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CLINIC AND SURGERY

Whole-slide imaging for evaluation of double-staining immunocytochemistry in canine lymphomas

Camila Benaduce Emanuelli Mello[1*, 2] (https://orcid.org/0000-0003-4425-5067)
Priscila Beatriz da Silva Serpa[3] (https://orcid.org/0000-0001-6073-2172)
Andrea Pires dos Santos[2, 4] (https://orcid.org/0000-0002-1037-970X)
Cinthia Melazzo de Andrade[1] (https://orcid.org/0000-0002-5579-5344)

[1]Departamento de Clínica de Pequenos Animais, Centro de Ciências Rurais (CCR), Universidade Federal de Santa Maria (UFSM), 97105-900, Santa Maria, RS, Brasil. E-mail: camilabemello@gmail.com. *Corresponding author.
[2]Department of Comparative Pathobiology, Purdue University, West Lafayette, IN, United States.
[3]Department of Population Medicine and Diagnostic Sciences, Cornell University, Ithaca, NY, United States.
[4]Purdue Institute for Cancer Research, West Lafayette, IN, United States.

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Camila Benaduce Emanuelli Mello[1*, 2] **Priscila Beatriz da Silva Serpa**[3]
Andrea Pires dos Santos[2, 4] **Cinthia Melazzo de Andrade**[1]

[1]Departamento de Clínica de Pequenos Animais, Centro de Ciências Rurais (CCR), Universidade Federal de Santa Maria (UFSM), 97105-900, Santa Maria, RS, Brasil. E-mail: camilabemello@gmail.com. *Corresponding author.
[2]Department of Comparative Pathobiology, Purdue University, West Lafayette, IN, United States.
[3]Department of Population Medicine and Diagnostic Sciences, Cornell University, Ithaca, NY, United States.
[4]Purdue Institute for Cancer Research, West Lafayette, IN, United States.

ABSTRACT: Lymphoma is the most common hematopoietic neoplasm in dogs, and cytopathology is widely used as a primary diagnostic tool. This evaluation is traditionally performed using light microscopy (LM). Alternatively, digital pathology (DP) is increasingly being adopted in veterinary medicine for cytopathologic diagnosis. However, no studies have evaluated the use of DP for immunocytochemistry (ICC). This study determined the agreement between digital smears and LM in interpreting double-staining ICC (dsICC) in canine lymphoma smears for a single evaluator (intra-observer). The second goal was to assess inter-observer agreement among two evaluators for DP. Twenty-eight Romanowsky-stained smears (RSSs) digitized before and after submission to dsICC underwent whole-slide imaging (WSI) scanning in Standard mode at 400x magnification. Markers CD20 and CD3ε were used for immunophenotyping. Glass and digital smears were evaluated by a single observer before and after dsICC. An additional evaluator independently analyzed the digitized slides. The Kappa Coefficient was used to determine dsICC intra-observer (LM vs Digital) and inter-observer (Digital-only) agreements. Each evaluator correctly phenotyped 24 smears. The intra-observer agreement was almost perfect (k = 0.86; 95% CI, 0.68–1.00). The inter-observer agreement for digital slides was similar (k = 0.87; 95% CI, 0.71-1.00). WSI scans demonstrated high agreement with LM and appear suitable for assessing and determining the immunophenotype of canine lymphomas subjected to dsICC.
Key words: CD20, CD3, digital cytopathology, dog, immunodiagnostic.

Whole-slide imaging para avaliação da imunocitoquímica de dupla marcação no linfoma canino

RESUMO: O linfoma é uma das neoplasias hematopoéticas mais comuns em cães, e a citopatologia é amplamente utilizada para o seu diagnóstico. Esta avaliação é comumente realizada por meio de microscopia óptica (MO). Por outro lado, a patologia digital (PD) vem sendo cada vez mais adotada na medicina veterinária para o diagnóstico citopatológico. No entanto, não há estudos que avaliem o uso da PD na análise de amostras submetidas à imunocitoquímica (ICC). Neste sentido, este estudo possui dois objetivos: 1) determinar a concordância entre slides digitalizados e MO na interpretação da ICC de dupla marcação no linfoma canino, em um único observador (intraobservacional); e 2) determinar a interobservância entre dois avaliadores em amostras digitais. Para realizar os objetivos, vinte e oito lâminas previamente coradas foram digitalizadas por meio de *whole-slide imaging* (WSI) scan, na metodologia *Standard mode*, com magnificação de 400x, antes e após a dupla marcação. Os marcadores CD20 e CD3ε foram utilizados para imunofenotipagem. Além disso, as lâminas convencionais e as digitalizadas foram avaliadas por um único observador. Igualmente, um segundo observador também avaliou todas as amostras digitais. O coeficiente de Kappa foi utilizado para determinar a concordância intraobservador (MO *versus* WSI) e interobservadores (WSI *versus* WSI). Os resultados mostram que o imunofenótipo foi estabelecido por ambos os avaliadores em 24 amostras digitalizadas. A concordância intraobservador foi κ = 0.86 (IC de 95%, 0.68-1.00) e a interobservador foi κ = 0.87 (IC de 95%, 0.71–1.00), ambas quase perfeitas. Amostras escaneadas digitalmente via *WSI* são adequadas para a determinação do imunofenótipo por dupla marcação em linfomas caninos.
Palavras-chave: CD20, CD3, citopatologia digital, cão, imunodiagnóstico.

