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CHAPTER 13

Laboratory Diagnosis of Viral Diseases

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Tests for the specific diagnosis of a viral infection in an animal are of two general types: (1) those that demonstrate the presence of the virus, and (2) those that demonstrate the presence of specific viral antibody. The provision, by a single laboratory, of a comprehensive service for the diagnosis of viral infections of domestic animals is a formidable undertaking. There are about 200 individual viral species, in some 20 different viral families, that infect the eight major domestic animal species (cattle, sheep, goat, swine, horse, dog, cat, and chicken). If antigenic types within an individual viral species are considered, and the number of animal species is broadened to include turkey, duck, and zoo and laboratory animals, then the number of individual viruses exceeds 1000. It is therefore not surprising that few single laboratories could have available the necessary specific reagents, skills, and experience for the diagnosis of such a large number of infections.

One consequence of this great variety of viruses is that veterinary diagnostic laboratories tend to specialize, e.g., in diseases of food ani-

mals, or companion animals, or poultry, or in "exotic" viruses. Within these specialized laboratories there is considerable scope for the development of rapid diagnostic methods that short-circuit the need for the isolation of viruses, which is expensive, time-consuming, and rarely necessary.

RATIONALE FOR SPECIFIC DIAGNOSIS

Many viral diseases can be diagnosed clinically, others with the assistance of the pathologist; but there are several circumstances under which laboratory confirmation of the specific virus involved is desirable or, indeed, essential.

Exotic Diseases

The industrialized countries of Europe, North America, Australasia, and Japan are free of many devastating diseases of livestock that are still enzootic in other parts of the world, such as foot-and-mouth disease, African swine fever, rinderpest, and fowl plague. All industrialized countries maintain or share the use of specialized biocontainment laboratories (such as those at Plum Island in the United States and Pirbright in the United Kingdom) devoted to diagnosis and research on such "exotic" viruses. Clearly it is of the utmost importance that the clinical diagnosis of a suspected exotic virus should be confirmed quickly and accurately (see Chapter 16).

Zoonoses

Several animal viral diseases such as rabies, Rift Valley fever, and eastern, western, and Venezuelan encephalomyelitis are zoonotic and are of sufficient human public health significance to require the maintenance of specialized diagnostic laboratories. For example, confirmation of the diagnosis of rabies in a skunk that has bitten a child provides the basis for postexposure treatment of the human patient (see Chapter 30). Confirmation and early warning of an equine encephalomyelitis virus epizootic allows implementation of mosquito control and other measures such as restriction of the movement of horses.

Certification of Freedom from Specific Infections

For diseases in which there is lifelong infection, such as bovine and feline leukemia, equine infectious anemia, and herpesvirus infections, a negative test certificate is often required as a condition of sale, particu-

larly export sale, for exhibition at a state fair, or show, or for competition, as at race meetings.

Artificial Insemination, Embryo Transfer, and Blood Transfusion

Males used for semen collection and females used in embryo transfer programs, especially in cattle, and blood donors of all species, are usually screened for a range of viral infections to minimize the risk of transmission to recipient animals.

Test and Removal Programs

For retrovirus infections, Marek's disease, pseudorabies, and certain other diseases, it is possible to reduce substantially the incidence of disease or eradicate the causative virus from the herd or flock by test and removal programs. Laboratory diagnosis is essential for the effective implementation of such operations.

Veterinary Health Investigations

Provision of a sound veterinary service in any state or country depends on a knowledge of prevailing viral diseases; hence, epidemiological studies to determine the prevalence and distribution of particular viral infections are frequently undertaken, usually based on the detection of specific antibody.

Clinical Management Dependent on Precise Diagnosis

Many relatively nonspecific disease syndromes, such as respiratory disease (e.g., kennel cough and shipping fever), diarrhea, and some skin diseases, may be caused by a variety of agents, viral and bacterial. Proper management of individual cases or infected herds or flocks may require specific viral diagnosis.

METHODS OF VIRAL DIAGNOSIS

Specific diagnosis of the kind outlined above can be achieved by one of three methods: (1) isolation and characterization of the causative virus, (2) direct demonstration of virions, viral antigens, or viral nucleic acids in tissues, secretions, or excretions, and (3) detection and measurement of antibodies (Table 13-1). Each group of methods has its place. Viral isolation remains the benchmark against which other methods are

TABLE 13-1
*Techniques for Identification of Viruses at Various
 Taxonomic Levels*

Taxonomic level	Techniques of choice
Family (sometimes genus)	Cytopathology in cultured cells Electron microscopy Complement fixation
Species (type)	Neutralization
Subtype	Kinetic neutralization Monoclonal antibody serology
Variant	Nucleic acid hybridization Oligonucleotide fingerprints Restriction endonuclease fragment patterns
Mutant (including by point mutation)	Nucleic acid sequencing

measured, and is essential when decisions of major economic importance depend on the diagnosis, e.g., with suspected exotic diseases such as foot-and-mouth disease or fowl plague. On the other hand, the direct demonstration of virions or viral components may provide a much more rapid and cheaper method of specific diagnosis than viral isolation, particularly when large numbers of samples must be tested. Epidemiological surveys, eradication programs, and the provision of certificates of freedom from specific infections are often based on serological methods or rapid tests for viral antigen.

COLLECTION, PACKAGING, AND TRANSPORT OF SPECIMENS

It requires at least as much effort, and often more, to process a negative specimen as it does one from which virus is isolated. The chance of isolating a virus depends critically on the knowledge, care, and attention of the veterinarian who collects the specimen (see Plate 13-1). Obviously such a specimen must be taken from the right place and at the right time. The right time is always as soon as possible after the onset of clinical signs; virus is usually present in maximum amount at about this time and diminishes, sometimes quite rapidly, in the ensuing few days. Specimens taken as a last resort when days or weeks of empirically chosen antibiotic therapy have failed are almost invariably a waste of effort.

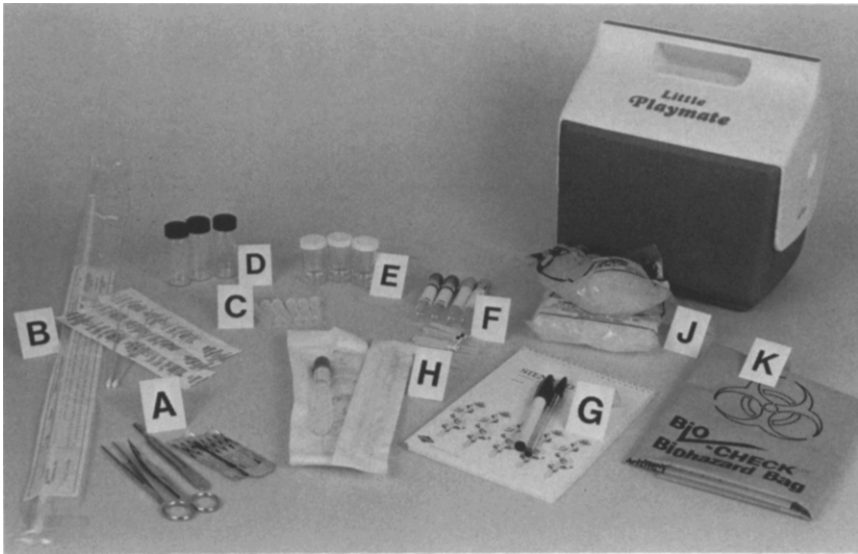


PLATE 13-1. Equipment required for collection of virus samples. (A) Sterile forceps, scissors, and scalpels. (B) Selection of sterile swabs. (C) Vials for containing virus transport medium for collection of samples for virus isolation or identification. (D) Bottles for collection of feces, blood, and other samples that do not require virus transport medium. (E) Bottles containing formol saline or Bouin's fixative for tissues to be examined histologically. (F) Blood collection equipment—without additive for serum, with anticoagulant for virus isolation. (G) Notebook and equipment for labeling specimens. (H) Swabs and transport medium for bacteriological investigation. (I) Cool box. (K) Heavy-duty plastic bags for postmortem material.

