

Lymphoma Immunophenotype of Dogs Determined by Immunohistochemistry, Flow Cytometry, and Polymerase Chain Reaction for Antigen Receptor Rearrangements

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Background: Immunohistochemistry (IHC), flow cytometry (FC), and PCR for antigen receptor rearrangements (PARR) are 3 widely utilized tests to determine immunophenotype in dogs with lymphoma (LSA).

Objectives: This study evaluated the ability of FC and PARR to correctly predict immunophenotype as defined by IHC and to determine the level of agreement among the 3 tests.

Animals: Sixty-two dogs with lymphoma.

Methods: Retrospective study. Medical records were searched to identify dogs with LSA that had concurrent IHC, FC, and PARR performed. Immunophenotype results were categorized as B-cell, T-cell, dual immunophenotype (B- and T-cell), or indeterminate. The results of FC and PARR were evaluated for correctly classifying B- and T-cell LSA as compared with IHC. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were evaluated in addition to concordance between each test.

Results: The sensitivity of FC was significantly higher than PARR for both B-cell (91% versus 67%; $P < 0.0072$) and T-cell (100% versus 75%; $P < 0.0312$) LSA. The percent agreement between FC and IHC was 94%, between PARR and IHC was 69%, between FC and PARR was 63%, and among all 3 tests was 63%.

Conclusions and Clinical Importance: Flow cytometry is superior to PARR in correctly predicting immunophenotype when evaluating lymph nodes from dogs already diagnosed with B- or T-cell LSA. If fresh samples are not available for FC, PARR is an acceptable assay for determination of immunophenotype given its high specificity.

Key words: Cancer; Flow cytometry; Lineage; Lymph nodes; Specificity.

Routine cytology and histopathology are frequently supplemented in the clinical diagnosis of lymphoma (LSA) in dogs by immunohistochemistry (IHC), flow cytometry (FC), and PCR for antigen receptor rearrangements (PARR). All 3 techniques are useful in determining immunophenotype, which is one of the main prognostic indicators for this disease.¹ Although histopathology and IHC provide valuable architectural and immunophenotypic information, respectively,^{2,3} FC has become more commonly utilized for lineage assignment in recent years with the increasing availability of antibodies against hematologic antigens of dogs.^{4–6} PARR, an assay that assesses clonality in a population of lymphoid cells through amplification of DNA encoding the variable regions of

Abbreviations:

FC	flow cytometry
IHC	immunohistochemistry
LN	lymph nodes
LSA	lymphoma
NPV	negative predictive value
PARR	PCR for antigen receptor rearrangements
PPV	positive predictive value

B- and T-cell receptors, is also frequently used in the clinical setting for lineage assignment.^{7,8}

Underscoring the importance of immunophenotyping results, 76% of veterinary oncologists requested lineage assignment after obtaining a diagnosis of LSA and 31% of clinicians reported treating B- and T-cell cases differently.⁹ Dogs with high-grade T-cell LSA have decreased remission durations and overall survival times when treated with CHOP-based chemotherapy potentially because of more rapid acquisition of drug cross-resistance,^{3,10,11} which has led to the evaluation and use of alkylating agent-heavy front-line protocols for these patients.^{9,12} In addition, immunophenotyping has been shown to change the diagnosis in up to 20% of cases with indolent lymphoma.^{13,14} This could alter prognosis and treatment recommendations, since certain forms (such as T-zone lymphoma) could be treated more conservatively.

An advantage of FC and PARR is that either test can be performed on cells obtained via fine-needle aspiration of enlarged peripheral lymph nodes (LN) in contrast to IHC, which requires LN biopsies that are more invasive and expensive to obtain and can require heavy sedation or anesthesia. In addition, FC can detect aberrant expression of B- and T-cell

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antigens,^{15,16} whereas PARR is the only test available to veterinarians that can confirm clonality in a population of lymphoid cells.¹⁷ Although samples for both FC and PARR are easily collected, FC requires live cells stored in saline or media, whereas PARR can be performed on any type of sample, including live or dead cells, effusions, formalin-fixed paraffin-embedded tissues, and cytologic samples.