INTRODUCTION

Canine lymphoma is the most common hematopoietic neoplasm in dogs, and cytopathological evaluation is widely used both as a primary diagnostic screening and as a confirmatory method (ZANDVLIET, 2016; RASKIN et al., 2019; MARTINI et al., 2022). Following a lymphoma diagnosis, immunophenotyping is often required to distinguish B-cell and T-cell neoplasms (CHILDRESS et al., 2025). Immunocytochemistry (ICC) has been widely used in veterinary medicine for the

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immunophenotyping of lymphocytes (CANIATTI et al., 1996; SAPIERZYŃSKI, 2010; FERNANDES et al., 2015; RASKIN et al., 2019). ICC can be performed on both stained and unstained smears, and it is typically carried out by incubating a single marker on separate areas of the sample (RASKIN et al., 2019). In addition, we recently optimized and validated a double-staining ICC (dsICC) assay that simultaneously phenotypes B and T lymphocytes on canine Romanowsky-stained smears (RSSs) (MELLO et al., 2026).

Digital pathology (DP) involves the acquisition and interpretation of digital images generated from glass slides, most commonly through whole-slide imaging (WSI), in which high-resolution images acquired in successive tiles are digitally stitched together to create a navigable virtual slide (BONSEMBIANTE et al., 2019; ZURAW & AEFFNER, 2022; PICCIONE et al., 2025; ANDERSON et al., 2026). The few veterinary studies evaluating WSI for cytological smear preparations have used the Z-stack scanning modality, which acquires multiple focal planes to better accommodate the three-dimensional nature of cytological samples (BONSEMBIANTE et al., 2018; BONSEMBIANTE et al., 2019). In contrast, Purdue University routinely uses the Standard scan mode available in the Motic EasyScan One scanner for training in digital cytology, which employs high-precision autofocus to capture a single autofocus-selected focal plane for each scanned area, generating a single-layer WSI.

WSI has been increasingly adopted in routine diagnostic practice, particularly in private laboratories (PICCIONE & BAKER, 2023; PICCIONE et al., 2025). Studies evaluating its application in cytopathology remain limited; although, available evidence indicated that WSI is suitable for cytological diagnosis and can reliably support lymphoma diagnosis, especially when performed by experienced evaluators (BONSEMBIANTE et al., 2018; BONSEMBIANTE et al., 2019). However, to the best of our knowledge, no studies have evaluated the use of Standard scan mode for interpreting ICC, and no reports have assessed digital evaluation of dsICC in canine lymphoma.

This study assessed intra-observer agreement between the conventional light microscopy (LM) and WSI scans of canine lymphoma cytological smears subjected to dsICC. The second goal was to assess inter-observer agreement of WSI scans only. We hypothesized that at least substantial intra-observer agreement between LM and WSI interpretations would be observed. We also hypothesized a similar inter-observer agreement for digital slides.

MATERIALS AND METHODS

Cytopathological samples

Twenty-eight RSSs of lymph nodes (n = 25), spleen (n = 2), and gastric mass (n = 1) aspirates from dogs with a cytological diagnosis of lymphoid neoplasm and further confirmed diagnosis and immunophenotype based on flow cytometry (FC) and/or immunohistochemistry (IHC) were included. PCR for antigen receptor rearrangement (PARR) genotyping was also used to confirm the diagnosis of lymphoma (RASKIN et al., 2019). The samples were submitted to a clinical pathology laboratory in a university veterinary hospital between May 2023 and May 2025. Smears were stained with Modified-Wright stain (Hematek® Stain Pak Siemens–Modified Wright's Stain) in an automated stainer (Hematek® 3000 System). The 28 samples included in this study were drawn from the same set used to validate our dsICC assay (MELLO et al., 2026). An almost perfect agreement was observed between the immunophenotype determined by dsICC and the results of confirmatory tests (MELLO et al., 2026). All slides were generated from excess clinical samples collected during routine veterinary care procedures, with client approval for their use.

Double-staining immunocytochemistry

The markers CD20 (Polyclonal; Thermo Fisher Scientific, Waltham, MA, USA) and CD3ε (Clone CD3-12; Bio-Rad, Hercules, CA, USA) were used at a 1:50 dilution for the dsICC. The patient smears were divided into two areas: one for simultaneous antibody application and the other as an internal negative control. Popliteal lymph node impression smears from a healthy Beagle were used as positive and negative control samples and included in each ICC run.

Slides were scraped with a blade to create two areas on a single smear, soaked in 99% ethanol for 10 minutes, fixed in cold acetone for 15 minutes, and dried at room temperature (RT). Heat-induced antigen retrieval (HIAR) was performed in a pressurized decloaking chamber (Biocare Medical, Concord, CA, USA) for 25 minutes at 95°C with a buffer solution (Diva Decloaker 1X; Biocare Medical). Afterward, a hydrophobic pen (ImmEdge® pen H-400; Vector Laboratories, Newark, CA, USA) was applied to the previously scraped areas of the smears. Blocking of endogenous peroxidase activity was achieved by incubating the slides with 3% H_2O_2 for 10 minutes, followed by two rinses in PBS 1X and blocking of non-specific epitopes with pooled horse and goat

