This article describes immunologic and molecular methods that are most commonly used at noncommercial veterinary diagnostic laboratories. Each approach has advantages in certain circumstances. Determining the best test to request ultimately depends on the case history and proper submission of appropriate specimens. When in doubt, practitioners should consult with a diagnostician at their local or regional veterinary diagnostic laboratory (see the box on page 739).

ABSTRACT:

For a busy practicing veterinarian, selecting the best diagnostic test(s) for a case can be very challenging. Moreover, knowing the principles of diagnostic procedures is important for choosing the most suitable diagnostics for a particular case. This article reviews direct approaches for diagnosing viral diseases, including several classic diagnostic methods that have been transformed by new technology, thereby improving specificity and sensitivity, turnaround times, and uniformity of results. Powerful new techniques for detecting and identifying infectious agents through advances in molecular biology are emphasized.

DIRECT DETECTION

A variety of approaches can be used for direct detection of viruses: cell culture (virus isolation), electron microscopy, fluorescent antibody (FA) testing, immunohistochemistry, ELISA, and nucleic acid testing. Virus isolation and electron microscopy can be used to identify the presence of an infectious virus or intact viral particles. FA testing, immunohistochemistry, and ELISA are conducted to provide a rapid diagnosis based on antigen detection and can be used to supplement virus isolation. Molecular-based techniques are valuable for detecting specific nucleic acid sequences in the genetic material of viruses that may be difficult or dangerous to propagate.

The primary advantage of direct detection methods is their ability to evaluate the current infection status of an animal (i.e., Is a viral pathogen present in clinically relevant specimens now?). A pitfall inherent to these methods is that the detected virus may not be the cause of the clinical disease. Nonpathogenic coronaviruses can be found in serum and tissues of cats that appear completely healthy. Travel may cause stress-induced shedding of latent, chronic, or persistent viruses in animals in which infection may not have been diagnosed. Recent vaccination with a modified-live virus vaccine can result in shedding of that virus for a period of time or sometimes establish a persistent infection. Cell culture and electron microscopy do not differentiate between natural ver-
sus vaccine strains of virus. This underscores the importance of taking a complete history from a client to determine the true cause of disease.

**Culture**

Culture of live virus from clinical specimens in cell monolayers, embryonated eggs, or experimental animals is considered the gold standard in diagnosing viral infections. Virus isolation in cell culture is the standard in evaluating nonculture tests that detect viral antigens or nucleic acids, assuming 100% specificity and sensitivity. Successful cultivation of virus in cell culture from affected tissues or body fluids is only the first step in the diagnostic process; in many cases and for many reasons, however, it is not possible to isolate a virus by culture (see box on this page). For example, it took several years to identify cell culture systems that would reliably propagate the porcine reproductive and respiratory syndrome virus (PRRSV). Many viruses have characteristic cytopathic effects (CPEs) in cell culture lines that aid diagnosticians in identifying the etiologic agent in a clinical specimen. However, neither demonstration of CPE nor histologic staining of inclusion body formations is sufficient to definitively identify a specific virus as the cause of disease. Many noncytopathic viruses can be successfully propagated in cell culture; however, as with cytopathic viruses, they can be definitively identified only with specific antibody testing, nucleic acid testing, or, in some cases, electron microscopy. Because numerous factors are involved in successfully culturing viruses from clinical samples, it is an imperfect technique for detecting all virus-containing specimens.

