ADVANCES IN MOLECULAR PATHOLOGY: DIAGNOSIS, PROGNOSIS, AND THERAPEUTIC SELECTION

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The term “pathology” originates from the Greek words “pathos” and “logos” and is therefore best translated as the “study of disease”. Pathology was established as an essential part of modern medicine that studies the etiology, pathogenesis and outcome of diseases by the Italian physician Giovanni Battista Morgagni in 1761. Utilizing postmortem examinations, he based his studies on empiric data that identified and localized causes of diseases in different organ systems rather than explaining disease processes by an imbalance of bodily fluids (humoral pathology) as proposed by the ancient Greeks. Rudolf Virchow revolutionized pathology and created the foundation of modern scientific medicine by introducing the concept of cellular pathology, which evaluates cell and tissue architecture to identify and classify morphologic alterations that form the basis of disease. These principles of cellular pathology are still relevant today. By identifying morphologic alterations, both gross and microscopically, pathologists have been able to determine disease processes, their cause and also predict the clinical outcome, thereby establishing pathology as the central science in modern medicine and especially disease investigations. Interestingly, the role of pathology has been previously challenged with the emergence of microbiology as a medical science. While Virchow believed that all disease processes were entirely attributed to cellular changes and ignored external effects by infectious agents, it quickly became clear that modern microbiology would not replace pathology, but on the contrary, underscore its importance. By being able to associate an infectious agent with a specific microscopic alteration, pathology became essential in both diagnostics and pathogenesis research of infectious diseases. However, classical cellular pathology has recently been shown to be limited as well. Classical gross and microscopic evaluations of tissues are insufficient to provide a precise prognosis for an individual patient or to determine the proper therapeutic approach. They also fail to show causality of lesions with infectious agents that are not microscopically identifiable. In the last decade, these needs have led to a shift in the paradigm of how pathologists analyze tissues for diagnostic purposes or pathogenesis research. In addition to microscopic examinations, we are now using molecular methods such as immunohistochemistry and in-situ hybridization to visualize targets of interest.
within microscopic lesions or with techniques such as laser capture microdissection we are able to extract target cells for further molecular analysis. The evolution of cellular pathology into molecular pathology is still in its infancy, but has already allowed us to not only link tissue alterations more precisely to the clinical course of disease, but also to molecular mechanisms of disease processes. In this talk, I will show a number of examples as to how modern molecular veterinary pathology has altered the way we perform daily diagnostic testing and contributed to our understanding of pathogenesis. Embracing molecular pathology as the future of our profession requires a shift in our training methods of young pathologists, a close collaboration with scientists across medical disciplines and education of the public on the benefits of integrating such new technologies into routine diagnostics, despite the higher costs.

**Molecular Pathology in Routine Surgical Biopsies**

Historically, the goal of histologic examination of tumor biopsies was to provide an accurate diagnosis and prognosis of a tumor entity and, in the case of excisional biopsies, to evaluate tumor margins. There is an increasing need for more accurate prognostic and predictive markers in veterinary oncology because of an increasing number of treatment options, the increased financial costs associated with treatment, and the emotional stress experienced by owners in association with the disease and its treatment. Over the last decade the ability of the Diagnostic Center for Population and Animal Health (DCPAH) to perform these services has been significantly advanced by integrating molecular tools such as immunohistochemistry (IHC) into the biopsy service to more accurately diagnose tumors, to provide an individualized prognosis, and to predict therapeutic responses. In addition, the use of modern technology allows us to deliver images to our clients that significantly enhance the assessment of tumor margins.

Canine cutaneous mast cell tumors (MCTs) are one of the most frustrating tumors for veterinary practitioners in terms of advising their clients. The inconsistency of the traditional histologic grading of MCTs has made it difficult to give an accurate prognosis and to select a treatment plan. We have published a new two-tier grading system for canine MCTs in order to more accurately identify more aggressive tumors and to eliminate interobserver variation. According to this system, MCTs are graded as high-grade or low-grade based on the number of mitoses, presence of multinucleated cells (3 or more nuclei) or bizarre nuclei, or karyomegaly. High grade MCTs have been shown to be significantly associated with a shorter time to metastasis, MCT associated mortality, and a shorter overall survival time. A low grade designation has been shown to be significantly associated with longer survival times and a decreased risk of metastasis. However, some low-grade MCTs may still exhibit aggressive biological behavior.

