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Laboratory techniques for diagnosis of virus infections

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An exact virological diagnosis is necessary for studies of the epidemiology of viral infections and for the control of epidemics and nosocomial infections. In some situations the clinical work is entirely dependent on the virological diagnosis, for example when rubella is suspected in early pregnancy, and for excluding hepatitis B carriers from becoming blood donors. The need for a rapid aetiological diagnosis will increase as more antiviral chemotherapeutics become available for clinical use, and the choice of drug and the development of drug resistance become problematic. Laboratory diagnosis of virus infections, including the specification of serotypes, has formed a necessary background to the development of viral vaccines and the continued control of the efficacy of vaccines.

Three different principles are used in the diagnosis of viral infections:

1. Isolation of infectious virus by inoculation into cell cultures or experimental animals.
2. Identification of virus or viral antigens directly in samples from patients. In the future the highly sensitive methods such as antigen detection by new serological tests or genome identification by nucleic acid hybridization techniques will probably be used routinely for direct identification of virus in samples from patients.
3. Serological analysis to demonstrate a specific antibody response.

Isolation of viruses

Most known viruses causing disease in man can be demonstrated by use of cell cultures or experimental animals. The virus infection is indicated by the more or less characteristic changes that the virus causes in cell cultures (cytopathic changes and cell death, the formation of viral antigens, haemagglutinin, or interferon etc.; see Chapter 11) or by the appearance of disease in experimental animals. In some cases, for example in myxovirus infections, the production of haemagglutinin is readily demonstrated by the addition of erythrocytes, which adhere to the surface of the infected tissue-culture cells (haemadsorption, HAd). An isolate of virus can be identified, typed, by neutralization tests or other antigen-antibody reactions, for example by immunofluorescence staining of infected cells, haemagglutination inhibition etc. (see description of methods below).
### TABLE 20.1. Scheme for collection of samples for virus diagnosis

| Disease                                      | Nasopharynx | Throat | Faeces | Cerebrospinal fluid | Urine | Other materials | Acute | Conv. 10–20 days | Conv. 4-6 weeks |
|----------------------------------------------|-------------|--------|--------|--------------------|-------|----------------|-------|-----------------|----------------|
| Aseptic meningitis, encephalitis, myelitis   | (+)         | +      | +      | (+)                | Vesicle fluid, Brain biopsy |
| Conjunctivitis, keratitis                    | +           | +      |        | (+)                | Conjunctival secretion, Corneal scraping |
| Mumps                                        | +           | +      |        | (+)\(^b\)          | Saliva |
| Respiratory infection                        | +           | +      | (+)\(^b\) |                   | +     |
| Stomatogingivitis                           | +           | +      |        |                    | Vesicle fluid |
| Pleuritis                                    | +           | +      |        |                    | Pleural fluid |
| Myocarditis-pericarditis                     | +           | +      |        |                    | Pericardial fluid |
| Hepatitis                                    | (+)         | +      |        |                    | +\(^c\) (+) (+) |
| Mononucleosis                                | (+)         | +      |        |                    | +     (+) |
| Cytomegalic inclusion disease                | +           | +      |        |                    | +     |
| Orchitis (without parotitis)                 | +           | +      |        | Saliva             | +     |
| Diarrhoeal disease                           | +           | +      |        |                    | +     |
| Maculopapular exanthema                      | (+)         | +\(^d\) |        |                    | +     |
| Exanthematous disease with vesicles          | +           | +      |        |                    | Vesicle fluid, scrapings from bottom of vesicles, crustal material |
| Rabies                                       | +           | +      |        |                    | Corneal imprint, mouth scraping or skin biopsy, saliva | + (+) |

\(^a\) Vesicle fluid
\(^b\) Conjunctival secretion
\(^c\) Corneal scraping
\(^d\) Scrapings from bottom of vesicles, crustal material
Supplementary material in special cases.

To be collected if an earlier convalescent serum has not provided etiological diagnosis. The time for collection of samples is calculated from the day of appearance of disease.

