RESEARCH ARTICLE

Application of Immunophenotyping and Heteroduplex Polymerase Chain Reaction (hPARR) for Diagnosis of Canine Lymphomas

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Abstract

Background: Canine malignant lymphoma is classified into B- or T-cell origin, as in the human case. Due to differences in prognosis, a suitable method needs to be developed for lineage identification. Aims: To determine the accuracy of immunophenotypic and molecular information between three methods: immunocytochemistry (ICC), immunohistochemistry (IHC) and heteroduplex polymerase chain reaction for antigen receptor rearrangements (hPARR) in spontaneous canine lymphomas. Materials and Methods: Peripheral blood, fine needle aspiration and tissue biopsies from enlarged peripheral lymph nodes prior to treatment of 28 multicentric lymphoma patients were collected. Cytopathology and histopathology were examined and classified using the updated Kiel and WHO classifications, respectively. Anti-Pax5 and anti-CD3 antibodies as B- and T-cell markers were applied for immunophenotyping by ICC and IHC. Neoplastic lymphocytes from lymph node and white blood cell pellets from peripheral blood were evaluated by hPARR. Results: In this study, low grade B-cell lymphoma accounted for 25% (7/28), high grade B-cell lymphoma for 64.3% (18/28) and high grade T-cell lymphoma for 10.7% (3/28). According to the WHO classification, 50% of all cases were classified as diffuse large B-cell lymphoma. In addition, ICC showed concordant results with IHC; all B-cell lymphomas showed Pax5+/CD3, and all T-cell lymphomas exhibited Pax5-/CD3+. In contrast to hPARR, 12 B-cell lymphomas featured the IgH gene; seven presented the TCRγ gene; five cases showed both IgH and TCRγ genes, and one case were indeterminate. Three T-cell lymphomas showed the TCRγ gene. The percentage agreement between hPARR and ICC/IHC was 60%. Conclusions: Immunophenotyping should not rely on a single method. ICC or IHC with hPARR should be used concurrently for immunophenotypic diagnosis in canine lymphomas.

Keywords: Canine lymphoma - diagnostic methods - hPARR - ICC - IHC

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Introduction

Immunophenotyping is essential for lymphocyte-cloned classification, prognosis and treatment option in human and canine lymphomas. Immunohistochemistry (IHC) is a classic method that is referred to a gold standard due to its accuracy (Valli et al., 2011; Thalheim et al., 2013). Regarding tissue specimens, it provides morphology of lymph nodes (LN) for histological assessment, but generalized anesthesia in dogs is required. Another immunophenotyping technique is immunocytochemistry (ICC). When compared to IHC, ICC is cheaper and easier. Fine needle aspiration biopsy (FNAB) can diagnose lymphoma by cytological classification; however, cell disruption, low cellularity or smear technique can lead to difficulty in interpretation (Aulbach et al., 2010; Sapierzynski, 2010). Nevertheless, liquid-based cytology, or keeping aspirated cells in a preservative fluid for multiple slide preparation, was developed to reduce these disadvantages (Fernandes et al., 2015; Wallace et al., 2015). The specific protein markers for B-cell identification were CD79a or Pax5, while T-cell marker was CD3. (Willmann et al., 2009; Valli et al., 2013). Flow cytometry is a good option for immunophenotyping data, but fresh sample from FNAB is needed, and specific antibody panels of B- and T- cells are required (Sozmen et al., 2005).

Polymerase chain reaction for antigen receptor rearrangements (PARR) showed effective results for diagnosis in confusing cases and detection of minimal residual disease (Burnett et al., 2003; Lana et al., 2006; Aresu et al., 2014). Not only samples from LN cytology, but also peripheral blood and formalin-fixed paraffin-embedded tissue were tested by this assay (Keller et al.,

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2007; Kaneko et al., 2009; Thilakaratne et al., 2010). However, false positive and false negative results by PARR are an obstruction when interpreting the results alone. In addition, pseudoclonality can occur as a result of immunologic diversity of immunoglobulin heavy chain (IgH) and T-cell receptor gamma (TCRγ) genes because a single copy is not amplified during PARR. Thus, duplicate, triplicate and heteroduplex analysis might be a helpful technique to rule out the pseudoclones, avoid misdiagnosis and resolve reliability of the results (Takanosu et al., 2010).