The site from which the specimen is collected will be influenced by the clinical signs and a knowledge of the pathogenesis of the suspected disease (Table 13-2). Having collected the appropriate specimen(s), it should be properly labeled and sent to the laboratory, with a history, including the provisional diagnosis. Where ambient temperatures are moderate and transit time to the laboratory is less than 1 day, ice or cold packs ($<4^{\circ}\text{C}$) in a styrofoam box are frequently used. If the environmental temperature is high and transit times longer than a day, dry ice (-70°C) may be used, although wet ice with provision to replenish it in transit is better. If exotic or zoonotic viruses are suspected, the styrofoam boxes must be replaced by or enclosed within sturdier, double-walled containers with absorbent padding. Appropriate permits must be obtained for interstate and international transportation, and in

TABLE 13-2
*Specimens Appropriate for Various
 Clinical Syndromes*

Disease	Live animal	Postmortem
Respiratory and ocular disease	Nasal and conjunctival swabs, blood ^a	Tissues from affected system, lymph nodes
Skin disease; lesions of mucous membranes	Scrapings of lesion, swab affected area, blood	Tissue from affected system, lymph nodes
Gastroenteritis	Feces, blood	Tissues from affected system, lymph nodes, intestinal contents
Systemic disease	Blood, nasal and urogenital swabs; feces	Tissues from various organs
Disease of the central nervous system	Blood, cerebrospinal fluid (if feasible); feces, nasal and urogenital swabs	Tissues from affected system, lymph nodes
Disease of the urinary tract	Urogenital swab; urine, blood	Tissues from affected system, lymph nodes
Abortion	Blood from dam, vaginal mucus	Tissues from placenta and fetus; blood from fetal heart; intestinal contents

^aBlood: refers to clotted sample for serology and sample with anticoagulant added for other tests. Large animals, 10–20 ml; small animals, 5–10 ml; others as appropriate. If possible remove clot before dispatch.

such circumstances the collection and transport arrangements need to be discussed with the laboratory and/or the appropriate government regulatory agency.

For particularly labile viruses such as respiratory syncytial virus, herpesviruses, or coronaviruses, it may be an advantage to take the cell culture to the animal.

VIRUS ISOLATION

Isolation and identification requires at least a week, usually longer, and it is expensive. However, it is probably the most sensitive available method, if properly collected material is used, and it provides material for further study.

Preparation of the Specimen for Inoculation

The sooner the specimen is processed and inoculated after arrival at the laboratory, the better. If delays of more than 1 day are anticipated,

the specimen may be frozen to -70°C . Swabs are processed by twirling them in the transport medium and expressing the fluid by pushing the swab firmly against the side of the container. Feces are dispersed on a vortex mixer. Tissue specimens are finely minced with scissors and homogenized in a glass or mechanical homogenizer.

Prior to inoculation, contaminating microorganisms are removed by filtering through membrane filters of average pore diameter $0.45\ \mu\text{m}$, although such filters allow the passage of mycoplasmas. Once virus is successfully isolated and grown to a high titer, the suspension can be refiltered through $0.22\ \mu\text{m}$ filters to exclude mycoplasmas. Feces and tissue homogenates should be diluted at least 1 : 10 and centrifuged at 1000 g for 15 minutes to obtain a supernate that can be filtered. If the concentration of virus is suspected to be very low, high concentrations of antibiotics may be preferable to filtration. Whatever the origin of the specimen, some of the original sample and some of the filtrate should be retained at 4°C or frozen until the isolation attempt is finalized.

Virus can be grown from the suitably prepared specimen by inoculation into cell cultures, laboratory animals, or the species of host animal from which the specimen was obtained. By far the most widely used substrate is cultured cells.

Growth of Virus in Cultured Cells

Choice of Cultured Cells. The choice of the optimal cell culture for the primary isolation of a virus of unknown nature from clinical specimens is largely empirical. Primary or low-passage, homologous, monolayer cell cultures derived from fetal tissues probably provide the most sensitive substrate for isolation of the greatest variety of different viruses. Continuous cell lines derived from the homologous species are almost as good. Often the nature of the disease from which the material was obtained will suggest what species of virus may be found, and in such cases the optimum cell culture for that virus can be chosen, in parallel, perhaps, with a second type of culture with a wide spectrum. Cell lines offer some advantages and are available for most domestic animals except avian species (Table 13-3).

Monolayer cell cultures for virus isolation should be grown in sealed containers, such as plastic flasks or glass tubes with screw caps. Open-culture systems such as petri dishes or microtiter trays should not be used because of the risks of cross-contamination. For some viruses, rolling the cultures on a drum improves isolation rates.

Special types of cultures are utilized for particular viruses. For example, betaherpesviruses and gammaherpesviruses may be recovered from monolayer cultures of tissue taken directly from the diseased animal,

TABLE 13-3
Some Commonly Used Cell Lines

Animal species	Cell line	
	Origin	Acronym
Cattle	Fetal kidney	MDBK
	Embryonic trachea	EBTr
Sheep/goat	None	—
Swine	Kidney	PK
Horse	Skin	EDerm
Dog	Kidney	MDCK
Cat	Fetal kidney	CrFK
	Embryo	FEmb
Chicken	None	—

whereas inoculation of established monolayer cell cultures with cell-free material may be negative. For some coronaviruses and rhinoviruses that do not grow well in monolayer cultures, growth may occur in explant cultures (i.e., small cubes of tissue from the trachea or gut), probably because these do not dedifferentiate in culture (see Chapter 3).

Recognition of Viral Growth. Cultures are usually incubated at 37°C, despite the fact that the normal body temperatures of all domestic animal species are somewhat higher. Cultures are observed daily for cytopathic effects. The speed and nature of the cytopathic effect caused by different viruses varies considerably. Cytopathic effect must always be based on comparison with uninoculated cell cultures; this is particularly important for viruses requiring incubation periods of longer than a week. Where none or a doubtful cytopathic effect is observed, it is usual to make a second or even a third ("blind") passage.

