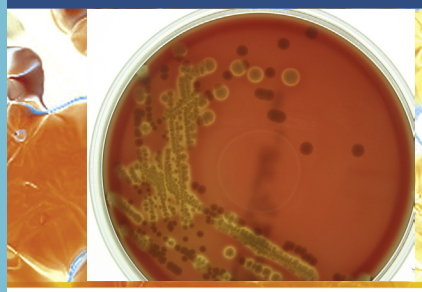
Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.



SECTION 1

Laboratory Diagnosis of Canine and Feline Infectious Diseases

Jane E. Sykes and Shelley C. Rankin

CHAPTER 1

Isolation in Cell Culture

Jane E. Sykes and Shelley C. Rankin

KEY POINTS

- With the increasing availability of nucleic acid–based testing, cell culture is decreasingly used for diagnosis of infections caused by obligate intracellular pathogens in dogs and cats.
- Cell culture remains an important technique for (a) confirmation of a diagnosis when the results of molecular testing or serology are unavailable or equivocal; (b) pathogen discovery; and (c) vaccine manufacture. For some pathogens, cell culture is the most sensitive and specific method for organism detection.
- Before collection of specimens, veterinary clinicians should communicate with the laboratory that is to perform the culture to discuss the patient signalment, history, immune status, travel history, nature of the suspected infection, and number of animals affected.
- Specimens are inoculated onto monolayers, and the infecting organism is identified based on the presence of characteristic cytopathic effect after a predictable incubation period, with or without confirmatory antigen staining, electron microscopy, or nucleic acid testing.
- False-negative results may occur as a result of inadequate specimen collection, deterioration of organisms during transport, or culture contamination with bacteria or fungi.
- Positive results do not imply that the organism detected is the cause of an animal's signs, because some organisms can be present without causing disease. This is especially the case for animals with respiratory or gastrointestinal disease.

INTRODUCTION

Cell culture refers to the culture of nucleated (eukaryotic) cells under controlled conditions within the laboratory. Infectious agents that require living host cells for replication can only be isolated in cell culture. With the advent of molecular diagnostic assays based on nucleic acid detection, cell culture is being used less often for routine clinical diagnostic purposes, because of the long turnaround times (days to weeks), cost, and requirement for significant technical expertise to perform cell culture and interpret results (Table 1-1). Nevertheless, isolation of viral and intracellular bacterial and protozoal pathogens in cell culture remains an important technique for the discovery of new pathogens, identification of organisms involved in disease when the results of molecular testing or serology are unavailable or equivocal, the propagation of isolates for research purposes, the generation of organisms for vaccination purposes, and the establishment of the efficacy of novel antimicrobial drugs. Vaccines for dogs and cats that are propagated in cell culture include those for canine distemper, canine adenovirus infections, parvovirus infections, rabies, and feline viral and chlamydial respiratory tract disease. Veterinary clinicians should remain aware of situations where cell culture may be the best technique to identify the presence of an infectious agent and the optimum methods for collection and submission of specimens. Knowledge of cell culture methods can help veterinary clinicians to submit the optimum specimens and to understand laboratory turnaround times, potential complications, and how to interpret results.

Specimen Collection and Transport

Although cell culture can be used to propagate intracellular bacteria and protozoa, it is most often used by clinicians for the diagnosis of viral infections. Active communication between the clinician and the laboratory that performs viral isolation is recommended. Successful detection of viruses is highly dependent on (a) collecting the appropriate specimens, (b) the timing of specimen collection, and (c) rapid and proper specimen

TABLE 1-1

Alternatives to Cell Culture for Diagnosis of Obligate Intracellular Pathogens That Infect Dogs and Cats

System Affected and Most Common Agents	Other Diagnostic Tests Available
Respiratory Tract	
Canine respiratory coronavirus	RT-PCR
Canine adenovirus-2	PCR
Influenza viruses	RT-PCR, antibody detection
Canine parainfluenza virus	RT-PCR
Canine distemper virus	RT-PCR, antigen detection using IFA
Canine herpesvirus	PCR
Feline herpesvirus-1	PCR, IHC
Feline calicivirus	RT-PCR, IHC
Eye	
Chlamydia felis	PCR
Central Nervous System	
Canine distemper virus	RT-PCR, direct IFA, CSF antibody detection
West Nile virus	RT-PCR
Encephalitis viruses	RT-PCR
Gastrointestinal Tract	
Coronaviruses	PCR
Canine distemper virus	PCR, direct IFA/IHC
Canine and feline parvovirus	PCR, IHC
Genital	
Canine herpesvirus	PCR
Congenital and Perinatal	
Canine herpesvirus	PCR
Feline herpesvirus-1	PCR
Blood	
Feline leukemia virus	PCR, antigen detection
Feline immunodeficiency virus	PCR, antibody detection
Anaplasma phagocytophilum	PCR, antibody detection
Rickettsia rickettsii	PCR, direct IFA on skin biopsies, antibody detection
Ehrlichia canis	PCR, antibody detection

