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# RAPID METHODS FOR THE MOLECULAR DIAGNOSIS OF INFECTIOUS DISEASES: CURRENT TRENDS AND APPLICATIONS

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Methods for the rapid diagnosis of infectious diseases have become increasingly common in the last decade. The impetus for the development of such techniques has stemmed from the need to provide clinically relevant information without the length of time and complexity inherent to traditional cultivation methods. This is particularly important in high-risk populations for which more effective antibiotics and antiviral compounds are now available.

Assay systems for the immunological detection of microbial agents occupy a central role in the molecular diagnosis of infectious diseases and are rapidly appearing on the market. Novel methods of organism detection by nucleic acid hybridization, long considered usable only in highly specialized laboratories, have the potential for use in routine microbiological laboratories and some systems are now commercially available.

As more efficient and rapid diagnostics systems are being developed, the selection of the optimal method will depend on the environment in which the system is to be used. Rapid methods for the detection of infectious agents might markedly improve health care in a variety of clinical, laboratory and epidemiologic situations.

## INTRODUCTION

Sensitive, specific and rapid diagnostic techniques for the detection of microbial agents in body fluids are important for the prevention, control and management of infectious diseases in clinical medicine as well as for the large scale study of epidemiology of infectious diseases.

Traditionally the diagnosis of infectious diseases has been accomplished by direct microscopic examination of clinical specimens or by the isolation of the infecting organism in culture. However direct microscopy suffers from limitations of low sensitivity and lack of specificity.

Cultivation is sensitive, specific, and capable of detecting agents whose presence is not suspected. Although it is considered the standard to which other methods should be compared, cultivation is often insufficiently rapid to provide information of practical use in clinical management or in the identification of outbreaks in close settings. The diagnosis by culture can also be limited by practical constraints in the case of viral agents for which the availability of numerous cell lines and sterile areas is required. Such requirements have so far limited the identification of viral agents to central laboratories. Furthermore cultivation of certain fastidious viruses such as rota-

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virus, Norwalk virus, hepatitis A and B virus and Epstein-Barr virus is problematic in generally available cell lines while important bacterial pathogens such as *Treponema pallidum* and Mycobacteria cannot be cultivated in artificial media. In the common clinical situation where antibiotics have been administered, cultivation may be inadequate in identifying a pathogen in antibiotic containing body fluids.

Finally, virulent, toxigenic strains such as *E. coli* and *Staphylococcus aureus* cannot always be distinguished in culture from avirulent strains.

In recent years a number of newly recognized viral, bacterial, fungal and parasitic agents have been associated with a wide range of human disease. In order to be clinically relevant such associations have to be tested in several different populations. It is important to ascertain the geographic and environmental range of potential pathogens and to identify populations at high risk for infections. The limitations of standard cultivation techniques have led to the development of more practical and more rapid methods for the diagnosis of human infections. In addition, in case of viral infections, the recent development of many antiviral compounds has provided the incentive for a more timely diagnosis of such infections in high-risk individuals like cancer chemotherapy and organ transplant recipients.

Advantages of rapid diagnosis in such settings include:

1. Aggressive pharmacologic management of potentially lethal infections.

2. Establishment of measures of isolation and patient cohorting, which are essential for the containment of communicable diseases.

3. Reduction of unnecessary use of antibiotics.

Most of the assays applied to the rapid detection of infectious organisms are based on the fact that such organisms express specific antigens and that antigen-antibody reactions can be completed and measured in a relatively short period of time. In addition, over the last few years other alternative techniques have been developed which do not rely on immunological interactions to detect microbial agents.

Nucleic acid hybridization, for example, has many features which make it a potentially efficient and practical system for the detection of microorganisms in a wide variety of health care settings.

