CHAPTER 4

Direct Antigen Detection

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A direct detection of viral particles, antigens or nucleic acids in clinical samples is the most straightforward strategy for specific viral diagnosis. Almost always, it is also the most rapid diagnostic method. Detection of virus particles requires electron microscopy, and detection of nucleic acids is done by hybridization methods, whereas antigen detection is done by immunologic methods using specific hyperimmune sera or monoclonal antibodies.

In antigen detection, complete virions are not necessarily required in the specimen. In fact, structural and even nonstructural proteins are usually the antigens that are detected since they are produced in large excess compared with full virion production. In immunofluorescence (IF), the specimen must contain infected cells, but in solid-phase immunoassays and in latex agglutination excreted antigens are also assayed. The detectable level of viral antigen varies according to immunoassay type used and the sensitivity of the specific assay. As an example, in IF, a single infected cell can be seen and the diagnosis made. Also, any immunoassay can be only as good as the quality of antibodies used in the test. In addition, because viral antigens are detected by specific antibodies, one has to know what he or she is looking for. If etiologic candidates are numerous (i.e., 100 different rhinovirus types in common cold), a type-specific antigen assay is not practical. A contrasting example is bronchiolitis of infants, where only a few viruses are candidates.

In diagnostic virology, the use of antigen detection has expanded steadily. Reagents have improved and quality control has been organized. The evolvement of monoclonal antibodies has, in particular, had great impact in the development of antigen assays, and numerous commercial kits are now available. The tests are usually simple to perform and often the results are provided in printout forms which are quick and relatively straight-forward to evaluate.

Immunofluorescence

Principles of Immunofluorescence in Antigen Detection

Since the introduction of fluorescein-labeled antibodies in 1941 (Coons et al., 1941), the method was applied to most areas of virology, and only recently has been partly replaced by methods using other labels. There are several recent reviews and books describing immunofluorescence methodology in great detail (Gallo, 1983; Gardner and McQuillin, 1980; Hanson, 1985). In this context only, some general outlines and examples of the most recent applications will be described.

The most frequently used fluorochrome dyes are fluorescein isothiocyanate (FITC) and rhodamine B. The emission maximum for FITC is 517 nm, and for rhodamine 595 nm. The fluorescence of FITC is apple green. The fluorescence of rhodamine is red when proper filter systems are used. The different colors make it possible to use double-labeling of the same specimen in some instances.

In antigen detection, a direct or indirect immunofluorescent method can be used. In a direct assay, antigen in infected cells is detected by virus-specific immunoglobulins conjugated with fluorochrome. In an indirect assay, virus-specific immunoglobulins are followed by anti-species-specific antibodies, which are labeled with fluorochrome. The direct assay is quicker, but on the other hand the sensitivity is better in the indirect assay because of the higher number of binding sites for the conjugate. The indirect method is also more convenient in practice if several viruses are searched for in the same specimen. For direct assays, each antiviral immunoglobulin must be separately conjugated. In indirect methods, only a few conjugated anti-species immunoglobulins are needed.

E. H. Lennette et al., *Laboratory Diagnosis of Infectious Diseases: Principles and Practice*

Sensitivity of IF assay may be further amplified by using biotin-avidin complexes (Nerurkar et al., 1983). Avidin anti-species conjugates are commercially available. Virus-specific biotin-labeled antibodies are more difficult to obtain.

Antibodies Used in Immunofluorescence

As in any antigen detection based on an immunologic reaction, in immunofluorescence the quality of antisera used is the most important single factor affecting the results. Conventional polyclonal antisera are still most commonly used, and are usually produced in rabbits or guinea pigs, but also in mice, hamsters, calves, goats, sheep, monkeys, horses, and even the egg yolk of chickens. In many instances, polyclonal antisera are still the only ones available, but monoclonal antibodies are now rapidly replacing them. In the case of a high quality polyclonal antiserum, a monoclonal antibody offers hardly any benefits in the actual assay, but the mass production and basically unchanged specificity of various batches of the same monoclonal antibody make the use of them practical. Difficulties may arise in a too narrow specificity of monoclonal antibody, and may require the use of pools. In that case, the situation resembles that of polyclonal antisera.

Despite the source of the antibody, the intended reagent must be carefully tested before use. Cross-reactions must be checked against related viruses, tissue culture cells in which immunizing antigen has been grown, other microbes or antigens possibly present in the clinical specimen, and negative clinical specimens. At least in case of polyclonal antisera, removal of undesired reactions by absorption is often necessary.

Conjugates Used in Immunofluorescence

The quality of fluorescein-labeled anti-species immunoglobulins ("conjugates") is just as important as the quality of antiviral antibodies. For indirect assays, conjugates are commercially available and can be used in almost any virus laboratory. In the indirect fluorescence assays, usually only a few conjugates are needed, and they are now commercially available.

As in the case of primary antiviral antisera, conjugates must always be checked by the final user. In principle, the controls are the same as those mentioned previously. Extensive check-ups may not be necessary between different batches. If problems arise, however, they very often are caused by the conjugate.

The preparation of a good conjugate, as noted, usually requires absorption and extensive testing to avoid nonspecific cross-reactions. Therefore, there are only a few commercial conjugates available for direct assays. Theoretically, monoclonal antibodies should make excellent conjugates because of their narrow specificity, which minimizes cross-reactions.

Nonspecific Fluorescence

In an ideal case, specific apple green fluorescence of FITC is seen against a practically dark background. Unfortunately, nonspecific fluorescence is avoided only in IF assays of highest quality. Nonspecific fluorescence is, as mentioned previously, generally caused by antiserum or conjugate that cross-reacts with the specimen or related antigen. Extensive quality controls and absorptions, if necessary, overcome this problem.

In some cases, nonspecific fluorescence might be caused by bacteria in the specimen. This is particularly the case with staphylococci in which protein A binds nonspecifically to the IgG class of immunoglobulins. Another common problem is the Fc receptors of *Herpes viridae*.

Autofluorescence of the clinical specimen itself is also possible. The color and intensity of autofluorescence may vary and cause some difficulties in interpretation. Therefore, when examining clinical material, it is necessary to use counter stains to diminish background fluorescence. Several different dyes are used for this purpose. Naphthalene black, for instance, produces an almost black background, but like Evans blue, it may give a reddish color to the noninfected cells, producing a good contrast to the green color of FITC (Gardner and McQuillin, 1980). Counter stain can be added as the last step of the staining procedure, but it is more convenient to add it directly to the conjugate.