When cytopathic effect is observed, there is a range of options:

1. The speed and appearance of the cytopathic effect, coupled with the case history, may immediately suggest the diagnosis.
2. After suitable manipulation, material from the cell culture may be examined by electron microscopy.
3. Infected monolayers on glass coverslips or special slide/culture chambers may be fixed and appropriately stained, and the cells examined for inclusion bodies, syncytia or other characteristic cell changes. Better, they may be stained with fluorescent antibody, which may provide an immediate definitive diagnosis. Where prior experience and knowledge suggest it, such slide cultures may be included at the time of primary inoculation, with a consequent saving in time.

Some viruses are relatively noncytopathogenic for cultured cells (see Chapter 6 and Table 3-1). Their growth in monolayer culture may sometimes be recognized by means of hemadsorption. Most viruses that hemagglutinate will also hemadsorb; the growth of paramyxoviruses, orthomyxoviruses, and, to a lesser extent, the flaviviruses and togaviruses, is routinely recognized in this way (see Plate 3-2).

Growth of Virus in Laboratory Animals

Nowadays laboratory animals play a minor role in the virus diagnostic laboratory. However, some virologists still regard intracerebral inoculation of baby mice as the method of choice for the isolation of rabies virus, flaviviruses, and togaviruses.

The developing chick embryo occupies a special place. Intraamniotic inoculation provides the most sensitive method for influenza viruses and for several avian viruses, and species diagnosis of orthopoxviruses can be made directly from the type of pock produced on the chorioallantoic membrane. In addition, chick embryos are extensively used as a source of primary monolayer cultures (fibroblasts, kidney cells) for the isolation of avian viruses.

Inoculation of the Natural Host Species

In veterinary medicine it is feasible to consider using the natural host species, especially susceptible young animals (e.g., calves, pigs, chicks), for the recovery of a virus from suspect material. Such animals, if free of antibodies, must be considered a highly sensitive substrate. However, their use would now be contemplated only for viruses not yet cultivable, or where cell culture procedures were negative in circumstances that strongly indicated a viral etiology, and/or where there might be serious repercussions if the diagnosis were missed.

Identification of Viral Isolates by Serology

A newly isolated virus can usually be provisionally allocated to a particular family, and sometimes to a genus or species, on the basis of the clinical findings, the host cell used for virus isolation, and the visible result of viral growth (cytopathic effect, hemadsorption, hemagglutination, electron microscopy of the cytopathogenic agent, etc.). Definitive identification, however, usually depends on serological procedures. By using the new isolate as antigen against known antisera, e.g., in a complement fixation test, the virus can often be placed into its correct family or genus. Having allocated it to a particular family (e.g., *Adenoviridae*), one can then go on to determine the species or serotype (e.g., canine

TABLE 13-4
Principal Serological Procedures Used in Diagnostic Virology

Technique	Principle
Virus neutralization	Antibody neutralizes infectivity of virion; inhibits cytopathology, reduces plaques, or protects animals
Hemagglutination inhibition	Antibody inhibits viral hemagglutination
Complement fixation	Antigen-antibody complex binds complement, which is thereafter unavailable for the lysis of hemolysis-sensitized sheep red blood cells
Immunoelectron microscopy	Antibody-aggregated virions are visible by electron microscopy
Immunofluorescence	Antibody labeled with fluorochrome binds to intracellular antigen; fluoresces by UV microscopy
Immunoperoxidase staining	Peroxidase-labeled antibody binds to intracellular antigen; colored precipitate forms on adding substrate
Enzyme-linked immunosorbent assay (ELISA)	Enzyme-labeled antibody (or antigen) binds to antigen (or antibody); substrate changes color
Radioimmunoassay	Radiolabeled antibody (or antigen) binds to antigen (or antibody), e.g., attached to solid phase
Immunodiffusion	Antibodies and soluble antigens produce visible lines of precipitate in a gel

adenovirus 1) by more discriminating serological procedures. This sequential approach is applicable only to families with a common family antigen.

The range of available serological techniques is now extremely wide (Table 13-4). Some are best suited to particular families of viruses. Each laboratory makes its own choice of favored procedures, based on considerations such as sensitivity, specificity, reproducibility, speed, convenience, and cost. Currently most serological procedures are carried out with "hyperimmune" sera comprising a polyclonal mixture of antibodies, sometimes after they have been absorbed to eliminate antibodies of certain specificities.

Monoclonal antibodies with defined specificity are now becoming available. These make it possible to proceed quickly to very specific diagnosis even to the level of subtypes, strains, or variants, e.g., rabies viruses from different geographical areas. Family-, genus-, and type-specific monoclonal antibodies are also being developed. As their properties are defined and they become commercially available, we can expect monoclonal antibodies to be widely used for all methods of serological identification.



PLATE 13-2. *Immunofluorescence, used here for determining the site of assembly of components of influenza virus. Antibody against the nucleoprotein antigen shows nuclear accumulation at 4 hours after infection of chick cells. Procedure: guinea pig antiserum to nucleoprotein antigen is added to a monolayer of infected chick cells, then fluorescein-conjugated rabbit anti-guinea pig IgG. (Courtesy Dr. N. J. Dimmock.)*

Immunofluorescence. The simplest way of identifying a newly isolated virus is by fluorescent-antibody staining of the infected cell monolayer itself (Plate 13-2). This can provide definitive diagnosis within an hour or so of recognizing the earliest suggestion of cytopathic effect. Immunofluorescence is best suited to the identification of monotypic genera, or genera of which only a single species affects that particular species of animal, or to epidemic situations when a particular virus is suspected; otherwise, replicate cultures must be screened with a range of antisera. The advantages and disadvantages of monoclonal antibodies, in comparison with polyclonal or "absorbed" sera, discussed below in the context of radioimmunoassays, apply equally to other serological procedures, including immunofluorescence and neutralization.

Electron Microscopy and Immunoelectron Microscopy. These procedures (see Plate 13-5) are most useful in the rapid identification of cell culture virus isolates, as well as directly on specimens (see below). Electron microscopy allows identification only to the level of family, whereas immunoelectron microscopy using suitable specific antibody may permit finer distinctions to be made.

Complement Fixation. For the complement fixation test, the acute and convalescent sera are heated (56°C for 30 minutes) to inactivate complement, then serially diluted in a plastic tray. Two to four units of antigen (e.g., a crude preparation of live or inactivated virus) are then added to each serum dilution together with 2 units of complement, derived from a guinea pig. The reagents are allowed to interact at 4°C overnight, to allow the complement to become “fixed.” Sheep erythrocytes, “sensitized” by the addition of rabbit antiserum to them (“hemolysin”) are then added and the trays are incubated at 37°C for 45 minutes. In those cups where the complement has been fixed by the virus-antibody complex, the hemolysin fails to lyse the sheep erythrocytes; where complement is still present, they are lysed.

