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Diagnostic Investigation of Emerging Viruses of Companion Animals

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Clinicians and laboratorians are usually the first to detect most outbreaks of emerging diseases in animals. Much attention is rightfully given to emerging diseases of commercial food animals; however, small animal practitioners also have an obligation to be vigilant to the possibility that new and devastating viral diseases might emerge that infect the companion animals in their charge. Canine parvovirus (CPV) type 2, emerged in 1978 and spread worldwide within less than 2 years [1]. In 2001, a new antigenic type, CPV-2c, was reported in Italy [2], which has since caused outbreaks in Western Europe, Asia, South America, and the United States [3] because current vaccines offer no protection for this type. In this article, the authors are specifically concerned with the timely and accurate detection of emerging diseases of small animals that are viral in origin. The term *emerging virus* is defined broadly and includes these categories:

- Variants of a known virus that has gained enhanced virulence or that is able to infect completely vaccinated animals
- A known virus that has reappeared in the population after a decline in incidence
- Novel or previously unidentified viral agents detected for the first time because of improved diagnostic capabilities
- ''Mystery diseases'' with large numbers of naive animals involved that are caused by previously uncharacterized viruses

Spread of an emerging virus among small companion animals is multifactorial and includes animal health and sanitation practices; migration of a pathogen from a wild reservoir to domestic animals because of changes in populations, trade, climate, land use, and the introduction of invasive species (eg, plant, animal, insect); and, finally, globalization, as was the case with West Nile virus (WNV). Emerging viral infections may take a heavy toll on the health of cats

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or dogs whenever they are brought into situations in which groups of animals are housed together, even temporarily, such as at greyhound racetracks, kennels, catteries, animal shelters, animal obedience training classes, dog parks, pet stores, pet day care facilities. This is especially true when pets are allowed by their owners to roam at will, commingling with ownerless feral dogs and cats and wildlife. For example, the rapid spread of CPV-2, which is extremely stable in the environment and highly contagious, was caused not only by the movement of dogs by their owners but by the transfer of fecal material on shoes and clothing of travelers and, unintentionally, through national and international mail [1].

According to the 2007 to 2008 National Pet Owners Survey conducted by the American Pet Products Manufacturers Association, the US pet cat population is estimated to be 88.3 million and the pet dog population is estimated to be 74.8 million [4]. Municipalities throughout the United States commonly pass animal control ordinances to protect the public health and safety and general welfare of the citizens and animals residing within the city. Typically, animal control codes limit the numbers of companion animals that individuals may own or keep on their private property, require that cats and dogs be licensed annually by owners and vaccinated against rabies, prevent animals from running at large, require proper disposal of animal waste, and prevent the feeding of wild or feral cats or dogs. Vaccination of dogs and cats by compliant pet owners for rabies prevention has, since 1960, dramatically reduced the occurrence of this disease; currently, most animal cases reported to the Centers for Disease Control and Prevention (CDC) now occur in wildlife [5]. Compliance with other animal control ordinances is variable, particularly among pet owners with respect to leash laws for dogs and cats and among well-intentioned individuals who maintain wild or feral colonies of cats and dogs by providing food, water, and shelter. Statistics from the Humane Society of the United States indicate that 6 to 8 million companion animals are admitted to shelters each year and nearly half are adopted or reclaimed by their owners, whereas the remaining animals are euthanized [6]. No census of ownerless dogs and cats is available. Estimates of the feral cat population in the United States range from 60 million to 100 million animals living primarily in or near urban settings with ample opportunity to interact with pets that are allowed to roam and with wildlife [7]. Thus, ownerless, wild, or feral dog and cat populations may transmit infectious and zoonotic diseases between wildlife and companion animals. From a public health standpoint, this is of particular importance because emerging viral infections from wildlife are often transmitted to human beings by means of a pet that is allowed to stray.

It is widely believed by virologists and public health epidemiologists that most viruses emerging from wildlife have an RNA or single-strand DNA genome [8] because they have a high propensity for mutation. Two significant canine viruses have emerged recently and meet this hypothesis: CPV and canine distemper virus (CDV). Canine distemper has re-emerged in the past decade [9,10] because of antigenic and genetic drift in the surface protein (H glycoprotein). In a multicontinent study, variant CDV strains, (but not

the vaccine strain of CDV virus) were the cause of illness within 2 weeks after vaccination. In 2005 and 2006, large outbreaks of CPV variants (CPV-2c and CPV-2b*) in kennels occurred in Oklahoma and other states [10]. Diagnostic and molecular studies detected mutations in the parvovirus isolates that explained the failures of current commercial CPV vaccines from conferring protection and of approved commercial diagnostic kits from detecting these new viral isolates. Another recent example is outbreaks of hemorrhagic symptoms associated with virulent feline calicivirus (FCV) in the United States [11]; however, molecular basis of gain of virulence in FCV is not yet understood. In addition to virus evolution, in some cases, the virus can be reintroduced back after the population immunity has declined after a period of disease-free status. Thus, diseases that have been eradicated from developed countries but are still circulating in developing countries [12] may re-emerge by reintroduction from trade or movement of animals.