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serum (both 10%) for 5 minutes. Primary antibodies CD20 and CD3ε were incubated simultaneously for 30 minutes. Slides were rinsed twice in PBS 1X, and the ImmPRESS® HRP Horse Anti-Mouse IgG Polymer and ImmPRESS® ALP Goat Anti-Rat IgG Polymer were concomitantly applied for 30 minutes. Then, after two rinses in PBS 1X, the ImmPACT NovaRED® chromogen was applied for 15 minutes, followed by one rinse in PBS 1X and two rinses in distilled H_2O. Vector® Blue chromogen was then incubated for 30 minutes. The slides were rinsed once in 1X PBS and twice in distilled H_2O, and cover slipped with an aqueous mounting medium (Agilent Dako, Santa Clara, CA, USA). Blocking procedures, primary antibodies, and polymer incubations were performed with slides kept in a humidified chamber (MELLO et al., 2026). All polymers and chromogens were from Vector Laboratories (Newark, CA, USA)

Digital slides – Whole slide imaging (WSI)

Each Romanowsky-stained smear (RSS) was digitized using the Motic EasyScan One scanner (Motic Digital Pathology, Emeryville, CA, USA) in Standard mode at 400x magnification (0.26µm/pixel) (Figure 1). The Standard scanning mode is routinely used for cytologic specimens in our diagnostic training, enabling rapid image acquisition. Scanning was performed by an experienced laboratory technician or by evaluator 1, who also reviewed all scanned RSSs prior to the dsICC assay to ensure appropriate image quality. Less than 24 hours after submission to dsICC and conventional LM evaluation, the smears were re-scanned by evaluator 1 using the same scanning mode (Figure 1). The control samples were also digitized. The resulting WSI scans were saved in SVS format. The scanning time for each lymphoma smear subjected to dsICC was retrospectively estimated from the creation timestamps of the sequentially generated WSI files.

Immunocytochemistry evaluation – light microscopy and digital

The dsICC evaluation was initially performed by a single evaluator (1) under LM (BX50, Olympus, Tokyo, Japan) immediately after dsICC. Following a 5-month washout period, the digitized smears were

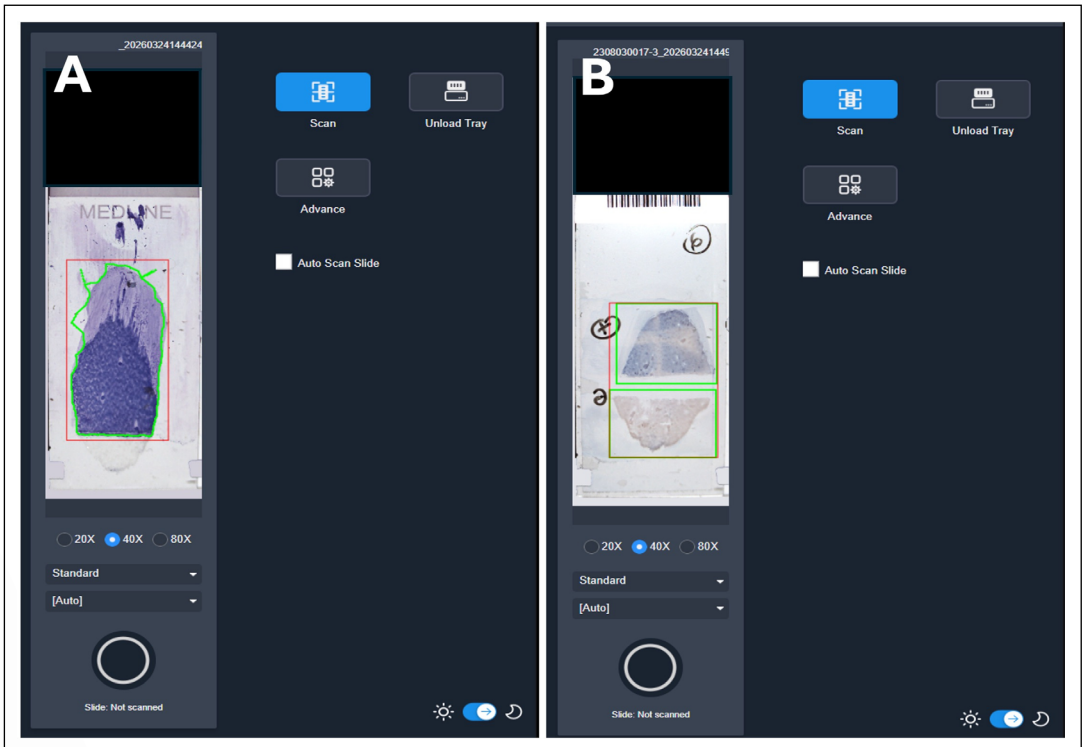


Figure 1 - The entire area of each Romanowsky-stained smear was scanned using Standard scan mode at 400× magnification before (A, red rectangle) and after (B, green rectangles) double-staining immunocytochemistry (dsICC) to immunophenotype CD20 and CD3-cells. The test/positive and negative areas were indicated by "+" and "-" symbols, respectively.

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analyzed. Evaluator 1 (CBEM) was a trained clinical pathologist with nearly 3 years of experience with digitally scanned cytological samples. A second evaluator (2; PBSS; board-certified clinical pathologist), blinded to the first observer's interpretations and with approximately 5 years of digital pathology experience, also performed analysis of digitized smears only. Both evaluators were blinded to the results of lymphoma confirmatory tests. The digital samples were analyzed using Aperio ImageScope software (Leica Biosystems, Lake Cook Road, IL, USA). Each evaluator used their own computer monitor, with no predefined specifications or color calibration (BONSEMBIANTE et al., 2019; ANDERSON et al., 2026).

