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Polymerase chain reaction for antigen receptor rearrangement: Benchmarking performance of a lymphoid clonality assay in diverse canine sample types

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Abstract

Background: Polymerase chain reaction for antigen receptor rearrangement (PARR) is a molecular diagnostic tool used for discrimination of lymphoid malignancies in dogs from benign processes. Assay variations have been described and are commercially available, but performance metrics are not uniformly reported.

Objectives: To describe performance (accuracy, sensitivity, specificity) and rigorous benchmarking of a PARR protocol (ePARR) in clinically relevant samples.

Animals: One hundred eighty-one client-owned dogs.

Methods: Lymphoma and benign tissues representative of the clinical spectrum with gold standard histopathologic and immunohistochemical diagnoses were collected. Assay development and benchmarking were performed on fresh frozen (FF) tissue, formalin-fixed paraffin-embedded (FFPE) tissue, flow cytometry pellets, and air-dried fine-needle aspirates (FNA). Assay performance was determined for FFPE from 56 dogs (18 B-cell lymphoma, 24 T-cell lymphoma, and 14 non-lymphoma), 80 frozen flow cytometry pellets (66 B-cell lymphoma, 14 T-cell lymphoma, 0 non-lymphoma), and 41 air-dried FNA slides (23 lymphoma, 18 non-lymphoma).

Results: For discrimination of lymphoma versus non-lymphoma, ePARR had 92% and 92% sensitivity and specificity on FFPE with 92% accuracy, 85% sensitivity from flow cytometry pellets (non-lymphoma was not evaluated to calculate specificity) with 85% accuracy, and 100% and 100% sensitivity and specificity for FNA with 100% accuracy. Stringent quality control criteria decreased assay success rate without significant performance improvement. Performance metrics were lower in most cases for discrimination of B- or T-cell versus non-B- or non-T-cell samples than for lymphoma versus non-lymphoma.

Abbreviations: CI, confidence interval; CLL, chronic lymphocytic lymphoma; CSU, Colorado State University; DIN, DNA integrity number; ePARR, PARR test developed in this study; FF, fresh frozen; FFPE, formalin-fixed paraffin embedded; FNA, air-dried fine-needle aspirates; IGH, immunoglobulin heavy chain gene; IHC, immunohistochemistry; PARR, PCR for antigen receptor rearrangement; PCR, polymerase chain reaction; QC, quality control; TBE, Tris-Borate-EDTA; TRG, T-cell receptor gamma gene.

E. J. Ehrhart, Shukmei Wong, William Hendricks, and Chand Khanna contributed equally to this study.

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Conclusions and Clinical Importance: These benchmarking data facilitate effective interpretation and application of PARR assays in multiple sample types.

KEYWORDS
clonality, lymphoma, molecular diagnostic, diagnosis, PCR for antigen receptor rearrangement (PARR)

1 | INTRODUCTION

As innovation is increasingly embraced in diagnostic veterinary medicine, molecular diagnostic tools are becoming more common. However, unlike conventional hematology and chemistry diagnostic tests, veterinary molecular diagnostic tests do not have a long history of benchmarking and standardization. Thus, a need exists for more rigorous assessment and reporting of molecular assay performance. For example, molecular diagnostic tests are increasingly utilized for differential diagnosis of lymphoma in dogs. Histopathologic discrimination of lymphoid malignancies in dogs from benign, reactive hyperplasia can be difficult in some cases, such as early lymphoma that does not efface nodal architecture, nodular lymphoma that mimics the architecture of a normal node, or lymphoma emerging in a patient with systemic inflammatory disease. Polymerase chain reaction (PCR) for antigen receptor rearrangement (PARR) is a molecular test for clonality that enables such discrimination.[1,2] Normal lymphocytes acquire unique antigen receptors during maturation through rearrangements of the V(D)J regions of T-cell and B-cell receptor genes (TRG [T-cell receptor gamma gene] and immunoglobulin heavy chain gene [IGH]) and are thus polyclonal at these genetic loci. However, lymphomas arise from clonal expansion of a single progenitor cell and therefore are characterized by monoclonal receptor loci. Lymphoma monoclonality can be detected using PARR, which incorporates PCR protocols to amplify specific sequences from lymphocyte DNA. Thus, PARR is based on identification of monoclonal lymphomas versus polyclonal benign or reactive tissues.

Canine T-cell lymphoma clonality assessment by PCR was first described in 1999[1] with the PARR assay itself, based on both TRG and IGH assessment for T-cell and B-cell lymphoma, published in 2003.[2] Several variations on the original protocols have since been reported.[3–10] The original PARR primer designs, based on cDNA amplification from normal canine spleen, have since been modified to (1) increase gene coverage after the publication of the canine genome and publication of deeper IGH and TRG loci annotation[11–13]; (2) correct primer mismatches[3,6,10]; and (3) optimize PCR conditions.[10] The PARR assays now are offered by multiple commercial, state, and academic laboratories. Sensitivity for PARR protocols has been reported to range from 72% to 100% and specificity reports range from 96% to 100%.[1–10] To facilitate standardization of PARR assays across multiple laboratories, an ongoing need exists for transparent reporting of conduct and performance metrics (ie, benchmarking) of PARR assays across diverse sample types (including flow cytometry pellets, formalin-fixed paraffin-embedded [FFPE] samples, low-cellularity samples, and instances of low DNA input) that have been rigorously characterized by gold standard diagnostic techniques. These needs continue to be incrementally addressed by various laboratories, and prior publications have comprehensively reviewed them. For example, a recent report summarized key elements and variables within the PARR workflow to provide a global perspective on needs both for increased PARR sensitivity through an expanded range of targeted genetic loci and also for harmonization of protocols and laboratory practices.[14] Another recent report also systematically evaluated and refined TRG and IGH primer sets in a large cohort of diagnostic samples.[10] Here, we build on these prior assessments to provide additional detailed deconstruction of the effects on PARR assay performance of multiple variables for which additional data will help facilitate harmonization. These studies include evaluation effects of sample quality, individual PARR primer sets, and diagnostic sample type on PARR sensitivity, specificity, and accuracy. To enable these assessments, we have developed and benchmarked a PARR assay that allows low input material and performs well on all common types of diagnostic samples, including low cellularity samples. For clarification from other similar PARR protocols reported in the literature, we will refer here to the protocol performed in our laboratory as "ePARR." We report on the performance metrics of the ePARR assay in a representative population of dogs with lymphoma or non-lymphoma using lymphoma histopathology review and complete immunohistochemistry (IHC) as a gold standard.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Sample numbers by cohort, sample type, and histology are summarized in Table 1. For initial development of ePARR, fresh flash frozen (FF) lymph node samples were obtained from the Colorado State University (CSU) Flint Animal Cancer Center Tissue Archiving Center. Samples were histologically confirmed as lymphoma or non-lymphoma with IHC phenotyping as described below to determine T- or B-cell subtypes. This cohort included 2 non-lymphoma lymph nodes from healthy non-tumor-bearing dogs, 1 B-cell lymphoma, and 1 T-cell lymphoma.

