Article

Clonality and phenotyping of canine lymphomas before chemotherapy and during remission using polymerase chain reaction (PCR) on lymph node cytologic smears and peripheral blood

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Abstract — Polymerase chain reaction (PCR) assays for the immunoglobulin and T-cell receptor genes were utilized to determine phenotype and clonality from lymph node cytologic smears and peripheral blood lymphocytes from 10 dogs with lymphoma, before chemotherapy and during remission. Results were compared with those from 13 dogs with a cytologic diagnosis of lymph node hyperplasia. Clonality was identified in 7 of the lymphomas on the basis of either lymph node cytology or peripheral blood lymphocytes before treatment. No lymph node hyperplasia samples were clonal. In 6 of the dogs with lymphoma, clonality was demonstrated during clinical remission. Detection of PCR clonality during clinical remission is an effective means of identifying minimal residual disease in canine lymphoma and thus additional work is warranted to determine if molecular remission is prognostic or predictive for outcome in well-controlled and well-defined lymphoma subtypes.

Résumé — Clonalité et phénotypage des lymphomes canins avant la chimiothérapie et durant la rémission à l'aide de la réaction d'amplification en chaîne par la polymérase (RCP) sur des frottis cytologiques des ganglions lymphatiques et du sang périphérique. Des essais par réaction d'amplification en chaîne par la polymérase (RCP) pour les gènes récepteurs de l'immunoglobuline et des cellules T ont été utilisés pour déterminer le phénotype et la clonalité à partir de frottis cytologiques des ganglions lymphatiques et de lymphocytes sanguins périphériques provenant de 10 chiens atteints d'un lymphome, avant la chimiothérapie et durant la rémission. Les résultats ont été comparés à ceux de 13 chiens avec un diagnostic cytologique d'hyperplasie des ganglions lymphatiques. La clonalité a été identifiée dans 7 des lymphomes en se fondant sur la cytologie des ganglions lymphatiques ou des lymphocytes sanguins périphériques avant le traitement. Aucun échantillon d'hyperplasie des ganglions lymphatiques n'était clonal. Chez 6 des chiens atteints de lymphome, la clonalité a été démontrée durant la rémission clinique. La détection de la clonalité de la RCP durant une rémission clinique représente un moyen efficace d'identifier une maladie résiduelle minime pour le lymphome canin et des travaux supplémentaires sont donc nécessaires pour déterminer si la rémission moléculaire représente un pronostic ou une prédiction des résultats dans des sous-types de lymphomes bien contrôlés et bien définis.

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Introduction

Lymphoma, a tumor caused by malignant clonal expansion of lymphocytes, is one of the most common neoplasms in dogs. It is also one of the malignancies most likely to have complete response to chemotherapy (1). Lymphoma is staged to determine the extent of disease; stage V is defined as neoplastic involvement of peripheral blood and bone marrow or other organ systems, or both (2). However, criteria for microscopic detection of neoplastic cells in peripheral blood are subjective and varied. More accurate detection of circulating tumor cells to determine pretreatment extent of the disease and post-treatment efficacy of therapy should result in improved outcome and monitoring of lymphoid neoplasms (3,4). Assessment and demonstration of clonality by molecular analysis of antigen receptor genes generally provides the most accurate diagnostic tool for lymphoma diagnosis (5–7). Polymerase chain reaction (PCR)

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clonality analysis, using DNA from cytologic specimens (8–10) and peripheral blood (4,11) provides a minimally invasive method to confirm neoplasia by demonstrating clonality, while concurrently providing the lymphocyte phenotype.

Many dogs with lymphoma have generalized peripheral lymphadenopathy, allowing for apparent ease of clinical monitoring. In the absence of other sites of disease, remission status is often determined through palpation and assessment of lymph node size. Persistent lymph node enlargement usually indicates lack of clinical remission, whereas normal size lymph nodes are often considered to represent complete remission (12,13). However, physical examination and measurement of lymph node volume may not be sufficient for accurate determination of remission (12,14) and cytologic examination of lymph node fine-needle aspirates (FNAs) is a more accurate means of determining remission status (14). While treatment of lymphoma with chemotherapy will achieve a complete clinical response for many patients, the majority will experience a recurrence of disease caused by residual cells present below the level of detection by standard light microscopy (15). More accurate methods of detecting residual neoplastic cells are needed in such instances. Minimal residual disease (MRD) can be detected by molecular techniques such as PCR (10,16,17).