There is a major commitment by the US Department of Agriculture (USDA) in this country and in cooperation with foreign governments and international agencies worldwide to monitor the health of food animals and certain wildlife but not of companion animals [13]. The primary mission of the CDC is to promote and protect human health. To this end, the CDC performs surveillance for noninfectious and infectious diseases, including zoonoses [14]; however, the only chosen reportable viral diseases of animals that are collected by the CDC are rabies and avian influenza (H5N1), and those that are reported to the CDC ArboNET system are avian, animal, or mosquito WNV infections. Largely, surveillance of companion animal diseases, many of which have zoonotic potential, has not been considered to be a priority until recently [15,16]. In 2004, the CDC partnered with the Purdue University School of Veterinary Medicine to establish a pilot surveillance system to monitor clinical syndromes and diseases of small animals [17] to determine whether animals can serve as sentinels of health hazards to human beings. The National Companion Animal Surveillance Program (NCASP) initially drew exclusively on the database of the privately owned organization, Banfield, the Pet Hospital, which provides medical care to approximately 1.6 million pet dogs and cats in 44 states, and it now integrates data from Antech Diagnostics to detect potential emerging and zoonotic infections. A long-term goal of the NCASP is to become a national resource in veterinary public health. In the meantime, the front line of companion animal surveillance for emerging diseases is at the home front, with astute small animal clinicians playing a major role.

It can be a challenge for busy and isolated veterinary practices to receive the information on emerging viruses. Linking to a health-related network for companion animals might fill the gap. Recently, a space-time permutation scan statistic, which was applied in the anthrax terrorist attacks in 2001 [18], WNV outbreaks [19], and enzootic raccoon rabies [20], has been applied to veterinary diagnostic data in the Unite States and Europe [21]. This analysis provides important information about potential clusters of medical conditions and issues medical alerts about the developing situations based on mortality and

confirmed diagnosis of important disease conditions. Earlier and more timely notifications should lead to more thorough investigations and reduce losses, especially from emerging viral diseases. It is important to keep in mind that clinical syndromes tend to be multifactorial, and it is essential to review the entire history, including environmental factors, with the specialist in a small animal specialty practice and also with a small animal teaching hospital before arriving at a conclusion about the case.

The purpose of this article is to encourage companion animal veterinarians to think outside the routine diagnostic plan when atypical cases of infectious disease are presented at their practices. Detecting emerging viral diseases of companion animals requires interaction and discussion among clinicians, pathologists, and virologists, and practicing small animal veterinarians must stay engaged in communication with these specialists through their state diagnostic laboratories or nearby colleges of veterinary medicine. Veterinary diagnostic medicine is rapidly progressing, and it is critical for the successful practitioner to stay abreast of new developments in small animal infectious diseases and their diagnosis through continuing education [22–24]. The development of monoclonal antibody technology in the 1980s and the advent of the polymerase chain reaction (PCR) assay in the 1990s have reshaped veterinary diagnostic strategies, especially in the subspecialty of virology. Now, these molecular techniques, which are becoming mainstream applications in routine viral diagnoses, are proving their merit in facilitating the diagnosis of emerging animal viruses. The authors offer practical information on the applications of diagnostic techniques for investigating viral disease outbreaks in companion animals. The authors provide this brief overview of diagnostic techniques in the modern virology laboratory that are used for routine diagnosis and in identifying novel and emerging viruses. Every step of diagnostic investigation–history, specimen collection, transportation, and laboratory examination–has to be carefully aligned for optimal outcome.

CLINICAL HISTORY AND SPECIMEN COLLECTION

Clinical History

Small animal clinicians are familiar with symptoms of common infectious diseases and are often the first to recognize the emergence of new disease problems. In some cases, there may be a history of vaccination compliance, yet some animals develop disease [25,26]. It is important to record the complete history, including the body system involved (eg, respiratory, gastrointestinal, reproductive tract, nervous system), clinical symptoms and their duration, the presence of lesions, and vaccination history. Particularly when the case is confounding, the client must be carefully and thoroughly interviewed as to how he or she manages the pet (ie, is the pet free to roam; has the pet traveled recently and where; if this is a new pet, where and how was it obtained; are there other pets in the household). Consulting a book on differential diagnoses can be useful to list the potential causes [27,28]. When a history of unusual symptoms is presented, clinicians, recognizing that these cases may be

important to individual and universal animal health, should refer these cases to an accredited veterinary diagnostic laboratory. It is convenient to attach copies of all relevant hospital records to the laboratory submission form to aid the diagnostician. Correct diagnosis depends on a thorough case history of the affected animal and submission of appropriate specimens that are collected and transported in a manner to preserve the integrity of the viral agent.