Crude cell culture supernatants often used for complement fixation tests contain not only mature virions but a range of soluble antigens, both structural and nonstructural. Since many of these are shared by many or all viruses within a particular genus or family, e.g., *Mastadenovirus*, they will cross-react with antibodies raised against any other member of the genus or family. This property makes complement fixation a useful method for preliminary screening of an isolate—to place it within the correct family or genus. Immune-adherence hemagglutination is basically a somewhat simplified version of the complement fixation test; currently it is applied more often to the detection of antibody than that of antigen.

Hemagglutination and Hemagglutination Inhibition. Virions of several viral families bind to red blood cells and cause hemagglutination. If antibody and virus are mixed prior to the addition of red blood cells, hemagglutination is inhibited (Table 3-4; Plate 13-3). The hemagglutination titer of certain viruses, e.g., canine distemper virus, may be increased by dissociation of the virions with detergents. Antisera may have to be pretreated to remove nonspecific inhibitors of hemagglutination (see Chapter 26).

The hemagglutination inhibition test is sensitive and, except in the case of the togaviruses, highly specific, since it measures antibodies binding to the surface protein most subject to antigenic change. Moreover, it is simple, inexpensive, and rapid, and is therefore the serological procedure of choice for identifying isolates of hemagglutinating viruses.

Virus Neutralization. The infectivity of a virus may be neutralized by specific antibody by a variety of mechanisms (see Chapter 9). Serum must first be “inactivated” by heating at 56°C for 30 minutes to remove nonspecific virus inhibitors. Serum-virus mixtures are inoculated into appropriate cell cultures, which are then incubated until the “virus

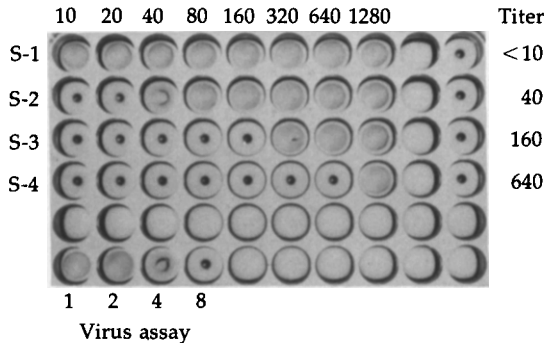


PLATE 13-3. Hemagglutination inhibition test, used for titrating antibodies to the viral hemagglutinin. Titers are expressed as reciprocals of dilutions. In the example illustrated, a horse was immunized against the prevalent strain of influenza virus. Serum samples S-1, S-2, S-3, and S-4 were taken, respectively, before immunization, 1 week after the first injection, 4 weeks after the first injection, and 4 weeks after the second injection. The sera were treated with periodate and heated at 56°C for 30 minutes to inactivate non-specific inhibitors of hemagglutination, then diluted in microtiter wells in twofold steps from 1/10 to 1/1280. Each well then received four hemagglutinating (HA) units of the relevant strain of influenza virus. After incubation at room temperature for 30 minutes, 0.05 ml of red blood cells was added to each well. Where enough antibody is present to coat the virions, hemagglutination has been inhibited; hence the erythrocytes settle to form a button on the bottom of the well. On the other hand, where insufficient antibody is present, erythrocytes are agglutinated by virus and form a mat. The virus assay (bottom line) indicates that the virus used gave partial agglutination (the end point) when diluted 1/4. Interpretation: The horse originally had no hemagglutinin-inhibiting antibodies against this particular strain of influenza virus. One injection of vaccine produced some antibody; the second injection provided a booster response. (Courtesy I. Jack.)

only" controls develop cytopathic effects (Plate 13-4). Antibody, by neutralizing the infectivity of the virus, protects the cells against viral destruction.

In keeping with the general trend toward miniaturization, most neutralization tests are now conducted in disposable nontoxic sterile plastic trays with, say, 96 flat-bottomed wells in each of which a cell monolayer can be established. Virus-antiserum mixtures can be added to established monolayers, or, more usually, serum dilutions are made in the wells, a standard amount of virus is added, and the mixture incubated, after which cells are added. In the standard neutralization test the end point is read by cytopathic effect, the titer of the serum being defined as the highest dilution that inhibits the cytopathic effect. In a version of the neutralization test known as the *plaque reduction* assay, cell monolayers

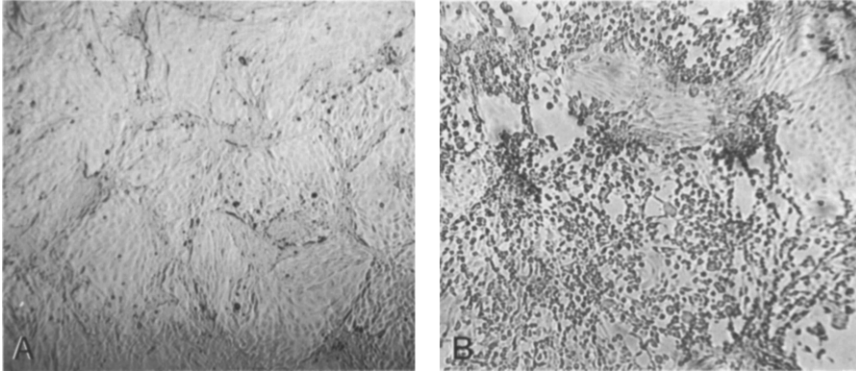


PLATE 13-4. *Virus neutralization test. A pig developed encephalitis during an epizootic of porcine enterovirus 1 infection. An enterovirus was isolated from the feces. One hundred times the TCID₅₀ of this virus was incubated at 37°C for 60 minutes with a suitable dilution of "inactivated" (56°C, 30 minutes) anti-porcine enterovirus 1 serum (a reference serum raised in a rabbit). The mixture was inoculated onto a monolayer of swine kidney cells in wells of a microculture tray (A). Virus similarly incubated with normal rabbit serum was inoculated into well B. The cultures were incubated at 37°C for several days and inspected daily for cytopathic effect (unstained, ×23). (Courtesy I. Jack.) Interpretation: The infectivity of this virus isolate has been neutralized by anti-porcine enterovirus 1 serum (no cytopathic effect); the control culture (B) shows typical cytopathic effect.*

inoculated with virus-serum mixtures are overlaid with agar or methylcellulose and incubated until plaques develop (see Plate 3-3); the end point is usually taken to be the highest dilution of serum reducing the number of plaques by at least 50%.

If a newly isolated virus proves to be "untypeable," i.e., not neutralizable by antisera against any of the known serotypes, it may be a novel serotype, or it may indicate a mixed infection with two distinct viruses, or aggregation of virions in the specimen. Aggregates can be removed by vigorous agitation, filtration, or, in the case of some nonenveloped viruses, dispersed with sodium deoxycholate, prior to repeating the neutralization test.