The purpose of this study was to evaluate immunophenotyping data between ICC and IHC, a gold standard method, using Pax5 and CD3. Heteroduplex PARR (hPARR) from peripheral blood and LN cytology was determined the consistency between samples and compared with immunophenotyping method. To achieve a goal, all results were analyzed and compared the agreement between ICC, hPARR and IHC for developing a diagnostic method for canine T- and B-cell lymphomas.

Materials and Methods

Animals and sample collections

Twenty eight dogs presented to the Oncology Clinic, Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, in 2013 to 2015 and clinically diagnosed as canine multicentric lymphoma were recruited for this study (IACUC Number 13310074). All cases were categorized according to the WHO clinical stage shown in Table 1, including peripheral LN measurement, thoracic and abdominal radiography and abdominal ultrasonography. Whole blood was collected for evaluation of complete blood cell count by automated, liver enzymes (ALT, ALP), kidney profiles (BUN, Creatinine) and blood parasite detection by blood morphology. The infiltration of atypical lymphoid cells was determined by buffy coated smear for clinical staging. If there were medium to large lymphoblasts and small lymphocytes more than 50%, it was classified as stage V. Microscopic diagnosis was based on cytological and histopathological results of the enlarged peripheral LN.

Cytology: FNAB from superficial LN was collected using 22 G needle with a 3 ml syringe. Cells from needle hub were flushed, smeared on silane-coated slides for cytopathological study and kept in sterile PBS-contained microcentrifuge tubes at -80°C for molecular study. For routine cytopathological assessment, at least two smears were fixed in absolute methanol, stained with Giemsa and classified as low or high grade lymphoma by cell morphology according to updated Kiel criteria, assuming that at least 80% of the cells presented on slides were blast cells excluding lymphocytic leukemia (Sapierzynski et al., 2010).

Histopathology: Biopsy specimen (popliteal, axillary or inguinal LN) was collected by a 6 mm diameter punch biopsy and preserved in 10% neutral buffered formalin for routine histopathological diagnosis. Histopathological changes in cell size and shape, nucleus size and mitotic figure, number of nucleoli, chromatin density, cytoplasm characterization of neoplastic lymphocytes and mitotic index were categorized based on the WHO classification (Vezzali et al., 2010; Valli et al., 2011) by a veterinary pathologist.

White blood cell pellet: Peripheral blood was also collected from patients. Buffy coat was separated from EDTA-anticoagulated blood by centrifugation at 5,000 rpm for 5 min and kept in sterile microcentrifuge at -80°C until used for determining lymphoid lineage with hPARR.

Immunocytochemistry

At least four cytological smears on silane-coated slides from enlarged LN of individual case were dried and fixed in cold acetone at 4°C for 10 min, and kept at -20°C until used or stained directly. Antibody control for both markers was a cytological smear from a normal canine lymph node.

Immunocytochemical Assays For B-Lymphocytes: Endogenous peroxidase in cells was blocked with 3% H_2O_2 and 1% bovine serum albumin (BSA, Merck Millipore, Germany) in 0.25% phosphate buffer saline-triton X-100 (PBST) used for non-specific blocking, followed by incubating with Pax5, monoclonal mouse anti-human antibody (1EW, Leica, UK) at a dilution of 1:50 at 4°C overnight. The samples were conjugated and amplified by modified avidin-biotin-peroxidase complex (LSAB) using Novolink polymer (Leica, UK) at room temperature for 15 min. The slides were washed in PBS; color developed by 3, 3'-diaminobenzidine (DAB, Leica, UK) and then counterstained with Mayer's hematoxylin for 1 min. Pax5 showed a nuclear staining in B cells. B-cell lymphoma was identified if at least 80% of the cells revealed expression of Pax5.

Immunocytochemical assays for T-lymphocytes: Peroxidase and non-specific background were blocked by 3% H_2O_2 and 1% BSA, respectively. Then, a pan T-cell marker, CD3 monoclonal mouse anti-human antibody (LN10, Leica), was incubated at 4°C overnight and Envision polymer (Dako, Denmark) was used to detect antigen with the LSAB method. The slides were developed in color with DAB (Dako, Denmark) and counterstained with Mayer's hematoxylin. Immunopositivity of CD3 was localized in cellular cytoplasm. T-cell lymphoma was diagnosed when at least 80% positive cells of a particular antibody were observed.