**Electron Microscopy**

Some viruses may be identified by electron microscopy based on size and unique structures directly from specimens that may be cytotoxic in cell culture (e.g., feces, stomach and intestinal contents, urine, body fluids). In fact, electron microscopy has been the test of choice in identifying enteric viruses, particularly parvovirus, rotavirus, coronavirus, and astrovirus, as well as herpesvirus, poxvirus, and picornavirus. When mixtures of two or more types of these enteric viruses are present, a virologist skilled in electron microscopy can easily differentiate them based on morphology. Electron microscopy can also be used to distinguish between nonenteric viruses such as cowpox (which is brick shaped) and pseudocowpox (which is oval shaped). Negative metallic stains such as phosphotungstic acid or uranyl acetate can be used to enhance the contrast between the viral structures and dark background. Most enteric viruses are excreted in large numbers early in the course of viral diarrheal disease. Sensitivity is the major limitation of electron microscopy, and at least $10^5$ to $10^7$ virus particles per milliliter must be present in the sample. Agglutinating viral preparations with specific antibodies before applying them to the electron microscope grid increases the sensitivity of this method. Few laboratories are equipped with this very expensive equipment, and the number of samples that may be prepared and

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**Limitations of Cell Culture**

- Appropriate specimens must be promptly retrieved and transported to preserve infectivity of the virus.
- Culture may yield little or no detectable viral replication when the specimen has:
  - Few viable organisms
  - Gross contamination with bacteria, fungi, or other viruses
  - Toxic substances within it
- No single universal cell culture line propagates all viruses equally well; thus stocks of many cell lines must be readily available.
- Not all viruses of diagnostic interest can be cultivated in cell culture; cell lines have not been identified or developed.
- Some viruses grow poorly in cell culture but well in embryonated hen eggs or laboratory animals.
- Virus isolation may require up to three blind passages of infective material through cell culture or eggs before detectable quantities of virus can be detected. Diagnosis may be delayed for 3 wk or longer.
- Protocols are not standardized; thus results may vary among laboratories.
inspected on a daily basis by highly trained personnel is very low (20 to 25 samples). Therefore, antigen detection methods such as ELISA are more often used to detect enteric viruses.

**Antigen Detection**

Antigen detection tests directly associate clinical signs with a virus in the specimen. Because isolating the virus is not required, most viral antigen detection assays may be conducted in as little as 10 minutes to 4 hours. Assay formats are based on using a characterized antibody coupled with a signal detection system, either a fluorochrome or an enzyme that catalyzes a chromogenic reaction that indicates the presence of specific viral antigen in a sample when the antibody reacts with its intended target. Other methods use solid particles (in lieu of stains), which form dense complexes visible to the naked eye as the means of detecting positive reactions (e.g., passive agglutination tests that use latex, immunogold, magnetic particles coated with antibodies to a specific antigen).

**ELISA**

Advances in the quality of ELISA substrates, flexibility of the ELISA test design, rapid turnaround time, and high sensitivity make ELISA for antigen detection a popular choice. The antigen-capture ELISA format (Figure 1) may be used to test a broad variety of specimens, including body fluids, secretions, excretions, scrapings, swabs, and tissues. Many commercial ELISA kits may be adapted to high throughput processing by using automated washers, optical density (OD) readers, and computerized data capture analysis for reporting test results. Small-budget laboratories that lack sophisticated OD readers can often visually judge color change against a color-coded interpretive chart that is often included with the kits. The color coding shows the relative abundance of viral antigen in the sample tested.

Most ELISA formats require 2 to 4 hours to complete and they are designed to be used by laboratories that test large numbers of specimens simultaneously. A number of rapid, self-contained ELISA kits approved by the USDA have been designed for point-of-care testing of specimens one at a time in clinician’s offices or in the field. Based on the principle of immunochromatography, the lateral-flow immunoassay format is essentially an ELISA conducted on a membrane with built-in positive and negative controls that yields test results in 5 to 30 minutes. Several commercial vendors offer rapid lateral-flow format tests for canine parvovirus, FeLV and FIV, and rotavirus. Preserving the infectivity of virus in specimens is not important for antigen detection by ELISA because dead organisms and those that have been phagocytosed and released by lysis buffer can be detected.