Oligonucleotide and Restriction Endonuclease Fingerprinting

For most routine diagnostic purposes it is usually not necessary to "type" the isolate antigenically, even to the degree just described. Sometimes, however, important epidemiological information can be obtained by going even further, to identify differences between "variants" or

subtypes within a given serotype (see Table 13-1). Short of determining the complete nucleotide sequence of viral nucleic acid, the most useful methods of doing this are by oligonucleotide fingerprinting of viral RNA or the determination of restriction endonuclease fragment patterns of viral DNA. With RNA viruses, viral RNA is labeled with ^{32}P during replication of the virus in culture; the labeled RNA is phenol-extracted from purified virions, digested with ribonuclease T1, and the resulting oligonucleotide fragments separated by two-dimensional polyacrylamide gel electrophoresis, or by cellulose acetate electrophoresis followed by DEAE-cellulose chromatography. Autoradiography reveals a "fingerprint" unique to that particular viral strain. An example of the epidemiological use of this technique to trace the origin and spread of foot-and-mouth disease virus in Europe in 1981 is described in Chapter 23.

Similarly, viral DNA prepared from virions or infected cells can be cut with appropriately chosen restriction endonucleases and the fragments separated by agarose gel electrophoresis. When stained with ethidium bromide or silver, restriction endonuclease fragment patterns (also called fingerprints) are obtained. The method has found application with all dsDNA virus families, particularly in epidemiological studies, but also in understanding pathogenesis. Depending on the viral family, the resolution of these methods is such that different isolates of the same viral species may be distinguishable, unless they come from the same epizootic. Minor degrees of genetic drift, often not reflected in serological differences, can sometimes be detected in this way.

Interpretation

The isolation and identification of a particular virus from an animal with a given disease is not necessarily meaningful in itself. Fortuitous subclinical infection with a virus unrelated to the illness in question is not uncommon. Koch-Henle postulates (see Chapter 2) are as apposite here as in any other microbiological context, but are not always easy to fulfill. In attempting to interpret the significance of any virus isolation, one must be guided by the following considerations:

1. The site from which the virus was isolated is important; e.g., one would be quite confident about the etiological significance of equine herpesvirus 1 isolated from the tissues of a 9-month-old aborted equine fetus with typical gross and microscopic lesions, or of distemper virus isolated from the cerebrospinal fluid of a dog with encephalitis, because these sites are usually sterile, i.e., they have no normal bacterial or viral flora. On the other hand, recovery of an enterovirus from the feces, or a

herpesvirus from a nasal or throat swab may not necessarily be significant, because such viruses are often associated with inapparent infections at these sites.

2. Interpretation of the significance of the isolation in such instances will be facilitated by recovery of the same virus from several cases of the same illness during an epizootic.

3. Knowledge that the virus and the disease in question are often causally associated provides confidence that the isolate is significant.

Laboratory Safety

It is appropriate to conclude this section with some remarks about safety precautions in virus diagnostic laboratories in general and regulations about exotic viruses in particular. Diagnostic virology is one of the less hazardous human occupations, but over the years a number of deaths have been caused by laboratory-associated infections. Some of the commoner hazards are listed in Table 13-5. It is important to note that many of the procedures that may be dangerous for laboratory workers, particularly aerosolization, may also be sources of laboratory contamination—something that may give rise to mistaken diagnoses and sometimes a great deal of misdirected administrative action.

Precautions to avoid these hazards consist essentially of good laboratory technique, but special measures may be needed. Mouth-pipetting is banned. Gowns must be worn at all times, and gloves for anything

TABLE 13-5
Laboratory Hazards

Hazard	Cause
Aerosol	Homogenization (e.g., of tissue in blender)
	Centrifugation
	Ultrasonic vibration
	Broken glassware
Ingestion	Pipetting
	Mouth pipetting
	Eating or smoking in laboratory
Skin penetration	Inadequate washing/disinfection of hands
	Needle prick
	Hand cut by broken glassware
	Leaking container contaminating hands
	Pathologist handling infected organs
	Splash into eye
	Animal bite

TABLE 13-6
*Summary of Recommended Biosafety Levels for
 Infectious Agents^a*

Biosafety level	Practices and techniques	Safety equipment	Facilities
1	Standard microbiological practices	None: primary containment provided by adherence to standard laboratory practices during open-bench operations	Basic
2	Level 1 practices, plus laboratory coats, decontamination of all infectious wastes, limited access, protective gloves and biohazard warning signs as indicated	Partial containment equipment (i.e., class I or II biological safety cabinets) used to conduct mechanical and manipulative procedures that have high aerosol potential that may increase the risk to personnel	Basic
3	Level 2 practices, plus special laboratory clothing, controlled access	Partial containment equipment used for all manipulations of infectious material	Containment
4	Level 3 practices, plus entrance through change room where street clothing is removed and laboratory clothing is put on, shower on exit, all wastes are decontaminated on exit from the facility	Maximum containment equipment (i.e., class III biological safety cabinet or partial containment equipment in combination with full-body, air-supplied, positive-pressure personnel suit) used for all procedures and activities	Maximum containment

^aFrom Centers for Disease Control and National Institutes of Health, "Biosafety in Microbiological and Biomedical Laboratories." U.S. Government Printing Office, Washington, D.C., 1984.

dangerous. Various classes of safety cabinets are available for procedures of various degrees of hazard; their use is summarized in Table 13-6.

Besides personal hazard, exotic animal viruses pose special community risks such that major developed countries with large livestock indus-

TABLE 13-7
Animal Viruses the Importation of Which is Restricted^a

Virus	Family	Virus	Family
African horse sickness virus	<i>Reoviridae</i>	Nairobi sheep disease virus	<i>Bunyaviridae</i>
African swine fever virus	Unclassified	Newcastle disease virus (velogenic strains)	<i>Paramyxoviridae</i>
Borna disease virus	Unclassified	Porcine polio-encephalomyelitis virus	<i>Picornaviridae</i>
Bovine ephemeral fever virus	<i>Rhabdoviridae</i>	Rift Valley fever virus	<i>Bunyaviridae</i>
Camelpox virus	<i>Poxviridae</i>	Rinderpest virus	<i>Paramyxoviridae</i>
Foot-and-mouth disease virus	<i>Picornaviridae</i>	Swine vesicular disease virus	<i>Picornaviridae</i>
Fowl plague virus	<i>Orthomyxoviridae</i>	Vesicular exanthema virus	<i>Caliciviridae</i>
Lumpyskin disease virus	<i>Poxviridae</i>	Wesselsbron disease virus	<i>Flaviviridae</i>

^aFor the United States. In other developed countries there are similar listings, some even longer; e.g., the Australian list includes, as well as these, bluetongue viruses, epidemic hemorrhagic disease of deer virus, hog cholera virus, malignant catarrhal fever virus, ovine progressive pneumonia virus, pseudorabies virus, rabies virus, the scrapie agent, and sheeppox virus, but it excludes bovine ephemeral fever virus, which is enzootic in Australia.

tries support special laboratories for their investigation. These are the so-called maximum containment laboratories, popularly designated by their location, e.g., Plum Island in the United States, Pirbright in the United Kingdom, and Geelong in Australia. "Restricted" animal viruses in the United States and Australia, the importation, possession, or use of which is prohibited or restricted by law or regulation, are listed in Table 13-7.