In connection with pharyngoconjunctivitis, herpangina or diarrhoea.

If HBsAg is demonstrated, certain rules for the follow-up should be applied (see Chapter 30).

With consideration taken of enterovirus and adenovirus infections.

Investigation of a suspected congenital or neonatal virus disease

The child: Blood sample (without additives).

Samples for virus isolation: Rubellavirus and CMV: pharynx swab, urine, cerebrospinal fluid. Herpes simplex virus or varicella virus: vesicle fluid, pharynx swab, cerebrospinal fluid. Enterovirus: faeces, cerebrospinal fluid, pharynx swab.

The mother: Blood sample (without additives). Indicate on the referral any diagnostic suggestions, symptoms of infections in the mother, and data about the child.

Samples for a direct diagnosis of viral antigen or virus particles in patient's material

Samples for immunofluorescence analysis (nasopharyngeal or tracheal secretions in respiratory infections, scrapings from conjunctiva and cornea in eye infections, and vesicular fluid from cases with vesiculated exanthema) should reach the laboratory within two hours, unless the cell smears are prepared at the hospital. Specimens for antigen detection by other methods or faecal samples for electron microscopy can withstand longer transport times.
Although virus isolation is still the technique most generally applicable for diagnosis of a virus infection, there are several important viruses which do not grow readily in ordinary cell culture systems (for example hepatitis virus A and B, rotavirus and certain strains of adenovirus), or which are not excreted or accessible at the time of disease (for example tick-borne encephalitis virus).

Samples for virus isolation must be collected by the correct method (Table 20.1) as early as possible during the disease since the concentration of virus is maximal at this stage and is often reduced rapidly as time passes. However, the infectious agent may in some cases be demonstrated for weeks, months or sometimes even years after the acute infection. Thus enteroviruses and adenoviruses can be excreted for a long time with faeces; CMV in urine and rubellavirus in pharynx and cataracts can be isolated for periods of months to years from congenitally infected children.

The time required for the laboratory to isolate a virus can vary between one to two days in the case of, for example, influenza, polio and herpes simplex viruses, to many weeks in the case of CMV.

Cell cultures

Virus isolation is most often performed in cultures of various kinds of cells (see also Chapter 5). By use of such techniques it has been possible to define the aetiology of many virus diseases, to isolate and characterize hundreds of previously unknown viruses, and to produce effective virus vaccines.

Cells of different origin and age may have a markedly varying susceptibility to different viruses. For diagnostic purposes a virus laboratory must therefore have access to a battery of cell lines from animals and man. As both diploid and heteroploid cell lines can be stored for an indefinite time at low temperature (liquid nitrogen - 196°C) the laboratory can easily keep appropriate cell lines in stock.

Some viruses grow poorly or not at all in cell cultures but can be isolated in organ cultures of highly differentiated tissue, for example some strains of coronaviruses in cultures of human nasal mucosa or tracheal epithelium. For isolation of virus from patients with persistent infection, cocultivation or even production of cell hybrids between susceptible cells and surviving infected cells from the patient may be needed. This technique has been used for the isolation of measles virus from explanted brain tissue in cases of subacute sclerosing panencephalitis (SSPE, see Chapter 16).

Experimental animals

In addition to cell cultures, laboratory mice and embryonated hen’s eggs are still used for routine isolation of viruses. Poxviruses and herpes simplex viruses can be isolated by infection of the chorioallantoic membrane of the embryonated egg, giving rise to localized tissue changes. These changes are frequently characteristic enough to be used not only as an index of infection but also for differential diagnosis, for example between smallpox and vaccinia virus. The amnion and the allantoic sacs of the embryonated egg were previously used extensively for diagnosis of certain myxovirus infections, primarily with influenza and mumps viruses. Today this method has to a major extent been replaced by cell culture or by the direct identification of viral antigens. Laboratory mice are still being used for isolation of many coxsackie A viruses and arboviruses.
Direct identification of virus or viral antigens in samples from patients

A specific diagnosis can be obtained within a few hours by direct demonstration of virus or viral antigens in samples from patients. The aetiological diagnosis will reach the hospital while the patient is still ill and it can thus give important assistance in the choice of treatment and the epidemiological measures to be taken. Viruses which have a restricted capacity to grow in cell cultures may be demonstrated by this technique. It can be predicted that methods of this kind will become of increasing importance in future diagnostic work in virus laboratories.

