MANUAL FOR RAPID LABORATORY VIRAL DIAGNOSIS

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THE MANAGEMENT OF NUTRITIONAL EMERGENCIES IN LARGE POPULATIONS

by

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CORRIGENDA

Page 16, footnote 1

Delete:

[1] UNICEF tablets specified as containing 0.2 g dried iron sulfate (equivalent to 368 mg of elemental iron) and 250 μg of folate are recommended for routine use—UNIPAC catalogue number 15 500 10 (bottles of 1000 tablets).

Insert:

[1] UNICEF tablets containing 300 mg of ferrous sulfate ($FeSo_4.7H_2O$), or about 60 mg of elemental iron, and 250 μg of folate are recommended for routine use—UNIPAC catalogue number 15 500 10 (bottles of 1000 tablets).

Page 20, Table 2, right-hand column, second entry (Xerophthalmia)

Delete: intramuscular injection of 55 000 μg water-miscible retinol palmitate (100 000 IU of vitamin A) followed the next day by oral administration of 110 000 μg (200 000 IU of vitamin A) ; adequate protein intake is essential

Insert: intramuscular injection of 55 000 μg water-miscible retinol palmitate (100 000 IU of vitamin A) followed the next day by oral administration of 68 000 μg of retinol acetate or 110 000 μg of retinol palmitate (200 000 IU of vitamin A) ; adequate protein intake is essential

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INTRODUCTION

During the past few years, the World Health Organization has paid particular attention to the essential role of rapid laboratory viral diagnosis in the management of virus diseases. Two WHO consultations have been held on the subject: at the Pasteur Institute, Paris, in December 1976 (1) and at the Ciba Foundation, London, in December 1977 (2). At both consultations, the need was expressed for a manual of rapid laboratory viral diagnostic techniques for use in training students. The techniques selected for training purposes should be suitable for use in all countries, especially those in which sophisticated tissue culture techniques and virus isolation procedures may not be practicable. It would also be desirable to encourage the use of techniques suitable for investigating problems mainly affecting developing countries - e.g., the diagnosis of rabies, viral hepatitis, and rotavirus infections.

Rapid viral diagnostic techniques fall into four main categories: electron microscopy, immunofluorescence, enzyme techniques, and radioimmunoassay. Although electron microscopy cannot be considered a major diagnostic tool for developing countries, many of these have centres equipped with electron microscopes. A section is therefore included on this subject to encourage the use of the method wherever the equipment is available. It was agreed at the above-mentioned consultations that, for immunofluorescence, the indirect method should be encouraged, and - except for rabies - only this method has been considered in the manual. The enzyme techniques described include the enzyme-linked immunosorbent assay (ELISA) for antigen and antibody detection, and also the immunoperoxidase method (non-solid phase) for the diagnosis of rabies. The use of the last-mentioned technique in other virus systems has been evaluated in a collaborative study (3). The manual also lays special emphasis on the detection of rotaviruses by ELISA, of respiratory virus by immunofluorescence, and of hepatitis A virus IgM antibody by radioimmunoassay. Hepatitis B is not considered because it has been fully dealt with in a previous publication issued by the Center for Disease Control, USA, in association with WHO (4). Radioimmunoassay also perhaps falls into the category of techniques, such as electron microscopy, that may not be suitable for all countries, and it may need to be replaced when other methods become available for the diagnosis of specific virus infections.

Although antibody detection by ELISA, except for IgM, cannot strictly be considered a rapid diagnostic technique, its ease of performance and its excellent correlation with conventional serological tests justify its inclusion.

The reagents used for all the recommended techniques need to be of assured purity. Organizations such as the European and Pan-American Groups for Rapid Laboratory Viral Diagnosis, in conjunction with WHO, will be publishing lists of suitable reagents from time to time. The safe handling of virological material, though not discussed in this brief book, is also of the greatest importance; a useful account is given in, for instance, chapters 1 and 2 of Lennette & Schmidt (5).

1. TECHNIQUES FOR PREPARING CLINICAL SPECIMENS FOR NEGATIVE STAINING AND IMMUNE ELECTRON MICROSCOPY

Almost any type of clinical specimen is acceptable for examination by the electron microscope technique of negative staining, although there are usually alternative methods of preparing specimens. Some of these methods offer the advantage of great speed, but there may be an associated drop in sensitivity. The most sensitive techniques usually require high-speed centrifugation.

Carbon-Formvar grids (400 mesh) are used for all methods. The phosphotungstic acid used for staining is adjusted to pH 6 with 1 mol/l KOH. This pH gives the best general results, but some viruses, e.g., rhinovirus and foot-and-mouth-disease virus, are acid-labile and should be stained with phosphotungstic acid adjusted to pH 8.

Direct method of handling virus in biological fluids

Such fluids include vesicle fluid, allantoic fluid, and faecal suspensions.

A drop of the virus-containing fluid is allowed to absorb to a grid but is not allowed to dry. The grid is then gently washed with a few drops of distilled water. The water is replaced with 20 g/l phosphotungstic acid adjusted to pH 6. Finally, the grid is blotted dry with the torn edge of a piece of filter paper and the grid is ready for examination in the microscope. The preparation time is approximately 10 min. (This method may be used also for preparing virus contained in gradient fractions, although rather longer washing may be required to get rid of caesium chloride or sucrose.)

Method of handling tissue culture preparations

Virus will probably be present in both the supernatant and the cell component of tissue cultures. It is therefore advisable to examine both, and this may be done with the least waste of time as follows. Remove cells from glass or plastic by scraping with a rubber-tipped rod. Since the cells will eventually be lysed, special care need not be taken in doing this. The cells and supernatant are transferred to a conical centrifuge tube and the cell component is pelleted at intermediate speed for 10 min in a clinical bench centrifuge. The supernatant is decanted from the tube and the cell pellet is drained upside down in a beaker containing absorbent material (Fig. 1). The supernatant is then transferred to a suitable tube and centrifuged for 1 h at approximately 12 000 g. The supernatant is then discarded and the pellet drained once again by placing the tube in an inverted position in a beaker as before. However, while the tissue culture supernatant is being centrifuged, the cell pellet may be examined in the electron microscope. If a diagnosis can be made, the supernatant need not be examined, thus saving time.

The cell pellet is handled as follows. With a Pasteur pipette, add a small amount of distilled water to the tube and resuspend the pellet. Continue adding distilled water until the suspension is only slightly cloudy. A drop of this suspension is then placed on a glass slide (Fig. 2) and an equal quantity of 40 g/l phosphotungstic acid at pH 6 is added. A drop of this suspension is then placed on a grid and the excess fluid is withdrawn with filter paper (Fig. 2).

Tissue culture supernatant

Wipe the excess fluid from inside the drained tube and add a small amount of distilled water. Resuspend the pellet, which may not always be visible, and stain and prepare a grid as before.

Allantoic fluid and urine

Either use the direct technique described previously or prepare as for tissue culture supernatant.

Faeces

Make a 100-g/l suspension of faeces in distilled water and clarify for 10 min in a bench centrifuge. Use the supernatant for the direct technique or prepare as for tissue culture supernatant.

Serum

Dilute the serum with an equal amount of phosphate-buffered saline (PBS) and centrifuge for 1 h at 15 000 g. Discard the supernatant and resuspend any pellet in the original volume of PBS. Recentrifuge for 1 h at 15 000 g and again discard the supernatant. Drain the tube as usual and carry out negative staining as before.

Nasal washings

Dilute to a suitable volume with PBS and process as for tissue culture supernatant.

Solid tissue

Soft tissue, e.g., brain or liver, is homogenized in a glass and PTFE homogenizer of the Tenbroek type with distilled water. The amount of water may vary, but as a rough guide a 100-g/l suspension is usually acceptable. This homogenate may then be clarified and, as for tissue culture, both the heavy debris and the supernatant may be processed for negative staining.

Hard tissue - e.g., mainly epidermal lesions, such as warts - is best ground in a pestle and mortar, using silver sand for greater efficiency. The ground tissue is then clarified, the supernatant centrifuged for 1 h at 12 000 *g*, and the pellet used as usual for negative staining.

Note: In preparing specimens for negative staining, large structures do not present a problem, but proteins of low relative molecular mass will overlay and obscure virus. This is the reason for draining tubes.

Immune electron microscopy

Again, many types of specimen may be used for this method. Tissue culture supernatant, clarified faeces, allantoic fluid, and serum are all acceptable. If the specimen has a large amount of background material, a light clarification is advisable.

Volumes of about 2 ml of virus antigen are suitable for use with this method, although as little as 0.2 ml and as much as 5 ml may be used, depending on availability.

If 2 ml of virus antigen are used, it is convenient to add 0.2 ml of antiserum at a suitable dilution. This dilution depends on the titre of the antiserum and the amount of virus in the antigen preparation. If the antiserum is uncharacterized, 0.2 ml of a 1:10 dilution is worth trying, subsequent adjustments being made when a result is obtained.

Antiserum is added to the virus antigen and thoroughly mixed with it. The mixture is then left to react for a suitable period. In case of urgency, this time may be half an hour at 37°C, but 1 h at room temperature or the whole night at 4°C are also satisfactory. After this reaction period, the specimen is centrifuged for 1 h at 12 000 g and the resultant pellet is handled as before for negative staining.

Note: The most important aspect of immune electron microscopy is judging the relative proportions of antigen and antibody. Too much antibody results in individual particles completely covered with antibody, while too little will not lead to complex formation. However, once an antiserum has been assessed, it will be found that considerable tolerance exists in the equivalence region for aggregation to occur.

Safety

Specimens for electron microscopy still contain active virus, and all materials used for preparing grids should be immersed in hypochlorite (Fig. 1).