To overcome our limitations in prognosticating canine cutaneous MCTs by using histopathology only, we developed an exclusive panel of tests that dramatically increases prognostic certainty and predicts response to targeted therapy. The panel includes cell proliferation analysis (Ki-67, AgNORs), c-Kit PCR to detect internal tandem duplication (ITD)
mutations in exon 11 and exon 8, and KIT IHC to analyze expression of this tyrosine kinase receptor. The functionality of each parameter is explained below.

**Assessing Proliferation Activity**

While histopathological examination of biopsy samples is used routinely for tumor diagnosis, molecular analysis of biopsy samples may provide more precise prognostication and in-depth guidance for effective therapeutic options. Evaluation of a variety of proliferation indices using histochemistry and immunohistochemistry (IHC) techniques have been suggested as prognostic indicators. When evaluating cell proliferation, two factors need to be assessed: how fast do cells cycle through the cell cycle (generation time) and what is the percentage of cells which are actively cycling through the cell cycle (growth fraction). Proliferation activity is a function of growth fraction and generation time. The cell cycle is composed of the G1, S, G2 and M phase. The mitotic index (MI) only represents a so-called phase index parameter that identifies cells in the M phase, but not the growth fraction. Using MI as the sole prognostic indicator, would actually result in misdiagnosing 30% of high-grade MCTs. The best parameter to assess the growth fraction is Ki-67, which only labels cells within the G1, S, G2, and M phase. The only parameter that assesses the proliferation speed is actually AgNORs. To better understand the different components of cell proliferation, consider the following analogy: When looking at a bridge, one could take a picture of that bridge. The photograph would tell us how many people are on that bridge at that millisecond when the photograph was taken. Such picture would not tell us how many people were entering or leaving the bridge. Such a picture would therefore represent the phase index, like MI. In contrast, a video of that bridge would be similar to the growth fraction by telling the observer how many people were entering and how many people were leaving that bridge, so how many people were crossing it. The growth fraction is best assessed by the Ki67 index. However, if to show the video, one would need to know at what speed to run it, since it could be run faster or slower than people were actually moving. The speed reflects the generation time best measured by AgNOR. So only if we measure the correct number of people crossing the bridge and the accurate speed of their crossing, can we truly assess how many people are at a given time on that particular bridge. We therefore developed a system which actually assesses both AgNORs and Ki-67 in association. This combination is used for prognostic evaluation of canine cutaneous MCTs. When evaluating Ki67 alone, MCTs with a high Ki-67 index have a high risk of mast cell related mortality, while those that have a low Ki-67 index have a very low risk. However, when combining AgNORs and Ki-67, the prognostic accuracy is even higher and allows us to better identify those MCTs that are associated with systemic disease and mast related mortality. Other examples of neoplastic entities where the proliferation activity is routinely assessed to better prognosticate the neoplasm include canine oral and cutaneous melanomas, canine lymphomas or canine mammary tumors.
Detection of c-Kit Mutations for Prognosis and Therapeutic Decision Making in Canine and Feline Neoplasms

One of the most widely used molecular tests for prognosticating neoplastic diseases in humans that also has remarkable clinical implications is the detection of c-Kit mutations by PCR. Somatic mutations in c-Kit have been associated with a number of neoplastic entities in humans, including gastrointestinal stromal tumors (GISTs), mast cell diseases, leukemia, myeloproliferative diseases, melanoma, and germ cell tumors. Activating mutations of c-Kit have been identified in mast cell tumors (MCTs) and GISTs in dogs and cats as well. Moreover, detection of mutations of c-Kit in these tumors has been associated with prognosis and targeted therapy.