In 1 study of over 600 dogs with LSA, IHC documented greater than 99% as consistent with either the B- or T-cell immunophenotype, with less than 1% null and no dual staining LSA noted.³ In contrast, up to 22% of cases using FC exhibited expression of both B- and T-cell antigens, and although only 2% of cases exhibited cross-lineage rearrangements using PARR in 1 study, this test has been shown to produce a false-negative result in 9–28% of cases.^{7,8,17} On the basis of this information and the added benefit of obtaining morphology and lymph node architectural information with IHC, we consider IHC the gold standard test in determination of LSA immunophenotype at our institution.

Only limited comparison of IHC, FC, and PARR in dogs with LSA has been reported^{7,8,15,16} and, to our knowledge, no study has evaluated concordance among these tests. The objectives of this study were to evaluate the ability of FC and PARR to correctly predict immunophenotype as defined by IHC, and to determine the level of agreement among the 3 tests.

Materials and Methods

Study Subjects

Samples and data were acquired from 62 dogs diagnosed with LSA between January 2008 and December 2010 that had been enrolled in a prospective study where an enlarged peripheral LN was surgically excised and analyzed for DNA aneuploidy and gene expression. IHC, FC, and PARR were utilized for immunophenotyping. Dogs had received no prior treatment, including prednisone. All clients signed a Veterinary Health Complex Hospital Board approved client consent form. The study protocol was approved by the Institutional Animal Care and Use Committee of North Carolina State University.

Lymph Node Processing

Approximately one-half of the LN (including cortex and medulla) was placed in 10% formalin and submitted for histopathologic examination. The remaining tissue was placed immediately in ice-cold RPMI 1640 culture medium^a /2% fetal bovine serum^a (FBS) for manual disassociation into a single cell suspension. After passing through a 70- μ m cell strainer,^b the cells were washed twice with ice-cold Dulbecco's phosphate buffered saline^a (PBS), incubated in 5 mL RBC lysis buffer^c at 37°C for 3 minutes, and washed an additional 2 times with PBS. The cells were quantitated using an automated cell counter.^d

Histopathology and Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections were created from excised LNs within 24–72 hours of collection. Mounting of tissue sections onto positively charged glass slides, sample prepa-

ration, and immunohistochemistry were performed as previously reported¹³ with the following modifications: Antibodies were utilized against CD3 (catalogue #: A0452^e) for identification of T-cell LSA and CD79a (catalogue #: M7051, clone: HM57^e) for identification of B-cell LSA. Target antigen retrieval for CD3 was performed by immersion in proteinase K for 10 minutes, whereas heat-induced epitope retrieval for CD79a was performed by an automated pressure cooker in EDTA/Tris buffer (pH 9.0). Three percent hydrogen peroxide was then applied to all tissue sections for 10 minutes to inactivate endogenous peroxidases. All slides were incubated for 30 minutes with FC Receptor Block.^f Primary antibodies were applied at a 1 : 150 dilution for CD3 (30 minute incubation) and at a 1 : 50 dilution for CD79a (10 minute incubation). Immunodetection was performed utilizing Dako Envision+Rabbit polymer^e for CD3 and Dako Envision+Mouse polymer^e for CD79a and an autostainer staining system (Dako Autostainer^e). Visualization of antibody binding was obtained via DAB+ chromogen^e (a 3,3-diaminobenzidine solution) for 5 minutes; tissue section counterstaining was performed using hematoxylin for 5 minutes, followed by progressive alcoholic dehydration and placement of a coverslip. Normal canine lymph node was utilized as a positive control, while commercially available mouse or rabbit nonimmune serum^g was used as a negative control for each experiment. Slides stained with hematoxylin and eosin (H&E) were reviewed alongside slides stained for CD3 and CD79a for determination of immunophenotype. All slides were evaluated by faculty members of the NCSU Anatomic Pathology Group using light microscopy for morphologic diagnosis and determination of immunophenotype based on the majority and distribution of cells positive for CD3 and CD79a. One pathologist (LB) performed a final review to confirm classification according to World Health Organization (WHO) criteria as previously reported.¹⁸