Specimen Collection

Submitting a comprehensive collection of specimens in a timely manner to the diagnostic laboratory from affected animals when the disease does not fit a familiar clinical picture, as is the case with emerging viral diseases, is of paramount importance. All the system(s) that are potentially involved and all the tissues with gross lesions should be sent to the diagnostic laboratory. It is important to check for concurrent infections. Viral diagnosis depends on the quality and type of specimen collected [29]. The best time for collection of specimens is immediately after symptoms of disease are first noticed. Samples from all body systems involved in the acute stage of the disease of affected animals should be submitted to the diagnostic laboratory in a timely manner by overnight delivery. At least 1 to 5 g or mL of each sample should be collected. Recovery of virus in cell culture depends on the condition of the specimen received by the diagnostic laboratory. Freezing specimens can be detrimental to virus isolation efforts (and also to electron microscopic identification) and should only be done (−70°C) if it is not possible to deliver the specimen to the laboratory within 48 hours. Use wet ice for shipping virology samples, because dry ice (solid carbon dioxide gas) can inactivate many viruses, preventing isolation in cell culture. Tissues intended for virus isolation should always be shipped in separate packages from specimens that are immersed in formalin to prevent fumes of formaldehyde from reaching the fresh tissues.

It is imperative that tissues and organs from animals that have died be harvested as soon as possible after death. Postmortem tissues should be placed in sterile containers with a small amount of transport medium (1–2 mL), if possible. When the clinician is unsure as to what specific organs and fluids should be retrieved, the entire carcass of the dog or cat may be delivered to the laboratory for examination. To obtain more specific details regarding specimen collection, packaging, and submission, contact the diagnostic laboratory of your choice by telephone or consult its specimen submission and fee schedule guidelines, which are often available on an Internet Web site.

Individuals who ship biologic substances for diagnostic testing are required by federal law to be in compliance with all regulations governing packaging and labeling of interstate shipments of causative agents. Failure to follow the regulations results in heavy fines (Fig. 1). Complete instructions on appropriate packaging for laboratory specimens to be mailed or shipped by a common carrier may be accessed in several sections of the Code of Federal Regulations (CFR). Health and Human Service regulations define such terms as *diagnostic specimen* and *etiologic agent* and describe requirements for packaging and labeling

Fig. 1. Improper packaging of clinical samples. This submission is unsuitable because no ice packs were used. Instead, Styrofoam peanuts were added with wooden shavings. These packing materials can be a source of contamination and do not provide any advantage. Recycled food containers are unsuitable because they are a source of food microorganisms.

of these materials for shipping in Title 42 CFR Part 72. Department of Transportation regulations for shipping and packaging are found in Title 49 CFR Part 173, including definitions of infectious substances (49 CFR 173.134) and requirements concerning shipments containing dry ice (49 CFR 173.217). Regulations for airline shipments of dangerous goods are also available through the International Air Transport Association (IATA) [30]. The US Postal Service and most commercial delivery services (eg, United Parcel Service [UPS]; Federal Express [FedEx]; and Dalsey, Hillblom, Lynn [DHL]) provide packing information on request.

LABORATORY METHODS

Viruses have a simple structure with a protein coat enclosed with only one type of nucleic acid (DNA or RNA) rather than both. Thus, methods for viral diagnosis target one of the components of the virus structure. For a definitive viral disease diagnosis, four basic approaches are used: direct detection by virus isolation or direct identification, viral serology for detection of a specific antibody, viral antigen detection, and molecular-based detection of genetic material. A brief discussion of the principles of diagnostic assays representative of each approach follows.

Gross Pathologic and Histopathologic Findings

Histologic (Fig. 2) and cytologic examination (Fig. 3) of tissues and fluids by a board-certified veterinary pathologist contributes valuable information about the pathologic signs, gross and microscopic, that distinguish infections caused by viral or bacterial pathogens and other possible etiologies. Tissue tropism, mononuclear infiltrates, development of inclusion bodies (intranuclear, cytoplasmic, or both), and the formation of syncytia are some of the characteristics that differ among viruses and can sometimes distinguish different viral infections. For example, most DNA viruses replicate in the nucleus, and thus

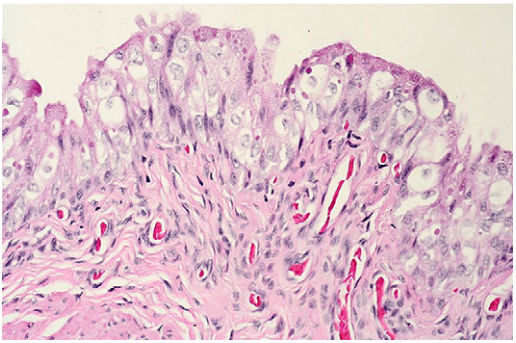

Fig. 2. Section of bladder from a dog with CDV. Eosinophilic inclusion bodies are present in the bladder epithelium. (*Courtesy of* Gregory Campbell, DVM, MS, PhD, Stillwater, OK.)

tend to produce intranuclear inclusions, whereas most RNA viruses form cytoplasmic inclusions, although there are exceptions. As part of the pathologist's examination, immunohistochemistry testing (Figs. 4 and 5), fluorescent antibody testing, and possibly in situ hybridization (ISH) studies on tissues may be ordered; these methods are considered elsewhere in this article. A complete histopathology report should include possible differentials for the lesions. The pathologist might note that some findings do not exactly fit the routine lesions he or she has observed in previously. In cases in which there are deviations in lesion type or distribution or when gross lesions and histopathologic findings