For assay benchmarking, ePARR performance was assessed in a diverse set of samples. Fifty-six FFPE samples from dogs with lymphadenomegaly and clinical suspicion of lymphoma were obtained from the CSU Diagnostic Laboratory. Two 20-μm-thick FFPE curls were cut from 18 B-cell lymphoma, 24 T-cell lymphoma, and 14 non-lymphoma

TABLE 1 Clinical cohorts

Sample type	Assay performed	BCL	TCL	Total Lym.	Non-Lym.	Total
FF	ePARR assay development	1	1	2	2	4
FFPE	ePARR assay benchmarking	18	24	42	14	56
FCP	ePARR assay benchmarking	66	14	80	0	80
FNA	ePARR assay benchmarking	NA	NA	23	18	41

Abbreviations: BCL, B-cell lymphoma; ePARR, PARR test developed in this study; FCP, flow cytometry pellets; FF, fresh frozen tissue; FFPE, formalin-fixed paraffin-embedded tissue; FNA, air-dried Fine-needle aspirates; Lym., lymphoma; NA, lymphoma subtype not determined; TCL, T-cell lymphoma.

paraffin blocks. Diagnosis of lymphoma was confirmed on all FFPE cases by histopathologic review by author E. J. Ehrhart. All lymphomas were immunophenotyped using CD3 and PAX5. An additional B-cell marker (CD79a) was utilized to confirm phenotype when PAX5 staining was equivocal. T-cell lymphomas included 16 peripheral T-cell lymphoma, not otherwise specified (PTCL-nos), 3 nodal T-zone lymphomas (NTZL), and 5 epitheliotropic T-cell lymphomas. The B-cell lymphomas included 12 diffuse large B-cell lymphomas, 3 nodal marginal zone lymphomas, and 3 intermediate size B-cell lymphomas. Eighty flow cytometry frozen pellets from patients with B- or T-cell lymphoma were provided by the Clinical Immunology Laboratory, CSU College of Veterinary Medicine and Biomedical Sciences. Flow cytometry included 14 T-cell lymphomas with 4 CD4+/CD8−, 5 CD4−/CD8+, and 5 CD4−CD8− cases. There were 66 B-cell lymphoma cases with 59 CD21+CD5+/− and 7 B-chronic lymphocytic lymphoma (B-CLL) cases. Flow cytometry and analysis was performed as previously described.[15] A diagnosis of lymphoma and immunophenotype by flow cytometry were made if the following criteria were met (Anne Avery, personal communication): (1) For B-cell lymphomas, >85% of the large cells in the lymph node expressed CD21 and >50% of the cells in the lymph node aspirate were larger than cells found in a normal lymph node; (2) for B-cell CLL, >10 000 B cells/μL were detected in peripheral blood with no increase in other lymphocyte subsets; (3) for T-cell lymphoma or leukemia, >50% of the cells in the sample (blood or node) expressed ≥1 T-cell antigens and had an abnormal phenotype (loss of CD5, lack of class II major histocompatibility expression) or were substantially larger than T cells found in normal or reactive blood.

Forty-one air-dried slide samples were obtained from Ethos Diagnostic Science including unstained and Wright-Giemsa or Diff-Quik (Fisher Scientific, Pittsburgh, Pennsylvania) stained air-dried fine-needle aspirate (FNA) slides of 18 non-lymphomas and 23 lymphomas (immunophenotyping not completed). Diagnosis of lymphoma was performed in these cases by cytological review by author C. Grimes. All prospective collections were approved by the Institutional Animal Care and Use Committees of Ethos Veterinary Health and TGen.

2.2 | Genomic DNA extraction

Genomic DNA was isolated from 30 mg each of FF lymph node biopsy specimens in the prospective development cohort. Frozen tissues first were disrupted and homogenized using the Bullet Blender Gold (Next Advance, Troy, New York) tissue homogenizer and QiaShredder (Qiagen, Hilden, Germany). Genomic DNA then was extracted using the Allprep DNA/RNA/miRNA Universal kit (Qiagen) according to the manufacturer's protocol. Quality of the DNA was determined using the TapeStation genomic DNA assay (Agilent Technologies, Santa Clara, California) according to the DNA integrity number (DIN) with DIN values ranging from 1 (highly degraded DNA) to 10 (mostly intact, high molecular weight DNA). Protein contamination additionally was assessed according to $A_{260/280}$ ratio measured by Nanodrop 1000 spectrophotometry (Thermo Fisher Scientific, Waltham, Massachusetts). Quantity of DNA was measured using the Qubit Fluorometer (Thermo Fisher Scientific) dsDNA Broad Range Assay. Genomic DNA from the FFPE cohort was extracted from two 20-μm FFPE curls. Excess paraffin was removed using a sterile scalpel blade and then disrupted and homogenized as described above. Genomic DNA was extracted using the GeneRead DNA FFPE kit (Qiagen) according to the manufacturer's protocol. Quality, quantity, and purity of extracted DNA were determined as described above. Following the manufacturer's protocol, genomic DNA was extracted from the flow cytometry cohort, frozen pellet cohort, and from Wright-Giemsa or Diff-Quik stained and unstained FNA slides using the QIAamp DNA Mini Kit and QiaAmp DNA Micro kit (Qiagen), respectively. Flow cytometry and frozen pellet samples were processed according to manufacturer protocol for tissue homogenization and lysis. The FNA slides were scraped using a sterile scalpel blade before performing extractions according to manufacturer instructions. Quality and quantity were determined as described above.

2.3 | ePARR protocol

We developed a PARR protocol (ePARR) using previously reported primer sets,[10] utilizing low DNA input, incorporating touchdown PCR,[16] and implementing electrophoretic peak analysis using the BioAnalyzer microcapillary gel electrophoresis system (Agilent Technologies, Santa Clara, California). The ePARR assay was performed according to the parameters described below in Sections 2.3.1 and 2.3.2.

