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Diagnosing infectious porcine diseases using immunohistochemistry

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Summary

This review describes the foundations, methodology, applications, advantages, and disadvantages of immunohistochemistry used for the diagnosis of swine infectious disease. Immunohistochemistry (IHC) relies on the binding of immunoglobulins to antigens present in tissues. Numerous proteins from infectious agents (viruses, bacteria, parasites, fungi) can be detected with this technique. The antigen-antibody reaction is revealed by an enzymatic reaction or the color emission of a fluorochrome. Due to the specificity and sensitivity of this reaction, IHC is an excellent alternative to more complex, expensive, and time-consuming laboratory procedures such as virus isolation or microbiological culture.

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Many factors—history, gross and microscopic findings, availability of certain techniques, cost, sensitivity, specificity, and speed—determine which diagnostic test practitioners and diagnosticians should use. Immunohistochemistry (IHC) is used increasingly frequently because it is relatively inexpensive, fast, and sensitive, and is less laborious than traditional microbiologic procedures. Immunohistochemistry uses immunologic techniques to detect specific proteins to identify an infectious agent. This paper describes the scientific basis of IHC, as well as its methodology, advantages, disadvantages, and application in the diagnosis of swine infectious disease.

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The basics of immunohistochemistry

Immunohistochemical techniques detect antigens in tissues or cells. Many compounds—mainly proteins, but also carbohydrates, nucleic acids, lipids, and other compounds—can act as antigens that will be recognized by antibodies.

Two types of antibodies are used in immunohistochemistry:

- polyclonal antibodies, and
- monoclonal antibodies.

Polyclonal antibodies are produced by immunizing rabbits or another species with the antigen to be detected. Polyclonal antibodies are multivalent, meaning that they contain antibodies for several regions of the antigen molecule, providing a strong detection capacity. However, polyclonal antibodies can cross-react with antigens from different organisms (e.g., polyclonal antibodies raised against bacterial lipopolysaccharide might also recognize different species of Gram-negative bacteria).

Monoclonal antibodies are produced in mice and are the result of fusing immunoglobulin-producing B cells with myeloma (plasma cell tumor) cells. This fusion results in hybrid cells, which continue to grow and divide in culture and which also produce antibodies. Monoclonal antibodies have the advantage of being highly specific for a single sequence or "epitope" of the antigen molecule. However, because monoclonal antibodies will bind only to one site (epitope) of the antigen molecule, fewer antibody molecules will bind to the antigen and be subsequently detected by the labeling method. Fixation may modify that particular binding site, making the antibody unable to recognize (bind to) the antigen; and thus fail to detect it. Deciding whether to use polyclonal or monoclonal antibodies, therefore, depends on the availability of reagents and how they perform in tissue sections. There is no rule of thumb—usually, it is necessary to try different antibodies to determine which one performs better under standard fixation and tissue processing methods. In theory, prolonged fixation will be more damaging to monoclonal antibodies than to polyclonal antibodies.

Most of the antibodies (immunoglobulins) used in IHC are of the immunoglobulin G (IgG) class (Figure 1). An IgG molecule consists of four polypeptide chains: two heavy chains and two light chains. At one end of the heavy chains is the constant fragment (Fc) of a particular