Determination of Immunophenotype using Flow Cytometry

Cells were suspended in 5 mL RPMI 1640/10% FBS, refrigerated, and analyzed by flow cytometry within 24–48 hours of sample collection. Samples were washed with PBS, spun for 10 minutes at 500 \times g, and the supernatant was discarded. The pellet was resuspended in PBS and the cells were stained with a panel of antibodies after counting (Table 1). The combination of antibodies in each tube for surface immunophenotypic LN analysis was as follows: anti-CD4/CD8/CD45, CD21/CD3/CD45, CD4/CD3/CD45, CD8/CD3/CD45, B5/CD45, CD14/CD45, CD11d/CD45, secondary antibody/CD45, isotype control antibodies, and a “cells only” control. For intracellular staining, 1 \times 10⁵ cells were treated with permeabilization reagents^h following the manufacturer's instructions with the following modifications: the irrelevant isotype control antibody, anti-CD3 antibody (catalogue #: MCA1477F, clone: CD3-12^h), and anti-CD79b (catalogue #: MCA2209F, clone: AT107-2^h) antibody were added for 10 minutes instead of 30 minutes, based on our laboratory's experience using these reagents on canine lymphocyte specimens. Antibodies were directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE), or 3-amino-9-ethylcarbazole (APC). Data were acquired on a FACSCalibur analyzer, FACSCalibur Systemⁱ; 15,000 events were acquired and stored for analysis using BD CellQuest software.^j

Cells were gated using forward versus side angle light scatter to identify lymphocytes, monocytes, and neutrophils, with side scatter versus CD45 staining to confirm these populations. Lymphocyte gating was used to evaluate the various surface and intracellular markers described above. Reference values for surface immunophenotyping were established using LN aspirates for

Table 1. Antibodies used for flow cytometric analysis of lymph nodes from dogs with lymphoma.

Antibody	Clone	Source
Anti-CD3	CA17.2A12	Serotec, Raleigh, NC
Anti-CD21	CA21D6	Serotec
Anti-CD4	YKIX302.9	Serotec
Anti-CD8	1.140	Tompkins, NCSU
Anti-CD45	YKIX716.13	Serotec
Anti-B5 antigen	B5	Tompkins, NCSU
Mouse IgG1	15H6	Southern Biotech, Birmingham, AL

6 healthy adult Beagles and the mean values were as follows: CD4 = 45%, CD8 = 21%, CD3 = 61%, CD21 = 22%, B5 = 12%, CD14 = 3%. Reference values for intracellular CD3 and CD79b (~60 and ~30%, respectively) were approximated using a combination of data from immunohistochemical studies and intracellular staining followed by flow cytometry on LN cells from healthy dogs. These values are similar to those previously reported.¹⁵ Atypical staining patterns, including an approximately 1.5 times greater (than reference value) percentage of a given lymphocyte subset with a concurrent decrease or loss of other lymphocyte subsets, atypical dual staining (eg, CD4+CD8+ or CD3+CD79b+ cells), and lack of cell surface marker staining (ie, intracellular CD3 staining only) were used to support a diagnosis of LSA. Immunophenotype was defined as B-cell, T-cell, dual immunophenotype (B- and T-cell), or indeterminate. Samples were categorized as dual expression only if cells expressed B- and T-cell receptor molecules simultaneously (>50% CD3+ cells and >50% CD79b+ cells). Samples that were nondiagnostic were not consistent with LSA (based on size, granularity, and staining patterns), or that displayed ambiguous staining patterns not definitively consistent with B-cell, T-cell, or dually expressing LSA were categorized as indeterminate.