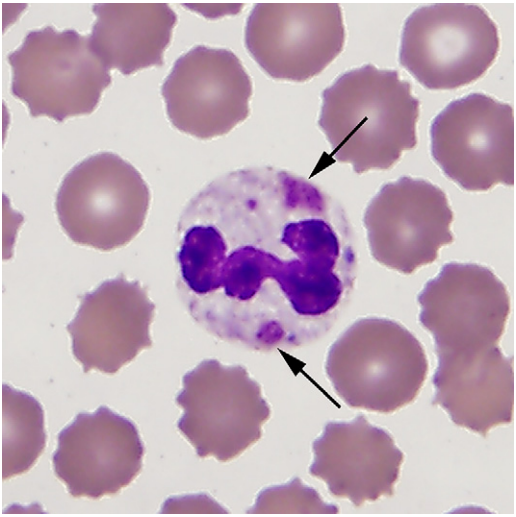

Fig. 3. Blood smear stained with aqueous Romanowsky stain shows intracytoplasmic inclusion bodies (*arrows*) confirmed to be positive for CDV.

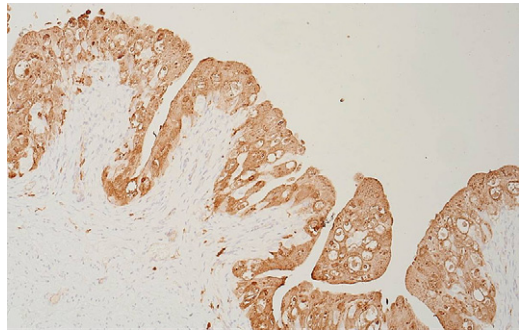

Fig. 4. Immunoperoxidase staining for CDV in the bladder of a dog. (*Courtesy of* Gregory Campbell, DVM, MS, PhD, Stillwater, OK.)

suggest the involvement of a viral disease but routine virology tests do not detect the expected conventional viral agents, variant or "emerging" viruses or even iatrogenic infections may be suspected. In early 1990, blue tongue virus serotype 11 was introduced in canine populations from a commercial modified-live multivalent canine vaccine that was associated with high mortality in dogs [31,32]. In some situations, second or even third opinions from pathologists at other laboratories who have special expertise should be solicited [33]. With the application of telepathology to veterinary case materials, networks of specialists, including veterinary pathologists, small animal clinicians, infectious disease specialists, and laboratory diagnosticians, are able to exchange patient histories, clinical data, and images (gross and microscopic) through the Internet for consultation, diagnosis, and education. This allows timely access to expert opinions at other locations throughout the world [34,35]. The use of telepathology can facilitate rapid intervention through the synergy of

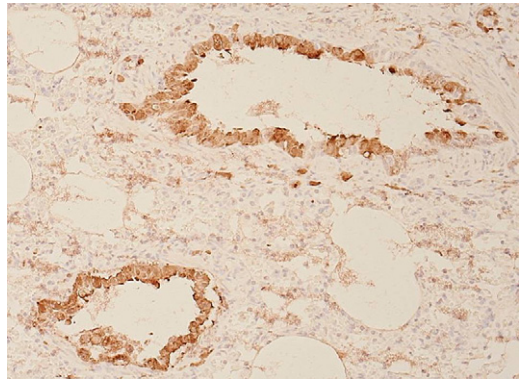

Fig. 5. Immunoperoxidase staining of a section of lung. The bronchiolar epithelium is positive for CDV antigen. (*Courtesy of* Gregory Campbell, DVM, MS, PhD, Stillwater, OK.)

computer technology and special pathology expertise (eg, system- and species-specific pathologic findings) to understand the lesions in difficult cases better.

DIRECT DETECTION

Virus Isolation

Conventional virus isolation techniques are often the backbone of investigation of novel viral diseases, provided that the virus is cultivable in available cell lines or primary cell cultures. Virus isolation may be relatively slow depending on the growth characteristics of the virus; however, roller culturing or centrifugation of samples onto cell monolayer(s) can enhance viral replication and recovery. In many of the recent emerging viruses from wildlife (eg, bats), the virus was first cultivated, allowing further characterization of the virus. It is important to keep in mind that virus isolation, even if the effort is successful, may have a slow turn-around time, approximately 2 to 3 weeks. Definitive identification of virus in cell culture can only be accomplished with specific antibody nucleic acid testing, and in the case of an "emerging" virus, existing reagents may not be reactive with the "new" virus. If culture is successful, however, the viral material may be studied by electron microscopy (EM) and by molecular techniques, as described in this article, to characterize the new isolate. Virus isolation requires fresh tissues and cannot be done on formalin-fixed tissues.