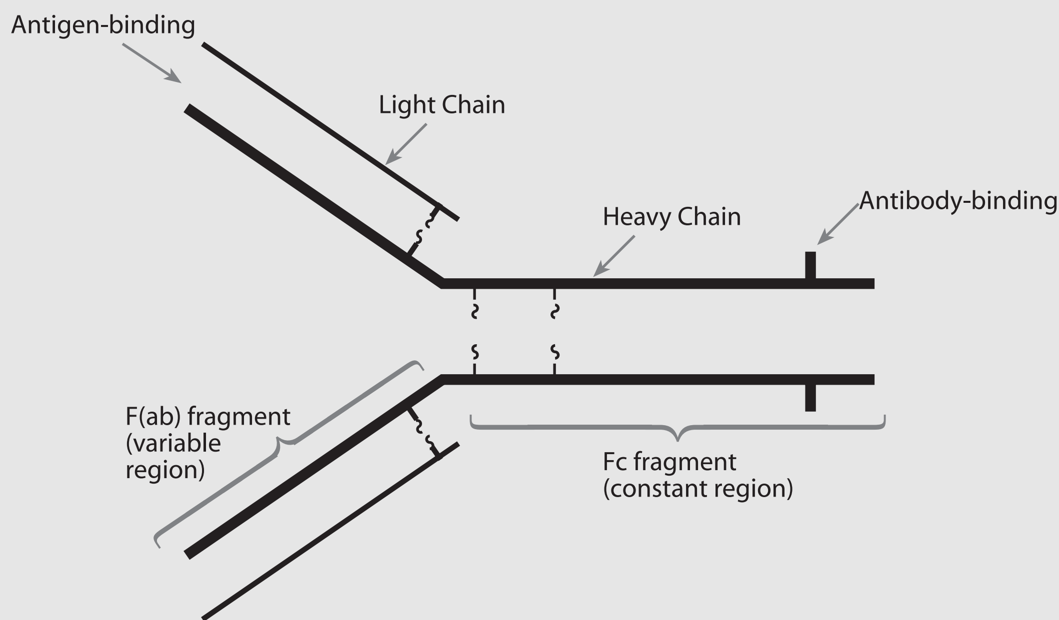


Figure 1

Structure of immunoglobulin G (IgG)

species; at the other end of the heavy chains and the two light chains are the variable regions of immunoglobulins (two variable regions per immunoglobulin). The variable region of the immunoglobulin is the binding site for antigens (antigen-binding fragment or F[ab]). Each immunoglobulin will recognize only one antigen through its variable regions. Infectious organisms are made of multiple proteins and other antigenic molecules. Ideally, IHC will detect a single protein, or a component thereof, that is specific to a given specific infectious agent.

Collection of samples

Immunohistochemistry is usually conducted on formalinized tissues. Immunohistochemical techniques that can be used on frozen samples are available but they are not widely used in field situations. It is critical that the time lapse between the death of the animal and the collection of tissues for IHC be as brief as possible. Postmortem decomposition can cause both false positive and false negative results.

Factors that influence tissue quality for IHC include:

- tissue type (tissues rich in enzymes such as the intestine or pancreas autolyze very fast),
- type of antigen (protozoa and fungi are probably more resistant to autolysis than viruses), and
- the distribution of the antigen.

Unfortunately, antigens are not always evenly distributed through a lesion or organ and, on occasion, the severity of lesions does not correlate with the localization of the infectious agent.

For practical purposes, the sooner the samples are placed in formalin, the better. Samples should include both the severely affected areas and the adjacent "normal looking" area, a sampling approach that should be used for any type of pathological examination. If a combined bacterial and viral infection is suspected (e.g., pneumonia), take different samples of the organ, including severely affected and less affected regions.

Preserving the tissues

Fixation can preserve both the antigenicity of proteins and the morphology of tissues; ideally, it preserves tissues as they were. The routine fixative used in histopathology is 10% neutral buffered formalin and it is also the standard fixative for IHC.

One additional advantage of formalin-fixed and paraffin-embedded tissues is that retrospective studies can be done using tissues stored for many years. Therefore, diseases not detectable several decades ago can now be retrospectively diagnosed with IHC on formalin-fixed, paraffin-embedded tissues.

In general, the sensitivity of a reaction depends on how many signal-generating molecules (e.g., peroxidase) are bound to an antigen-immunoglobulin complex in the tissue section. Formalin cross links proteins that can limit access of antibodies to antigenic sites and therefore may cause false negative results even in the presence of large amounts of antigen. Therefore, it is important to limit the time the sample spends in the fixative. This interval should be limited to no more than 2 days, although response to fixation might be different depending on the type of antigen examined.