Immunofluorescence Microscope

The development of the IF microscope since the early days of immunofluorescence has been huge. Improvement of filter and lens systems is still taking place, partly as a result of modern computer technology. Major manufacturers have developed several models, which are fully ready for use. The best models have built-in interference and barrier filters for one or more fluorescence systems so that they can be changed by one movement of the revolver.

For most applications, incident light is recommended. A brighter and sharper fluorescent pattern is achieved because the light beam does not go through the specimen. The price of mercury bulbs has become reasonable and their life-time lengthened so that they are superior to halogen bulbs.

Opinions differ concerning the use of objectives. In many cases, dry objectives are more practical, but some experts prefer oil objectives. In either case, one should not be content with standard objectives, but obtain at least plain-corrected ones.

Interpretation of Results

Immunofluorescence is regarded as a demanding technique that requires careful attention to detail throughout the procedure. As always, the collection of the specimen is the first critical point. When possible, the same trained personnel should take the samples. Handling and transportation requirements vary according to the specimen, but in general for tissues or nasopharyngeal aspirates, it is good practice to pack them on ice if the virus laboratory is far away. Antigenicity of the virus may be readily lost if the specimen is stored at room- or higher temperatures. Various smear-type specimens are preferable to be spread directly on the microscope slide and fixed with acetone before shipment. It should also be noted that all microscope slides used for immunofluorescence must be acid-washed before use to remove grease.

The quality of antisera and conjugates has already been emphasized. Any reagent giving nonspecific reactions should be disregarded or further improved (that is, by absorptions).

When interpreting the results, both positive and negative controls must naturally work in a desired way. It is not uncommon to find that the positive control is no longer positive. The reason for this lies most probably in the conjugate, which has lost its activity. Reasons include bacterial growth, aggregation of globulin molecules, or repeated freezing and thawing (among others). It is good practice to sonicate and/or centrifuge the conjugate at least once a week.

The next step is to evaluate the specimen and make sure that it contains a sufficient amount of cells. If this is not the case, no result should be given and a new specimen should be requested. Even when the specimen has been correctly taken, cells can be lost during washing steps as a result of poor drying and fixation or a greasy objective glass.

When the specimen seems to be positive, the microscopist must evaluate the intensity, amount of the fluorescence, and background. In addition, the fluorescent pattern must be specific for the virus being looked for.

Immunofluorescence microscopy must be done by a trained and experienced microscopist. If the strict qualifications of the IF test itself are fulfilled, it does not take an excessively long time to train a microscopist. However, everybody involved in the entire procedure, including the microscopist, should continuously keep up their skills. This can be achieved only by ensuring a sufficient and steady flow of specimens.

Applications of Immunofluorescence in Antigen Detection

Applications Utilizing Conventional Antisera

Since the first description of immunofluorescence by Coons et al. (1941), it took almost three decades before the technique really took wing. In research laboratories, most viruses have been examined with immunofluorescence, but for diagnostic purposes, its golden period really started in the late 1960s. Recent developments, however, have led to the replacement of immunofluorescence with other methods, such as radioimmunoassay and enzyme immunoassay, which can be automated and are far less labor-consuming. On the other hand, the measuring of light (fluorescence) is still one of the most sensitive detection systems, and methods such as time-resolved fluoroimmunoassay that are measured by special equipment will be seen in the future for viral diagnostic purposes. The major applications of immunofluorescence in the diagnosis of viral diseases can be divided into three categories on the basis of the sample (Table 1).

The first important application utilizing infected tissue was the demonstration of rabies in the salivary glands of rabid animals (Goldwasser et al., 1959). In the diagnosis of rabies, IF has maintained its position to the present day. In the case of a suspected rabid animal, both brain and salivary gland tissue can be collected postmortem. A reliable diagnosis is achieved in a simple way by preparing an impression smear of a small (1 to 2 mm) piece of the tissue and squeezing it between two objective slides (Gardner and McQuillin, 1980), then fixing and staining in a routine way.

The same impression smear technique can be readily used for any tissue specimen taken at autopsy and biopsy. In pneumonia or other lower respiratory tract infections, a lung biopsy may be considered,

Table 1. Examples of immunofluorescence in viral antigen detection of clinical specimens

Specimen	*Type of viral infection*
Tissue (biopsy or sample from autopsy)	Encephalitis, pneumonia, carditis hepatitis, pancreatitis, exanthemas, etc.
Exfoliated cells	Respiratory infections
Vesicles	Herpes and varicella

although a positive result is not always obtained even in a case of real viral infection.

Encephalitis caused by herpes simplex virus is a life-threatening situation. A rapid diagnosis is needed to determine the specific treatment, but the diagnostics are hampered by several other differential possibilities. A brain biopsy, although possible only in the largest hospitals, seems to be the only way to obtain the specific diagnosis in the early phase of the illness. Again, a negative result does not exclude the possibility of herpes simplex infection.

A special skin punch biopsy technique has been used for diagnosis of varicella zoster and other erythematous viral infections (Olding-Stenkvist and Grandien, 1976). This technique also includes a simple and rapid preparation of the clinical specimen.

One of the most extensive uses of immunofluorescence is in the rapid diagnosis of viral respiratory infections. Gardner and McQuillin (pioneers in this field) have established the techniques (Gardner and McQuillin, 1968; McQuillin et al., 1970), that are widely applied in virus laboratories worldwide (Ørstavik et al., 1984). Viral antigens are looked for in exfoliated epithelial cells of nasopharyngeal aspirate, which is collected by suction through the nostrils. For an ideal result, the cells must be extensively washed to rid them of mucus, which may cause autofluorescence or physically cover the antigenic sites and, thus, diminish the specific fluorescence. The method is especially useful for respiratory syncytial viral infections where a common problem in infected children is the considerable excretion of mucus in the respiratory tract. Other viruses include influenza viruses A and B, parainfluenza viruses 1 to 4, adenoviruses, and measles virus. Some examples of fluoresceing cells from nasopharyngeal aspirates are shown in Figure 1A to D.

For the first time, it was also possible to make a relatively rapid viral diagnosis in the specimens sent from small and distant laboratories. Technical personnel were trained to collect the sample, wash the cells, and fix them on the objective slide, which could then be mailed to a special laboratory for staining and evaluation of the immunofluorescent pattern. The results from our laboratory for the first 5 years of the use of immunofluorescence in the diagnosis of respiratory infections are summarized in Table 2. An important factor behind this success is the availability of controlled, high quality reagents either commercially or through the European and Pan American Groups for Rapid Viral Laboratory Diagnosis (W.H.O. Scientific Group; 1981).