The dsICC evaluation assessed the proportion of immunolabeled neoplastic cells and residual non-neoplastic lymphocytes, as well as the staining intensity (absent, weak, moderate, or strong) of both CD20 and CD3ε. Background reaction was also graded. Final immunophenotypic interpretation was based on the predominant staining pattern of the neoplastic cell population and the cytomorphologic findings. The proportion of immunolabeled cells was recorded. Digitized RSSs were reviewed during the dsICC evaluation.

Statistical analysis

Based on the dsICC results, each smear was classified as B-cell lymphoma**,** T-cell lymphoma**,** or inconclusive. These classifications were used to determine agreement. Intra-observer agreement (LM vs WSI) and inter-observer agreement for WSI were assessed using unweighted Cohen's kappa (κ) (BERTRAM et al., 2018; FISH et al., 2026). Agreement was interpreted as poor (< 0.00), slight (0.00-0.20), fair (0.21-0.40), moderate (0.41–0.60), substantial (0.61-0.80), and almost perfect (> 0.80) (LANDIS & KOCH, 1977). Statistical analyses were performed using R version 4.6.0.

RESULTS

Evaluator 1 successfully phenotyped 24 of 28 digitally scanned smears, being 17 B-cell and 7 T-cell lymphoid neoplasms (Table 1). Among the 4 inconclusive samples, two were also classified as inconclusive, and the other two were assigned as B-cell lymphoma on LM. Intra-observer agreement between LM and WSI interpretations was almost perfect (κ = 0.86; 95% CI, 0.68-1.00), with an observed agreement of 92.9%.

Most digitally scanned smears showed similar signal intensity and background reaction to LM, with a predominant neoplastic cell population (> 50%) positive for CD20 or CD3ε (Figure 2). Minor differences in signal intensity were observed in three B-cell lymphomas (Cases 8, 12, and 17), but these differences did not affect the final immunophenotypic interpretation. The background on digitally scanned smears was similar across all 28 smears, except for four samples. Two smears (Cases 11 and 16) showed increased background staining on WSI compared with LM, while the other smears (Cases 2 and 17) showed decreased background. Differences in background did not compromise the dsICC either. As observed on LM, residual small lymphocytes were readily recognized, except in the smear from Case 6, which showed no evidence of residual lymphocytes on the WSI scanned sample.

Among the two inconclusive smears on WSI but with a definitive phenotype on LM, one smear (Case 9) showed no evidence of CD20+ neoplastic cells; however, residual CD3ε+ small lymphocytes were scattered (< 25%) and showed a weak signal. On LM, the preparation showed a population of medium-to-large lymphocytes, described as weakly CD20+. These intact cells were in low numbers and were mixed with a few small residual CD3+ lymphocytes. The same inconclusive interpretation was made on WSI by evaluator 2, who described a few residual CD3+ cells and CD20+ neoplastic cells with weak cell signal in low numbers.

The other inconclusive sample (Case 19) was characterized by medium- to large-sized neoplastic lymphocytes with a weak CD20+ cell signal; however, at the edges of the smear, the medium- to large-sized neoplastic cells also showed a weak CD3ε+ cell signal, along with residual small CD3ε+ and CD20+ cells. Interestingly, the LM evaluation was consistent with B-cell lymphoma, given the presence of large neoplastic cells with moderate-to-marked CD20 immunoreactivity, whereas the presumptive neoplastic CD3ε+ cells were not identified.

Evaluator 2 was also able to phenotype 24 of 28 digitized smears. Inter-observer agreement for WSI interpretation was almost perfect (κ = 0.87; 95% CI, 0.71–1.00), with an observed agreement of 92.9%. Inter-observer disagreement was observed in two cases. Case 19 was given a T-cell phenotype by evaluator 2, who described a presumptive predominant population of neoplastic lymphocytes with a weak CD3 signal, mixed with a few CD20+ lymphocytes. Case 20, given evaluator 1's interpretation of a B-cell neoplasm, was deemed inconclusive by evaluator 2, who observed about 60% of CD20+ neoplastic cells but described the signal as granular.

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Table 1 - Double-staining immunocytochemistry immunophenotyping results for canine lymphomas assessed by light microscopy (LM) and whole-slide imaging (WSI), with corresponding confirmatory diagnostic test results. All samples were obtained from fine needle aspirates.

Case	Tissue	Confirmatory tests	dsICC LM evaluator 1	dsICC WSI evaluator 1	dsICC WSI evaluator 2
1	LN	BCL	BCL	BCL	BCL
2	LN	BCL	BCL	BCL	BCL
3	LN	BCL	BCL	BCL	BCL
4	LN	BCL	BCL	BCL	BCL
5	LN	BCL	BCL	BCL	BCL
6	Spleen	TCL	TCL	TCL	TCL
7	LN	BCL	BCL	BCL	BCL
8	LN	BCL	BCL	BCL	BCL
9	LN	BCL	BCL	I	I
10	LN	BCL	BCL	BCL	BCL
11	LN	TCL	TCL	TCL	TCL
12	LN	BCL	BCL	BCL	BCL
13	LN	BCL	BCL	BCL	BCL
14	LN	BCL	BCL	BCL	BCL
15	GM	TCL	I	I	I
16	LN	TCL	TCL	TCL	TCL
17	LN	BCL	BCL	BCL	BCL
18	LN	BCL	BCL	BCL	BCL
19	LN	BCL	BCL	I	TCL
20	LN	BCL	BCL	BCL	I
21	LN	TCL	TCL	TCL	TCL
22	LN	BCL	I	I	I
23	LN	TCL	TCL	TCL	TCL
24	LN	BCL	BCL	BCL	BCL
25	LN	BCL	BCL	BCL	BCL
26	LN	BCL	BCL	BCL	BCL
27	LN	TCL	TCL	TCL	TCL
28	Spleen	TCL	TCL	TCL	TCL

dsICC: double-staining immunocytochemistry; LM: light microscopy; WSI: Whole-slide imaging; LN: lymph node; GM: gastric mass; I inconclusive; Confirmatory tests comprised flow cytometry, immunohistochemistry (IHC), and/or PCR for antigen receptor rearrangement (PARR).