Immunohistochemistry

Four to six micrometer-thick sections were immunophenotypically classified using anti-CD3 and anti-Pax5 to identify T- and B-cell lineage, respectively. Briefly, antigen retrieval for CD3 was achieved by heating the slides with 10 mM citrated buffer (pH 6.0) in a microwave oven. To block endogenous enzymes, the slides were incubated in 3% H_2O_2 for 10 min and 1% BSA at 37°C for 10 min, respectively. After washing, the slides were incubated with ready-to-use monoclonal mouse CD3 antibody (LN 10, Leica, UK) at 4°C for 12-14 h. Then, the slides were incubated with modified avidin-biotin-peroxidase complex or Envision polymer at room temperature for 45 min. Finally, they were immersed in DAB to develop an immunological reaction and then counterstained with Mayer's hematoxylin before mounting. T-cell lymphoma was interpreted when at least

80% of the neoplastic cells were stained with CD3.

Similarly, for the immunostaining of anti-Pax5 (clone 1EW) to determine B-cell lineages, antigens were retrieved by heating the slides in Tris/EDTA (pH 9.0) with an autoclave oven (121 °C, 5 min). Subsequently, they were incubated with anti-Pax5 antibody (dilution 1:50) at 4°C overnight. The slides were rinsed with PBS, blocked peroxidase and non-specific background with 3% H_2O_2 at room temperature for 10 min and 1% BSA at 37°C for 10 min. Novolink detection was conjugated on tissues at room temperature for 15 min. DAB was used as chromogen and Mayer's hematoxylin was used for counterstaining.

Interpretation of positive B-cell lymphoma was achieved when at least 80% of the neoplastic cells stained positively with Pax5.

hPARR

Genomic DNA of the cytological pellet and white blood cell (WBC) pellet was extracted with a mammalian genomic DNA miniprep kit (Sigma-Aldrich, USA). DNA concentration was measured by a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA). Conventional PARR protocol was performed for both specimens, followed by heteroduplex analysis (Takanosu et al., 2010). Specific primers were chosen, the Cμ gene for controlling DNA amplification, IgH gene for B lymphocytes and TCRγ gene for T lymphocytes (Burnett et al., 2003). DNase/RNase-free distilled water was used as a negative control. ExcelBand 100 bp+ 3K DNA ladder (Smobio Technology, Taiwan) was used for sample size identification. Primer concentrations were 200-300 nM. The PCR cycling protocol was 94°C for 45 s, 60°C for 30 s and 72°C for 30 s. After 35 cycles of PCR amplification, each PCR product was divided into two aliquots and each was run for heteroduplex analysis: incubation at 95°C for 5 min and re-annealing at 4°C for 30 min. Afterward, every PCR sample was visualized in 2% agarose gel electrophoresis with 10% ethidium bromide staining by AlphamanagerTM (Alpha Innotech, USA). A monoclonal band of each gene indicated a positive result. Biclonal or triclonal might have been observed and indicated two or three neoplastic clones. A negative result appeared as a polyclonal or no bands. B- and T-cell positive controls were histopathological diagnosis as B- and T-cell lymphoma.

Statistical analysis

Results of these three tests were classified as B-cell lymphoma if Pax5+/CD3-/distinct IgH gene or T-cell lymphoma if Pax5-/CD3+/ TCRγ gene. Sensitivity, specificity and percent agreement of ICC and hPARR compared with IHC were calculated. Cohen's K test was run to determine the agreement between hPARR and ICC and IHC, respectively with SPSS version 22.0.

Results

Signalment

Twenty eight dogs consisted of 50% (14/28) male and 50% (14/28) female with the average age of 10 years (range 3-15 years). 78.6% (22/28) was pure and 21.4% (6/28) was mixed breeds as shown in Table 2. The majority of pure breeds were Golden Retriever and Shih Tzu. All cases were multicentric lymphoma. 28.6% (8/28) was classified as clinical stage III; 53.6% (15/28) had clinical stage IV and 17.9% (5/28) had clinical stage V. Only seven dogs with clinical stage III and IV showed normal blood profiles, whereas others had abnormal blood results such as neutrophilic leukocytosis, thrombocytopenia, and increased ALT or ALP. Three dogs with thrombocytopenia showed Ehrlichia canis infection. Five dogs with clinical stage V presented lymphocytosis and leukemic appearance.

Cytopathology and histopathology

After cytopathological and histopathological grading based on the updated Kiel and WHO classifications, 25% (7/28) was classified as low grade lymphoma and 75% (21/28) were categorized as high grade lymphoma (Table 2).