2. DIAGNOSIS BY THE INDIRECT IMMUNOFLUORESCENCE TECHNIQUE ON CLINICAL SPECIMENS, WITH PARTICULAR REFERENCE TO RESPIRATORY VIRUSES

Only the methods used for examining clinical material for virus infection are described here. The success of the methods depends on a number of factors, including the adequate preparation of specimens and the availability of tested reagents free from nonspecific activity.[1]

Direct detection of virus

Preparation of specimens. The best specimen for the investigation of respiratory virus infection is a nasopharyngeal secretion taken from the nasopharynx by suction and aspirated into a mucus extractor (Fig. 3). Secretions should be rapidly transported on melting ice to the virus laboratory. Cells are separated by centrifugation at 350 *g* at 4°C. The supernatant is removed and the cell deposit is used for rapid virus diagnosis. This deposit is resuspended in 3-4 ml of PBS and is gently pipetted with a wide-bore Pasteur pipette. Thick fragments of mucus that will not break up are discarded. The cell suspension is transferred to a test-tube and a further 4 ml of PBS are added. The contents are mixed and centrifuged again at 350 *g* for 10 min. The deposit is resuspended in PBS to dilute any mucus still present and to make sufficient numbers of preparations on the slides to be able to test for a number of viral antigens. The slides may be precoated with PTFE, exposing squares for the cell suspension, or squares may be etched on to ordinary slides. One drop of cell suspension is spread evenly in each square and is allowed to dry in the air. When dry, the cell preparation is fixed in acetone for 10 min at 4°C.

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[1] The European Group for Rapid Laboratory Viral Diagnosis has tested the following Wellcome reagents and found them satisfactory for use.

Antisera: influenza A, respiratory syncytial virus, herpesvirus hominis, and measles.

Antispecies conjugate: antirabbit, antibovine.

Cough swabs (see 6) are far less suitable than nasopharyngeal secretions. The cells present on such swabs are gently eluted into PBS by pipetting the fluid over the swab repeatedly. Cells smeared from cough swabs directly on to slides are useless for this technique. Cells eluted from such swabs are treated in exactly the same way as described for nasopharyngeal secretions.

Skin scrapings are taken from the base of vesicles. Crusted lesions are less suitable for immunofluorescence diagnosis. Eye scrapings may be taken from the conjunctivae, particularly from the area of dendritic ulcers. Scrapings taken from these lesions are teased with two dissecting needles in a drop of PBS on a slide. When the tissue has been sufficiently broken up for free cells to be suspended in the PBS, the cell suspension is air-dried and fixed in acetone as described above.

In the case of biopsy and post-mortem material, secretions taken from the respiratory tract are treated as indicated above. The most suitable method of examining tissues is that of impression smears. Pieces of tissue 2-3 mm^3 in size are pressed between glass slides to make the impression. These impression smears are then fixed in acetone after drying, as described above.

Reagents. Immunofluorescence diagnosis requires reagents adequately tested for specificity and freedom from unwanted antibodies. Stringent testing is needed, not only on tissue culture but also on clinical material, to ensure that the reagents are free from all nonspecific reactions. Most antisera require elaborate cleansing from unwanted antibodies. This usually involves absorption with tissue culture cells in which the virus was grown for the preparation of antiserum, and also with cells of human origin. Most virus antisera are produced by their users. However, certain commercial firms are now producing specific virus antisera, e.g., Wellcome Reagents produce a bovine anti-respiratory-syncytial (RS) virus serum and a bovine anti-influenza A serum. They also produce rabbit anti-herpesvirus serum. Many laboratories have been preparing their own antisera because of the limited number available commercially. Among the viruses that have been detected in clinical material by the indirect immunofluorescence technique are RS virus, influenza A and B, parainfluenza viruses 1, 2, 3, 4a, and 4b, adenovirus group, measles, mumps, rubella, herpesvirus hominis, varicella zoster, and cytomegalovirus (CMV).

Conjugates are available commercially, but these also require absorption before use. Suitably absorbed antispecies conjugates are available (Wellcome).

The optimum fluorescent antibody titres of antisera and conjugates must be assessed by titration on appropriate virus-infected tissue cultures and clinical material. All procedures may be carried out by diluting the antispecies conjugate in 0.1 g/l Naphthalene Black in PBS. This is especially valuable in diminishing the autofluorescence of human cells in clinical material.

Light source and microscope. A high-intensity light source with strong emission of ultraviolet and violet-blue wavelengths is required. The lamp most generally used for this purpose is the mercury vapour burner HBO 200. Where only blue-light fluorescence is required, iodine quartz with interference filters may be used.

Transmitted and incident light are both suitable for fluorescence microscopy, but with the development of reliable interference filters incident-light illumination has become more universally available. Each type of microscope and method of illumination requires its own filter system.

Staining techniques. Slides, with usually up to three cell preparations per slide, are treated with a number of appropriate antisera. For example, cells from a child with croup would usually be tested for parainfluenza 1, 2, 3, 4a, and 4b, and also for influenza A and B and RS virus when they are epidemic. The cell preparations on each slide are covered with the predetermined optimum dilution of antiserum and incubated in a moist chamber at 37°C for 30 min. The slides are then washed gently and given three separate washings of 10 min each in dishes of PBS. The slides are allowed to dry, and then the appropriate antispecies serum conjugated with fluorescein isothiocyanate is added to each square; the slides thus stained are again incubated in a moist chamber for 30 min at 37°C. The washing procedure is exactly the same as before except that there is a final rinse in distilled water to remove any crystal deposits from PBS that might occur when the slide is dried. The slide is air-dried and examined under oil immersion. There is no need for any mountant or coverslip.

Controls. Usually, more than one antiserum, from several rabbits, is used as a control. There is no real negative control for a specimen of clinical material. However, the pre-inoculation serum of the animal in which the antiserum has been prepared is a useful additional control in cases of doubt. The best guide to positive fluorescence is as follows.

1. Intracellular fluorescence.
2. The distribution of fluorescence in the cell should be characteristic of the particular virus as learnt from experience.
3. Fluorescence should be apple green. Any deviation from this colour should be treated with suspicion.

When one is introducing a new technique, one must not discard conventional techniques until a sufficiently large number of comparisons (at least 100) has been made between positive immunofluorescence and a conventional technique. The most usual conventional technique is virus isolation. Obviously, there should be a high correlation between the two techniques: of the order of 95-100%.

Quality control of reagents.

(a) Infected and control cells should be tested to determine the optimum dilution of the antiserum for detecting the antigen. With a previously standardized and appropriate antispecies conjugate, this antiserum dilution must be at least four times as high as that revealing any nonspecific fluorescence with uninfected cells. The control cell cultures should include a continuous human line, a continuous monkey kidney line, a diploid cell strain, and a primary monkey kidney line.

(b) A limited titration for final evaluation should be made on positive clinical material appropriate for the virus being tested.

(c) Antisera should be tested at their optimum dilutions on negative pharyngeal secretions and, where possible, on other negative human clinical specimens.

(d) Antisera should be tested on cell cultures infected with a representative collection of viruses, e.g., RS virus and parainfluenza 1, 2, 3, 4a, and 4b, mumps, influenza A and B, measles, herpesvirus, CMV, and at least two enteroviruses. There should be no nonspecific reaction with these antisera at their optimum dilutions, and the antisera should show no nonspecific activity at their optimum dilutions when tested against heterologous virus antigens.

Quality control of antispecies conjugate, e.g., antirabbit or antibovine.

(a) Dilutions of antiglobulin conjugate with an appropriate antiserum should be tested on positive tissue cultures and positive human specimens to find the optimum dilution for use. A preliminary optimum dilution may be obtained on tissue culture, but must be modified for final use on material taken direct from the patient.

(b) The dilutions are then tested at their optimum for any nonspecific reaction on cell lines likely to be used in the laboratory, including those mentioned above for antisera.

(c) Antiglobulins should then be tested at the optimum dilution on negative human material, including nasopharyngeal secretions, lung material, and biopsy material.

These criteria will apply only under the conditions of the test system, and it is therefore of great advantage to users of the reagents to use the staining technique including counterstain as suggested above.

Fuller details of these techniques have been given elsewhere (6).

Detection of specific IgM class of antibody by immunofluorescence techniques

The appropriate cell line is infected with the virus. Slides may be prepared with infected cells fixed in acetone for 5 min, and then used. Fixed cells are treated for 3 h at 37°C with dilutions of patient's serum taken in the acute phase. The slides are washed for 10 min three times (half an hour in all) as described previously, and are then stained with antihuman IgM for half an hour at 37°C, washed again, and examined under oil immersion. Unfixed cells may be used ("membrane fluorescence"), 0.02 ml of serum dilution being placed in each well of a microtitration plate, followed by 0.015 ml of virus-infected-cell suspension. Incubation is carried out for 1 h at room temperature. Excess serum is then removed by washing the cells three times in Hanks' balanced salt solution, using a plate centrifuge at 100 g. Then 0.015 ml of antihuman IgM is placed in each well and the plates are incubated at room temperature for 1 h, the cells being washed as before. The cells are then resuspended in 0.03 ml of Hanks' solution and spotted on to slides covered with coverslips. The slides are sealed with wax and then read. Specific IgM may be masked on occasions by high levels of specific IgG. However, with rapid diagnosis early in the illness, this should not present much difficulty because IgG titres should not have risen. Among the many methods of separating IgM are absorption by Staphylococcus aureus Cowan type 1, fluorescence staining of specific IgM in sucrose gradients, thin-layer gel filtration, and affinity chromatography.

The results may also be influenced by rheumatoid factor. If this is present, it may be removed by absorption with aggregated IgG complexes.

3. IMMUNOFLUORESCENCE IN RABIES DIAGNOSIS

Rabies is a disease with a long incubation period. Its treatment also is long. Rapid and specific examination of the suspected material is therefore necessary for treatment to be undertaken and continued.

The fluorescent antibody (FA) technique, first developed in 1958 (7), is one of the best available, because of its specificity and rapidity. It has largely replaced diagnosis by inoculation of animals. It may be used in both its direct and indirect forms, with the same sensitivity.

The direct FA technique has been successfully applied for the titration of neutralizing antibodies on tissue cultures, with results comparable to those obtained by the neutralization technique in mice (8).

Materials and methods

Antisera. It is known that the rabies virion contains two major antigens: an internal antigen, the nucleocapsid, and a peripheral antigen, the glycoprotein. In rabies, antibodies against the nucleocapsid are responsible for the immunochemical staining (fluorescence and immunoperoxidase), complement fixation, and precipitation.

