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Effects of pre-analytical variables on flow cytometric diagnosis of canine lymphoma: A retrospective study (2009–2015)

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ABSTRACT

Flow cytometry (FC) is increasingly being used for immunophenotyping and staging of canine lymphoma. The aim of this retrospective study was to assess pre-analytical variables that might influence the diagnostic utility of FC of lymph node (LN) fine needle aspirate (FNA) specimens from dogs with lymphoproliferative diseases. The study included 987 cases with LN FNA specimens sent for immunophenotyping that were submitted to a diagnostic laboratory in Italy from 2009 to 2015. Cases were grouped into 'diagnostic' and 'non-diagnostic'. Pre-analytical factors analysed by univariate and multivariate analyses were animal-related factors (breed, age, sex, size), operator-related factors (year, season, shipping method, submitting veterinarian) and sample-related factors (type of sample material, cellular concentration, cytological smears, artefacts). The submitting veterinarian, sample material, sample cellularity and artefacts affected the likelihood of having a diagnostic sample. The availability of specimens from different sites and of cytological smears increased the odds of obtaining a diagnostic result. Major artefacts affecting diagnostic utility included poor cellularity and the presence of dead cells. Flow cytometry on LN FNA samples yielded conclusive results in more than 90% of cases with adequate sample quality and sampling conditions.

Introduction

Lymphoma is the most common haematopoietic malignancy in dogs, with an incidence of about 84 cases/100,000 dogs per year (Dorn et al., 1970). Classification schemes, and diagnostic and therapeutic approaches, tend to reproduce those applied in human medicine, with only a few exceptions, including a higher prevalence of multicentric lymphomas and diffuse versus nodular lymphoma (Marconato et al., 2013a). These features favour the use of minimally invasive diagnostic techniques, such as fine needle aspirate (FNA) cytology and subsequent flow cytometry (FC) (Comazzi and Gelain, 2011).

Currently, cytology of FNAs is considered to be the primary diagnostic approach for canine lymphoma (Marconato et al., 2013a), since it is cost-effective, minimally invasive and well accepted by the owners. The information provided by cytology in association with other ancillary techniques sets the basis for therapeutic decisions in the majority of cases (Regan et al., 2013). FC is being increasingly used in veterinary medicine as more canine-specific monoclonal antibodies become available. Flow cytometry is routinely used for immunophenotyping of lymphoma and to refine the diagnosis of specific lymphoma subtypes (Seelig et al., 2014; Martini et al., 2015). It can also be used for lymphoma staging (Marconato et al., 2013b) and to evaluate minimal residual disease after treatment (Aresu et al., 2014).

However, veterinary FC facilities are currently limited to a few reference laboratories, mainly for economic reasons. Most of these laboratories are part of academic institutions and provide services for internal veterinary hospitals, external veterinary laboratories and private practices. The specific requirements for sample preparation and shipping may limit the utility of FC. We hypothesised that different sampling techniques, shipping and storage conditions might bias the results and influence the diagnostic performance of FC. To the authors' knowledge, only one study on the influence of pre-analytical variables on diagnostic performance of FC is available in cats (Martini et al., 2017), but a

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study evaluating the effects of such variables in a high number of dogs is still lacking.

The aim of this retrospective study was to assess whether pre-analytical variables influence the diagnostic utility of lymph node (LN) FNA samples from dogs with clinically suspected lymphoproliferative disease analysed by FC. The goal was to create recommendations for sampling techniques, sample storage and shipping, in order to decrease pre-analytical errors, and to increase the diagnostic utility of FC for the diagnosis of lymphoma and leukaemia in dogs.

Materials and methods

Inclusion criteria

The FC database of the Department of Veterinary Medicine, University of Milan, Italy, was searched retrospectively and canine cases were identified over a period of six years (2009–2015). The inclusion criterion was a LN FNA submitted for flow cytometric immunophenotyping. If other sample types, such as peripheral blood (PB), bone marrow (BM) aspirates, body cavity effusions, FNA from mass lesions, spleen, liver or other tissues, were submitted, these cases were included in the study only if a LN FNA from the same animal was analysed, regardless of the diagnostic pathway used and what tissue (e.g. PB or BM for leukaemias) was considered first in the diagnostic pathway. Exclusion criteria included cases composed of tissues other than LN aspirates and cases submitted for minimal residual disease analysis.

Sample collection

Samples were collected from one or more enlarged LNs by multiple aspirations, with or without suction, using a fine needle (21–22 G). The collected material was suspended in 1 mL transport medium (Roswell Park Memorial Institute, RPMI 1640, recommended; phosphate buffered saline/saline solution, not recommended), refrigerated at 4–6 °C and shipped to the laboratory within 24 h of sampling with a cold pack in the envelope to maintain sample cooling.

