Hematologic malignancies, particularly lymphoma and leukemia, are a diverse group of diseases with a myriad of different presentations. Although the diagnosis of these diseases can be straightforward, there are many cases in which the diagnosis is difficult to establish with conventional methods. Molecular diagnostic testing to identify oncogenes and clonal lymphocyte populations can aid in resolving ambiguous cases, and several of these tests are routinely available for canine patients. Sensitive polymerase chain reaction–based methods are also useful for answering a variety of research questions. Here, detection of mutations of the c-kit gene in mast cell tumors, the bcr-abl fusion gene in myelogenous leukemias, and clonality of lymphocyte populations for the diagnosis and monitoring of lymphoma and leukemia are discussed.

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Detection of wild type and mutated c-kit gene in a patient with a mast cell tumor. A PCR product revealed the presence of internal tandem duplication in all the tumors, as well as a second, larger PCR product, indicating that one copy of the gene was longer. Sequencing of the large PCR product showed only the wild type gene. Lanes 4 and 5 are draining lymph node and the primary tumor, showing both the wild type gene and the larger product of the mutated gene.

Each tumor (the ITD are different in each patient), detection of c-kit ITD has now been used for diagnosis, determining relatedness between mast cell tumors, prognosis, and monitoring treatment. Investigators at Michigan State University studied 2 large series of mast cell tumors to determine if the presence of the c-kit ITD correlated with stage and outcome. They found that only about 10% of all mast cell tumors have ITD, but the presence of these mutations correlated strongly and inversely with outcome, both in cases treated with surgery alone, and cases treated with surgery plus chemotherapy. C-kit ITDs were never found in grade I mast cell tumors, but in both grade II and III MCT. Other factors, including histologic grade, mitotic index, and other measures of proliferation, can also be used to predict survival. Together, these tools can provide a thorough picture of this frequently encountered tumor type.

The observation that, in some cases, mast cell tumors can recur in distant sites sometimes a year or more after the first one is removed led Zavodovskaya and coworkers to ask whether these tumors are derived from the same clone, or are newly developed tumors. They describe 2 patients with mast cell tumors that recurred over a period of 3 years. One of these patients had one mast cell tumor occur each year for 3 years, with no therapy between the appearance of each tumor. These were completely excised each time and recurred in a different location. The investigators amplified c-kit DNA from each of the 3 tumors. A PCR product of the correct size relative to the wild type c-kit gene was identified in all the tumors, as well as a second, larger PCR product, indicating that one copy of the gene was longer. Sequencing of the large PCR product revealed the presence of internal tandem duplications, which were identical in all 3 tumors, indicating that the tumors were all derived from the same clone. The investigators could conclude that the neoplastic mast cells were never eradicated. A second case also involved a dog with multiple mast cells, which had a cytologically more aggressive appearance (grade III). Again, each of the individual tumors had the same tandem duplication, indicating they all arose from the same neoplastic clone.

Although not reported in this article, the sensitive nature of PCR-based assays would allow clinicians to determine if neoplastic mast cells could be found in the blood, marrow, liver, and spleen using tissue aspirates, and the number of such cells could be quantified (for example, as in Fig 1). Studies aimed at demonstrating that the extent of disease determined by molecular methods is correlated to prognosis would be useful, and may result in more aggressive treatment in some cases, and less aggressive treatment in others. In addition, the efficacy of chemotherapy could also be assessed by quantifying tumor burden pretreatment and posttreatment in those cases that harbor the c-kit mutation.

Another potential use for detecting the c-kit mutation is to establish lineage when round cell tumors with an ambiguous origin are identified. Finding the ITD would support the diagnosis of a mast cell tumor, although it should be emphasized that other types of round cell tumors have not been tested for c-kit mutations, an important step in using c-kit ITD for diagnostic purposes.