CSF, Cerebrospinal fluid; IFA, fluorescent antibody; IHC, immunohistochemistry; PCR, polymerase chain reaction; RT, reverse transcriptase.

transport and processing. Thus the actions of the veterinary clinician play a critical role in ensuring positive test results when a virus is present.

The clinician should discuss with the laboratory what types of viruses are suspected in light of the animal's clinical presentation. The patient signalment, history, clinical signs, immune status, travel history, and number of animals affected should be discussed to generate conclusions regarding the nature of the suspected infection (Box 1-1). Some viruses, such as feline coronavirus (FCoV), are difficult to isolate in cell culture or grow slowly, whereas others, such as feline calicivirus (FCV), replicate readily and rapidly in cell culture, and the sensitivity of cell culture is high. Viruses differ in respect to the cell type they prefer to replicate within. As a result, specimens should be sent to the laboratory with information on the specific viruses that are suspected.

BOX 1-1

Factors That Should Be Discussed with the Laboratory before Collection of Specimens for Pathogen Isolation in Cell Culture

Patient species, breed, age, and environment
Number of animals affected
History and clinical signs
Immune status of the patient
Geographic location and travel history
Suspected infectious agents
Timing of specimen collection
Type and amount of specimen to be collected
Transport conditions, including timing and method of transportation

The timing of specimen collection is particularly important for viral infections. Specimens should be collected as early as possible following the onset of clinical signs, optimally within the first week, because viral shedding may commence before the onset of signs and continue for only a few days. The duration of viral shedding depends on the type of virus and the anatomic site sampled. When multiple animals are affected, collection of specimens from more than one animal may increase the chance that an isolate will be obtained. If possible, antibody testing using acute and convalescent phase serology should be performed concurrently to help confirm the diagnosis (see Chapter 2).

Selection of the best specimen and collection site for culture is optimized based on knowledge of the pathogenesis of the infectious agent involved, because the optimum specimen collection site may not be the site where clinical signs are most severe. Attempts should be made during specimen collection to prevent contamination of the specimen with normal flora, although this is not always possible. Specimen size should also be maximized (for example, at least 5 mL of blood, body fluids, or lavage specimens, and ideally 8 to 10 mL of blood) to increase the chance of a positive isolation. In general, nasal or nasopharyngeal washes have been preferred over nasal swabs in human patients for isolation of respiratory viruses, but one study showed that nasal swab specimens were just as sensitive as nasopharyngeal washes for isolation of most respiratory viruses.[1] Nasal or oropharyngeal swab specimens are collected by placing a long-shafted swab in the area to be sampled, rotating the swab against the mucosa, and allowing the secretions to be absorbed for approximately 5 to 10 seconds.

Swabs and small tissue specimens for virus isolation should be placed in buffered virus transport medium, which contains antibiotics and protein. This can be obtained from the laboratory or purchased from other commercial sources. It is important that the medium used has not reached its expiry date.

TABLE 1-2

Specimen Collection Guide for Diagnosis of Viral and Intracellular Bacterial Infections of Companion Animals

