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Methodology in diagnostic virology

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Definitive identification of viral infections can be challenging. Sometimes, history and clinical presentation are sufficient to narrow, if not solidify, the diagnosis. Laboratory testing often is required for confirmation of the identity of the infectious agent. Although commitments of time and money are involved, verification of the pathogen identity is important. Many diseases present with vague or similar symptoms and lesions. Depending upon the agent, aspects such as prognosis, treatment protocol, and control measures will be impacted by the diagnosis. In addition, identification of the infecting pathogen will aid surveillance and may be required by regulations that pertain to trade and transport. Finally, some agents have zoonotic potential [1,2].

Impediments to viral diagnoses exist. The variety of techniques and methods for detection of viral infection is nearly as diverse as the viruses themselves. Deciding which assay is most suitable in each case can be daunting. In addition, there is little, if any, standardization among diagnostic laboratories; this impacts the interpretation of results. The advent of newer molecular assays also offers challenges to the practitioner in terms of understanding the technique and assessing the results appropriately. Finally, proper interpretation of results can be difficult, especially with regard to serologic assays.

Diagnostic virology in its simplest form involves two avenues—detection of the virus itself and characterization of the host antibody response to the infecting virus. The assays that are involved vary in methodology, expense, availability, sensitivity, and specificity. This article provides an overview of some of the more common techniques that are used and their respective advantages and disadvantages.

First, it is important to remember that the results that are received from the various assays depend, in large part, on the quality of the specimen that

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was submitted. For antibody assays, this is straightforward, because only serum is required. It may be advantageous to ship these samples cooled in warm climes or during hotter time periods to prevent bacterial growth and antibody destruction. Proper sampling and shipment for virus detection may be more involved. For many assays (eg, antigen detection assays), the virus need not remain viable. In these situations, rapid shipment and cooling during transport are not required. For some assays, however, degradation of the virus during transport will necessitate cooling during shipment. This often is true for genetic detection that uses polymerase chain reaction. Many viral genomes, especially those of RNA viruses, are labile. For virus propagation, the virus must remain infectious from the moment it is collected until it reaches the laboratory. This requires rapid, cooled transport [1,2]. Some viruses are inactivated by freezing; therefore, as long as shipment is speedy, refrigeration/cold packs generally are sufficient [1]. Communication with the laboratory regarding shipping requirements will assist in the proper transport.

Virus detection

Detection of the virus that causes an infection uses one of several methods: The virus may be propagated in the laboratory and characterized; the virus may be visualized by electron microscopy; the viral proteins may be detected using specific antibody; the viral nucleic acid may be detected; or an activity of the virus, such as red blood cell agglutination, may be assayed. Identification of the virus that is associated with the disease is good evidence for identification of the etiologic agent. One must remember that the relationship between the agent that is detected and the disease is not always causal. Some of the more common assays are discussed below.

Virus isolation

For many viruses, virus isolation remains the gold standard for identification [1,3]. This assay requires collection of a sample that contains viable virus and propagation of the virus in a cell culture system. For some avian viruses, propagation in embryonated eggs is required [1]. After growth in the in vitro or in vivo system, the virus is characterized and identified.

Sample collection and transport are critical steps in successful virus isolation [1,2,4]. Usually, samples are collected from the disease lesions, using swabs, fluids, and tissue samples. Various secretions/excretions can be collected, depending on the mode of virus shedding. Viruses in which a viremic phase occurs following exposure may be isolated from blood; however, this most often is possible only in the early stages of infection. Collection of virus for virus isolation optimally is done in the acute phase. Later in infection or during chronic infection, the presence of host antibody may inhibit virus growth in the laboratory. The speed of shipment that is

required depends on the lability of the virus; nonenveloped viruses tend to be hardier and less susceptible to inactivation than enveloped viruses [4]. Because the identity of the virus usually is not known, overnight shipment on cold pack is ideal. Certain information should be provided to the laboratory to facilitate the chances of successful propagation (eg, species, history, clinical presentation). Cell lines that are used vary with the suspected virus; any information that can assist the diagnostician in the appropriate selection of propagation medium is helpful.

After viral growth has been detected, identification of the virus is done. This can be accomplished in several ways. Some viruses produce characteristic cytopathic changes (eg, syncytia) in cell culture (Fig. 1). For viruses whose identity is completely unknown, electron microscopy allows visualization and classification of the virus into a family based on morphology. Specific identification usually is done through the use of virus-specific antibody in an assay, such as immunofluorescence. Alternatively, a virus activity may be exploited to identify the virus. For example, the virus of Newcastle disease, avian paramyxovirus–1, agglutinates chicken red blood cells (RBCs). Its presence in cell culture can be detected by agglutination of RBCs using the cell culture supernatant.

Virus isolation offers several advantages. It remains the gold standard for many viruses. It is more sensitive than the antigen detection assays (eg, immunofluorescence) because the virus is amplified effectively by propagation in the laboratory. Virus isolation is a nonspecific assay (ie, any virus present in the sample) may be propagated. This is in contrast to antigen identification, where the assay is specific. For example, submission of cells from a tracheal wash for the virus of infectious laryngotracheitis (herpesvirus) of birds will be negative if the agent is that of infectious bronchitis (coronavirus); however, if the sample is submitted for virus isolation, either virus may be isolated.

Virus isolation also has certain disadvantages. Generally, it is more time-consuming and expensive than most routine antigen detection assays [1]. It may take 1 to 2 weeks for the virus to be detectable and the increased material and labor increases the cost. Obviously, the virus must remain viable during shipment which can be a problem with certain labile viruses (eg, herpesviruses). Rapid shipment in refrigeration is required which may add to the cost. The presence of other components (eg, bacteria, toxins) can interfere with the ability of the virus to grow in the laboratory. For example, hepatic viruses can be difficult to isolate because of the presence of cell culture toxins in the liver tissue [2]. This also is true if neutralizing antibodies are present. Additionally, some viruses simply do not grow well in an in vitro system. This is true for some enteric viruses, for example.