Although immunofluorescence is still the most widely used method for respiratory viral infections and it has some advantages over other methods, in many laboratories it has been replaced by enzyme immunoassays for practical reasons.

In its early stages of development, it was already possible to examine the vesicles on the skin of herpes simplex or varicella zoster virus-infected patients by immunofluorescence for the presence of antigen (Biegeleisen et al., 1959; Schmidt et al., 1965). An optimal specimen is taken by scraping off cells from the bottom of the vesicle on the drop of saline solution on the objective slide, air-drying it, and processing it normally. Immunofluorescence of the herpes virus family is challenging. It is not always easy to collect enough cells to make a representative sample. In addition to common cross-reactions between different herpes viruses, Fc receptors may also cause problems. Monoclonal antibodies, also commercially available (Balkovic and Hsiung, 1985; Fung et al., 1985), should make the interpretation easier and give the specific type as well.

Applications Utilizing Monoclonal Antibodies

Only 10 years after the original report of Köhler and Milstein (1975), monoclonal antibodies had found a permanent place in all fields of biological sciences. Great expectations have also been placed for their use in diagnostic virology. In theory, monoclonal antibodies make an excellent reagent, but many problems in their use remain to be solved. Some benefits and pitfalls are listed in Table 3.

Although monoclonal antibodies have several beneficial features, they may cause problems when used as diagnostic reagents. Obviously, restricted specificity may result in the loss of some prevalent viral subtypes. This might necessitate the use of a pool of two or more monoclonal antibodies. It is not possible to produce a cocktail of reagents with exactly the same characteristics from batch to batch.

Table 2. Results of respiratory viral antigen detection by indirect immunofluorescence in nasopharyngeal aspirates from 1978 to 1982, Department of Virology, University of Turku, Finland

No. of specimens	*Positive*		*RSV*	*Influenza*		*Parainfluenza*			*Adenovirus*
	No.	*%*		*A*	*B*	*1*	*2*	*3*	
6,190	1,388	22	826	91	13	80	53	101	224

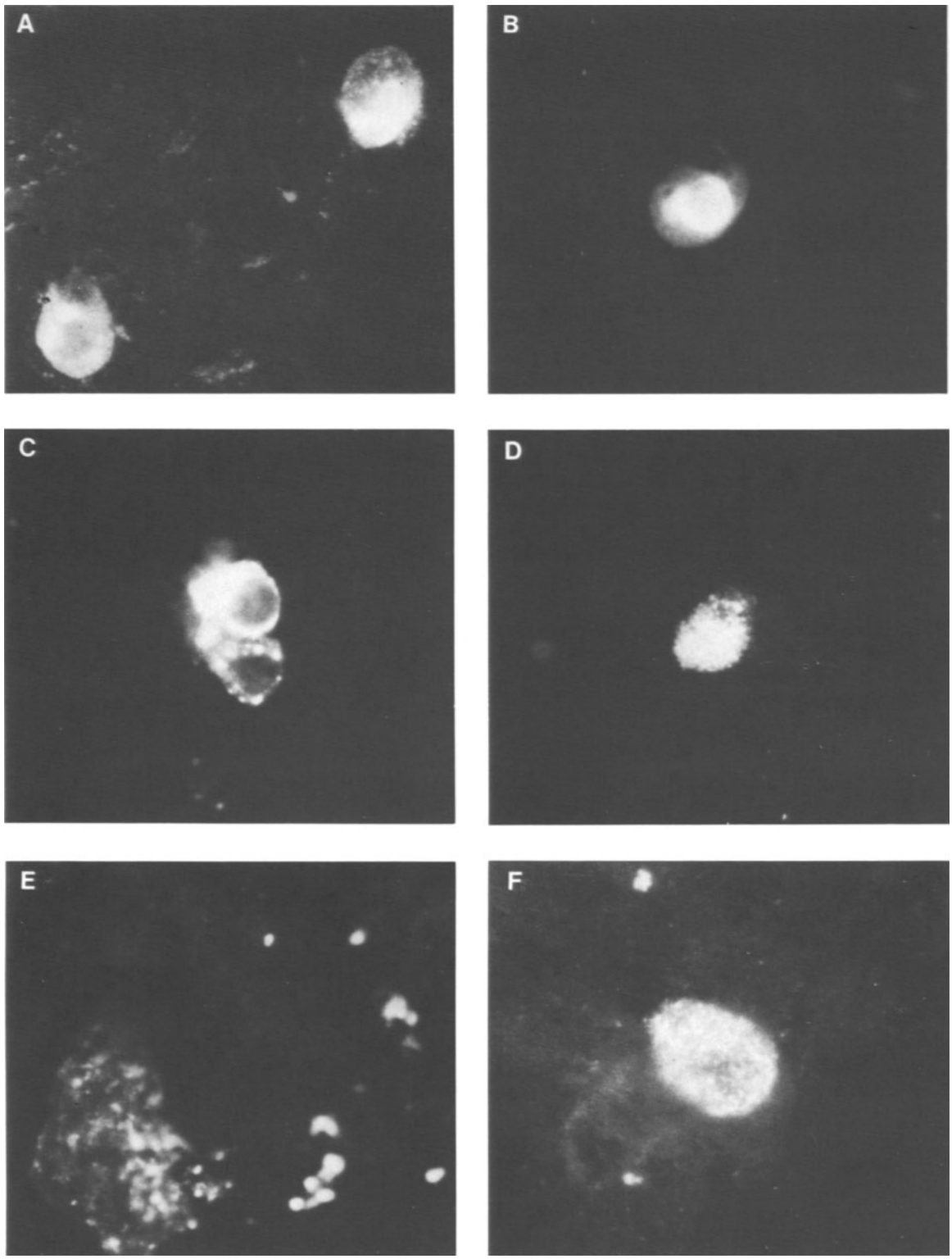


FIG. 1. Immunofluorescence of virus-infected cells from nasopharyngeal aspirates (A to E) and of an impression smear from a brain biopsy specimen (F). (A) Respiratory syncytial virus. (B) Influenza A virus. (C) Parainfluenza 3. (D) Adenovirus. (E) Nonspecific staining with mucus. (F) Herpes simplex virus. A color plate of this figure appears in Color Plate 1 at the beginning of this volume.

TABLE 3. General properties of monoclonal antibodies in immunoassays

Benefits	*Pitfalls*
Monospecificity due to monoclonality	Too narrow specificity; problems in group detection or due to antigenic variation
Homogenicity	Need for pooled reagents
Good typing reagent	Random success
In vitro production	Problems in characterization, labeling, and stability
Mass production	Unpredictable properties
Low production costs	Low avidity
	Variations between ascites batches

Even the repetitive production of a single monoclonal antibody with an identical character may be impossible because of variation in the animal stock used for raising ascites fluid.