DIRECT IDENTIFICATION OF VIRUS, VIRAL ANTIGEN, OR VIRAL NUCLEIC ACID

We use the term direct identification, in contrast to virus isolation, to encompass a variety of methods that can be used to detect and often identify the etiological agent by the direct demonstration of virions or

viral constituents in the tissues, secretions, or excretions of infected animals. Although they do not provide the laboratory worker with a culture of the causative virus for further study, these direct methods have great advantages in terms of speed, cost, and the number of samples that can be examined. They can be subdivided into methods used to detect virions, viral antigens, or viral nucleic acids.

Direct Detection of Virions by Electron Microscopy

The introduction of negative staining procedures, together with a realization that in many clinical situations the concentration of virions frequently exceeds the critical lower limit of 10^6 per milliliter required for visualization in the electron microscope, has led to the use of this instrument for rapid viral diagnosis (Plate 13-5). The procedure is particularly suited to enteric infections, in which a crude fecal suspension can be clarified by low-speed centrifugation, followed by high-speed centrifugation to yield a pellet for negative staining. In addition to its value in the recognition of known viruses, this technique has led to the discovery of new viruses of etiological importance in diarrheal diseases which were, and in some cases remain, uncultivable (e.g., some adenoviruses, astroviruses, caliciviruses, coronaviruses, parvoviruses, and rotaviruses).

The procedure is also suited to viral infections of the skin and mucous membranes, the appropriate specimen being scabs, vesicular fluid, or scrapings made with a scalpel. Also, as described earlier, electron microscopy can be used for the rapid identification of viruses isolated in cell culture, allowing immediate and definitive diagnosis to the family or sometimes the genus or species level.

The sensitivity of electron microscopic methods can be enhanced by the use of immune serum, by a procedure known as immunoelectron microscopy. The sample, usually clarified by low-speed centrifugation, is mixed with antibody, and after overnight interaction, the immune complexes are pelleted by low-speed centrifugation and the pellet negatively stained. The antibody used may be serum from an old animal hyperimmune to a large number of viruses, or it may be type-specific polyclonal or monoclonal antibody, or such antibodies may be used sequentially. Solid-phase immunoelectron microscopy procedures have also been developed, in which virus-specific antibody is first bound to the plastic supporting film on the copper grid. Sensitivity is enhanced by a double-layering procedure, in which staphylococcal protein A (which binds the Fc moiety of IgG) is bound to the film, then virus-specific antibody, to which the sample is then added.

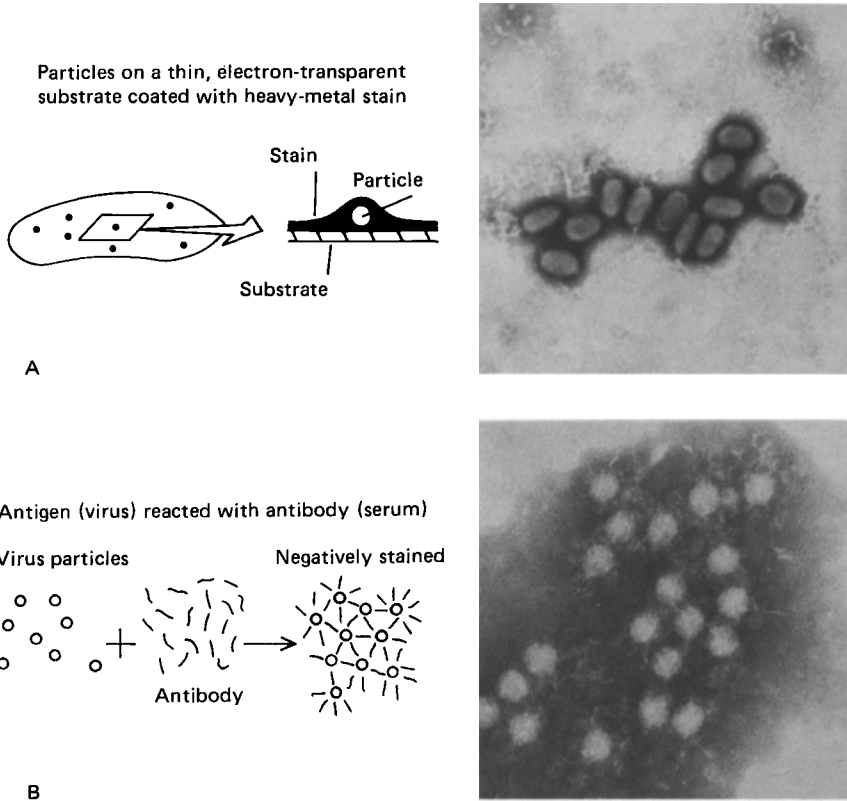


PLATE 13-5. *Negative staining for electron microscopy.* (A) *Direct staining:* virions of bovine papular stomatitis virus. An electron-opaque stain (1% phosphotungstic acid, pH 7.3) was mixed with scrapings from the lesion and applied to plastic film supported by the copper electron microscope grid ($\times 13,000$). (B) *Immunolectron microscopy:* an isolate of foot-and-mouth disease virus type O was incubated with homotypic antiserum and stained with phosphotungstic acid ($\times 100,000$). Note aggregation of virions by antibody. [From E. P. J. Gibbs et al., *Vet. Rec.* **106**, 451 (1980).]

Direct Detection of Viral Antigen(s)

These methods are based on direct interaction between virions or viral antigens, *in situ* in tissues or in excretions or secretions, and specific antibodies which are pre-labeled in some way so as to permit the ready recognition of the interaction. The methods are specified by the method of labeling used: immunofluorescence, immunoperoxidase staining, radioimmunoassay, or enzyme-linked immunosorbent assay (ELISA). Vir-

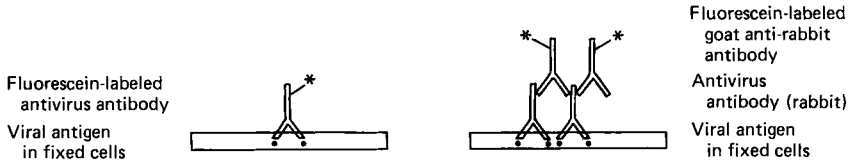


FIG. 13-1. Immunofluorescence. Left, direct. Right, indirect.

al antigens can also be detected by such time-honored serological procedures as precipitation and complement fixation.

Immunofluorescence. Its specificity, sensitivity, rapidity, and relative simplicity make immunofluorescence a procedure of singular importance in the rapid diagnosis of viral infections. The prototypic example of immunofluorescence is the diagnosis of rabies, for which it has been the standard test for more than 20 years (see Chapter 30). It is now being used for a wide range of viruses. Immunofluorescence can be applied to smears and frozen sections of tissues or organs.