For the cytopathology of low grade B-cell lymphoma, lymphocytic lymphoma presented small round cells (1

Table 1. World Health Organization (WHO) Staging Criteria for Canine Lymphoma

Stage	
I	A single lymph node is involved
II	Regional lymphadenopathy (restricted to one side of diaphragm)
III	Generalized lymphadenopathy
IV	Hepatosplenomegaly (with or without lymphadenopathy)
V	Involvement of bone marrow, central nervous system or extranodal sites
	Substage a: no clinical signs
	Substage b: clinical signs of illness

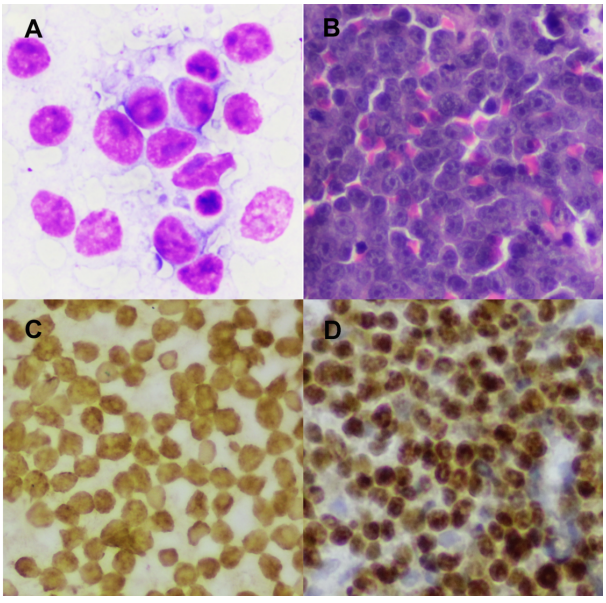

Figure 1. Diffuse Large B-cell Lymphoma (High Grade). A) Cytopathology revealed typical large B cells with a large nucleus and large centrally located nucleolus (Giemsa, 400x) B) Histopathology displayed round, oval or cleaved nuclei, with reticular chromatin and large central nucleolus (H&E, 400x) C) Pax5 was immunolabelled in nucleus of B cells (ICC, 400x) D) Pax5 showed positive staining in nucleus of malignant B lymphocytes (IHC, 400x)

to 1.5 red blood cell in diameter) with small densely basophilic round nuclei, no nucleoli and minimal cytoplasm; prolymphocytic lymphoma had slightly larger and more vesicular nuclei; macronucleolated medium-sized cell (MMC) lymphoma showed medium-sized cells with fine chromatin, prominent nucleolus and a moderate amount of weakly basophilic cytoplasm; centroblastic-centrocytic lymphoma revealed a mixture of centrocytes (irregular notched or non-cleaved nuclei, dense chromatin and narrow cytoplasmic rim) and centroblasts (round nuclei, dispersed chromatin, prominent multiple nucleoli and narrow basophilic cytoplasm). For the high grade B-cell lymphoma, malignant lymphocytes were mainly large-sized cells. Polymorphic centroblastic lymphoma was composed of a few MMCs, a few immunoblasts and mostly centroblasts. Immunoblastic lymphoma had large size, large nucleolar volume with central or multiple nucleoli, extended basophilic cytoplasm and moderate to high mitotic figures (Figure 1A). Lymphoblastic lymphoma had small to medium-sized cells with convoluted nucleus, fine chromatin, discrete nucleoli and moderate extension of basophilic cytoplasm. For T-cell lymphoma, pleomorphic small cell type illustrated small cells with irregular indented nuclei, clumped chromatin and scant cytoplasm; pleomorphic mixed type contained small-sized and large-sized cells with irregular nuclei and

Table 2. Signalment, Stage, Cytological and Histological Classifications, Immunophenotype and hPARR Results of Twenty-eight Lymphoma Cases