Above all, there is concern about the availability of monoclonal antibodies. Only rather advanced laboratories are able to produce their own reagents, and commercially there is a tendency toward complete kits, not just single reagents. For the same reasons, it may prove difficult to freely exchange information on the quality control of the reagents.

Monoclonal antibodies have been raised for practically all important human viruses, but for pure diagnostic purposes, only a few monoclonal antibodies have been used in immunofluorescence. In the diagnostics of respiratory syncytial viruses, monoclonal antibodies have proved to be equal to polyclonal antibodies (Bell et al., 1983; Routledge et al., 1985). By using monoclonal antibodies, it has been shown that subtypes of respiratory syncytial viruses may occur alternately or concomitantly (Åkerlind and Norrby, 1986). Influenza A and B viruses can also be detected by monoclonal antibodies (McQuillin et al., 1985; Shalit et al., 1985). Antigenic changes of both viruses may cause problems in subsequent epidemic seasons.

Cytomegalovirus (CMV) pneumonia is a growing problem in immunocompromised patients. Monoclonal antibodies have been used successfully in the diagnostics of CMV pneumonitis in bronchoalveolar lavage material and in open-lung biopsies (Emanuel et al., 1986; Hackman et al., 1985).

Reliable typing of herpes simplex viruses is now available as a result of monoclonal antibodies (Pereira et al., 1982). Most laboratories use enzyme immunoassay (Ziegler et al., 1985) for this, but the reagents work equally well in immunofluorescence (Balkovic and Hsiung, 1985). Herpes viral antigen has been detected directly by immunofluorescence in erythema multiforme patients (Orton et al., 1984). Commercial FITC-conjugated monoclonal antibodies for the detection of herpes virus are also now available (Fung et al., 1985).

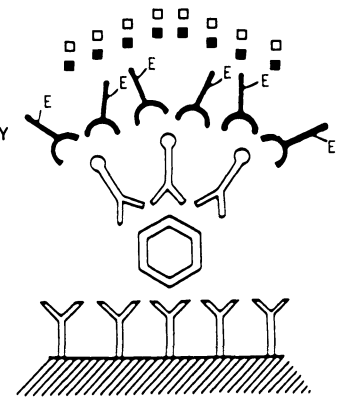
Solid-Phase Immunoassays

Principles and Solid-Phase Immunoassays

Solid-phase immunoassays are highly sensitive in the direct detection of viral antigens in clinical specimens, and are widely used both in commercial kits and in homemade tests. They have many advantages including: 1) stability of viral antigens in clinical specimens and accordingly no special requirement for transportation to the laboratory; 2) the possibility of semiautomated bulk testing of specimens; 3) tests can be standardized, resulting in acceptable daily variations; and 4) availability of technologically advanced measuring equipment with printout results and the possibility of analyzing the results by flexible computer programs. The applications in diagnostic virology include hepatitis B (HBsAg) antigen in serum (Lander et al., 1971, Ling and Overby, 1972), gastroenteritis viruses (rotaviruses and adenoviruses) in stool specimens (Halonen et al., 1979; Kalica et al., 1977; Middleton et al., 1977; Sarkkinen et al., 1979; Sarkkinen et al., 1980), respiratory viruses (RSV, influenza A and B, parainfluenza 1, 2, and 3, adenoviruses, and coronaviruses) in nasopharyngeal aspirates (Berg et al., 1980; Chao et al., 1979; Halonen et al., 1985; Sarkkinen et al., 1981a, 1981b, 1981c), herpes simplex and varicella zoster in vesicle fluid (Forghani et al., 1974; Ziegler, 1984; Ziegler and Halonen, 1985), and HIV in blood (Allain et al., 1986; Goudsmit et al., 1986; Paul and Falk, 1986).

The principle of the assays is simple. The catching antibody is a solid phase, which is usually a polystyrene microtitration well, bead, or tube. The specimen is incubated with solid-phase antibody, the nonbound material is washed away, and labeled or nonlabeled (or biotinylated) antibody (followed by labeled anti-immunoglobulin or enzyme-labeled avidin) is added. After another wash, the bound label is measured directly (RIA) or through a color (EIA) or enhancement (TR-FIA) reaction.

Earlier polyclonal immunoreagents were used in solid-phase immunoassays, and one of the most commonly used test configurations is a four-layer or antispecies principle (Fig. 2). The sensitivity of 0.1 ng of viral protein/ml of the specimen can be reached if the first incubation of the specimen with solid-phase antibody is extended to overnight (16 h) at 37°C. This assay principle is simplified by labeling the highly purified secondary antibody. More recently, mono-

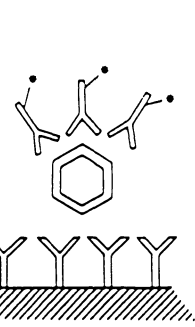


Layer	Reagent	Incubation
Stopping solution	1N HCl	
Substrate	O-phenyldiamine	30 min at RT
Anti-species indicator antibody	Swine anti-rabbit IgG antibody; HRPO-labelled	1 hour at 37°C
Secondary antibody	IgG fraction of rabbit anti-viral hyperimmune serum	1 hour at 37°C
Specimen	Virus antigen (structural protein)	16 hours at 37°C
Primary catching antibody	IgG fraction of guinea pig anti-viral hyperimmune serum	
Solid phase	Polystyrene microtiter strip	

FIG. 2. Principle of the indirect anti-species enzyme immunoassay for the detection of respiratory viruses in sonicated nasopharyngeal aspirates from patients with acute respiratory disease.

clonal antibodies have been used as immunoreagents and one-incubation (one-wash) assays can be built (Fig. 3). In these 1-h assays, the specimen and the labeled antibody are incubated simultaneously. If the monoclonal antibodies are of high quality and screened for this particular purpose, the sensitivity of the assay can be 0.1 ng of viral protein/ml. However, the very high sensitivity of the assay is not always a critical factor. For instance, gastroenteritis viruses are usually excreted in stool in large quantities, often more than 1 μg/g of stool, and 0.1-ng sensitivities are not required. On the other hand, HSV can occur in lower concentrations, particularly in genital specimens. Respiratory viral antigens are often excreted in hundreds of nanograms in nasopharyngeal secretions, but they can also be found in lower concentrations. Minimal concentrations of HBsAg can be detected in blood. Finally, one of the most critical assays in terms of sensitivity is HIV detection directly in blood specimens.