Electron microscopy

Electron microscopy provides a means for visualizing virus from samples or laboratory propagation. Samples are homogenized, pelleted by
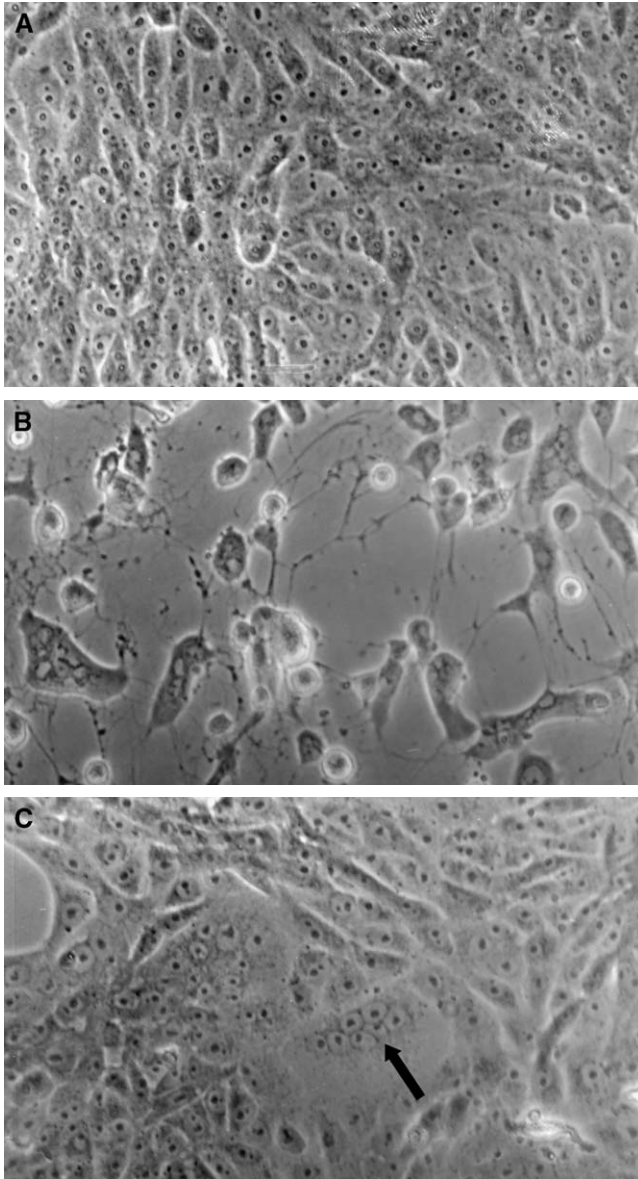
Fig. 1. Photomicrograph of cell culture exhibiting cytopathic effects. (*A*) Normal uninfected cells. (*B*) Cell death; note stringy appearance of cells, cells with vacuoles, and rounded detached cells. (*C*) Multi-nucleated syncytia (*arrow*).

centrifugation, and a small amount is mixed with a stain (eg, phospho-tungstic acid) [1,5]. This is aerosolized onto a small grid and viewed by transmission electron microscopy. The virus, if present, is shadowed by the stain which gives an image that is similar to a negative photograph. The

identification is based on morphology of the virion which allows classification into the virus family (Fig. 2). A more specific identification is provided by the addition of virus-specific antibodies to the mixture. This results in clumping of the virus [4,5].

Like virus isolation, electron microscopy is a nonspecific assay (ie, it looks for any virus that is present in the sample). For example, in diarrheic samples, any virus could be seen if present, whether parvovirus, coronavirus, or rotavirus, whereas an assay that is specific for one will miss the others. Where available, this assay is rapid and generally takes less than 1 day. In addition, the virus need not remain viable for detection and samples may be stored before shipment. Because feces is the most common sample that is submitted, storing and shipping under cooled conditions to keep down bacterial growth is recommended.

The equipment that is required for electron microscopy is extremely expensive as is the maintenance. Therefore, this service may not be widely
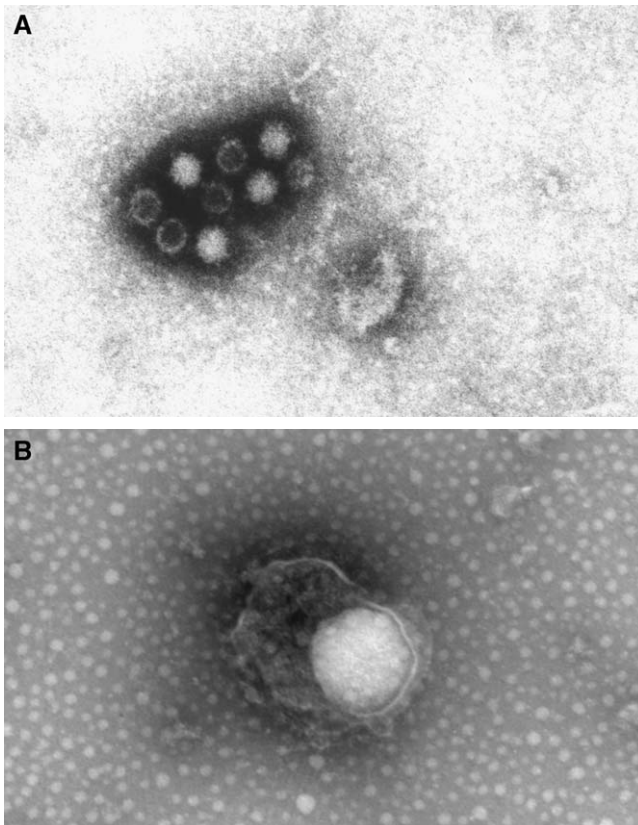