2.3.1 | Primer design and PCR

Primers were synthesized (Integrated DNA Technologies, Coralville, Iowa) and each 5′ primer was paired with its 3′ partner as published previously (Table S1).[10] These primers were optimized based on improved annotation of and by matching to canine IGH and TRG genomic regions.[12,13] Primer sets 1-6, 11, and 12 target IGH rearrangements, whereas primer sets 7-10, 13, and 14 target TRG rearrangements. For determining quality of sample DNA, control primer set Cμ and γ-actin sets a, b, and c, which

target the IgM constant region and canine γ-actin gene, respectively, were utilized. To enable the utilization of low cellularity samples, a range of DNA inputs from 6.25 to 100 ng were assayed to determine the minimum DNA input that would still provide a diagnostic result. Of note, 12.5 ng was the lowest input that still provided comparable banding patterns to the standardly reported 100 ng input (Figure S1). Therefore, samples with total DNA yielding <225 ng were omitted from ePARR analysis (12.5 ng per primer set with 18 primer sets total). The ePARR reactions in a total volume of 20 μL were performed with 12.5 ng of DNA, 500 nM per primer set, and 1× HotStarTaq Plus Master Mix (Qiagen). To increase the sensitivity, specificity and yield, touchdown PCR was utilized (Figure S3).[16] Thermal cycling conditions for ePARR for primer sets 1-8, 11-12, Cμ, and γ-actin primers were performed on the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California) as follows: 95°C for 5 minutes, followed by 3 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds; 3 cycles of 95°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds; 3 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds; 3 cycles of 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds; 18 cycles of 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds, with a final extension of 72°C for 2 minute. For primer sets 9, 10, 13, and 14, thermal cycling conditions were 95°C for 5 minutes, followed by 3 cycles of 95°C for 30 seconds, 52°C for 30 seconds, 72°C for 30 seconds; 3 cycles of 95°C for 30 seconds, 51°C for 30 seconds, 72°C for 30 seconds; 3 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds; 3 cycles of 95°C for 30 seconds, 49°C for 30 seconds, 72°C for 30 seconds; 18 cycles of 95°C for 30 seconds, 48°C for 30 seconds, 72°C for 30 seconds, with a final extension of 72°C for 2 minutes. Where sufficient DNA was available (all FF samples as well as 11 samples from each sample type including FFPE, FNA, and cell pellets), each sample was evaluated in duplicate and by heteroduplex analysis to assess reproducibility and account for possible pseudoclonality. Heteroduplex analysis was performed as previously described.[17] Briefly, each ePARR PCR reaction was divided into 2 aliquots. One aliquot was incubated at 95°C for 5 minutes and reannealed at 4°C for 30 minutes. Both intact and processed PCR products were run on a nondenaturing 10% Tris-Borate-EDTA (TBE) gel (Thermo Fisher Scientific) for 1.5 hours at 100 V. The gel was stained with 1× GelStar Nucleic Acid Gel Stain (Lonza, Rockland, Maine) for 30 minutes with gentle rocking and visualized by transillumination.

2.3.2 | Fragment analysis, peak scoring, and interpretation

The PCR fragment analysis was carried out utilizing microcapillary electrophoresis to provide high PCR product resolution (Figure S2). Fragment analysis was carried out using the Agilent 2100 BioAnalyzer and Expert Software and using the High Sensitivity DNA kit according to the manufacturer's protocol, similar to the GeneScan microcapillary electrophoresis analysis previously described.[6] Prior reports have utilized PCR multiplexing,[8] PCR product pooling with fluorescent labeling, or both before electrophoresis.[6,10] We evaluated individual primer sets both in our PCR reactions and also in our BioAnalyzer readouts, thereby enabling assessment of individual primer sets in the setting of overall assay performance. Each DNA sample was amplified using multiple PARR primers in parallel, and PCR products from a single case were simultaneously loaded onto a single BioAnalyzer chip for analysis. Peak interpretation was performed by author S. Wong, blinded to sample type. Characteristics of the Agilent BioAnalyzer readout are summarized in Table S2 with representative electropherograms shown in Figure 1. Observed clonal peaks were at least several times taller than their width, usually 5× taller with no or very few polyclonal peaks at the peak base in expected size regions in the electropherograms, and a clear sharp band was observed in the pseudogel images without nonspecific ladder banding or smear patterns (Figure 1A, B). Non-lymphoma samples had a polyclonal phenotype of multiple small broad peaks that followed a normal distribution in expected size regions seen in the electropherograms alongside a ladder banding pattern or smear in the pseudogel images (Figure 1C). In keeping with a previously described approach[10] and in order to balance cost and sensitivity, only primer sets 1-10 and Cμ were visualized on the BioAnalyzer unless these primer sets gave negative results in which case reactions from primer sets 11 to 14 and γ-actin then were visualized. Primer sets 1-10 and Cμ provided a clear diagnostic result in 170 of 181 (94%) cases with alternate primers utilized in 11 of 181 (6%) cases. We also implemented a quality control (QC) cutoff based on (1) sufficient input DNA to run at least 4 experimental primer sets (2 of the best-performing B-cell-specific and 2 T-cell-specific primer sets with 1 being primer set 7) and Cμ control primer set and (2) successful amplification of a control primer. If insufficient DNA was available or control primer amplifications failed, the sample was considered a QC failure. In keeping with peak calling parameters described in prior publications,[9,10] all tested samples were scored based on height and width of peaks within expected product size ranges (Table S2). For each primer set, 1 or 2 tall sharp narrow peaks that were taller than wide at the expected product size range with minimum polyclonal peaks at the peak base were determined to be clonal for that particular primer set. Samples with multiple small peaks spread across primer size range in a Gaussian distribution or peaks that were as wide as they were tall were deemed to be polyclonal (ie, a reactive non-lymphoma sample).