The specificity of the antigen assay is not usually a problem when immunoreagents have been pre-



Layer	Reagent		Incubation
	Type	Concentration	
Enhancement solution	15 uM 2-naphtoyltrifluoroacetone 50 uM tri-N-octylphosphino oxide, 0.1% Triton X-100 in 0.1M acetate buffer, pH 3.2 with potassium hydrogen phtalate		15 min at RT
Anti-viral indicator antibody	Eu-chelate-labelled Mouse monoclonal anti-RSV (nucleoprotein) antibody (clone A1)	10 ng/assay	1 hour at 37°C
Specimen	Nucleoprotein of influenza A	0.1 - 10.000 ng/ml	
Catching antibody	Mouse monoclonal anti-influenza A (nucleoprotein) antibody (clone A3)	500 ng/well	
Solid phase	Polystyrene strip		

FIG. 3. Principle of the monoclonal one-incubation time-resolved fluoroimmunoassay for the detection of influenza A virus.

pared from monoclonal antibodies. In contrast, extensive evaluations must be made to ensure that the particular epitope on viral antigen being assayed is well conserved in all clinical "isolates" and may require continuous monitoring.

Immunoreagents

Immunoreagents are prepared from antibody-positive human serum, hyperimmune serum, and monoclonal antibodies. Purified IgG or total immunoglobulin fractions (Ig) are better solid-phase catching antibody preparations than are diluted serum or ascites fluid, and IgG or Ig are equally effective. A simple method to prepare Ig from hyperimmune serum or ascites fluid is to precipitate by sodium sulphate, followed by Sepharose G-200 chromatography for desalting. Protein A-sepharose adsorption of IgG is also often used. Mouse immunoglobulins in ascites fluid are efficiently purified by high pressure liquid chromatography (HPLC) (Burchiel et al., 1984).

Total immunoglobulin fraction (Ig) is adsorbed on a polystyrene microtitration well, tube, or bead in carbonate buffer, pH 9.6 (Voller, 1986) by overnight incubation at ambient temperature. However, the binding reaction is not critical for pH or temperature, and binding may be complete in a few hours. The coated plates, strips, or beads can be stored in carbonate buffer for many weeks at 4°C. If longer storage is required, a postcoating with gelatin or bovine albumin, sometimes with carbohydrates, increases the stability.

The optimal concentration of Ig in a solid-phase immunoassay is always pretitrated against increasing concentrations of labeled or secondary antibodies and antigens. The concentrations required depend on the specific activity of the IgG preparation. In optimal conditions, it is about 250 ng of Ig per well, but it may vary from 100 to 1,000 ng per well. Often, several optimal concentrations can be used; if a low concentration of Ig is used in solid phase, an increased concentration of the labeled antibody is required (and vice versa).

Microtitration strips with 8 or 12 wells are convenient in daily routine diagnosis. Depending on the number of specimens tested each day, the correct number of precoated strips are included in the test. An additional advantage is that the immunoreactions are more homogeneous in each well of the strips than in 96-well plates.

The purity of the labeled or secondary antibody is more critical than that of the catching antibody on solid phase. High pressure liquid chromatography-purified monoclonal antibody is an ideal preparation. Polyclonal IgG of high-titered hyperimmune serum can be used, but often an optimal test requires immunosorbent purified polyclonal antibody.

Labeling of antibody by iodine-125 for radioimmunoassay is usually done by modifications of the Hunter and Greenwood (1962) method. Use of antigen-bound antibody for labeling may have some advantages, particularly with polyclonal antibodies, when immunosorbent purification is combined with labeling (Pelkonen, 1982). Enzyme labeling is usually done according to Wilson and Nakane (1978) (horseradish peroxidase) or Engvall and Perlmann (1972) (alkaline phosphatase). An isothiocyanate reaction is used for labeling with Europium chelate (Hemmilä et al., 1984).

Monoclonal Antibodies

The use of monoclonal antibodies in the detection of viral antigens by solid-phase immunoassays is rapidly increasing and they may soon replace polyclonal antibodies. The problem in their use both on solid phase and as a labeled antibody has been their weak binding capacity. This has been at least partially overcome by using new screening assays in selecting the hybridomas. The screening assay must be as close as possible to the final use of antibodies. As an example, when hybridomas are screened for the production of monoclonal antibodies used in one-step (one-wash) assays, the well is coated with polyclonal antiviral antibody and the screening antigen and the diluted hybridoma cultures are added into the well simultaneously without washing in between (Fig. 4). About half of the monoclonal antibodies are highly reactive in this screening assay and can be used as labeled indicator antibody. The yield of the hybrido-

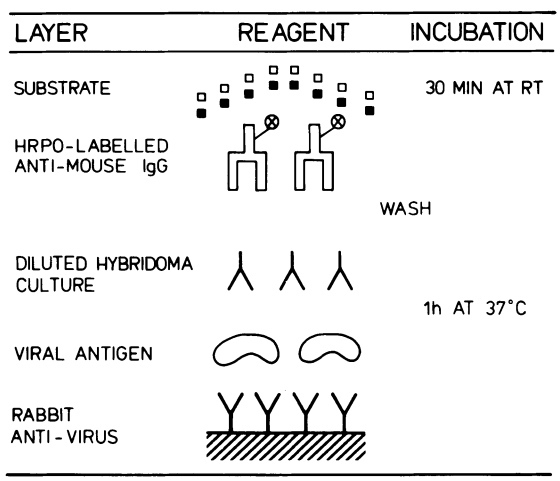


FIG. 4. The screening assay of hybridoma cultures for selecting monoclonal antibodies in one-incubation (one-wash) assays.

mas producing these highly reactive monoclonal antibodies per fusion experiment is considerably lower than that with a standard screening assay using antigen bound directly on solid phase. After cloning, there may finally be only 0 to 5 reactive clones per 1,000 hybridoma wells of the initial fusion experiment.

Primary screening of monoclonal antibodies for solid phase is more difficult, but fortunately monoclonal antibodies, which are highly reactive in the screening assay for the indicator antibody, are often reactive on solid phase as catching antibody. Once the prospective indicator antibody has been labeled, it must be tested against several nonlabeled antibodies on solid phase, which have been reactive in the screening assay (Table 4). In these optimization tests, positive and negative specimens and a purified reference viral preparation must be included.

Comparison of Solid-Phase Immunoassays

When the sensitivity and specificity of radioimmunoassay, enzyme immunoassay, and time-resolved fluoroimmunoassay are compared, the differences are minimal when polyclonal immunoreagents are used in these tests. Actually, the sensitivities and specificities are dictated by the quality of the antibodies used to prepare the immunoreagents. The label material (radioisotope, enzyme, euchelate) has only a marginal effect on the final performance of the tests. Other factors, such as practicality, safety considerations in the laboratory, and availability of commercial kits or reagents, must be taken into account when these tests are selected for the antigen assays in daily diagnosis.