Since it may not always be possible to limit the duration of fixation to less than 2 days, laboratories use a variety of antigen unmasking techniques (such as enzymes and/or heat) to recover antigen expression. Samples to be fixed should not be thicker than 0.5 cm and ideally the ratio of fixative to sample should be 10:1 (v:v). If you collect samples of good quality (fresh, of the right size, and representative of the lesion) and place them in formalin immediately, you have

preserved the specimen indefinitely. If for any reason fresh tissues do not arrive at the diagnostic laboratory in good condition, making isolation or fluorescent antibody procedures impossible, IHC can still be used to detect pathogens on fixed tissues.

Processing of fixed samples

Once tissue samples are fixed, they are embedded in paraffin or resins, sectioned in a microtome and mounted on glass slides. Immunohistochemical procedures are then performed on these unstained tissue sections.

Immunohistochemical techniques

There are several steps in an immunohistochemical reaction. In general, it is necessary to use procedures that allow antigens in formalin-fixed tissues to be recognized by antisera. These methods are generically referred to as antigen unmasking or antigen retrieval. Sometimes it is also necessary to reduce nonspecific reactions (background) before starting the immunohistochemical reaction.

There are several systems to retrieve antigens:

- detergents (e.g., Tween 20, saponin, Nonidet p40) and chaotropic substances (guanidine, sodium thiocyanate);
- enzymatic digestion (trypsin, proteinase K, pronase E); and
- heat-induced antigen retrieval.

A major advance in immunohistochemistry was the discovery in the early 1990s that some previously nonreactive antigens in formalin-fixed, paraffin-embedded tissues, even after enzymatic treatment, could be "retrieved" by heating sections in a buffer solution. Microwave ovens, pressure cookers, and steamers have all been successfully used as sources of heat. It has been hypothesized that heating provides the energy not only to rupture the hydroxyl bonds formed by the fixative with the protein antigen, freeing some antigens, but also releases tissue-bound calcium ions that contribute to tighter bonds with the fixative. Heat-based retrieval systems not only permit some antigens to be detected that otherwise go undetected, but also increase the sensitivity of some methods, allowing the antibody to be further diluted. However, every antigen has to be tested to find the best retrieval conditions to optimize results.

The use of blocking agents for endogenous enzyme activity (e.g., peroxidase, alkaline phosphatase, etc.) are also necessary when using enzyme-based methods. A similar problem may exist with avidin-biotin methods due to the presence of endogenous biotin activity in many tissues.

Signal-generating systems

Visualization of the antigen-antibody reaction site depends on a signal-generating system which is conjugated to the antibody, or other molecules such as avidin. There are three types of signal-generating systems:

- fluorochromes, which are visualized by excitation with light of the appropriate wavelength (excitation wavelength) and imaging the emitted fluorescence (emission wavelength) with appropriate filters. Although fluorochromes are not popular on fixed and paraffin-embedded samples due to autofluorescence, they are frequently used on frozen tissue sections. Some of the fluorochromes currently in use include fluorescein isothiocyanate, rhodamine, and Texas red.
- enzymes, such as horseradish peroxidase (most commonly), or alkaline phosphatase, glucose oxidase, and b-galactosidase. With their specific histochemical substrates and a variety of capturing chromogens, enzyme markers can produce different colored end-products—usually brown, blue, or red—that are visible by light microscopy. The reaction with diaminobenzidine (DAB), a chromogen, results in a brown color (Figures 2–7, following page). The reaction when using alkaline phosphatase is usually visualized in blue.
- metals, such as colloidal gold. Antigen-antibody reaction is detected with a developer that contains a reducing agent (usually hydroquinone) and a silver solution (usually silver acetate). The sensitivity of this technique is similar to or higher than that of enzyme-based methods.