Electron microscopy (EM)

EM analysis is used for estimation of the size, form and structure of virus particles. It is thus possible to identify to which family a virus belongs. For further characterization immune EM with specific antibodies is used.

For diagnostic EM the negative contrast technique is commonly used. The virus particles are surrounded by an electron-dense contrast which reveals the surface structure of the particles. If the outer part of the virion is damaged the contrast may penetrate and allow identification of internal components. The investigation is readily performed by first allowing the test material and then the contrast to spread over a carbon and plastic coated copper grid for 15–30 seconds whereafter excess fluid is removed by a filter paper. The analysis can be performed in less than one hour. However, a relatively high concentration of virions is needed to give a positive result. Particles which occur in a low concentration in material from patients may be concentrated before examination, for example by ultracentrifugation of agglutination by specific antibodies.

Figure 20.1. EM picture of rotavirus in a faecal sample from a child with diarrhoea.
(Magnification: ×—160 000. Photo: L. Svensson)
EM analyses are often performed on faecal samples (e.g. rotavirus and adenovirus; Figure 20.1) but also on vesicle fluid (poxviruses and herpesviruses) and on brain biopsies (herpesvirus and rabies virus). Samples of serum and respiratory secretions are not suitable for routine EM analysis.

**Immunological methods for identification of virus-specific antigens**

For diagnostic purposes the presence of viral antigens may be demonstrated in cells as well as in suspension.

If collected at the proper time and from the appropriate locality most specimens will contain infected cells. The immunofluorescence (IF) technique has been shown to be a reliable method for specific identification of viral antigens in such cells. Immune peroxidase staining is a similar method, but since certain tissues contain endogenous peroxidases and artifacts may be encountered thereby it is less often used.

**Immunofluorescence (IF) analyses**

These are performed with sampled cells placed on a microscope slide. Fixation in acetone is used to bind viral antigens and cell components in cells and to make the cell membrane permeable to antibodies. The presence of viral antigens is demonstrated with specific antibodies which are either themselves fluorescein-conjugated (the direct method) or which are traced by fluorescein-conjugated antiglobulins (the indirect method). Fluorescein gives a yellow-green light in the fluorescence microscope. The more-or-less-typical topographical distribution of viral antigens in infected cells may give additional support to the diagnosis (Figure 20.2). A diagnosis can be given within three hours.

*Figure 20.2. Cells containing RS virus antigen visualized by immunofluorescence, from a nasopharyngeal sample of a six-months-old child with obstructive bronchiolitis*
Analysis of antibody response in patients

The method is useful for the routine diagnosis of viral infections in the respiratory tract, skin, eyes and brain. In the case of very labile viruses, some of which are difficult to isolate, the method occasionally is the only practical means of obtaining a diagnosis. In experienced hands it is considered to be as reliable as virus isolation for the diagnosis of rabies infections.

Other immunological techniques

Viral antigens in suspension may be demonstrated by enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA) as well as by the less sensitive techniques of immunodiffusion (ID) and immunolectroosmophoresis (IEOP). In ELISA and RIA specific antibodies attached to a plastic surface bind viral antigens present in the patients’ sample. The antigen is then demonstrated by specific antibodies coupled to a marker, i.e. an enzyme or a radioactive compound. Alternatively, the presence of antigen may be indicated through its competition with labelled antigen which is added instead of the labelled serum (see Figure 20.5).

ELISA and RIA methods are currently used for demonstration of hepatitis B antigens in serum, for rotavirus, adenovirus and hepatitis A virus antigen in faecal samples, but also for the demonstration of herpesvirus antigens and antigens of certain respiratory viruses. The sensitivity of the tests is high. For this reason, strict requirements have to be applied on the specificity and purity of the reagents used. Results of the tests may be obtained within a day.