2.4 | Statistical analysis

Performance of the ePARR assay was assessed by calculating sensitivity, specificity, and accuracy (true positives + true negatives/total samples). Because the ePARR assay classifies patients as B-cell lymphoma, T-cell lymphoma, or non-lymphoma, these measures were calculated after eliminating samples with inconclusive results or insufficient material (sample failures) and collapsing classifications as follows:

- Combine B-cell and T-cell lymphoma classifications, and assess classifications of lymphoma versus non-lymphoma
- Combine T-cell lymphoma and non-lymphoma classifications, and assess B-cell lymphoma versus T-cell lymphoma or non-lymphoma

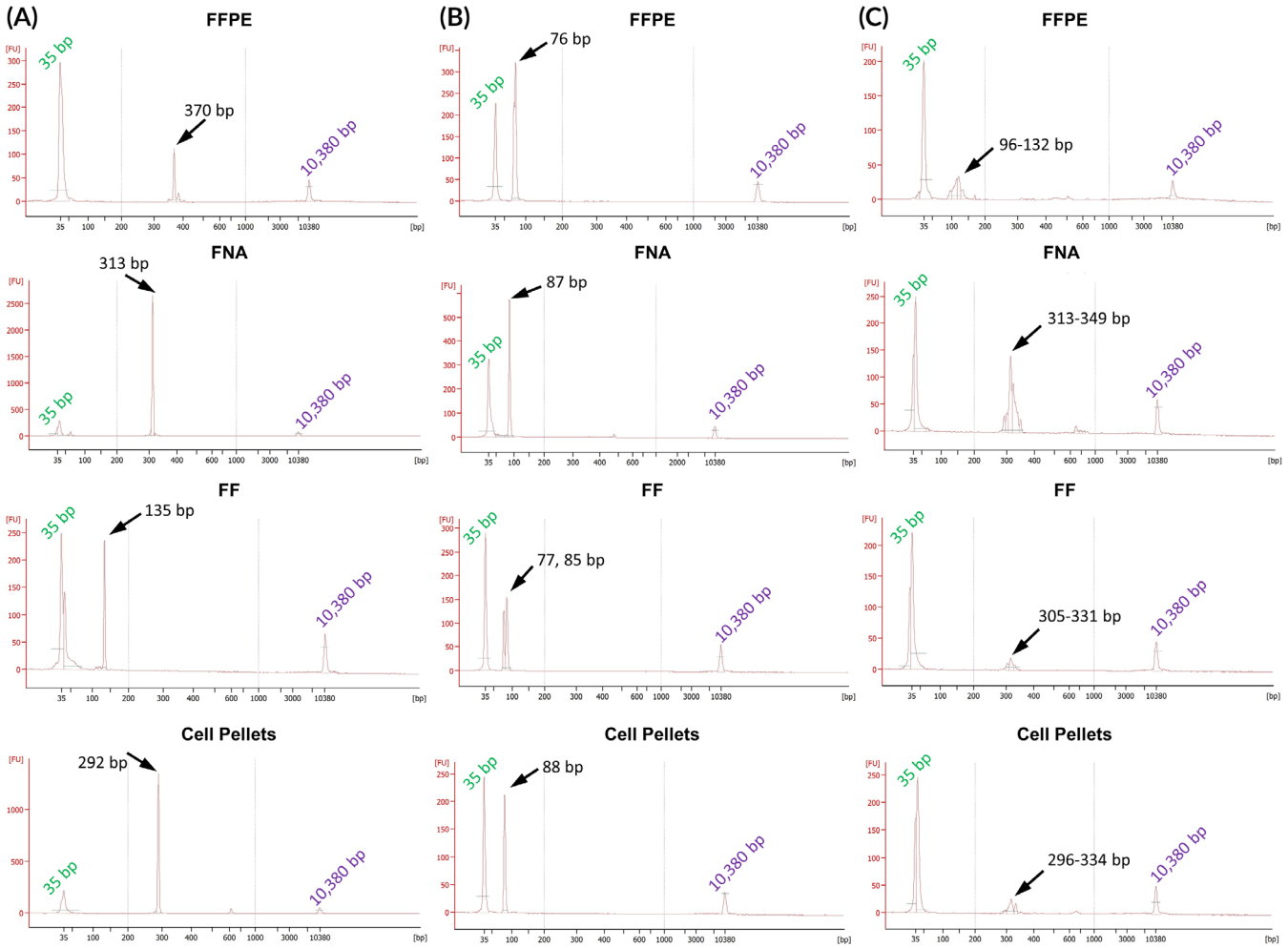

FIGURE 1 Representative ePARR electropherograms of clonal and polyclonal products from various sample types. Sample types assayed are shown on top of the figure. Fragment size is shown on the x-axis and fluorescence value is on the y-axis. The number on top of the peak in black indicates fragment size (bp). Peaks at 35 bp (in green) and 10 380 bp (in purple) represent the lower and upper size marker, respectively. Arrows indicate either clonal or polyclonal peaks. A, Representative clonal peaks of B-cell lymphoma DNA from FFPE, FNA, FF, and flow pellets were assayed with ePARR B-cell primer sets. Note the sharp tall peak with minimal polyclonal peaks near the clonal peak base for each electropherogram. B, Representative peak for T-cell primer sets. T-cell lymphoma DNA from FFPE, FNA, FF, and flow pellets were assayed with ePARR T-cell primer sets. The sharp tall peaks seen at the size range 55-88 bp without any nonspecific peaks at the peak base are indicative of a clonal phenotype. C, Representative polyclonal peaks. Non-lymphoma DNA samples from FFPE, FNA, FF, and flow pellets were assayed with ePARR primer set 1-10. Note multiple small peaks without any sharp peaks within the size markers. ePARR, PARR test developed in this study; FF, fresh frozen tissue; FFPE, formalin-fixed paraffin-embedded tissue; FNA, air-dried fine-needle aspirates

- Combine B-cell lymphoma and non-lymphoma classifications, and assess T-cell lymphoma versus B-cell lymphoma or non-lymphoma

The 95% confidence intervals (CI) were calculated for sensitivity, specificity, and accuracy based on exact binomial distributions. All analyses were performed using Stata, version 14.2.

3 | RESULTS

3.1 | ePARR performance benchmarking

We benchmarked the performance of the ePARR test in diverse sample types with a focus on a rigorously clinically characterized cohort of 56 FFPE tissues that included gold standard histopathologic diagnosis including immunophenotyping. This was followed by expanded benchmarking of the assay in 80 flow cytometry samples and feasibility assessment in 41 air-dried FNA slides (Table 1). Performance of the ePARR assay for identification of lymphoma versus non-lymphoma as well as discrimination of B-cell versus non-B-cell or T-cell versus non-T-cell lymphoma for samples passing QC was assessed by calculating sensitivity, specificity, and accuracy for each of the 3 sample type cohorts. The ePARR calls and performance (sensitivity, specificity, and accuracy) in these sample types are summarized in Tables 2–4.

