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Virus isolation and quantitation

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This chapter outlines the two most commonly used methods of virus isolation, namely tissue cultures and embryonated eggs. We have chosen to present the first section by describing the methodology involved for the predominant virus groups, which cover the

majority of viruses encountered in the clinical situation. Virus isolation is a prelude to the diagnostic methodology described in a later chapter. We also describe selected methods of virus assay and the calculation of virus titers.

Primary isolation of viruses

For clinical samples, the type of specimen and the manner of collection are dependent on the laboratory methods anticipated (see Chapter 15). Ideally specimens should be collected within two days of onset of symptoms, because most viruses are shed only in the initial stages of illness. (Exceptions are adenovirus types 8, 19, and 37 in keratoconjunctivitis in which the virus is shed from the eye for 14 days; mumps virus, which is shed from the parotid gland and saliva for up to 12 days; and various adenoviruses and picornaviruses which are shed in the stool for weeks or months after onset, particularly in children). Nasal swabs are the easiest specimens to collect for respiratory viruses and are also the best specimens (i.e., they contain the most virus) for the majority of the respiratory viruses described here. For nasal swabs, urogenital calginate swabs are inserted into the nasal passages, gently rotated to absorb mucus and cells, and then vigorously twirled into 2 ml of transport medium (such as tryptose phosphate broth with 0.5% gelatin, veal heart infusion broth, or trypticase soy broth), preferably without antibiotics. Throat swabs can be obtained with cotton-tipped wooden applicator sticks that are rubbed against the posterior nasopharynx and then placed in the transport medium. The stick can easily be broken off to leave the cotton tip in the medium. Nasopharyngeal aspirates are collected with a neonatal mucus extractor and mucus trap to which transport medium is added. Swabs or scrapings of vesicular lesions are likewise carefully obtained and placed in transport medium. Urine and stool specimens are collected as for any pathogen. For more unusual viruses, the preferred specimen may be a lesion scraping, cerebrospinal fluid, biopsy or autopsy specimens, or serum or blood cells. Which specimen to collect is often determined by the sites exhibiting clinical symptoms (see Chapter 15).

Specimens should be placed on wet ice and transported to the laboratory for immediate

testing. This is particularly critical for specimens for fluorescent antibody tests, because the epithelial cells must remain intact for a reliable test result. If testing is not possible within 5 days after collection, the specimens should be frozen on dry ice and stored at -70°C until processed, although this may decrease the amount of viable virus.

When processing for viral isolation, the specimens are treated with antibiotics, vigorously mixed, clarified at 1000 g for 3 min at 4°C to remove cell debris and bacteria, and inoculated onto appropriate cell culture monolayers in glass tubes. The cultures should include a continuous human epithelial line (e.g., HEp2, A549, HeLa), a human embryonic lung diploid fibroblast cell strain (e.g., HLF, HELF, MRC5, WI38), human lung mucoepidermoid cells (NCI-H292) to replace MK cells for most applications (Castells et al 1990; Hierholzer et al 1993b), and human rhabdomyosarcoma cells (RD) for the broadest coverage of viruses within practical limitations (Hierholzer 1993; Hierholzer and Hatch 1985; Matthey et al 1992; Meguro et al 1979; Smith et al 1986; Woods and Young 1988). The Epstein-Barr virus (EBV)-transformed marmoset cell line is particularly useful for isolation of measles virus. The inoculum (0.5 ml tube^{-1}) is adsorbed to the cell monolayers (whose growth medium has been decanted) for 1 h at ambient temperature, and the cultures are then fed with maintenance medium and incubated at $35\text{--}36^{\circ}\text{C}$ for several weeks, with subpassaging as required.

Some cell types and certain viruses require roller cultures, while others do best with stationary cultures during their incubation period. In general, all tube cultures of primary monkey kidney cells (MK, AGMK) and their derivative cell lines (Vero, BSC-1, LLC-MK2, etc.), all diploid fibroblast cell cultures, and NCI-H292 cells should be rolled in roller drums or agitated on rocker platforms to remove toxic by-products from the cell surface and to replenish critical nutrients to the cells more

quickly. Cultures of HEK, HEp2, KB, A549, and HeLa do not need to be rolled except for measles virus isolation in HEK and RSV isolation in HEp2.

Small variations in media composition and incubation temperature are also important for the successful isolation of certain viruses. For instance, some viruses require prior treatment with trypsin for successful cultivation, and some cells require the presence of trypsin in the maintenance medium for them to be sensitive to certain viruses. Cooler temperatures (33–35°C) are required for certain, less-invasive viruses, such as coronaviruses, rhinoviruses, and those enteroviruses causing hemorrhagic conjunctivitis (Hierholzer 1976; Hierholzer and Hatch 1985; Lennette et al 1995). These details are best found in current reference works concentrating on the virus families and their diagnostic tests (Fields et al 1990; Hierholzer 1993; Lennette et al 1988, 1995; Mandell et al 1990). One other caution is that any time primary monkey kidney cells are used, such as for influenza, measles or rubella, the laboratory worker must be acutely aware of the probable presence of simian adventitious agents which will confound the isolation and identification of human viruses (Arya 1975; Hsiung 1968, 1969; Hull 1968). Details of these and of the media required for different cells and viruses can be obtained elsewhere (Murray et al 1995; Rose et al 1992; Lennette et al 1995). Viral growth is seen as CPE in the cell monolayer visible under light microscopy (see Chapter 1) or by other means (see page 33).

Primary isolation of viruses in cell cultures

Herpesviridae

As a Family, the herpesviruses are spread by aerosolized droplets, fomites, and direct contact, and cause a wide variety of ocular, oropharyngeal, genital and generalized diseases in man. Herpes simplex viruses types 1 and 2 grow well in many cell types, notably primary rabbit kidney, HEK, HEp2, HeLa, A549, HLF

and NCI-H292 cells; type 2, usually a genital isolate, grows more slowly than type 1. Roller cultures are not necessary. Cytomegalovirus (CMV), a notably labile virus, is the only herpes virus that is shed in great amounts in the urine, and replicates slowly in roller cultures of diploid fibroblast cells, producing giant cells in 12–30 days. Varicella-zoster (VZV), the cause of chickenpox and shingles, also grows slowly in fibroblast cells on roller culture.

EBV, associated with infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma; human herpesvirus type 6 (HHV-6), the cause of roseola infantum (exanthema subitum or fourth disease) in children; and HHV-7, the probable cause of some roseola cases, require special conditions and cells for successful cultivation in the laboratory. In culture, EBV infects both B-lymphocytes and epithelial cells with the CD21 receptor (Fields et al 1990; Lennette et al 1995), HHV-6 grows best in primary CD4⁺ T-lymphocytes rendered more susceptible by the presence of antibody to CD3 (Hall et al 1994; Inoue et al 1994; Pellett et al 1992), and HHV-7 also replicates in CD4⁺ T-lymphocytes (Black and Pellett 1993; Black et al 1993; Tanaka et al 1994; Yasukawa et al 1993).