The remaining two inconclusive smears on WSI and LM were characterized by neoplastic cells with absent cell signals. Evaluator 2 also classified both samples as inconclusive for the same reasons, although reduced CD20 expression was suspected in Case 22. The results of each sample for evaluators 1 and 2 are provided in the supplementary material (Table S1).

Additional observations made by both evaluators during the analysis of WSI scans included: 1) a heterogeneous population of CD20+ and CD3ε+ lymphocytes readily recognized in positive-control samples; 2) cells displaying no signal in negative areas on digitized smears (Figure 3); and 3) the ability to display digital RSSs alongside the dsICC-evaluated image on the same screen, enabling direct image comparison and overlay (Figure 4), thereby helping and improving confidence in the digital interpretation.

Interestingly, five digitally scanned RSSs were described as having poor diagnostic quality, a finding consistent between the two pathologists. Two samples were noted as markedly thick, with a monolayer and better cell morphology at the edges of the smear, while three samples were annotated as having low intact cellularity, despite clear identification of neoplastic lymphocytes on scanned slides. Among them, two smears were inconclusive

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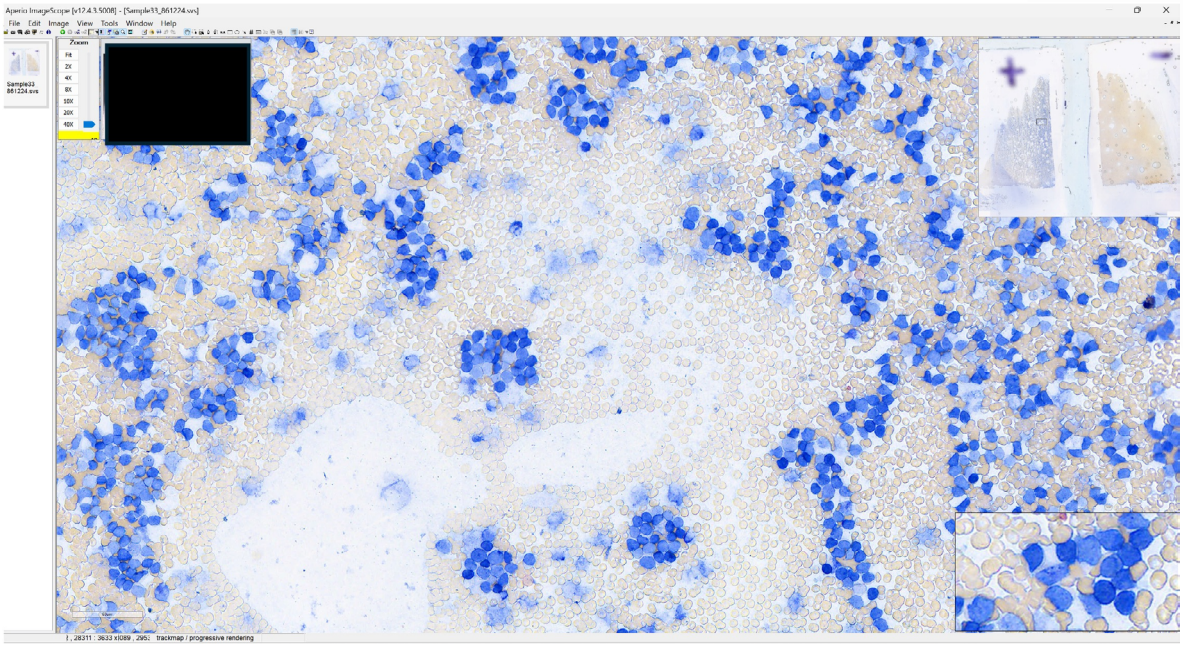


Figure 2 - Digitally scanned smear of a lymph node aspirate from a dog after being submitted to double-staining immunocytochemistry (dsICC) and correctly interpreted as T-cell lymphoma (Case 23). The sample showed a predominant population of intermediate- to large-sized neoplastic lymphocytes with, overall, moderate-to-marked CD3ε reaction (blue signal). The smear was submitted to whole-slide imaging scanning in Standard mode at 400× magnification.

on WSI evaluation, whereas the remaining three samples showed a definitive phenotype that agreed with LM.

WSI file sizes averaged 1.59 ± 0.79 GB for RSSs and 1.38 ± 0.58 GB for dsICC slides. The dsICC whole-slide images were significantly smaller than the corresponding Romanowsky whole-slide images (paired t-test, $t(27) = 2.49$, $P = 0.019$), with a mean paired difference of 0.21 GB (95% CI, 0.04-0.39 GB). The estimated scan duration for dsICC WSI was 15.4 ± 7.1 minutes (median, 13.5 minutes; range, 4-29 minutes).