System Affected	Possible Agents	Specimen Type
Respiratory tract	Dogs: coronaviruses, canine adenovirus, influenza viruses, parainfluenza virus, CDV, canine herpesvirus Cats: FHV-1, FCV, influenza viruses, FCoV	Oropharyngeal swabs Nasal flushes, transtracheal wash or bronchoalveolar lavage specimens: ideally 5 to 10 mL of fluid Lung tissue obtained at biopsy or necropsy, including an area adjacent to affected tissue
Eye	Dogs: canine herpesvirus, canine adenovirus Cats: FHV-1, FCV, *Chlamydia felis*	Conjunctival swab, scraping or biopsy
Central nervous system	Dogs: CDV, West Nile virus, arboviruses	Cerebrospinal fluid: ideally at least 0.5 to 1 mL Blood: 8 to 10 mL Brain at necropsy
Gastrointestinal tract	Dogs: CDV, CPV, rotaviruses, canine coronavirus Cats: FCoV, FCV, FeLV, rotaviruses, toroviruses	Feces: ideally an olive-sized portion of formed feces or 10 mL of liquid stool Intestinal biopsies obtained using endoscopy or surgery, or intestinal tissue obtained at necropsy
Genital	Dogs: canine herpesvirus Cats: *Chlamydia felis*	Vesicle scrapings, vaginal swabs
Congenital and perinatal	Dogs: canine herpesvirus Cats: FHV-1, FeLV	Blood, tissues obtained at necropsy
Blood	Dogs: *Anaplasma phagocytophilum, Rickettsia rickettsii, Ehrlichia canis* Cats: FeLV, FIV, FCoV	Blood: ideally 8 to 10 mL

CDV, Canine distemper virus; CPV, canine parvovirus; FCoV, feline coronavirus; FCV, feline calicivirus; FeLV, feline leukemia virus; FHV-1, feline herpesvirus-1; FIV, feline immunodeficiency virus.

Liquid specimens such as blood, cerebrospinal fluid, and bronchoalveolar lavage fluid do not need to be placed in transport media. Blood samples should be collected using sterile technique, with antiseptic preparation of the site of venipuncture, and can be submitted in EDTA anticoagulant tubes.

All specimens should be refrigerated on collection and transported as quickly as possible (preferably within 24 hours) to the laboratory, because delayed transport can lead to loss of organism viability. If delays in excess of 2 to 3 days are anticipated, the specimen can be frozen. Freezing should be avoided whenever possible, as it may lead to dramatic loss of virus viability. If freezing is unavoidable, freezing at −70° C is preferable to freezing at −20° C, and shipping on dry ice is preferable, if possible. The laboratory's submission guide should be checked for specimen handling recommendations.

Table 1-2 provides a guide to the recommended specimen types for isolation of viruses or obligate intracellular bacteria from companion animals. Specimens should be labeled with the patient data, the site(s) from which the specimen(s) was collected, specific organisms suspected, and the time and date of specimen collection. Contained specimens should be placed inside leakproof triple packaging and transported on wet ice or cold packs to the laboratory, especially if transport is expected to take longer than 1 hour. Absorbent materials should be placed within the secondary container in order to absorb any spills. If specimens are to be shipped, the specimen must be labeled and handled according to governmental and International Air Transport Association (IATA) regulations for shipping materials known to contain infectious substances, which are categorized as Category A or Category B. Category A infectious substances are those capable of causing permanent disability or life-threatening or fatal disease in otherwise healthy animals and humans.[2] Most specimens submitted by veterinarians fall under Category B, which are those that do not fall under the criteria for inclusion in Category A. Updated documents providing guidance on regulations for the transport of infectious substances are provided online by the World Health Organization (WHO).[2] Import permits may be required for interstate and international transportation.

Diagnostic Methods

Maintenance of Cell Cultures in the Laboratory

In general, cells are grown as a monolayer on a plastic plate. The cells in the monolayer can be derived directly from an animal *(primary cell culture)*, which tend to have a limited life span, or they may be immortalized *(continuous cell lines)*. Primary cell cultures are needed for the isolation of some viruses, because the cells more closely resemble those present in vivo, and the replication of these viruses occurs more efficiently in primary cell lines than in continuous cell lines. Further subculture of primary cell lines often reduces their sensitivity to viral infection. Primary cell cultures are generated by placing tissues in cell culture media, often after treatment of the tissue with an enzyme such as trypsin or collagenase. Primary white blood cell cultures (such as peripheral blood mononuclear cell cultures) are generated by separation of the white cells from the other cellular elements using density gradient centrifugation, and adding them to a culture medium. Ficoll, a highly branched polysaccharide,

TABLE 1-3

Examples of Continuous Cell Lines Used for Isolation of Viruses and Intracellular Bacteria That Infect Dogs and Cats