Fig. 2. Electron micrographs of two viruses (original magnification ×40,000). (*A*) Parvovirus. (*B*) Iridovirus.

available; it is primarily found at academic and research institutions. Also, expertise is required to identify the virus which also may impact availability. The fee for this service varies with the laboratory, but usually is reasonable, despite the expensive equipment. A drawback to electron microscopy is its relative lack of sensitivity. Generally speaking, approximately 10^6 particles per gram of sample are required for visualization [1,4,5]. More may be required for small viruses, such as parvovirus. Because of this, electron microscopy usually is restricted to testing of feces in cases of enteritis where virus concentration is significant in the acute phase of disease. For example, in diarrhea that is caused by parvovirus, the animal may be shedding more than 10^9 particles per gram. It also is used to identify viruses in cell culture, where the virus has been amplified through growth in the laboratory. It is too insensitive to detect virus in most tissue, fluid, or swab samples. These require propagation before detection. One exception to this may be poxviruses. Because of their large size, they may be seen in samples from lesions.

Antigen detection

Antigen detection assays involve the use of virus-specific antibody to detect or bind to the viral protein or antigen in the sample. The assays vary in the sample substrate, how the binding of antibody to antigen is visualized, sensitivity, and specificity. Availability of these assays for the various viruses depends largely on the availability of antibody to the pathogen. This can be a challenge for many viruses of exotic species because antibody to them often are not commercially feasible to develop. In these cases, one may find testing available at laboratories that are involved in research on the particular pathogen. We will consider several of the more common assays that are available.

Immunofluorescence assays (IFA)

Immunofluorescence assays (IFAs) are used in many diagnostic laboratories. They are fast and economical and can be done on a wide variety of samples. These include tissue impression smears or scrapings, cells pelleted from fluids, and cell cultures. Generally, slides are made by the practitioner and submitted to the laboratory. Neither fixation nor special storage is required before submission. The quality of the sample does affect the results. Because the assay involves detection of infected cells, the more cells that are on the slide, the more likely it is that infected cells will be identified. Most IFA assays use fluorochrome-labeled antibody, such as fluorescein (Fig. 3; direct) [1,3]. Therefore, it is important that before collection of ocular samples, fluorescein staining of the eye must be avoided. Also, purulent discharge will result in background fluorescence because of the autofluorescence of granules in white blood cells and should be removed before collection of the sample. Bacteria also may lead to background fluorescence.
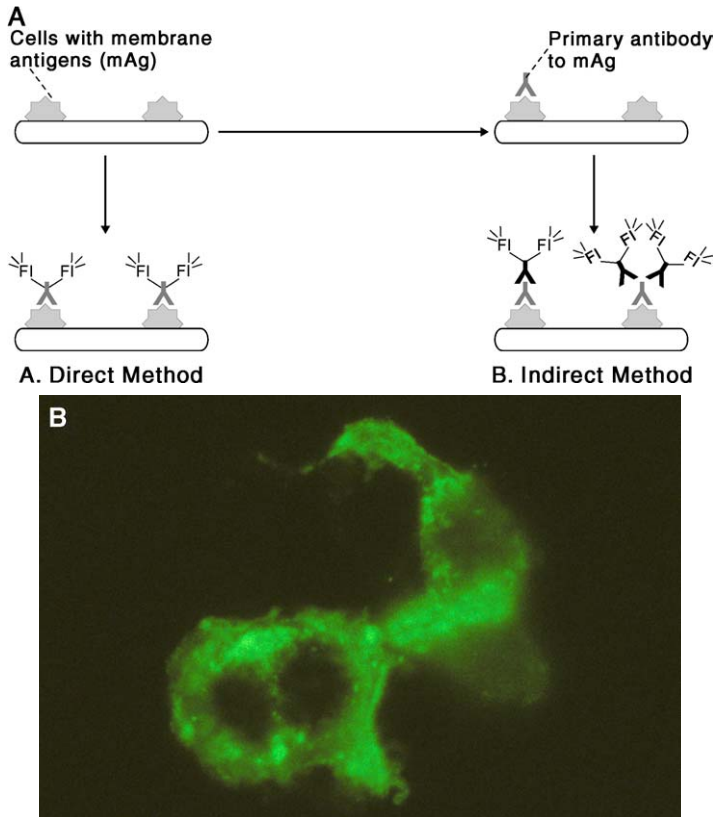
Fig. 3. (*A*) Direct and indirect immunofluorescence assays using fluorescein-labeled antibody. (*B*) Photomicrograph of immunofluorescence assay of virus-infected cells using fluorescein-labeled antibody.

These assays may be direct or indirect (see Fig. 3). In direct assays, the pathogen-specific antibody is labeled. In indirect assays, the virus-specific antibody is followed with an antibody to the first antibody (antiglobulin) that is labeled. This extra layer of antibody may increase the sensitivity of the antigen detection assays as a result of increased fluorescence relative to direct assays [4].

Immunofluorescence assays are intermediate in sensitivity. This sensitivity is even lower in chronic infections where the presence of host antibody can block the binding of the detecting antibody [6,7]. In general, a positive result ''rules in'' but a negative result does not ''rule out.'' Also, because it is a specific assay, it does not detect the presence of other pathogens (eg, an IFA assay for felid herpesvirus on a conjunctival scraping will be negative if the pathogen involved is calicivirus). Finally, technical expertise is required to interpret IFA assays. The reader must be familiar with the pattern of fluorescence and have the ability to distinguish viral fluorescence from

background or nonspecific fluorescence. Experience is thus required for proper interpretation of IFA assays.