DISCUSSION

This study demonstrated that evaluation of RSSs subjected to a dsICC assay and digitally scanned using the Standard mode is suitable for immunophenotyping canine lymphoma, with high agreement compared with LM. Furthermore, the almost perfect inter-observer agreement in WSI interpretation supports the feasibility of DP for dsICC evaluation. Most digitally scanned smears showed similar signal intensity and background staining to LM; however, a few discrepancies were noted, resulting in differences in inter- and intra-observer agreement regarding dsICC interpretation.

The two interobserver disagreements observed during WSI evaluation appeared to have different underlying causes. The disagreement involving Case 19 (B-cell lymphoma on LM) may have reflected a scanning-related effect. Both evaluators identified a weak CD3ε signal within the neoplastic population on the digital slide; however, evaluator 1 considered this finding equivocal and classified the case as inconclusive, whereas evaluator 2 interpreted it as sufficient to support a T-cell phenotype. In addition, the presumptive CD3+ neoplastic population was not identified on LM by evaluator 1. This isolated discrepancy may have resulted from differences in the areas selected by each observer to evaluate the smears or in the visual appearance of the digital image, including signal intensity and background staining, leading to a false-positive interpretation. In contrast, the disagreement in Case 20 appeared to result from subjective interpretation of the staining pattern rather than differences in image appearance. While evaluator 1 interpreted the case as a B-cell lymphoma, evaluator 2 considered it inconclusive because the CD20 immunoreactivity was perceived

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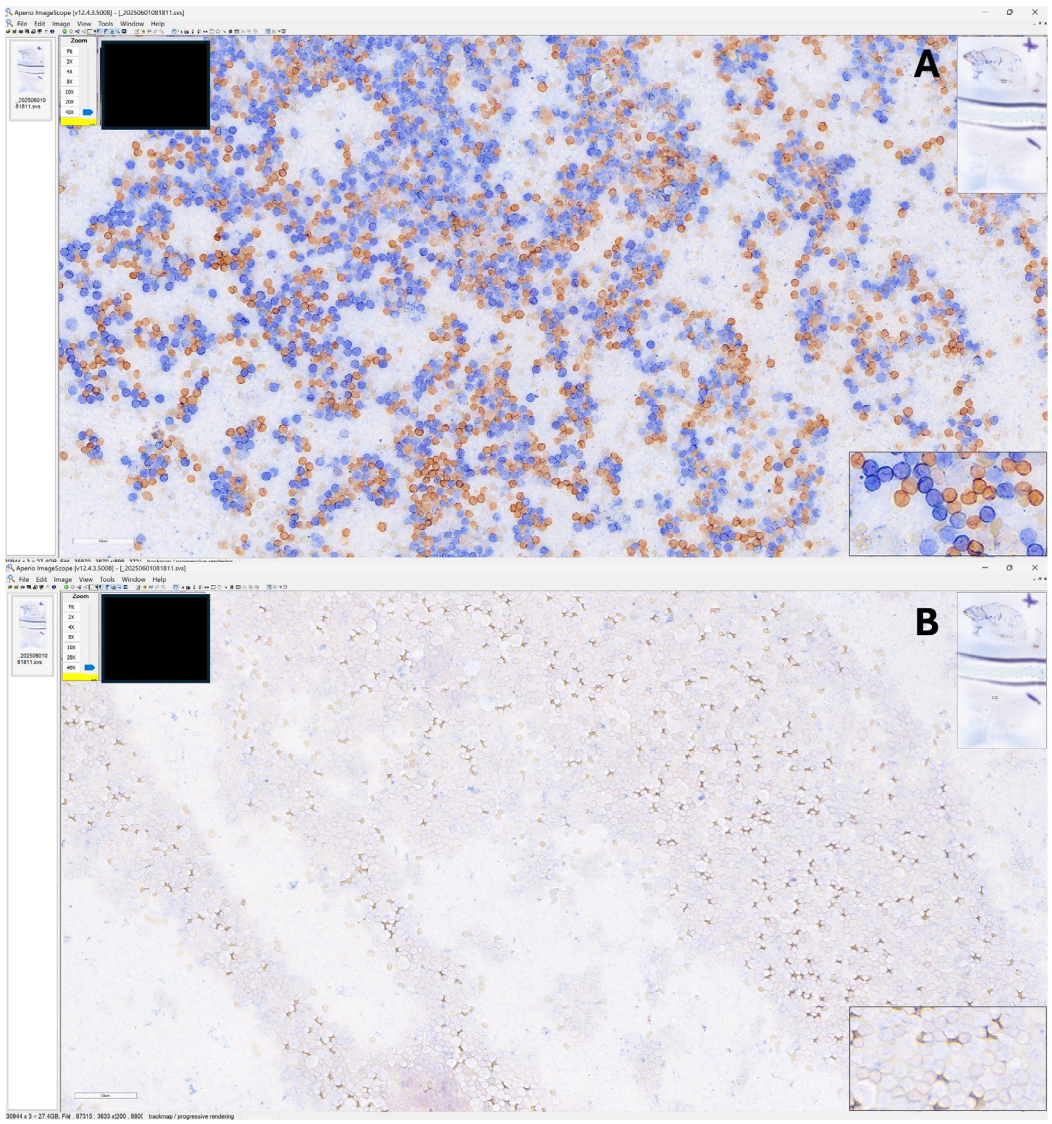


Figure 3 - Digitally scanned popliteal lymph node impression smear from a healthy Beagle used as a positive and negative control for a double-staining immunocytochemistry (dsICC). A, Positive area characterized by a heterogeneous population of CD20+ (red signal) and CD3ε+ (blue signal). B, Negative area characterized by cells displaying no cell signal. The smear was submitted to whole-slide imaging scanning in Standard mode at 400× magnification.

as granular and therefore non-specific. Together, these findings suggested that the few discrepancies observed between evaluators reflected both image-related characteristics and the inherent subjectivity of ICC interpretation.

