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Cultivation and Characterization of Viruses

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Methods have been developed for the storage, visualization, quantification (direct and indirect), and propagation of viruses. There are also methods for the laboratory diagnosis of viral diseases, many of which are serological, based on detection of the response of the host to infection. These diagnostic methods are discussed in Chapter 7.

Historically it was noted that the contagion of some diseases was able to pass through a filter that bacteria could not. The filtrates obtained failed to yield growth on bacterial media and were eventually shown experimentally to be infective and contain viruses. Except for large poxviruses, viruses could not be seen with the light microscope. Eventually viruses were observed with the electron microscope. Some of the important methods used in the basic study of viruses are described below. As indicated in the previous chapter, there is considerable diversity in the physical characteristics of animal viruses. The feature that most reflects the properties of the virions is the presence or absence of a viral envelope. As outlined in Table 2.1, nonenveloped viruses are in general sensitive to ultraviolet radiation, are relatively thermostable, and susceptible to ice crystal damage. Due largely to the presence of the membrane envelope, enveloped viruses are inactivated by lipid solvents (such as chloroform and ether) and detergents (such as deoxycholate), sensitive to ultraviolet and gamma radiation, relatively thermolabile, and more extensively damaged by ice crystal formation than nonenveloped viruses.

Table 2.1. Important Biological, Physico-chemical Properties of Enveloped and Nonenveloped Virions

Characteristic	Nonenveloped Virus	Enveloped Virus
Ultraviolet radiation	Sensitive	Sensitive
Gamma radiation	Sensitive	Sensitive
Thermostability	Thermostable	Thermolabile
Susceptibility to ice crystal damage	Yes	Extensive
Inactivation by lipid solvents and detergents	No	Yes

Viral Propagation Methods

To isolate, characterize and identify viruses and to produce virus vaccines a considerable amount of virus particles is

required. This is accomplished by several propagation procedures as follows:

Host Animals
Viral propagation in uninfected susceptible host organisms was once the only way to obtain large quantities of virus. Currently, the use of experimental host animals for virus propagation is for ethical reasons limited. Virus propagation in animals is more useful for those viruses that do not grow easily in cell culture. For example, vaccine strains of turkey hemorrhagic enteritis virus can either be propagated in live birds or in cell culture. However, splenic (live-bird) propagated product appears to be the more widely used of the two.
For diagnostic purposes, inoculation of animals is a means of detecting virus in clinical samples, such as rabies virus inoculation of suckling mice.

Embryonated Eggs
The use of embryonated eggs for propagation of viruses was one of the first alternatives to the use of host organisms for animal viruses prior to the development of cell and tissue culture techniques. Embryonated eggs are still the preferred method for the propagation of influenza A viruses and many avian viruses. Embryonated eggs are also useful in the differentiation of some viruses that produce similar lesions, such as cowpox and pseudocowpox viruses. Although bluetongue virus (BTV) is a virus of mammals, it replicates well in embryonated eggs and this system is used for virus propagation for diagnostic and research purposes.
When using embryonated eggs, one needs to consider the possibility of the presence of maternal antibodies (IgY) in the egg yolk sac. Consequently, it is often preferable to obtain embryonated eggs from specific pathogen-free (SPF) flocks. Passage through embryonic eggs is useful in the attenuation of certain viruses for modified live virus vaccines.

Cell/Tissue Culture
Tissue culture refers to the growth and maintenance of living tissue cells in vitro. There are two basic types: explant culture and cell cultures. Explant cultures are small tissue fragment samples from the host that are maintained in culture, while cell cultures result from the breaking down of various host tissues into individual cells. Most systems used in virology are indeed cell cultures and not tissue culture, though both terms are used interchangeably. Cell cultures can be further subdivided into primary, semi-continuous, and continuous cell cultures.