Human patients with higher levels of residual cells by PCR analysis in the post-induction period relapsed earlier than the comparison group (15). Moreover, there was a higher relapse rate among patients with high levels of MRD, compared with those with intermediate levels or no detectable MRD. Molecular assessment is now commonly used as a measure of outcome in clinical trials of novel therapies for the treatment of human lymphomas (15). Polymerase chain reaction has been used to assist in the diagnosis, staging, prognostication, and detection of clonality of canine lymphomas. However, studies to detect minimal residual disease using PCR clonality in canine lymphomas are rare (10). Although PCR is reported to be superior to microscopic evaluation for detecting circulating neoplastic lymphocytes at the time of lymphoma diagnosis (4,11), similar studies to assess remission status are lacking in the veterinary literature.

The objectives of the current study were to compare PCR clonality and phenotype results in dogs with lymphoma to those with hyperplastic lymph nodes, and to compare PCR clonality and microscopic blood smear evaluation for the detection of residual neoplastic lymphocytes during clinical remission.

We hypothesized that the PCR clonality assay would differentiate lymphoma from hyperplastic/reactive lymphocytes using cells scraped from lymph node smears or from peripheral blood (when abnormal lymphocytes are present) and that clonality detected during clinical remission would indicate minimal residual disease. We also hypothesized that the PCR clonality assay would be more sensitive than microscopy for the detection of clonal lymphocytes in peripheral blood from dogs in clinical remission and therefore more effective in screening dogs for minimal residual disease following chemotherapy.

Materials and methods

Lymphoma case selection

Client-owned dogs presented to the Western College of Veterinary Medicine (WCVM) and Canada West Veterinary Specialists and Critical Care Hospital during 2006–2008 and diagnosed with multicentric lymphoma and no concurrent diseases were used in this study. Due to client constraints (such as, financial) to proceed with chemotherapy, the sample number was limited to 10 during the collection period. Multicentric lymphoma was diagnosed based on lymph node cytology (18). According to this protocol all cases were classified in the high grade lymphoma category. The World Health Organization (WHO) staging system (2) was used to classify the extent of the disease (Table 1). Dogs that had received treatment for lymphoma or immunosuppressant drugs for any reason were excluded from the study. Clients' consent to enroll the dogs into the study and a commitment to permit chemotherapy for their dogs were necessary selection criteria. All dogs in the study were treated using the modified University of Wisconsin — Madison chemotherapy protocol (UW19) for lymphoma (19) after initial samples were collected.

Table 1. World Health Organization (WHO) clinical staging of canine lymphosarcoma

Clinical stage*	Criteria
I	Involvement limited to single node or lymphoid tissue in single organ (excluding bone marrow)
II	Involvement of many lymph nodes in a regional area (± tonsils)
III	Generalized lymph node involvement
IV	Liver and/or spleen involvement (± stage III)
V	Manifestation in the blood and involvement of bone marrow and/or other organ systems (± stage I–IV)

* Stages are further classified to clinical substage a (without systemic signs) or substage b (with systemic signs).

Sample collection

The protocol for this study was approved by the University Committee on Animal Care and Supply (UCACS) and all samples were collected according to UCACS guidelines. Cytologic samples consisted of smears from lymph nodes obtained by FNA. Air dried Romanowsky stained (Wright-Giemsa; Bayer Diagnostics, Toronto, Ontario) smears that were initially examined by a clinical pathologist to establish a diagnosis of lymphoma were used. The cytology smears were reevaluated for morphology, cell density, and degree of cell disintegration by one of the authors (DT). Before onset of treatment approximately 3 mL of whole blood was collected from each animal in an EDTA tube for a complete blood (cell) count (CBC) (Cell Dyn 3500; Abbott Diagnostics, Abbott Park, Illinois, USA), blood smear preparation and buffy coat separation for PCR. Smears were prepared using a push slide technique, air dried, and stained with Wright-Giemsa stain.

Repeat peripheral blood samples and cytology samples (where FNA was possible from regressing lymph nodes) were collected from each animal when in complete remission, approximately 5 wk after onset of therapy. Complete remission was defined as the disappearance of all clinical evidence of disease on the basis of physical examination (normal lymph node palpation, no organomegaly), CBC, and/or biochemical analyses. Dogs

Table 2. Canine lymphosarcoma: PCR clonality results from peripheral blood and lymph node smears (pretreatment and during remission)