Two types of antiserum may be used: a horse antiserum with a high neutralizing and antinucleocapsid titre, prepared against the Pasteur virus strain produced in cell culture; and a rabbit antiserum against a pure preparation of nucleocapsid.

Purification of antibodies. Two methods may be used: (1) purification with the help of an immunoadsorbent (this method has been described in detail) (9); and (2) chromatography on QAE Sephadex 50, which permits the recovery of pure IgG from the antinucleocapsid sera (10).

Conjugation with fluorescein isothiocyanate (FITC). After dialysis against a carbonate buffer, pH 9, for 5 h, the IgG preparations are conjugated with FITC in the proportion of 1 mg of FITC per 100 mg of protein.

Preparation and examination of specimens. Material from animals suspected of having rabies, consisting of frozen sections and brain smears, is examined as follows.

To make frozen sections, organs of the suspected animals are harvested under sterile conditions, cut rapidly into 1-mm^3 fragments, and frozen on dry-ice. The fragments are placed on the specimen holder, kept at -20°C, and, the following day, cut into sections 0.004 mm thick. The preparations are fixed with acetone for 10 min and stained with the FA technique.

Smears are prepared from the hippocampus (Ammon's horn) and from the medullary bulb of the suspected animals, one smear of each being spread on each slide. The slides are then dried, sterilized under ultraviolet light for 5 min, and fixed with cold acetone for 10 min. One of the smears on each slide is then stained with labelled antirabies globulin and a 200-g/l suspension of rabid mouse brain. The other smear is stained with labelled antirabies globulin and a 200-g/l suspension of normal mouse brain. The slides are kept in a humid atmosphere for 30 min at 37°C, after which they are washed for 10 min with buffered saline (pH 7.2), for 2 min with distilled water, and for a further minute with another lot of distilled water. After being dried again, the slides are treated with poly(vinyl alcohol) or 100 g/l glycerol for 2 h at 37°C, mounted, and examined immediately under the fluorescence microscope. An absence of fluorescence in the preparation exposed to rabid mouse brain indicates a specific reaction. In the case of the preparation exposed to normal mouse brain, specific fluorescence indicates positivity, and an absence of fluorescence, negativity.

Conclusions

With the technique of column chromatography on QAE Sephadex 50, good purification may be obtained and a high titre of IgG conjugated with FITC.

In addition, the use of antisera against purified viral nucleocapsids makes it possible to obtain conjugates with very high titres.

The direct FA technique is highly sensitive for frozen sections or smears.

As a diagnostic technique for rabies antigens, immunofluorescence satisfies all criteria of sensitivity and specificity.

The use of an antirabies nucleocapsid antiserum, purified on a column of QAE Sephadex 50 for the FA technique, is very convenient because of its high sensitivity, lack of background, and low cost.

4. IMMUNOENZYME TECHNIQUE FOR THE IDENTIFICATION OF RABIES ANTIGEN AND ANTIBODY

Viral antigens have been demonstrated by means of antibodies labelled with various readily detectable substances. The first substances used were fluorescein with the ultraviolet microscope and ferritin with the electron microscope. Subsequently, enzymes linked with antibodies were used to demonstrate the presence of antigens and for assaying antibody in a large number of parasitic, bacterial, and viral diseases. The enzymes most frequently used are alkaline phosphatase, glucose oxidase, and peroxidase. Peroxidase, which is detectable with the light and electron microscopes, is the enzyme most used in virology.

Peroxidase (relative molecular mass: 40 000) may be linked with antibody by means of glutaraldehyde or periodate without impairing the functions of either the antibody or the peroxidase (11,12). The peroxidase-labelled antigen-antibody complex is revealed by a staining reaction caused by the peroxidase in the presence of hydrogen peroxide and benzidine or o-dianisidine.

Identification of the rabies antigen

The direct technique makes use of peroxidase-linked rabbit antirabies immunoglobulins derived from immune sera obtained with the nucleocapsid or glycoprotein antigens. The indirect technique requires peroxidase-linked anti-rabbit IgG or peroxidase-linked sheep antihuman IgG and IgM.

Direct technique. Histological slides bearing two impression smears of rabid brain (preferably from the hippocampus region) are fixed in acetone cooled to -20°C. A dilution of antirabies nucleocapsid serum labelled with peroxidase in a 100-g/l solution of normal mouse brain is added to the smear near the label and a 100-g/l solution of normal mouse brain in phosphate buffer is added to the other smears. The slides are incubated for 1 h at 37°C and washed in phosphate buffer for 10 min. They are then treated with 5 mg of diaminobenzidine dissolved in 10 ml of Tris-HCl buffer, pH 7.4. To this solution is added one drop of 30-volume hydrogen peroxide. One drop of this reagent is placed on each smear, and after 5 min at room temperature the preparations are washed in distilled water. They are examined under the light microscope at different magnifications and lastly under oil.

The small and large brown-stained inclusions found close to the label represent the specific antigen. They are absent from the control (13).

Indirect technique. A suitable dilution of antirabies nucleocapsid serum (preferably from a rabbit) is added to the smear near the label and normal rabbit serum is added to the other smear. The preparations are incubated for 30 min at 37°C and washed with a phosphate buffer solution, pH 7.2. Peroxidase-labelled antirabbit globulins are then added in the appropriate dilution. After 30 min at 37°C, the specimens are washed and the substrate (hydrogen peroxide and benzidine) is added in the same proportions and at the same time as for the direct technique. The results are read in the same way as for the direct technique. Small and large brown inclusions represent the rabies antigen. The control should be negative.

Assay for rabies antibody

The immunoenzyme (IE) technique for the assay of soluble substances was developed recently (14). This technique has made it possible to follow the dynamics of the appearance of rabies antibodies in subjects vaccinated against rabies and in subjects suffering from rabies who have received no antirabies treatment. Specific IgM and IgG are readily identified in the latter subjects.

Principle. Polystyrene tubes are coated with rabies antigen (if microtitration plates are used, 0.3 ml of antigen is placed in each well). Corresponding antiserum is then added, followed by an enzyme-labelled anti-immunoglobulin preparation. The amount of enzyme remaining in the tube (or in the wells of the plate) after washing reflects the amount of specific antibody present in the serum (15).

Materials. The antigen used is the Pasteur rabies virus strain adapted to cell culture $BHK_{21}C_{13}$, which has a titre of about 10^8 plaque-forming units (PFU) per millilitre. This virus, grown in the presence of bovine albumin, is concentrated 25 times by precipitation with zinc acetate. The precipitate, dissolved in Tris-EDTA buffer, is then centrifuged at 60 000 g for 150 min. The sediment is reconstituted in 0.05 mol/l carbonate buffer (pH 9.6) with 0.2 g/l NaN_3. This constitutes the starting antigen.

The technique requires polystyrene tubes (50 mm high and 11 mm wide at the base) or microtitration plates (Greiner type M-129-A), which are readily available commercially, and peroxidase-labelled sheep antihuman immunoglobulin.

Two human control sera should be used: a positive serum, which is a pool of several sera with a high serum neutralization titre (5-10 IU) in the mouse, and a negative serum pool (titre in serum neutralization test: $< 1/5$).

Method. The optimum dilution of the antigen (between 1:100 and 1:200) in 1 ml of carbonate buffer is placed in the tube and incubated for 3 h at 37°C, then at 4°C until the next day, when the test is performed. (If microtitration plates are used, 0.3 ml of antigen in buffer is placed in each well of the plate.) Each tube or plate is washed 5 times with 5 ml of PBS containing 0.5 g/l polysorbate 20 (Tween 20). This mixture is used for all the washings.

All the sera for testing and the control sera are diluted 1:200 in PBS containing 0.5 g/l bovine albumin and 1 ml of this mixture is placed in each tube (or 0.3 ml is placed in each well, if microtitration plates are used). The tubes or plates are incubated for 1 h at 37°C and washed 5 times. Then 1 ml of a 1:500 dilution (same diluent) of peroxidase-labelled antihuman IgG is added (or 0.3 ml is placed in each well, if microtitration plates are used). After incubation for 1 h at 37°C, followed by washing, the amount of enzyme attached to the tube or well is determined by testing with a specific substrate. This substrate is prepared on the spot as follows: 1 ml of 1 mol/l phosphate buffer (pH 6), 1 ml of a 10-g/l solution of o-dianisidine in methanol, and 0.1 ml of 30-volume H_2O_2 are made up to 100 ml with distilled water. Then 2.5 ml of the substrate are added to each tube (or 0.3 ml is placed in each well, if microtitration plates are used) and the tubes or plates are incubated for 1 h at 37°C. The reaction is stopped with a drop of 5 mol/l HCl and the absorbance is read in the spectrophotometer at 403 nm. The difference in absorbance at 403 nm between the test serum and the negative control gives the result.

The advantages of the IE technique described are: stability of conjugates, easy measurement, and a sensitivity much greater than that of the serum neutralization test, particularly in untreated subjects with rabies encephalitis, since specific IgM can be detected in the serum in the very first days of the disease.

5. THE POTENTIAL APPLICATION OF THE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TO DIAGNOSTIC VIROLOGY

Immunosorbent assay (ELISA) appears to show great promise in the diagnostic laboratory (14,16,17). Most applications of ELISA in the viral field must still be considered as being in the research and development phase.

The obvious use of ELISA is for the detection of antibody, which may be done by the "indirect method" (Fig. 4). Antigens, e.g., viruses or their products, may be measured by the double antibody sandwich method (Fig. 5).

In principle these methods are simple, but there are many variables that may affect the results. Some of these will be considered here.

Carrier surface

Tubes, discs, and beads may be used. Disposable haemagglutination plates are particularly convenient (see Annex 1). It is essential to use a solid phase carrier that has a high binding capacity and is constant within each batch and from one batch to another. Considerable variation may occur within and between different production batches of all the plastic - e.g., polystyrene, poly(vinyl chloride) - beads, tubes, and microtitration plates used. For tests relying on passive adsorption of reactant, chemical composition and surface characteristics are particularly important. In addition, special treatment, e.g., sterilization by radiation, may affect the reactivity of the solid phase. Therefore, each batch of material should be tested prior to use as a solid phase. This testing should also include the determination of within-batch variation.