**Agglutination Reactions**

Viral hemagglutination, a passive agglutination reaction between the hemagglutinin (HN) molecule present on the viral capsid and corresponding HN receptors present on the membranes of erythrocytes, is a technique to detect the presence of these specific viruses in cell cultures. The hemagglutination technique is insensitive because large numbers of viral particles are required to cause visible agglutination and precipitation of erythrocytes; however, it is the basis for a more powerful and sensitive serologic technique—hemagglutination-inhibition (HAI). Reverse passive agglutination testing is a popular alternative to ELISA for detecting viral antigen, and latex agglutination testing may be one of the more familiar protocols. This qualitative or semiquantitative technique uses solid carrier particles instead of enzyme-mediated chromogen formation as the means of detecting positive reactions. Carrier particles (e.g., latex beads) are coated with antibody to specific viral antigen. The latex beads are mixed with a test specimen on a microscope slide and observed after a brief incubation of 2 minutes or less. Specimens that show particulate agglutination and precipitation observable by the naked eye are positive for the pres-
ence of viral antigen. Rotavirus detection by latex agglutination testing is nearly as sensitive as electron microscopy but less sensitive than most ELISA kits. In addition to latex beads, carrier particles for antiviral antibody include charcoal, gold, silver, gel, plastic beads, and tanned erythrocytes.

**Fluorescent Antibody Testing**

Viral structural antigens may be quickly detected in cytology preparations, tissue impressions, frozen tissue sections, and cell culture monolayers using fluorescein-conjugated monoclonal or polyclonal antibodies. Indirect immunofluorescent antibody (IFA) testing is the gold standard for diagnosing persistent infection with FeLV. Tropism of viruses for particular cell types and the appearance of inclusions and antigens in specific locations during viral replication within the cell are characteristic for each type of virus. This information is important in interpreting the test. Direct and indirect FA test protocols are illustrated in Figures 2 and 3. A quick turnaround time of approximately 2 hours or less and a relatively high specificity are the prime advantages of FA testing. However, slides must be interpreted within a few hours of preparation because the fluores-
cein label fades with repeated exposure to light. False-positive results may occur because of nonspecific binding of the FA test reagents when the preparation is from necrotic or connective tissue or mucous is present. Polyclonal fluorescent antibody conjugates have occasionally been reported to react with infectious agents other than the virus of interest.

**Immunohistochemistry**

Viral antigens may be detected in paraffin-embedded tissue sections using enzyme-conjugated antibodies. Although the concept behind the procedure is similar to FA testing of fresh tissues for antigen, immunohistochemical staining is considerably more complicated and time-consuming. Because the tissue fixation process is harsh, often causing degradation or masking of antigenic sites that are of diagnostic interest, preliminary steps are required to rehydrate the tissue, inactivate endogenous tissue enzyme activity, and retrieve the antigen of interest. After the tissues have been prepared, the immunohistochemical detection steps proceed as shown in Figure 4, which illustrates the avidin–biotin complex method using a biotinylated secondary antibody; this process is analogous to indirect IFA testing. It is also possible to carry out the avidin–biotin complex method using biotinylated antiviral antibodies in a process analogous to direct IFA testing. Figure 5 shows bovine coronavirus-infected spiral colon cells stained by immunohistochemistry. Advantages of immunohistochemistry compared with FA testing are as follows:

- Results are read by light microscopy.
- Infected cells are readily seen in relation to lesions and tissue morphology.
- Slides may be stored indefinitely for future reference.

Developing a test protocol for each antibody detection system is labor intensive, but once parameters have been determined, the process may easily be automated. Endogenous peroxidase and other enzymatic reactions characteris-
Antiviral antibodies and sample tissue
Incubate and wash