Detection of Antigen Receptor Gene Rearrangements using PCR

DNA was isolated^j from 5×10^6 cells per the manufacturer's protocol within 24 hours of collection. DNA was quantitated^k and stored at -80°C until the PCR reactions were run. To assess clonality, each sample in duplicate was subjected to PCR^l using standard reagents and primers as previously described.⁷ The reaction products also underwent heteroduplex analysis¹⁷ before separation using capillary gel electrophoresis.^m The output of results was in the form of a gel view (a computed pseudogel image) and an electropherogram view (fluorescence intensity versus time/amplicon size). A canine T-cell cell line¹⁹ was used as a positive T-cell control, while a previously B-cell PARR positive LSA sample was used as a positive B-cell control. Negative controls consisted of PCR reaction mixtures with no DNA added. A clonal sample was reported if one or more discrete bands of the appropriate size⁷ were seen on the gel view and a distinct peak was seen in the electropherogram view. A polyclonal sample was reported if either no or multiple bands or peaks were seen. In all samples where no bands or peaks were produced, another PCR using C μ primers was performed⁷ to prove the presence of DNA in the sample. Samples were classified as B-cell (if clonality was detected with B-cell primers only), T-cell (if positive with T-cell primers only), dual immunophenotype (if positive with B- and T-cell primers), or indeterminate (if negative or polyclonal).

Categorization of Results and Statistical Analysis

For all 3 tests, immunophenotype results were categorized as B-cell, T-cell, dual immunophenotype (B- and T-cell), and indeterminate. The results of FC and PARR were evaluated for correctly classifying B- and T-cell lymphomas as compared with IHC (considered the gold standard test). The sensitivity and specificity for both of the tests were calculated, in addition to positive and negative predictive values (PPV and NPV, respectively). A DeLong's test was used to compare the ability of FC and PARR to correctly predict immunophenotype as defined by IHC. This tested the null hypothesis that the AUC of both tests was the same. As additional summary measures, the concordance among FC, PARR, and IHC was calculated. All analyses were performed in Stata v10 (www.stata.com).

Results

Immunophenotyping Results

Sixty-three cases met inclusion criteria. Of these, 1 dog was excluded because of poor quality of the lymph node and artifact during processing. Using IHC, 46 (74%) cases were identified as B-cell LSA and 16 (26%) cases were identified as T-cell LSA. No cases were negative for staining or showed staining with both anti-B- and T-cell antibodies. Using routine H&E combined with IHC, 34 (55%) cases were classified as diffuse, large B-cell (DLBCL) LSA, 10 (16%) cases as peripheral T-cell LSA not otherwise specified (PTCL-NOS), 7 (11%) cases as late marginal zone (MZL) LSA, 6 (10%) cases as T-zone LSA, 4 (6%) cases as MZL LSA, and 1 (2%) case as follicular LSA.

Using FC (Fig 1), 42 (68%) cases were classified as B-cell LSA, while 17 (27%) and 2 (3%) cases were classified as T-cell or dually expressing LSA, respectively. One (2%) case was placed in the indeterminate category, because it could not be confirmed if it was a dually expressing or B-cell LSA. Using PARR (Fig 2), 31 (50%) cases showed a clonal rearrangement using B-cell primers, 13 (21%) cases showed a clonal rearrangement using T-cell primers, 2 (3%) cases showed clonal populations with both B and T-cell primers, and 16 (26%) cases were negative using both sets of primers and placed in the indeterminate category. Immunophenotype results within each WHO subtype for all three tests (IHC, FC, and PARR) are summarized in Table 2.

Comparison of IHC, FC, and PARR

For B-cell LSA, the sensitivity of FC in correctly determining immunophenotype (as defined by IHC) was significantly higher than PARR (91% versus 67%; $P < .0072$), although there was no difference in specificity (100%). For T-cell LSA, the sensitivity of FC was also significantly higher compared with that of PARR (100% versus 75%; $P < .0312$), although the difference was not as strong as when examining B-cell LSA ($P < 0.0312$ versus $P < .0072$). Similar to B-cell LSA, there was no significant difference in specificities between the 2 tests (98%). For PARR, the overall