Dog	Sex[a]	Age (years)	Breed[b]	Clinical stage	Cytopathology (Updated Kiel)	Histopathologyc (WHO)	Grade	Immunophenotype				PARR
								IC		IH		
								Pax5	CD3	Pax5	CD3	
1	M	10	GR	III	Lymphocytic	B-SLL	Low	+	-	+		TCRγ
2	M	15	ST	V	Lymphocytic	B-SLL	Low	+	-	+	-	IgH
3	M	9	GR	V	Prolymphocytic	LLI	Low	+	-	+	-	Dual
4	F	13	Mixed	IV	Centroblastic-centrocytic	FC	Low	+	-	+	-[d]	TCRγ
5	M	8	CH	V	Centroblastic-centrocytic	FC	Low	+	-	+	-[d]	IgH
6	F	14	Mixed	IV	Centroblastic-centrocytic	FC	Low	+	-	+	-[d]	TCRγ
7	F	10	GR	III	MMC	NMZ	Low	+	-	+	-	IgH
8	F	11	LR	IV	Centroblastic monomorphic	DLBCL	High	+	-	+	-	IgH
9	M	14	ST	V	Centroblastic monomorphic	DLBCL	High	+	-	+	-	IgH
10	F	8	ST	III	Centroblastic polymorphic	DLBCL	High	+	-	+	-	Dual
11	F	10	Beagle	III	Centroblastic polymorphic	DLBCL	High	+	-	+	-	IgH
12	M	9	ST	IV	Centroblastic polymorphic	DLBCL	High	+	-	+	-	IgH
13	M	8	GR	IV	Centroblastic polymorphic	DLBCL	High	+	-	+	-	TCRγ
14	F	10	MP	IV	Centroblastic polymorphic	DLBCL	High	+	-	+	-	IgH
15	M	9	GR	V	Centroblastic polymorphic	DLBCL	High	+	-	+	-	IgH
16	F	14	GR	IV	Immunoblastic	DLBCL	High	+	-	+	-	TCRγ
17	F	12	ST	IV	Immunoblastic	DLBCL	High	+	-	+	-	IgH
18	M	7	ST	III	Immunoblastic	DLBCL	High	+	-	+	-	Dual
19	M	7	Mixed	III	Immunoblastic	DLBCL	High	+	-	+	-	TCRγ
20	F	15	CS	III	Immunoblastic	DLBCL	High	+	-	+	-	Dual
21	M	10	Mixed	III	Immunoblastic	DLBCL	High	+	-	+	-	Indeterminate
22	M	10	Mixed	IV	Lymphoblastic	B-LBL	High	+	-	+	-	IgH
23	F	7	Poodle	IV	Lymphoblastic	B-LBL	High	+	-	+	-	IgH
24	F	12	GR	IV	Lymphoblastic	B-LBL	High	+	-	+	-	Dual
25	F	6	ST	IV	Anaplastic	B-ALCL	High	+	-	+	-	TCRγ
26	M	3	BT	IV	Pleomorphic mixed	PTCL	High	-	+	-	+	TCRγ
27	M	6	Mixed	IV	Pleomorphic mixed	PTCL	High	-	+	-	+	TCRγ
28	F	15	GR	IV	Pleomorphic large cell	PTCL	High	-	+		+	TCRγ

[a]M=male; F= female, [b]BT= Bull terrier; CH= Chihuahua; CS= Cocker spaniel; GR= Golden retriever; LR= Labrador retriever; MP= Miniature pinscher; ST= Shih Tzu, [c]B-SLL= B-cell small lymphocytic lymphoma; LLI= B-cell lymphocytic lymphoma of intermediate type; FC= follicular lymphoma; NMZ = nodal marginal zone lymphoma; DLBCL = diffuse large B-cell lymphoma; B-LBL= B-cell lymphoblastic lymphoma; PTCL= peripheral T-cell lymphoma, [d]CD3 positive T-cells were located in the paracortical area

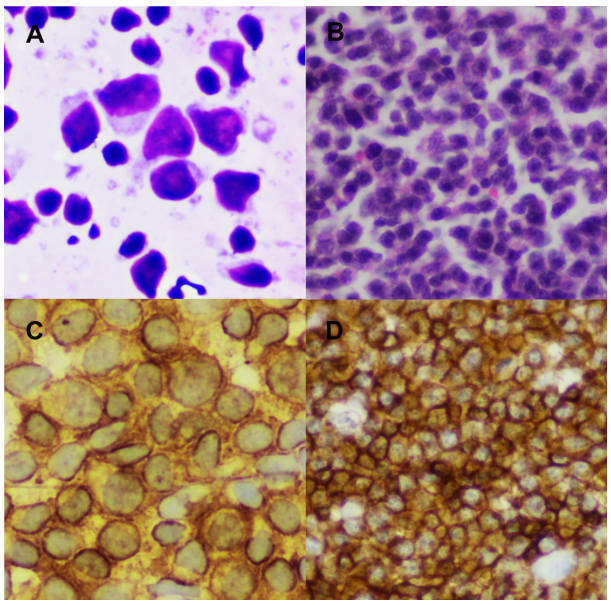

Figure 2. Peripheral T-cell Lymphoma (High Grade). A) Cytopathology presented pleomorphic lymphoblastic cells with irregular nuclei, fine chromatin and pale basophilic cytoplasm. (Giemsa, 400x) B) Histopathology showed various sizes of T-cell lymphoma, small to large malignant T- cells, with irregularly cleaved nuclei and various amounts of cytoplasm (H&E, 400x) C) Positive immunostaining of CD3 revealed in cytoplasm of T-lymphoblasts (ICC, 400x) D) CD3 immunostaining was observed in the cytoplasmic border of the T lymphoblasts (IHC, 400x)