Primary antibody reaction
Incubate and wash

Antibody binds to virus-infected tissue

Fluorescence = virus-infected tissue

Secondary antibody reaction
Incubate and wash

Fluorescein antispecies globulin

**Figure 3. Indirect immunofluorescent antibody testing.** Two antibodies are used for indirect IFA testing: a primary, unlabeled antiviral antibody and a secondary, fluorescein isothiocyanate–labeled antibody generated against the immunoglobulin of the primary antibody source (e.g., if the primary antibody is raised in mice, the secondary antibody could be antimouse antibody generated in goats). Using a secondary antibody requires one additional incubation period and washing for indirect IFA testing; otherwise, the procedure is similar to that described in Figure 2. Indirect IFA testing is more sensitive than direct IFA testing; several fluorescein isothiocyanate–labeled antibodies may bind to a single antiviral antibody; thus the fluorescein signal observed on indirect IFA testing preparations is usually more intense. Interpreting indirect IFA testing slides is the same as for direct IFA testing.

Viable viral particles are not necessary for nucleic acid testing techniques to detect their presence in a specimen. For optimum results, however, it is best to handle specimens for nucleic acid–based tests with the same care and following the same guidelines as for specimens collected for cell culture or antigen testing. Nucleic acid testing
Antiviral antibodies and sample tissue

Incubate

Antibody binds to virus-infected tissue
Excess antibody is washed off after incubation

Add biotinylated antiglobulin

Binding of secondary biotinylated antibody

Add avidin–biotin complex formation

Add substrate

Chromogen precipitates on the infected tissue in the area of antibody–antigen binding

Precipitate

Figure 4. Immunohistochemical staining for antigen detection. **Step 1:** After the paraffin-embedded tissue sections have been prepared to unmask antigenic sites of interest, a primary, antiviral antibody is applied. Following an incubation period, the unbound antibody is washed from the slide. **Step 2:** A secondary, biotin-labeled, antiglobulin antibody is applied to the tissue. The slide is incubated and then washed to remove excess antibody. **Step 3:** Avidin and a preformed, biotinylated, horseradish peroxidase complex are applied to the tissue; the avidin–biotin complexes amplify the intensity of the enzyme-based detection system. **Step 4:** An appropriate substrate, usually a colorless soluble benzidine derivative, is added. **Interpretation:** Tissues that are positive for the presence of viral antigen show a colored precipitate where the specific antibody has bound. As with FA testing, knowledge of the tropism of viruses for particular cell types is necessary to properly interpret the preparations and thus avoid reporting false-positive and false-negative results.
The primary advantage of direct detection methods is their ability to evaluate the current infection status of an animal.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a method of detecting a specific characterized, 100 to 1,000 base pair fragment of genetic material. The viral genome must initially be extracted from the clinical specimen. For double-stranded DNA (dsDNA) viruses, the following are added to a single reaction tube: an aliquot of extracted DNA, two single-stranded oligonucleotide primers complementary to and flanking a specific target region of DNA, a thermostable DNA polymerase, deoxyribonucleotide triphosphates, and a buffer containing a defined solution of salts. Three steps are repeated in a series of 25 to 50 cycles (Figure 6):

- Denaturation of the dsDNA
- Annealing of the primers to complementary regions of the single-stranded DNA
- Extension of the primers in the 3’ to 5’ direction to form two new dsDNA molecules

This reaction results in exponential accumulation of amplified target dsDNA of a predetermined size. Theoretically, the copy number of DNA amplicons doubles with each cycle of the PCR: If \( n \) equals the number of PCR cycles, then \( 2^n \) fragments may be formed. Appropriately positive and negative controls are run in separate reactions each time a sample is tested. After completion of the PCR, aliquots of the amplified DNA are electrophoresed into an agarose gel next to positive and negative controls and molecular mass markers. The nucleic acid fragments are stained with ethidium bromide, which appears bright orange when exposed to ultraviolet light.