## SELECTION OF DIAGNOSTIC SYSTEMS

Although assay systems have been developed for the detection of a large number of infectious agents relevant in human pathology, most are utilized as research tools and relatively few of them have become commercially available. This reflects the fact that high level of test repro-

ducibility is necessary before such assays can be applied in diverse situation and compare favorably with more cumbersome but highly reproducible standard microbiologic techniques. The efficiency of assay systems for microbiological diagnosis must also be clearly established when they are to be used extensively for screening purposes in high-density urban areas or under field conditions.

It is apparent that the degree of efficiency which is required from an assay system i.e. sensitivity, specificity, predictive value as well as speed of performance and practically varies widely upon clinical, laboratory and epidemiologic environments. Thus, as more rapid diagnostic systems are being developed and marketed, the choice of the appropriate method to achieve an optimal yield in the diagnosis of infectious diseases will depend on the individual characteristics of such environments.

## IMMUNOASSAY SYSTEMS

Assay systems which make use of the specific interactions of labeled antibodies with microbial antigens have followed the successful application of radioimmunoassay, fluorescent immunoassay, enzyme immunoassay for the detection of drugs and hormones in body fluids (10, 32, 39, 45).

Many of the principles involved in the detection of microbial organisms are essentially the same as for the detection of these compounds and involve the binding of an antigen molecule to an antibody molecule and the detection of this reaction through another labelled ligand. In most applications a solid phase surface, generally of plastic material, is used to separate bound from unbound immune reagents. However, there are differences in the molecular composition and steric presentation of the antigen which may account for lower degrees of efficiency at times present in the detection of infectious agents. The relative large size (often greater than 200,000 MW) and polymeric nature of microbial antigens as well as the variable form in which the antigen is present in body fluids make it difficult to apply the same separation and detection techniques as for smaller and chemically defined molecules. Fortunately, antigen-antibody reactions generally occur under a wide range of condition of temperature, ionic strength and pH, thus making it possible to detect microbial antigens in media as different as blood, cerebrospinal fluid, urine, gastrointestinal and respiratory secretions.

To increase the efficiency of antigen detection in such media it is at times necessary to use antibodies which react with a large number of different antigenic determinants to insure reaction with the form of the antigen which is expressed during the course of the infection. This is particularly problematic when monoclonal antibodies

are used to recognize specific, individual epitopes on the infectious antigen.

The kinetics of the antigen-antibody reaction in the non-competitive solid phase assays commonly used to detect microbial antigens are described by the law of mass action (7, 32):

(Ag Ab) / (Ag) (Ab) = Ka

in which (Ag) = concentration of the antigen,

(Ab) = concentration of the antibody,

(AgAb) = concentration of antigen-antibody complex, Ka = affinity constant of the antibody. As the unknown antigen can only be measured when complexed with the specific antibody, the sensitivity of an immunoassay largely depends on two factors: 1) concentration of the antibody; and 2) avidity of the antibody (sum of the affinities of the different monospecific antibodies forming a polispecific antiserum). The total antibody concentration available for the detection of the antigen is a function of the concentration of the immunoglobulin in the antiserum and the proportion of immunoglobulin specifically directed at the antigen to be measured; this is, for example, markedly lower in post-infectious compared to hyperimmune sera.

The second most important limiting factor of immunoassay for the detection of complex antigens in the specificity of the antibody which is used.

Antigens prepared from tissue culture or animal sources often contain antigenic determinants of host origin that resist purification attempts by physiochemical techniques. Immunization of animals with such antigens can lead to the production of antibodies directed against host components (37). Non-specific reaction can also occur through the F (c) part of the antibody molecule, when it reacts with rheumatoid factor-like substances present in serum or other body fluids (16).

Most development work in immunoassays for detection of infectious microorganisms has focused on formats which permit an increase of the signal without a concomitant increase of the background activity (7, 15, 44). This can be achieved in two ways: 1) improvement of the kinetics of antigen-antibody interactions, for example, by devising liquid-phase rather than solid-phase formats; and 2) utilization of highly purified immunoreagents. The achievement of higher sensitivity levels is critical to be able to rapidly detect microbial antigens at different stages of infections. Comparative studies (22) utilizing different commercially available immunoassay systems for the direct detection of rotavirus in stools have shown clearcut differences in the ability to detect rotavirus antigens in diarrheal stool after the first 2-3 days of overt disease.