The herpes virus group is readily visualized by electron microscopy (EM), with the cubic icosahedral shape of the capsid being prominent (Palmer and Martin, 1988). The viruses are commonly speciated by indirect fluorescent antibody (IFA), enzyme immunoassay (EIA), DNA probes, and polymerase chain reaction (PCR) (Fields et al 1990; Hall et al 1994; Inoue et al 1994; Hierholzer 1991; Lennette et al 1988, 1995; Mandell et al 1990; Pellett et al 1992).

Adenoviridae

Adenoviruses are another group of viruses associated with diverse clinical syndromes. They are spread by droplets, fomites and the fecal–oral route; some serotypes are also spread venereally. Most serotypes replicate readily in HEK, HEp2, A549 and NCI-H292 cells, with or without rolling. Types 40 and 41, associated with infantile gastroenteritis

grow best in an Ad5-transformed HEK line (Graham-293 cells) or in HEp2 under a fortified Opti-MEM medium (Gibco BRL) containing low serum (0.4% fetal calf serum) and 0.1% 2-mercaptoethanol. The adenoviruses are among the easiest viruses to identify because they are unique in producing prodigious quantities of soluble antigens as they grow in cell culture, and these antigens possess many type- and group-specific properties that lend themselves to a wide variety of diagnostic tests. Adenoviruses are differentiated from other viruses which may grow in the same cells and with similar cytopathic effects (CPE) by EM, complement fixation (CF), latex agglutination (LA), IFA, EIA, time-resolved fluoroimmunoassays (TR-FIA), DNA probes, restriction enzymes, PCR with suitable primers and cytological and inclusion-body staining methods; they are then serotyped by hemagglutination (HA)/hemagglutination-inhibition (HI) and neutralization (SN) tests (Adrian et al 1986; Hierholzer 1991, 1993; Hierholzer et al 1990; Lennette et al 1988, 1995; Murray et al 1995).

Papovaviridae

The papovaviruses consist of two genera, but only members of one can be isolated in cell culture. Polyoma BK virus can be recovered in HEK, NCI-H292, and diploid fibroblast cells, and the polyoma JC virus replicates in primary human fetal glial cells, but both viruses may be missed because their CPE develops slowly and is vague. BK virus is identified by HA/HI tests at 4°C with human 'O' erythrocytes and specific antiserum; BK and JC virus are identified by EIA, SN, and probes. The papillomaviruses, forming the other genus, cannot be propagated in cell culture or identified serologically, so are only identified in biopsied tissues by various DNA hybridization techniques (Fields et al 1990; Lennette et al 1988, 1995).

Poxviridae

Vaccinia virus and certain other pox viruses can be isolated in MK, Vero, NCI-H292, and

diploid fibroblast cells, and identified by the type of plaques formed and by EM, HI, EIA and various DNA tests (Murray et al 1995; Ropp et al 1995).

Reoviridae

Reovirus 1, 2 and 3 are frequently isolated from throat and rectal specimens in NCI-H292, MK and HeLa cells under stationary or roller conditions after 5–14 days of culture, but they have never been clearly associated with human disease. They are easily visualized by EM and serotyped by HA/HI tests with human 'O' erythrocytes and antisera and by SN tests.

Paramyxoviridae

The Family Paramyxoviridae comprises the genera *Paramyxovirus* (parainfluenza virus types 1, 2, 3, 4A, 4B and mumps virus), *Morbillivirus* (measles virus or rubeola), and *Pneumovirus* (respiratory syncytial virus [RSV]). The parainfluenza and mumps viruses replicate well in roller cultures of NCI-H292 cells under a fortified medium containing trypsin for optimal sensitivity, and in MK cells without trypsin (Castells et al 1990; Meguro et al 1979). The CPE induced by these viruses may develop in 4–7 days but the cultures must generally be blind-passaged and held an additional week to ensure viral growth. The cells rarely become detached, and may not show obvious CPE at all, so that NCI-H292 or MK cultures for these viruses must be hemadsorbed with guinea pig, human, or monkey erythrocytes at the end of the culture period, and the viruses then typed by HI, IFA, EIA, or TR-FIA tests (Hierholzer et al 1993a; Lennette et al 1995).

Measles virus is most easily isolated using the marmoset cell line, B95/8 (Bellini and Rota 1995). The virus also causes CPE in primary African green monkey kidney (AGMK), Vero and HEK cells after 7–14 days of roller culture. But because the CPE usually develops slowly and may not be recognized, the monolayers should be hemadsorbed with vervet

monkey erythrocytes to ensure detection of the virus. The virus is then further identified by HI tests with vervet erythrocytes, IFA, EIA and SN tests, or by probe or PCR tests (Fields et al 1990; Hummel et al 1992; Lennette et al 1995; Mandell et al 1990; Murray et al 1995).

RSV produces distinct syncytia in HEp2, HeLa and NCI-H292 cells in 5–12 days in roller cultures. Group A and B strains of RSV are readily identified by IFA, EIA and TR-FIA tests (Anderson et al 1985; Hierholzer et al 1990, 1994b; Lennette et al 1995; Murray et al 1995).

Orthomyxoviridae

Influenza A, B and C viruses are spread by aerosol droplets and fomites and are best recovered in roller cultures of MK and MDCK cells and in embryonated eggs. Chick embryo, MDCK and other cells require a fortified medium containing trypsin for optimal sensitivity (Frank et al 1979, Klenk et al 1975; Lazarowitz and Choppin 1975; Meguro et al 1979). The viruses are detected in MK cells by hemadsorption and in MDCK cells by HA, and are then identified by IFA, EIA, HI or SN tests (Grandien et al 1985; Lennette et al 1988, 1995; Mandell et al 1990; Murray et al 1995).

Picornaviridae

This very large Family includes the genus *Enterovirus* (polio virus 1, 2, 3; 23 Coxsackie A viruses; 6 Coxsackie B viruses; 31 echoviruses; and 5 more recent enteroviruses), and the genus *Rhinovirus* (125 serotypes). The enteroviruses are spread by aerosolized droplets, fomites and the fecal–oral route, whereas the rhinoviruses are spread by aerosols and fomites only (Dick et al 1987; Mandell et al 1990). These viruses produce CPE in NCI-H292, MK, RD, trypsin-treated MA-104 and diploid fibroblast cells, preferably in roller cultures, although some Coxsackie A viruses grow only in suckling mouse brain. Enteroviruses are distinguished from rhinoviruses by

acid- and chloroform-lability tests in which both genera are chloroform-stable but only enteroviruses are acid-stable; the viruses are identifiable to serotype only by type-specific SN tests (Agbalika et al 1984; Hierholzer and Hatch 1985; Hyypia and Stanway 1993; Lennette et al 1995; Murray et al 1995).