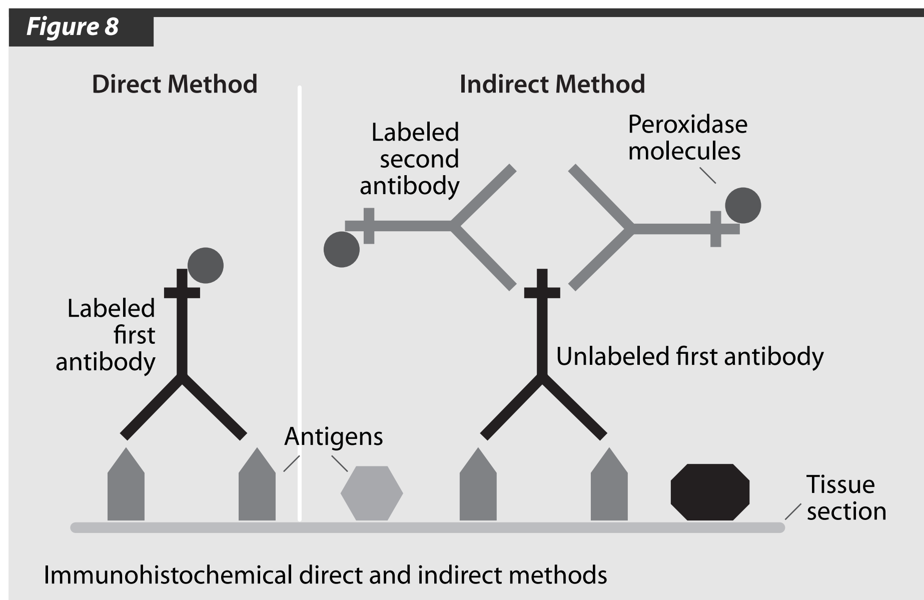
Methods

The primary goal of an IHC test is to be sensitive enough to demonstrate a specific antigen-antibody binding in tissue sections. Since Coons first described an in situ immunocytochemical technique, new methods have been developed that dramatically increase sensitivity while maintaining the specificity of the reaction.

Immunohistochemistry can use a direct, indirect, or multiple layer method:

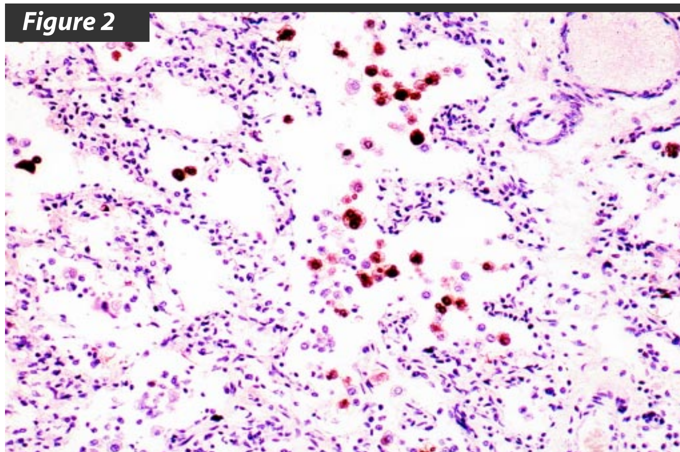
- The direct method (Figure 8) is the simplest of immunohisto-

Figure 8



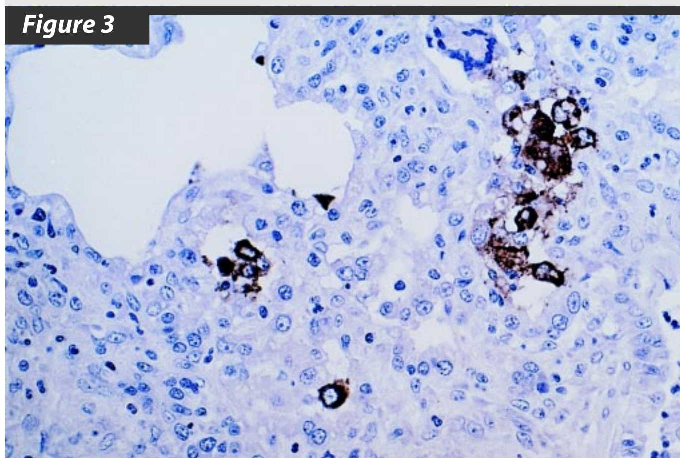
Immunohistochemical direct and indirect methods

