not allow to establish subtype and immunophenotype of examined lymphoma cells diagnosis was described as indistinctive.

For immunocytochemical assays at least two smears from every case examined were stained as it was described previously (Caniatti et al. 1996, Sapieryński 2010). Immunocytochemical stains were conducted using commercially available antibodies (Dako® Denmark) to the pan T-lymphocyte marker CD3 (Polyclonal Rabbit Anti-Human) and B cell antigen receptor complex CD79 alpha (Monoclonal Mouse Anti-Human). One smear was stained with anti-CD3 antibody, and second was stained with CD79 alpha antibody. B cell lymphoma or T cell lymphoma was recognised if at least 80% of cells revealed expression of CD79 alpha or CD3, respectively.

The cytopathological and immunocytochemical diagnoses were then compared; in order to measure the accordance between immunocytochemistry result and Giemsa stain result of the same sample the Cohen's kappa coefficient was calculated. The calculations were following the formulas, for kappa by  $\kappa = p_o - p_c / 1 - p_c$ , where  $p_o$  is observed agreement, and  $p_c$  is a chance agreement, and for the approximate 95% confidence interval for kappa by  $SE(\kappa) = \sqrt{p_o(1 - p_o) / (n(1 - p_c)^2)}$ .

## Results

On the basis of routine cytopathology B cell lymphoma was diagnosed in 37 of examined cases and T cell lymphoma was diagnosed in 30 of examined cases; in 3 other cases subtype and immunophenotype of tumors were not established and the diagnosis was indistinctive. On the basis of the results of immunocytochemistry 42 of 70 cases of canine lymphoma (60%) were recognized as B cell lymphoma and 28 (40%) as T cell lymphoma. Additionally,

Table 1. Detailed data on preliminary diagnosis (based on Giemsa stained slides only) and final diagnosis (based on Giemsa stained slides and IC) in cases with any type of discrepancy. (CblPpsc – Centroblastic pleomorphic predominantly small cell; Pmsl – Pleomorphic mixed small and large cell; UTcell – Unclassifiable T cell).

No.	Preliminary diagnosis	CD 79a expression (% of cells)	CD3 expression (% of cells)	Final diagnosis
1.	CblPpsc	5%	95%	UT cell
2.	Pmsl	90%	10%	CblPpsc
3.	Clear cell	90%	10%	Lymphocytic
4.	Clear cell	85%	15%	Prolymphocytic
5.	Equivocal (small blastic cells)	5%	95%	Lymphoblastic T
6.	Equivocal (small cells)	5%	95%	Pleomorphic small cell
7.	Equivocal (large and small cells)	90%	10%	CblPpsc

low-grade lymphomas were recognized in 16 (22,8%) cases and high-grade lymphomas were recognized in 54 (77,1%) cases. Full accordance between the results of routine cytopathology and IC was obtained in 63 out of 70 examined dogs (90% of cases). Some discrepancies between cytopathology and immunocytochemistry results were noted in 7 out of 70 cases (10% of all cases). In 4 cases the subtype and immunophenotype of lymphomas established on the base of Giemsa stained slides were different than immunophenotype of lymphomas confirmed according to immunocytochemistry. In other 3 cases during routine cytopathology the diagnosis of subtype and immunophenotype of tumors were only suspected; immunocytochemistry allowed to confirm previous cytopathological diagnosis in all of these cases. Detailed data on preliminary diagnosis based on Giemsa stained slides examination and final diagnosis based on Giemsa stained slides and immunocytochemistry results in cases with any type of discrepancy are listed in Table 1. Among lymphoma cases with some discrepancy between routine cytopathology results and IC most of the tumors consisted of small lymphocytes without characteristic morphology. As for the measure of the agreement between immunocytochemistry and Giemsa stain results the calculated kappa value was equal to 0.802 (0.731, 0.873).

## Discussion

Correct diagnosis of lymphoma in veterinary patients and detailed characteristics of recognized growth are essential for professional discussion with animal's owner focused on sufficiency of proposed medical treatment and long-term prognosis (Marconato et al. 2011). Unfortunately, even in cases when besides routine cytopathology some additional tests are introduced to diagnostic process, final diagnosis

can be reached in majority, but not in all animals diagnosed. As recently published study revealed, even histopathologic examination of lymph node samples conducted by experienced pathologists allow to obtain correct final diagnosis in most, but not all cases of canine lymphoma (Valli et al. 2011). Besides clinical staging and histologic grade, also cells' immunophenotype in cases of canine lymphomas is one of the most prognostically important tumors' features (Valli et al. 2011). Presumed immunophenotype of lymphoma cells can be easily established on the basis of microscopic examination of slides stained with routine methods, but it can be difficult or impossible in some cases. Application of the immunostaining methods in diagnosis of canine cancers, especially in the case of lymphoma, is widely described in literature and the usefulness of lymphoma immunophenotype recognition during planning of therapy and prognosis determination is fully accepted in veterinary oncology (Fisher et al. 1995, Vali and Young 2007).

It seems to be reasonable that lymphoma diagnosis based on cytopathologic examination of cytologic samples collected during low-invasive fine-needle biopsy can be excellent alternative in cases when tissue sample collection during anesthesia cannot be performed due to various reasons. (Sapierzynski 2010, Comazzi and Gelain 2011). In human medicine cytopathological analysis of fine-needle aspirates of enlarged lymph nodes allowed to diagnose non-Hodgkins lymphoma in 80-93% cases, additionally in 67-86% patients the determination of the type and subtype of tumour is possible (Das 1999, Alam et al. 2010). However, in some difficult cases this differentiation is impossible or difficult and necessity of additional tests, and among others repeated fine-needle biopsy or histopathology, exists.