Viruses difficult to isolate

Togaviridae, Flaviviridae, Rhabdoviridae, Filoviridae, Bunyaviridae and Arenaviridae families contain the formerly-known arboviruses, so named because they are spread by insect and/or rodent vectors, plus many rare and unusual viruses that cause disease in geographically distinct parts of the world. These viruses are numerous and have too many specialized isolation requirements to enumerate here. Certain arboviruses can be isolated in primary cells or derivative cell lines like BHK-21, Vero and LLC-MK2, after 2–10 days of culture. The CPE may be difficult to detect, particularly in mosquito suspension cultures (see Chapter 1). Many arboviruses can only be recovered in whole animal systems such as embryonated chicken eggs, suckling mice or hamsters, or mosquitoes. Fortunately, most diagnostic laboratories will not encounter these viruses, either because of their rarity in most parts of the world or their requirement for Biosafety Level 3 or 4 containment facilities. Other works detail the isolation requirements and systems, the specific identification tests and the laboratory precautions for these viruses (Fields et al 1990; Lennette et al 1988, 1995).

Of particular interest is the isolation in deer mice of the causative agent of the newly-described Hantavirus Pulmonary Syndrome. The virus, named Sin Nombre virus, is a member of the Bunyaviridae and can be propagated in the Vero-E6 cell line after growth in deer mouse lungs (Elliott et al 1994).

The gastroenteritis viruses also belong to diverse virus families and have unique requirements for culture. Rotaviruses, after treatment with trypsin, will replicate in BSC-1, MA104 and primary AGMK cells under roller conditions

(Babiuk et al 1977). Caliciviruses and astroviruses may replicate in primary HEK or LLC-MK2 cells under a fortified medium with trypsin (Sanchez-Fauquier et al 1994). The human enteric coronaviruses and toroviruses cannot be isolated in the laboratory (Koopmans and Horzinek, 1994; Lennette et al 1988, 1995), and the enteric adenoviruses were discussed above.

The human parvovirus B19 causes fifth disease (erythema infectiosum) and is easily spread by aerosolized droplets and fomites in schools, and vertically to the fetus during the first trimester of pregnancy. The virus has been reported to grow in specialized erythropoietic stem cell cultures, but will not be recovered in standard culture systems. Thus, B19 infection is documented by EIA testing of the patient's acute-phase serum sample, where both virus and antibodies are sought; EM, Western blots, DNA hybridization probes and PCR have also been used extensively (Anderson 1987; Durigon et al 1993; Fields et al 1990; Mandell et al 1990; Murray et al 1995).

The human coronaviruses cause a significant proportion of 'common colds' and are spread by droplets, but are best identified directly in nasal and throat specimens by IFA, EIA and TR-FIA because the viruses are extremely labile and difficult to recover in the laboratory. The peplomers constitute the primary antigen detected in all tests, including

the HI test for strain OC43 (Hierholzer et al 1994a; Lennette et al 1988, 1995; Schmidt et al 1979).

The HIV and HTLV viruses (family Retroviridae) also require specialized systems for isolation and identification, and will not be recovered by standard culture methods (Lennette et al 1995; Warfield and Feorino 1992).

The interpretation of any cell culture isolation result or of any test used to identify the isolate is dependent on the methods used. An isolate's association with human disease is dependent on the patient data obtained with the specimen and on the known epidemiology of the virus. All of these interpretive elements are reviewed elsewhere (Fields et al 1990; Hierholzer 1993; Lennette et al 1988, 1995; Mandell et al 1990; Murray et al 1995; Rose et al 1992).

Primary isolation of viruses in embryonated eggs

In most cases the advent of tissue culture techniques has superseded the need to isolate viruses in embryonated hen eggs. However, for some viruses, e.g., influenza and avian species, the embryonated egg is still

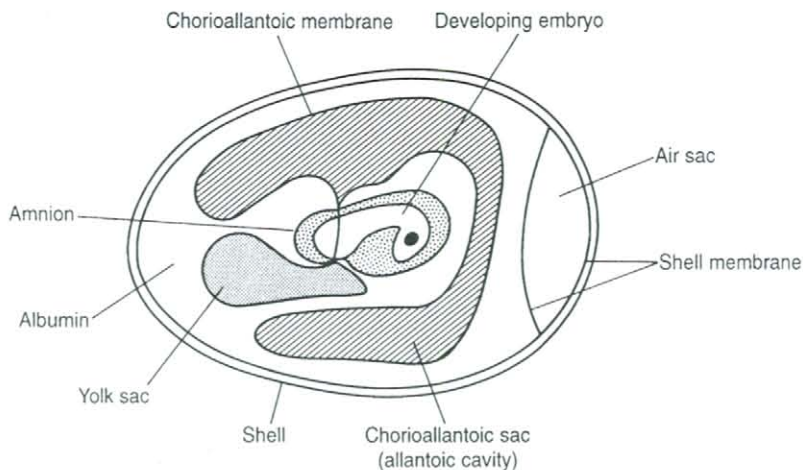


Figure 2.1. The embryonated egg

often used for virus isolation. As will be determined later, the embryonated egg is also used for infectivity (pock) assays. The embryonated egg provides an ideal 'receptacle' in which to grow viruses, as it is sterile and has a range of tissue types and cavity fluids which both support the replication and allow the concentration of infectious virus. The anatomy of the egg is shown in Fig. 2.1.

Fertile hens' eggs are acquired from a suitable hatchery and incubated at 37°C in an atmosphere of about 62% humidity with a forced (usually fan driven) air circulation. This prevents drying out of the egg and allows for good air exchange in the developing embryo. More elaborate incubators also have a mechanism for gently rocking the egg at frequent time intervals. The embryo and developing membranes and cavities go through a variety of anatomical changes up to hatching. Particular sites of injection are therefore optimal at various times in the development of the embryo, e.g., infectious bronchitis virus is propagated in the yolk sac of a 5–6 day old embryo, whereas Newcastle Disease and Influenza viruses are inoculated into the chorioallantoic sac of a 9–11 day embryo.

The procedure of egg inoculation can be divided up into a series of steps.

1. Eggs are 'candled' to check for viability and to determine the positions of the embryo, membranes and blood vessels. Dead eggs will have little or no vasculature and have a characteristic translucent appearance. Darkly stained eggs are usually heavily contaminated. Candling is carried out in a darkened room using a light box which has one small egg-shaped hole surrounded by a piece of foam on which the egg is placed. Rotating the egg immediately reveals its anatomical make-up.

2. Eggs are disinfected with alcohol and marked on the shell in preparation for the drilling of holes, care being taken to avoid areas rich in blood vessels.
3. The virus is injected via the appropriate route, and the hole is covered with tape, glue or wax.
4. Contaminated eggs (which appear 24 h post inoculation) are discarded – such eggs can often be detected by their smell!
5. Eggs are chilled and harvested 2–5 days post infection

Procedure

We have chosen to describe the procedure for chorioallantoic sac inoculation, a common method of isolating influenza virus. Inoculation onto the chorioallantoic membrane for pock formation is described on page 40. In addition, Fig. 2.2 shows diagrammatically