ELISA and immunohistochemistry

ELISAs are available for some viral pathogens. These assays normally are provided as commercial kits and availability is dependent on marketability. Some available assays include feline leukemia virus, canine parvovirus, rotavirus, equine influenza virus, and bovine viral diarrhea virus. Most use pathogen-specific antibody for capture and detection as for IFA assays (Fig. 4B). The antibody is anchored in a solid substrate, such as a membrane or plastic well. The sample, usually in fluid form, is added. The initial sample collected may be fluid (eg, serum for feline leukemia virus) or the sample may be solubilized in a diluent using a swab (eg, feces for parvovirus). If the virus is present, it will be bound by the anchored antibody. This is followed by soluble pathogen-specific antibody with enzyme attached, which will bind the bound virus. Addition of the substrate for the enzyme leads to a color change. Washing is a critical step in many
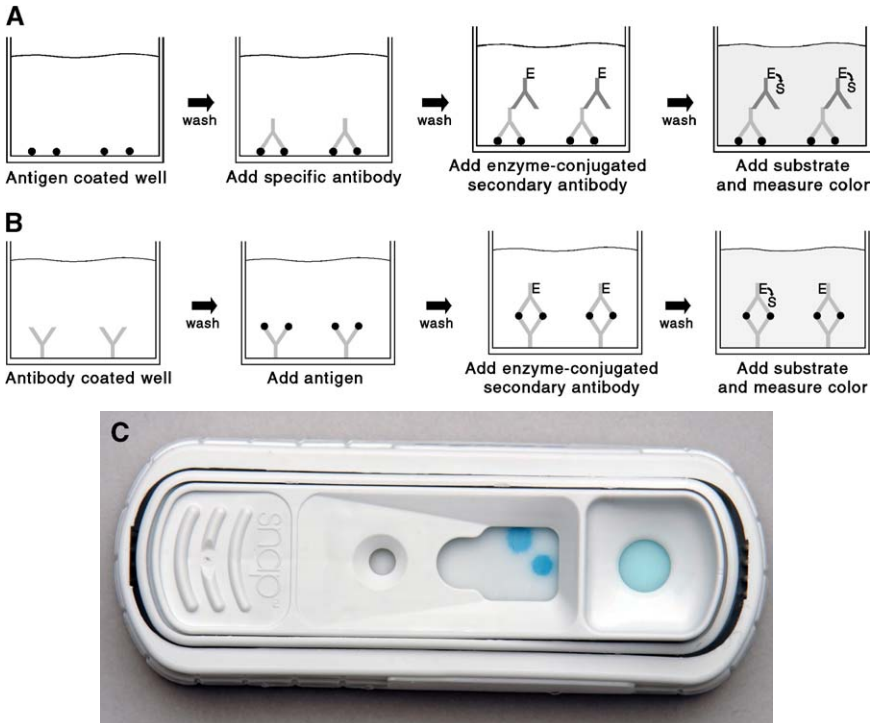

Fig. 4. (*A*) ELISA for antibody detection. (*B*) Diagram for ELISA for antigen detection. (*C*) Example of ELISA for Feline Immunodeficiency Virus (antibody) and Feline Leukemia Virus (antigen) (IDEXX Snap Test, Westbrook, Maine); FIV is positive (indicated by top blue spot).

ELISA assays to ensure unbound antibody is removed. Variations on this protocol may be found with different assays, but the underlying technique of pathogen-specific antibody binding the virus in the sample is similar in all (Fig. 4) [1,3,4].

ELISAs offer several advantages. Generally, they are rapid assays that require only minutes to 1 to 2 hours, at most, for completion and most are economical. They are simple and often can be done patient-side. Usually, ELISAs are more sensitive than IFAs. Because protein antigen is detected, the agent need not remain viable for identification.

ELISAs, like IFAs, are specific assays. Thus, a negative result will not provide information on the presence of other pathogens. Sensitivity of ELISA is usually good; however, negative results may occur early or late in infection when concentrations are low. Also, if the animal has large amounts of antibody present to the pathogen, these may bind and mask the agent and prevent binding by the assay antibody. False positive results may occur for a variety of reasons. The most common may be insufficient washing. Other factors can lead to false positive results. For example, some feline leukemia virus ELISAs use antibody to feline leukemia virus that is prepared in mice as the anchored and soluble antibody. Some cats have antibody to mouse antibody from hunting behavior; this can be bound by the anchored and soluble assay antibodies and lead to false positive results. Tests that use rabbit antibody can lead to the same result in felids with antirabbit antibody [8].

Immunohistochemistry (IHC) is similar to ELISA in that an enzyme-bound antibody is used to detect the virus in the sample (Fig. 5). The major difference is that the sample used is fixed tissue [9]. Sampling thus requires biopsy or necropsy. This allows identification of the virus associated with the microscopic lesion, if present.
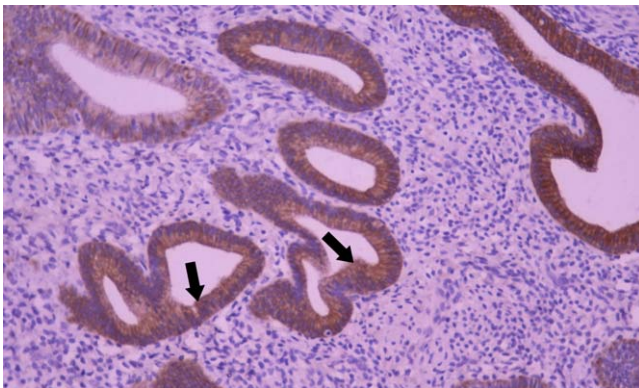

Fig. 5. Photomicrograph of uterine tissue labeled by immunohistochemistry (antibody to epithelial cell marker); note brown color labeling of epithelia (*arrows*). (Courtesy of R. Donnell, DVM, PhD, DACVP, Knoxville, Tennessee).