The KIT protein, the product of the c-Kit proto-oncogene, is classified as a tyrosine kinase (TK) receptor subclass III. Activation of KIT occurs through binding of the KIT ligand, stem cell factor (SCF), which is produced by fibroblasts and endothelial cells. Binding of SCF to KIT results in receptor dimerization and change in conformation of the receptor, leading to the stabilization of the receptor-receptor interaction. In non-neoplastic human and canine mast cells, KIT activation results in various cellular responses, including differentiation, proliferation, growth, survival, adhesion, and chemotaxis. Gain-of-function mutations of c-Kit cause constitutional tyrosine phosphorylation and downstream activation independent of ligand binding.

Immunohistochemistry for KIT labeling is a standard test to differentiate canine GISTs from soft tissue sarcomas. GISTs are derived from the interstitial cells of Cajal that express KIT, while all other non-angiogenic non-lymphogenic intestinal sarcomas are negative for KIT. Because GISTs can exhibit a more aggressive biological behavior than non-angiogenic non-lymphogenic intestinal sarcomas, detection of KIT expression or a c-Kit mutation is necessary to accurately diagnose, prognosticate, and select proper therapeutic strategies. While canine mast cells normally express KIT, different KIT expression patterns have been reported in canine MCTs. Nonetheless, although there is significant correlation between these KIT expression patterns and disease-free and overall survival times of affected dogs, KIT expression patterns alone cannot reliably predict the biological behavior of each individual canine MCT.

Activating mutations in c-Kit can easily be detected within tumor tissue by gel-based PCR using primers specific to exons that carry the mutation. Activating mutations have most commonly been reported in exons 8, 9, 11 and 17. While the test can easily be performed on freshly-collected tumor samples, including fine needle aspirates, DNA from tumor samples can also be extracted from formalin fixed paraffin embedded (FFPE) tissues. The main limitation of detecting c-Kit mutations by PCR are inadequate numbers of tumor cells and failure of proper extraction. Selection of FFPE blocks that contain sufficient tumor tissue is crucial for accurate detection of mutations. If there are not enough neoplastic cells with mutations in the tumor sample, wild type alleles from non-neoplastic cells could be amplified and result in a false negative result.
Ordinarily, the juxtamembrane domain of KIT (encoded by exon 11) serves as a negative control domain, which prevents kinase activation in the absence of ligand. Mutations in the juxtamembrane domain disrupt the negative regulation of the kinase domain and thereby result in uncontrolled receptor activation. Mutations in the juxtamembrane domain have been reported in up to 33% of canine cutaneous MCTs and in 35.3% of canine GISTs. In canine MCTs, there is significant association between c-Kit mutations in exon 11 and a high grade designation. Dogs with MCTs harboring such a c-Kit mutation have been reported to have significantly shorter overall survival and disease-free survival times. Moreover, c-Kit mutations are also significantly associated with aberrant KIT protein localization. Interestingly, the DNA sequences of c-Kit mutations observed in canine GISTs are similar to mutations in human GISTs. Recently, a similar mutation has also been detected in a cat with a gastric GIST.

Another location for activating c-Kit mutations in canine and feline MCTs that cause receptor phosphorylation and activation without binding of ligand is in exon 8. In canine MCTs, mutations in exon 8 are far less common than mutations in exon 11 and reportedly occur in less than 3% of affected dogs. While they are a predictor of therapeutic response, they have not been shown to be associated with a more aggressive biological behavior. In contrast to dogs, c-Kit mutations in feline MCTs have only been identified in exon 8 and have been proposed to play an important role in feline mast cell tumorigenesis.

In addition to KIT expression patterning that results in accurate tumor prognosis, detection of c-Kit mutation will provide the information necessary for predicting proper therapeutic response. Recent therapeutic breakthroughs in the treatment of KIT signaling-driven tumors involve the development of tyrosine kinase inhibitors (TKIs). TKIs are promising new agents for selective, specific inhibition of malignant cell growth, promotion of apoptosis, and inhibition of angiogenesis and metastasis of tyrosine kinase receptor signaling mediated tumors. Canine MCTs with activating mutations in exon 11 respond well to TKIs that are now readily available for dogs. Furthermore, high-grade MCTs lacking mutations or aberrant KIT expression have been reported to respond better to a chemotherapy protocol composed of vinblastine and prednisone than to TKI. Similar to the results in dogs, cats with MCTs harboring a mutation in exon 8 have also been reported to have a favorable response to TKIs. In conclusion, molecular analysis using PCR to detect c-Kit mutations in FFPE tumor samples allows more accurate prediction of clinical behavior, determination of the risk of metastases, and assistance in determining appropriate treatment strategies for KIT signaling-driven tumors.