3.1.1 | Primer performance variability

Individual primer set performance is summarized in Table S3. Primer sets were considered informative when they showed a definitive

TABLE 2 ePARR performance for lymphoma versus non-lymphoma[a]

		FFPE	Flow cytometry pellets	FNA
Control primer QC pass	Passed QC	50/56 (89%) = 37 lymphoma, 13 non-lymphoma	79/80 (99%) = 79 lymphoma, 0 non-lymphoma	37/41 (90%) = 19 lymphoma, 18 non
	Sensitivity	34 ePARR positives/37 lymphoma (92%; 95% CI, 78%-98%)	67 ePARR positives/79 lymphoma (85%; 95% CI, 75%-92%)	19 ePARR positives/19 lymphoma (100%; 95% CI, 82%-100%)
	Specificity	12 ePARR negatives / 13 non-lymphoma (92%; 95% CI, 64%-100%)	ND	18 ePARR negatives/18 non-lymphoma (100%; 95% CI, 81%-100%)
	Accuracy	46 ePARR positives and negatives/50 QC passes (92%; 95% CI, 81%-98%)	67 ePARR positives and negatives/79 QC passes (85%; 95% CI, 75%-92%)	37 ePARR positives and negatives/37 QC Passes (100%; 95% CI, 91%-100%)
Control primer, DIN and input DNA QC pass	Passed QC	28/56 (50%) = 22 lymphoma, 6 non-lymphoma	79/80 (99%) = 79 lymphoma, 0 non-lymphoma	28/41 (68%) = 16 lymphoma, 12 non-lymphoma
	Sensitivity	22 ePARR positives/22 lymphoma (100%; 95% CI, 85%-100%)	67 ePARR positives/79 lymphoma (85%; 95% CI, 75%–92%)	16 ePARR positives/16 lymphoma (100%; 95% CI, 79%-100%)
	Specificity	6 ePARR negatives/6 non-lymphoma (100%; 95% CI, 54%-100%)	ND	12 ePARR negatives/12 non-lymphoma (100%; 95% CI, 74%-100%)
	Accuracy	28 ePARR positives and negatives/28 QC passes (100%; 95% CI, 88%–100%)	67 ePARR positives and negatives/79 QC passes (85%; 95% CI, 75%–92%)	28 ePARR positives and negatives/28 QC passes (100%; 95% CI, 88%-100%)

Abbreviations: CI, confidence interval; DIN, DNA integrity number; ePARR, PARR test developed in this study; FFPE, formalin-fixed paraffin-embedded tissue; FNA, air-dried fine-needle aspirates; ND, no non-lymphoma samples were assessed and thus specificity was not determined; QC, quality control as defined by successful amplification with control primer sets.
[a]95% confidence intervals are shown.

TABLE 3 ePARR performance for B-cell lymphoma versus T-cell lymphoma and non-lymphoma[a]

		FFPE	Flow cytometry pellets	FNA
Control primer QC pass	Passed QC	50/56 (89%) = 17 B-cell, 33 T-cell and non-lymphoma	79/80 (99%) = 65 B-cell, 14 T-cell and non-lymphoma	37/41 (90%) = unknown lineage
	Sensitivity	16 ePARR positives/17 B-cell (94%; 95% CI, 71%-100%)	58 ePARR positives/65 B-cell (89%; 95% CI, 79%-86%)	NA
	Specificity	32 ePARR negatives/33 T-cell and non-lymphoma (97%; 95% CI, 84%-100%)	14 ePARR negatives/14 T-cell and non-lymphoma(100%; 95% CI, 77%-100%)	NA
	Accuracy	48 ePARR positives and negatives/50 QC passes (96%; 95% CI, 86%-100%)	72 ePARR positives and negatives/79 QC passes (91%; 95% CI, 83%-96%)	NA
Control primer, DIN and input DNA QC pass	Passed QC	28/56 (50%) = 11 B-cell, 17 T-cell/non-lymphoma	79/80 (99%) = 65 B-cell, 14 T-cell/non-lymphoma	28/41 (68%) = unknown lineage
	Sensitivity	10 ePARR positives/11 B-cell (91%; 95% CI, 59%-100%)	58 ePARR positives/65 B-cell (89%; 95% CI, 79%-86%)	NA
	Specificity	17 ePARR negatives/17 T-cell and non-lymphoma (100%; 95% CI, 80%-100%)	14 ePARR negatives/14 T-cell and non-lymphoma (100%; 95% CI, 77%-100%)	NA
	Accuracy	27 ePARR positives and negatives/28 QC passes (96%; 95% CI, 82%-100%)	72 ePARR positives and negatives/79 QC passes (91%; 95% CI, 83%-96%)	NA

Abbreviations: DIN, DNA integrity number; ePARR, PARR test developed in this study; FFPE, formalin-fixed paraffin-embedded tissue; FNA, air-dried fine-needle aspirates; NA, B-cell and T-cell lymphoma gold standard diagnosis not available for these samples; QC, quality control as defined by successful amplification with control primer sets.
[a]95% confidence intervals are shown.

TABLE 4 ePARR performance for T-cell lymphoma versus B-cell lymphoma and non-lymphoma[a]

		FFPE	Flow cytometry pellets	FNA
Control primer QC pass	Passed QC	50/56 (89%) = 20 T-cell, 30 B-cell, and non-lymphoma	79/80 (99%) = 14 T-cell, 65 B-cell, and non-lymphoma	37/41 (90%) = unknown lineage
	Sensitivity	17 ePARR positives/20 T-cell (85%; 95% CI, 62%-97%)	9 ePARR positives/14 T-cell (64%; 95% CI, 35%-87%)	NA
	Specificity	29 ePARR negatives/30 B-cell and non-lymphoma (95%; 95% CI, 83%-100%)	65 ePARR negatives/65 B-cell and non-lymphoma (100%; 95% CI, 94%-100%)	NA
	Accuracy	46 ePARR positives and negatives/50 QC passes (92%; 95% CI, 81%-98%)	74 ePARR positives and negatives/79 QC passes (94%; 95% CI, 86%-98%)	NA
Control primer, DIN and input DNA QC pass	Passed QC	28/56 (50%) = 11 T-cell, 17 B-cell, and non-lymphoma	79/80 (99%) = 14 T-cell, 65 B-cell, and non-lymphoma	28/41 (68%) = unknown lineage
	Sensitivity	11 ePARR positives/11 T-cell (100%; 95% CI, 72%-100%)	9 ePARR positives/14 T-cell (64%; 95% CI, 35%-87%)	NA
	Specificity	16 ePARR negatives/17 B-cell and non-lymphoma (94%; 95% CI, 71%-100%)	65 ePARR negatives/65 B-cell and non-lymphoma (100%; 95% CI, 94%-100%)	NA
	Accuracy	27 ePARR positives and negatives/28 QC passes (96%; 95% CI, 82%-100%)	74 ePARR positives and negatives/79 QC passes (94%; 95% CI, 86%-98%)	NA