A method of testing a representative sample of a batch of beads, tubes, or microtitration plates is as follows.

1. Coat with a laboratory reference human Ig.
2. React with enzyme-labelled anti-human Ig.
3. Add substrate and read the results after appropriate incubation.

Such a procedure may indicate the suitability and variability of the solid phase material as well as any patterns of uptake. A standard deviation of 0.05 with an absorbance reading of 1.0 may be considered acceptable.

Although the results of coating with human Ig seem to be fairly representative for many materials (antigens or antibodies), one may also choose a predetermined concentration of another material as a laboratory reference.

For the detection of antibodies the carrier surface must be coated with an antigen solubilized with an appropriate buffer (see Annex 1). The antigens are usually attached to the solid phase by passive absorption. Covalent linking might be advantageous in some situations. A variety of commercially available antigens have proved to be satisfactory. However, viral antigens prepared and standardized for reactivity in other antibody tests, such as complement fixation or passive agglutination, may not be satisfactory for ELISA. Such antigens are often diluted in solutions of other proteins, e.g., albumin, or processed for anticomplementary activity. This inevitably affects ELISA reactivity. Where the relevant viral antigen is known and can be purified in a practical and economical manner, it should be used in lieu of crude preparations. However, most viral antigens are crude extracts and cannot always be satisfactorily characterized. The only reasonable way of standardizing these is by carrying out checker-board ELISA titrations with at least one reference serum containing antibody to the antigen in question, under defined conditions, with defined anti-immunoglobulin conjugates. Subsequently, such antigens must be tested against a panel of sera, so that spurious nonspecific reactivity may be detected. Such a panel should include strongly positive, weakly positive, and negative samples, and samples known to cause problems in other serological tests. The use of "control antigen", i.e., material prepared like the viral antigen but from uninfected material, is often necessary. True positivity may be concluded only when there is a sufficiently great difference between the reactions with viral and control antigens.

The suitability of antisera for the preparations of solid phase antibody to be used for antigen testing depends on specificity and titre. Requirements for specificity do not seem to differ considerably from those for other immunoassay systems. If the antiserum has a sufficiently high titre, a crude immunoglobulin fraction may be used for coating. Sensitivity must be determined with a dilution series of at least one positive reference preparation. Sensitivity and specificity should be assessed with a panel of positive, weakly positive, and negative test samples.

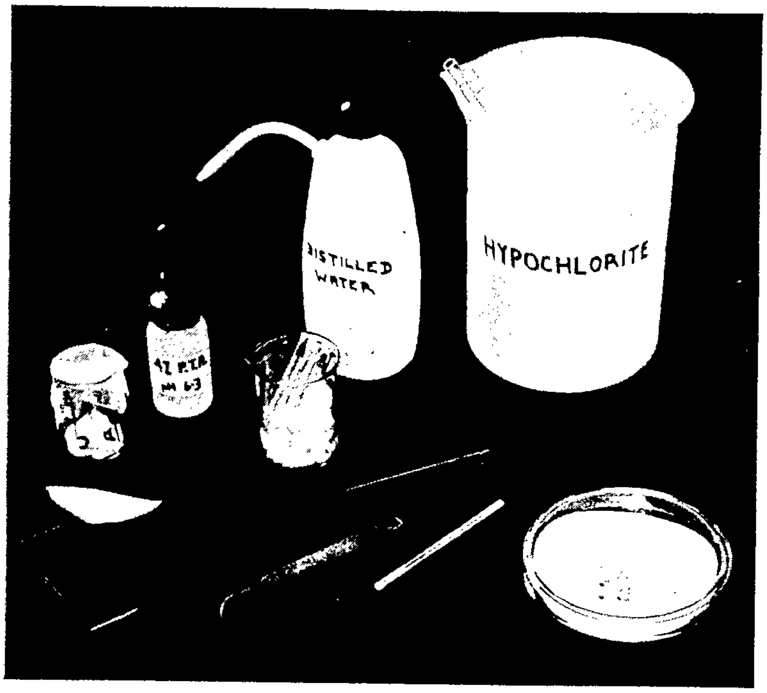

DISTILLED WATER
HYPOCHLORITE

Fig. 1 Material required for carrying out negative staining

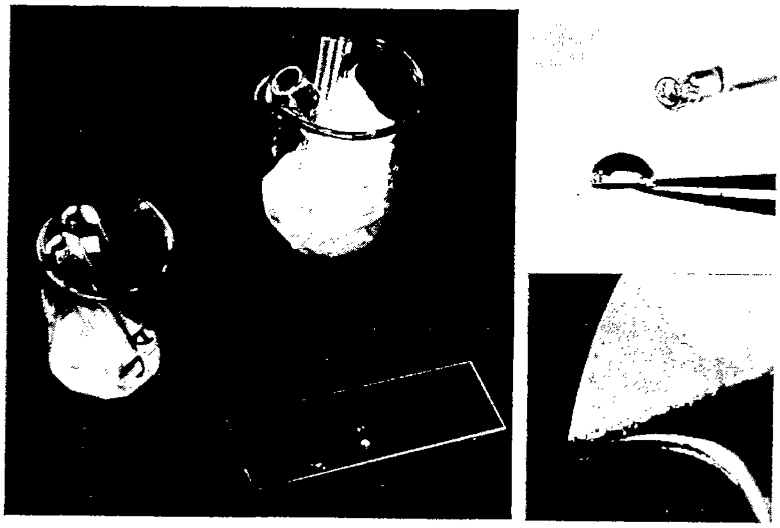
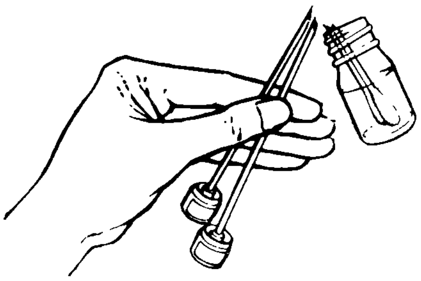


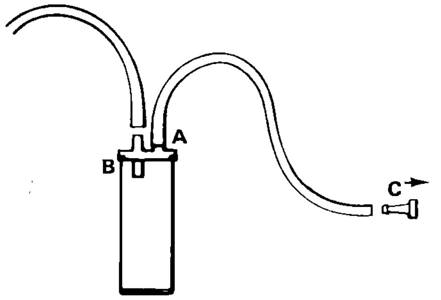
Fig. 2 Method of handling specimens and grids for negative staining

Fig. 3 Transport of cough swabs; aspiration and transport of nasopharyngeal secretions



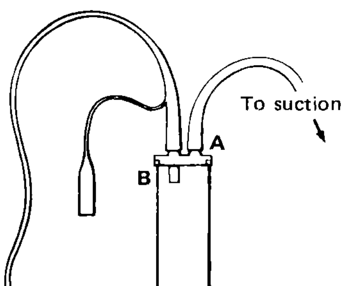
Transport of cough swabs

Cough swabs are placed in the same bottle of Hanks' medium

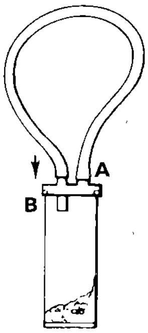


Apparatus for aspirating nasopharyngeal secretions

(i) Remove and discard tube at nozzle B and mouthpiece at C



(ii) Attach size 8 nasogastric tube to nozzle B and attach tube A to suction pump



Transport of nasopharyngeal secretions

Remove and discard nasogastric tube; detach end of tube from suction pump and attach it to nozzle B

WHO 79155

Fig. 4 The indirect method of ELISA for assaying antibody

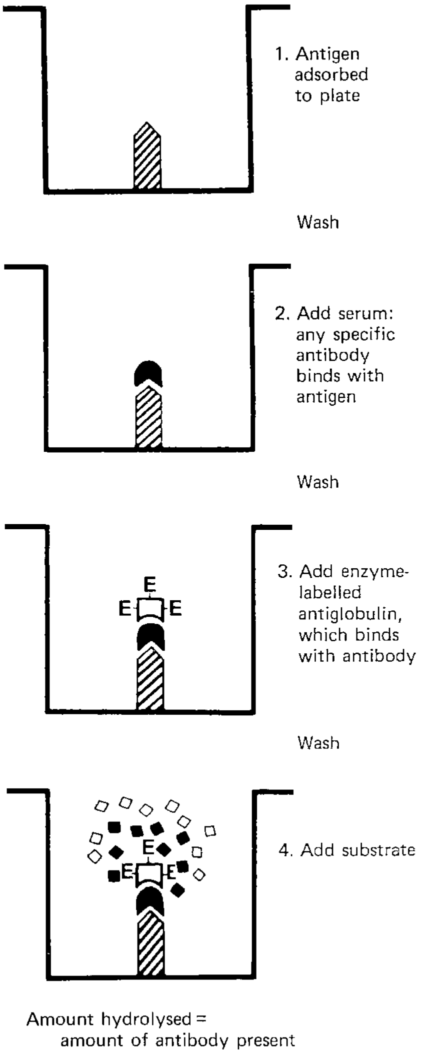

1. Antigen adsorbed to plate
Wash
2. Add serum: any specific antibody binds with antigen
Wash
3. Add enzyme-labelled antiglobulin, which binds with antibody
E
Wash
4. Add substrate
Amount hydrolysed = amount of antibody present

Fig. 5 The double antibody sandwich method of ELISA for assaying antigens or viruses