Physical and Chemical Methods That Aid in Identification of Viruses

EM is often used in veterinary diagnostic laboratories to detect enteric viruses in fecal samples retrieved during the course of viral diarrheal disease. Additionally, EM is indispensable for identification of emerging and previously unidentified viruses in clinical samples [36], and this method has helped in the identification of many new viruses, including, most recently, bat Lyssavirus [37]. Viruses can be classified up to the virus family based on size, shape, and distinctive structural features, such as envelopes or protein spikes, particularly for parvovirus, rotavirus (Fig. 6), coronavirus, astrovirus, herpesvirus,

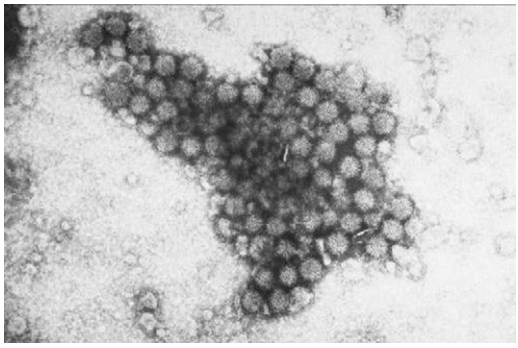

Fig. 6. Detection of rotavirus particles by EM. Most virus particles are similar in size and shape. The picture shows a few empty rotavirus particles.

poxvirus, and picornavirus. EM allows detection of multiple viruses simultaneously. Application of antibodies to supplement the EM diagnosis provides higher sensitivity and further confirmation of the viral diagnosis. Sensitivity is the major limitation of EM, and at least 10^5 to 10^7 virus particles per milliliter must be present in the sample being examined. Because the electron microscope is an expensive piece of equipment that requires special technical skills and a high level of expertise, it is not available in many laboratories. Viral components can also be determined by several basic biochemistry experiments.

Acridine orange (AO) staining can determine the nature of the nucleic acid of purified viral particles [38]. Differentiation as to whether the nucleic acid is single- or double-stranded in nature is based on the color developed on AO staining; double-stranded DNA or RNA nucleic acids stain yellow green, whereas single-stranded DNA or RNA acids stain flame red. Nuclease susceptibility of the purified virions differentiates DNA from RNA. The presence of envelope on viruses can be determined by susceptibility to the virus to heat, ether, or other lipid solvents [39]. The titrated virus preparation is treated with ether or chloroform. A decrease in virus titer of greater than 1 log is considered to be significant to indicate the presence of envelope on the virus. The presence of envelope indicates that virus is susceptible to common disinfectants. Lack of envelope indicates that the virus is resistant to the use of common disinfectants.

ANTIBODY DETECTION METHODS

Serology

Classic serology tests indirectly determine the viral etiology of disease by detecting the presence of antibody in serum (red-topped tube) to a specific test viral antigen, and thus provide retrospective evidence of an immune response or exposure to a virus. Serologic methods still provide powerful tools in the virology laboratory of today for diagnosing viral diseases that are seen routinely and for discovering and characterizing novel viral diseases. Serologic tests are now used to detect antibody or antigen in serum and body fluids. Typically, methods used in the virology laboratory are serum neutralization (SN), hemagglutination-inhibition (HAI) test, indirect fluorescent antibody test (IFAT), and ELISA. Serologic results require interpretation by an expert diagnostician based on critical clinical observations, confirmation by pathology examination, virus isolation, and mass screening of the populations by serology. If animals in populations that have never been exposed to or vaccinated against a given virus have specific antibodies detected in their serum, it is expected that this is most likely attributable to recent exposure to the emerging virus. Paired serum samples are important to demonstrate a fourfold significant increase in antibody titers, which indicates that the diagnosis of recent exposure may be attributable to infection as opposed to previous exposure or vaccination depending on the vaccination history. Serology is also useful to study the antigenic distance of the emerging virus and provides clues as to whether the newly emerged agent is or is not likely to be protected by an available vaccine(s), such as heterologous virus in another species of animal.

Hemagglutination Inhibition

Viral hemagglutination (HA) occurs between the viral protein; hemagglutinin (HN), which is present on the viral capsid or envelope of only certain families of viruses; and specific receptors on red blood cells (RBCs) that bind to HN, causing their agglutination and precipitation from solution. This phenomenon is the basis for a powerful and sensitive assay, the HAI test. When a hemagglutinating virus is mixed with serum containing antibodies specific to that virus, RBCs that are added to the mixture do not agglutinate and precipitate from solution. Feline panleukopenia, CPV, influenza A, and parainfluenza antibodies may be detected by HAI testing. The HAI method may also be used to identify unknown virus utilizing antibodies of known specificity; however, most often, this test is applied to detect the presence of antibodies in a serum sample against specific hemagglutinating viruses. Variants of CPV and feline parvovirus can differ in the hemagglutinating activity of swine erythrocytes [40,41].