Similar differences between LM and WSI have been described previously, with digitally scanned cytological smears tending to exhibit darker backgrounds and increased basophilia than conventional microscopy (BONSEMBIANTE et al., 2018). Although that study evaluated slides scanned with the Z-stack modality, we similarly observed increased basophilia in cytological smears digitized with the Standard mode at 400x magnification. These variations may have contributed to the minimal differences in cell signal and background reaction, as well as to the interpretation observed in Case 19. Nevertheless, the Standard scan mode demonstrated almost perfect agreement with LM, indicating that these occasional differences had minimal impact on the

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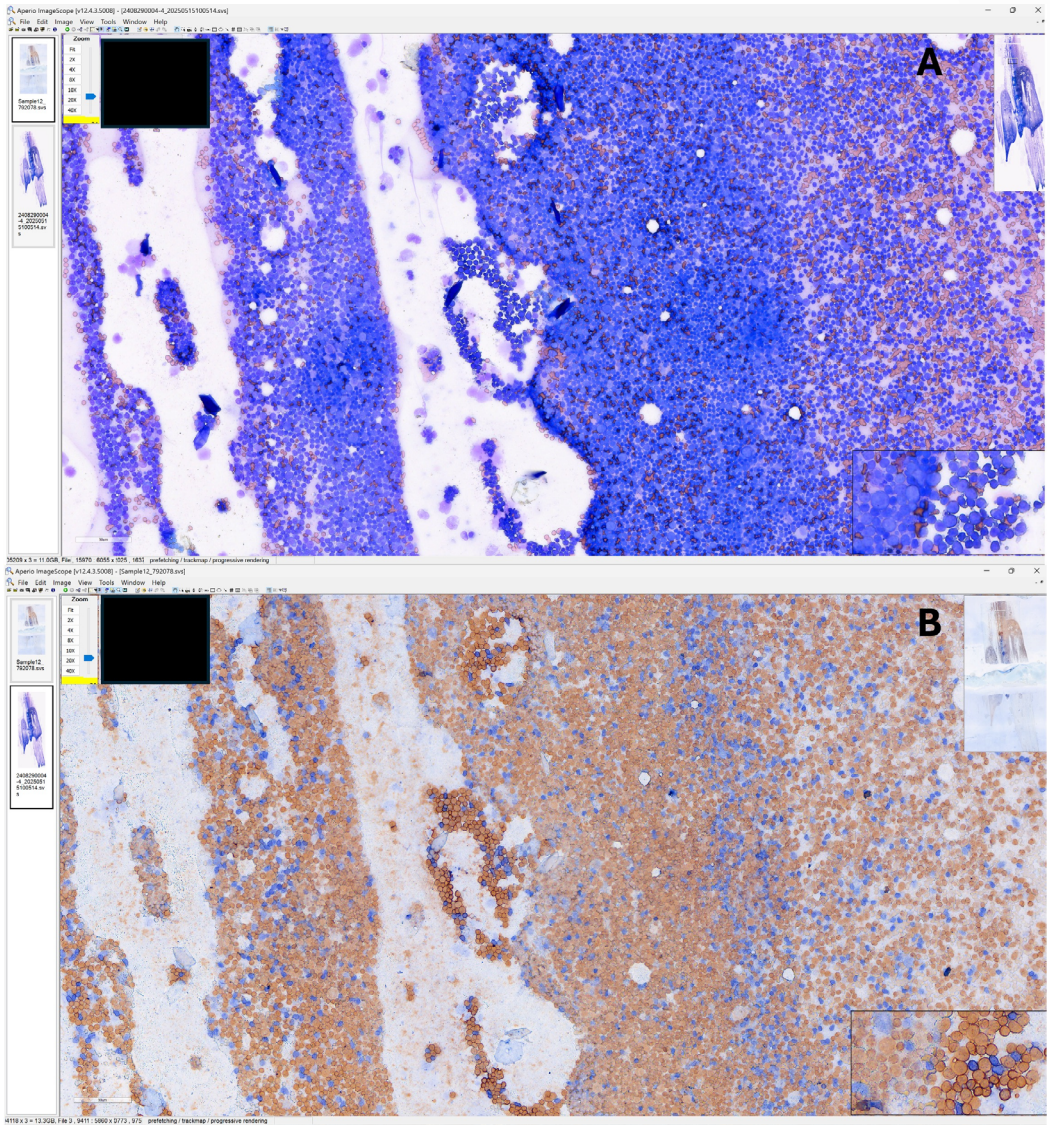


Figure 4 - Digitally scanned smear of a lymph node aspirate from a dog with a confirmed diagnosis of B-cell lymphoma. A. Romanowsky-stained smear characterized by a predominant population of intermediate to large neoplastic lymphocytes. B. The same sample was submitted to double-staining immunocytochemistry (dsICC). The smear was characterized by a predominant population of medium- to large-sized neoplastic lymphocytes displaying moderate to marked CD20+ cell immunoreactivity (red signal) mixed with a residual population of small CD3ε+ cells (blue signal) in lower numbers. Both pictures represent the same area, allowing image overlay. The smears were submitted to whole-slide imaging scanning in Standard mode at 400× magnification.

overall immunophenotypic interpretation. Whether the additional focal planes provided by Z-stack imaging would reduce these isolated discrepancies remains unknown and warrants direct comparison with the Standard scan mode in future studies.