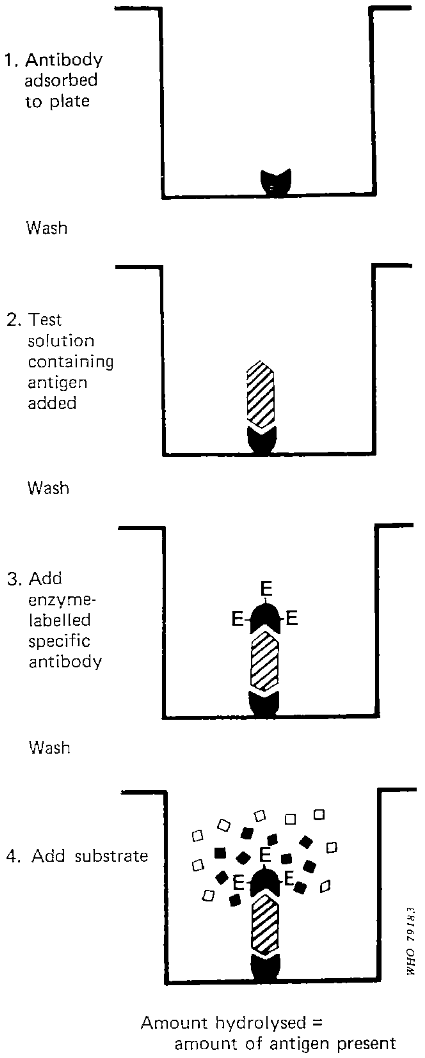
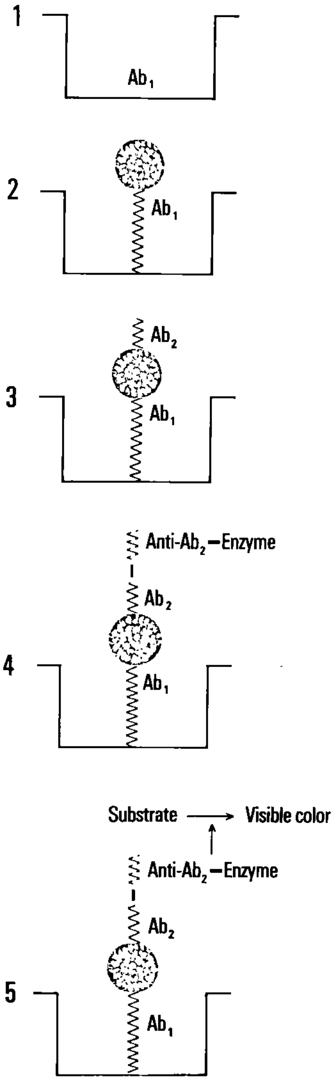

1. Antibody adsorbed to plate
Wash
2. Test solution containing antigen added
Wash
3. Add enzyme-labelled specific antibody
E
Wash
4. Add substrate
WHO 79183
Amount hydrolysed = amount of antigen present

Fig. 6 Indirect ELISA for antigen measurement


1
Ab₁
2
Ab₁
3
Ab₂
Ab₁
4
Anti-Ab₂–Enzyme
Ab₂
Ab₁
5
Substrate → Visible color
Anti-Ab₂–Enzyme
Ab₂
Ab₁

1. Antibody directed against the antigen to be measured is adsorbed to the well of a microtitre plate.

2. The test material is added. Any antigen will bind with an antibody directed to it.

3. Unlabelled antibody from a different animal than Ab_1 is added. This will react with any antigen that is bound with Ab_1.

4. Enzyme-labelled antibody directed against the globulins of the animal source of Ab_2 is added.

5. A substrate is added. The enzyme adhering to the well will convert the substrate to a visible form. The amount of colour is proportional to the amount of antigen in the test material.

In general, the quality of the solid phase antigen or antibody should be such that the difference in response between specific and nonspecific binding is maximal. This difference should be expressed as a ratio, and should also be in the accurate range of the equipment, e.g., spectrophotometer, used for measuring the response.

Test sample

The optimum dilution for sample testing must be determined by checker-board titrations with the reference positive, weakly positive, and negative samples.

The dilution of the samples giving the greatest difference in response from negative samples is used for routine testing. This difference may be expressed as a ratio, but should also be related to the total length of the scale of the equipment used for measuring the response. For reading with the naked eye, the optimum contrast between (weakly) positive and negative should be sought.

It may be necessary to test samples at two or more dilutions in some instances.

Clinical samples, such as faeces or cell scrapings, may have to be processed prior to the test proper. This procedure should be optimized by checker-board titrations, as indicated above. The effect of this processing on the specificity should be assessed.

Conjugates

Antisera of high specificity and titre are necessary for the production of conjugates. It is best to obtain large batches of antisera and to conjugate the necessary amounts of this stock. The change from one batch to another should be accompanied by the testing of sensitivity and specificity.

Antisera against immunoglobulins to be used for antibody testing should be free from antibodies against the viral and control antigens on the solid phase. Furthermore, it is often preferable to employ immunoglobulin class-specific antisera (e.g., anti-IgG, anti-IgM). Their specificity in any new assay system should be checked.

Antisera against viral antigens should be carefully checked for specificity, e.g., with antigen preparations of related viruses and with control antigens.

The enzyme-labelled first or second antibody should preferably be free from both nonlabelled antibody and unconjugated enzyme. The conjugated enzyme should have retained much, preferably all, of its enzyme activity. The labelled antibody should have retained much, preferably all, of its original affinity. To what extent these ideals have to be reached depends on the requirements of the final test system. The significance of physicochemical characteristics, such as relative molecular mass, for the suitability of conjugates in immune tests is still uncertain.

Various enzymes may be used in this system (see Annex 1). So far, peroxidase, alkaline phosphatase, and beta-galactosidase have been the most frequently used.

Anti-immunoglobulin conjugates may be assessed:

(a) on solid phase materials coated with the relevant immunoglobulin (positive control);

(b) if class specificity is important, on solid phase material coated with other immunoglobulin classes (negative controls);

(c) on solid phase material coated with viral (and control) antigen(s) (negative controls);

(d) with reference positive sera in the test system in which the conjugate is to be used subsequently (positive controls); and

(e) with reference negative sera in the test system in which the conjugate is to be used subsequently (negative controls).

Antivirus conjugates may be assessed:

(a) with reference positive samples in the test system in which the conjugate is to be used subsequently (positive controls) and

(b) with reference negative samples in the test system in which the conjugate is to be used subsequently (negative controls).

The optimum conjugate concentration should be such as to ensure an optimum difference in response between positive and negative samples.

Substrates

Enzyme substrates should be cheap and safe, and should produce an easily observed colour change on degradation (see Annex 1). 4-Nitrophenyl phosphate is suitable for alkaline phosphatase, and 5-aminosalicylic acid has been widely used for peroxidase, but orthophenylenediamine with H_2O_2 is to be preferred as a substrate for peroxidase.

Results

The results may be assessed visually, samples yielding an obvious coloration being regarded as positive. This coloration may be quantified if serial dilutions of the test sera are made.

For more precise measurement, a simple spectrophotometer is needed. With a microcell these machines may be used even with reagent values as small as 0.2 ml.

Test conditions

The immunological specificity of enzyme-immunoassay, like that of other immunoassays, is based on the specificity of the reagents in the final test combination. However, even if the quality of the individual reagents is satisfactory, it cannot be taken for granted that the combination of reagents is sufficiently specific, precise, and sensitive for a particular purpose. Consequently, standardization and quality control measures should aim particularly at the final reagent combination. This should comprise at least the various tests mentioned for the individual reagents.

The use of well characterized reference panels (international, national, interlaboratory, or intralaboratory) is essential.

A useful way to determine sensitivity is by means of a dilution series of one or more positive samples. The diluent should be such that the composition of the diluted sample is similar to that of the sample tested routinely, e.g., a positive test serum may best be diluted with a (diluted) negative serum from the same species.

The best way of checking the validity of results is to include reference positive and negative sera in each group of tests.

Positive results in antigen tests should be confirmed in blocking tests by means of human or primate antiserum of established specificity.

Never should more than one bulk reagent be changed at any time. Each change should be accompanied by testing as pointed out in the section on conjugates.

Monitoring test performance

Any new ELISA test should be monitored under varying conditions and on several occasions after its introduction. For validation, it is necessary both to carry out comparative studies with one or more of the established serological methods and to relate results to clinical observations.

The new test system should be investigated on a representative sample of the study population. Care should be taken in extrapolating the test results to a different population since antibody or antigen levels, as well as (sub)specificities, may differ and criteria for positivity and negativity may have to be reconsidered.

Stability of reagents

It must be ascertained that reagents and reagent combinations retain their properties regarding specificity and sensitivity for a long time under the conditions prevailing in the laboratory. If reagents are to be sent from one laboratory (or country) to another, storage conditions may be difficult to control. Therefore, it may be useful to carry out accelerated stability testing at elevated temperature and humidity. Storage conditions of reagents and reference materials should be clearly stated. For standardized reagents and reference materials, an expiry date should also be given.

Standardization needs

Reference reagents. Such reagents are particularly useful in the establishment of new tests in a laboratory.

The provision of viral antigen and control antigen suitable for coating, on a national or preferably international scale, would allow laboratories to establish the quality of their own antigen reagents.

The specifications of an anti-immunoglobulin horseradish peroxidase conjugate are being drawn up by a subcommittee of the International Union of Immunological Societies/WHO Standardization Committee.

Reference preparations. Positive and negative "known" reference preparations must be available in large quantities for each viral system. Each laboratory will need its own reference materials for day-to-day use, but both national and international reference standards should be made available.

Reference panels. Such panels are already being introduced in the USA and some other countries (e.g., for HBsAg and rubella serology). National or interlaboratory panels should be established in other countries.

The origin of the reference materials and the criteria used in their selection should be publicly known, so that it may be possible to judge their suitability for particular test samples.

Example of an indirect ELISA for antibody to cytomegalovirus (CMV)

1. CMV antigen is diluted in coating buffer to an optimum concentration (predetermined by checker-board titration against reference positive and reference negative sera). Polystyrene micro-haemagglutination plates are divided into four sections of three rows each. Then 0.2 ml of CMV antigen is added to each well of the first two rows of each section. The third row contains 0.2-ml amounts of control antigen (extract from uninfected tissue culture, used at the same dilution as the CMV antigen). The plates are kept at $4^{o}C$ (or at room temperature) in a humid chamber for 5 h and are then washed by emptying and refilling with PBS-polysorbate 20 (Tween 20) from a wash bottle. This washing process is carried out three times at intervals of 3 min.

2. Test and reference sera are diluted 1:200 in PBS-polysorbate 20. Then 0.2-ml amounts of each serum sample are tested in duplicate against the CMV antigen and singly against the control antigen. Several replicates of the reference positive and negative sera are included on each plate. The sera are incubated at room temperature for 2 h. The plates are then washed as before.