Analysis of antibody response in patients

Serological methods are used for demonstration of specific antibody responses in patients with an infection.

Usually two sera are needed to perform a serological analysis. The first sample is taken as early as possible after appearance of symptoms, the other 1–2 weeks later. A methodologically significant increase of the antibodies against a certain viral antigen between the two samples, indicates that the patient at that time has been infected with the specific agent tested for or at least with an agent that is antigenically related. The serum titre (antibody concentration) is given as the highest dilution of serum which gives a positive reaction (for example 1:512), alternatively as the inverted value of this dilution (512).

Antibodies of IgM class appear earlier than, or at the same time as, IgG antibodies, but disappear more rapidly. With prolonged or chronic infections the IgM may remain detectable for a longer period of time. Virus-specific IgM antibodies frequently can be demonstrated in the first serum sample, which makes rapid diagnosis possible. If the first sample has been taken late the patient may already have developed a pronounced IgG antibody response. Identification of specific IgM antibodies may then provide the only possibility of serological diagnosis. As pointed out in Chapter 15, IgM analyses are of particular importance in the diagnosis of congenital infections since maternal IgM, contrary to maternal IgG, does not pass the placental barrier. Presence of specific IgM antibodies in the blood of a newborn child demonstrates, therefore, that the child must have been infected before birth.

Viruses induce the synthesis of several different antigenic proteins, and the antibody response in the infected individual is therefore complex. Antibodies
against antigens on the surface of the infectious particle are usually type-specific and antibodies of this kind may neutralize virus infectivity and consequently also provide immunity against infection. IgG antibody responses of this kind are usually durable. Antibody responses against other antigens, for example internal structural antigens and non-structural antigens, may be less durable and may cross-react with the corresponding antigens from related viruses. Different antigens may dominate in different serological techniques if one and the same antigen material has been used. It is therefore necessary to comment on the principles of some of the more important techniques and for the interpretation of results obtained.

The neutralization test (NT)

This measures antibodies which react with the surface of virions and neutralize their infectivity. The test is usually performed by incubating a series of dilutions of serum with a fixed dose of infectious virus, usually 100 ID$_{50}$ (100 times the dose of virus which can infect 50 per cent of inoculated cell cultures), during a fixed time, for example one hour at +37°C or overnight at +4°C. The mixtures are then inoculated into susceptible cultures or experimental animals. The dilution of serum which can inhibit virus growth in 50 per cent of the infected cultures or animals is taken as the neutralization titre. In order to obtain reproducible results it is important that constant time and temperature for incubation of virus and antibodies are maintained, that the same cell type is used, and that the tests are read after a fixed time period. Preparations of certain virus strains contain aggregates of virions which are difficult to neutralize and therefore have to be removed, for example by ultrafiltration, before the neutralization test is carried out. The serum samples are usually inactivated at +56°C for 30 minutes before use, but for an effective neutralization of certain enveloped viruses, for example rubellavirus, there is need for the presence of a complement which may be added in the form of fresh serum.

Several technical variations of the neutralization test may be mentioned. The inoculated cultures can be covered by a layer of agar-containing medium and the antibody titre scored as the dilution which gives a 50 per cent reduction in the number of plaques. In order to allow automation and to save reagents, the cells may be cultivated and the neutralization test performed in the small wells of a microtitre plate made of disposable plastic. The end point of the serum titration can often be read on the bases of metabolic inhibition: cells which are actively metabolizing acidify the medium and the colour of the pH indicator is changed. This occurs in the wells where the virus has been neutralized but not in cultures where the virus infection has interfered with the cellular metabolism or destroyed the cells. If needed, the type-specificity of the neutralization tests can be increased to allow a comparison between closely related virus strains. This is performed either in the classic way by crosswise absorption of sera with the respective antigens or by kinetic neutralization. In the latter case the neutralization effect is measured by inoculation into cell cultures after varying times of incubation of the virus–serum mixture.