At admission, samples were examined visually and cellularity was evaluated using an automated analyser (XT-2000iV, Sysmex). Cellularity was considered to be suitable for analysis if the cell concentration in 1 mL was $>5\times10^9$ cells/L (corresponding to $>5\times10^6$ cells in total), whereas samples with $<1\times10^9$ cells/L (corresponding to $<1\times10^6$ cells in total) were excluded from processing, although slight variability may have occurred due to the preferences of the operator dealing with the sample.

Flow cytometry

Processing for FC was performed as described by Gelain et al. (2008). The erythrocyte lysis step was not considered to be necessary for LN FNA samples unless gross haemodilution was detected at visual inspection. For PB and BM samples, red blood cells were lysed by adding a lysis solution containing 8% ammonium chloride.

The panel of antibodies applied to LN samples was adapted over time as more conjugated antibodies against canine leucocytes became available. Samples processed before 2011 were analysed using mainly a two-colour approach. Starting from 2011, a multicolour approach was applied, with the addition of CD45 as tracking label in all tubes. The diagnostic algorithm varied throughout the years, but a basic panel included antibodies against CD5, CD21, CD34, and CD45. On the basis of the staining results obtained with this panel, expression of other antigens was evaluated, including CD3, CD4, CD8, CD11b, CD14, CD18, CD20, CD25, CD44, CD79a, CD117 and major histocompatibility class (MHC) II. The antibody panel used to label PB and BM samples varied based on the phenotype of neoplastic cells identified in the LN sample. If the LN sample was not adequate for FC, the basic antibody panel was applied to PB and BM, with the addition of CD4 and CD8. Specificity, sources and clones of antibodies are listed in Novacco et al. (2015). Samples were acquired using a flow cytometer (FACScalibur, Becton Dickinson) and analysed using Cell Quest software (Becton Dickinson). All FC interpretations and reports were made by one clinical pathologist.

The final diagnosis was based on a comprehensive evaluation of all the data provided by the referring veterinarian, including different combinations of history, presenting complaint, clinical signs, LN cytological smear, haematology data and LN/PB/BM FC analysis. Cases included in the present study were classified based only on the FC report, without any re-evaluation of the raw FC data.

Criteria for diagnosis

The final diagnosis was derived from the flow cytometry report and was based on cytology findings, if available, combined with a comprehensive interpretation of flow cytometric results obtained from LN aspirates and other sample material. Samples were considered to be 'non-diagnostic' if one or more of the following criteria were present: (1) insufficient cellularity ($<1\times10^6$ cells in total); (2) sample composed primarily of dead cells; and (3) if flow cytometric results strongly differed from cytological evaluation, for example, when neoplastic cells were disrupted during processing and only a small residual non-neoplastic population was labelled. Dead cells were identified by gross evaluation of the sample (based on colour, odour or the presence of tissue debris) and/or with a viability stain (propidium iodide), which was included in the last 2 years of the study period.

Samples were considered to be 'negative for lymphoid tumour' if: (1) a mixed population of predominantly small cells was present with a cytology supporting a reactive/hyperplastic lymph node; or (2) other causes of lymphadenomegaly were identified by immunophenotyping and cytology, for example, LN metastasis from solid tumours, histiocytic tumours and plasma cell tumours. Samples were considered as 'likely lymphoid tumour' if: (1) flow cytometry from a lymph node showed a prevalent population (>65%) of lymphoid cells with a single phenotype, but only a reduced panel of antibodies was allowed and/or the lack of a good quality cytological smear precluded a definitive diagnosis; (2) flow cytometry from a lymph node was poorly cellular ($<1\times10^6$ cells in total) or provided equivocal results, but LN cytology was highly suggestive of lymphoma and immunophenotyping of PB and/or BM was suggestive of a lymphoid tumour. Cases were classified as 'lymphoid tumour' if a definitive diagnosis of lymphoma or leukaemia was made based on the results of immunophenotyping of LN, PB, or BM. In many cases, extended subtyping of lymphoid neoplasia was possible based on immunophenotyping of different tissues and cytological evaluation: (1) B cell lymphoma (irrespectively of the grade); (2) high grade T cell lymphoma (based on immunoreactivity to T cell markers and cytological aspects including high numbers of mitotic figures); (3) low grade T cell lymphoma (based on typical T zone pattern staining on FC and/or distinctive cytological features); (4) acute leukaemia (starting with PB or BM immunophenotyping of precursor cells confirmed by LN infiltration); and (5) chronic lymphocytic leukaemia (starting with PB or BM immunophenotyping confirmed by LN infiltration).

Statistical analysis

Statistical analysis was applied to identify pre-analytical variables possibly affecting the likelihood to reach a diagnosis;