Usually, the sensitivity and specificity of IHC are good and it is the gold standard for some diseases, such as chronic wasting disease of cervids and feline infectious peritonitis [10,11]. Availability of this assay depends upon availability of the pathogen-specific antibody. Also, expertise in sample preparation and processing is required which also may impact availability.

Agglutination assays

Some pathogens have the ability to agglutinate RBCs. This assay usually is used to detect virus in cell culture [1,3]. Newcastle disease virus can be detected in the laboratory after propagation by detection of RBC agglutination. This assay is not done routinely on clinical samples for virus detection.

Agglutination of viruses also can be accomplished by the presence of pathogen-specific antibody in the assay; the immune complex formation can be visualized under certain conditions. One method is to attach the antibody to a solid substrate, such as a latex bead; addition of the sample with the antigen leads to clumping of the substrate which can be seen. The most common mechanism is through agar gel immunodiffusion (Fig. 6). In this instance, the immune complexes precipitate out in the agarose and form
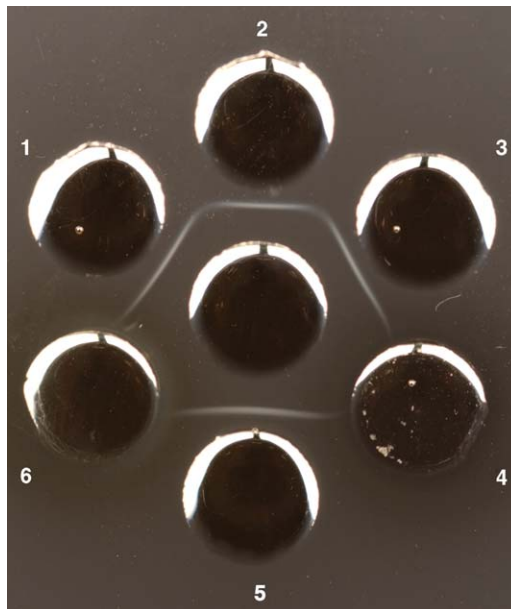

Fig. 6. Example of agar gel immunodiffusion for antibody to equine infectious anemia virus. Center well contains viral antigen; wells 1, 3, and 5 contain positive control sera; wells 2, 4, and 6 contain patient sera. Well 2 is positive, well 4 is weak positive, and well 6 is negative.

a precipitin band. This assay is low in sensitivity and is not used widely for detection of viral pathogens [1].

Genetic detection

The assays that were described above involve detection of whole virus or protein components of the virion. An alternative method is detection of the genetic material of the virus. A variety of techniques is available to accomplish this and are described below.

In situ hybridization

In situ hybridization (ISH) uses genetic probes to detect nucleic acid within the cell. Samples are as for IHC and usually involve fixed tissues, but pretreatment protocols differ from IHC. DNA or RNA probes are designed to hybridize to a portion of the pathogen genome or mRNA; their binding is detected through the labeling of the probe. The labels that are used can vary and include radioactive isotopes, fluorescent dyes, or chemiluminescent materials. The presence of the agent within the cell of the tissue sample can be visualized [9].

This technique allows visualization of the agent that is associated with the lesion and provides good evidence for identification of the etiologic agent of disease. There are several factors to consider with ISH to ensure appropriate interpretation of results. The sensitivity of the assay depends primarily upon two factors—the probe design and the abundance of the target. The probe design includes such parameters as the probe composition and length. The abundance of the target influences directly whether binding can be detected. Generally, for targets of low abundance, probes of increased length are required to increase the amount of label. The specificity also is impacted by the probe design. Is the genetic region that is targeted highly conserved or heterologous among isolates? Problems with nonspecific cross-hybridization also can be encountered.

The availability of this assay for particular pathogens depends on the presence of the required expertise and on the availability of sequence data for the virus of interest that is necessary for probe design. This technique may not be widely available for some pathogens of nondomestic species. Additionally, biopsy or postmortem tissue collection is required [12].

Polymerase chain reaction

Polymerase chain reaction (PCR) has become more common and accessible for detection of many pathogens. This technique involves the extraction of nucleic acid from the sample and amplification of a portion of the genetic material of the agent of interest. This amplification is
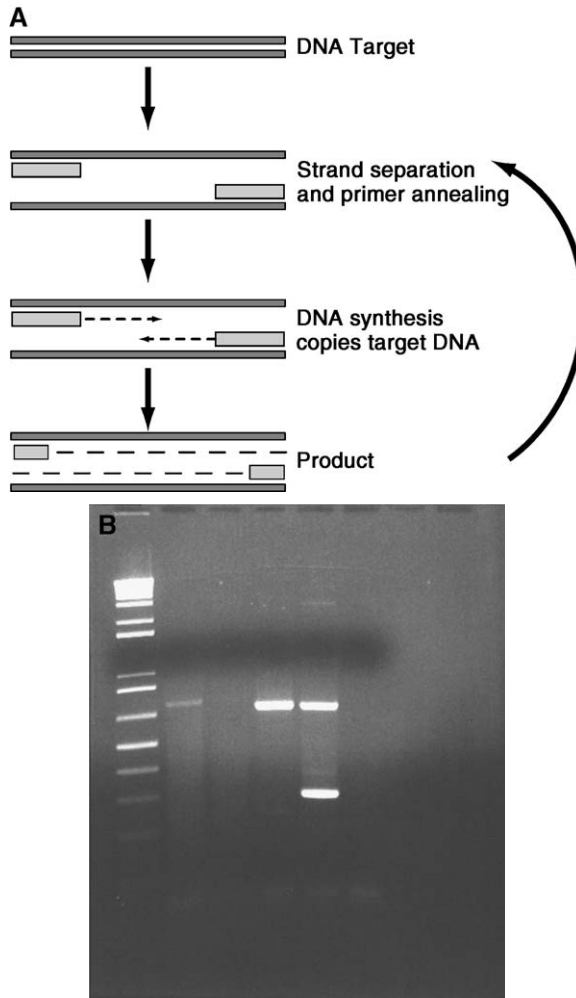
Fig. 7. (*A*) PCR. (*B*) Agarose gel electrophoresis analysis of DNA products. From left: lane 1, molecular weight markers; lane 2, weak positive; lane 3, negative; lane 4, positive; lane 5, positive control; lane 6, negative control.