The haemagglutination-inhibition (HI) test

Many viruses can agglutinate red blood cells from one or more species at a suitable pH and this reaction can be blocked by antibodies directed against the antigen on the surface of virions which is attached to cellular receptors (see also Chapter 4).
Either whole virus particles or isolated haemagglutinin is used as antigen in the test. By use of the latter kind of antigen, somewhat higher antigen and antibody titres may be obtained. It is important as a first step to remove non-specific inhibitors of the haemagglutination which are present in most sera. These inhibitors are of a varying nature for the different viruses; glycoproteins and thermolabile inhibitors in the case of influenza virus; lipoproteins in the case of rubellavirus and other togaviruses etc. Consequently, the methods used to remove these inhibitors vary. Serial dilutions of sera which have been properly treated are incubated with an antigen amount corresponding to 4 times the minimum haemagglutinating dose. After these two ingredients have been allowed to interact, red blood cells are added and allowed to sediment. They form different bottom patterns depending upon whether they are agglutinated or agglutination has been blocked by antibodies. The serum titre is the highest 2-fold serial dilution of serum capable of inhibiting the haemagglutination (see Figure 20.3).

![Figure 20.3. Demonstration of antibodies by a haemagglutination-inhibition (HI) test.](image)

The HI test is relatively sensitive and only indicates antibodies against the haemagglutinin. In most cases this reaction is type-specific and correlates well with neutralizing antibodies and immunity. However, togaviruses represent an exception since different alphaviruses and flaviviruses cross-react in the test.

**The complement fixation (CF) test**

This test has a broad application and has therefore been one of the most extensively used serological techniques in virus-diagnostic laboratories.

The test is performed by incubating dilutions of heat-inactivated serum (56°C for 30 minutes) with 2–8 units of viral antigen and 2 units of complement (guinea pig serum) at +4°C overnight. An indicator system which contains sheep erythrocytes sensitized with amboceptor (antibodies against the erythrocytes) is then added and the mixture is incubated at +37°C. If the patient’s serum contains antibodies against the viral antigen a complex is formed which ‘fixes’ the complement during the first antigen–antibody reaction. Consequently, no lysis of the sensitized red
blood cells added later will occur. Various purified antigens may be used in the CF test, but usually relatively crude and complex preparations are employed and the test therefore frequently measures antibodies against antigens other than the surface antigens of the virus. As already mentioned, these antigens are often group-specific rather than type-specific. Several controls have to be included in each CF test. In one control, no antigen is added to the serum dilution in order to exclude the possibility that the serum by itself fixes complement. This may occur if it has been contaminated by bacteria or contains circulating antigen–antibody complexes. Another control includes antigen but not serum, to exclude the possibility that the antigen by itself reacts with complement. Finally, a control serum and non-infectious antigen is included, to exclude the possibility that the antibody detected reacts with non-viral antigen from the cells or medium used for antigen preparation.

CF tests with viral antigens primarily measure IgG antibodies, rarely IgM antibodies. The CF antibody response therefore develops relatively slowly and an increase in antibody titres may be shown later than by the HI test, for example, which demonstrates both IgG and IgM antibodies. The titres obtained in CF tests are relatively low and the reaction is less sensitive than the HI test.

The immunodiffusion (ID) test

The ID test is relatively insensitive. In spite of this it has been used extensively in virological work, since it has the capacity to discriminate between several simultaneous reactions of different antigen–antibody systems.

When antigen and antibodies diffuse against each other from two wells punched in a thin agarose gel, a precipitate line can form where the two reactants meet in optimal proportions. Identical precipitate lines fuse to a continuous curve whereas lines representing different antigen–antibody complexes cross each other. Both the speed and the sensitivity of immunodiffusion tests can be increased by driving antigen and antibodies against each other in an electrical field, immunoelectrophoresis (IEOP).