As present study revealed in majority of canine lymphoma cases (90% of cases) microscopic examin-

ation of smears stained with Giemsa solution allowed to recognize the subtype and the immunophenotype of growth. However, in 10% of presented cases establishment of neoplastic cells immunophenotype was not correct or the result of examination was not unequivocal, while the application of specific antibodies revealed high percentage of cells with the same expression pattern (85-95% of cells with the same immunophenotype) and allowed previous suspicion to be confirmed or previous incorrect diagnosis to be verified. The kappa value that represents the measure of agreement between two methods applied in subtyping and immunophenotyping of the lymphomas was equal to 0.802 (0.731, 0.873). Some interpretation guidelines for evaluating observed kappa values have been proposed in several textbooks. According to Fleiss et al. (2003) kappa value  $\geq 0.75$  indicates excellent agreement, when Everitt (1989) suggests kappa value  $> 0.81$  as almost perfect agreement and between 0.61 and 0.80 as a substantial agreement. Altman (1991) suggests that kappa value  $> 0.80$  refers to a very good agreement, and kappa value from 0.61 to 0.80 a good agreement. Following these guidelines the kappa value observed in this study should be considered as an evidence of good or even very good agreement between Giemsa-stained cytology slides and immunocytochemistry.

Depending on cause and type of inflammatory response in majority of cases population of nonneoplastic lymphocytic cells within stimulated lymph node is characterized by presence of pleomorphic lymphocytes of both subpopulations (B and T cells) with predominance of one immunophenotype. Lymphomas are neoplastic proliferations that origin from one transformed cell, so at least some features (immunophenotype of neoplastic cells) should be identical. In study that used immunophenotyping in classification of canine lymphomas, B cell or T cell tumors were recognized if more than 50 – 60% of cells within a given growth showed expression of CD79 alpha antigen or CD3 antigen, respectively (Guija de Arzacochaga et al. 2007). However, these analyses were conducted on samples of neoplastic tissues, but not on cytologic smears. Unfortunately, studies that determine percentage of cells with chosen immunophenotype within given lymphoma immunophenotyping subtypes are lacking. That is why, similarly to Caniatti et al. (1996), for augmentation of probability of correct diagnosis in the presented work the cytologic diagnosis of blastic lymphoma was based on the presence of at least 80% positively stained cells with immature, blastic cells morphology. In cases of small-cell lymphomas diagnostic criteria were more rigorous – diagnosis of lymphoma with given immunophenotype was established based on presence of

at least 90% positively stained small cells. Obviously, the presence of normal population of lymphocytes with different immunophenotype scattered among neoplastic cells have to be taken into account. Thus it is essential to remember that final cytopathologic diagnosis have to be established on the basis of results of routine cytopathology (morphology of neoplastic cells) and immunocytochemistry (percentage of cells with specific morphology that express given immunophenotype).

Some discrepancies between routine stained slides and these stained with IC were observed in 7 cases (10%); among these cases 5 were lymphomas consisting mainly of small cells and 2 other cases were medium and large cells lymphomas. Neoplastic cells of small-cell lymphomas are often characterized by less typical morphology with respect to cells immunophenotype and immunocytochemistry seems to be especially important and useful in these cases. When microscopic observation of smears stained with Giemsa solution does not allow the subtype and immunophenotype to be determined these cases of tumors are defined as general group – small cell lymphomas. Small cell lymphomas can be both B cell and T cell tumors and in some cases immunocytochemistry can be the only method of immunophenotype recognition. In 2 of 7 cases the discrepancy concerned medium- and large-cells tumors; lymphomas that consist of such cells possess quite typical morphology and usually both diagnosis of tumors subtype and immunophenotype are not difficult. However, the causes of such discrepancies can be various, for example moderate quality of examined smears, or presence of inflammatory response within lymph node examined.

Contrary to Working Formulation or WHO system classification, which describe three grades of malignancy of growth (low-grade, intermediate-grade and high-grade), the updated Kiel classification system, that was applied in the present study, allows for dividing in two, low-grade and high-grade, subtypes of the tumors. However, based on the current literature it can be stated that in most of the canine lymphoma cases this system of cytological classification is sufficient and evaluation of architectural pattern of growth, as in WR and WHO systems is not always necessary (Comazzi and Gelain 2011). Additionally, different therapeutic protocols in cases of different histologic subtypes of canine lymphoma in veterinary medicine are still not established and it seems that the updated Kiel classification system is sufficient enough in veterinary oncology (Vail and Young 2007, Comazzi and Gelain 2011).

It can be concluded that cytopathological examination of Giemsa stained smears is helpful in determining the lymphoma cells immunophenotype and combi-



nation of routine cytopathology and immunocytochemistry in cases of canine lymphomas allows to obtain the precise diagnosis in majority of cases. Connection of these two quite simple methods of examination of smears collected during low-invasive procedure allows to obtain some important information (type and subtype of lymphoma, cytologic grading and lymphoma cells immunophenotype) that are necessary for planning of appropriate therapy and to determine prognosis. Additionally, it should be mentioned that routine microscopic examination of the cytologic slides stained with Giemsa solution is excellent, cheap and simple method of diagnosis of lymphoma in dogs. In the present work the subtype of tumor and its immunophenotype was established on the basis of Giemsa stained cytologic slides in 90% of examined cases, and the agreement between this method and IC was represented by kappa value equal to 0.802. Additionally, the application of immunocytochemistry in these ambiguous cases allowed to avoid the need of repeated collection of cytological samples. Finally, what is especially important, this routine procedure allowed to eliminate the need of collection of tissue samples during surgery or core-biopsy and thus time, cost and patient discomfort related to more complex and invasive medical procedures can be easily reduced.

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