Figure 2



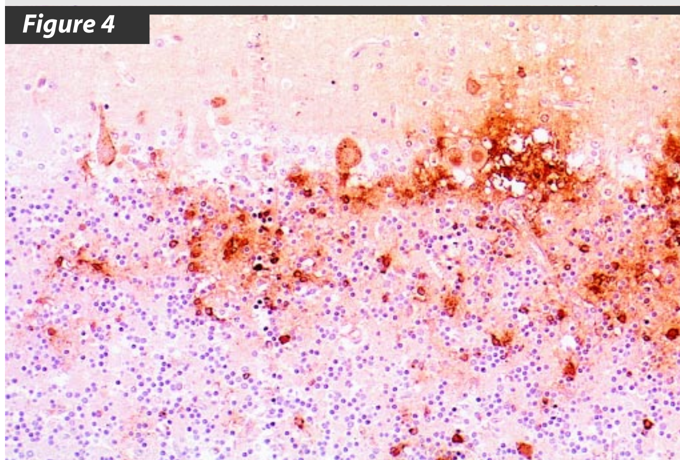
Lung. Numerous alveolar macrophages (stained in brown) in alveolar lumina contain antigen for porcine reproductive and respiratory syndrome virus (PRRSV). Avidin-biotin-peroxidase. Hematoxylin counterstain.

Figure 3



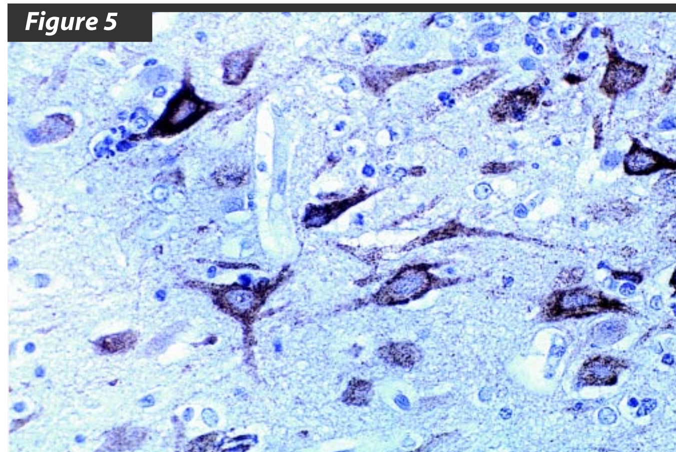
Lung. Antigen specific for PRRSV is observed in alveolar macrophages colored in brown. Avidin-biotin-peroxidase. Hematoxylin counterstain.

Figure 4



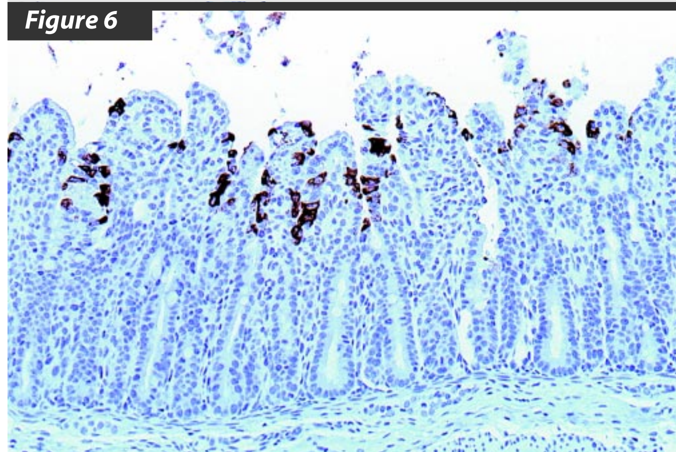
Cerebellum. A brown color indicates presence of antigen for pseudorabies virus in Purkinje cells and cells of the granular layer of a naturally infected pig. Peroxidase-antiperoxidase. Hematoxylin counterstain.