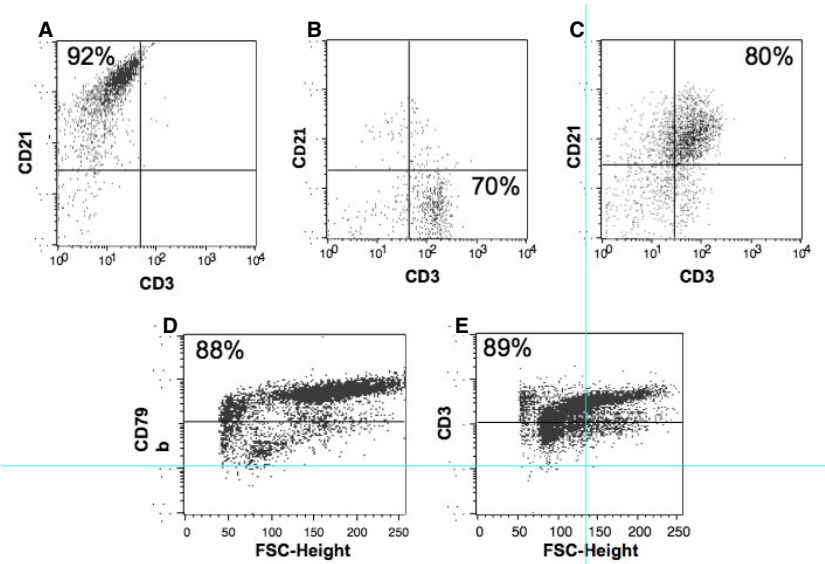


Fig 1. Immunophenotyping of canine lymph nodes using flow cytometry. Peripheral lymph node cells were stained and examined by flow cytometry as described in the methods section. Results are reported as the percentage of positive cells for the antigen in question using flow cytometry dot plots. (A) B-cell lymphoma, approximately 92% of the cells exhibit surface CD21 expression (upper left quadrant). (B) T-cell lymphoma, approximately 70% of the cells exhibit cell surface CD3 expression (lower right quadrant). (C) Dual lineage lymphoma, approximately 80% of the cells exhibit dual CD21 and CD3 expression (upper right quadrant, dual lineage confirmed by PARR). (D) The dot plot represents the percentage of positive cells (above line) versus forward scatter (FSC). Approximately 89% of the cells are positive for intracellular CD79b, indicative of a B-cell neoplasm. (E) Approximately 88% of the cells are positive for intracellular CD3 (above line), indicative of a T-cell neoplasm. Dogs classified as dual positive by intracellular staining have intracellular immunophenotyping profiles similar to both “D” and “E” concurrently. Gating for percent positive cells was based on isotype control antibodies and a “cells only” control (data not shown).

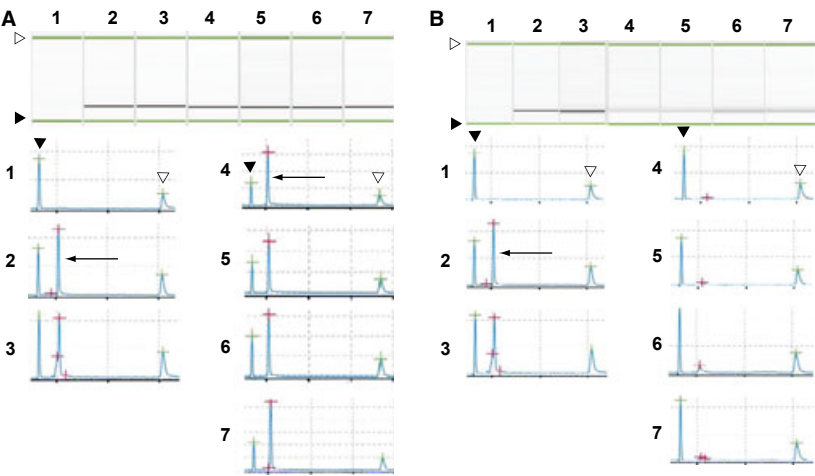


Fig 2. Immunophenotyping of canine lymph nodes using PARR. DNA was extracted from excised LN cells and subjected to PARR analysis. (A) Representative B-cell PARR + case. The top image is a computed pseudogel while the bottom images are electropherograms corresponding to each lane. Lanes: 1) No DNA negative control, 2 & 3) known B-cell PARR + control in duplicate, 4 & 5) LN cells in duplicate, 6 & 7) heteroduplex analysis of samples in lanes 4 & 5. (B) Representative B-cell PARR – case. Lanes are as in (A). Samples were deemed B-cell PARR + if a distinct band of the appropriate size in duplicate was seen in the pseudogel image and a distinct peak was seen in the corresponding electropherograms. Samples were deemed B-cell PARR – if a “smear” of amplicons was seen in the pseudogel image and a distinct peak was not seen in the corresponding electropherograms or if all lanes were blank. Arrowheads: black, 50 bp; white 500 bp. Arrows: positive clonal peaks.

sensitivity in detecting a clonal rearrangement with B-cell, T-cell, or both primer sets regardless of immunophenotype was 74%. For FC, the overall sensitivity of definitively confirming a diagnosis of lymphoma was 98%.