accomplished by repeated cycles of DNA synthesis primed by small pieces of nucleic acid primers that are designed to target a specific genomic region (Fig. 7A). The resultant product is identified through gel electrophoresis (Fig. 7B), restriction enzyme digestion, probes, or nucleotide sequencing [3,4].

There are several variations to this assay. If the starting material targeted is RNA rather than DNA—such as with RNA viruses or assays that target mRNA of the agent—a reverse transcription reaction to convert the RNA to DNA is required before the actual amplification. Nested PCR involves two rounds of multiple amplification steps using two primer sets, one

internal to the other. This allows significantly more amplification of the starting material. Quantitative PCR, also known as Real Time or TaqMan, allows quantification—either relative or absolute—of the starting material. Multi-plex PCR uses multiple primer pairs to allow detection of more than one pathogen in a single reaction [1].

One of the hallmarks of PCR is its exquisite sensitivity, especially with nested PCR, which can be a double-edged sword. It allows the detection of small amounts of the agent in the sample, which can be advantageous if low amounts are present [13–15]. Contamination or carry-over can lead to false positive results. In addition, subclinical or even latent infections (eg, herpesviruses) may be detected [16,17]. Real Time or quantitative PCR provides information on the amount of virus that is present in the sample. This can assist in distinguishing clinical from subclinical amounts of the virus in the sample.

False negative results also can occur and may be due to a variety of reasons. These may include degradation of the sample during transport, presence of PCR inhibitors in the sample, or insufficient genetic homology between the primers that are used and the target. This last reason can be a problem with viruses that undergo significant strain variation. In this case, primers should target highly conserved regions ideally [18]. For example, this has been an impediment to development of PCR assays that consistently work with all strains of FIV. RNA templates require reverse transcription before amplification; this step can be inefficient and may lead to false negative results [18].

Specificity of PCR also depends upon primer design, but generally, specificity of PCR is high. Depending upon the target, the assay can be family-, genus-, species-, or strain-specific.

Stringent controls and a significant level of expertise are required for accurate results using PCR. Contamination of samples can lead to false positive results. Nested PCR assays, because of their enhanced sensitivity and increased chance of sample contamination, are more likely to give false positive results than single rounds of PCR. Inquiries to the laboratory personnel regarding these parameters can provide useful information that can aid in interpretation of results. Often times, certain laboratories have more experience and expertise with certain pathogens. One laboratory that can test for all relevant pathogens is probably not feasible.

A variety of sample types can be used for PCR; it depends, in large part, on the pathogenesis of the agent. For example, viruses that are shed in feces can be tested for using fecal material; viruses that produce significant viremia may be tested for using serum or whole blood; viruses that are shed in feather dander (eg, psittacine beak and feather disease virus) may be detected in feathers [19]. Again, communication with the diagnostic laboratory will provide helpful information. Shipment of the sample usually requires refrigeration to prevent degradation of the genetic material of the agent. This is of particular concern with RNA targets because RNA is much more labile than DNA.

Serology

Evaluation of the host immunologic response involves detection of antibody to a specific pathogen. This provides, at the least, a historical perspective of the pathogens to which the animal has been exposed. By doing paired antibody assays—testing in the acute and convalescent phases of disease—the current infection status can be determined. A fourfold increase between acute and convalescent samples indicates active infection. Serology on herds or flocks can be used to establish the prevalence of the agent in the population [1].

The assays that are used to detect antibody are similar to ones for antigen detection—the antigen that is detected in serology is a pathogen-specific antibody. Unlike some antigen detection assays (eg, as virus isolation, electron microscopy), all serologic assays are specific (ie, they only detect antibody to a certain infectious agent). The assays for antibody detection can be divided into three basic categories [20]: (1) direct detection of the antibody, (2) detection of antibody-facilitated activity, and (3) detection of antibody-facilitated inhibition.

The first category involves binding of the animal's antibody to antigen anchored to a solid substrate (eg, membrane, slide). Detection of the bound antibody is done through anti-immunoglobulin (eg, antifeline IgG) that is labeled with a dye or enzyme [3,4]. These assays include immunofluorescence, ELISA, and western blot. This can be an obstacle in testing exotic species because there is limited availability of antiglobulin for most species of animals. Some that are available include antibody to immunoglobulin of ferrets, raccoons, llamas, psittacines, deer, bears, rabbits, monkeys, and several species of rodents. Testing for antibodies in other species may be available in some laboratories that are involved in research on the species [21]. If no antiglobulin is available, then testing is restricted to one of the other two categories.

The second category exploits the facilitation of an activity by the formation of the immune complex (patient's antibody; capture antigen, or virus); an activity occurs in response to formation of the immune complex formation. These may include complement fixation, agglutination, and precipitation. These assays detect IgG and IgM. Because IgM is an efficient activator of complement and enhances agglutination because of its pentameric structure, these assays are useful for detection of IgM [22,23]. The third category exploits the inhibition of an activity by the formation of an immune complex (ie, an activity that the virus normally is capable of effecting does not occur because of binding of the patient's antibody to the assay virus). These include hemagglutination inhibition (HI) and virus neutralization. These two assays measure antibody to specific surface epitopes of the pathogen, usually viral attachment proteins [3,4].