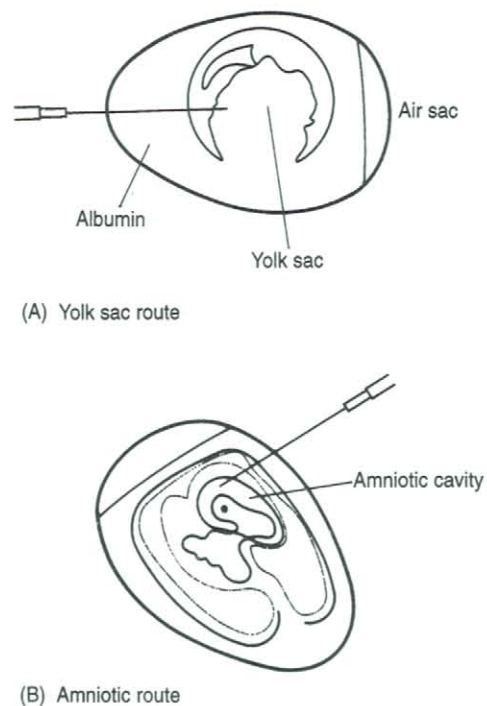


Figure 2.2. Yolk sac and amniotic routes of inoculation.

the routes for amniotic and yolk sac inoculations

1. Candle 9–11 day old eggs to determine viability and sterilize with alcohol. Place the egg pointed end down and drill a small groove in the blunt end of the egg.
2. Mark, with a pencil, an area towards the pointed end of the egg above the embryo and free of blood vessels. Carefully drill a groove in the shell, without damaging the chorioallantoic membrane, at the point of the mark.
3. Inject 0.1 ml virus suspension using a tuberculin syringe fitted with a 26G- $\frac{3}{8}$ needle into the chorioallantoic sac through the groove created in step 2 above.
4. Seal both grooves with adhesive tape.
5. Incubate the egg with the blunt end uppermost in a humidified incubator at 33°C.
6. Collect fluids 2–4 days after inoculation.
7. Place the eggs at 4°C overnight in order to kill the embryo.
8. With the blunt end uppermost, insert scissors into the groove and cut away the area of shell above the air sac taking care not to puncture membranes.
9. This reveals the secondary shell membrane which should be carefully removed with forceps.
10. Gently lift the chorioallantoic membrane to the top of the egg and introduce a pasteur pipette into the sac by piercing the membrane. Remove the allantoic fluid, taking care not to puncture the yolk sac.
11. The fluid should be clear. If the fluid contains erythrocytes centrifuge at 500 g for 10 min and collect the supernatant.
12. The fluid can be assayed using the haemagglutination test (see page 41). Store at -70°C.

Detection of viruses in cell cultures in the absence of CPE

Hemadsorption tests

Hemadsorption is a fast and convenient method of detecting orthomyxoviruses (influenza A, B, C) and non RSV paramyxoviruses (parainfluenza 1, 2, 3, 4A, 4B; mumps; measles) in cell cultures in which the CPE can vary from obvious to minimal. It is even used in the presence of CPE to obtain a quick delineation from other virus groups which may cause similar CPE in the same types of cultures. The method conserves the virus and viral antigens, because the supernatant fluid is decanted from the cell culture into a sterile tube at the end of the incubation period (7–10 days); the monolayer is washed twice with 2–3 ml of plain Hanks Balanced Salt Solution (HBSS) at room temperature; 1 ml of fresh HBSS followed by 0.2 ml of 0.4% mammalian erythrocyte suspension from the appropriate species is added to the monolayer; the tube is incubated stationary with the fluid covering the monolayer; and the test is read 3 times at 20-min intervals by agitating the tube in a sideways motion and then observing the monolayer at 40–100× magnification to see if the erythrocytes are firmly attached to the cultured cells or are floating free in the fluid (see Fig. 15.4). The hemadsorbed monolayer should be regarded as contaminated at this point because the erythrocyte suspension would more than likely not be sterile; subpassaging and virus-specific identification tests should be carried out with the decanted and saved supernatant fluid.

Viral interference tests

Rubella virus was originally detected only by its ability to prevent another virus from infecting the same cells it was replicating in. Current diagnostic testing for rubella may employ specialized cultures in which CPE may become evident; rubella can, under certain culture conditions, cause a vague and variable CPE in rabbit cornea, rabbit kidney and Vero cells. Most commonly, however, rubella specimens are inoculated onto Vero, BHK-21, or primary vervet monkey kidney cell cultures for recovery of the virus, under roller conditions for 4–10 days. Virus is detected by the viral interference test, in which a known quantity of another virus (such as echovirus-11 or coxsackievirus-A9, both enteroviruses) is added to the culture fluid at the end of the culture period allowed for rubella, and the culture is then reincubated for 3 days and observed for enterovirus CPE. The challenge virus is chosen and standardized to give 3+ CPE in 3 days in that particular cell line. In the test, cultures containing rubella are refractory to the enterovirus infection while control cultures (not containing rubella) are sensitive and exhibit the expected degree of CPE within 3 days.

Cellular toxicity by viral autolysis

The human respiratory coronaviruses growing in diploid fibroblast cells (229E virus) or in RD cells (OC43 virus) do not cause any visible CPE during their replication phases. Only

when viral replication is complete and the virus titer has peaked (at about 10^7 Tissue Culture Infective Doses (TCID₅₀) per 0.1 ml), after 26–30 hours of roller incubation, no changes begin to be evident in the monolayer. For the next several days, CPE is seen as a degeneration evenly across the monolayer. The CPE increases to 4+ concomitant with autolysis of the newly-formed virions; by the time complete CPE is achieved, there is very little infectious virus left in the culture (Hierholzer, 1976; Schmidt et al., 1979).

Standard tests for viral antigens and nucleic acids

Arboviruses replicating in mosquito suspension cell cultures at 20–30°C may be detected by subpassaging to monolayer cultures in which CPE may be evident or by HA/HI, EIA, complement-fixation, or nucleic acid tests for the specific viruses suspected. Hepatitis A virus replicates in primary rhesus MK cultures in 1–4 weeks but is non-cytolytic; it is detected by EIA or hybridization tests. The standard tests for viral antigens and viral RNA or DNA, such as these, can of course be applied to any culture of any suspected virus, regardless of the type and degree of CPE observed. CPE by itself should never be considered pathognomonic.

Quantitation of virus

For most virology experiments it is essential to know the concentration of the total or infectious virus particles present in any given virus suspension. Such quantitation forms the basis for example of determining one-step growth curves, examining the neutralization of virus infectivity, assessing the activity of chemotherapeutic agents, monitoring the stages of virus purification, and assessing virus pathogenicity. Animal viruses are quantified by either an infectivity assay (e.g., TCID₅₀, Egg Infective Dose₅₀ (EID₅₀), Lethal Dose₅₀ (LD₅₀), pock assay), other biological/chemical assays (e.g., haemadsorption, haemagglutination, total protein), or by direct total virus particle counting using the electron microscope. Most virologists however, would consider the plaque assay to be the easiest, most accurate and sensitive form of assaying virus infectivity. The plaquing efficiency of some viruses however is often poor and hence other methods are needed (e.g., haemagglutination by influenza).

Those readers who teach virology at a practical level may find the comparative titration methods of e.g., herpes simplex virus, a useful class experiment. This virus can be titrated by plaque, pock, TCID₅₀, LD₅₀ and total particle counting methods, thus allowing the accuracy, sensitivity and practicality of each method to be demonstrated.