RNA viruses initially require transcription of the RNA genome into complementary DNA (cDNA), a hybrid RNA/DNA molecule, by a process called reverse transcriptase PCR. This process requires mixing the following in a reaction buffer: RNA-directed DNA polymerase (reverse transcriptase), sequence-specific oligonucleotide primers, deoxynucleotide triphosphates, and viral RNA template. A common two-step format begins with annealing the DNA primers to RNA denatured at 158°F (70°C) for a few minutes followed by standardization and quality control are important features required of any USDA-licensed diagnostic kit, and a few kits based on nucleic acid testing techniques have been approved for use in food safety testing and veterinary medicine. In an effort to harmonize molecular-based methods in veterinary diagnostic laboratories, the American Association of Veterinary Laboratory Diagnosticians (AAVLD) sponsored a workshop and compiled a manual of nucleic acid testing assays that are being used in AAVLD-accredited laboratories (see box on page 739).
extension incubation at 98.6°F to 107.6°F (37°C to 42°C) for 30 to 60 minutes. The cDNA molecule serves as a template in the PCR reaction. Although there are slight differences in extracting nucleic acids from DNA and RNA viruses and an initial reverse transcriptase PCR step is required to amplify RNA viruses, the post-PCR analysis is identical for both types of viruses.

PCR tests are becoming popular in diagnostic laboratories. When no other method of viral detection is available (i.e., no culture system has been developed for a particular virus; a class 3 virus is suspected, thus requiring use of a biosafety level 3 facility; or the sample amount is limited), PCR is the preferred test. PCR format can be used to distinguish subtypes of some viruses (e.g., bovine viral diarrhea [BVD] virus 1 and 2 and untypeable BVD virus isolates). Currently, most PCR assays are conducted when a single, specific virus is suspected to be present in the specimen. Few multiplex PCR assays have been developed that can detect more than one type of virus in a single assay. Many research and commercial laboratories are attempting to develop tests that can simultaneously identify the presence of several pathogenic viruses that are common causes of disease in specific body systems (e.g., reproductive, respiratory, or gastrointestinal PCR panels). One dilemma of using nucleic acid testing methods is avoidance of false-positive test results due to amplicon contamination of control and test specimens. Strict workflow practices must be observed in the labora-
tory to control amplicon contamination. Both the practi-
tioner, at the point of specimen collection, and the labo-
ratory technologist conducting the analysis must follow
meticulous quality-control measures. An inaccurate, mis-
leading test result is worse than not conducting any test-
ing at all. However, false-negative results may occur
when the specimen is poor quality or substances are
present that inhibit nucleic acid amplification, especially
in urine, feces, blood, sputum, saliva, milk, tears, semen,
and necrotic tissues. The cost of conducting PCR is gen-
erally two to three times that of cultural or serologic
assays, but the sensitivity and relatively shorter turn-
around time compared with cell culture followed by FA
testing can be advantageous in many situations. Depend-
ing on the PCR protocol design, testing may take as lit-
tle as 3 hours or as long as 2 days for final results.

**Restriction Fragment Length Polymorphism**

Numerous restriction endonuclease enzymes have
been characterized that cut dsDNA at short, specific,
unique sequences. Use of these enzymes to cut either
full-length genomic DNA or PCR-amplified fragments
is applied in diagnostic laboratories as a means of
authenticating the amplified PCR sequence as virus-spe-
cific or differentiating viruses and viral subtypes that
cannot be readily typed using monoclonal antibodies.
Each virus or viral subtype is cut into a given number
of fragments of a characteristic length that distinguishes it
from other viruses or subtypes; hence the name *restriction
fragment length polymorphism* (RFLP). After elec-
trophoresis of the enzyme cleaves fragments into an
agarose gel, slight differences in fragment lengths (poly-
morphisms) may be detected and compared with known
controls by staining with ethidium bromide. In veteri-
nary diagnostic laboratories, RFLP has had limited use,
but this format has been used to distinguish subtypes of
BVD (i.e., BVD1, BVD2, untyped BVD isolates), rabies
virus, and infectious bronchitis virus among others.