Figure 5



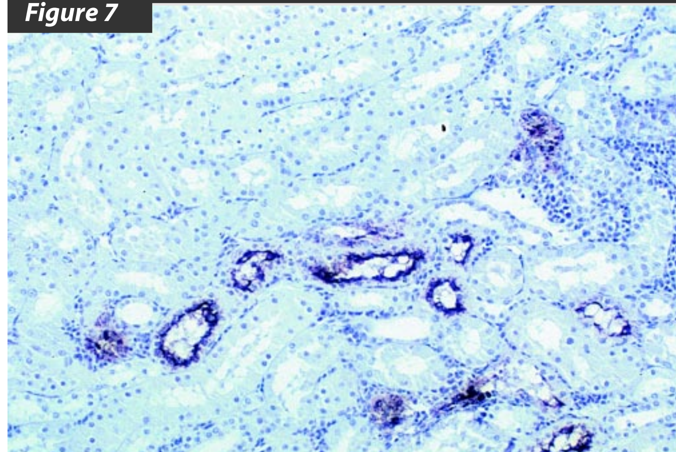
Brain. Diffuse staining for pseudorabies virus antigen within the cytoplasm of neurons. Peroxidase-antiperoxidase. Hematoxylin counterstain.

Figure 6



Small intestine. Apical enterocytes (stained in brown) contain antigen for transmissible gastroenteritis virus. Peroxidase-antiperoxidase. Hematoxylin counterstain.

Figure 7

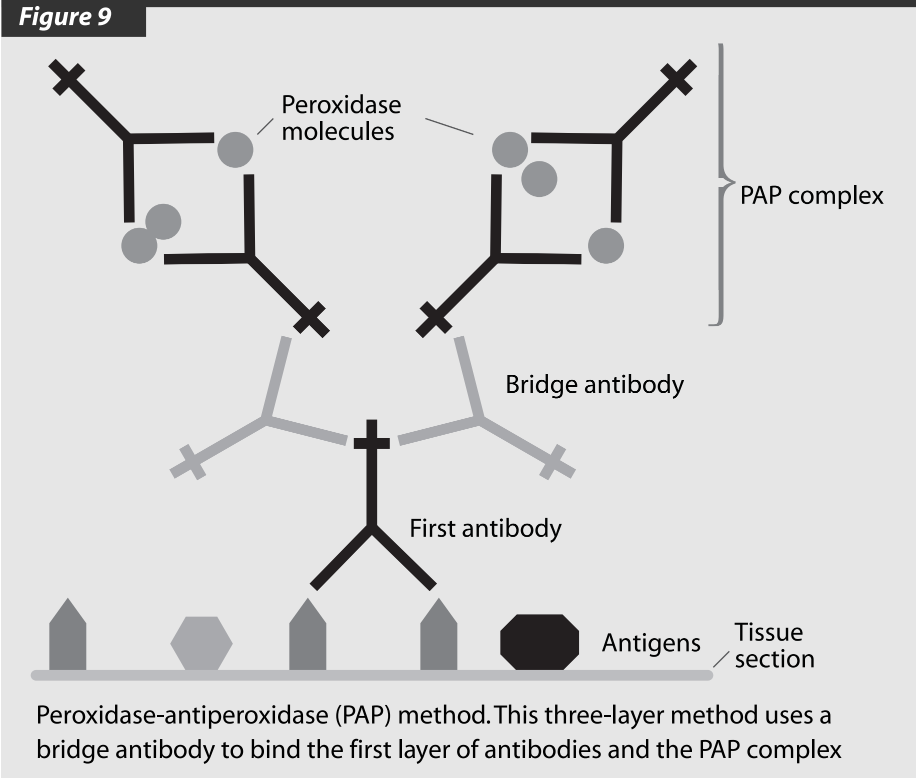


Kidney. Interstitial nephritis. Leptospira antigen is demonstrated (brown color) in the apical border of tubular epithelium. Peroxidase-antiperoxidase. Hematoxylin counterstain.

chemical methods. The reaction is a one-step process with a labeled primary antibody. The antibody molecules bind the antigen through the F[ab] fragment (Figure 1). It is also frequently used in diagnostic laboratories as a quick screening with fluorescent-labeled antibodies and frozen sections.