Analogous assay formats can thus display different degrees of sensitivity when low amounts of antigen are present due to the rapid decrease of viral replication and the complexing of antigen with endogenous antibody in the form of immunocomplexes.

Furthermore, the direct detection of antigenic molecules in body fluids by specific antibodies can be problematic in the case of intracellular antigens; these are difficult to extract from sites inaccessible to labeled antibodies without altering their antigenicity (27). Thus, more sensitive assays need to be developed which can detect infectious antigen molecules at the sub-nanogram level and in microenvironments that are unfavorable to antigen-antibody reactions,

## ROLE OF ANTIBODIES IN IMMUNOASSAYS. MONOCLONAL ANTIBODIES

While the development of high quality immunoreagents such as affinity purified antibodies for a number of infections agents have provided the basis for a more widespread usage of rapid assay systems, advances in monoclonal antibody production has allowed for the development of highly specific, reproducible assay systems (13). Advantages of monoclonal antibodies for the detection of defined antigens include unlimited supply of a reagent which can unequivocally distinguish infectious from host antigens (17); in addition, the fact that each molecule of monoclonal antibody is directed at the target antigen can result in an effective increase of the active antibody concentration available for interaction with the antigen. There are however potential problems with the use of monoclonal antibodies in immunoassay systems.

One such problem is the lower affinity often exhibited by monoclonal antibodies (9, 48) which may offset the advantages derived from increased antibody concentration. The kinetics of the antigen-antibody reaction requires that antibody reacts at different sites of large proteins antigen to produce the high affinity binding necessary in immunoassay.

In addition some monoclonal antibodies that are directed at a very narrow range of antigenic determinants might not recognize a large percentage of natural isolates of a microorganism and might lead to false negatives reactions. This can occur, for example, with agents such as Enteroviruses which display great antigenic variability. Screening of relatively broadly reactive clones is thus necessary to enhance the range of detectable antigenic determinants.

## ENZYME IMMUNOASSAYS

Enzyme immunoassays (EIA or ELISA) which utilize enzyme-linked immunoreactants bound to a solid phase by a series of antigen-antibody have gained a widespread usage as practical systems for the detection and quantitation of infectious antigens (49). This can be attributed to the high sensitivity of EIA systems due to the fact that a single molecule of enzyme catalyzes the conversion of a large number of molecules of substrate. Thanks to such extensive magnification of the antigen-antibody reaction, the results can be interpreted visually or objective measurements of the colored reaction products can be obtained by simple colorimetric instrumentation.