This section of the chapter highlights the essential features of these methods drawing upon specific virus assays as examples. The reader should be aware however, that this book is not a manual for all viruses but serves to outline the basic techniques. Specific details for individual viruses can be obtained from the published literature.

Infectivity assays

Probably the most important attribute of a virus is its ability to infect and replicate within

a cell. The virus replicative cycle is accompanied by a number of biochemical and morphological changes within the cell which usually culminates with cell death. These morphological changes, which are often readily visualized with the naked eye, but may require light microscopy are, as stated earlier, referred to as the virus cytopathic effect (CPE) and may take several forms e.g., cell rounding, cell fusion (syncytia formation) or total cell lysis. A few viruses (e.g., selected Retroviruses) do not kill infected cells or cause cytopathic effect but instead transform the cells into rapidly growing foci, capable in many cases of forming tumors in animals. Such foci of infection can be observed in infected monolayers in a similar way to the detection of virus CPE.

Infectivity assays, like other forms of assay, are designed so as to allow the calculation of a virus 'titer' (the number of infectious units per unit volume, e.g., plaque forming units per milliliter). Infectious units are usually thought of as being the smallest amount of virus that will produce a detectable biological effect in the assay, e.g., a plaque forming unit, a pock forming unit). Infectivity assays are of either the quantal or focal type. Quantal assays detect the presence of infectious virus by use of an 'all or none' approach. Does a tissue culture monolayer show CPE? Is an egg infected? Has an animal died? Focal assays rely on the detection and counting of foci of infection, e.g. a focus of CPE (plaque) or a focus of inflammatory response (pock) which allows for the quantitative determination of the number of infectious units as opposed to the qualitative approach of the quantal assay.

Virus dilution

Virus titers are determined by making accurate serial dilutions of virus suspensions, the diluent usually being either tissue culture maintenance or growth medium. Such serial dilutions

are usually done using factors of 2, 5 or 10, the former obviously giving a more precise titer. For routine use 10-fold dilutions are usually carried out.

When setting up such a dilution series consideration should be given to the final volume of diluent needed for the assay and thus aliquots of 0.9, 4.5, 9.0 ml are usually made in a series of sterile tubes or bottles. In order to conserve virus stocks it is usual for the first (lowest) dilution in the series to be achieved by the use of 0.1 ml stock suspension. With subsequent dilutions it is very important to use a new sterile pipette for each transfer and to thoroughly mix each virus dilution before further transfer. Use of the same pipette will transfer millions of virus particles along the series, resulting in a very large dilution error. Once diluted, virus should be assayed as soon as possible although, if necessary, some viruses will withstand storing at 4°C for a few hours before assay. The use of such a storage procedure should obviously be checked to determine if it is suitable for the virus under assay.

TCID₅₀

The TCID₅₀ is defined as that dilution of a virus required to infect 50% of a given batch of inoculated cell cultures. The assay relies on the presence and detection of cytocidal virus particles (i.e., those capable of causing CPE).

Host cells are grown in confluent healthy monolayers, usually in tubes or 96-well tissue culture grade plastic plates, to which aliquots of virus dilutions are added. The method becomes more accurate with increasing numbers of tubes or wells per dilution, but it is usual to use either 5 or 10 repetitions per dilution.

On incubation the virus replicates and progeny virions are released into the supernatant, these infecting healthy cells in the monolayer. The CPE is allowed to develop over a period of days (depending on the virus and cell type) at which time the cell monolayers are observed microscopically (they can be fixed and stained

if necessary). Tubes are scored for the presence or absence of CPE.

It is thus a quantal assay in that each tube provides only one piece of information, i.e., is there CPE or not? The data is used to calculate the TCID₅₀ of the initial virus suspension by one of two ways – the Reed-Muench and the Spearman-Kärber methods (see below). The calculation does not tell us how many infectious units are present in the original virus suspension but what dilution of virus will give CPE in 50% of the cells inoculated.

Procedure

1. Seed tissue culture tubes/wells at a density of cells which will be confluent on the day of virus assay.
2. Make serial dilutions of virus suspension in appropriate diluent.
3. Remove tissue culture growth medium from healthy confluent monolayer and replace with appropriate dilution of virus. This would usually be 1 ml of virus dilution in a tissue culture tube and 0.1 ml in the well of a 96-well plate. Set up at least 5 tubes/wells per virus dilution.
4. Also include at least 5 control tubes/wells which contain diluent alone, i.e. no virus.
5. Incubate at appropriate temperature in either a closed or open incubator system and monitor the development of CPE. Record CPE after a designated time, having observed the cell control tubes/wells first.
6. CPE is usually graded on a 0–4 system; 0 (no CPE) 1 (less than 50% of cells showing CPE) 2 (about 50% of cells showing CPE) 3 (about 75% of cells showing CPE) 4 (the monolayer is totally destroyed or shows 100% CPE).
7. Calculate the TCID₅₀ counting all the tubes/wells with 1–4 CPE as being positive.

Table 2.1. Data used to calculate TCID₅₀ using Reed-Muench or Spearman-Kärber method

Log of virus dilution	Infected test units	Cumulative infected (A)	Cumulative non-infected (B)	Ratio of A/(A + B)	Percent infected
-5	5/5	9	0	9/9	100.0
-6	3/5	4	2	4/6	66.7
-7	1/5	1	6	1/7	14.3
-8	0/5	0	11	0/11	00.0

Calculation of TCID₅₀

The data shown in Table 2.1 will be used to demonstrate the calculation by either the Reed-Muench or Spearman-Kärber methods.

Reed-Muench method (Burlison et al 1992, Reed and Muench 1938)

The dilution in Table 2.1 that corresponds to the 50% end point obviously lies somewhere between the 10⁻⁶ (66.7% infected) and 10⁻⁷ (14.3% infected) dilutions. The proportionate distance between these two dilutions is calculated in the following manner:

$$\frac{(\% \text{ positive above } 50\%) - 50\%}{(\% \text{ positive above } 50\%) - (\% \text{ positive below } 50\%)}$$

= Proportionate distance

$$\text{i.e., } \frac{66.7\% - 50\%}{66.7\% - 14.3\%} = 0.3$$

Given that the log of the dilution above 50% is -6, the proportionate distance is 0.3 and the log of the dilution factor is -1 (i.e., serial 10 fold dilutions were used) the 50% end point is now calculated in the following way:

$$(\log \text{ dilution above } 50\%) + (\text{proportionate distance} \times \log \text{ dilution factor}) = \log \text{ ID}_{50}$$

$$(-6) + (0.3 \times -1.0) = -6.3$$

$$\text{Therefore ID}_{50} = 10^{-6.3}$$

This is the end point dilution, i.e., the

dilution that will infect 50% of the test units inoculated.