Two alternative staining procedures are used: (1) direct immunofluorescence, in which the antiviral antibody is conjugated to the fluorescent dye, fluorescein, and (2) indirect ("sandwich") immunofluorescence, in which an antiimmunoglobulin specific for the animal species providing the antiviral antibody is conjugated to fluorescein (Fig. 13-1). For instance, an acetone-fixed smear or frozen tissue section is treated with virus-specific antibody (prepared, say, in rabbits), then rinsed before the second antibody, a fluorescein-conjugated anti-rabbit immunoglobulin made in goats, is added. Indirect procedures have two significant advantages over direct-staining procedures:

1. If antibodies to different viruses are raised in a single animal species, e.g., rabbits, then only a single conjugated antibody is required.
2. The amount of bound labeled antibody is greatly augmented, hence the method is much more sensitive. Although simple in principle, the effective use of immunofluorescence demands careful attention to many technical details if false positive results are to be avoided.

In addition to immunofluorescent staining of specimens taken directly from clinical cases, the method is an important adjunct in the identification of viruses isolated in cell cultures. It may also be used in reverse, for the detection of antibody in serum. Slides containing viral antigen, either smears, sections, or, more usually, cell cultures, are prepared in large numbers and stored at -70°C . For use, they are flooded with the serum under test and a second fluorescein-conjugated antispecies antibody is used to detect the bound antibody.

Special care needs to be exercised in the application of immunofluorescence to herpesviruses, in that some herpesvirus-infected cells are known to express Fc receptors on their plasma membrane; such receptors bind all IgG molecules, not only those with herpesvirus specificity, hence additional controls are required.

Immunoperoxidase Staining. An alternative method for locating and identifying viral antigen in infected cells employs an enzyme-labeled antibody. The procedure requires less expensive equipment than immunofluorescence—an ordinary light microscope is used—and produces a morphologically clearer, nonfading, permanent preparation. The procedures and principles are similar to those of immunofluorescence. The conjugated antibody, bound to antigen by a direct or indirect procedure, is detected by adding a substrate appropriate to the particular enzyme; in the case of peroxidase this is H_2O_2 mixed with a benzidine derivative which forms a colored, insoluble precipitate in the presence of enzyme. A disadvantage of the technique is that endogenous peroxidase present in the cells of many tissues, particularly leukocytes, produces false positive results. This problem can be circumvented by meticulous technique and adequate controls.

Radioimmunoassay. In radioimmunoassay the label is a radioactive element, commonly ^{125}I . The method is exquisitely sensitive, enabling viral antigens to be detected at concentrations as low as $10^{-12} M$. Many protocols for radioimmunoassays have been devised. Both direct and indirect methods can be used, the principles being the same as for immunofluorescent staining (Fig. 13-2). Most are solid-phase procedures in

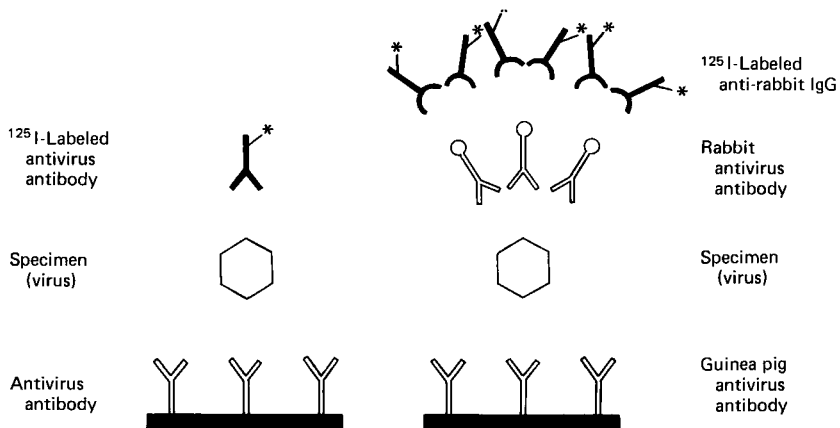


FIG. 13-2. Radioimmunoassays for detection of virus and/or viral antigen. Left, direct. Right, indirect.

which the “capture” antibody (or antigen) is bound to a solid substrate, typically a polystyrene tube or bead, or to the wells of a plastic microtiter plate. In the simplest format (Fig. 13-2, left) the sample suspected to contain virus or viral antigen is allowed to bind to the bound antibody, then after washing, ¹²⁵I-labeled antiviral antibody (“detector” antibody) is added. After a further washing, the bound labeled antibody is measured in a gamma counter. A more commonly used protocol is the indirect radioimmunoassay, in which the detector antibody is unlabeled but a further layer, ¹²⁵I-labeled anti-IgG, is added as “indicator” antibody. (The antiviral antibodies constituting the capture and detector antibodies must be raised in different animal species; see Fig. 13-2, right.)

Enzyme-Linked Immunosorbent Assays (ELISA). ELISA (also known as enzyme immunoassay, EIA) offers the same sensitivity as radioimmunoassay without the inherent disadvantages of expensive isotopes of short half-life and the need for safe handling and disposal and a costly gamma counter. The basic principles are similar to those of radioimmunoassay (Fig. 13-3). Antibody is bound to a solid phase, usually the wells of a microtiter tray. Samples suspected to contain antigen are added to the wells. After an appropriate reaction time, the wells are rinsed and a second virus-specific antibody that has been conjugated to an enzyme is added. After allowing this to bind, the contents of the well are rinsed and a substrate for the enzyme is added. The assay is read by

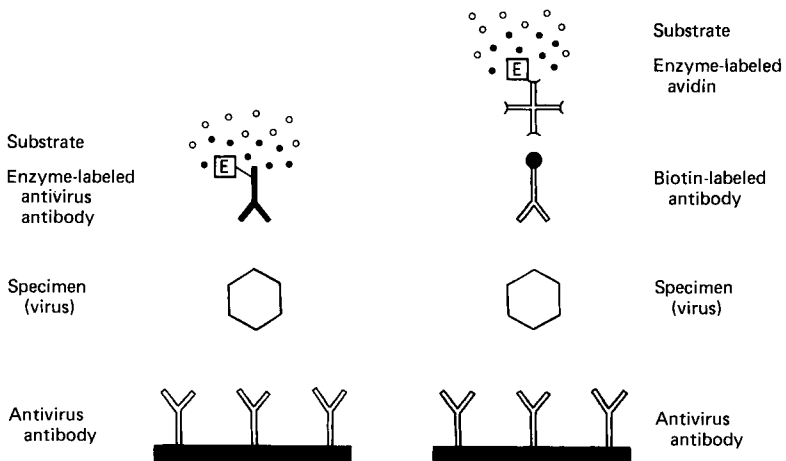


FIG. 13-3. Enzyme-linked immunosorbent assay (ELISA) for detection of virus and/or viral antigen. Left, direct. Right, avidin–biotin.

a color change in the substrate and can be made quantitative by serially diluting the antigen to obtain an end point or by photometrically reading the amount of color change, a reflection of the amount of enzyme-conjugated antibody bound. As in radioimmunoassay, there are many variations in protocol, e.g., exploiting the very high affinity of avidin for biotin (Fig. 13-3, right). Moreover, if antigen is bound to the plate first, the procedure is equally suitable for the detection and quantitation of viral antibody.

