

Immunohistochemistry: Forging the links between immunology and pathology

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Abstract

The technique of immunohistochemical staining allows the visualization of epitopes in situ in histological tissue sections. A series of innovations in the methods and reagents and the introduction of mechanization have enhanced the ease and technical reliability of this technique resulting in widespread application in veterinary diagnostics and research. This brief overview will highlight some of the applications for immunohistochemical staining with an emphasis on the use of the technique in diagnostic veterinary medicine, particularly for the detection of infectious disease agents.

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1. Introduction

Immunohistochemical (IHC) staining is a laboratory technique that utilizes specific antibodies for visualization of the quantity, tissue distribution and cellular location of immunogenic epitopes in histological tissue sections. The concept was introduced as early as the 1940s when fluorescein dye (visible under ultraviolet light) was tagged to antibodies directed

against pneumococci for identification of this organism with specific anti-serum (Coons et al., 1941). This method, often abbreviated IFA for “immunofluorescence assay” has been widely used for the detection of specific pathogens, viral as well as bacterial and protozoal in “fresh”/unfixed tissues in both human and veterinary medicine.

Since its inception, the ease, accessibility and reliability of IHC staining has increased enormously due to a series of advances in methodology. The introduction of “enzyme-”, rather than fluorescein-labeled antibodies enabling IHC staining to be viewed with the light microscope arguably had the greatest impact (Nakane and Pierce, 1967). This innovation removed IHC techniques from the exclusive purview of specialized laboratories with fluorescence microscopes to the realm of a much wider group of scientists

Abbreviations: IHC, immunohistochemistry; BVDV, bovine viral diarrhea virus; IFA, immunofluorescence assay; FeLV, feline leukemia virus; ABC, avidin–biotin complex; PMWS, porcine postweaning mortality and wasting syndrome; PCV2, porcine circovirus 2; CWD, chronic wasting disease

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and histopathologists. The discovery of antigen retrieval methods (Huang et al., 1976) and highly amplified detection methods (Hsu et al., 1981) were instrumental in allowing IHC methods to be used in conjunction with tissues subjected to tissue fixatives such as formalin and thus increased the applicability for routine diagnostics. The final development that has advanced routine use of IHC methods was the innovation of robotic staining systems. These machines have removed the tedium and increased the reliability of IHC methods. Together these advances in technique have resulted in the use of IHC in many research and diagnostics laboratories as integral parts of the routine technique repertoire. This brief review will touch on some of the many applications for IHC in veterinary immunology and pathology with examples primarily of work from the Diagnostic Immunology Laboratory of Prairie Diagnostic Services at the Western College of Veterinary Medicine at the University of Saskatchewan, Saskatoon, Canada (Haines and Clark, 1991; Haines and Chelack, 1991).

2. Application of immunohistochemistry in diagnostic pathology

Many of the first reports of applications for IHC staining were of detection of immunoglobulin in tissue sections from patients with suspect immune-mediated skin disease, and as technical improvements developed these were often first reported for this application (Burnham et al., 1963; Michel et al., 1973). In particular, the use of antigen retrieval methods and the amplified detection methods that enabled IHC to be used in formalin-fixed sections has been instrumental in improving the utility and reliability of the test for diagnosing these patients (Berti et al., 1983; Haines et al., 1987). This improved reliability is attributable to the use of serial sections from a single biopsy for determination of the histological appearance of the lesions and for demonstration of the presence and pattern of immunoglobulin deposition. The ability to make this correlation assists in confirming the pathogenesis of these autoantibody-induced disease processes and increases the confidence of the clinician and histologist in the diagnosis. As veterinarians gain more experience with responses to various therapies

among the plethora of immune-mediated diseases, the ability to accurately distinguish disorders such as bullous pemphigoid, systemic lupus erythematosus and the various pemphigus disorders will be of increasing importance. More recently, as the antigenic targets for the immune-mediated skin diseases have become better understood, IHC methods have been used to demonstrate changes in the patterns of these molecules in lesional tissues. The best example is alteration in the pattern of desmoglein 1 expression in patients with pemphigus foliaceus; this antigen is the target for antibody-mediated destruction in a significant number of both human and veterinary patients. Antibody binding to this antigen (part of the desmosome involved in cell to cell adhesion), prompts the phenomena of antibody-induced patching, capping with subsequent shedding and/or internalization. Demonstration of these alterations in distribution of desmoglein can be used to study pathogenesis of these lesions as well as to assist in confirmation of the diagnosis (Carlotti et al., 1993; Steeves et al., 2002).