Cell Line	Cell Origin	Pathogen(s)
Vero cells; recombinant Vero-SLAM cells	African Green monkey renal epithelial cells	CDV[11,12] *Rickettsia rickettsii*[13] *Toxoplasma gondii*[14]
Madin-Darby canine kidney cells (MDCK)	Kidney	CDV[8,15] Canine adenovirus[8,15] Canine herpesvirus-1[8,15] Parvoviruses[8,16] Canine parainfluenza virus[8] Canine calicivirus[4] Rotaviruses[17] Influenza viruses[18] FeLV[19] *Neospora caninum*[20]
Crandell-Reese feline kidney cells	Fetal kidney	FHV-1[21] FCV[21,22] FCoV[23] Parvoviruses[24] FIV[25]
HL-60	Human leukemia	*Anaplasma phagocytophilum*[26]
A-72	Canine fibroma	Canine adenovirus[27] Canine coronavirus[27] Canine parainfluenza virus[27] Canine herpesvirus[27]
McCoy	Mouse fibroblast	*Chlamydia felis*[28]
FCWF	*Felis catus* whole fetus, has characteristics of macrophages	FCoV[29] FHV-1[30]
DH-82	Monocyte/macrophage	*Ehrlichia canis*[31]

CDV, Canine distemper virus; FCoV, feline coronavirus; FCV, feline calicivirus; FeLV, feline leukemia virus; FHV-1, feline herpesvirus-1; FIV, feline immunodeficiency virus.

is an example of a medium used commonly for density gradient centrifugation. Primary cell cultures have been used widely for the isolation of intracellular pathogens of dogs and cats.[3-6]

Low-passage cell lines remain viable and sensitive to viral infections for 20 to 50 passages. *Continuous cell lines* are the type of cell line used most commonly for diagnostic, research, and commercial purposes. These are derived from cancer cells (such as the widely used HeLa cell line, derived from human cervical cancer cells of a patient named Henrietta Lacks),[7] or they result from experimental induction of cellular mutations (for example, using a carcinogen). Continuous cell lines representing a wide variety of cell types are available from commercial suppliers (Table 1-3). Laboratories that perform virus isolation for disease diagnosis may need to simultaneously inoculate multiple cell lines, because

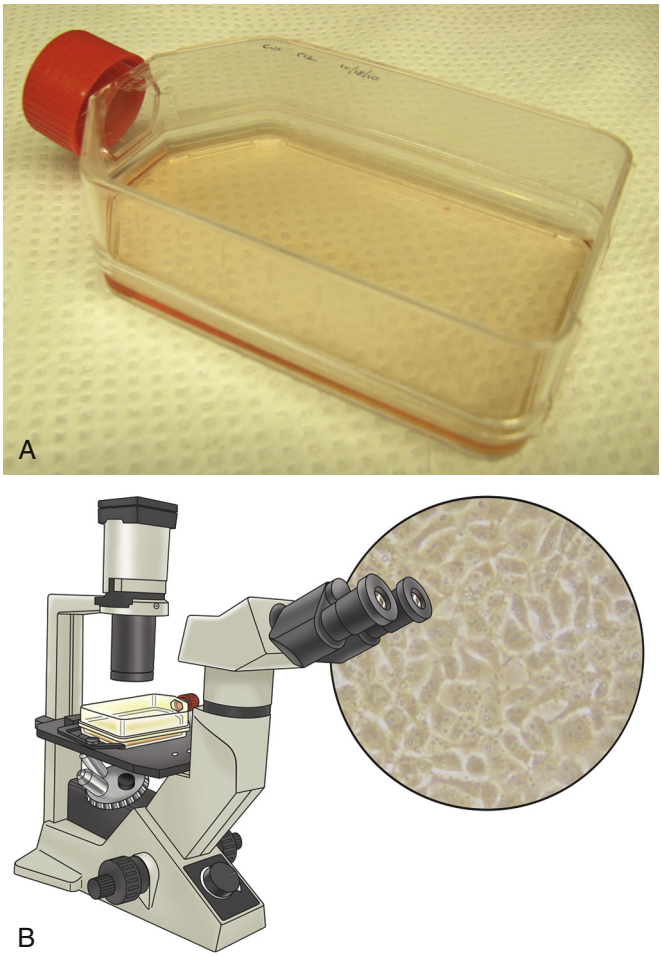


FIGURE 1-1 **A,** Plastic flask that contains cell culture medium. **B,** A confluent cell monolayer is present on the bottom of the flask and can be visualized through the top of the flask using a binocular inverted microscope.

different viruses prefer to replicate in differing cell types. Mixed cell cultures are also now available commercially to simultaneously facilitate isolation of multiple different viral pathogens.