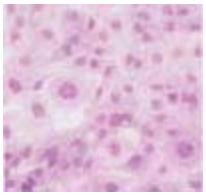

Figure 2-1. Normal cell culture. *Courtesy of A. Wayne Roberts*. - To view this image in full size go to the IVIS website at www.ivis.org . -

Explant Culture
These are cultures of small fragments of specific tissue removed from the host animal directly. Explant cultures are useful for virus isolation and required for the isolation of some coronaviruses. Demonstration of latency by human and animal alpha herpesviruses may require explant cultures of nerve sensory (i.e., trigeminal) ganglia.

Primary Cell Cultures
These are derived from fresh tissues that have been enzymatically digested with trypsin or other protease to release individual cells. As a result, primary cultures are often composed of many different cell types. Under in vitro conditions, primary culture cells rarely divide or divide at a very low frequency. Therefore, they have a finite lifetime, referred to as the Hayflick limit. In spite of the life span of the primary cultures, they are ideal for the isolation of some viruses. Primary cultures rarely survive beyond passage 20th in vitro.

Semi-continuous Cultures
Also known as diploid cell lines, as they contain the normal diploid chromosome characteristic of the species from which they were derived. Semi-continuous cultures are primary cultures that have some cells that can be nurtured to survive beyond the Hayflick limit. Semi-continuous cultures tend to die out between the 30th and 50th passage in vitro. In spite of this limitation, semi-continuous cultures are useful in the propagation of a wide range of viruses. Semi-continuous cultures are usually fibroblastic.

Continuous Cell Cultures
Also known as heteroploid cell lines, as the cells have an abnormal number of chromosomes. These cell cultures are derived from normal or neoplastic tissue and are characterized by their ability to be propagated in vitro indefinitely. In general, continuous lines are not as sensitive as the other types for viral propagation. However, they facilitate the large-scale growth of some viruses for vaccines and research. Many continuous lines are available from repositories, such as the American Type Culture Collection (ATCC).

Most virology laboratories freeze early stocks of the continuous cells as cell lines in continuous culture may change their cellular characteristics. Causes of changes in cellular characteristics may be infection by Mycoplasma spp. or contaminating viruses (e.g., swine circovirus and bovine viral diarrhea virus).

Concentration and Purification of Viruses

Once a virus has been adequately propagated, it needs to be recovered from host cells and debris and purified. This is accomplished by a number of processes that involve differential centrifugation (various speeds), dialysis, precipitations, chromatography and density gradients. The initial step of this process is differential centrifugation; a slow speed (~2,000 x g) is used to remove large cellular debris. This is followed by high-speed centrifugation (40K to 80K x g) to concentrate the virus for small volumes; by dialysis and precipitation for larger volumes; and by cold (-70°C) methanol or polyethylene-glycol precipitation, also for large volumes. Purification is achieved through chromatography and centrifugation through density gradients. Enveloped viruses can be purified by velocity sedimentation through sucrose gradients. Nonenveloped viruses can be purified by centrifugation through cesium chloride gradients.

Infectivity and Storage

Infectivity

Infectivity is a virus particle's ability to infect a host cell. The temperature outside of a host cell readily affects the virus' ability to retain its infectivity, particularly in the case of enveloped viruses. As viruses have no metabolic activity of their own, infectivity is the best means to evaluate the integrity of the viral particle following exposure at a particular temperature. The following are important considerations:

- At 60°C, infectivity of the virus will decrease rapidly within seconds.
- At 37°C, infectivity will decrease dramatically within minutes.
- At 20°C, infectivity decreases within hours.
- Infectivity at the above temperatures influences viral spread by direct contact (at 37°C) and by fomites (at 20°C).
- At 4°C, infectivity in tissues is lost over days. Clinicians should keep this in mind regarding clinical specimens.

Temperatures below freezing are often used for long-term storage. The important consideration is keeping ice crystal formation to a minimum.
It should be kept in mind that viruses vary greatly in their resistance and lability. Some are able to survive for hours, days, and even months under environmental conditions, while others are inactivated in a few minutes under similar conditions.