Since microtiter plate colorimeters that can measure a large number of reactions in a short period of time are available, EIAs can be adapted to large scale testing of infectious agents in large hospitals and to epidemiologic surveys. In addition, central laboratories are often equipped to measure high energy substrates such as fluorescent and chemoluminescent compounds. Such substrates can be detected in much smaller quantities than chromogenic substrates (39, 45, 50), ultimately allowing for a faster detection of clinically relevant pathogens. On the other hand, microtiter plates in which antibody to different infectious agents have been previously bound can be stored at 4°C for periods up to several months and can then be utilized even in small laboratories
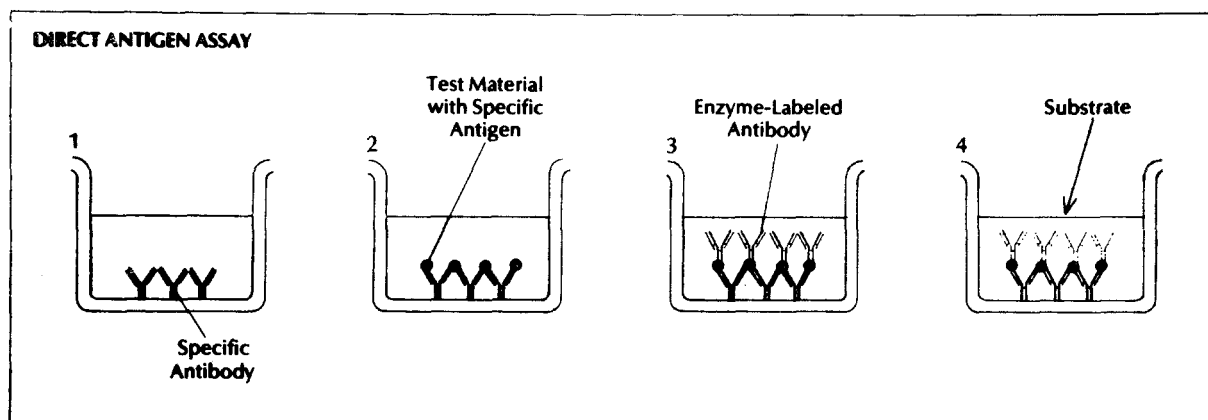


Direct (or "sandwich") ELISA for antigen detection begins with binding of specific antibody to microtitration plate well (1). When test specimen is added, antigen to which the antibody is directed will adhere to the antibody (2). Next, enzyme-labeled specific antibody is added, completing the sandwich (3). In the final step, addition of enzyme's substrate results in a color change (read by eye or measured by machine) proportional to the amount of antigen in test specimen (4).
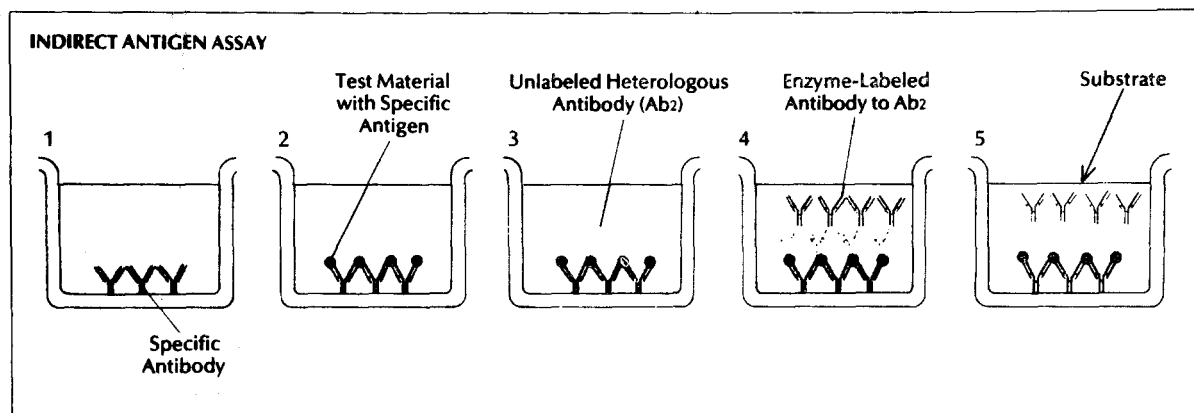
Unlike direct ELISA, indirect ELISA for antigen detection uses enzyme-labeled antiglobulin and unlabeled specific antibody from two different animal sources. Thus, after antibody from one animal species is bound to well (1) and the test specimen is added (2), unlabeled antibody from another animal species is introduced (3). Addition of enzyme-labeled antibody directed at second animal species' globulin (4) sets the stage for a color change when enzyme's substrate is introduced (5).

Figure 1. - Enzyme immunoassay: Direct and indirect antigen assay.

to investigate, for example, outbreaks of gastro-intestinal or upper respiratory disease in settings such as day care centers and nursing homes.