Cells for cell culture are stored in the laboratory in liquid nitrogen tanks. The cells are thawed, dispersed in cell culture medium, and allowed to settle on the bottom of a plastic flask (Figure 1-1). The cell culture medium keeps the cells moist and provides the cells with nutrients. *Minimum essential medium* (MEM), also known as *Eagle's minimum essential medium*, and *Dulbecco's medium* are examples of widely used synthetic cell culture media. The cell culture medium contains a balanced salt solution, essential amino acids, glucose, vitamins, and a bicarbonate buffering system. Variations of MEM are available, some of which contain nonessential amino acids, the pH indicator phenol red, and the pH buffering agent HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), which helps to maintain a physiologic pH despite an increasing concentration of CO_2 that is produced as a result of cellular respiration. The medium often requires supplementation with animal serum, most commonly fetal calf serum, which helps the cells to attach to and spread on the plate, although serum-free medium has also been used successfully for culture of canine and feline viruses.[8] Media for cell culture growth differ from media for maintenance of cells in culture, the former generally containing a higher concentration of animal serum (10% versus 2% for maintenance medium).

To prevent bacterial or fungal contamination when viral pathogens are cultured, careful attention to sterile technique is required. Manipulation of cells in the flask is performed in a laminar flow hood, and the culture medium is sometimes supplemented with antibiotics (generally ampicillin or an aminoglycoside), with or without an antifungal agent (amphotericin B or nystatin). Cell culture flasks are placed in a humidified incubator that maintains temperature usually at 37°C, and a gas mixture that typically contains 5% CO_2.

The cell culture flasks are removed from the incubator and examined daily using an inverted binocular tissue culture microscope (see Figure 1-1), which allows examination of unstained cells through the bottom of the flask. The medium is replaced with fresh medium if the pH indicator suggests it is becoming too acidic. Once the cells have multiplied sufficiently to form a semiconfluent monolayer, the cells can either be passaged or be allowed to reach confluence before inoculation with specimen. In order to passage the cells, the medium is removed, and a small volume of trypsin or EDTA solution is added to the monolayer. The monolayer is incubated with the solution for several minutes, after which the cells detach from the flask and can be resuspended in medium, which is then added to new flasks.

Occasionally, cell lines become cross-contaminated with other cell lines. The HeLa cell line is an example of a prolific and hardy cell line that commonly contaminates other cultures. Major cell line repositories such as the American Type Culture Collection (ATCC) use genetic typing methods to verify the identity of the cells in their collection. Cell lines can also become easily contaminated with *Mycoplasma* spp., which pass through filters used to exclude other bacteria. The presence of contaminating *Mycoplasma* spp. can interfere with replication of other pathogens and cause alterations of cellular morphology. *Mycoplasma* contamination is detected using stains such as Hoechst stain, culture for *Mycoplasma*, and commercially available PCR assays that specifically detect *Mycoplasma* DNA for the purpose of cell culture quality assurance.[9]

Inoculation of Cell Cultures

Infection of a cell monolayer is accomplished by removal of overlying medium and inoculation of the monolayer with a suspension of viral or bacterial organisms. If tissue specimens are provided for culture, the specimens are homogenized in culture medium before they are inoculated onto the monolayer. Specimens may first be centrifuged to remove cell debris and bacteria. Passage through a filter can also be used before inoculation to remove bacteria. The inoculum is added to the monolayer. Organisms within the inoculum are then allowed to settle on the monolayer for approximately 1 hour before the residual inoculum is removed and fresh maintenance medium is added.

For isolation of virus, the monolayer is then examined daily for evidence of cytopathic effect (CPE). Medium is replaced weekly or biweekly. CPE refers to the cellular changes induced by viral replication in the cell culture monolayer and includes cell lysis or cell fusion (syncytium formation). The presence of a particular viral pathogen is indicated by the appearance of characteristic CPE for that pathogen after a predicted incubation period (Figure 1-2). For example, CPE induced by FCV is characterized by cell rounding and detachment that can occur within 24 hours, and within 16 hours for highly virulent strains.[10] The presence of CPE is confirmed through the comparison of inoculated cultures to uninoculated (control) cultures. Examination of the cell monolayer using light microscopy after fixation and staining can reveal additional diagnostic features, such as the presence of inclusion bodies and syncytium formation. Some viruses are relatively noncytopathogenic. In some cases, the presence of these viruses can be demonstrated by adding washed erythrocytes to the monolayer and examination of the monolayer for evidence of hemadsorption (see Figure 1-2, B). Hemadsorption results from incorporation of viral surface hemagglutinin molecules into the plasma membranes of the cell culture monolayer. Examples of hemadsorbing viruses that produce minimal CPE include influenza and parainfluenza viruses. Other, more sensitive and specific methods for identification of