3. A 0.2-ml amount of dilute antihuman immunoglobulin labelled with alkaline phosphatase is added to each well and the plates are kept at 4°C overnight. The plates are then washed as before.

4. A 0.2-ml amount of enzyme substrate (4-nitrophenyl phosphate) solution is added to each well. The reaction is allowed to proceed at room temperature until the standard positive serum reaches an extraction value of 1.0 at 405 nm (read in a spectrophotometer). The reaction is then stopped with 0.05 ml of 3 mol/l NaOH.

The absorbance of the contents of each well is then read at 405 nm in a spectrophotometer. The substrate solution containing NaOH must be used as the blank.

The values obtained with each serum on the control antigen are subtracted from those obtained on the positive antigen. The resultant value is a measure of the reaction with the CMV component of the antigen. The values above those obtained on a pool of negative sera are considered as positive.

For other viruses, the optimum conditions must be established. Not all commercially available antigens are satisfactory, and each new batch should be tested before being used routinely.

Applications to viruses of man (18)

The first use of ELISA in the viral field was for the measurement of antibody to rubella (19,20,21). The indirect ELISA yielded results comparable to those obtained by haemagglutination inhibition. Crude antigens may be satisfactory for most purposes, but, for some special applications (e.g., detection of antibody to CSF), highly purified antigens gave better results (22). If enzyme-labelled anti-IgM conjugate is used as the indicator, it is possible to measure specific rubella antibody in the IgM fraction of sera. It is recommended that the sera should be fractionated prior to such testing, e.g. on Sephadex G200. The existence of rheumatoid factor together with IgG specific antibody often leads to false positive IgM antibody results. The rheumatoid factor may be removed by prior absorption with aggregated IgG.

By means of ELISA methods essentially the same as those developed for rubella, it has been possible to assay antibody to CMV (23,24). Results obtained with ELISA correlated well with IFA, and ELISA was found to be more sensitive than CFT. CMV IgM antibody has also been assayed by ELISA (25).

The fact that very little antigen is used in ELISA tests has permitted the use of scarce, highly purified antigens in ELISA tests for herpes 1 and herpes 2 antibody (26). These could be clearly differentiated. Antibody to Epstein-Barr virus (EBV) has also been measured in a similar manner with extracts of P3HR1 cells as antigen (27).

Antibody to influenza A virus and to respiratory syncytial virus (RSV) was detected by ELISA only a few days after infection, and the test was shown to be highly specific (22,28). The very early detection of antibodies by ELISA after rabies vaccination suggests that the test may be a good way of monitoring the efficacy of this vaccine (29).

It is clear from preliminary studies that ELISA may be used to measure antibody to adenoviruses, arboviruses, coxsackieviruses, measles, mumps, and varicella zoster virus (30). An important recent application has been for the detection of IgM antibody to hepatitis A (31).

The double antibody sandwich version of ELISA has been used to assay rotaviruses and for the detection of HBsAg. For the latter purpose, microplates, plastic discs, and beads have been used as the carriers sensitized with antibody to HBsAg (32,33). The sensitivity of these ELISA tests is higher than that of haemagglutination and is comparable to radioimmunoassay (RIA). The reagents are stable for at least one year, so that these assays are ideally suited for areas where RIA is impracticable. Recently an ELISA technique for the detection of HBeAg has been described (34).

6. ELISA FOR THE DETECTION OF HUMAN ROTAVIRUS AND ESCHERICHIA COLI HEAT-LABILE TOXIN

The human rotavirus is a major cause of diarrhoea in infants and children in many parts of the world. Because the virus does not grow efficiently in tissue culture systems, the usual virological techniques cannot be used to diagnose infection with this agent. Rather, the antigen must be identified directly in faecal material. Radioimmunoassay and counter-immunoelectrophoresis (CIE) systems have been developed, but none has proved to be practical for use in widespread epidemiological studies. For this reason an ELISA method was developed for the direct detection of rotavirus in stools. While initially a simple sandwich technique similar to that used for the detection of hepatitis B surface antigen was employed, an indirect technique with enzyme-labelled antiglobulin has been found more useful (Fig. 6) (35). Such a technique has the advantage of being more sensitive and of conserving specific antirotavirus reagents. In addition, indirect systems allow for the use of a single enzyme-labelled antiglobulin in a wide variety of tests.

This assay is outlined in Procedure 1, page 34. Poly(vinyl chloride) is the best carrier system for this sort of assay. While other plastics may be used, they are less sensitive and require the use of the reagents in higher concentration. It is best to avoid using the outer wells of poly(vinyl chloride) microtitration plates, since they may give falsely elevated values. The most accurate results are obtained by using the inner wells of the plate and filling the outer wells with water at each step. With this technique, there is a less than 5% well-to-well variation throughout the plate and visual interpretation of colour is quite accurate. Visual reading is made easier by incorporating weakly positive controls in several wells in the plate, so that the observer may compare the test specimen with these controls and read the test under a wide variety of time and temperature conditions. Dilutions are made in fetal bovine or goat rotavirus-negative sera to prevent nonspecific cross-reactivity with antibovine or anticaprine antibodies, which occasionally are present in stools (36). In addition, each specimen is tested in wells coated with normal goat serum to rule out potential false positive results due to this anti-rotavirus antibody. The specific reagents for rotavirus assay are available from the Research Resources Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA.

The results of ELISA for rotavirus may be made highly accurate by confirming the positivity of specimens with a blocking test, as outlined in Procedure 2, page 35. Use of the blocking test allows for extremely accurate visual determination, because less colour appears in the specimen treated with convalescent serum than in that treated with acute serum. Thus, each specimen may serve as its own control. With this method, visual interpretation is at least as sensitive as electron microscopy. In fact, when the assay is performed in this way, rotavirus antigen not detectable by electron microscope techniques may be detected.

Because of the frequency of *E. coli* in infants, an ELISA technique for the detection of *E. coli* toxin is included. Antibody to purified heat-labile toxin is difficult to obtain. Therefore it is easier to establish the assay by means of immunologically related cholera toxin (*37*). The technique is outlined in Procedure 3, page 36. The same conjugate, enzyme-labelled anti-guinea-pig globulin, may be used in ELISA for *E. coli* toxin and for rotavirus. When performed in this way, the former is at least as sensitive as the standard bioassay systems, such as *y-1* adrenal cell assay.

Procedure 1. ELISA for Determining Rotavirus (HRVLA) Antigen

Note: Use only the inner 60 wells of the microtitration plates. Fill the outer wells with PBS containing 0.5 ml/l polysorbate 20 (Tween 20) (38) at each step to ensure even heat distribution (see also Annex 1). Reconstitute the reagents with 1 ml of sterile PBS, pH 7.4.

1. Dilute goat anti-human rotavirus serum 1:20 000 in carbonate buffer. Add 0.1 ml to the inner wells of soft, round-bottomed poly(vinyl chloride) microtitration plates. Store at 4°C until use (at least 24 h). Also coat control well with the same dilution of normal goat serum.

2. Just before use, wash 3 times in PBS-polysorbate 20.

3. Add 0.05 ml of stool suspension (20-100 g/l) and 0.05 ml of polysorbate 20 + 10 g/l fetal calf serum + 5 g/l goat serum. Add stool suspension to duplicate anti-rotavirus wells and control wells. Incubate overnight at 4°C or for 2 h at 37°C.

4. Wash 3 times with PBS-polysorbate 20.

5. Add 0.1 ml of guinea-pig anti-human rotavirus serum diluted 1:500 in a mixture of PBS-polysorbate 20 + 10 g/l fetal calf serum + 5 g/l goat serum. Incubate for 1 h at 37°C. Higher dilutions of guinea-pig serum may be tried (depending on the strength of specimens).

6. Wash 3 times with PBS-polysorbate 20.

7. Add 0.1 ml of alkaline-phosphatase-labelled anti-guinea-pig serum diluted 1:400 in a mixture of PBS-polysorbate 20 + 10 g/l fetal calf serum + 5 g/l goat serum. Incubate at 37°C for 1 h.

8. Wash 3 times with PBS-polysorbate 20.

9. Add 0.1 ml of substrate solution (see Annex 1).

10. Incubate at room temperature until a yellow colour appears. A positive specimen will have visibly more colour in the anti-rotavirus wells than in the control wells. Compare the yellow colour with that of a weakly positive standard (or dilution of a positive serum pool). Nebraska calf diarrhoea virus should be weakly positive at dilutions of 1:20 to 1:50. Questionable samples should be confirmed by means of a blocking test.

Procedure 2. Blocking Test for Confirmation of Rotavirus Antigen

1. Add 0.075 ml of stool filtrate to 0.075 ml of preinfection serum diluted 1:10 in PBS containing 0.5 ml of polysorbate 20 (Tween 20) per litre.[1] (Fetal calf serum may also be used.) Add 0.075 ml of stool filtrate to 0.075 ml of standard postinfection serum diluted 1:10 in PBS-polysorbate 20.[1]

2. Incubate this mixture for at least 1 h at 37°C.

3. Transfer 0.05 ml of the mixture to duplicate wells of a microtitration plate coated with goat antirotavirus, and process as for stool specimens.

4. Rotavirus-containing stools should demonstrate at least a 50% reduction[2] in the amount of yellow colouring when the postinfection serum is added, in comparison with the preinfection serum.

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[1] This dilution should be adjusted according to the strength of the standard serum added.

[2] If a 50% reduction is not achieved, the stool specimen should be diluted to make sure that this is not due to an excess of antigen.

Procedure 3. Detection of Cholera Toxin and E. coli Heat-labile Toxin

1. Dilute horse anticholera toxin 1:10 000 in carbonate buffer pH 9.6.[1] Add 0.1 ml to the inner 60 wells of a poly(vinyl chloride) microtitration plate. Incubate for at least 14 h at 4°C. Store the plates at 4°C until use.

2. Wash. Add 0.1 ml of broth or culture. Incubate at room temperature for 2-14 h. Use strain 408-3 (lyophilized E. coli that produces labile toxin (LT)) as a standard control. Dilute for 0.1, 0.01, and 0.001 g/l of E. coli toxin.