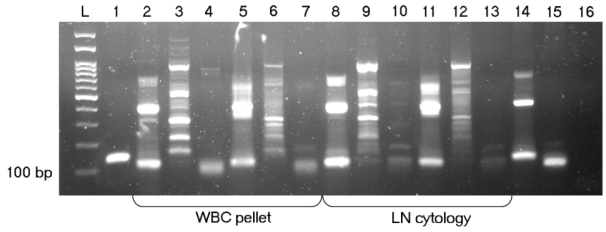

Figure 3. Conventional and Heteroduplex PARR Results from both Peripheral white Blood Cells and Lymph Node Cytologic Specimens Showed Distinct Bands of Immunoglobulin Heavy Chain (IgH) Genes. L = ExcelBand 100bp ladder; 1 = Cμ gene (130 bp); 2, 5, 8, 11 = IgH major gene (120 bp); 3, 6, 9, 12 = IgH minor gene (120 bp); 4, 7, 10, 13 = TCRγ gene (90 bp); 1-7 = sample from peripheral white blood cells; 8-13 = sample from lymph node cytology; 5-7 and 11-13 = heteroduplex analysis; 14 = B-cell lymphoma positive; 15 = T-cell lymphoma positive; 16= negative control

pale cytoplasm (Figure 2A). The pleomorphic large cell lymphoma had irregular nuclei, fine chromatin and pale to moderately basophilic cytoplasm.

The histopathology of lymph node punch biopsy was mainly classified into diffuse large B-cell lymphomas or DLBCL (Figure 1B) for 50% of cases. It was composed of centroblastic (multiple periphery nucleoli) and immunoblastic (single central nucleolus) lymphoma. A starry-sky pattern was frequently observed. Moreover, the mitotic index (MI) varied with the average number 8 MI/HPF. 10.71% of cases were follicular lymphoma (FC), which showed a mixture of centrocytes and centroblasts

Table 3. Results and Comparison Between Immunophenotype and Clonal Gene Rearrangements in Twenty-eight Lymphoma Dogs

Clonal gene rearrangement	Pax5 (+), CD3 (-) (n = 25)	Pax5 (-), CD3 (+) (n = 3)
IgH (+), TCRγ (-)	12	0
IgH (-), TCRγ (+)	7	3
IgH (+), TCRγ (+)	5	0
IgH (-), TCRγ (-)	1	0

located throughout the follicles and CD3-positive T cells located between interfollicular or paracortical areas.

Three cases of B- lymphoblastic lymphoma (B-LBL) were immunostained with Pax5. The morphology presented a diffused pattern with homogenous medium-sized cells, round nuclei, fine chromatin and indistinct small nucleoli. Starry-sky and high MF were obviously seen.

B small lymphocytic lymphoma (B-SLL) was presented less in this study (two cases). It illustrated small B lymphocytes with small round basophilic nuclei and scant cytoplasm, while B-cell lymphocytic lymphoma of intermediate type (LLI) showed slightly larger nuclei and more vesicular chromatin. One sample revealed nodal marginal zone cell lymphoma (NMZ). Macronucleolated medium-sized cells, small to medium nuclei, fine chromatin, prominent nucleolus and a moderate amount of cytoplasm were characteristic of this type. One anaplastic plasmacytoid B-cell lymphoma (APL) showed diffuse infiltration by medium to large cells with pleomorphic nuclei, prominent nucleoli and eccentric basophilic cytoplasm. Peripheral T-cell lymphoma (PTCL) was reported in three cases. It was apparent that there was an intermixing of small to large cells with irregular indented nuclei and different amounts of cytoplasm (Figure 2B).