**PCR to Detect Antigen Receptor Rearrangements**

Detection of clonal expansion, representing the hallmark of cancer, has been proven to be clinically useful for the diagnosis of lymphoma based on the detection of specific and dominant T-cell receptor and/or immunoglobulin gene arrangements in both animals and humans. When histology and immunohistochemistry cannot distinguish between a reactive (polyclonal) population and a neoplastic (clonal) population of lymphocytes, PCR to detect antigen receptor
rearrangements (PARR) can be helpful for both B- and T-cell lymphomas. PARR testing to
determine clonality of lymphocytic proliferations has recently become a common practice in
canine and feline medicine. This assay has been used clinically in many different situations, such
as distinguishing between: canine indolent nodular lymphoma and atypical hyperplasia, intestinal
lymphoma and inflammatory bowel disease (IBD), hepatic lymphoma and
cholangitis/cholangiohepatitis, leukemia and reactive, non-neoplastic conditions, and cutaneous
lymphoma and lymphocytosis, erythema multiforme, or other lymphocytic inflammation.

Neoplastic lymphocytes of a particular neoplasm contain unique DNA regions that are
found primarily within the CDR3 region of both immunoglobulin and T-cell receptor (TCR)
genes. This region encodes the antigen binding region of each receptor, which in B-cells is
formed by the recombination of V, D, and J segments, and in T-cells is formed by the
recombination of V and J segments. Primers that are specific for conserved regions of V and J
segments can be used to amplify CDR3. Then, the PCR products are separated by size and, if
there is a proliferating lymphocyte population with a single sized receptor (clonal population), a
dominant band will be present in the gel view or a dominant peak will be present in the
histogram view.

One of the PARR assay’s most common applications in diagnostic practice is to
differentiate between IBD and intestinal lymphoma in cats. Specific histologic features that aid
in this distinction have been well-established in cats. However, in some cases, histology alone, or
histology plus immunophenotyping, is insufficient for diagnosis. PARR testing is especially
helpful when only superficial endoscopic biopsy samples are available or in early cases of
lymphoma that lack submucosal invasion or marked epitheliotropism. A diagnostic algorithm,
using histologic features, immunophenotyping, and PARR testing, has been established to aid in
the diagnosis of feline intestinal lymphoma.58

Just like any of the molecular tools described here, PARR testing should never be used as
a stand-alone test. The pathologist needs to interpret the histologic features,
immunophenotyping, and clinical findings in conjunction with one another. There are several
reasons for this. First, the sensitivity of the primers to detect rearranged immunoglobulin heavy
chain (IgH) or TCR genes must be taken into consideration. Qualitative sensitivity is defined as
the ability of the PARR assay to detect a purely clonal population of lymphocytes. Quantitative
or analytical sensitivity is defined as the assay’s ability to detect a clonal rearrangement in a
background of non-neoplastic lymphocytes. In some cases, a neoplastic population of
lymphocytes is present within an inflamed tissue and the clonal lymphocytes can be, in effect,
“diluted out” by the polyclonal inflammatory cells. Thus, different tissues may also influence the
sensitivity of the assay if they are more prone to having a concurrent inflammatory or reactive
population of lymphocytes. For example, PARR testing has a higher sensitivity in nonlymphoid
tissues compared to lymphoid tissues. It has been speculated that “the rearranged sequences from
normal lymphocytes compete with amplification of the neoplastic DNA.” We routinely receive
blocks of tissue or unstained slides for clonality testing that contain multiple organs, such as
intestine, liver, and lymph node. In order to increase the quantitative sensitivity of the PARR test in these cases, we only use paraffin shavings that contain the tissue of concern.