Detection of Chromosomal Translocations

bcr-abl in Chronic Myelogenous Leukemia

One common feature of hematopoietic neoplasia is the presence of translocations that bring together 2 genes to create a single fusion gene and protein product. These new fusion genes provide a growth advantage to the tumor cell, and are an essential feature of oncogenesis. The best studied of these fusion genes, the “Philadelphia chromosome,” is the bcr-abl fusion gene found in more than 90% of all human chronic myelogenous leukemias and occasionally ALL and AML [reviewed in1]. Abl is a tyrosine kinase that has myriad activities involved in cell growth and differentiation. It is encoded on human chromosome 9, and in chronic myelogenous leukemia (CML) is translocated to chromosome 22. The site of the translocation varies within bcr (breakpoint cluster region) gene, so that a new fusion gene, bcr-abl, is formed. The new fusion protein formed allows for the constitutive activation of the abl tyrosine kinase, which, in turn, promotes the development of CML. The bcr-abl translocation can also be found, sporadically, in some acute B-cell leukemias.

Most cases of human CML can be diagnosed by findings in the peripheral blood, which include a high neutrophil count, the presence of neutrophils at all stages of differentiation, and basophilia, and can also include lymphocytosis, eosinophilia, and thrombocytosis. However, in cases where there is extreme neutrophilia and other causes are suspected, such as infection and paraneoplastic expansion of neutrophils associated with some cancers, finding the bcr-abl translocation is considered diagnostic for CML, and its absence virtually rules out the disease.
Patients with fewer copies of the bcr-abl translocations in a particular remission achieved is a strong indicator of prognosis. The process of producing a diverse repertoire of antibodies to combat a complex universe of pathogens involves the breakage and recombination of DNA encoding immunoglobulin (Ig) genes. These events also create the opportunity for aberrant recombination (ie, recombination between Ig genes and genes on different chromosomes), and, as such, many human B-cell lymphomas, leukemias, and plasma cell tumors are characterized by translocation between Ig heavy chain genes (IgH) or light chain genes (Igκ or Igλ), and oncogenes such as c-myc and members of the bcl family (reviewed in15). The enhancer elements that promote transcription of Ig genes are brought into proximity of the oncogenes, causing their transcription to be dysregulated. Different forms of lymphoma are characterized by different translocations: for example, 100% of mantle cell lymphomas in people have a translocation that brings together the IgH and CCND1 loci.16 Diffuse large B-cell lymphomas have rearrangements between IgH, k, or l and bcl-2, bcl-6, or c-myc, and Burkitt’s lymphoma often has IgH-c-myc translocations.16

Breen and Modiano14 describe a case of canine Burkitt-like lymphoma that has an IgH-c-myc translocation. In addition, genomic gain of chromosome 13, which contains the canine c-myc gene, was seen in a series of B-cell lymphomas in dogs, suggesting that amplified c-myc expression may play a role in the pathogenesis of these more common kinds of B-cell lymphomas.17 Assays to detect such translocations are in common use in human oncology, and can also be developed for canine lymphoma. When available, these assays can be used...
to aid in the diagnosis of difficult cases, subclassify B-cell lymphomas into more meaningful prognostic subgroups, and monitor chemotherapy. It is important to note that these assays are not expensive or technologically complex tests—in fact, their addition to the diagnostic palette may make diagnosis and prognostication less expensive and invasive.

Clonality Assays

**Principals of Clonality Assays**

Any diagnostic test that demonstrates that a group of cells is derived from a single clone can be considered a clonality assay. The term is usually used to refer to detection of the unique genes found in each individual B- or T-cell: Ig genes in B-cells and T-cell receptor genes in T-cells. The portion of these genes that encodes the antigen-binding region is the portion that varies between cells, both in size and sequence. Once a B- or T-cell is mature and divides in response to antigenic stimulation, the Ig and T-cell receptor genes are passed on to the daughter cells.

Clonality assays are accomplished by isolating DNA from cells suspected to be neoplastic, and then using PCR primers directed at the conserved regions of T-cell receptor or Ig genes that flank the hypervariable regions of these genes, amplifying the variable regions. The PCR products are then separated by size, and, in some cases sequence, using a variety of possible methods. The presence of a single-sized PCR product is indicative of clonality, whereas the presence of multiple PCR products supports a reactive process (Fig 3).