Serum Neutralization

SN measures the inhibitory activity of a hyperimmune serum against viral isolates in cell culture. Commonly performed in a cell culture microwell format, this is a long-standing method for quantifying virus-specific antibodies, and it is usually performed to test for antibodies to viruses that typically cause cell damage (cytopathic effect [CPE]) to the host cell culture they infect. When a virus is mixed with hyperimmune serum containing antibodies specific to that virus, the antibodies bind the virus, preventing infection of the cell culture. The SN test can diagnose current infection using acute and convalescent serum samples from individual animals. It may also be used to determine immune status conferred on vaccinated animals. Vaccination antibody titers often differ from antibody titers developed in response to natural infection. Usually, vaccination titers are lower relative to infection titers, and maximal titers occur approximately 21 to 30 days after vaccination. SN assays are commonly performed to detect antibodies to FCV, herpesvirus, enteric coronavirus, and syncytial viruses and to canine herpesvirus, CDV, coronavirus, parainfluenza virus, and adenovirus.

ELISA

This is useful for screening large numbers of samples for the presence of antibodies against viruses. The ELISA format is flexible, and it may be used to detect antibody or antigen in clinical specimens. In either case, the detection system is an antibody conjugated to an enzyme. When the enzyme-linked antibody binds to the analyte being measured, the enzyme reacts with a chromogenic substrate, causing a color change to occur that may be measured spectrophotometrically or evaluated visually. Several ELISA kits are available to detect antiviral antibodies in companion animals, including CPV and CDV, feline leukemia virus (FeLV), feline immunodeficiency virus (FIV), and feline coronavirus. The immunoglobulin M (IgM) ELISA is a method used to distinguish current infection from past infection. During acute disease or immediately after vaccination with modified-live viruses, IgM is the first class of

immunoglobulin produced in response to infection, appearing 1 to 2 weeks before there are detectable levels of IgG in the serum. Because it is short-lived, IgM levels typically disappear 3 months after infection. A single acute-phase serum test sample is sufficient to diagnose current infection with an IgM ELISA. Testing of IgM titers is available for several viral agents, including CDV and CPV among others. ELISA is useful for screening naive animal populations for the presence of antibodies against viruses to track the origin and spread of emerging infections. Antibodies to WNV have recently been detected in dogs and cats by IgM-capture ELISA [42]. A related method known as virus neutralization can be used to identify the serotype of a newly discovered virus.

Western Blot Assay (Immunoblot Assay)

Western blot (WB) may be used as a supplementary test to confirm antibody ELISA results for FIV testing [43]. To perform the assay, purified virus is disrupted using detergent; the constituent proteins are then separated on the basis of molecular weight by electrophoresis in a polyacrylamide gel. The proteins are transferred (blotted) from the gel to a nitrocellulose or polytetrafluoroethylene (PTFE) membrane for stabilization. The electrophoretically separated proteins are the antigen substrates for analyzing the test sera for the presence of specific antibodies. As with the ELISA format, the Western immunoblot uses an enzyme-labeled antispecies antibody that binds to the test serum antibodies that have bound to the separated viral antigens. Substrate reacting with the enzyme-labeled antibody in the presence of a colorless soluble benzidine derivative results in conversion to colored insoluble precipitate at the protein bands where test serum antibodies are bound. The molecular weight of the protein detected is characteristic for a particular viral component. Immunoblot results of the unknown test antisera are compared with positive control test sera for interpretation. A major advantage of the immunoblot technique is that a full antibody profile of a single serum sample is made simultaneously, identifying each of the individual particulate viral antigens that patient antibodies bind. As an epidemiologic tool, WB analysis may be used to detect currently circulating viral subtypes within a population and to characterize new emerging viral subtypes. Immunoblotting is also a valuable research technique for antigen detection that is often used to characterize novel viruses by comparing them with known related viral family members using standard antisera or monoclonal antibodies.

ANTIGEN DETECTION METHODS

Immunofluorescence Assays

Immunofluorescence assays on cells from clinical samples can be applied for rapid diagnostic investigations (30–45 minutes), provided that the fluorescent microscope and expertise are available in a laboratory. With the pooling of primary monoclonal antibodies against potential viral agents, the assay can be used as a screening tool and the sample tested again with individual conjugates to obtain specific virus diagnosis (Fig. 7).

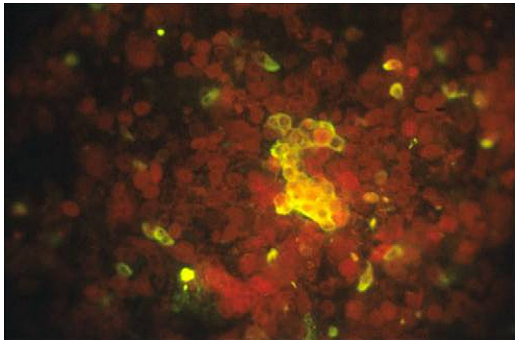

Fig. 7. Direct fluorescent antibody test. Cells show intracytoplasmic staining for coronavirus multiplying in the nasal cells. The negative cells stain brick red. The positive cells stain apple green.