The first clonality assays that were developed to detect clonal populations of T-lymphocytes in dogs were based on limited sequence data and therefore they often miss certain V and J segment rearrangements. Based on more recent studies that have identified additional less commonly expressed rearrangements and that have provided more detailed characterization of the complete canine TRC locus, a new multiplex PARR assay has been developed for assessment of canine T-cell proliferations that has been shown to have an improved sensitivity. In addition to using multiplex primers, the sensitivity of the PARR assay has also been improved by using capillary gel electrophoresis. Multiplex primers generate more complex electrophoretic gels, therefore, capillary gel electrophoresis is particularly important to accurately interpret the more complex gel patterns; conventional gels may not provide sufficient resolution to identify clonal bands in a polyclonal background. Furthermore, knowledge of primer design and expected bands is crucial to avoid interpretation errors.

False negative results can occur in some cases of lymphoma that are immunophenotypically of either B- or T-cell origin, but that do not have a rearrangement in their respective gene, IgH or TCR. For example, some B-cell lymphomas may have a polyclonal result with PARR testing for rearrangements in IgH, but may actually have a rearrangement in TCR. This is an example of lineage infidelity. If only PARR testing for IgH rearrangement is performed, cases such as these may be misdiagnosed as reactive or inflammatory rather than neoplastic. Thus, for some cases that are suspected to be neoplastic, PARR testing for both B- and T-cell clonality is required. Also, in some cases of canine and feline lymphoma, dual clonality has been reported. This can occur when a lymphoma has a dual genotype with lineage infidelity or, rarely, when a composite lymphoma is present, which is defined as concurrence of two monoclonal malignant B- and T-cell populations. Laser capture microdissection can be used to separate specific cell populations for clonality testing, but this is a costly method that is not routinely available at most veterinary laboratories. It is important to remember that the immunophenotyping defines the type of lymphoma as B- or T-cell, not the gene rearrangement. Thus, in the example above, the diagnosis would be a B-cell lymphoma with TCR gene rearrangement.

Specificity of the PARR assay also needs to be considered. Specificity is defined as a test’s ability to correctly identify negative results. In general, most PARR assays for assessment of feline and canine B-cell and T-cell proliferations are highly specific. Sample size is one factor that may influence specificity. For example, a very small endoscopic intestinal biopsy with a poorly cellular inflammatory lymphocytic infiltrate may result in a clonal PCR result. It is possible that such samples with very small numbers of lymphocytes may contain non-neoplastic, reactive lymphocytes that were derived from the same precursor cell. Another instance where a false positive result can occasionally occur in dogs is in cases of Ehrlichia canis infection. E. canis infection has been reported to produce a clonal expansion of T-lymphocytes. It is also important to remember that not all clonal populations are malignant. For example, a canine
cutaneous plasmacytoma can have a clonal immunoglobulin rearrangement but these tumors are considered to be benign in dogs.

Specific procedural measures must also be taken to avoid false positive (clonal) results. All samples should be run in duplicate, along with both native and denatured forms; if this practice is not followed, pseudoclones can occur, resulting in false positive (clonal) results. Pseudoclones are characterized by 1 or 2 bands that are of different size or non-reproducible when run side by side. It is important to inquire whether or not a laboratory uses these methods; if they do not, results should be interpreted with caution. Oligoclonal results can also be somewhat difficult to interpret. Oligoclonal samples have 3 to 5 reproducible bands in duplicate analysis and up to 3 bands are considered to be most consistent with a neoplastic cell population. Oligoclonality can occur in some cases of chronic inflammatory disease, early neoplasia, neoplasms involving more than a single clone, certain infections, and other types of neoplasia. Thus, again, it is important to use PARR testing in conjunction with clinical findings, histopathology, and immunophenotyping.

Ultimately, despite a polyclonal PCR result, if histologic features and immunohistochemical results are consistent with lymphoma, a diagnosis of a suspected lymphoma should still be made, and additional biopsy samples obtained at a later time point should be tested with PARR. Similarly, in cases where histology and immunophenotyping are inconclusive, but PARR testing indicates a clonal proliferation of either B- or T-lymphocytes, continued patient follow-up is required to determine if the clonal result is predictive of the patient developing lymphoma. As the characterization of both feline and canine immunoglobulin genes of B-cells and TCR genes of T-cells continues to improve, assays with improved sensitivity and specificity will continue to be developed but should still never be used as a stand-alone test.