Abbreviations: DIN, DNA integrity number; ePARR, PARR test developed in this study; FFPE, formalin-fixed paraffin-embedded tissue; FNA, air-dried fine-needle aspirates; NA, B-cell and T-cell lymphoma gold standard diagnosis not available for these samples; QC, quality control as defined by successful amplification with control primer sets.
[a]95% confidence intervals are shown.

clonal peak or peaks matched to gold standard diagnostic phenotype (ie, clonal peaks for IGH or TRG primers in lymphomas, clonal peaks for IGH primers in B-cell lymphomas, and clonal peaks for TRG primers in T-cell lymphomas). Concordance between duplicate runs as well as the absence of pseudoclones in heteroduplex analysis was also criteria for a primer set to be considered informative. The primer sets most frequently informative for overall lymphoma versus non-lymphoma diagnosis were primer set 1 (IGH, 50%), 5 (IGH, 46%), 4 (IGH, 44%), and 7 (TRG, 23%). Overall performance in part reflects the composition of this cohort by B-cell (58%), T-cell (27%), or unknown phenotype (16%). Additionally, although alternate B- and T-cell primer sets 11-14 were not routinely used in ePARR (utilized in 11/181 or 6% of cases), none of these primer sets (with 1 exception for primer 14 in a single case) provided an informative result. When considering B-cell phenotype alone, primer set performance from the most to the least informative was primer 1 (69%), 5 (66%), 4 (61%), 3 (14%), 2 (11%), 6 (8%), 11 (0%), and 12 (0%). When considering T-cell phenotype alone, primer set performance from the most to the least informative was primer set 7 (67%), 9 (23%), 8 (10%), 10 (8%), 14 (9%), and 13 (0%). For determination of non-lymphoma, IGH primer sets most effective and informative were as follows: primer sets 1 (94%), 4 (91%), 5 (88%), and 7 (85%). In a small subset of cases, IGH primers produced clonal peaks in T-cell lymphomas and TRG primers produced clonal peaks in B-cell lymphomas. Five B-cell lymphomas (6%) diagnosed as such using ePARR (because of multiple clonal peaks with IGH primers) also showed a small clonal peak with a T-cell primer set (primer set 7). Only 1 T-cell lymphoma (3%) diagnosed as T-cell lymphoma by ePARR (because of multiple clonal peaks with TRG primers) also showed a small clonal peak with a B-cell primer set, primer set 3 (3%).

3.1.2 | DNA yield and quality considerations across sample types

To determine if DNA yield, quality, or both might impact the performance of the ePARR assay, total DNA (in ng) and DNA quality were measured for each of the 181 samples. As shown in Table S4, total DNA varied greatly among sample types, with median values of 18 550 ng per 30 mg FF tissue (13 600-28 300 ng/30 mg), 4160 ng per flow cytometry cell pellet (341-25 600 ng/pellet), 722 ng for two 20 μm FFPE scrolls (7-24 630 ng/2 scrolls), and 7760 ng per FNA slide (400-40 000 ng/slide). The average $A_{260/280}$ ratio for all samples was 1.9. As previously mentioned, a minimum of 12.5 ng of input DNA may be optimal for each ePARR primer set to balance analyte consumption with PCR specificity. Therefore, at least 225 ng DNA yield was necessary to enable the utilization of all 18 primer sets. However, multiplexing or utilization of fewer primer sets would decrease this requirement. The 12.5 ng minimum also was not absolute because individual primer set performance is variable. All FF, flow cytometry pellets, and FNAs yielded sufficient DNA, but 68% of the FFPE samples yielded sufficient DNA for all analyses.

The DNA quality was highest for FF and flow cytometry pellets (medians = 6.6 and 6.9, respectively) and lower for FFPE and FNA (medians = 3.9 and 4.8, respectively). Notably, some FFPE and FNA had a DIN of 1. Although not all samples with low DINs failed,

approximately 60% of the inconclusive assay results did have a DIN ≤ 3.5. Therefore, a DIN of ≥3.5 was additionally selected as a criterion for a QC pass to evaluate the impact of QC cutoffs on assay performance. All FF and flow cytometry pellets satisfied the DNA quality criterion, compared to only 63% of the FFPE samples and 78% of the FNAs. When both DNA yield and DNA quality criteria were applied, 100% of FF samples, 100% of flow cytometry pellets, 52% of FFPE samples, and 78% of FNAs satisfied the DNA QC criteria.

3.1.3 | ePARR performance benchmarking in an FFPE cohort

Fifty-six FFPE samples were evaluated by ePARR (18 B-cell lymphoma, 24 T-cell lymphoma, and 14 non-lymphoma) with gold standard diagnoses. The performance of ePARR on FFPE samples before and after applying DNA QC criteria (total DNA ≥ 225 ng and DIN ≥3.5) is shown in Tables 2–4. Six samples failed control primer QC, whereas 50% (n = 28) of samples passed stringent QC and input criteria. After application of these stringent QC criteria, accuracy of ePARR on FFPE was 100% (95% CI, 88%-100%) in identifying lymphoma versus non-lymphoma, 96% (95% CI, 82%-100%) in identifying B-cell versus non-B-cell lymphoma, and 96% (95% CI, 82%-100%) in identifying T cell versus non-T-cell lymphoma. After application of stringent QC criteria, failure rates increased, but sensitivity and specificity for classifying a sample as lymphoma versus non-lymphoma increased from 92% and 92% to 100% and 100% with the accuracy increasing from 92% to 95% (Table 2). The sensitivity and specificity for classifying a sample as B-cell lymphoma versus T-cell lymphoma or non-lymphoma increased from 94% and 97% to 91% and 100% with an accuracy of 95% regardless of QC adjustment (Table 3). The sensitivity and specificity for classifying a sample as T-cell lymphoma versus B-cell lymphoma or non-lymphoma increased from 85% and 95% to 100% and 94% with the accuracy increasing from 92 to 96% (Table 4) although the failure rates and the CI widths increased in most cases. However, these differences were small and did not reach statistical significance potentially because of small sample sizes and as evidenced by wider sensitivity, specificity, and accuracy CIs.