The reciprocal of this number gives rise to the virus titer in terms of infectious doses per unit volume. If the inoculation of virus dilution was 0.1 ml the titer of the virus suspension would therefore be:

$$\begin{aligned} &10^{6.3} \text{ TCID}_{50} \text{ } 0.1 \text{ ml}^{-1} \\ &= 10 \times 10^{6.3} \text{ TCID ml}^{-1} \\ &= 10^{7.3} \text{ TCID}_{50} \text{ ml}^{-1} \end{aligned}$$

Spearman Kärber method
(Spearman, 1908; Kärber, 1931)

Again using the data from Table 2.1 the following formula is used to directly estimate the 50% end point:

$$\frac{\text{Highest dilution giving } 100\% \text{ CPE} + \frac{1}{2} - \text{total number of test units showing CPE}}{\text{number of test units per dilution}} = \text{TCID}_{50}$$

$$\begin{aligned} &-5 + \frac{1}{2} - \frac{9}{5} = -6.3 \text{ TCID}_{50} \\ &\text{or } 10^{-6.3} \text{ TCID}_{50} \text{ unit volume}^{-1} \end{aligned}$$

The titer, given a volume of 0.1 ml, is therefore:

$$\begin{aligned} &10^{6.3} \text{ TCID}_{50} \text{ } 0.1 \text{ ml}^{-1} \\ &= 10 \times 10^{6.3} \text{ TCID ml}^{-1} \\ &= 10^{7.3} \text{ TCID}_{50} \text{ ml}^{-1} \end{aligned}$$

The principle involved in the TCID₅₀ experiment is the same for either animal deaths (LD₅₀) or infection of a developing fertile hen's egg (EID₅₀).

Plaque assay

The plaque assay is an infectivity assay that quantifies the number of infectious units in a given virus suspension. Plaques are localized discrete foci of infection denoted by zones of cell lysis or CPE within a monolayer of otherwise healthy tissue culture cells. Each plaque originates from a single infectious virion thus allowing a very precise calculation of the virus titer.

Plaque assays are essentially of two types, suspension assays and monolayer assays. In suspension assays a high concentration of healthy tissue culture cells, in a small volume, are shaken with a suitably diluted aliquot of virus to allow virus adsorption to take place; cells are seeded onto a tissue culture grade vented petri dish. The monolayer assay on the other hand requires a small volume of virus diluent to be added to a previously seeded confluent tissue culture cell monolayer for virus adsorption to take place. In both assays, prior to incubation, an overlay medium is added to the cell suspension or cell monolayer. Overlay media, composed of either agar/agarose or methylcellulose solution prevent the formation of secondary plaques by forcing those virus particles released from the initial infected cell to invade adjacent cells as opposed to spreading to other areas of the cell monolayer.

Following the addition of overlay medium the assay dishes are incubated at an appropriate temperature until plaques are readily discernible. At this point petri plates or microplates are 'fixed' with formol saline solution and stained with crystal violet solution. Plaques are observed either macro- or microscopically.

For statistical reasons 20–100 plaques per monolayer are ideal to count, although the actual number is often dependent on the size of the plaque and the size of the vessel used for the assay.

The infectivity titer is expressed as the number of plaque forming units per ml (pfu ml^{-1}) and is obtained in the following way:

plaque number \times reciprocal of dilution \times
reciprocal of volume in ml

e.g. if there is a mean number of 50 plaques from monolayers infected with 0.1 ml of a 10^{-6} dilution there are:

$$50 \times 10^6 \times 10 = 5 \times 10^8 \text{ pfu ml}^{-1}$$

It is essential that the reader experiments to determine the most sensitive and suitable plaque assay for their own virus/cell system as the methods vary in relation to detail. Such details include:

- sensitivity of the cell to virus infection – plaquing efficiencies varying from cell to cell
- the time required for virus adsorption to cells
- the type of agar used in the overlay medium, i.e. some can be inhibitory for virus replication
- the time of incubation, i.e. plaques must be visible but discrete
- the constituents of the medium in which the overlay is dissolved, i.e. some viruses require high Mg^{2+} concentrations for plaque formation
- the ability of the virus to cause a detectable CPE in tissue culture
- the ability of the cells to form a confluent monolayer
- the need to add a protease (e.g. trypsin) to the overlay medium for plaque formation.

Monolayer assay procedure

This method, modified from Burleson et al 1992, is for the assay of EMC virus in BHK cells.

- Seed an appropriate number of tissue culture grade vented petri dishes with a sufficient concentration of tissue culture cells to reach confluence on the day of assay.
- On the day of assay observe the cell monolayers, which must be healthy and confluent. Thin monolayers are not suitable for assay purposes.

3. Prepare an overlay medium using Difco agar:
 - Eagle's minimal essential medium (8.3 ml)
 - FBS (1.7 ml) } (Solution A)
 - TPB (1.7 ml) }
 - Agar (30% in Hanks Basal Salt Solution and autoclaved) (5.0 ml) (Solution B)
 - adjust pH with sterile NaHCO_3 solution
 - a) melt the agar and maintain in the 48°C water bath
 - b) prepare solution A and equilibrate at 48°C in the water bath
 - c) combine solutions A and B in a sterile bottle and adjust the pH
 - d) keep in the water bath until needed
 - e) do not use the overlay medium if it is 'clumpy'
4. Prepare appropriate virus dilutions.
5. Discard the medium from the previously seeded petri dishes.
6. Carefully add 0.2 ml virus dilution to each of duplicate tissue culture monolayers and gently rock the dish to achieve an even distribution of virus.
7. Allow the virus to adsorb for 30 min at 37°C in a CO_2 incubator or atmosphere of CO_2 . The monolayers may be tilted after 15 min to avoid the cells drying up, although this step may not be necessary.
8. Remove unadsorbed virus – if many assays are to be done use a suction apparatus. Many virologists wash the monolayer at this stage with sterile PBS but others consider this step optional.
9. During the last five minutes of virus adsorption remove the agar overlays from the water bath, thus permitting them to cool. Beware of solidification – trial and error will give experience of how long this takes. The agar should be warm but not uncomfortable to the touch.
10. Add 5 ml of appropriate overlay/plate and allow to solidify. Incubate at 37°C for 48 h.
11. Fix plates in formal saline (10%) for at least 60 min. Plates can be left in this solution until they can be conveniently stained.
12. Remove agar overlay using something similar to a 'bent pin' and stain the monolayer with crystal violet solution (10%) for 10 min.
13. Invert and drain the plates and count using, if necessary, a stereomicroscope.

Suspension assay method

The method outlined below was devised by Russell (1962) for herpes simplex virus assayed in BHK cells.

1. Dilute virus to the appropriate dilution in Eagle's medium containing tryptose phosphate broth (10%) and calf serum (10%) (ETC).
2. Remove 2 ml virus dilution to a fresh Universal or McCartney bottle to which are added a total of 8×10^6 BHK cells.
3. Shake the cell-virus suspension at 37°C for 30 min. This is normally achieved by using a Luckhams Shaker or something similar.
4. Following adsorption add 8 ml overlay medium (ETC containing 0.8% carboxymethyl cellulose solution) and thoroughly mix with the infected cell suspension.
5. Remove 5 ml of the resulting suspension to each of two 60 mm vented tissue culture plastic petri plates and incubate in a CO_2 atmosphere at 37°C .
6. Plates may be observed microscopically after 1 day to observe the development of plaques.
7. After 2 days incubation the medium is decanted into a disinfectant solution

and the cell sheet fixed in formol saline solution (10%). If time does not permit this, plates can be fixed with formol saline solution prior to decanting the overlay medium.