Localization of Infectious Agents within Microscopic Lesions Using Immunohistochemistry (IHC) and In situ Hybridization (ISH)

To establish the cause or pathogenesis of certain diseases, it is often necessary to determine the presence of infectious agents or abnormal gene expression in association with morphologic changes within tissues. While PCR is a highly sensitive tool and often considered the gold standard for detection of an infectious agent in the tissues, it does not allow for the correlation of the microscopic lesions directly with the causative agent. This can be crucial because tissues may harbor vaccine, latent, or otherwise clinically asymptomatic virus that could potentially be misinterpreted as the cause of disease. A good example is the recent concern about canine circovirus (CaCV-1) infection in dogs. Only demonstrating CaCV-1 infection via PCR is not sufficient for the diagnosis of CaCV-1 associated diseases and detection of CaCV-1 by ISH allowed us to associate the virus with distinct microscopic lesions and will help to elucidate its pathogenesis and pathogenic potential. Ultimately, an accurate diagnosis requires localization of the virus within characteristic lesions.
IHC is presently the most frequently used molecular tool for detection of a specific protein target within microscopic lesions of FFPE tissue. Some of the main disadvantages of IHC are the limited availability of species-specific antibodies and the continued supply and batch variation of some antibodies. Another issue is the specificity of some antibodies, mostly in regards to infectious agents. ISH is another powerful molecular tool that allows detection of a particular DNA or mRNA sequence within cells and tissue sections. In essence, any nucleic acid sequence can be specifically detected by the use of a probe that is the “antisense” or reverse complementary sequence of the target.

ISH can be used to identify and localize nucleic acid of infectious agents within lesions, as well as to localize genes, their transcripts, and chromosomal aberrations. Any nucleic acid sequence can specifically be detected by the use of chemically labeled DNA or RNA probes. Non-radioactive probes, generally labeled with digoxigenin, have the advantage that their antibody detection has no cross reactivity with animal tissues and that they can be visualized using a chromogen such as nitroblue-tetrazolium (NBT), similar to IHC. With digoxigenin-labeled oligonucleotide probes, the synthesis is easy to achieve through automated manufacturing and endlabeling. High-performance liquid chromatography (HPLC) purification of the labeled probe is highly desirable to increase probe sensitivity by removing non-labeled probe. These probes are also more stable and are easier to work with because of their resistance to degradation by the ubiquitous, contaminating RNase. Nonetheless, due to lower labeling efficiency and decreased target size, oligonucleotides are less sensitive than, for instance, RNA probes (riboprobes). In our experience using non-amplified detection systems, digoxigenin-labeled oligoprobes are usually limited to detect target concentrations that would be recognized by up to 25 cycle thresholds by a complementary PCR reaction.

Historically, ISH is regarded as a time-consuming, labor-intensive, and technically complex method, the optimization of which can be difficult. A proposed rational design of oligonucleotide probes that takes into account melting temperature, runs of identical nucleotides, guanine-cytosine (GC) content, length, and secondary structures allows for a standard ISH protocol that keeps constant basic parameters such as tissue fixation, hybridization conditions, and washing procedures. This approach has worked well in our laboratory and represents another great advantage of oligonucleotide probes compared to riboprobes. Automation of the ISH procedure has further improved consistency, reliability, and feasibility of this assay, with increased throughput capability.