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We have termed the clonality assay used to detect antigen receptor rearrangements the PCR for antigen receptor rearrangements (PARR) assay to distinguish it from other types of clonality assays. It should be noted, however, that this term is not used in the human literature.

**PARR Can Aid in the Diagnosis of Lymphoma in Cytologically and Histologically Ambiguous Cases**

In practice, the PARR assay is most commonly used to aid in distinguishing reactive (polyclonal) from neoplastic (monoclonal) lymphocytes when these distinctions are difficult to make with other means. The assay probably differs in sensitivity and specificity between laboratories, and these values need to be established by each institution offering the assay. Contact information for each laboratory performing these assays is provided in Table 1. Our laboratory detects approximately 75% of histologically or cytologically confirmed lymphomas, when the assay is performed on the cells in question (based on testing of approximately 800 cases). Those cases that cannot be detected are most likely cases where the PCR primers cannot detect the particular Ig or T-cell receptor genes used in that tumor. When human clonality assays were first developed, they reported a similar sensitivity, but the addition of detection of light chain rearrangement has increased the sensitivity to close to 100%.

Is the presence of a clonal population diagnostic for lymphoma or leukemia? Because the PARR assay can detect as few as 1 neoplastic cell per 100 cells, it provides the opportunity to detect neoplastic lymphocytes earlier than cytology
or histology. Therefore, to determine the specificity of a positive (clonal) finding, patients with a positive result but no histopathologic or cytologic evidence of lymphoma must be followed up for some period of time to determine if the presence of a clonal population predicted development of lymphoma/leukemia. Different laboratories may choose to establish a specificity number in different ways, but in our laboratory we obtained 1-year follow-up information for 141 dogs with a positive PARR result, but no definitive cytologic or histologic diagnosis at that time (unpublished data). Six percent of these patients did not develop additional evidence of lymphoma in the subsequent year; the remaining 94% had a definitive cytologic or histologic diagnosis of lymphoma or leukemia within the next year. The only non-neoplastic disease in dogs that has been reported in the literature to cause a clonal lymphocyte expansion is *Ehrlichia canis* infection,21,22 and 2 dogs in this group of 6% false positives were shown by serology and follow-up to have *E. canis*. Surprisingly, *E. canis* only appears to cause clonal T-cell expansion. One case each of tentatively diagnosed Lyme, Bartonellosis, and Rocky Mountain spotted fever was associated with a clonal B-cell population (although we do not have definitive evidence that these diseases cause the expansion), and the remainder of cases remain idiopathic. Thus, overall, this test in our laboratory has 75% sensitivity and 94% specificity. We have not yet developed data on the performance of the assay on different tissues (such as cerebrospinal fluid, lymph node aspirates, blood, intestinal biopsies, and so forth) but hope to be able to provide this data within the next year.

**PARR Can Be Used to Determine the Relatedness of Lymphocyte Populations**

Other than being used to distinguish reactive from neoplastic lymphocyte expansions, the PARR assay has been used in human medicine to detect lymphocytes that will eventually become lymphoma in preneoplastic tissues. The best example of such a study was the demonstration that the lymphoid hyperplasia seen in early *Helicobacter pylori* infection contains the B-cells that eventually become neoplastic in MALT-lymphoma associated with this infection. This association was established by sequencing the hypervariable regions of B-cells present in the preneoplastic lesions, and demonstrating that one of these sequences was identical to the hypervariable region seen in the B-cell lymphoma that eventually developed.29

Our laboratory used this same principle to demonstrate the progression of a multicentric B-cell lymphoma to multiple myeloma.25 In this case, the dog originally presented with cytologically confirmed B-cell lymphoma and normal Ig levels. The dog was treated with a CHOP chemotherapy protocol and achieved clinical remission. Several months later the dog re-presented with a monoclonal gammopathy, lytic rib lesions, and plasma cells in multiple sites. The progression from multicentric B-cell lymphoma to multiple myeloma had not been reported in the literature, so we wanted to determine if these were 2 separate tumors. Sequencing of the hypervariable region of the Ig gene demonstrated that the B-cell lymphoma and the multiple myeloma were derived from the same clone.