8. Wash the cell sheet gently with water from a beaker and stain for 10 min with crystal violet solution (10%).
9. Decant the stain into a beaker, invert the plates to dry them and count the plaques with the aid of a stereomicroscope.

Pock assays

As discussed earlier the fertile hen's egg is routinely used in virology for both virus growth and virus assay. It has also been used in the diagnostic laboratory for the typing of virus strains, often using the criterion of pock size or pock type. A pock is essentially an area of inflammatory response which results from the virus invasion of an epithelial cell on the chorioallantoic membrane (CAM) of a fertile hen's egg. Like the plaque assay the method is quantitative and each pock results from the infection caused by an infectious unit of virus (pock forming unit). The method is considered by some to be very messy and time consuming and recent animal licencing reforms have restricted the use of the fertile hen's egg (particularly in the UK) to animal licence holders.

As a result the egg is no longer used to the same degree as it was several years ago.

The method requires the creation, in the egg, of a false air sac, onto which is inoculated an aliquot of diluted virus. Following incubation the membrane is observed and the pocks counted. Fig. 2.3 shows a diagram of the egg before and after the creation of the false air sac.

The infectivity titer is obtained in the following way:

$$\text{pock number} \times \text{reciprocal of dilution} \times \text{reciprocal of volume in ml}$$

e.g. if there is a mean number of 50 pocks from eggs inoculated with 0.1 ml of a 10^{-6} dilution there are:

$$50 \times 10^6 \times 10 = 5 \times 10^8 \text{ pkfu ml}^{-1}$$

Pock assay procedure

The procedure outlined below is used routinely in our laboratory for demonstrations of the herpes simplex virus pock assay:

1. Candle 9–11 day old embryonated hen eggs to determine viability (described earlier).
2. Mark the air sac and an area for inoculation over the centre of the chorioallantoic membrane free from blood vessels.

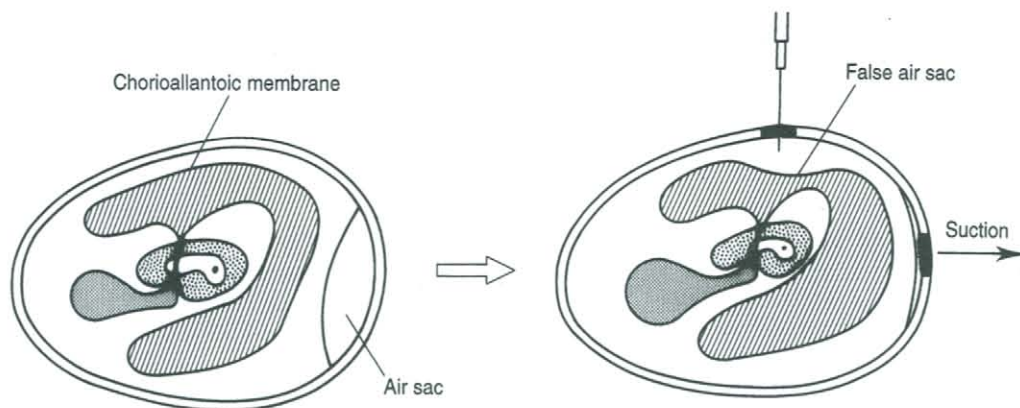


Figure 2.3. Creation of false air sac in embryonated egg.

3. Swab both areas of the egg with alcohol and drill a 3–4 mm groove in the shell at centre of the air sac and also over the CAM (position already marked). Avoid damage to the shell membrane.
4. Using a sterile mounting needle puncture the shell membrane over the air sac.
5. Place a small drop of sterile saline on the exposed shell membrane over the CAM.
6. Place the tip of a mounting needle or hypodermic syringe, through the drop of saline, on the exposed shell membrane, vertically to the long axis of the egg, and press gently but firmly to split the fibres of the shell membrane. *Avoid puncturing the CAM which lies directly beneath.*
7. Using a rubber teat apply suction to the air sac hole. The chorioallantoic membrane should detach and drop away from the shell membrane, the drop of saline acting as a 'wet wedge'.
8. The contents of the egg become transposed and an artificial air sac forms beneath the hole over the CAM. Check this by candling.
9. Using a 1 ml syringe filled with an appropriate virus dilution, inoculate the eggs (by inserting the needle into the artificial air sac through the hole over CAM to depth of about 4–5 mm) with 0.1 ml of virus suspension. Inoculate two eggs with each dilution of the virus.
10. Rock the eggs gently to distribute the inoculum.
11. Seal the holes in the shell with adhesive tape or wax.
12. Incubate the eggs at 37°C for two days in a humidified environment with the false air sac uppermost.
13. Chill eggs at 4°C overnight prior to harvesting.
14. Place each egg in a petri dish with the inoculated area uppermost.
15. Cut the egg into two halves around the long axis using sterile scissors.
16. The chorioallantoic membrane should remain in the top half of the shell and can be removed with forceps. Discard the rest of the egg contents.
17. Place the harvested membrane in a fresh petri dish containing saline.
18. Wash the membrane free of yolk, shell, etc., and transfer to a further petri dish containing 10% formol saline.
19. Examine the membranes for presence of pocks. This is easier if they are placed over a black background. Count the pocks and calculate pock forming units per millilitre of original virus suspension.

Haemagglutination

The ability of some viruses to aggregate various species of red blood cells (RBCs) is referred to as haemagglutination. This effect is brought about by the interaction of specific virus glycoproteins with surface receptors present on the plasma membrane of RBCs. Not all viruses are capable of causing this reaction and those that do may only react with RBCs of particular species and may do so only under stringent conditions of pH and ionic strength. Other viruses, however, may react with a whole range of RBCs in a basic saline solution.

For the reaction to occur, the virus should be in sufficient concentration to form cross-bridges between RBCs, causing their agglutination. Thus, RBCs left in a hemispherical well unagglutinated will fall to the bottom of the well and form a well-defined RBC pellet. Agglutinated RBCs on the other hand will form a lattice-work structure which coats the sides of the well. The two morphological appearances (pellet and lattice) are easily discernible with the naked eye.

The assay, one of the most common indirect methods of determining virus titer, is not a measure of infectivity. Indeed, virus replication does not take place during this assay. Instead it measures those particles of virus in

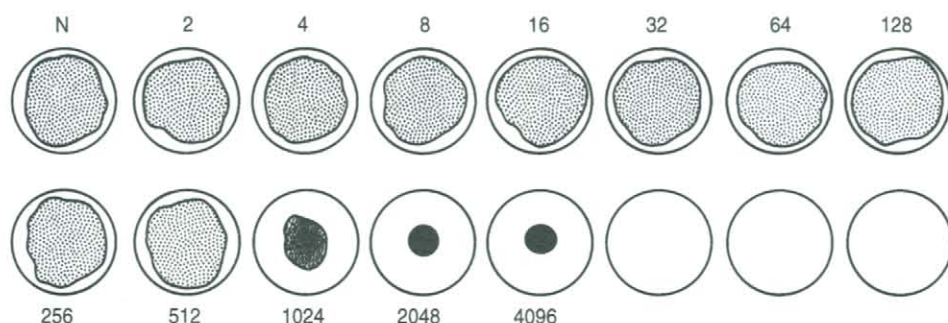


Figure 2.4. Diagram of a sample haemagglutination assay. Serial doubling dilutions of virus shows complete agglutination end point at 1:512 and 50% end point at 1:1024.

a given suspension capable of causing haemagglutination and as such the assay is not at all sensitive, as a very large number of particles are needed to produce the effect.