Serologic assays may be qualitative or quantitative [3]. The former determines only the presence of antibody, not the level. This can be useful

for agents that cause life-long infection, such as the retroviruses; the presence of antibody would indicate infection. Quantitative assays determine the relative concentration of antibody by testing serial dilutions of the patient's serum. In this case, the titer is the highest dilution at which the presence of antibody is detected. Detection of seroconversion or a fourfold increase in titer between paired samples indicates active infection [1]. Paired titers can be circumvented in populations by screening a proportion of the animals. For example, testing exposed animals, clinically ill animals, and recovered animals can mimic acute and convalescent sampling. Basing a diagnosis on the magnitude of a single titer is unreliable; however, the laboratory may be able to provide some interpretation of a single titer.

The usefulness of serology depends, in part, on the virus and disease. If onset of clinical signs coincides with antibody production, active disease may be diagnosed. For some pathogens, disease occurs before antibody production; serology would be retrospective. Some pathogens cause disease months or years later, such as FIV; detection of antibodies is sufficient to diagnose infection [8].

Direct detection of the antibody

Most of these assays use antibody to the patient's immunoglobulin for detection. They vary in the capture antigen matrix (eg, slides, membrane, plastic wells) and label (eg, fluorescein, enzyme, chemiluminescent). Because these assays use species-specific antiglobulin, availability for exotic species is limited. Antiglobulin to antibody of domestic species may be used in some situations (eg, anticanine IgG in nondomestic canids like wolves). For those that are available, detection across species line is not always known. For example, in snakes, antiglobulin to IgY of boids does not detect IgY of elapids [21].

These assays vary in sensitivity and specificity. IFA assays are intermediate in sensitivity, whereas ELISAs and western blots are higher in sensitivity [22]. Generally, specificity depends upon the capture antigen that is used. For example, assays that use antigen that is shared with other agents that are nonpathogenic may detect antibody to these pathogens. Such an assay is antigen slides that contain whole *Rickettsia rickettsii* (Rocky Mountain spotted fever) organisms for detection of antibodies [24]. Because this agent shares antigenic epitopes with nonpathogenic rickettsial organisms, the presence of antibody does not confirm infection with the pathogenic agent [24]. Western blots have the highest specificity because antibody to the individual proteins of the agent, including antigens that are specific for the pathogenic species, is detected. For example, the western blot for Lyme disease can distinguish antibody to the vaccine from natural infection because of the difference in antibody profiles (vaccine: antibody to OSP A; natural infection: antibody to outer surface protein (OSP) C) [25].

IFA assay

The basis for immunofluorescence for serology is the same as for antigen detection. In this case, the antigen, usually virus-infected cells, is fixed to a glass slide. The anchored antigen "captures" the antibody that is present in the patient's serum (see Fig. 3; indirect). The bound antibody is detected with fluorescein-labeled antiglobulin. IFA assays are quantitative assays. The titer is reported as the highest dilution at which fluorescence is detected.

ELISA

The antigen in ELISAs is anchored to a membrane or plastic well. As with IFA assay the patient's antibody is captured and detected with antiglobulin, to which an enzyme is attached. Addition of the enzyme substrate leads to a color change (see Fig. 4). Most ELISAs are qualitative, rather than quantitative, assays with results reported as positive or negative. Most ELISAs are provided as kits by commercial suppliers and may be limited for diseases of exotic species. Some may be adaptable to exotic species, such as the FIV antibody ELISA, but one must recognize that these kits are not approved for use on nondomestic species and may not work consistently in all situations.

Western blot

Western blot is similar to ELISA. With western blot, however, the agent's proteins are separated on a gel before attachment to a membrane. In this way, the antibody response to specific proteins can be evaluated (Fig. 8) [22,23]. This can be helpful to confirm results of other assays. For example, FIV ELISA positive results are confirmed through western blot [8]. Because western blot detects antibody to individual pathogen proteins, it is more specific than other assays. False positive results that occur as a result of detection of antibody to nonspecific proteins are avoided. As with ELISAs, western blots do not quantitate antibody levels.
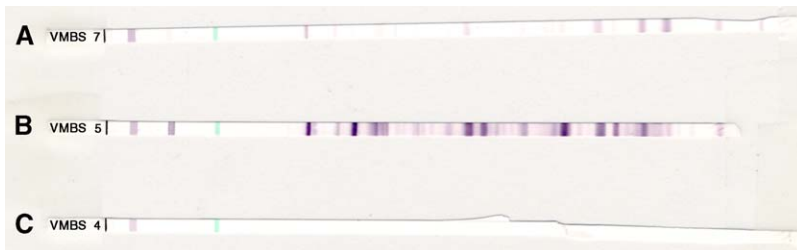

Fig. 8. Photograph of western blot for detection of antibody to Lyme disease agent. (*A*) Positive sample. (*B*) Positive control. (*C*) Negative control.

Detection of antibody-facilitated activity

Complement fixation

This assay is confined to detection of virus-specific antibodies. Although it has been invaluable in the past, it is not used commonly in most diagnostic laboratories. Formation of an immune complex by the binding of patient antibody to the virus leads to activation of the complement cascade. In the assay system, the incubation of virus with patient's serum is done with the addition of guinea pig complement. If antibody is present, the complement is activated and effectively exhausted. After the incubation, sheep RBCs, to which antibody has been attached, are added. These are, in effect, immune complexes. If the complement has been exhausted, the RBCs are not lysed by complement. If no antibody was present in the patient's serum, addition of the sensitized RBCs leads to complement activation and RBC lysis [1,3]. Most complement fixation assays quantitate the amount of antibody. Because IgM and IgG can activate complement, both will be detected. Generally, complement fixation is not used as widely as other antibody detection assays.