ELISA procedures have been developed for a wide variety of applications in veterinary medicine. At one level, kits have been marketed for the rapid diagnosis of a number of important viral diseases by veterinary practitioners themselves. At another level ELISA procedures have been automated by the introduction of automatic dispensing, washing, and spectrophotometric reading and recording instruments, that permit hundreds of samples to be processed in a day, e.g., in the testing of swine for pseudorabies antibodies.

Immunodiffusion (Precipitation-in-Gel). If wells are cut in agar and antibody and antigen are placed in separate wells, the two diffuse toward each other (immunodiffusion) and form visible bands of precipitate (Plate 13-6). Several ingenious applications of the procedure have been developed and the test is widely used for the diagnosis of some diseases of domestic animals (e.g., bovine leukemia, equine infectious anemia; see Chapter 31).

Complement Fixation. Although now considered too cumbersome a procedure for general use in the rapid detection of viral antigen, the complement fixation procedure is still employed for the rapid and specific detection of foot-and-mouth disease viral antigen in vesicular fluid, providing both rapid diagnosis and specific typing of the virus involved.

Direct Detection of Viral Nucleic Acid

If dsDNA is separated ("melted") into single strands by heat or alkali treatment, the single strands will, under appropriate conditions, reanneal to each other, or competitively to an identical or related complementary strand. If the original DNA is labeled with either ^{32}P or ^{35}S , it may be used as a probe for the detection of related DNA in infected cells. This procedure, known as *in situ* hybridization, can be made even more specific by using probes that are shorter than the full-length genome. It can also be made more sensitive by maximizing the amount of label incorporated into the probe. Hybridization is detected by autoradiography. Recently, nonradioactive hybridization procedures, based on the

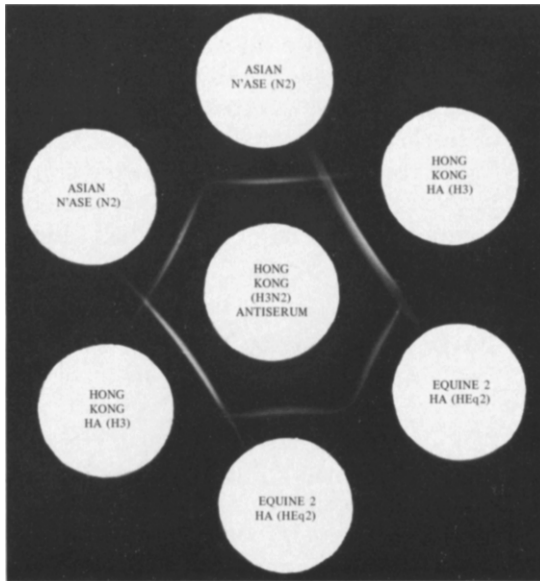


PLATE 13-6. Immunodiffusion test. Example illustrates its use to analyze relationships between envelope antigens of influenza A virus. Center well: antiserum to Hong Kong influenza virus (H3N2). Peripheral wells, purified antigens: Hong Kong hemagglutinin (H3), Asian neuraminidase (N2), and equine 2 hemagglutinin (HEq2). Antiserum to Hong Kong virus contains antibodies to all the antigens tested. Note (1) two pairs of antigens (N2 and HEq2) each show fusion of precipitin lines ("reaction of identity"), (2) neuraminidase N2 and hemagglutinin H3 show complete crossing over of precipitin lines ("reaction of complete nonidentity"), and (3) equine (HEq2) and Hong Kong (H3) hemagglutinins show partial fusion of lines ("reaction of partial identity") indicating serological cross-reactivity. (Courtesy Dr. R. G. Webster.)

incorporation of biotin-conjugated nucleotides into the DNA probe, have been developed; avidin, which binds strongly to biotin, is subsequently added. The avidin is detected by ELISA, immunofluorescence, or immunoperoxidase staining.

In situ hybridization procedures are particularly useful when viral DNA is present in cells but is not expressed, as with integrated retroviral DNA, or episomal DNA in some papovavirus-infected cells. Probes can be made highly specific by selection from a collection of cloned fragments of the whole viral genome. The probes are labeled by *in vitro* nick translation procedures, and are then applied to nitrocellulose blot transfers of the animal tissue (active hybridization) or to nitrocellulose blots

taken from gels on which viral nucleic acid has been separated (Southern blotting), or from nitrocellulose onto which viral nucleic acid-containing samples have been spotted (dot-blot hybridization). These procedures have proved of great value in virus research, but it remains to be seen to what extent nucleic acid probes and hybridization procedures displace other methods for rapid diagnosis of viral infections. It may have advantages over virus isolation in the case of viruses that are noncultivable, slow growing, dangerous, or nonviable as a result of suboptimal conditions of transport or storage.

DETECTION AND MEASUREMENT OF ANTIVIRAL ANTIBODIES

Detection of viral antibody can be used for the diagnosis of viral infections, either in individual animals or in populations. The method is particularly useful in the latter context, since serum samples are readily obtained with simple equipment, in contrast to special requirements, time, and effort needed for collecting samples for virus isolation. Furthermore, tests for antibody such as ELISA lend themselves to automation, so that large numbers of samples can be tested. They form the basis of epidemiological surveys and of control and eradication programs, but have major limitations in diagnosis.

For diagnosis in the individual animal, paired sera are tested for specific viral antibody, the first sample being taken when the animal is first examined (acute-phase serum), and the second sample 2–4 weeks later (convalescent-phase serum). A rise in antibody titer between the first and second samples is a basis, albeit in retrospect, for a specific viral diagnosis. Sometimes the demonstration of antibody in a single serum sample is diagnostic of current infection, e.g., with retroviruses and herpesviruses, since these viruses establish lifelong infections. However, in such circumstances there is no assurance that the persistent virus was responsible for the disease under consideration.

Detection of antiviral antibody in presuckle newborn cord or venous blood provides a basis for specific diagnosis of *in utero* infections. It was used, for example, in showing that Akabane virus was the cause of arthrogryposis–hydranencephaly in calves (see Chapter 29). Since transplacental transfer of immunoglobulins is rare in domestic animals (see Table 9-3), the presence of either IgG or IgM is indicative of exposure of the fetus to antigen.

Serological methods based on the detection of virus-specific IgM may also be used for the specific diagnosis of recent viral infection, since antibodies of the IgM class appear first after primary infection and de-

clines to relatively low levels, compared to IgG, by about 3 months after infection. However, the method has not yet been much exploited in veterinary medicine.

Technical advances such as miniaturization (microtiter plates), automation for large numbers of samples, monoclonal antibodies, and the development of diagnostic kits such as latex agglutination assays for detecting specific IgM, have resulted in a revolution in the approach to diagnostic serology in human medicine. The costs, coupled with the technical problems associated with the large number of animal hosts and their many viruses, have delayed the development of these procedures in veterinary medicine, but their use can be expected to expand considerably in the future. However, screening programs to establish regional or national prevalence rates for particular viruses, based on detection of specific antibody in single serum samples, are an essential feature in defining the epidemiology of viruses of domestic animals.

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