3. Wash. Add guinea-pig anticholera toxin serum diluted 1:2000 in a mixture of PBS containing 0.5 ml of polysorbate 20 (Tween 20) per litre + 10 g/l fetal calf serum + 10 g/l normal horse serum.[2] Incubate for 1 h at 37°C.

4. Wash. Add enzyme-labelled anti-guinea-pig serum diluted 1:800 in the above-mentioned mixture. Incubate for 1 h at 37°C.

5. Wash. Add substrate and read by comparing with the 408-3 standard control. Read as positive specimens with a colour intensity at least as great as that of the standard control E. coli LT 408-3. The usual sensitivity of the assay is between 0.01 and 0.001 g/l.

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[1] If supplied at a 1:5 dilution, use at 1:2000.
[2] If supplied at a 1:5 dilution, use at 1:400.

7. MEASUREMENT OF ROTAVIRUS ANTIBODY BY ELISA

The ELISA technique may be used for the measurement of antibody to rotavirus. A problem that must be overcome in adapting the standard ELISA antibody technique for use in measuring serological response to rotavirus involves the antigen. The virus does not grow well in tissue culture; hence, it is difficult to obtain virus pure enough for use as the antigen. If impure antigen is used, cross-reactivity with nonrotavirus antigens interferes with the test reactions. While the virus may be purified from faecal specimens by sedimentation in sucrose and density banding in caesium chloride, this is difficult to do on a mass scale. An alternative method is first to coat poly(vinyl chloride) plates with hyperimmune antirotavirus antibody, as is done in the antigen assay, and then add a standard source of rotavirus antigen diluted in a nonionic detergent such as polysorbate-20 (Tween 20) and in an excess of a neutral protein such as fetal calf serum (39). With this method, only rotavirus antigen will bind to the solid phase. The cross-reacting antigens will be removed in the washing procedures. The technique of performing the antibody assay with this method is outlined in Procedure 4, page 38. The results may be expressed either as an end-point of the titration or as a ratio of positive to negative (P/N) computed by dividing the absorbance of the test serum by that of a standard negative serum at the same dilution. For greater accuracy, a set of dilutions may be used, and a mean P/N value may be determined. When performed in this way, ELISA is more sensitive than the standard complement fixation or fluorescent antibody assay, while possessing the other advantages of ELISA assay systems.

Procedure 4. Rotavirus Antibody Determination Binding Method

1. Coat a Microelisa plate (Cooke 220-29) or equivalent microtitration plate with goat antirotavirus diluted 1:10 000 in carbonate buffer. Incubate at least overnight at 4°C.

2. Wash the plate. Add antigen diluted in PBS containing 0.5 ml of polysorbate 20 (Tween 20) per litre. Incubate for 2 h at 37°C or overnight at 4°C. Store the plates at 4°C with the antigen still on them. This antigen should be a 20-100 g/l bacteria-free filtrate or suspension stored with 2 g/l sodium azide. The optimum dilution should be determined by checker-board titration. The antigen used is a 20 g/l calf stool suspension of human rotavirus strain 75-80 diluted 1:10 (10 antigen units should be used routinely).

3. Wash the plate on the morning of the test. Add serum or fluid diluted in a mixture of PBS-polysorbate 20 + 10 g/l fetal calf serum + 5 g/l goat serum, the latter being free of rotavirus antibody. Sera may be diluted 1:100, 1:400, 1:1600, and the dilutions may be made in the pre-coated plate by means of a Titertek multichannel pipette. Incubate for 2 h at 37°C or overnight at 4°C.

4. Wash the plate. Add an appropriate enzyme-labelled conjugate diluted in a mixture of PBS-polysorbate 20 + 10 g/l fetal calf serum + 5 g/l goat serum. Incubate 1-2 h according to the conjugate at 37°C.

5. Wash, then add the substrate. Compare the yellow colouring with that of a known positive control at the same dilutions. (Virtually any adult serum may be used.)

8. SERODIAGNOSIS OF ACUTE VIRAL HEPATITIS A BY RADIOIMMUNOASSAY FOR IgM

Unlike hepatitis B virus (HBV), infection with hepatitis A virus (HAV) does not result in a viraemia that can be detected easily by serological methods. Furthermore, since the bulk of HAV is often shed in stool well before the onset of clinical symptoms, it is neither feasible nor practical to assay acute-illness-phase stool suspensions for the presence of HAV for diagnostic purposes (40). Like many acute virus infections, however, hepatitis A results in the production of early (acute-phase) antibody that is primarily of the IgM class of immunoglobulins (41). Radioimmunoassay (RIA) procedures have recently been described for the differential detection of IgM- and IgG-specific anti-HAV (41), thus making it possible to discriminate between acute-phase (IgM) and convalescent, or pre-existing, (IgG) antibody. This means that it is now possible to serodiagnose an acute case of viral hepatitis A by testing a single acute-phase serum specimen.

The only commercially available test for anti-HAV is a competitive binding RIA procedure in which HAV is bound to the surface of a polystyrene bead as part of the test system. In this test, solid-phase HAV can combine with either IgM or IgG anti-HAV in the patient's serum or with ^{125}I-labelled IgG anti-HAV used as the test "probe". If a patient's serum is negative for anti-HAV, the addition of an HAV-coated polystyrene bead to a mixture of this serum and the probe will result in maximum binding of ^{125}I-IgG. If a patient's serum is positive for anti-HAV, however, the addition of an HAV-coated bead to a mixture of this serum and the probe will result in decreased binding of ^{125}I-IgG. The patient's anti-HAV, whether it is of the IgM or IgG class of antibody, will compete with ^{125}I-IgG anti-HAV for HAV binding sites. Presumably, high-titred sera (or sera with high avidity antibody) will compete more effectively with ^{125}I-IgG anti-HAV for HAV binding sites in the competitive RIA. This procedure has been termed the "competitive binding assay" (CBA).

Since most acute-phase antibody is IgM anti-HAV, any test that can detect the presence of IgM or IgG anti-HAV differentially should be suitable for the serodiagnosis of acute viral hepatitis A. The commercially available CBA for anti-HAV, in its standard form, detects both IgG and IgM anti-HAV. Thus CBA, as such, cannot be used for the serodiagnosis of acute hepatitis A. Therefore, a modified CBA procedure has been developed so that it may detect IgM and IgG anti-HAV differentially. The modified CBA is based on the principle of preferential absorption of IgG anti-HAV from a patient's serum with protein A, a membrane component of Staphylococcus aureus cells. Since protein A has a much higher affinity for IgG than for IgM, any serum that contains mostly IgM anti-HAV (acute-phase antibody) should remain positive for anti-HAV in the standard CBA test after absorption. Diagnosis of acute hepatitis A by use of the modified CBA procedure, therefore, is dependent on the effects of protein A absorption on the patient's anti-HAV activity. The modified CBA procedure is performed as outlined in Procedure 5, page 41. Test results are evaluated by the criteria listed in Procedure 6, page 42. The standard CBA test, on which the modified CBA test is based, is performed and evaluated as outlined in Procedure 7, page 43.

It is important to note that the modified CBA procedure yields the most consistent test results when acute-phase sera are collected within 3-4 weeks of the onset of clinical illness. Sera collected after this time may contain substantial titres of IgG anti-HAV, which will result in ambiguous test results. It has also been found that sera should not be tested at dilutions of less than 1:2, since the quantity of protein A used in the modified CBA procedure may not have the capacity to absorb the bulk of the IgG. Under these conditions, the absorbed test sample may still be positive for IgG anti-HAV and the R value may be less than 2.5 (Procedure 6, page 42). Sera that are negative for anti-HAV at a dilution of 1:2 cannot be typed. It has also been found that the diluted, unabsorbed serum should yield one-half (or less) of the cut-off value in the standard CBA test in order for the modified CBA test to be valid. Unabsorbed sera with a count/min greater than one-half of the cut-off value may give falsely low R values, since the quotient $(NC\bar{x})/(\text{sample count/min})$ has a constant (fixed) numerator (see Procedure 7, page 43). Diluted, unabsorbed sera that fall into this category should be retested at a lower dilution.

Procedure 5. Modified CBA Procedure for the Differential Detection of IgM and IgG Anti-HAV

1. Suspend 1 g of dried staphylococcal cells (Newman D_2C strain) in 10 ml of 0.01 mol/l PBS, pH 7.2, containing 0.25 g/l sodium azide as a preservative. This is the protein A suspension.

2. Pipette 0.1 ml aliquots of the protein A suspension into 1-ml microcentrifuge tubes (one aliquot per serum dilution).

3. Centrifuge the aliquoted cell suspensions for 1 min at top speed in a microcapillary centrifuge; discard the supernatant and use the cell pellets as described below.

4. Dilute the test serum in 0.01 mol/l PBS, pH 7.2 (containing 0.25 g/l sodium azide), to a final volume of 0.15 ml. The volume of serum added depends on the percentage competition of the undiluted serum in the standard CBA procedure. If the serum gave 90% competition or more it should be tested at a dilution of 1:15. If the serum yielded less than 90% competition, it should be tested at a dilution of 1:5.

5. Add 0.1 ml of diluted serum to the cell pellet, mix, and incubate for 30 min at room temperature (25°C).

6. Centrifuge the mixture to re-pellet the cells.

7. Assay 0.01 ml of the above-mentioned supernatant by means of the standard CBA procedure (Procedure 7, page 43). This is the absorbed test serum.

8. Assay 0.01 ml of the unabsorbed, diluted test serum (see step 4 above) with the standard CBA procedure (Procedure 7, page 43).

9. Evaluate the test results (Procedure 6, page 42).

Procedure 6. Evaluation of Modified CBA Test Results

1. Test results should be evaluated by two criteria:

(i) Positivity of the diluted serum after protein A absorption.

(ii) Ratio of diluted test serum count/min before (-A) and after protein A (+A) absorption (ratio, R = (+A)/(-A)). This is the most important criterion.