The three principal methods of storing viruses are:

- Freezing at -70°C with or without a cryopreservative.
- For long term storage, freezing in liquid nitrogen (-196°C).
- Lyophilizing or freeze drying with storage in a freezer or at room temperature.

Virus Visualization

The two major methods mainly used to visualize the structure/morphology of viruses are electron microscopy and atomic force microscopy. Other types of microscopy are used to observe changes induced by virus replication in virus-infected cells. Without a means to visualize viruses, it is difficult to obtain information about structure or virus-cell interactions. Furthermore, being able to visualize viral particles allows one to estimate the number of particles present in a suspension directly. There are other methods that allow one to estimate the number of viruses indirectly. In either case, direct or indirect, enumeration quantification is always an estimate of numbers. This estimate is important when preparing vaccines, when determining the minimum number of virions required to produce disease, and in viral research procedures.

Light Microscopy

While the light microscope is not useful for the direct examination of viruses (except poxviruses), it is useful for observing the effects of viral infection on the host cell. The virus-caused cell damage or destruction is referred to as the cytopathic effect (CPE). Observable cytopathic effects include:

1. Cells rounded up and aggregated in grape-like clusters, as with adenoviruses;
2. Cells round up, shrink, and lyse, leaving large amounts of cellular debris, as with enteroviruses;
3. Cells become swollen and round up in focal areas, as with herpesviruses; and
4. Cells fuse producing multinucleate cells (syncytia), as with paramyxoviruses.

Additionally, inclusion bodies, characteristic of some viruses, can be visualized.

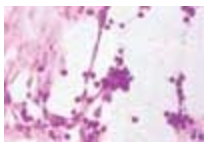

Figure 2-2. Equine "slow herpesvirus" cytopathic effect. *Courtesy of A. Wayne Roberts*. - To view this image in full size go to the IVIS website at www.ivis.org . -

Fluorescence Microscopy

Fluorescence microscopy can be used to visualize virus-infected cells or tissues using virus antigen-specific fluorochrome tagged antibody. The antibody binds specifically to virus antigens within the cells or tissues and thus labels them with a fluorescent tag (usually fluorescein). The fluorescent tag is then visualized with a UV microscope that excites the fluorochrome, which one sees as a colored focus with a relatively dark background. Alternatively, visualization can be performed indirectly by using unlabeled antibodies (as found in convalescent serum) followed by fluorochrome labeled antibodies that bind the first antibody. Fluorescent antibody based assays are commonly used in viral diagnosis and research.

Electron Microscopy

Electron microscopy involves the acceleration of electrons to high energy and magnetically focusing them into the sample. The high-energy electrons have very short wavelengths and thus provide better resolution of very small structures. Electron microscopy has enough resolution power to visualize large polymers, such as DNA, RNA, and large proteins.

To facilitate visualization, samples may be coated with heavy metals, such as osmium, prior to examination by electron microscopy. The electrons hit the heavy metals, which are then visualized on a fluorescent screen. Electron microscopy yields 3-dimensional images of virions and their localization within the host cell (nuclear or cytoplasmic) at a given point in time following infection. As the samples are treated with heavy metals, observing virions within live cells is not possible.

Atomic Force Microscopy

The atomic force microscope works by measuring a local property (such as height, optical absorption, magnetism, etc.) with a probe placed very close to the sample. This makes it possible to take measurements over a small area of the sample. Electrons are able to "tunnel" between atoms, resulting in a small, but measurable force. The result of these measurements is a detailed contour map of the surface of a structure.

The advantages of atomic force microscopy are minimal sample preparation and use on living specimen. This method has been useful for detailed images of capsid structures and virus-cell interactions.