TABLE 4. A representative parainfluenza type 2 TR-FIA test for selecting an optimal monoclonal antibody on solid phase as a catching antibody

		Monoclonal antibodies in solid phase		
Controls		*4-2D/2g*	*1-6D/9H*	*10-4F/8E*
Reference virus	1,000 ng/ml	73.238[b]	103.676	217.288
	100	10.043	16.576	19.510
	10	2.224	2.961	2.657
	0	1.827	1.953	873
Positive NPS		7.479	8.329	15.982
Negative NPS		1.984	1.975	1.591

[a] Eu-labeled monoclonal antibody (1-6D/9H) was tested with the same and two other monoclonal antibodies (4-2D/2g and 10-4F/8E) on solid phase. Three dilutions of reference virus and positive and negative nasopharyngeal specimens (NPS) were included in the optimization test.
[b] Counts per second value.

Radioimmunoassay (RIA)

This technique is still the test with the highest reproducibility. It has less daily variations than do the other solid-phase immunoassays, and the variations between laboratories are minimal and the counting equipment (gamma counters) are reliable. Automation of RIA tests is easier than with EIA tests, and this is one reason why many blood banks with a large number of specimens to be tested for HBsAg still use RIA technology.

The major disadvantages, of course, are the health hazard of handling radioactive material and problems in waste disposal. Actually, in many countries, government regulations rather than real health hazards have limited the use of RIA in diagnostic virology. The short expiration time in commercial RIA kits and the need for frequent labeling of your own reagents are additional factors that have decreased the use of RIA in viral antigen detection.

Enzyme Immunoassay

The enzyme immunoassay (EIA) is the most commonly used solid-phase immunosassay for viral antigen detection. Commercial kits are available for HBsAg and HIV antigen detection in blood, and for rotavirus detection in stool. One of the most advanced tests is the AUSZYME monoclonal test (Abbott Laboratories, North Chicago, Ill.), which is based on monoclonal antibodies both on solid phase and as the HRPO-labeled indicator antibody. The test can be done in one incubation; the serum specimen and the labeled antibody are incubated simultaneously with a bead coated with the catching antibody. One of the benefits of EIA is the possibility for quick visual evaluation of the test.

Many laboratories have reported the development of EIA tests for viral antigen detection, including rotavirus, adenovirus, Norwalk agent, caliciviruses, and astroviruses in stool; herpes simplex in vesicle fluid and genital specimens; varicella zoster in vesicle fluid; cytomegalovirus in urine; arboviruses in blood; and respiratory viruses in nasopharyngeal aspirates and throat swabs. However, the use of these tests in daily diagnostic work has not always been indicated, although this is the most important challenge for the new tests. In our diagnostic unit, rotavirus and adenovirus detection by polyclonal EIA (Halonen et al., 1979; Sarkkinen, 1981) has been performed since 1982, herpes simplex and varicella zoster virus since 1985, and respiratory viruses (influenza A and B, parainfluenza 1, 2, and 3, respiratory syncytial virus, and group-reacting hexon antigen of adenovirus) during 1984 to 1986. Representative results of these EIA tests are shown in Table 5, 6, and 7, and the principle of the indirect anti-species

TABLE 5. A representative indirect enzyme immunoassay for the detection of rotavirus and adenovirus in 1/20 diluted stool specimens from 10 patients with acute gastroenteritis

Controls and specimen no.	*Rotavirus*	*Adenovirus*	*Conclusion*	
Reference antigen[b]	100 ng/ml	1.066[a]	1.271	
	10	0.185	0.205	
	1	0.074	0.087	
	0	0.060	0.054	
Specimen no.				
11340		2.407	0.055	Rotavirus
11341		1.372	0.055	Rotavirus
11345		1.832	0.054	Rotavirus
11429		0.059	0.054	Negative
11430		0.065	0.053	Negative
11450		0.072	0.056	Negative
1048		0.041	0.755	Adenovirus
1431		0.042	0.040	Negative
1470		0.040	1.470	Adenovirus
1471		0.041	0.058	Negative

[a] Optical density.
[b] Purified Nebraska calf diarrhea virus (rotavirus) and crystallized hexon (adenovirus).

TABLE 6. A representative enzyme immunoassay for the detection of herpes simplex virus (HSV) and varicella zoster virus (VZV) in vesicle swabs from patients with vesicular rash

Controls and specimen no.	*HSV*		*VZV*		*Conclusion*
	Normal serum	*Immune serum*	*Normal serum*	*Immune serum*	
Reference virus	0.049	0.977	0.040	0.333	
Control antigen	0.044	0.045	0.044	0.040	
Specimen no.					
3310	0.039	0.047	0.166	1.150	VZV
3311	0.046	0.051	0.059	1.001	VZV
3314	0.042	0.051	0.057	0.057	Negative
1879	0.042	0.046	0.052	0.062	Negative
2065	0.068	0.314	0.059	0.064	HSV

TABLE 7. A representative indirect enzyme immunoassay for the detection of respiratory syncytial virus (RSV), influenza A and B, parainfluenza types 1, 2, and 3, and adenovirus in nasopharyngeal aspirates from seven patients with acute respiratory disease

Reference antigen and specimen no.	*RSV*	*Influenza*		*Parainfluenza*			*Adenovirus*	*Conclusion*
		A	*B*	*1*	*2*	*3*		
Reference								
1,000[a] ng/ml	0.985[d]			0.893	1.790	1.894		
100[b]		1.517	0.870				1.995	
0[c]	0.149	0.036	0.087	0.059	0.039	0.085	0.087	
Specimen no.								
101911	0.129	0.049	0.127	0.090	0.053	0.111	0.083	Negative
101920	0.141	0.080	0.123	0.162	0.098	0.125	0.135	Negative
101942	1.906	0.041	0.090	0.273	0.087	0.104	0.116	RSV
101948	0.087	0.046	0.100	0.079	0.062	1.918	0.105	Parainfl. 3
101954	1.751	0.112	0.131	0.181	0.121	0.129	0.099	RSV
101992	0.116	0.244	0.073	0.093	0.092	0.085	0.084	Influenza A
NIT 39	0.134	0.082	0.121	0.127	0.094	0.098	0.086	Negative

[a] Nonpurified cell lysate antigen.
[b] Purified virus.
[c] Pool of negative specimens.
[d] O.D. value.