While the demonstration of autoantibody deposition in immune-mediated skin disease is the historical focus for IHC diagnostics, infectious disease diagnosis is currently the most widely used application of the technique in veterinary diagnostic medicine. The classical technique, using fluorescein labeled antibody is still the basis for specific pathogen identification in fresh/frozen tissues in most virology and bacteriology laboratories. Through the use of antigen retrieval methods and amplified detection systems such as the avidin–biotin complex (ABC) immuno-peroxidase methods, the applicability of IHC to formalin fixed tissues has increased the convenience and the spectrum of applications for identifying specific pathogens in tissues. There are specific antibodies available either commercially (Linscott, 2002) or from researchers or government agencies to most of the known pathogens of importance in veterinary medicine. The reagents for production and amplification of the visible signal (i.e. the secondary antibodies directed to immunoglobulin and avidin–biotin complex reagents) are widely available in “kit” format and of excellent quality.

IHC staining has been proven to be rapid and reliable to readily detect a wide array of agents, bacterial, viral and protozoal, including those that are nonviable or inherently difficult to isolate or culture.

An early and important example of this is the IHC detection of bovine respiratory syncytial virus, this agent is notoriously difficult to isolate and yet can be readily detected in formalin fixed tissues in paraffin blocks; even in archival tissue blocks stored for many decades (Bryson et al., 1988; Haines et al., 1989). There are many examples of other agents (influenza A virus, canine distemper virus, chlamydia, mycobacteria sp., neospora, etc.) where the agent is easily detected by IHC but classical isolation is difficult, expensive and/or involves considerable time delays (Haines et al., 1992, 1993; Bildfell et al., 2000). Thus, IHC has become a front-line diagnostic test replacing or supplementing, for routine diagnosis, many classical isolation and culture techniques.

There are many additional reasons why IHC staining has become a widely adopted technique for veterinary infectious disease diagnostics. Of considerable importance in some locales is the convenience of submission of a single stable tissue sample that can be used for histological diagnosis as well as for subsequent detection of infectious agents. Many veterinary diagnostic laboratories, especially those performing diagnostics for food-producing animals are located considerable distances from the submitting veterinary clinics, often hundreds of miles. The ability to ship “fixed” tissues, a single set of samples, suitable for both histopathology and specific pathogen detection without concern about precision of time and temperature is very convenient.

Additionally, the ability to specifically select the tissues with consistent histological lesions for IHC investigation for suspect infectious agents may function to increase the sensitivity of the diagnosis—particularly in instances of agents that are sparse or focal in the tissues; agents such as rabies virus or protozoan pathogens such as *Toxoplasma gondii*, *Neospora caninum* or *Sarcocystis neurona* (Cruickshank et al., 1990; Bryan et al., 1994; Martínez-Burnes et al., 1997).

The ability to visualize the presence and distribution of the putative pathogen in association with histological lesions is often instrumental in forming confident diagnoses and in implying causality. This is true of many routine diagnoses where the histological lesions are suggestive but not pathogemonic and it is instrumental in helping to link pathogens to new disease manifestations or to determine the scope of

syndromes associated with putative new pathogens. Some examples where IHC has been definitive in linking an infectious agent to a new disease manifestation include the use of IHC staining to demonstrate antigens of the feline leukemia virus (FeLV) in skin tissues of cats with giant cell dermatosis (Gross et al., 1993) or to link papilloma-viruses to lesions such as aural plaques in horses or generalized dermatitis in large cats (Fairley and Haines, 1992) or the morbillivirus (related to canine distemper virus) to death losses in large cats (Wood et al., 1995).

Immunohistochemical staining has been used to study and assist in the understanding of the pathogenesis of infectious agents as the quantity, tissue and cellular locations of the agent can be visualized. The pathogenesis of acute severe BVDV infections has been at least partially revealed through the study of the tissue distribution of the virus in naturally occurring and experimental disease (Ellis et al., 1998a).

Immunohistochemical staining has also been applied fruitfully in the description of disease due to new pathogenic agents. An important example is the discovery of the link between the disease described as porcine post weaning mortality and wasting syndrome (PMWS) and porcine circovirus II (PCV2) (Ellis et al., 1998b). The first compelling evidence of a role for this virus in this syndrome was the finding of abundant viral antigen in the lesional tissues of affected pigs (Ellis et al., 1998b; West et al., 1999). Subsequent studies describing the involvement of PCV2 in other syndromes (reproductive losses, myocarditis, etc.) have also used IHC demonstration of the viral antigens in affected tissues as an indicator for the involvement of this virus in production of the disease process (O'Connor et al., 2001).