		Pretreatment				During remission				
		IGH — Clonality		TCRG — Clonality		IGH — Clonality		TCRG — Clonality		
Case number	Clinical stage	Peripheral blood	Cytology	Peripheral blood	Cytology	Peripheral blood	Cytology	Peripheral blood	Cytology	Relapse (FRD)
1	Va	+	−	−	−	+	−	−	−	+ (62)
2	IVa	−	+	−	−	+	NA	−	NA	+ (180)
3	IIIa	−	+	+	−	−	NA	+	NA	−
4	IIIa	−	−	−	−	−	−	−	+	−
5	IVa	−	+	−	+	−	−	−	−	−
6	Va	−	+	−	+	−	+	−	−	−
7	Va	−	−	−	−	−	−	−	−	−
8	IVa	−	−	−	+	−	−	+	−	+ (150)
9	IVa	−	−	−	+	NA	−	NA	−	−
10	IVa	−	−	−	−	−	−	−	−	−

+ — Positive, − — Negative, NA — Not available, TCRG — T-cell receptor gene, IGH — Immunoglobulin heavy chain, FRD — First remission duration in days.

were monitored for another 4 to 6 mo after collection of a second sample.

Lymphoid hyperplasia case selection

Specimens from 13 client-owned dogs presented to WCVM, between 2006–2008 with the cytologic diagnosis of lymphoid hyperplasia were used in the study. Lymph node cytologic samples and peripheral blood were collected from each dog as described above for lymphoma cases.

Blinded study design

All samples were assigned numbers and arranged in a random manner by a technologist so that their identities would be unknown for the procedures to follow. The smear evaluation, PCR procedure and interpretation of PCR results were done without the investigators having knowledge of the diagnosis. Blood smears were reevaluated by 2 authors (DT, BK) for the presence of abnormal circulating lymphocytes. The abnormal lymphocytes were identified by previously published criteria with minor modifications (4).

DNA extraction — cytologic samples

One lymph node cytology smear from each case was soaked in xylene to remove the coverslip and then rehydrated through decreasing concentrations of ethanol (30 s in each 100%, 90%, and 80% ethanol solutions). Cells were recovered from the slides using a scalpel blade and DNA was extracted using the DNeasy extraction kit (Qiagen, Valencia, California, USA) according to the manufacturer's extraction protocol for animal cells. DNA was eluted with ultra pure water and stored immediately at −20°C until PCR analysis.

DNA extraction — peripheral blood

Whole blood samples were spun down at 5000 rpm in a Sorval centrifuge (Thermo Scientific, Waltham, Massachusetts, USA) at room temperature for 5 min and 200 μL of the buffy coat was removed. Then DNA was extracted and stored as described above until PCR analysis.

Primers and PCR conditions

Polymerase chain reaction amplification for Ig and TCR genes was performed in duplicate in our laboratory using previously published methodology and primers (6) with few modifications as described below. All PCR amplifications were performed in a MJ Research PTC-200 thermocycler (GMI, Minnesota, USA). The PCR mixes contained 2.5 μL of DNA in a total reaction volume of 47.5 μL. The initial activation step was performed by using HotStar *Taq* Plus DNA polymerase (Qiagen) at 95°C for 5 min. Gels were visualized with a UV transilluminator (Alpha Innotech Corporation, California, USA). To ensure that DNA was amplifiable when no product was visualized, positive control primers (Sigmf1, Srμ3) ~ 130 bp were used to amplify the constant region of IgM. A negative control (ultra pure water) was included for each primer set in each run of the PCR assay to ensure no DNA contamination was present in the reaction mix. Positive controls included DNA from cytology smears obtained from 2 dogs with lymphoma in which the phenotype (B or T cell) was previously determined by immunohistochemistry. The heteroduplex duplicate result was used to determine the clonality status of the sample. Clonality analysis was repeated when results were equivocal; absence of any PCR products in 1 of the duplicate heteroduplex lanes or when both TCR and Ig clonal rearrangements were noted.

Interpretation of PCR clonality results was done by 2 of the authors (DT, BK) without knowledge of the initial diagnosis; results were reviewed and a final agreement was made.

Results

The PCR clonality assay detected polyclonality in all 13 cytologic and peripheral blood samples of lymphoid hyperplasia cases. Follow-up information on the clinical course for a period of 6 to 24 mo was obtained by review of medical records or correspondence with veterinarians confirming that these dogs did not develop malignancy.

Clonality and blood smear evaluation results of the 10 lymphoma cases are summarized in Tables 2 and 3. Of the 10 lymphomas assayed before chemotherapy, 7 had clonal product

Table 3. Canine lymphosarcoma blood smear evaluation and PCR clonality results (pretreatment and during remission).