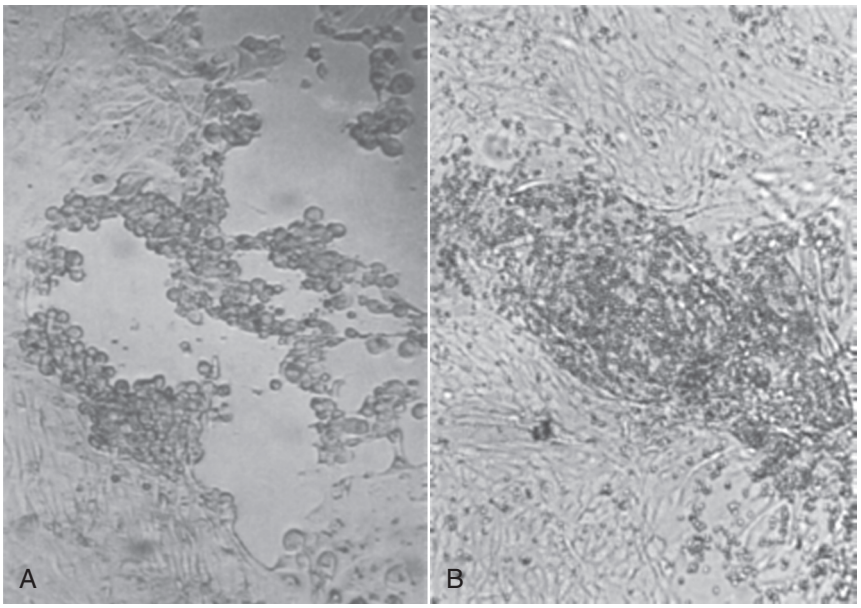


FIGURE 1-2 **A,** Cytopathic effects produced by a herpesvirus as viewed in the laboratory. Note the focal areas of rounded and detached cells. **B,** Hemadsorption: erythrocytes adsorb to infected cells that have incorporated hemagglutinin into the plasma membrane. Magnification ×60. (Courtesy of Jack I; from MacLachlan NJ, Dubovi EJ eds. Fenner's veterinary virology, 4 ed. New York: Academic Press, 2011.)

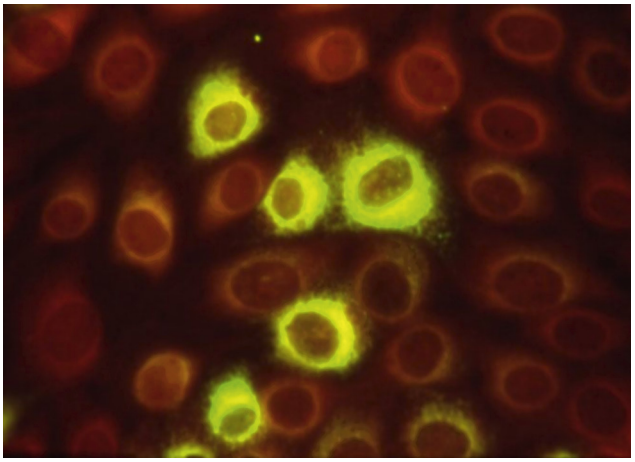


FIGURE 1-3 Indirect fluorescent antibody detection of noncytopathic virus infected cells. A cell monolayer exposed to virus for 72 hours was fixed with cold acetone. Fixed cells were stained with a mouse monoclonal antibody specific for bovine viral diarrhea virus (BVDV) followed by a goat anti-mouse serum tagged with fluorescein isothiocyanate. (From Maclachlan NJ, Dubovi EJ. Fenner's Veterinary Virology, 4 ed. New York: Academic Press, 2011.)