- Indirect methods (Figure 8) rely on the addition of a second layer of antibody directed against the first layer (which is specific for the antigen being detected). The antigen-binding fragment (F(ab)) of the second layer of antibodies will usually bind to the first layer through its Fc segment. In this case, it is the second layer of antibodies that is labeled, not the first. The indirect method is more sensitive than the direct method (the number of signal-generating molecules, (e.g. enzymes) bound to a single molecule of antigen is higher than with the direct method), but the procedure is more laborious.
- Multiple-layer methods, including the peroxidase-antiperoxidase (PAP) method and the avidin-biotin complex (ABC) method, are those most commonly used in a diagnostic laboratory when working with formalin-fixed, paraffin-embedded sections.

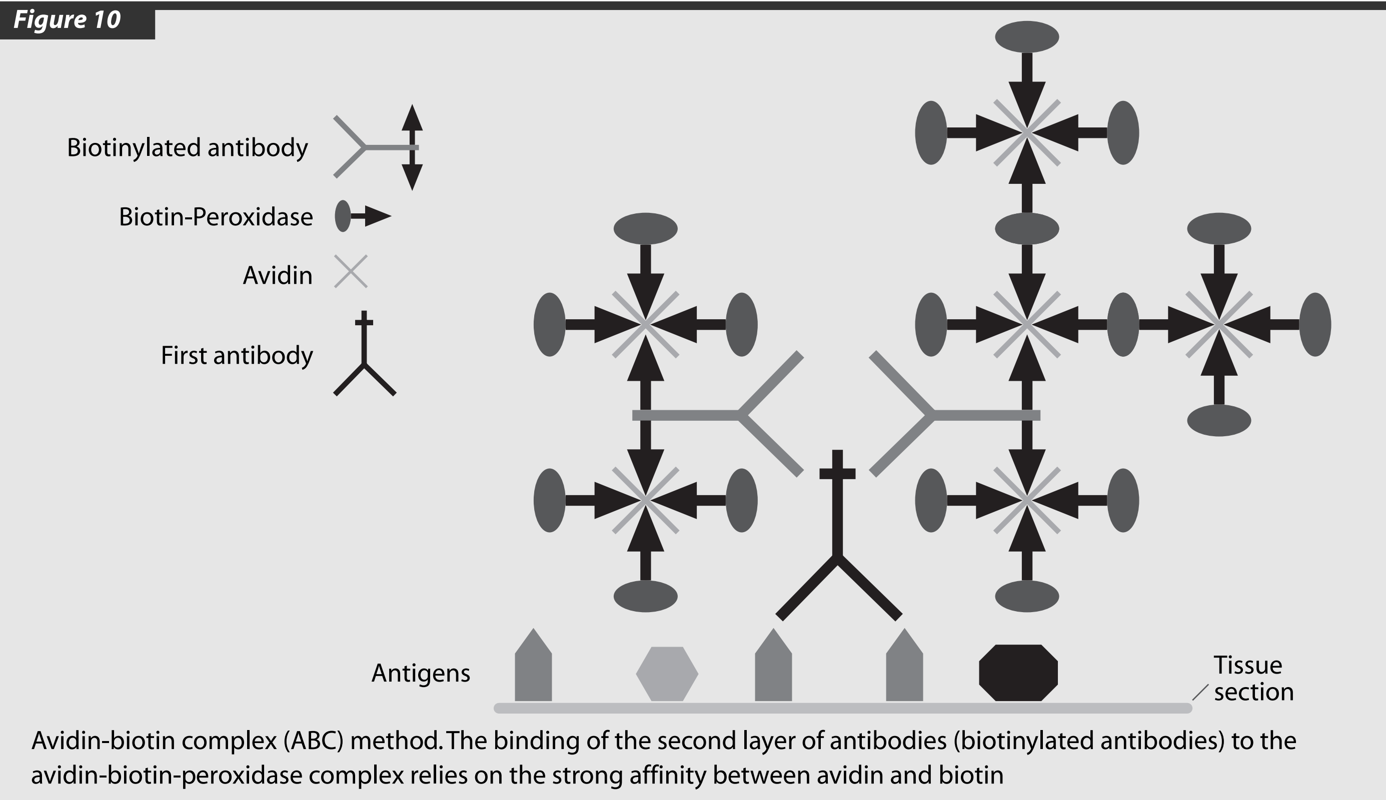
Figure 9



Peroxidase-antiperoxidase (PAP) method. This three-layer method uses a bridge antibody to bind the first layer of antibodies and the PAP complex