Case number	Pretreatment				Remission			
	CP1	CP2	CP3	PCR	CP1	CP2	CP3	PCR
1	+	−	+	+ B	−	−	−	+ B
2	+	+	+	−	−	−	−	+ B
3	−	−	−	+ T	−	−	−	+ T
4	−	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−	−
6	+	+	+	−	−	−	−	−
7	−	+	+	−	−	−	−	−
8	−	−	−	−	−	−	−	+ T
9	+	+	+	−	−	−	−	NA
10	−	−	−	−	−	−	+	−

CP1, CP2, CP3: Clinical pathologists 1,2,3, + — atypical lymphocytes present, − — atypical lymphocytes absent.

identified from DNA from either cytology or peripheral blood lymphocytes and clonal product was not identified in samples from the remaining 3. Of the 7 cases, 6 demonstrated clonality on cytologic specimens and 2 demonstrated clonality on peripheral blood; 1 of these produced clonal bands on both peripheral blood and cytologic samples.

Of the same 10 dogs assayed during remission, 6 had clonal product identified in either cytology (2/6) or peripheral blood (4/6) samples and 4 had polyclonal PCR product identified in either cytology or peripheral blood samples. Of these 6 dogs that demonstrated clonality, 3 had relapsed (cases 1, 2, and 8) and their first remission durations were 62 d, 180 d, and 150 d, respectively. Cytologic smears obtained from FNA of lymph nodes during remission revealed lymphocytic hyperplasia. However in 2 cases, cytology smears were not available for cytologic evaluation or PCR during remission due to an inability to obtain representative material from regressing lymph nodes.

Based on blood smear evaluation at the time of diagnosis, abnormal lymphocytes were detected by microscopy (at least 2 of 3 clinical pathologists) in 5 cases; 2 of these had clonal results. During remission abnormal lymphocytes were detected by microscopy in 1 case and 4 had clonal results. None of the lymphoid hyperplasia cases had abnormal circulating lymphocytes detected by any of the clinical pathologists.

Discussion

Cytologic assessment of samples obtained by FNA is often the 1st line of morphological investigation of lymphoma. However it can be difficult to differentiate reactive and neoplastic lymphocyte populations, particularly early in the course of disease. This distinction is a fundamental prerequisite for an accurate diagnosis, successful therapy and patient management (20,21). Polymerase chain reaction based clonality testing for lymphocyte populations has been developed for dogs and these tests may aid in differentiating between lymphoma and lymphocytic hyperplasia. Phenotype was assigned in our study based on the type of clonal rearrangement detected in the sample. The presence of a clonally rearranged Ig gene indicates a B-cell phenotype and a clonally rearranged TCR gene indicates a T-cell phenotype. Presence of both Ig gene and TCR gene rearrangement (dual rearrangements) is consistent with a variety of possibilities, the most likely being aberrant rearrangement of the genes in a tumor that retains recombinase activity, and additional tests would be required to establish a phenotype and to properly classify the malignancy (6,22). In our study, clonal rearrangements of either Ig or TCR genes, or both, were found in 7/10 cytologically confirmed lymphomas before treatment. Data in this study are consistent with other reports (6,8,9). Careful histologic examination of tissues and immunophenotyping are warranted for the accurate interpretation of clonality results (6). Application of the WHO-REAL classification system for lymphomas has shown that expected outcomes for lymphoma are likely to vary by subtype (6). Definitive classification of lymphomas into each subtype would require histopathology (22) and this was not done in the present study.

In lymphoid hyperplasia cases, the PCR clonality assay detected polyclonality in all 13 cytologic and peripheral blood samples. It was not possible to obtain a definitive diagnosis for these dogs and these cases likely represent a variety of inflammatory conditions.

Occasionally, interpretation of native PCR products is hampered by the presence of a background smear or faint banding pattern that obscures monoclonal bands in the target range. In these situations, PCR products can be denatured, reannealed, and then resolved on polyacrylamide gels. This technique is referred to as heteroduplex analysis and it enhances the specificity of the assessment of PCR amplification of the target region, since it provides a means to confirm that bands consisted of molecules of identical size and sequence (23).

The 3 samples that gave a negative result, despite the presence of cytologically confirmed lymphoma, could be samples that contained nucleotide sequences in their variable (V) or joining (J) region genes to which our primers did not bind (9,24). This limitation may be overcome by using newly designed PCR primers that are directed to the conserved or specific nucleotide sequence for each subgroup of V and J genes (7,25). Based on information from the canine genome database and bioinformatics analysis, Yagihara et al (7) designed 4 combinations of primers for the TCR gene. Tamura et al (25) discovered conserved sequences within the vast majority of 61 V segments and 1 J segment of the immunoglobulin heavy chain (IgH) gene and designed optimal primers to detect the monoclonality of IgH in canine B-cell lymphoma (25). Tumors that have deleted or only partially rearranged antigen receptor genes or chromosomal defects can also give negative results (9,24).