ELISA for Antigen Detection

The ELISA is also a means for detecting viral antigens present in clinical specimens, and it offers a relatively quick turn-around time. Antigen test ELISA kits are available to detect antiviral antigens in companion animals, including CPV, FeLV, and FIV. Additionally, it is a common practice by many veterinary diagnostic laboratories to appropriate the use of some rapid antigen test kits intended for the human diagnostic market, specifically, rotavirus test kits. When monoclonal antibodies are used as capture antibodies in ELISA test kits, however, they fail if there is a mutation in the epitope of the viral surface protein present in the specimen that is being tested. Lateral flow immunoassay is a special application of the ELISA that provides a rapid, economic, portable, sensitive, and specific technique that is convenient for performing testing outside of the laboratory. It is the technique of choice for emerging viral infections [44,45], and it has gained attention for use in diagnosing foreign animal diseases and zoonotic and emerging viral infections of animals, such as influenza virus and WNV, in the field. The test kits are small in size (size of credit cards), extremely stable at ambient temperature (25°C), and take minutes to perform.

MOLECULAR-BASED METHODS

An advantage of nucleic acid–based testing is that specimens submitted for analysis do not have to have viable viral particles present to be detected by this means. There is a trend toward application of molecular or gene sequence–based techniques to routine virology testing in diagnostic laboratories, which is justified under several circumstances. First, a molecular technique may be the test of choice if conventional methods of diagnosis are technically weak, such as when a viral agent is noncultivable or there are biocontainment concerns with culturing the virus, the virus has amorphous morphology by EM, antibodies are unavailable or not specific to the virus, and serologic tests result in a confounding diagnosis. Second, molecular techniques may be essential to

detect and classify the sequence type or genotype of a virus. Third, a viral agent may be characteristically slow to replicate, such as γ-herpes virus; thus, a molecular method might provide a better turn-around time for diagnosis. In this instance, a rapid diagnosis might be achieved by pan-herpesvirus PCR. Finally, a novel viral isolate that cannot be definitively identified by the routine diagnostic methods described previously may merit investigation and characterization by molecular-based techniques, which are indispensable in the classification of new and emerging viruses. These advanced techniques may confirm a diagnosis of viral etiology when other tests have failed; however, they are, unfortunately, relatively expensive. Furthermore, the presence of nucleic acid does not equate to infection, and infections are attributable to subclinical, latency-associated nucleic acids or defective interfering virus particles, such as in paramyxoviruses, produced in nonproductive infections in genetically resistant hosts. Clients, who bear the financial burden, should be counseled as to the benefit and shortfalls of this testing before ordering molecular-based tests. An excellent review of molecular-based techniques for diagnostic testing of infectious diseases has appeared in a previous issue in this series [46].

Polymerase Chain Reaction

The most familiar nucleic acid testing technique, PCR, has been used for more than a decade; however, over the past few years, real-time PCR has taken its place, revolutionizing diagnostic virology. In this procedure, the PCR chemistry may be combined with detection using a single-stranded DNA probe with a fluorescent label [47]. Moreover, the procedure may be completed within an hour, and it allows for quantitation of results. Because the hands-on steps are reduced and the PCR reactions are not opened, it eliminates the chances of cross-contamination in the laboratory. Real-time PCR protocols are gaining more acceptance in routine veterinary diagnosis.

In Situ Hybridization

ISH involves using nucleotide probes with an attached label. Non–isotope-labeled probes (digoxigenin or fluochrome) can be applied in veterinary diagnostic laboratories. Diagnostic applications of ISH involve identification of virus-specific sequences (DNA or RNA) in the tissues or cells [48]. Although uncommon in veterinary diagnostic laboratories, ISH is in routine use in human diagnostic laboratories for detection of the genotype of human papilloma viruses in cervical samples. For ISH, smears and tissues (fresh, unfrozen, and fixed tissues) are suitable.

Electropherotyping and Restriction Fragment Length Polymorphism

In electropherotyping and restriction fragment length polymorphism (RFLP), double-stranded DNA (RFLP) or RNA (electropherotypes) is purified and size-separated on agarose or acrylamide gel electrophoresis. Because nucleic acids are charged and double-stranded molecules bind more ethidium bromide compared with single-stranded nucleic acids, under the electric field, the nucleic acids migrate and larger sized molecules separate out higher than smaller sized

molecules. For DNA molecules to be tested, the double-stranded viral DNA- or PCR-amplified fragments are digested with restriction enzymes. These techniques allow quick differentiation of viral genomes (DNA or RNA). Both techniques have applications in molecular epidemiology of rotaviruses [49].

NEW GENERATION MOLECULAR TECHNIQUES

Viral Genome Sequencing Technologies

Viral genome or mRNA sequencing is a powerful molecular epidemiologic tool and has been applied for epidemiology of rabies virus [50]. Sequences of novel or emerging viruses may be derived based on known conserved sequences of previously characterized viruses within the same family. Although virus sequencing is gaining more routine application in veterinary laboratories, it does add cost, and thus should be used judiciously. When these methods fail to identify a newly discovered virus, which is truly novel, metagenomic analysis, which is largely used in research laboratories, may be applied. Pyrosequencing is a recent variation on sequencing short stretches of PCR-generated DNA without the need for labeled primers, labeled nucleotides, and gel electrophoresis [51]. Although this variation on PCR and nucleic acid sequencing is currently used exclusively as a research tool, it is likely to be adapted for clinical diagnostic work in future years because it has been demonstrated to detect many different unrelated viruses simultaneously in a single reaction and to identify viral serotypes and detect viral isolates that could not previously be typed by classic procedures [52,53].