Immunoelectron Microscopy

This technique allows the visualization of antibody/antigen complexes that are specific to a particular virus. In this method, ultrathin sections are cut and incubated with antibody that is specific for the virus. Following a washing step, the section is incubated with Protein A conjugated gold particles (size range is 5 to 20 nm). The Protein A gold particles bind to the Fc portion of the antibody and are detected by electron microscopy.

Direct Enumeration of Viruses

Estimating the number of viruses has a number of important uses including research and vaccine production. Electron microscopy is used for the enumeration of viral particles in a cell free solution. A known volume of sample is examined and the number of virions counted. This number is then used to estimate the number of viruses. One limitation is that empty capsids, thus non-infective particles, are also counted. In research, the number of infectious particles and the total number are compared and establish a ratio of total particles/infectious particles for a given virus.

Indirect Enumeration of Viruses

Indirect methods of viral enumeration are those that utilize factors associated with infectivity (biological activity). The three principal methods used to indirectly assess viral concentrations are hemagglutination assays, plaque forming assays, and the limiting dilution method.

Hemagglutination

This assay is based upon the property of many enveloped viruses to agglutinate red blood cells (RBCs).

The assay is carried out by adding red cells to dilutions of the virus sample in a microtiter plate, then observing for hemagglutination. It takes many viruses to coat RBCs and result in hemagglutination. For example, it takes approximately 10^4 influenza virions per hemagglutination unit (HA unit). An HA unit is defined as the highest dilution of the viral sample that causes complete hemagglutination.

Hemagglutination is useful in the concentration and purification of some viruses, and as a rapid presumptive test for the presence of these viruses in fluids from infected cell cultures and chicken embryos. It is especially useful for assaying viral activity of cell cultures infected with hemagglutinating viruses that produce little or no discernible cytopathic effect (CPE). Clinical specimens such as feces can also be directly examined for hemagglutinating activity of particular viruses (discussed further in Chapter 7).

Similar type assays that test for enzyme activity of a particular virus (such as one producing reverse transcriptase) can be performed in a similar manner.

Plaque Forming Assay

This assay involves the inoculation of susceptible host cells with virus and using their biological activity to estimate the

number of virions present.
In the procedure, ten-fold serial dilutions of virus sample are used to inoculate monolayers of host cells. Following incubation to allow the virions to adsorb to the surface of the host cells, the monolayer is overlaid with a gel composed of host cell medium and agarose. The presence of the agar prevents viral spread in the culture of host cells on a large scale, but allows localized cell-to-cell spread. With cytopathic viruses, host cell destruction results in the development of clear zones called plaques, which can be visualized 24 to 72 hours of incubation. A calculation involving the number of plaques observed, the dilution factor of the sample, and the volume of sample dilution used, yields the plaque forming units (PFU) per milliliter of sample.

The Limiting Dilution Method
This titration-based assay measures an effect on cells in vitro, such as CPE, when exposed to various dilutions of a virus containing solution. If possible, a known concentration of reference virus culture is used as a positive control. Depending upon the virus, either two-fold or ten-fold serial dilutions of the viral material are made and placed with the cells. The infectivity titer (reciprocal of the highest dilution showing 50% CPE of the infected cultures) is expressed as the $TCID_{50}$/ml (tissue culture infectious dose). This assay may be used with cultured cells, embryonated eggs or even in laboratory animals.

Miscellaneous Methods Used for Characterization

There are some methods used in virology that are helpful in the identification and classification of an unknown virus. Some of the techniques will be briefly mentioned here, but explained in greater detail later if they are used in the laboratory diagnosis of a particular virus.

Sensitivity to Lipid Solvents
The sensitivity of viruses to lipid solvents, such as chloroform and ether, aids in the taxonomy of some viruses. Any viruses that possess a membranous outer envelope are susceptible to lipid solvents. All enveloped animal viruses, except some poxviruses, are ether sensitive.

Identification of Nucleic Acid Type
This is performed by examining nucleic acid synthesis in cell cultures in the presence of DNA synthesis inhibitors, such as 5-bromo-2-deoxyuridine (BRU). If viral synthesis is inhibited, then virus multiplication will likewise be decreased. In the event that virus growth is not inhibited, the virus is presumed to contain RNA.