In order to detect contamination resulting in false positives, a negative control was used throughout the DNA extraction and PCR protocol for each run. The negative control for each primer set was consistently devoid of PCR product. Again, in the absence of duplicate PCR clonality analysis, amplification of a residual background population of normal lymphocytes could produce a false positive result (24). This possibility was excluded in our study since we conducted the PCR assay in duplicate loaded in adjacent lanes in the same gel.

Two cases in our study had rearrangements of both Ig and TCR genes. Approximately 25% to 30% of high-grade lymphocytic tumors in humans (26,27) and some canine lymphoma cases have been reported to have rearrangements of both Ig and TCR genes (6,9,10,11). This is likely due to true cross lineage

rearrangement in which some lymphomas can retain recombinase activity and thus aberrantly rearrange antigen receptors irrespective of their lineage (6). Additionally, the presence of a benign population of T lymphocytes of limited diversity reacting against a B-cell lymphoma may result in clonal TCR gene rearrangement (24). Immunophenotyping is required to determine lineage commitment in such cases.

The second aim of this study was to evaluate the usefulness of the PCR clonality assay for identification of residual neoplastic cells and compare PCR results with clinicians' assessment for determination of remission status of canine lymphomas. The PCR clonality assay was positive for 6/10 of dogs in complete clinical remission. Three dogs that had clonal results during remission had recurrence of lymphoma at a later date. Our results suggest that physical examination (normal lymph node size, no organomegaly) may not be sufficient for accurately determining remission, and that PCR is a useful tool for identifying recurrence or detecting minimal residual disease after chemotherapy. Since routine microscopic examination is often unable to recognize a residual neoplastic population whenever it accounts for $<$ 1% of the total cells, molecular tools are mainly used for the detection of minimal residual disease in humans and PCR has been successfully used for this purpose (15,28). Further, human patients with follicular lymphomas who have had chemotherapy have longer disease-free survival if their peripheral blood/bone marrow becomes negative rather than remains PCR positive, and PCR negativity is strongly predictive of continued clinical remission (29,30). Polymerase chain reaction clonality testing performed at more frequent times during the course of treatment of canine lymphoma may be more useful to assess treated dogs for continued clinical remission. Complete clinical remission is one of the primary goals in treating lymphomas in dogs and lack of complete clinical remission is undesirable (31,32). Accurate and reliable assessments of remission status early in the course of disease may enable optimization of treatment for the individual, in which patients not achieving complete clinical remission with appropriate treatment periods are treated with alternate protocols (32). The use of clonal rearrangement to detect minimal residual disease should characterize the clonal rearrangement by precise size or identical sequencing at both the time of diagnosis and at relapse (28). Future studies to investigate the specific nucleotide sequence of each clonal rearrangement are warranted in relation to canine lymphomas. The use of molecular remission as a surrogate marker of clinical response and the significance of minimal residual disease in determining prognosis and planning treatment strategies are extensively addressed in the human literature (33,34).

Some of our results failed to show correlation between the detection of neoplastic cells in peripheral blood at diagnosis and during clinical remission, and again there was no correlation with absence of molecular remission in lymph nodes. These findings are inconclusive and not easily explained. Further, 1 lymphoma case yielded confusing results (case 3); T-cell clonality was detected from peripheral blood while B-cell clonality was detected from cytologic specimens. In remission, T-cell clonality was again detected from peripheral blood. These findings are also inconclusive and problematic, perhaps our blinded study design may have accounted for these unexplainable results as biases regarding interpretation were eliminated.

Diagnosis and clonality of canine lymphoma using PCR of cytologic and peripheral blood samples has been previously reported in the veterinary literature. However, our study used both cytologic specimens and peripheral blood from the same animal for clonality analysis in pretreatment and clinical remission states.

Polymerase chain reaction is sensitive and applicable to a small amount of DNA obtained from cytologic specimens and peripheral blood by minimally invasive methods. This assay can be used to detect minimal residual disease and is therefore useful in monitoring response to therapy, remission, or detecting early relapses. In summary, detection of PCR clonality during clinical remission is an effective means of identifying minimal residual disease in canine lymphoma, and thus additional work is justifiable to determine if molecular remission is prognostic or predictive for outcome in well-controlled and well-defined, uniform populations of lymphoma subtypes.

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