TABLE 8. Monthly results of respiratory viral antigen detection in nasopharyngeal aspirates by enzyme immunoassay during the respiratory epidemic period from September 1985 to August 1986, Department of Virology, University of Turku, Finland

Month	No. of specimens tested	Positive		RSV	Influenza		Parainfluenza			Adenovirus
		No.	(%)		A	B	1	2	3	
September 1985	168	15	(9)	4						11
October	199	33	(17)	22			2		1	8
November	269	75	(28)	63					1	11
December	365	146	(40)	137					1	8
January 1986	361	128	(35)	113	2		3		1	9
February	310	72	(23)	64		1				7
March	280	88	(31)	21	38	3	5		12	9
April	254	55	(22)	8	4	4	2	2	22	13
May	190	31	(16)	1			2		22	6
June	138	19	(14)		1			1	4	13
July	111	15	(14)				1	1	1	12
August	109	5	(5)							5
Total	2,754	682	(25)	433	45	8	15	4	65	112

EIA used in the respiratory virus antigen detection is shown in Figure 3. Monthly results of respiratory virus EIA tests during the respiratory epidemic year from September 1985 to August 1986 are shown in Table 8.

Time-Resolved Fluoroimmunoassay

A new interesting immunoassay, time-resolved fluoroimmunoassay (TR-FIA) (Soini and Kojola, 1983), combines many advantages of RIA and EIA. Some of the limitations of the earlier solid-phase immunoassays are avoided in TR-FIA, but some new requirements, such as being very labor intense, have resulted with this new technology.

The measurement principle of time-resolved fluorescence is shown in Figure 5. The principle is based on new probes (Europium chelate) with a long fluorescent decay time and on a short pulsed excitation of the probes. One-step monoclonal TR-FIAs have been developed for the detection of HBsAg (Siitari et al., 1983) and respiratory viruses (Walls et al., 1986). The principles of this test are presented in Figure 3. In the assay, two monoclonal antibodies are used, each against the same structural protein, but with a different epitope specificity. In some assays, the same monoclonal antibody used as the catching antibody in solid phase can be used as the labeled monoclonal antibody. The catching antibody is coated on the wells of polystyrene microtitration strips, and the

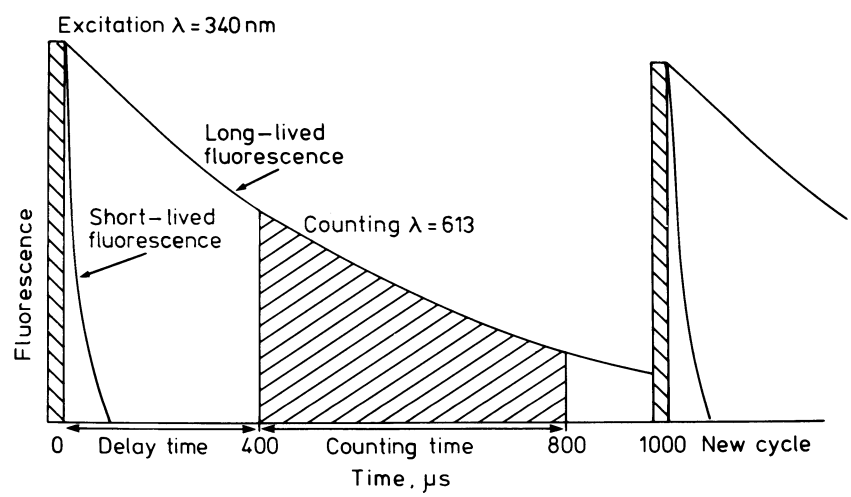


FIG. 5. The detection principle of time-resolved fluorescence. Shown are fluorescence of Eu-chelate and the short decay background fluorescence. Actual decay time is less than 1 μs.

FIG. 6. The 1230 Arcus fluorometer with a disc unit and printer.

indicator antibody is labeled with Europium chelate (Hemmilä et al., 1984). The specimen is simultaneously incubated with the indicator antibody in the well coated with the catching antibody. The incubation time is 1 h at 37°C, followed by a six-cycle washing and the addition of enhancement solution, which dissociates the Europium ion from the antigen-bound antibody and forms a new, highly fluorescent chelate in solution. Fluorescence is measured for 1 s with a single-photon counting fluorometer. Fluorescence activity is expressed as counts per second (cps), and these values are printed out (Fig. 6).

The one-step TF-FIAs of respiratory viruses have been in daily use in our diagnostic unit since January 1, 1987. Typical test results are shown in Table 9, and all results in 1987 are shown in Table 10. The sensitivity of the one-step monoclonal TR-FIA is usually higher than those of the indirect EIAs with polyclonal antibodies when measured with purified viral preparations. However, the clinical sensitivities have been improved only marginally, except with the influenza A virus TR-FIA in which the number of positive specimens has increased by 20% compared with polyclonal EIA.

The advantage of TR-FIA is clearly in the assays that use monoclonal antibodies. After labeling, monoclonal antibodies are in the monomer form, and the small molecular weight (400) of Eu-chelate ensures the biological activity of antibody molecules (Waris et al., in press). In contrast, horseradish peroxidase labeling of monoclonal antibodies results in aggregates of IgG and HRPO molecules, with a molecular weight of 500,000 to 1,000,000 resulting in decreased biological activity.

Time-resolved fluoroimmunoassays require high working standards. The six-cycle washing is critical because the high input activity in labels (millions of cps) must be reduced to a few hundred of cps in negative specimens. The label must be pipetted precisely into the right place in the well to avoid nonspecific binding of the label. In addition, the relatively expensive fluorometer and difficulties in obtaining the Eu-chelate for labeling have limited the use of TR-FIA.

Enzyme Immunofiltration Staining Assay

In this assay, the infected cells in the clinical specimens are filtered on glass Fiber disk placed in a 48-well plate with a vacuum manifold (Cleveland and

TABLE 9. A representative monoclonal one-step time-resolved fluoroimmunoassay for the detection of respiratory syncytial virus (RSV), influenza A and B, parainfluenza types 1, 2, and 3, and adenovirus in nasopharyngeal aspirates from five patients with acute respiratory disease

Reference antigen, cut-off value, and specimen no.	*RSV*	*Influenza*		*Parainfluenza*			*Adenovirus*	*Conclusion*
		A	*B*	*1*	*2*	*3*		
Reference								
1,000 ng/ml[a]		140.534[c]						
100 ng/ml[b]	8.712		12.251	15.467	10.490	22.670	33.320	
0 ng/ml	534		496	1.454	455	767	721	588
Cut-off	1.157		1.391	4.544	1.632	2.169	1.998	1.360
Specimen no.								
6233	486	441	2.880	387	945	701	585	Negative
6235	513.760	662	1.558	10.72	1.823	789	496	RSV
6237	540	508	1.518	1.195	770	769	672	Negative
6243	8.584	399	1.041	439	663	813	426	RSV
6318	369	431	356.536	1.009	667	873	399	Influenza B

[a] Nonpurified cell lysate antigen.
[b] Purified virus.
[c] Mean counts per second value of duplicates.