A quantitative determination of antibody concentration is most readily performed by inclusion of antigen in the gel. Under these conditions the precipitate occurs around the well in a circular zone, the diameter of which is proportional to the antibody titre. A variant of this technique which has been found very useful in serological diagnosis and immunity determination in rubella is the single radial diffusion haemolysis test (Figure 20.4). In this test red blood cells covered by rubella haemagglutinin are mixed into the agarose gel. After diffusion of antibodies into the gel it is overlaid with complement. This leads to development of zones of haemolysis since red blood cells carrying antigen–antibody complexes are destroyed.

The immunofluorescence (IF) test

The IF test has been used for determination of antibodies in routine diagnostic work and for research purposes it has been used extensively, for example in the characterization of different antigen–antibody systems in infections with EBV.

IF methods offer possibilities for discrimination between antibodies belonging to different immunoglobulin classes. Thus the test can be used for demonstration of IgM antibodies against, for example, EBV and CMV. Virus-infected cells fixed with acetone are used as antigen. They are incubated first with dilutions of the
Analysis of antibody response in patients

Figure 20.4. Single radial diffusion haemolysis test for determination of antibodies against rubellavirus. The uppermost row includes one negative specimen (only the well itself can be seen) and three positive control samples (clear zones around the wells). Rows 2 and 3 show test results with serum pairs from patients with rubella. Each convalescent serum in row 3 has a matching acute serum just above it in row 2. The bottom row includes sera from patients analysed for immunity against rubella; there are four positive samples.

patient’s serum and then, after washing, with fluorescein-conjugated antibodies against human IgM. If IgM is adsorbed to the cells it can then be visualized by fluorescence microscopy. A false-positive reaction may be encountered in this test if the patient’s serum contains specific IgG and rheumatoid factor, i.e. IgM autoantibodies directed against IgG. False-negative results may be encountered in the presence of high concentrations of specific IgG since this may block the binding of IgM to the antigen.

RIA and ELISA

These techniques are as a rule more sensitive than other techniques for antibody determinations. The tests may be designed on different principles, employing either labelled antibodies or labelled antigen as an indicator of the possible occurrence of specific antibodies in the serum of a patient (Figure 20.5).

Because of the high sensitivity of the tests, it is necessary that the indicator reagent (antigen or antibodies) which is coupled to a radioactive isotope (usually iodine) or an enzyme (alkaline phosphatase or peroxidase) is available in a highly purified form in order to give the test a satisfactory specificity. Commercial RIA and ELISA tests are becoming available to an increasing extent, and many virus laboratories have developed their own RIA and ELISA techniques for various viruses. Once the specificity requirements are met with, these assays can readily be standardized and automized. The RIA technique involves the use of radioactive
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isotopes, and therefore carries certain disadvantages for the working environment as well as for the stability of the reagents. Also, the reading of results of the RIA technique requires the availability of more expensive equipment (i.e. gamma counter) than does the ELISA test.

Both techniques can be used for determination of specific antibodies of different Ig classes. However, when these sensitive methods are used for indirect determination of IgM by use of antibodies against human IgM, it is particularly important to be aware of the risk of non-specific reactions because of the presence of rheumatoid factors. In order to avoid this problem the factor or IgG has to be eliminated before performance of the test. Alternatively, antibodies against human IgM adsorbed onto the plastic surface may be employed to capture IgM from the test sample. Specific IgM is then tested for by incubation with viral antigen labelled with the indicator (radioactive isotope or enzyme). This variant also excludes the competition by high-titre specific IgG present in patient serum.

**Evaluation of laboratory results**

It should be pointed out that demonstration, by use of laboratory techniques, of a virus infection in a patient does not necessarily imply that this infection is the cause of the patient's disease. Many viruses often give inapparent infections or are excreted for a long time after the acute disease. Serological cross-reactions between related viruses should also be considered, for example between mumps and other paramyxoviruses, between herpes simplex and varicella-zoster virus and between different enteroviruses. These cross-reactions, which most often are encountered in CF tests, are common if the patient has been previously exposed to a related virus. On the other hand, cross-reactions are used routinely for group diagnosis of certain infections such as those caused by influenza and adenoviruses.
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