There are a number of ways in which EIA systems can be formulated. Figure 1 depicts the two most common forms of EIA for antigen detection. It should be noted that even in their simplest formulation EIA require about 4 hours to complete and such a time period may be too long for the effective management of serious bacterial or viral infections of the childhood such as Haemophilus influenzae meningitis or Herpes simplex encephalitis. This is due to the fact that most of the currently available immunoassays systems require fairly lengthy incubation and washing steps. Recently assays have been developed which attempt to eliminate such steps, thus simplifying the test procedure and reducing the performance time. Thus EIA formats have been devised in which only one incubation step is necessary. The double determinant assay (51) is particularly suitable for use with monoclonal antibodies because clones can be selected that are directed at different determinants of a macromolecular antigen. EIA utilizing this format for the detection of rotavirus in stool can be completed in about 40 minutes.

## PARTICLE AGGLUTINATION ASSAYS

In peripheral environments, in which methods of rapid diagnosis can be most advantageously used, even the simplest instrumentation may be lacking. Although objective quantitation of antigen content in clinical specimens is highly desirable, in that it reduces the erroneous interpreation of borderline results, qualitative assay systems can be valuable in such environments.

Particle agglutination is one such system which makes use of antibody immobilized onto visible particles such as latex beads. Latex agglutination assays have been applied to the detection of various pathogens (2, 18, 19, 21, 38), thanks to simple reaction protocols, rapidity and favorable diffusion kinetics. However agglutination assay have not obtained widespread usage as they have exhibited a lower degree of sensitivity than analogous enzyme immunoassay systems (22, 30). In addition latex agglutination systems, unlike EIA, are difficult to apply to large numbers of samples.

A centrifugation-augmented-solid-phase-immunoassay (CASPIA) has recently been developed which combines the advantages of enzyme immunoassays and particle agglutination assays, but avoids their individual disadvantages (23).

The CASPIA assay is based on the binding of antigen to a solid phase surface like in traditional isotopic, enzymatic and fluorescent immu-

noassay systems (Fig. 2). However, antibody-coated latex microspheres also bind to the antigen captured on the microplate solid-phase. As the binding of a relatively small antibody molecule to solid-phase antigen results in the attachment of relatively large, opaque latex microspheres, this systems offers the potential for the magnification of the initial solid phase antigen-antibody reaction without the need for enzyme-substrate reactions. Thus visual determinations are as accurate as instrument measurements in distinguishing positive from negative specimens.

The CASPIA assay has been applied to the measurement of antigens of two bacterial pathogens that frequently cause serious disease in children, H. influenzae and group A beta hemolytic streptococcus, and of the commonest cause of infantile diarrhea, rotavirus (23). By using commercial latex reagents CASPIA was able to achieve sensitivity levels that were significantly greater than those of traditional slide agglutination assays and at least equivalent to those of ELISA systems.

The CASPIA system makes use of improved diffusion kinetics of the immunoreagents to constitute an example of an efficient, low cost method that might be an alternative to traditional microbiological techniques in a wide range of clinical, laboratory and epidemiological circumstances.

## NUCLEIC ACID HYBRIDIZATION TECHNIQUES

The ability to rapidly identify microorganisms without the need for them to replicate has made immunoassays a very attractive system in diagnostic microbiology. The use of immunoassays in diverse clinical situations has become particularly widespread for the diagnosis of common infections such as respiratory infections caused by group A beta hemolytic Streptococcus (6, 11, 12, 21).

However, attempts have been made to overcome the inherent limitations of immunoassays i.e. the kinetics of the antigen-antibody reaction by non-immunological techniques which rely on the detection of a microorganism's genome rather than its protein end products.

These techniques are based on nucleic acid hybridization i.e. on the fact that a nucleic acid single strand lines up precisely with another single strand containing a complementary base sequence, adenine opposite thymine, guanine opposite cytosine. Thus nucleic acid nucleotide sequences in a target organism hybridize complementary sequences of a specific nucleic acid probe.