3.1.4 | ePARR performance benchmarking in a flow cytometry cohort

Eighty samples were evaluated (66 B-cell lymphoma, 14 T-cell lymphoma, and 0 non-lymphoma) with gold standard diagnoses. Performance is shown in Tables 2–4. One B-cell lymphoma sample failed control primer QC, and the additional 79 samples passed the stringent QC and input criteria. After application of stringent QC criteria, ePARR accuracy on flow pellets was 85% (95% CI, 75%-92%) in identifying lymphoma versus non-lymphoma, 91% (95% CI, 83%-96%) in identifying B-cell versus non-B-cell lymphoma, and 94% (95% CI, 86%-98%) in T cell versus non-T-cell lymphoma. When B-cell and T-cell lymphomas were combined, the sensitivity for classifying a sample as lymphoma versus non-lymphoma was 67 of 79 (85%), with 95% CI ranging from 75% to 92% and accuracy of 85% (Table 2). Because none of the flow cytometry pellets were derived from patients without lymphoma, specificity could not be calculated. Additionally, all flow pellets met QC criteria. The sensitivity and specificity for classifying a sample as B-cell lymphoma versus T-cell lymphoma were 89% (95% CI, 79%-96%) with accuracy of 91% and 100% (95% CI, 77%-100%), respectively (Table 3). The sensitivity and specificity for classifying a sample as T-cell lymphoma versus B-cell lymphoma were 64% (95% CI, 35%-87%) and 100% (95% CI, 94%-100%), respectively, with accuracy of 94% (Table 4).

3.1.5 | ePARR performance benchmarking in an air-dried slide FNA cohort

The classifications of the 41 FNA samples were 23 lymphoma with unknown B-cell or T-cell phenotype and 18 non-lymphoma. Four lymphoma samples were inconclusive because of poor amplification of control primers (90% passed QC), and 9 additional samples did not pass stringent QC criteria including DIN and input cutoffs. A total of 28 (68%) samples passed stringent QC criteria. Accuracy of ePARR was 100% (95% CI, 91%-100%) and 100% (95% CI, 88%-100%) before and after application of stringent QC criteria (Table 2), whereas sensitivity and specificity for classifying a sample as lymphoma versus non-lymphoma were 19/19 (100%; 95% CI, 82%-100%) and 18/18 (100%; 95% CI, 81%-100%), respectively.

4 | DISCUSSION

Our primary goal was to provide an example of what we believe is necessary transparency in the conduct and performance of a molecular diagnostic assay, PARR. Collective transparency will facilitate interlaboratory comparisons until more rigorous approaches are implemented such as development of reference standards to share among laboratories, prospective multi-laboratory studies conducted in a diagnostic cohort or both. Such steps are particularly important for molecular diagnostic tools such as PARR that increasingly are being adopted in diverse laboratories. Although overall reported PARR assay performance is strong across laboratories (Table S5),[1–10] ongoing needs include detailed assessment of key assay components such as DNA yield and quality, individual primer set performance, and performance benchmarking across sample types. Additionally, although sensitivity typically is reported across studies, specificity and PARR performance in non-lymphoma are less commonly evaluated.[10] Furthermore, even similar protocols conducted in different laboratories may result in altered performance because of user variability and minor differences in protocol including variations in primer design and PCR conditions, multiplex versus singleplex PCR, and assay readout variations from standard gel electrophoresis of individual primer sets to microcapillary electrophoresis of pooled primer sets. We aimed to address some of the key needs for PARR assay benchmarking by building on a recent comprehensive assessment of a PARR protocol[10] to conduct performance assessments across diverse sample types for the ePARR assay in our laboratory.

We report on the performance of ePARR in a representative population of 181 samples from dogs with lymphoma using histopathologic review by a pathologist with lymphoma expertise (E. J. Ehrhart) and complete IHC as a gold standard. This reporting has been conducted in FF tissue, FFPE tissue, air-dried FNAs, and flow cytometry pellets and includes assessment in samples with low cellularity. We evaluated accuracy and performance (sensitivity and specificity) of the ePARR assay to establish important performance metrics to guide clinical interpretation and application of test results and to inform future areas of assay development. These metrics allow 2 critical questions to be addressed: (1) In what percentage of cases will ePARR give a clinically relevant result? and (2) When ePARR provides a result, how often is that result correct? Not only are ePARR sensitivity (true-positive rate) and specificity (true-negative rate) critically important, but accuracy (true positives + true negatives) is also a critical metric. We have chosen to focus on reporting accuracy in lieu of positive and negative predictive values because of dependence of these latter metrics on disease prevalence in the tested population and because our selection of cases is not representative of the natural population disease prevalence. As others have also recently emphasized,[10,14] large, blinded prospective studies that control for these biases are needed to robustly assess PARR performance metrics. Specificity is of particular concern for veterinary molecular diagnostic tools such as PARR insofar as incorrect diagnoses can lead to costly treatments with high morbidity and potential for lethal events. Such false-positive PARR reactions can be the result of both flawed assay performance and disease biology that can yield potentially unexpected forms of lymphocyte expansion after inflammatory stimuli.[2,10] Although a specific mechanistic explanation for the false-positive and false-negative ePARR results is beyond the scope of our study, we believe that, like any PARR reaction, our reported ePARR false negatives can be explained by assay inadequacies, whereas false positives are reasonably the result of both assay performance and disease biology.