Immunocytochemistry and immunohistochemistry

In this study, B-cell lymphoma accounted for 89.3 % (25/28), whereas T-cell lymphoma accounted for 10.7% (3/28) of the cases. With immunophenotyping by ICC and IHC, all B-cell lymphomas showed immunostaining with anti-Pax5 antibody (Figure 1C and 1D). Though, CD3 showed positive staining in all cases of T-originated lymphoma (Figure 2C and 2D).

hPARR

In this study, DNA concentration of all WBCs and cytological pellets was between 4 ng to 260 ng with A260/280 equal to1.18 to 1.98. The conventional and hPARR classified clonal rearrangements into four types: IgH gene, TCRγ gene, dual genes and indeterminate. 12 dogs (42.9%) showed IgH gene rearrangements (Figure 3); 10 cases (35.7%) presented TCRγ gene rearrangements (Figure 4); five cases (17.9 %) showed dual genes rearrangement and one dog (3.6%) presented a polyclonal result of both genes (Table 3). Both peripheral WBC and LN cytology revealed similar results. However, two WBCs and one LN sample from three dogs did not show any distinct gene, so the result was restricted to the only apparent gene from the other samples.

Statistics data

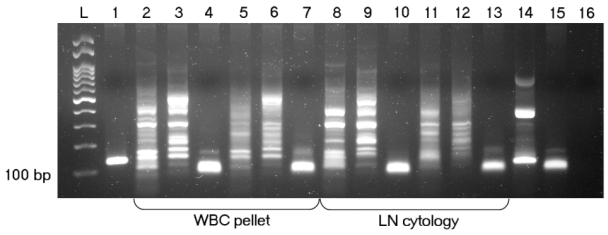

Figure 4. Conventional and Heteroduplex PARR Results from Both Peripheral White Blood Cells and Lymph Node Cytologic Specimens Showing T-cell Receptor Gamma (TCRγ) Genes. L = ExcelBand 100-bp ladder; 1 = Cμ gene (130 bp); 2, 5, 8, 11 = IgH major gene (120 bp); 3, 6, 9, 12 = IgH minor gene (120 bp); 4, 7, 10, 13 = TCRγ gene (90 bp); 1-7 = sample from peripheral white blood cells; 8-13 = sample from lymph node cytology; 5-7 and 11-13 = heteroduplex analysis; 14 = B-cell lymphoma positive; 15 = T-cell lymphoma positive; 16= negative control

The sensitivity and specificity of ICC and IHC for B- and T-cell lymphomas were similar. However, the sensitivity and specificity of IgH primer sets were 48% and 100%, respectively. The sensitivity and specificity of TCRγ primer sets were 100% and 52%, respectively (Table 3). The percentage agreement between ICC/IHC and hPARR was 60%. There was a slight agreement between ICC/IHC and hPARR, κ=0.2 (95%CI 0.002, 0.398), P<0.05.

Discussion

In this study, high grade B-cell lymphoma was 64.3% and high grade T-cell lymphoma was 10.7%, while low grade B-cell lymphoma was 25% with no low grade T-cell lymphoma. High grade lymphomas, especially DLBCL and PTCL, were the most common type, which is similar to other studies. DLBCL is more regularly reported than PTCL in canine multicentric lymphoma (Wilkerson et al., 2005; Willmann et al., 2009; Ponce et al., 2010; Valli et al., 2013). In addition, immunophenotyping is necessary because B- and T-originated lymphoma have a different prognosis. High grade T-cell lymphoma has the shortest survival time; however, clinical stage or histopathological grading also has an impact on overall survival time (Kiupel et al., 1999; Valli et al., 2013). According to the WHO classification, the 28 dogs had multicentric lymphoma in at least clinical stage III. At the same time, all low grade lymphomas were in advanced stages, but the canine patients did not show any symptom at the presentation unless generalized lymphadenopathy. In contrast, most high grade lymphomas appeared as a systemic disease at the first visit.

Nowadays there are general criteria for canine lymphoma classification based on cellular morphology, lymph node architecture, and phenotype, for example, the updated Kiel (Fournel et al., 1997; Fournel et al., 2002), Working formulation (Guija de Arespacochaga et al., 2007), and WHO classification (Vezzali et al., 2010; Valli et al., 2011). As a result of emphasizing cytomorphology and immunophenotype data, this study used the updated Kiel scheme for categorizing LN cytology as was done in previous reports (Sozmen et al., 2005; Sapierzynski et al., 2010). On the other hand, the WHO system showed high accuracy and suitability for diagnostic classification of canine lymphoma tissues even if veterinary pathologists were not hematopathologists (Valli et al., 2011).