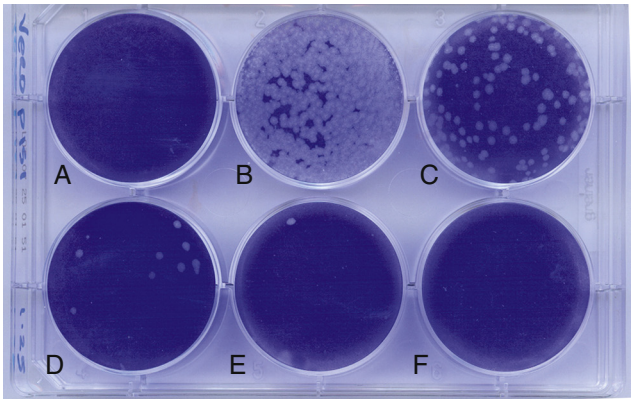


FIGURE 1-5 Determination of the concentration of infectious virus using a plaque assay. Vero cell monolayers were inoculated with serial 10-fold dilutions of vesicular stomatitis virus. After a 1-hour adsorption period, cultures were overlayed with 0.75% agarose in cell culture medium containing 5% fetal bovine serum. Cultures were incubated for 3 days at 37°C in a 5% humidified CO_2 atmosphere. The agarose overlay was removed and cultures fixed and stained with 0.75% crystal violet in 10% buffered formalin. **A,** Control culture. **B-F,** serial 10-fold dilution of virus: **B,** 10^{-3}; **C,** 10^{-4}; **D,** 10^{-5}; **E,** 10^{-6}; **F,** 10^{-7}. (From Maclachlan NJ, Dubovi EJ. Fenner's Veterinary Virology, 4 ed. New York: Academic Press, 2011.)

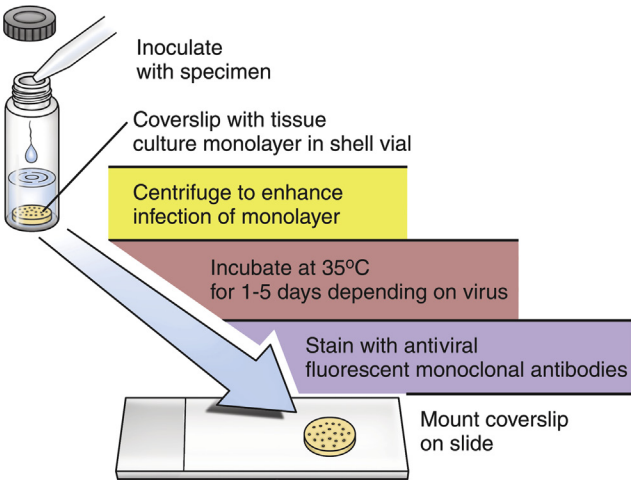


FIGURE 1-4 Schematic of the shell vial virus isolation technique. (Modified from Shelhamer JH, Gill VJ, Quinn TC, et al. The laboratory evaluation of opportunistic pulmonary infections. Ann Intern Med 1996;124(6):585-599.)

an organism that has infected a cell culture monolayer include fixation and staining of the monolayer with fluorescent antibodies that are specific for certain viruses (Figure 1-3), use of nucleic acid detection techniques such as PCR, or use of electron microscopy.

For *spin-amplified cell culture*, the monolayer is centrifuged at low speed after inoculation. This can enhance recovery of certain viruses and intracellular bacterial pathogens such as chlamydiae. Either an entire plate of cell culture wells can be spun in a plate centrifuge, or *shell vials* can be used (also known as the *shell vial technique*). Shell vials are small, flat-bottomed bottles (Figure 1-4). A monolayer is grown on a glass coverslip at the bottom of the vial, and after inoculation, the vials are centrifuged at low speed. Using this method, fluorescent antibody staining for viral or chlamydial antigen is used to detect the pathogen before CPE occurs. After 48 to 72 hours, the coverslips are fixed, stained with virus-specific fluorescent-labeled antibodies, removed from the vials, and examined with a fluorescence microscope.

Plaque Assays

Overlay of the monolayer with agar or methylcellulose after inoculation with virus minimizes subsequent viral movement through the monolayer and restricts damage by each replicating viral particle to a small area. This results in the formation of "holes" in the monolayer, or *plaques* (Figure 1-5). The monolayer can then be stained and the number of plaques can be counted in order to obtain information regarding the amount of virus in the inoculum. Plaque assays are commonly used by researchers to assess the efficacy of antiviral treatments, through reduction of plaque formation.

Laboratory Safety Concerns That Relate to Cell Culture

The processing of specimens in a biological safety cabinet not only serves to protect cultures from contamination but also acts to protect the laboratory worker from laboratory-acquired infections. Most viruses grown in veterinary diagnostic laboratories are classified as biosafety level 2 (BSL 2) agents. Biosafety levels range from 1 through 4 (Table 1-4). In the United States, the Centers for Disease Control and Prevention (CDC) assigns these levels. It is important that veterinary clinicians be aware that isolation of hazardous pathogens can only be conducted in specially designed and accredited laboratories.