2. Interpretation of test results:

Minus protein A (-A)	Plus protein A (+A)	R	anti-HAV
-	-	NC[a]	neg(untypeable)[b]
+	+	< 2.5	IgM primarily[c]
+	+	$\geqslant 2.5$	IgM+IgG mixture[c]
+	-	> 2.5	IgG primarily[d]

[a] NC = not calculated

[b] Sera that are negative for anti-HAV at a dilution of 1:5 are untypeable.

[c] Sera that are positive for anti-HAV after protein A absorption contain primarily IgM anti-HAV if $R < 2.5$, and a mixture of IgM and IgG if $R \geqslant 2.5$. These latter sera are generally from patients in the late acute phase or early convalescent phase of hepatitis A.

[d] Sera that are negative after protein A absorption, or have an R value > 2.5, contain primarily IgG (convalescent or pre-existing) anti-HAV.

3. Sera may yield ambiguous test results if:

(i) They are tested undiluted.

(ii) They are collected from suspected cases of hepatitis A more than 4 weeks after the onset of clinical symptoms.

(iii) The diluted serum yields more than one-half of the cut-off value in the CBA test (see Procedure 7, page 43).

Procedure 7. CBA Procedure for the Detection of Anti-HAV

1. Place 0.01 ml of each serum specimen (or diluted serum) in the bottom of each well of a 20-well reaction tray by means of a micropipette.

2. Add 0.2 ml of ^{125}I-anti-HAV to each of the wells containing serum specimens.

3. Carefully add one HAV-coated bead to each well, cover the reaction tray with a cover sealer, and gently tap the tray to ensure that each bead is covered with the reaction mixture.

4. Incubate the trays at 45°C for 4 h or at room temperature for 18-24 h.

5. Remove and discard the cover sealer, aspirate the contents of the wells, and wash the beads with two 5-ml aliquots of distilled or deionized water.

6. Transfer the beads to counting tubes and count for 1 min in a well-type gamma scintillation counter.

7. The presence or absence of anti-HAV is determined by comparing the net count rate per minute (count/min) of the serum specimen with a cut-off value (calculated as shown in step 8).

8. Calculate the cut-off value by dividing the sum of the negative control mean count rate (NC$\bar{x}$; average of 5 negative controls) and the positive control mean count rate (PC$\bar{x}$; average of 3 positive controls) by 2. For example, (NC$\bar{x}$ + PC$\bar{x}$)/2 = (20 000 + 950)/2 = 10 475 count/min.

(a) Serum specimens (diluted or undiluted) with count/min values greater than the cut-off value are negative for anti-HAV.

(b) Serum specimens with count/min values below the cut-off value are positive for anti-HAV.

(c) Percentage competition of the test serum is calculated as follows: (1-(test sample count/min/NC$\bar{x}$)) x 100.

REFERENCES

1. Laboratory techniques for rapid diagnosis of viral infections: a Memorandum. Bulletin of the World Health Organization, 55: 33 (1977).

2. Progress in the rapid diagnosis of viral infections: a Memorandum. Bulletin of the World Health Organization, 56: 241 (1978).

3. GARDNER, P. S. ET AL. Bulletin of the World Health Organization, 56: 105 (1978).

4. Laboratory techniques for the detection of hepatitis B surface antigen. Atlanta, GA, Department of Health, Education and Welfare, Center for Disease Control, 1977 (HEW Publication).

5. LENNETTE, E. H. & SCHMIDT, N. J., ed. Diagnostic procedures for viral and rickettsial infections, 4th edition. New York. American Public Health Association, 1969 (5th edition in preparation).

6. GARDNER, P. S. & McQUILLIN, J. Rapid virus diagnosis - application of immunofluorescence. London, Butterworth, 1974.

7. GOLDWASSER, R. A. & KISSLING, R. E. Proceedings of the Society for Experimental Biology and Medicine, 98: 219 (1958).

8. DEBBIE, J. G. ET AL. Infection and immunity, 5: 902 (1972).

9. AVRAMEAS, S. & TERNYNCK, T. Immunochemistry, 6: 53 (1969).

10. ATANASIU, P. ET AL. Annales de microbiologie, 125B: 85 (1974).

11. AVRAMEAS, S. & URIEL, J. Comptes rendus hebdomadaires des séances de l'Académie des sciences, 262: 2543 (1966).

12. NAKANE, P. K. & PIERCE, G. B. Journal of histochemistry and cytochemistry, 14: 929 (1966).

13. ATANASIU, P. ET AL. Annales de l'Institut Pasteur, 121: 247 (1971).

14. ENGVALL, E. & PERLMANN, P. Immunochemistry, 8: 871 (1971).

15. ATANASIU, P. & PERRIN, P. Annales de microbiologie, 130A: 257 (1979).

16. ENGVALL, E. & PERLMANN, P. Journal of immunology, 109: 129 (1972).

17. VAN WEEMEN, B. K. & SCHUURS, A. W. W. M. FEBS letters, 15: 232 (1971).

18. SEVER, J. L. & MADDEN, D. L., ed. Journal of infectious diseases, 136, Suppl. Oct. 1977.

19. VOLLER, A. & BIDWELL, D. E. British journal of experimental pathology, 57: 243 (1976).

20. GRAVELL, M. ET AL. Journal of infectious diseases, 136: (Suppl.) S300, (1977).

21. VEJTORP, M. Acta pathologica et microbiologica Scandinavica, B86: 387 (1978).

22. LEINIKKI, P. & PASSILA, S. Journal of clinical pathology, 29: 1116 (1976).

23. VOLLER, A. ET AL. In: Rose, N. R. & Friedman, H., ed. Manual of clinical immunology. Washington DC, American Society of Microbiology, 1976, p. 506.

24. CASTELLANO, G. A. ET AL. Journal of infectious diseases, 136: (Suppl.) S337 (1977).

25. SCHMITZ, H. ET AL. Journal of clinical microbiology, 5: 629 (1977).

26. VESTERGAARD, B. F. ET AL. Acta pathologica et microbiologica Scandinavica, B85: 466 (1977).

27. WALLEN, W. C. ET AL. Journal of infectious diseases, 136: (Suppl.) S324 (1977).

28. BISHAI, F. R. & GALLI, R. Journal of clinical microbiology, 8: 648 (1978).

29. ATANASIU, P. ET AL. Annales de microbiologie, 128A: 489 (1977).

30. BIDWELL, D. E. ET AL. Journal of infectious diseases, 136: (Suppl.) S274 (1977).

31. DUERMEYER, W. & VAN DER VEEN, J. Lancet, 2: 684 (1978).

32. WOLTERS, G. ET AL. Journal of clinical pathology, 29: 873 (1976).

33. HALBERT, S. P. & ANKEN, M. Journal of infectious diseases, 136: (Suppl.) S318 (1977).

34. VAN DER WAART, M. ET AL. Journal of medical virology, 3: 43 (1978).

35. YOLKEN, R. H. ET AL. Lancet, 2: 263 (1977).

36. YOLKEN, R. H. ET AL. Lancet, 2: 819 (1977).

37. YOLKEN, R. H. ET AL. Infection and immunity, 14: 471 (1978).

38. ROSE, N. R. & FRIEDMAN, H., ed. Manual of clinical immunology. Washington D.C., American Society of Microbiology, 1976, p. 511.

39. YOLKEN, R. H. ET AL. Journal of clinical microbiology, 6: 439 (1977).

40. DIENSTAG, J. L. ET AL. Lancet, 1: 765 (1975).

41. BRADLEY, D. W. ET AL. Journal of clinical microbiology, 5: 521 (1977).

ANNEX 1

ELISA - MATERIALS AND METHODS

Carrier surface

Disposable polystyrene micro-haemagglutination plates.

Coating buffer

Carbonate-bicarbonate (pH 9.6): 1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$, and 0.2 g of NaN_3 made up to 1 litre with distilled water. Store at 4°C for not more than 2 weeks.

PBS-polysorbate 20 (Tween 20)

Consists of 8.0 g of NaCl, 0.2 g of KH_2PO_4, 2.9 g of Na_2HPO_4 $12H_2O$, 0.2 g of KCl, 0.5 ml of polysorbate 20, and 0.2 g of NaN_3 in 1 litre of distilled water. The pH is 7.4. Store at 4°C.

Conjugates (about 20 ml of diluted conjugate will be needed for each plate).

(a) Alkaline-phosphatase-labelled sheep antihuman IgG. Store in concentrated form at 4°C with sodium azide as a preservative. Dilute stock solution in PBS-polysorbate 20 immediately before use.

(b) Horseradish-peroxidase-labelled sheep antihuman IgG. Store in lyophilized state or in solution at -20°C. Make up the amount needed and dilute in PBS-polysorbate 20 immediately before use.

Substrates

(a) For alkaline phosphatase conjugates:

Diethanolamine buffer (100 g/l) consists of 97 ml of diethanolamine, 800 ml of water, 0.2 g of NaN_3, and 100 mg of $M_gCl_26H_2O$. Add 1 mol/l HCl until the pH is 9.8. The total volume is made up to 1 litre with water. Store at 4°C in the dark. Remove a sufficient amount (20 ml per plate) 1-2 h before the substrate solution is to be used, and allow it to warm to room temperature.

Substrate solution is 4-nitrophenyl phosphate (1 g/l). Tablets (5 mg) are stored at -20°C in the dark until used. Immediately before use, one tablet is dissolved in each 5 ml of 100 g/l diethanolamine buffer, which has been warmed to room temperature. (20 ml per plate will be needed.) It must be used the same day.

Reaction-stopping solution: 3 mol/l NaOH.

(b) For peroxidase conjugates:

Phosphate-citrate buffer pH 5.0 consists of 24.3 ml of 0.1 mol/l citric acid (192 g/l), 25.7 ml of 0.2 mol/l phosphate (28.4 g Na_2HPO_4/l), and 50 ml H_2O.

Substrate solution orthophenylenediamine to be made up freshly immediately before use. Dissolve 40 mg of orthophenylenediamine in 100 ml of the above-mentioned buffer and add 0.15 ml of 300 g/l H_2O_2.

This substrate is light-sensitive and must be used at once.

Reaction-stopping solution: 2 mol/l H_2SO_4.