FL was the least common canine lymphoma. The incidence rate was 1% (Ponce et al., 2010; Vezzali et al., 2010). It can be divided into grade I to grade III, depending on the predominance of centrocytes or centroblasts. This study revealed a co-expression of B- and T-phenotype similar to previous studies (Wilkerson et al., 2005; Vezzali et al., 2010). Another rare subtype was anaplastic plasmacytoid, which was reported in less than 1% (Ponce et al., 2010; Vezzali et al., 2010). It was diagnosed in one Shih Tzu dog and shown very aggressive disease with no response to standard chemotherapy.

To diagnose neoplastic lineage, IHC was used, as it is a standard and accurate method for histomorphology. In this study, all lymph node samples collected by punch biopsy were valuable for immunophenotyping by Pax5 and CD3. The Pax5 antibody was selected as a B-cell marker because it was expressed in pro B-cell throughout the activated B-cell stage, not in plasma cells and not accidently stained in T lymphocytes (Willmann et al., 2009). In addition, ICC was developed for lineage identification, as it is easier and cheaper than IHC. The ICC results in this study showed agreement with the IHC results with both the anti-Pax5 and anti-CD3 antibody. The evaluation of positivity for these two techniques was run blindly with the cut-off value 80% because of avoiding misdiagnosis with reactive lymph node. Few cytological smear glass slides produced a poor collection technique, such as a thick smear, low cellularity, and slow air dry or high watery sample, led to challenging for interpretation when compared with IHC. Other reports also found some complications using ICC with cytological smear samples, such as background staining, cell distortion and poor cellular specimen (Sapierzynski, 2010; Sapierzynski et al., 2012); however, the cytospin preparation technique showed advantageous immunocytochemical data with regard to little or no background staining (Valli et al., 2009; Aulbach et al., 2010).

PARR was a molecular assay used for the identification of a clonal proliferation, which could detect variable regions of B- and T-cell receptor genes (Burnett et al., 2003; Takanosu et al., 2010). It was also used to determine minimal residual disease in lymphoma cases during and after chemotherapy (Calzolari et al., 2006; Thilakaratne et al., 2010; Manachai et al., 2014; Aresu et al., 2014). The difficulty of this assay in this study was the presence of false positivity, false negativity and dual rearrangements. It was found that 13 B-cell lymphoma cases presented TCRγ (28%), dual (20%), and indeterminate (4%) genes by both conventional and hPARR. In this study, IgH primer sets had lower sensitivity than TCRγ primer sets. This might be because primers cannot bind to V and J regions due to immunologic diversity of target genes, presence of mutations or aberrant gene rearrangements of tumor cells (Takanosu et al., 2010; Thilakaratne et al., 2010; Boone et al., 2013). Dual gene rearrangements might be caused by the presence of two clones of neoplastic

cells, the transformation of single neoplastic clone to multiple clones or the presence of rearrangements on both chromosomes (Burnett et al., 2003). When comparing the sensitivity and specificity of ICC with IHC, a gold standard for immunophenotyping, there was no variation. Conversely, PARR showed lower sensitivity for IgH primers and lower specificity for TCRγ primers when compared to ICC/IHC. Thalheim et al. (2013) also reported the percent agreement between IHC and PARR which was 69%, while our result was 60%. It might be because the caseload in this study was limited; especially the number of T-cell lymphoma case, thus sensitivity, specificity and percent of agreement values might be controversial. Due to the unclear results in some cases, capillary electrophoresis or Gene Scan analysis in the detection of PARR products were developed for a superior solution (Jeon et al., 2007; Goto et al., 2015).

In this study, both WBC-based and LN-based specimens were collected for PARR analysis. Both samples illustrated a parallel result of the clonal amplification. However, one sample of LN cytology and two samples of WBC pellet did not reveal clonal rearrangements; even though every patient had clinical stage III to V. Lana et al. (2006) also reported that 28% of cases with stage III to V had no detectable amplification gene from either peripheral blood or LN samples. This might be because samples did not contain specific DNA sequences, or current primers could not attach to receptor rearrangement genes in that sample.

Further investigation needs specific primers to individual genomic information of a particular clone of B- and T-cell receptor genes (Tamura et al., 2006; Yagihara et al., 2007; Keller and Moore, 2012). Thus, immunocytochemical and immunohistochemical techniques should be used for identifying lymphocyte lineage. However, concurrent methods such as heteroduplex PARR could be applied as an adjunct diagnostic tool either with ICC or IHC for sup-typing confirmation.

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