As this probe is bound to labels analogous to those utilized in immunoassays, objective measurements of the detected sample nucleic acid can be obtained. Figure 3 depicts schematically the different steps involved in DNA hybridization assays.
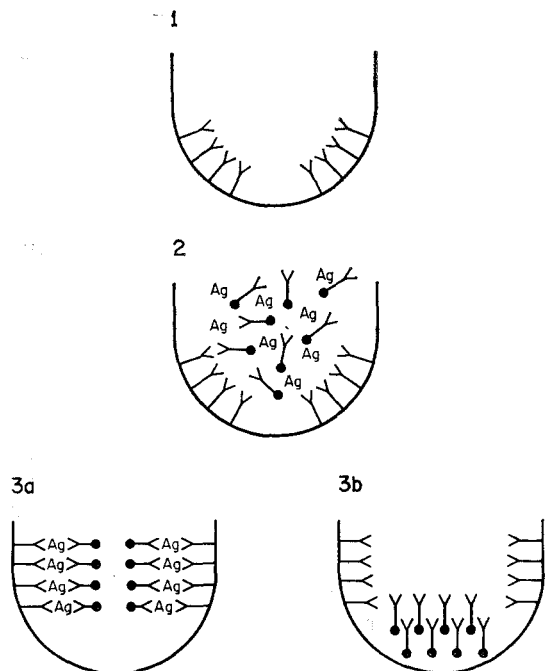
Figure 2. – Principle of CASPIA. (1) The wells of micro-titer plates are coated with antibody directed at the antigen to be measured. (2) Particulate antibody to the antigen and the sample to be tested are added. If there is antigen (Ag) in the sample, it will bind to the solid-phase antibody and to the particulate antibody causing the particles to adhere to the solid phase. (3) The microplate is centrifuged at ~ 1,500g for 15 min. The presence of antigen in the solid phase is manifested by particles remaining on the solid phase (3a). Specimens not containing antigen will result in the particles migrating to the bottom of the wells (3b). The presence of the particles on the side of the wells can be seen macroscopically, recorded by a photocopying device, or measured by a microplate color-imeter. (From reference #23).

These steps involve the chemical extraction of the nucleic acid from the clinical sample and its denaturation by physical or chemical methods to make it single stranded. The extracted single stranded nucleic acid is then fixed, by heating, to a membrane (most commonly nitrocellulose) so as to avoid self-annealing and incubated with labeled single stranded nucleic acid probe. After hybridization the filter is washed to remove unbound probe and, if a radiolabeled probe has been used, exposed to an X-ray film. This format has become the most universally utilized and is known as mixed-phase hybridization as it employs a liquid phase probe to bind a target nucleic acid immobilized on a solid phase (the nitrocellulose filter).

Approximately similar relationships apply to RNA-DNA hybrids and RNA-RNA hybrids. As such reactions have very efficient kinetics in the presence of a large number of hydrogen bonds between single strands of target and probe nucleic acid, there is a potential for rapid detection of very small quantities of microbial nucleic acid (1).
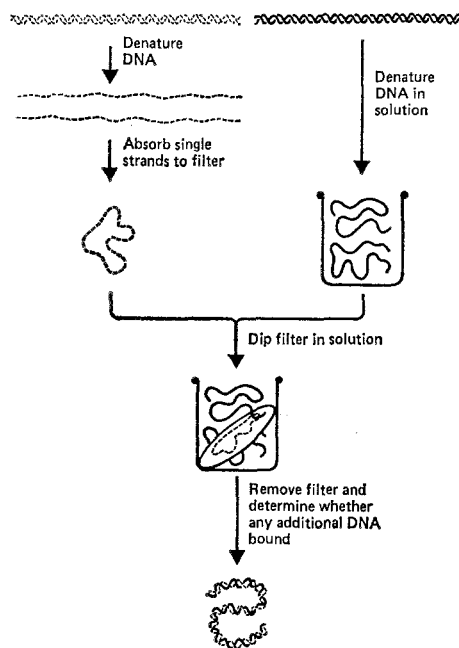


Figure 3. – DNA hybridization assay. Reaction between microbial DNA extracted from a clinical sample (LEFT) and labeled DNA probe (RIGHT).