Interpretation of Results

Negative Results

Reasons for negative test results using virus isolation are numerous, so the clinician should not assume that a virus is not present when a negative test result is obtained (Box 1-2). Negative test results can occur as a result of inadequate specimen size, lack of viral shedding by the animal at the time of specimen collection, the binding of antibodies to viruses within the specimen,

TABLE 1-4

Biosafety Levels Set for Diagnosis of Laboratory Practices

Level	Risk	Examples	Example Precautions
BSL 1	Minimal potential hazard to human health and the environment	Canine adenovirus-1 Nonpathogenic *Escherichia coli*	Gloves, facial protection. Standard microbiologic practices using bench-top techniques. Routine decontamination practices (hand washing, routine bench disinfection, autoclaving of infectious waste).
BSL 2	Moderate potential hazard to human health and the environment. Organisms cause mild disease or are difficult to contract as laboratory aerosols.	Most veterinary viruses, including influenza viruses	Access to the laboratory restricted when work is taking place; extreme precautions with sharp contaminated materials; use of appropriate biosafety cabinets when generation of aerosols possible. No requirement for directional airflow into the laboratory.
BSL 3	Dangerous agents that can be transmitted by aerosol within the laboratory but for which effective vaccines or treatments exist.	West Nile virus Equine encephalitis viruses *Rickettsia rickettsii* *Coxiella burnetii* *Mycobacterium tuberculosis*	Laboratory is located away from high-traffic areas. Restricted laboratory access when work in progress; double door entry, ventilation providing airflow into the room, exhaust air not recirculated; special practices and protective clothing for BSL 3, including biosafety cabinet use; special floor and ceiling materials specified.
BSL 4	Dangerous and exotic agents that pose a high risk of aerosol-transmitted laboratory infections, or which produce severe or fatal disease in humans	Ebola virus Marburg virus Smallpox	Hazmat suit and self-contained oxygen system, entrance containing multiple showers, a vacuum room, an ultraviolet light room, and multiple airlocked doors. Strict control of laboratory access to authorized personnel.

BOX 1-2

Reasons for Negative Test Results following Isolation of Viruses in Cell Culture

Virus not causing the disease
Virus causing the disease, but:
- Specimen size inadequate
- No viral shedding at the time of sampling
- No viral shedding at the site of sampling
- Antibody interference with viral infectivity for cell culture
- Inadequate organism numbers at the anatomic site of specimen collection
- Loss of organism viability during transportation to the laboratory
- Overgrowth of one viral pathogen by another
- Overgrowth by bacterial or fungal agents
- Lack of cell sensitivity for the virus (wrong cell type inoculated)
- Laboratory inexperience with techniques required for virus isolation and identification

inadequate organism numbers at the anatomic site of specimen collection, or loss of organism viability during transportation to the laboratory. Loss of viral viability is more likely to occur with enveloped viruses such as canine distemper virus than with non-enveloped, hardy viruses such as canine parvovirus or FCV.

Negative test results can also occur when the cell line inoculated is not sensitive to the virus present in the specimen. Low-passage cells may also lose their infectivity for viral infection if they have undergone multiple serial passages.

False negatives can also occur if the plates are overgrown by bacteria or fungi. Although treatment of the viral transport and culture media with antimicrobials can help prevent this, resistant bacteria or fungi may still be present. Sometimes, multiple viruses are present within a specimen, and one virus overgrows another. For example, this can occur with mixed infections with FCV and feline herpesvirus-1 (FHV-1) in cats with upper respiratory tract disease. FCV rapidly infects Crandell-Reese feline kidney cells and produces CPE, which obscures the concurrent presence of FHV-1.

Positive Results

It is imperative that veterinary clinicians be aware that the detection of a virus in a specimen does not always imply that the organism is the cause of the animal's clinical signs. This is especially true for specimens collected from the respiratory or gastrointestinal tracts, where multiple infectious agents may be present concurrently, and in many cases, viruses can replicate in these locations without causing clinical signs of illness. In some animals, the development of severe clinical signs is more likely when there is simultaneous presence of multiple infectious agents. As noted previously for specimens that test negative, the presence of one agent may also obscure the presence of another, more significant organism (such as with FCV and FHV-1 co-infections), which could result in the incorrect assumption that only one organism is the cause of an animal's disease.

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