**Gene Sequencing**

Gene sequencing (cycle sequencing) of PCR amplifica-
tion products is an extremely sensitive technique used to
genotype viral isolates. Gene sequencing can be used to
identify minor differences between isolates that would be
missed by PCR and RFLP analysis conducted in tandem.
Only two laboratories offer the service for a very limited
number of viruses: California Animal Health Food Safety
Laboratory System identifies infectious bronchitis virus,
and Iowa State University Veterinary Diagnostic Labora-
tory identifies porcine reproductive and respiratory virus.

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**基因测序**

基因测序（循环测序）用于PCR扩增产物，是极其敏感的技术，用于测
genotype病毒隔离。基因测序可以用来识别不能通过PCR和RFLP分析
检测到的病毒亚型之间的少量差异。只有两家实验室提供服务，
用于有限数量的病毒：加州动物健康食品安全实验室系统
识别传染性支气管炎病毒，爱荷华州立大学兽医诊断实验室
识别猪繁殖与呼吸道病毒。

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1. Which statement regarding diagnosis of viral disease is true?
   a. Detecting inclusion bodies in histochemically stained cells or tissues is sufficient for a definitive diagnosis of viral disease.
   b. Serology of single serum samples, an indirect diagnostic method, is sufficient to diagnose current viral infection.
   c. Immunohistochemical staining for viral antigen can eliminate or confirm a viral cause of disease.
   d. Detecting CPE in a cell culture indicates viral infection in the test specimen.
   e. Passive hemagglutination provides a definitive diagnosis of viral disease.

2. A definitive diagnosis for feline viral rhinitis, which is caused by feline herpesvirus 1, can be made based on
   a. clinical history and signs alone.
   b. histochemical staining, revealing the presence of characteristic intranuclear inclusions in respiratory epithelial cells.
   c. cytopathic effect seen in cell culture.
   d. FA testing.
   e. a and b.

3. Which category of commercial viral antigen detection test is available for office-based testing of some viral pathogens?
   a. PCR
   b. lateral-flow immunoassay
   c. FA testing
   d. hemagglutination testing
   e. electron microscopy

4. Viral antigen is commonly detected by
   a. Western blot.
   b. histopathologic staining.
   c. ELISA.
   d. PCR.
   e. reverse transcriptase PCR.

5. The use of two antibodies is required to detect viral antigen by
   a. immunohistochemistry.
   b. complement-fixation test.
   c. indirect IFA testing.
   d. direct IFA testing.
   e. a and c

6. Which test has the quickest turnaround time for detecting viral antigen?
   a. indirect IFA testing
   b. immunohistochemistry
   c. Western blot
   d. PCR
   e. gene sequencing

7. Which statement regarding PCR is false?
   a. Only DNA may be amplified by PCR.
   b. Extremely small amounts of sample may be used.
   c. Viable virus specimens are required to conduct PCR or reverse transcriptase PCR.
   d. Test results may be obtained in 1 working day.
   e. For RNA viruses, a cDNA template must be transcribed by reverse transcriptase PCR before conducting PCR.

8. Nucleic acid testing methods may be used to detect virus in clinical specimens. Which statement is true?
   a. Sequencing PCR products is the best way to definitively identify a virus.
   b. Either PCR alone or RFLP of PCR products may be conducted to subtype viruses.
   c. DNA-dependent DNA polymerase is used to make cDNA from RNA in reverse transcriptase PCR.
   d. Each cycle of PCR results in a twofold increase of target DNA.
   e. Specimen quality is not an important factor.

9. For indirect IFA testing and immunohistochemical staining, the secondary antibody
   a. is specific to the species of animal being evaluated for disease.
   b. is specific to the species of animal from which the primary antibody was derived.
   c. is specific to the viral antigen being tested for.
   d. has a signal detection marker.
   e. b and d

10. Methods of direct detection of viral disease
    a. require collecting acute and convalescent phase serum samples.
    b. evaluate sera for the presence of antiviral antibodies.
    c. depend on culture and isolating viral pathogens.
    d. may be based on antiviral antibodies or PCR.
    e. must be conducted using only fresh tissues.