In addition to describing performance of ePARR on FFPE samples using the application of gold standard diagnosis on a large set of lymphoma and non-lymphoma samples, we described performance on typical diagnostic samples of FNA and flow cytometry. Critically, the design of these studies also allowed us to assess the influence of DNA quality as well as individual primer performance (ie, the fraction of diagnoses in which each primer set produced a clonal peak consistent with the known clinical phenotype) on ePARR performance. Striking variability was observed ranging from 0%-94% with a small number of primer sets accounting for the majority of diagnostic results (Table S3). Primer sets 1 (IGH, 69%), 4 (IGH, 61%), 5 (IGH, 66%), and 7 (TRG, 67%) were the only sets to provide an informative result in >50% of cases within B- or T-cell phenotypes. The remaining primer sets rarely, if ever, provided a diagnostically informative result including primer sets 2 (IGH, 11%), 3 (IGH, 10%), 6 (IGH, 6%), 8 (TRG, 10%), 9 (TRG, 23%), 10 (TRG, 8%), 11 (IGH, 0%), 12 (IGH, 0%), 13 (TRG, 0%), and 14 (TRG, 9%). Most primer sets were overall more often informative for identification of non-lymphoma as evidenced by 94% of runs with primer set 1 identifying a polyclonal peak in this setting. In a small proportion of samples (6% of B-cell lymphomas and 3% of T-cell lymphomas), small clonal peaks were seen in primers targeted to the opposing lineage (eg, clonal peaks in IGH primers in a T-cell lymphoma). This occurred at low frequency in 1 TRG primer set and 4 IGH primer sets. Importantly, these single small clonal peaks were always present amidst multiple higher clonal peaks for phenotype-specific primer sets and specificity for B-cell or T-cell versus non-B-cell or non-T-cell lymphoma was 94%-100% for all sample types. These small clonal peaks may be a consequence of either primer-specific artifacts or actual cross-lineage rearrangement. The T- or B-cell spikes appeared clonal and thus were unlikely to be a result of infiltrating cross-lineage polyclonal populations. Further studies are required, however, to assess the source of these results and refine understanding of their impact on PARR execution.

We evaluated the impact of strict QC criteria on performance because reports of DNA quality in PARR samples, particularly FFPE samples, and the impact of reported quality on performance have been limited to date. In terms of overall performance and sample quality, ePARR performed best in high-quality FF and FCP samples with the highest per sample yields, DINs, and control primer success rates. Final success rates after application of stringent QC (control primer, DIN, and input cutoffs) were 99% for FCP, 68% for FNA, and 50% for FFPE. In addition to utilization of control primer success criteria, despite reduction in the percentage of successful assays, inclusion of more stringent QC criteria increased accuracy, sensitivity, and specificity for FFPE from 95%, 92% and 92% to 100%, 100% and 100% for discrimination of lymphoma versus non-lymphoma, from 95%, 94% and 97% to 95%, 91% and 100% for discrimination of B-cell lymphoma versus non-B-cell lymphoma, and 92%, 85% and 95% to 95%, 100% and 94% for discrimination of T-cell lymphoma versus non-T-cell lymphoma. Although, like FFPE, FNA samples less commonly passed QC, performance for these samples was nonetheless high (100%, 100% and 100%) even before implementation of more stringent QC criteria. These data suggest that implementation of more stringent QC guidelines might not be advantageous in a diagnostic setting despite increased sensitivity and specificity. However, sample type determinations might be warranted (eg, more stringent QC in FFPE, but not FNA). In addition to our assessment of control primer, DIN, and input cutoffs, further assessment of the impact of additional QC steps such as technical replicates, heteroduplex analysis,[2,9,17] or implementation of $A_{260/280}$ cutoffs on assay logistics and performance metrics should prove valuable for harmonization and optimization of PARR protocols.

Overall, the ePARR assay, in keeping with other PARR protocols, shows strong performance for discrimination of lymphoma from non-lymphoma as well as B-cell lymphoma from non-B-cell lymphoma and T-cell lymphoma from non-T-cell lymphoma across sample types. After implementation of stringent QC, accuracy, sensitivity and specificity in FFPE were 95%, 100% and 100% for lymphoma versus non-lymphoma, 95%, 91% and 100% for B-cell lymphoma versus non-B-cell lymphoma, and 95%, 100% and 94% for T-cell lymphoma versus non-lymphoma, respectively. For flow pellets, accuracy and sensitivity after stringent QC cutoffs were 95% and 85% for lymphoma versus non-lymphoma, 95% and 89% for B-cell lymphoma versus non-B-cell lymphoma (with specificity of 100% for B-cell lymphoma versus T-cell lymphoma), and 95% and 64% for T-cell versus non-T-cell lymphoma (with specificity of 100% for T-cell lymphoma versus B-cell

lymphoma), respectively. Finally, accuracy, sensitivity and specificity after stringent QC in FNAs were 95%, 100% and 100% for lymphoma versus non-lymphoma, respectively. Overall, ePARR is 95% accurate across sample types and diagnostic settings with trends that suggest high sensitivity in FFPE and FNA across diagnostic settings (95%-100%) that is somewhat decreased in flow pellets (64%-85%) and high specificity across sample types and diagnostic settings (94%-100%). Thus, application of ePARR on routine diagnostic samples (eg, FNA, flow cytometry) also confirms high accuracy, specificity, and sensitivity (Tables 2–4). These performance metrics are comparable to those previously described (Table S5). As described earlier, reported PARR sensitivities range from 74% to 100%, whereas specificities range from 96% to 100%, both of which are dependent on protocol, sample type, and cohort variability. Importantly, specificity and PARR performance in non-lymphoma have not been as commonly reported as sensitivity. Overall, differences in protocols, cohorts, sample types, and other variables often confound comparative assessments.

Our study builds on substantial prior data that PARR is a valuable diagnostic tool with high accuracy, specificity, and sensitivity for the diagnosis and exclusion of lymphoma in dogs. It focuses on the value of rigorously detailing the assay methods and variables including data interpretation alongside the importance of reporting performance metrics to inform critical evaluation and clinical application. Evaluation of PARR assay performance will continue to be critical as modifications in current PARR protocols are introduced that may claim improved primer coverage, improved primer performance, improved workflow with similar or better performance at decreased cost, or improved performance with low-quality DNA. In addition to the opportunity for PARR assay innovation to further improve performance, ongoing needs include those for PARR reference standards, prospective assessment in diagnostic cohorts, and assessment of interlaboratory variability. Even more broadly, in the setting of molecular diagnostic testing, it is necessary that the conduct and performance of an assay be reported with transparency. In our study, we have provided an example of such transparency. We believe that these data support that the publication of molecular diagnostic benchmarking (ie, transparent reporting of assay performance and conduct), perhaps even above and beyond the evidence of novelty, should be a shared priority in veterinary medicine. It is also important to recognize, as previously suggested,[14] that collective benchmarking through broader multi-institutional efforts faces substantial challenges including funding and logistical hurdles. Therefore, although some harmonization is currently possible by following accepted general best practices for clonality testing, it nonetheless remains critically important to continue to publish and share benchmarking data among laboratories.

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CONFLICT OF INTEREST DECLARATION

E. Ehrhart, K. Richter, C. Grimes and C. Khanna are employees of Ethos Discovery, funders of the study. Ethos Discovery did not influence the study.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

All work was completed under appropriate review and IACUC guidelines for Ethos Veterinary Health and TGen.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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