**PARR Is Not Useful for Clinical Staging, but Can Be Used to Quantify Tumor and Monitor Response to Treatment**

Clinical staging can be a subjective process, relying on the opinions of cytologists and radiologists about the extent of involvement of blood, bone marrow, and other organs, and the degree to which each of these methods is used can change.
the way any individual patient is staged.\textsuperscript{30} We hypothesized that detection of tumor in the blood using the PARR assay would be a more objective method of staging—specifically that the presence of PARR-detectable tumor cells in the blood at presentation would predict a poorer outcome. However, we found in a study of 62 patients with either B- or T-cell multicentric lymphoma, clinical stage but not PARR stage was associated with outcome. Furthermore, we found that although 75\% of clinical stage III B-cell lymphomas had tumor in their blood by PARR but not cytology, there was no difference in outcome between these dogs and 25\% of the dogs did not have PARR-detectable tumor. Therefore, in its current form, PARR is not a useful tool for staging lymphoma.

PARR was recently shown, however, to provide some useful insights into the response of dogs to treatment with chemotherapy.\textsuperscript{27} The investigators in this study sequenced the hypervariable regions of the Ig gene involved in each case of B-cell lymphoma they followed. They then designed primers that would anneal specifically to the hypervariable region of each tumor; this is in contrast to the primers used for the standard PARR, which bind to the conserved regions to detect as many tumors as possible with as few primer combinations as possible. The advantage of making tumor-specific primers is that the sensitivity for detecting tumor cells increases substantially, and the number of tumor cells can be quantified.

Only one dog was tested before treatment, so it is not possible to evaluate the magnitude of the initial response to chemotherapy. All dogs were tested during treatment, and, at all times, tumor was detectable in the peripheral blood, even if the patients achieved full clinical remission. In most cases, the number of tumor cells was between 1 and 10 cells/\( \mu \)L during remission, but those numbers increased drastically when coming out of clinical remission—achieving concentrations of 1000 cells/\( \mu \)L. An increase in the number of cells in the blood correlated very well with increasing lymph node size and loss of clinical remission.

Although the production of tumor-specific primers may not be practical or affordable for a large number of owners, detecting MRD with this method can be useful in a research setting. For example, if it can be shown that the degree of tumor burden reduction in the earlier stages of treatment is correlated with eventual outcome, then quantifying tumor burden can be a useful way of testing the efficacy of novel chemotherapies without needing to follow up with a patient through the entire course of treatment. Quantifying the response to treatment may also be a first step toward understanding why some tumor types have an overall poorer outcome.

A Note about Cats

Of the various assays discussed above, only the PARR assay has been reported in cats.\textsuperscript{31-33} The assay has been applied to the characterization of a T-cell lymphoma in a cat to confirm T-cell origin,\textsuperscript{34} and it has also been applied to a series of cases of feline inflammatory bowel disease and lymphoma.\textsuperscript{31} The assay was able to detect 78\% of histologically confirmed lymphomas. Specificity data were not reported in this study, but the assay has the potential to help distinguish between inflammatory bowel disease and lymphoma in those cases that are histologically unclear.

Summary

There are several applications that are not appropriate for any of the tests described above. These are not intended as a first-line diagnostic procedure, and often are not necessary. For example, although PARR testing can provide lineage information (B- vs T-cell) when it is positive, if the diagnosis of lymphoma or leukemia is unambiguous, flow cytometry, immunocytochemistry, or immunohistochemistry are better tests to determine the phenotype because more information can be obtained. Therefore, PARR testing is less useful for confirmed cases of lymphoma. These assays are also not intended as screening tests for healthy animals, because they have not been evaluated in this capacity. These assays can, however, provide a wealth of clinically useful information (in resolving ambiguous cases) and be useful research tools for better understanding the biology of hematologic malignancy.

References