Using FC, immunophenotype of 4 (9%) and 0 cases of B- and T-cell LSA, respectively, were incorrectly determined (Table 3). Using PARR, immunophenotype of 15 (33%) and 4 (25%) cases of B- and T-cell LSA, respectively, were incorrectly determined (Table 4).

Table 2. Distribution of immunophenotype results within WHO subtype for IHC, FC, and PARR.

	Morphologic Diagnosis Based on Histopathology – Number of Cases						Total = 62
	DLBCL	FL	MZL	MZL (late)	PTCL-NOS	TZL	
IHC							
B	34	1	4	7	0	0	46
T	0	0	0	0	10	6	16
FC							
B	31	1	4	6	0	0	42
T	1	0	0	0	10	6	17
Dual	2	0	0	0	0	0	2
Indeterminate	0	0	0	1	0	0	1
PARR							
B	22	1	2	6	0	0	31
T	1	0	0	0	7	5	13
Dual	1	0	0	0	1	0	2
Indeterminate	10	0	2	1	2	1	16

IHC, immunohistochemistry; FC, flow cytometry; PARR, PCR for antigen receptor rearrangements; DLBCL, diffuse large B-cell; MZL, marginal zone lymphoma; FL, follicular lymphoma; PTCL-NOS, peripheral T-cell lymphoma not otherwise specified; TZL, T-zone lymphoma.

Table 3. Immunophenotype results using FC.

Immunophenotype on FC	Immunophenotype on IHC	
	B-cell No. of Cases (%)	T-cell No. of Cases (%)
B-cell	42 (91)	0 (0)
T-cell	1 (2)	16 (100)
Dual B/T-cell	2 (4)	0 (0)
Indeterminate	1 (2)	0 (0)
Total	46 (100)	16 (100)

IHC, immunohistochemistry; FC, flow cytometry.

Table 4. Immunophenotype results using PARR.

Immunophenotype on PARR	Immunophenotype on IHC	
	B-cell No. of Cases (%)	T-cell No. of Cases (%)
B-cell	31 (67)	0 (0)
T-cell	1 (2)	12 (75)
Dual B/T-cell	1 (2)	1 (6)
Indeterminate	13 (28)	3 (19)
Total	46 (100)	16 (100)

IHC, immunohistochemistry; PARR, PCR for antigen receptor rearrangements.

PPV and NPV of FC and PARR for B- and T-Cell Lymphoma

The PPV and NPV of FC in determining immunophenotype for B-cell LSA were 100 and 80%, respectively, whereas for T-cell LSA PPV and NPV were 94 and 100%, respectively. The PPV and NPV of PARR in determining immunophenotype for B-cell LSA were 100 and 52%, respectively, whereas for T-cell LSA PPV and NPV were both 92%.

Concordance among IHC, FC, and PARR

The percent agreement between FC and IHC was 94%, between PARR and IHC was 70%, between FC and PARR was 63%. The percent agreement among all 3 tests was 63%.

Discussion

The results of this study demonstrate that, although there is no difference in specificity between the 2 techniques, FC is more sensitive (91% versus 67%) than PARR in correctly determining immunophenotype as defined by IHC. The increased sensitivity of FC over

PARR, most evident in B-cell LSA, is most likely caused by the high number of PARR negative B-cell cases (28%) compared with PARR negative T-cell cases (19%). PARR also demonstrated a low NPV (52%) when evaluating B-cell LSA. Although our results might have been influenced by sample size variation (46 B-cell cases versus 16 T-cell cases), the distribution presented here closely resembles that observed in previous reports.^{4,20}