Restriction Enzyme Analysis
Restriction enzymes (RE) are endonucleases that cut double-stranded DNA at specific recognition sites, ranging from four to eight base pair palindromic sequences.
Restriction endonuclease analysis is particularly useful in the "subserotypic" classification of viruses, in the differentiation of modified-live virus of vaccines from virulent virus, and in the epidemiologic tracking of disease outbreaks. Procedurally, the method entails treating viral DNA with one or more REs, and then separating the resulting fragments according to size by polyacrylamide gel electrophoresis.
RNA viruses can be similarly analyzed by first making a complementary DNA (cDNA) strand from the RNA using the enzyme reverse transcriptase, and then amplifying this cDNA by the PCR method described in Chapter 7.

Hemadsorption
Membrane-bound viruses such as orthomyxoviruses and paramyxoviruses obtain their outer envelope by budding through the cell membrane. Prior to budding, viral coded proteins (hemagglutinins) are incorporated into the cell membrane. Such cells will adsorb erythrocytes to their surfaces, and the resulting foci of hemadsorption can be detected microscopically.

Immunological Methods
Animals infected with viruses respond by producing specific antibodies. Detection and measurement of these antibodies, which reflect disease status, are useful in planning herd health programs and studying the epidemiology of disease outbreaks.
While detection of antibodies is also useful in disease diagnosis, it is often a time-consuming process requiring the comparative measurements of antibody in acute and convalescent sera, usually collected 10 to 14 days apart. A more rapid approach is to use specific antiviral antibodies to detect viral antigens directly in clinical specimens. These antibodies are usually obtained by hyperimmunizing rabbits or goats with a specific virus. Alternatively, monoclonal antibodies may be used, if available.
Monoclonal antibodies (mAbs) are prepared in mice by first exposing the mouse to the viral antigen, which sensitizes B cells of the spleen. These cells are collected and chemically fused with a mouse plasmocytoma cell line that secretes IgG. These hybrid cells are then cloned and the resulting hybridomas, which are derived from a single cell, are analyzed for secretion of the specific antiviral IgG. Selected hybridoma cells are injected back into mice intraperitoneally, where the cells grow rapidly, and cause an accumulation of ascitic fluid containing a high concentration of mAb. Figure 2.1 depicts

the steps involved in preparing monoclonal antibodies.
Monoclonal antibodies are particularly useful in typing and subtyping viruses. When coupled to a fluorochrome, mAbs are widely used for the detection of viruses in tissues. They are also used in a number of commercial ELISAs for identification of viruses.
The more common tests that are mainly used in diagnostic or clinical virology are discussed in Chapter 7.

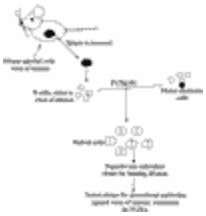

Figure 2-3. The steps associated with the development of specific monoclonal antibodies. - To view this image in full size go to the IVIS website at www.ivis.org . -

Glossary
Cytopathic viruses: These are viruses that alter the microscopic appearances of cells in culture. The changes may include rounding up of cells, cell fusion, cell detachment, production of inclusion bodies, etc.
Density gradients: This is a procedure for separating cells or large molecules, such as proteins and nucleic acids, usually by centrifugation through a density gradient. The latter consists of a solution in which there is a range of densities with the solute (generally sucrose or cesium chloride) less concentrated at the top and more concentrated at the bottom. As a result of centrifugation the cells or molecules move through the gradient and form a band at the density where their specific gravity is equal to that of the medium.
Palindromes: Sequences that read the same in both directions. Most recognition sites of restriction endonucleases are palindromes, e.g., the recognition sequence of *EcoRI* (E. coli) is:
5' GAATTC 3'
3' CTTAAG 5'