With the PAP method (Figure 9), two first layers of antibodies are similar to the indirect method but are not labeled. The third layer consists of immunoglobulins that recognize (bind) the peroxidase molecules (PAP complex) and they are raised in the same species as for the first antibody layer. Therefore, the second antibody layer will act as a "bridge" between the first and the third layer of antibodies. This method is 100–1000 times more sensitive than the indirect method (due to the increased number of signal-generating molecules per molecule of antigen) without loss of specificity.

Figure 10



Avidin-biotin complex (ABC) method. The binding of the second layer of antibodies (biotinylated antibodies) to the avidin-biotin-peroxidase complex relies on the strong affinity between avidin and biotin

Table 1

Porcine infectious agents that can be detected by IHC

Viral diseases	Bacterial and other infectious diseases
Transmissible gastroenteritis virus (TGEV) [17–19]	*Haemophilus parasuis* [38,39]
Pseudorabies virus (PRV, Aujesky's disease virus) [20–23]	*Actinobaciluus pleuropneumoniae* [38,40]
Porcine reproductive and respiratory syndrome virus (PRRSV) [24,25]	*Streptococcus suis* [41]
Swine influenza virus (SIV) [26,27]	*Mycoplasma hyorhinus* [38,42]
Porcine respiratory coronavirus (PRCV) [28,29]	*Mycoplasma hyopneumoniae* [43]
African swine fever [30]	*Salmonella* spp. [44]
Porcine rotavirus [31,32]	*Pasturella multocida* [38,45]
Porcine epidemic diarrhea [33]	*Seruplina* spp. [46]
Hog cholera [34]	*Escherichia coli* [38,47]
Swine vesicular disease virus [35]	*Chlamydia* spp. [48]
Adenovirus [36]	*Leptospira* spp. [49]
Porcine paramyxovirus [37]	*Staphylococcus hyicus* [50]
	Erysipelothrix rhusiopathiae [51]
	Mycobacterium spp. [52]
	Toxoplasma gondii [53]

The avidin-biotin complex (ABC) method (Figure 10) is a multiple-layer method that relies on the extremely high affinity between avidin, a glycoprotein from egg white, and biotin, a vitamin. In this method, the second antibody is biotinylated and the third layer is a complex of avidin mixed with biotin that is labeled with a marker (enzyme, fluorochrome, etc.).

Interpreting results

Immunohistochemistry is intended to help in diagnosing the etiologic agent of an infectious process. The presence of a colored reaction (provided that it is specific according to the controls used) indicates the presence of components of the infectious agent tested for. Whether the appearance of a specific color is significant or not in the context of the case is open to interpretation by the diagnostician. A careful assessment of the clinical history, lesions, and all test results should be made before attempting to formulate a definitive diagnosis. Conversely, a negative result by immunohistochemistry does not completely rule out the presence of a particular infectious agent or its potential significance to the case. Results by immunohistochemistry, like those obtained by other diagnostic methods, must be supported by clinicopathologic data.

Table 1 lists porcine infectious agents that can be detected by IHC. Because not every laboratory has the resources to detect all of these or other infectious agents by immunohistochemistry, it is advisable to contact your local diagnostic laboratory to know what tests are available in your area.

Immunohistochemistry is a valuable technique for diagnosing infectious diseases of pigs. It is sensitive, specific, fairly inexpensive, and easy to perform. Although in most diagnostic laboratories it is not considered the "gold standard," it is as specific as bacterial and virus isolation, provided adequate controls are used.

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