Microarray Platform

A biochip or microarray is small solid support, such as a nylon membrane, silicon chip, or glass slide, on which nucleic acid fragments, antibodies, or proteins are immobilized in an orderly arrangement. Thousands of different molecules, referred to as probes, may be machine-printed as spots on the support, allowing for high throughput of samples using lower volumes of analyte in less time than conventional laboratory techniques take to complete. Microarrays are essentially miniaturized laboratories that can perform hundreds or thousands of simultaneous biochemical reactions that are most commonly detected through the use of fluorophores. The fluorescent signal patterns formed by each analyte are then compared by the computer software using complex algorithms to make an identification of its contents. Biochips enable researchers to screen large numbers of biologic analytes quickly for a variety of purposes, ranging from disease diagnosis to detection of bioterrorism agents. Biochip technology is still relatively new and has not yet entered the mainstream of clinical diagnostics techniques, although it is widely used in research institutions. As an epidemiologic tool, the use of nucleic acid microarrays was instrumental in the rapid identification of the first severe acute respiratory syndrome (SARS) coronavirus outbreak in China [54]. Coronavirus protein microarrays have been used to screen Canadian sera [55] for specific antibodies to SARS and to other coronaviruses in a comparative study with the traditional ELISA.

Scientists around the world are assessing the feasibility of using microarrays as tools for surveillance and diagnosis of influenza viruses [56,57]. Once issues of sensitivity and assay validation have been addressed satisfactorily and the cost of the technology has become more affordable, microarray technology may find a place in clinical diagnosis.

ESTABLISHING VIRAL DISEASE CAUSATION

Pathogenic Virus or "Orphan" Virus or "Vaccine-Source" Virus

Molecular methods for detecting and identifying viral pathogens are powerful. It is possible to detect a virus in a specimen, but it may have no association with the clinical condition. These types of viruses are called "orphan viruses." Minute virus of canine is a parvovirus, and it causes no clinical disease [58]. As a result of the advent of sensitive molecular techniques, it is quite common to detect viral sequences of agents that may be present in a sample but not associated with the disease (orphan viral agents). It is possible to study the association of the viral agent with the pathologic findings observed to support the diagnosis. Moreover, the PCR protocols targeting structural genes that are expressed only during active infection are useful and avoid the potential false-positive results attributable to latency or persistent viral infections. Moreover, the sense and antisense probes offer the opportunity for resident and replication intermediates of viruses. Obviously, the history of recent vaccination should be known, and the vaccine virus from the same lot of vaccine should be simultaneously included in the testing run and sequenced over critical regions to ensure that the virus in the sample is the same or different from the vaccine.

Failure or Lack of Correlation Between Diagnostic Techniques

When fluorescent antibody testing or immunohistochemistry testing is performed, false-negative findings result even when a related virus is present. Because of changes in the sequence of the target protein epitopes, antibody-based detection methods may fail to provide the diagnosis; monoclonal antibodies used may fail to react and polyclonal antibodies may cross-react weakly when a variant strain of virus is present. Thus, a sudden trend in lack of correlation between tests may signal an emerging variant of the virus. If a new variant of the virus arises, it may be associated with a change in the clinical profile and we may or may not understand the molecular basis of this shift. It is possible that the polyclonal antibodies may react weakly with the new variant of the virus. In many cases, the PCR primers may fail to amplify the new variant if the mutation occurs in the hypervariable region of the target gene amplified. For example, in the recent emergence of CPV variants, many practitioners noted clinical symptoms compatible with CPV but the commercial field tests were not working. If a new variant of virus emerges, a polyclonal antibody antiserum prepared in a heterologous species (rabbit or goat) can be used as a primary antibody against the whole virus, because it is possible that the monoclonal antibody might fail. The molecular techniques are more likely to fail compared with the antibody-based techniques because of the

degeneracy of codons. It is important to keep in mind that factors other than emerging viruses can also affect the performance of USDA-approved tests. For example, local anesthetic can also affect the outcome of antibody tests. In one study, the use of lidocaine was recommended over oxybuprocaine to avoid false-positive results [59].

SUMMARY

It should be clear to the readers that veterinarians are bound to encounter emerging viruses in their practice. The problem is unavoidable because viruses are "perfect" obligate parasites. Even the immune response dictates the nature of virus that evolves in a host. Thus, vaccines are to be viewed as preventive tools rather than as a cure for emerging viruses. In some situations, the best vaccine is bound to fail. Similarly, the diagnostic methods have to be tailor-fitted to keep up with the emerging viruses. If the clinical signs and diagnostic methods fail to correlate, the veterinarian should work with diagnostic laboratory to solve the diagnostic puzzle. Your state veterinary diagnostic laboratory may be the first place that issues an alert to veterinary professionals and the public at large to possible emerging viral diseases. Newsletters from your state diagnostic laboratory can be a good source of information about emerging viral diseases in your area. Additional sources that are dedicated to dog and cat health issues and public health are available on the Internet [60–68].

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