The development of robotic staining systems has diminished the tedium of performing IHC techniques and improved their overall reliability. Some of the robotic staining systems can process up to 60 slides simultaneously (Brigati et al., 1988). The ability to stain very quickly large numbers of tissues has encouraged the application of IHC staining to both retrospective and large prospective survey studies. Studies of disease agents in feedlot cattle in particular may be best done using this method as the tissues can be collected, stored in formalin, shipped in batches

and tested during periods that are convenient for the laboratory. An example of this application is the study of the involvement of various infectious agents in death losses among feeder cattle. There has been considerable controversy as to the roles of acute infection with bovine viral diarrhoea virus (BVDV) and *Mycoplasma bovis* in disease and death in feeder cattle. IHC has been instrumental in enabling surveys of tissues of affected animals to assist in revealing pertinent associations between disease and these and other pathogens (Haines et al., 2001, 2004; Shahriar et al., 2002).

Robotic IHC staining is also considered the gold standard test for surveillance for important pathogens such as the prion protein-induced transmissible spongiform encephalopathies (Miller et al., 1994). IHC stains can be completed almost as quickly as other assays advocated as “rapid” and the results have greatly increased reliability compared to methods where the benefits of viewing the distribution of the agent in the context of the lesion is absent. New generation robotic stainers can efficiently process up to 80 cases/day. In the autumn of 2003 our laboratory performed (in addition to other routine diagnostics) IHC testing of over 14,000 tests for chronic wasting disease (CWD) prion in tissues harvested from wildlife.

Many of the applications of IHC in infectious disease diagnostics have been for post-mortem tissues, however biopsies from living animals may also be tested for some agents. In particular, methods have been developed to diagnose systemic infections in living patients using skin biopsies as test tissue. As an example, canine distemper virus infects a wide variety of tissues in the body including the epidermis (Appel, 1996). Attempts to demonstrate acute infections with this virus using conjunctival scrapings or other difficult-to-sample tissues have been fraught with technical problems, however IHC staining of haired skin has been shown to be a reliable method for demonstration of the virus in acutely infected dogs (Haines et al., 1999). Similarly, BVDV is known to be present in virtually every tissue in the body in persistently infected cattle and IHC testing of skin biopsies has been demonstrated to be an efficient and effective alternative to other test methods for the detection of this important source of infective virus (Njaa et al., 2000) and is now widely used for individual and herd screening purposes.

While infectious disease diagnostics has been the focus of IHC development in our laboratory and many others in veterinary medicine, in human diagnostic medicine the primary application of this method, aside from diagnosis of immune-mediated skin diseases, has been in the demonstration of tumor-associated antigens to better diagnose and to provide prognostic information in cancer patients. The foundation of these efforts has been to determine the cellular origin and/or differentiation of the tumor cell population. The use of antibodies recognizing cell and tissue-specific intermediate filament proteins has been fundamental for this purpose and are often used as initial/triaging IHC stains to determine if undifferentiated neoplastic cell population is of mesenchymal or epithelial origin prior to staining for antigen with greater selectivity (Taylor, 1986; Desnoyers et al., 1990). Antibodies to intermediate filament proteins and to many other cell and tissue-specific antigens, developed for use in tissues from people, can be used in veterinary species as many of these important molecules are highly conserved. In addition, some laboratories have developed and validated markers specifically for veterinary species (Danilenko et al., 1992). As the sophistication of treatment of neoplasia in veterinary medicine increases, the incentive to better determine the characteristics of tumor populations will continue. Of particular importance are lymphoid neoplasms and other tumors of round cells often occurring as skin masses in pets. It is increasingly critical that these tumors are accurately identified as cells of similar lineage may have different therapeutic and prognostic indications depending on the terminal differentiation (B versus T lymphocytes for example) of the cell population (Monteith et al., 1996; Fernandez et al., in press). It is also desirable that good reagents are identified for determination of neoplasms of other round cell tumors of the skin (e.g. melanoma) as tumors of vastly different lineage (and prognosis) can be morphologically indistinguishable (Berrington et al., 1994).

In addition to the application of IHC staining for determining cell origin in tumor diagnosis more sophisticated use can be made of the IHC technique for determining the malignancy of the neoplasm and/or for other useful indicators. For example, normal p53 protein is undetectable in tissues however mutant p53, consistent with carcinogenesis is readily demonstrated

by IHC and can be used in a variety of applications including determining relative malignancy, in defining tumor margins and in detecting micro-metastasis (Nambiar et al., 2001).

Beyond diagnostic medicine, immunohistochemistry has also been shown to be a useful technique with a multitude of applications in human and veterinary disease research. There is a plethora of well-defined antibodies to important cytokines, receptors and signaling molecules and IHC methods are widely applied for the study of physiology, immunology and pathogenesis of disease processes. Both the art and science of immunohistochemistry have developed enormously since the inception of the method and as a consequence the scope of the applications has expanded to address the many questions that can be asked by visualization of specific epitopes in situ in tissue sections.

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