The haemagglutination assay is done by end point titration. Serial two fold dilutions of virus suspension are mixed with an equal volume of RBCs of known concentration and wells are observed for the presence or absence of a lattice. RBCs are routinely used at 0.3% or 0.5%. The end point of the titration can be interpreted in two ways: (a) the last dilution showing complete agglutination or (b) the dilution which shows 50% agglutination. Most laboratories use the former and by definition such a dilution is said to contain 1 HA unit. The HA titer of a virus suspension is therefore defined as being the reciprocal of the highest dilution which causes complete agglutination and is expressed as the number of HA units per unit volume for a given concentration of RBCs. An example upon which a calculation of the HA titer can be made is shown in Fig. 2.4. The end point in this figure, assuming this to be the highest dilution capable of complete agglutination, is $\frac{1}{512}$. If 0.2 ml of virus were added per well the HA titer would be 512 HA units 0.2 ml^{-1} or 2560 HA units ml^{-1} . Using the 50% end point calculation the titer would be 1024 HA units 0.2 ml^{-1} or 5120 HA units ml^{-1} .

Burleson et al (1992) document a method whereby the number of haemagglutinating virus particles can be obtained, given the HA titer and the fact that the number of RBCs in the well approximates the number of HA particles. In the above example, knowing that a

0.3% RBC suspension gave rise to a titer of 512 HA units 0.2 ml^{-1} , the number of haemagglutinating virus particles in the original suspension can be calculated. A 10% solution of RBCs is defined as having 8×10^8 RBCs ml^{-1} . A 0.3% solution therefore contains 2.3×10^7 RBCs ml^{-1} . As 0.2 ml of RBC were added to each well this represents 4.8×10^6 RBCs per well. At a 1:512 dilution there were 4.8×10^6 haemagglutinating virus particles in the well. As 0.2 ml of virus was used this represents 2.4×10^7 virus particles ml^{-1} . When multiplied by the dilution factor (512) this means that 1.2×10^{10} haemagglutinating virus particles ml^{-1} are present in the original virus suspension.

Procedure

The procedure outlined below is routinely used in our laboratories to assay influenza virus using chicken RBCs.

1. Chicken RBCs are 'brought in' in preservative solution. Alternatively, acquire by bleeding the wing vein of a chicken.
2. Prepare a stock solution of RBCs (10%) in phosphate buffered saline. Once washed away from preservative these cells will last for 1–2 weeks only at 4°C . The 10% solution can be achieved by a haemocytometer count, i.e. a suspension of 8×10^8 cells ml^{-1} , or by use of a haematocrit tube.
3. Carry out the assay in a large 80 well

WHO plate or in a 96 well plate. For the former 0.2 ml volumes are used, for the latter 0.1 ml. We will assume a WHO 80 well plate is being used.

4. Add 0.2 ml saline to all wells in the two rows of the plate (apart from well 1).
5. Add 0.2 ml virus suspension to well 1. This represents neat virus.
6. Add 0.2 ml virus suspension to well 2 and mix by pipetting up and down into the pipette tip. Transfer 0.2 ml to well 3 and repeat the mixing step. Carry out the serial two fold dilutions along the row and transfer 0.2 ml to row 1 of column 2. Again dilute along the row, this time discarding the tip (+0.2 ml) into disinfectant after the last well.
7. Add 0.2 ml RBCs (0.3% or 0.5%) to each well. Gently tap the plate to ensure good mixing of RBCs and virus.
8. Leave for 1 h at ambient temperature and read the end point

As will be seen in later chapters virus haemagglutination can be inhibited by specific antisera, this forming the basis of the haemagglutination-inhibition test.

Particle counting

A later chapter of this book discusses and elaborates upon the electron microscope and its uses in virology. In this chapter we highlight the basic methodology of virus particle counting using the technique of negative staining. This procedure was excellently described by Watson et al (1963).

The counting procedure relies on the use of a 'reference particle'. The principle is that if viruses can be mixed with reference particles of known concentration (i.e. number per unit volume) a simple determination of the ratio of virus to reference particles will yield the virus count. The Dow Corning Company, many years ago, made this possible by producing suspensions of spherical polystyrene latex particles of uniform diameter, with physical

properties allowing calculation of their count. However, early problems in virus particle counting arose because of the inability to differentiate, in some cases, between the latex beads and the virus particles when observed in the electron microscope!

The problem of identification was easily overcome by the incorporation in the reference particles/virus mixture of a negative stain, phosphotungstate (PTA), which reveals the characteristic structures on virus particles (Watson 1962).

The mixture of virus, negative stain and latex beads is deposited, in droplet form using a bacteriological loop, onto a specimen grid. Upon observation in the electron microscope a virus/latex ratio can be derived by examination of substantial parts of the grid.

Procedure

The loop drop procedure, described below, may be used for any virus suspension containing approximately 10^9 particles ml^{-1} , and was developed for herpes simplex virus.

1. Portions of latex and virus suspensions (0.1 ml) each diluted to contain about 10^9 particles ml^{-1} are mixed with 0.1 ml of 0.5% PTA and 0.1 ml of 0.5% bovine serum albumin. The dilution of virus if no infectivity titer is known, will be by trial and error.
2. Introduce a drop of the above mixture, using a platinum loop onto a carbon Formvar grid held carefully with fine tweezers.
3. Wait for 1–2 minutes and then dry the grid by touching it with blotting paper.
4. Scan the grid in the electron microscope at a screen magnification of $\times 40,000$.
5. Virus particles and latex beads are clearly visible and counts are made of the number encountered using five groups of 20 latex beads.
6. The number of particles is calculated by the method outlined below:

Table 2.2.

	Latex	Virus ($\frac{1}{50}$ dilution)
	20	9
	20	8
	20	8
	20	9
	20	6
mean	20	8

Assuming the latex to have been used at a concentration of (for example) 2.84×10^9 beads ml^{-1} , the total number of virus particles per millilitre is given by:

$$\frac{8}{20} \times 2.84 \times 10^9 \times 50 = 5.7 \times 10^{10} \text{ particles ml}^{-1}$$

If the infectivity titer, e.g. pfu ml^{-1} , is known it is possible from the above to calculate the particle:infectivity ratio of the virus. Assuming the titer of the above virus suspension to be 5.0×10^8 pfu ml^{-1} the particle: infectivity ratio would be:

$$\frac{5.7 \times 10^{10}}{5.0 \times 10^8} = 114$$

Determination of this ratio is important for many areas of virology, e.g. monitoring virus purification regimes, looking for defective interfering particles, determining the state or age of a virus suspension.

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