TABLE 10. Monthly results of respiratory viral antigen detection by monoclonal one-incubation time-resolved fluoroimmunoassay in nasopharyngeal aspirates

Month	No. of specimens tested	Positive		RSV	Influenza		Parainfluenza			Adenovirus
		No.	(%)		A	B	1	2	3	
January 1987	334	44	(13)	1	32			1	1	9
February	284	36	(13)	16	3		1		6	10
March	323	76	(24)	41		1	2		22	10
April	230	64	(28)	37			6		15	6
May	246	55	(22)	21			8		13	13
June	191	52	(27)	38			2		4	8
July	189	32	(17)	15			2	1	1	13
August	223	24	(11)	2			4			18
September	226	11	(5)	3			1			7
October	380	29	(8)	17				3		9
November	471	106	(23)	94				1		11
December	672	253	(38)	225				4		24
Total	3,769	782	(21)	510	35	1	26	10	62	138

Richman, 1987). Viral antigens are detected by short incubations with biotinylated monoclonal antibodies, streptavidin-HRPO conjugate, and the substrate aminoethylcarbazole. Infected cells and cell debris are stained red, and the test is read with a microscope. The total time required is 30 min. As few as two virus-infected cells per swab can be detected. Many viruses including herpes simplex, varizella zoster, adenoviruses, and chlamydial antigens have been detected in clinical specimens or cell cultures.

Latex Agglutination Tests

The sensitivities of latex agglutination test used for viral antigen detection are not as high as those of solid-phase immunoassays. However, if the specimen contains large amounts of viral antigen, latex agglutination tests can be used. These simple and very rapid tests are based on small beads coated with viral antibody. The antigen, preferably virus particles in the specimen, forms bridges between the beads, which are agglutinated, and the reaction can be read macroscopically. Such tests have been developed for the detection of rotaviruses (Cevenini et al., 1983; Haikala et al., 1983; Julkunen et al., 1985; Pai et al., 1985; Sanekata et al., 1981) and adenoviruses (Bricout et al., 1987; Grandien et al., 1987) in stool. The sensitivities are 80 to 90% compared with the most sensitive EIAs and 90 to 95% compared with electron microscopy. The specificity is almost 100% when calculated from the specimens that can be tested by this method. However, about 5 to 15% of stool specimens agglutinate control beads coated by normal serum and these specimens must be tested by EIA. New modifications in some commercial kits have reduced the agglutination reactions with control beads.

Recent developments in commercial latex agglutination tests include slides where beads are dried on the spot, and the only step in the test is addition of diluted stool specimens. Previously, the reading of the weak positive specimens required considerable expertise, but in the new tests the evaluation of the results is easier. These tests are quite practical in smaller hospital outpatient laboratories and can even be practiced in the doctor's office.

Future Prospects

A question often asked is whether viral antigen assays or nucleic acid hybridization will be used in the future for the detection of virus directly in clinical specimens. In the next few years, antigen detection, when available and most likely with monoclonal one-incubation (one-wash) assays, will find increasing use. However, the type-specific assay of papillomaviruses is an example where DNA hybridization has no competition from antigen assays. In group- or subgroup-specific enterovirus assays, the prospect that it will be done with nucleic acid hybridization is better than with antigen detection. In addition, we must realize that immunoassays have been used in viral antigen detection for almost 20 years with constant improvements in the tests as compared with only a few years in the use of hybridization technology. We can expect similar improvements in the DNA assays both in the form of increased sensitivity and in more simple and faster tests.

Other questions many investigators ask concern the future developments in rapid diagnosis of respira-

tory infections. Should we use immunofluorescence or solid phase immunoassays in the detection of respiratory viral antigens? What is the role of antibody assays in the retrospective diagnosis of these infections in relation to antigen detection?

We suggest that choice depends on the previous experience of the laboratory, the number of specimens tested daily, how the transportation of specimens is organized, and the available expertise for IF microscopy. If the laboratory has a large number of specimens and previous experience with EIAs, solid-phase immunoassays may be more practical, whereas in small hospital laboratories, IF may be the better choice. The diagnostic efficiency of these two techniques is almost the same if expertise and good reagents are available. Further developments in respiratory viral antigen detection will include coronavirus and rhinovirus assays, preferably group- or subgroup-specific rhinovirus assays.

Serologic diagnosis of respiratory viral infections by IgG EIA in paired serum specimens is slightly more efficient than antigen detection, with the exception of in adenovirus infections. We suggest that antigen detection is the primary diagnostic method, but that EIA IgG serology should be performed in selected cases if antigen detection is negative and the continued search for an etiologic agent is justified.

Further improvements in antigen detection will be the addition of calicivirus, astrovirus, and coronavirus antigen detections in gastroenteritis virus assays; the addition of a cytomegalovirus assay in antigen detection in genital swabs; increased sensitivity of herpes simplex virus assays (particularly for screening of nonsymptomatic pregnant women); and increased sensitivity of HIV antigen assays.

Latex tests do not compete with the more sensitive assays, but they may find increased use in small hospital laboratories, doctor's offices, and field studies.

Monoclonal antibodies may replace polyclonal immune sera in the near future, but only larger diagnostic centers can afford to produce their own monoclonal antibodies even though they have the expertise to build the immunoassays or immunofluorescence tests if reagents are available. For this reason, the commercial availability of antigen detection kits based on monoclonal antibodies as well as monoclonal reagents is highly desirable.

Laboratories that produce their own monoclonal antibodies should realize that for each specific use, monoclonal antibodies must be produced and screened separately. Reagents that have been produced for immunofluorescence usually are not suitable for solid-phase immunoassays. The screening assays must be designed to be as close as possible to the final use of the monoclonal antibodies. A common mistake is that monoclonal antibodies that will be used in solid-phase antigen assays are screened against viral antigen bound on solid phase. In the final assays, the viral antigen in the specimen is never bound directly on solid phase; there is always a catching antibody first on solid phase. Once the right type of monoclonal antibodies are available, viral antigen detection will be greatly improved independently of the technology used.

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