Agglutination/precipitation

Formation of immune complexes can be visualized through agglutination or precipitation of the complexes. In the former, the viral antigen may be attached to a substrate that can be visualized (eg, latex bead) or clumping of the organism itself may be seen [26,27]. In the latter, the formation of immune complexes leads to precipitation in an agarose medium at optimal concentrations, which can be visualized [28]. This is used commonly for detection of antibodies to caprine arthritis encephalitis virus and equine infectious anemia virus (see Fig. 6).

Detection of antibody-facilitated inhibition

Hemagglutination inhibition

This assay is used with certain viruses that have the ability to agglutinate RBCs. This assay is mediated by the surface proteins of the virus [1,3]. The patient's serum is incubated with the virus in a well. If present, binding of the patient's antibody to the virus will prevent the RBC agglutination by the virus. Therefore, the RBCs will pellet in the well. The highest dilution at which agglutination of the RBCs is inhibited is reported as the titer (Fig. 9). This assay measures antibody to epitopes on the surface protein of the virus. These proteins are under immunologic pressure for variability; thus, virus strains may vary in the antigenicity of these proteins [29]. Antibodies to highly conserved proteins (eg, polymerase or core proteins) are not detected
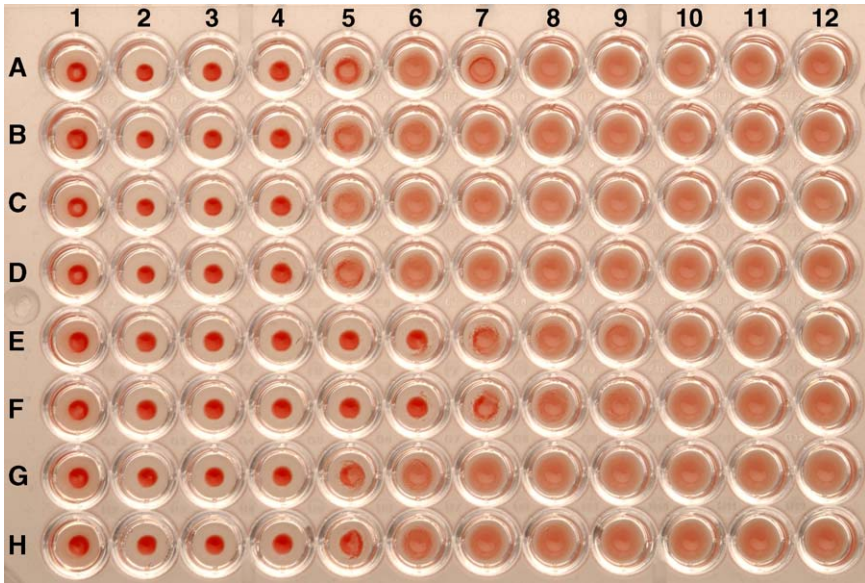
Fig. 9. Photograph of HI assay. Column 1: serum control with RBC and patient serum only. Columns 2–11: RBC, virus, patient sera in dilutions 1:20 to 1:5120. Column 12: virus control with RBC and virus only. Rows A–H: duplicates of patient serum. A/B: antibody titer of 1:80. C/D: antibody titer of 1:80. E/F: antibody titer of 1:320. G/H: antibody titer of 1:80.

by this assay. As a result, antibody that recognizes these specific epitopes on one virus strain may not recognize a different strain in this antigenic region. For example, HI is used often to measure antibodies in reptiles to OPV. We found that by using different isolates of ophidian paramyxovirus (OPV) in the assay, different antibody titers will result with the same serum because of variability of the epitopes among virus strains (unpublished data).

Serum virus neutralization

This assay is considered to be the most definitive serologic assay and may correlate with protection [1]. Binding of antibody to certain virus proteins inhibits the ability of the virus to infect a cell or neutralizes the virus. In this assay, the patient's serum is incubated with the virus, after which susceptible cells are added. If antibody is present, no infection will occur as evidenced by lack of cytopathic effects or detection of the virus in the cells by IFA assay. The absence of antibody allows the virus to infect the cells and this infection can be detected. The highest dilution at which no evidence of cellular infection (or infection of 50% of the cells) occurs is reported as the titer. As with HI, this assay commonly measures antibody to epitopes of surface proteins, and as a result, virus strain variation in these epitopes impacts the results.

Interpretation

Interpretation of serologic results can be a challenge. For quantitative assays, assigning an infection status to an animal based solely on the magnitude of a single titer is unreliable. Antibody may be absent or decreased in the acute stage of disease. Conversely, past infections with some agents lead to persistently elevated titers for significant periods of time [1]. In addition, titers vary depending upon the type of assay and capture antigen that are used. Finally, because laboratories vary in the methodology that is used, titers vary among them. Paired titers offer the most information, but are not always feasible. Communication with laboratory personnel can assist interpretation. For qualitative assays, a positive result does not correlate necessarily with active infection. Exceptions to this are those that cause lifelong infections (eg, FIV).

Summary

Selection of proper assays and appropriate interpretation of results can be a challenge to the veterinary clinician. Assays vary in methodology, sensitivity, and specificity. These assays can be invaluable in attaining the correct diagnosis, but a clear understanding of the assay and the results is essential. To this end, communication with the laboratory personnel is crucial. Optimal sample selection, shipping recommendations, assay selection, and interpretation should be discussed with the laboratory staff.

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