The overall sensitivity of PARR in our study was 74%; this is lower than the sensitivity of 91% noted in initial studies evaluating PARR in diagnosing lymphoid neoplasia,⁷ but similar to the sensitivity of 72–75% reported when a larger sample size was evaluated.^{8,17} It is important to note that only lymph node tissue was evaluated in our study, compared with blood, bone marrow, cavity fluids, and aspirates of internal organs that were included in the original study by Burnett et al. The lower limits of detection of clonality using PARR can be influenced by the tissue from which the sample is taken because the assay is reported to be less sensitive when examining lymphoid versus nonlymphoid tissue (1 neoplastic cell in 1,000 cells versus 1 neoplastic cell in 100 cells, respectively).⁷

PARR had discordant results with IHC in 15 (33%) B-cell and 4 (25%) T-cell cases. Interestingly, the immunophenotype of all 19 discordant cases was correctly determined by FC. Although the majority of these cases were classified as indeterminate attributable to a negative PARR test result, 2 cases (1 B-cell and 1 T-cell) exhibited dual clonality while 1 B-cell case exhibited clonality using T-cell primers only. This uncommon phenomenon, referred to as cross-lineage rearrangement,^{7,15,16,21} has been previously reported in 5% of dogs with T-cell LSA and 21% of dogs with MZL.^{7,14} Potential causes of cross-lineage rearrangement include postoncogenic events resulting from persistent activity of the V(D)J recombinase system, a benign population of lymphocytes with limited diversity emerging in the milieu of immune dysregulation that accompanies proliferation of neoplastic cells, or the true presence of 2 neoplastic populations within 1 sample.^{14,21–23}

Flow cytometry correctly identified all but 4 (9%) of the 46 B-cell samples. One (2%) sample expressed T-cell antigens, 2 (4%) samples expressed both B- and T-cell antigens, and 1 (2%) sample was indeterminate. All 4 of these samples, when evaluated using PARR, had concordant results with IHC as they exhibited clonality (ie, exhibited IgH monoclonal rearrangements) using B-cell primers. Histopathologically, 3 of these samples were classified as DLBCL, whereas 1 (the indeterminate sample) was classified as MZL. FC results of this MZL case revealed a large percentage of CD21+ cells and a small percentage CD3+ cells; however, with intracellular staining, 48% were CD79b+ and 37% CD3+. As this particular sample exhibited *IgH* monoclonal rearrangement using PARR (in addition to CD79a staining with IHC), it is likely that the CD3+ positive cells were resident effector or regulatory non-neoplastic T-cells. A recent study evaluating molecular profiles of canine lymphoma subtypes showed enrichment of gene sets associated with activated T-cells in MZL samples, in addition to an excess of T-cells seen by using FC.²⁴

Dually expressing lymphomas/leukemias have been detected in up to 22% of cases using FC, although previous studies have included cases coexpressing CD21 or CD79a with CD3.¹⁵ This phenomenon, proposed to be a consequence of lineage infidelity, occurs more frequently in people with T-cell (versus B-cell) lymphoma/leukemia, and these cases tend to have unusual cytogenetic abnormalities and exhibit a poor response to treatment.^{25,26} This was not the case in our study, although 2 dogs with DLBCL exhibited dual expression. Lineage infidelity characterized by coexpression of CD79a and CD3 has been reported in only a few people with more differentiated lymphoid B-cell neoplasms, including DLBCL (frequently in association with Epstein-Barr virus).²⁷ Possible reported mechanisms of CD3 expression in B-cell neoplasms detected with FC include neoplastic transformation occurring at a precursor level, derepression of genetic material during neoplastic transformation, and neoplastic expansion of a subpopulation of normal B-cells that express T-cell antigens.²⁸

FC correctly identified all cases determined to be T-cell using IHC. One of these samples showed 90% CD3+CD4+CD21+ cells, although 91% of all cells were CD3+ with intracellular staining (no cells were CD79b positive). This case was not categorized as a dual expression lymphoma, as CD21 (also known as the complement receptor type 2 or CR2) is expressed on many cell types (thymocytes, a subset of peripheral T-lymphocytes, follicular dendritic cells, astrocytes, some epithelial cells) in addition to B-cells.^{29,30} This particular sample was classified as T-zone lymphoma (TZL) based on histopathology, a subtype of lymphoma shown to express high levels of the CR2 gene.²⁴