The inconclusive smear in Case 22, resulting from the absence of detectable immunolabeling, highlights a limitation of relying on a single B-cell marker for lymphocyte immunophenotyping. Although this case was confirmed as a B-cell lymphoma by confirmatory diagnostic methods, a detailed discussion of this limitation is beyond the scope of the present study, whose primary objective was to evaluate the feasibility of DP for dsICC interpretation.

Although five digitally scanned slides were considered of poor quality, three still yielded

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the correct immunophenotypic interpretation. The suboptimal quality likely reflected the inherent challenges of digitizing three-dimensional cytological preparations, as well as the use of archived surplus clinical material rather than the highest-quality diagnostic slides. In our routine diagnostic practice, the highest-quality smears are reserved for clinical archiving and potential future use, leaving only surplus slides available for research purposes. Consequently, evaluator 1 may have been more accepting of suboptimal slides during both LM and WSI evaluation, recognizing that higher-quality replacement smears were unavailable. Because the Standard scan mode acquires a single focal plane, its contribution to these observations cannot be ruled out and warrants further investigation in studies that directly compare Standard and Z-stack scanning modalities. Finally, one intra-observer discrepant case (Case 9) involved only a few weakly immunoreactive neoplastic cells on LM and absent neoplastic cells on WSI, suggesting that sparse immunolabeling may occasionally limit confidence in digital interpretation.

The time required to scan a single smear using Z-stack increases significantly, taking approximately 3-4 hours at 400x magnification (BONSEMBIANTE et al., 2018; BONSEMBIANTE et al., 2019). This period is strongly discouraging for inclusion in diagnostic routines (BONSEMBIANTE et al., 2018). Although, direct comparisons should be interpreted with caution due to differences in study design and scanning conditions, the estimated mean scan time in the present study (15.4 ± 7.1 minutes, not counting non-scan intervals) was shorter than the previously reported Z-stack scanning time, suggesting a practical advantage for routine diagnostic use (BONSEMBIANTE et al., 2018; BONSEMBIANTE et al., 2019).

An independent second evaluator was included in the digital assessment, and LM evaluation by this evaluator was also intended. However, when the archived glass slides were re-examined approximately 13 months after the initial dsICC assay, the neoplastic and residual lymphocytes no longer exhibited detectable CD20 immunoreactivity, precluding independent LM interpretation. Consequently, the second evaluator participated only in the WSI assessment. This observation illustrates an additional advantage of DP: immunoreactivity on archived glass slides may diminish over time, whereas digitally archived WSI scans preserve staining characteristics. Accordingly, digital archiving of both RSSs and dsICC slides may provide additional benefits for long-term record keeping, retrospective case review, education, and legal documentation (PICCIONE & BAKER, 2023). Nevertheless, the substantial digital storage requirements remain an important practical limitation that warrants further optimization (PICCIONE et al., 2025).

Our study design is consistent with a simple modality comparison; however, it still has limitations (BERTRAM et al., 2018). The small sample size, which does not meet the recommended minimum of 60 cases, and the absence of at least three evaluators to assess intra-observer agreement between LM and WSI preclude formal validation of WSI for dsICC evaluation under current ASVCP guidelines (FISH et al., 2026). Consequently, these findings should be interpreted as a proof-of-concept agreement study demonstrating feasibility rather than as a formal validation study. The limited sample size also resulted in relatively wide 95% confidence intervals around the κ estimates, indicating some imprecision in the estimated agreement despite the high observed concordance. Therefore, these findings should be confirmed in larger studies.

Although the samples were not randomized for further digital assessment by evaluator 1, the washout period was able to eliminate any possible bias, as it is much higher compared to what has been recommended and described (BONSEMBIANTE et al., 2018; BERTRAM et al., 2018; BONSEMBIANTE et al., 2019; FISH et al., 2026; ANDERSON et al., 2026).

Lastly, this study evaluated the agreement between WSI and LM in interpreting dsICC-stained smears but did not assess the analytical performance of the dsICC assay itself. Therefore, the results support the feasibility of DP for interpreting dsICC preparations but should not be interpreted as a validation of the immunocytochemical assay.

CONCLUSION

WSI-scanned smears obtained in Standard scan mode at 400x magnification appeared suitable for immunophenotyping canine lymphomas following dsICC, demonstrating high agreement with conventional LM. A study with a larger sample size and intra-observer agreement for LM and WSI across three evaluators is still necessary to validate digital dsICC using the same digital system and scanning modality.

SUPPLEMENTARY MATERIAL

Supplementary Table S1 is available in the Zenodo repository at <https://doi.org/10.5281/zenodo.21415048>.

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DATA AVAILABILITY STATEMENT

All relevant data are within the paper.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTION

All authors critically reviewed the manuscript and approved the final version.

DECLARATION OF USE OF ARTIFICIAL INTELLIGENCE

The authors declare that they have not used any generative artificial intelligence in the writing of this manuscript.

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