Probes for infectious agents can either be generic or species-specific. By screening tissues first with a generic probe (e.g. papillomavirus, Leishmania, Chlamydiophila), it is possible to search for a wider range of agents, followed by the use of species-specific probes at a later stage to speciate the agent of interest (e.g. L. infantum, C. psittaci). ISH can also be used to localize mRNA and to determine expression levels of specific transcripts such as oncogenes. This allows for information on the distribution and expression pattern of the target within tissues, providing spatial and temporal information about infectious agents and genes as well as information on disease progression and levels of cell differentiation and functional activity.
Careful selection of the proper test is crucial for successful identification of the target. For instance, papillomaviral mRNA codes for structural proteins that lead to accumulation of large number of virions in the stratum granulosum and corneum. Thus, non-keratinizing epithelial neoplasms associated with papillomavirus infection may be negative with IHC for this virus since commercial antibodies target viral proteins that are produced only in the late stages of viral replication. Detection of papillomavirus within equine and feline sarcoids will similarly require testing for the viral nucleic acid rather than for viral proteins. ISH can also differentiate sites of productive versus nonproductive viral infection when using either multiple probes that target mRNA and DNA or probes in combination with IHC. Additionally through dual ISH/IHC labeling, cells that are actively producing virus may be able to be distinguished from those solely involved in phagocytosis or uptake of the target. Depending on the probe design, ISH can also be used to exclusively detect the replicative form of a virus. Similarly, combining ISH and IHC is useful to identify, respectively, which cells are producing and which cells are the targets of secreted proteins such as cytokines.

The major pitfalls with both IHC and ISH relate to the differentiation between specific and non-specific or background labeling. Some probes will bind non-specifically to neurons, glandular epithelial cells and collagen. Likewise, certain immunohistochemical detection methods will label endogenous enzymes or other molecules such as biotin. Besides the specificity and sensitivity of the antibodies or probe, other variables that can affect the labeling results include tissue processing, retrieval methods, and the detection system. For instance, secondary polyclonal antibodies may cross-react with pathogens that are morphologically similar to the target, with the potential for incorrect interpretation. Toxoplasma gondii cysts and tachyzoites in tissues cross reacted with a rabbit polyclonal anti-digoxigenin antibody in ISH for Leishmania, probably because the antiserum was raised in an animal subclinically infected with T. gondii. This problem, which could easily lead to false-positive diagnosis considering the similar morphology between Leishmania and T. gondii, was solved by replacing the polyclonal antibody with a monoclonal anti-digoxigenin antibody. Differences in labeling intensity and variable assessment of the degree of positivity, mainly in the case of some nuclear antigens such as Ki-67, p53 and estrogen receptor, may also lead to significant interobserver variation. Knowledge of the expected labeling pattern within the tissue and subcellular compartment is therefore essential for correct interpretation, along with the need for proper controls to ensure accurate results.

Conclusions

The provided examples clearly show that the integration of molecular techniques into routine histopathology can easily be achieved on FFPE samples and allows the pathologist to provide the client with diagnostic, prognostic and therapeutic information far beyond the knowledge gained from routine histologic examination. To allow such integration of molecular testing, it is important for a laboratory performing molecular assays on FFPE tissues to
implement and adhere to protocols that minimize nucleic acid damage. The process of nucleic acid preservation should start with minimizing the interval between tissue collection and fixation. The formalin buffering system, tissue size, length of fixation, and the embedding procedure can subsequently all affect nucleic acid quality.

Most importantly, one diagnostic test is unlikely to provide a complete picture of the disease. A combination of selecting the proper tissue samples and applying the appropriate molecular tests allows for better understanding of the pathogenesis, identification of the target cells, monitoring of disease progression, and determination of the most appropriate patient management. The use of FFPE material in this setting is fundamental because this may be the only material available, in addition to enabling retrospective analyses using archived material. In modern diagnostic settings, communication between the different laboratory sections including both the molecular biologists and the pathologists is crucial. For molecular pathology be to successful in a routine diagnostic setting, it will require the next generation of pathologists being trained in the use of molecular methods, a close collaboration between pathologists and scientists working in the field of molecular biology and education of veterinarians and animal owners to understand not only the benefits of such testing, but also its associated costs.

Acknowledgement

I would like to thank my long standing collaborators Roger Maes, Rebecca Smedley, Annabel Wise, Ingeborg Langohr and Tuddow Thaiwong who all contributed to this work. A more comprehensive review of integrating nucleic acid-based analysis into diagnostic pathology has been published by Maes et al., 2014.

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