Although the results of this study suggest that FC might be the more accurate test for determination of immunophenotype, PARR is the only assay that can detect true clonality of a population of lymphoid cells and should be considered as a front-line test in ambiguous cases. In humans, detection of B-cell clonality using FC can be done by evaluating the expression ratio of immunoglobulin light chains kappa and lambda; clonality is generally identified when the kappa:lambda light chain ratio is greater than 4 : 1 or less than 1 : 2.³¹ This approach cannot be used in dogs, as the kappa : lambda ratio is much wider in this species.^{5,32}

Although the majority of LSA cases in dogs do not require IHC, FC, and PARR to obtain a diagnosis, there are instances where each test would add additional information to the overall clinical picture. For example, as indolent lymphoid malignancies such as follicular, early marginal zone, or T-zone LSA tend to contain moderate numbers of non-neoplastic resident lymphocytes, IHC and FC may be inconclusive. In addition, cytology of fine-needle aspirates of enlarged peripheral LNs can be suggestive of a reactive population or an 'emerging' LSA, as low numbers of neoplastic lymphocytes may be present. In both of these instances, PARR should be performed to confirm clonality and immunophenotype. Finally, in cases where both FC and PARR are performed and the results are either discordant or indeterminate, IHC is recommended as immunophenotype can be assessed in the context of cellular morphology and LN architecture.

Limitations of this study include sample size and a limited panel of antibodies used with FC. Utilization of a wider array of antibodies (such as CD34, CD5 and MHC II) would allow for more accurate characterization of each sample, and may have helped rule out the presence of 2 neoplastic cell populations. Also, this study only evaluated the use of FC and PARR on samples from lymph nodes. Although the general sensitivity of PARR (regardless of immunophenotype) has not yet been reported using samples exclusively from extranodal sites, only 70% of extranodal sites evaluated with FC tested positive in confirmed cases of LSA in a recent study.³³ Another limitation of this study is that all testing was performed at only 1 institution (North Carolina State University). Interpretation of each assay (particularly FC and PARR) is highly dependent on the skill

and experience of the operator and it is possible that different results could be obtained if testing was performed elsewhere. Therefore, the results presented here are specific to only our institution.

Overall, we determined that FC is superior to PARR in correctly predicting immunophenotype when examining samples obtained from enlarged peripheral LN of dogs with LSA. Despite this, fresh samples for FC might not always be available and the results of this study suggest that PARR is also an acceptable assay for determination of immunophenotype and a good follow-up test for lymphoma cases determined to be dual or indeterminate with FC. As PARR is the only available test to determine clonality in cytologically and histologically ambiguous cases of canine LSA, it can be utilized as a front-line test to confirm a diagnosis of lymphoid neoplasia; however, the results of this study suggest that FC be employed as a primary diagnostic technique for the determination of immunophenotype once a diagnosis of LSA has been made. Further studies are warranted to confirm these findings in a larger population of patients, and to characterize the ability of these tests to correctly determine immunophenotype of LSA at extranodal sites.

Footnotes

- ^a Mediatech, Inc., Herndon, VA
^b BD Falcon, BD Biosciences, Bedford, MA
^c Roche, Indianapolis, IN
^d Nexcelom Bioscience, Lawrence, MA
^e Dako Corporation, Carpinteria, CA
^f Innovex Biosciences, Inc., Richmond, CA
^g BioGenex, San Ramon, CA
^h LeucoPerm Reagent A, LeucoPerm Reagent B; AbD Serotec, Raleigh, NC
ⁱ BD Biosciences, Mountain View, CA
^j Qiagen DNeasy Kit, Qiagen, La Jolla, CA
^k Nanodrop 2000, Thermo Scientific, Wilmington, DE
^l Eppendorf Mastercycler ep gradient S, Eppendorf, Hamburg, Germany
^m QIAxcel Electrophoresis System, Qiagen

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Conflict of Interest Declaration: Authors disclose no conflict of interest.

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