Furthermore, unlike in immunoassays, the same preparation procedures can be used for clinical samples of different nature, as microorganisms' nucleic acids are chemically alike. Table 1 describes applications and main advantages offered by hybridization techniques in diagnostic microbiology. Several reviews cover in detail principles and practices of nucleic acid hybridization (1, 8, 24, 41).

TABLE 1.
Properties of nucleic acid probes
in diagnostic microbiology.

1. High sensitivity due to efficient kinetics of hybridization reactions.

2. High specificity due to detection of highly conserved genetic sequences in antigenically different strains of the microbial agent.

3. Simultaneous detection of nucleic acid in large number of clinical specimens in epidemiologic studies.

4. Ability to detect sequences coding for virulence factors (e.g. E. coli toxins).

5. Ability to detect microorganisms in latent phase, complexed with antibody, intracellularly sequestered or difficult to cultivate.

6. Ease of production of probes of predetermined specificity.

The final efficiency of this tecnique when applied to the detection of microbial nucleic acids in clinical samples depends largely on the conditions under which hybridization reactions occur. Hybridization of complementary strands, a concentration-dependent reaction, is especially influenced by factors such as length of nucleotide sequence, ionic strength, temperature and viscosity. The performance time of hybridization reactions is also affected by the practical constraints of the amount of radiolabeled probe which can be used.

Furthermore, the hybridization principles can be applied with variable degrees of efficiency in different assay formats. In dot hybridization, which is the most frequently used technique, nucleic acids are extracted from the specimen and applied directly to nitrocellulose membranes. This minimizes any preparation steps and allows for the processing of large number of specimens using multichannel microfiltration units which can apply several samples on membranes. In addition, the labeled probe can be removed and the membranes containing the original sample nucleic acid can be rehybridized with probes of different specificity.

Clinically relevant applications of dot hybridization assays include the identification of E. coli in stools, of Neisseria gonorrhoeae in urethral swabs, of Varicella-zoster in skin vescicles, of Cytomegalovirus in urine, of Epstein-Barr virus in throat washings and of Human Immunodeficiency Virus in cultures of peripheral blood cells (3, 15, 25, 26, 31, 36).

In Southern hybridization analysis the sample nucleic acid is cut by specific restriction endonucleases, electrophoresed in gel to separate DNA pieces of different molecular weight and then transferred to nitrocellulose (40). Although this technique does not lend itself to immediate clinical usage, it has been applied to the epidemiologic study of the relationship between infections and tumor development. For example integration of hepatitis B virus DNA into host DNA has been demonstrated in individuals with hepatocellular carcinoma (35). A relationship has been established between chronic Epstein-Barr infection and development of nasopharyngeal carcinoma (46).

The sandwich hybridization assay (47) is another format which makes use of two nucleic acid fragments, one immobilized onto nitrocellulose and the other added as a soluble labelled probe. Both fragments are complementary to the sequence to be identified.

The sandwich format offers the potential of a higher sensitivity due to higher concentration of nucleic acid reagents; furthermore it can display higher specificity because the test sample is kept in solution, unlike most hybridization methods in which the sample DNA is fixed to a solid support along with other interferenig substances present in the sample. This is particularly important for the detection of nucleic acids in crude clinical samples such as nasopharyngeal aspirates and urine (33). Overnight tests based on sandwich hybridization have been constructed for detection of Adenovirus, Chlamydia trachomatis, Cytomegalovirus (29, 34, 43).

Despite the potential for widespread application, nucleic acid hybridization techniques have suffered the major drawback of utilizing radioisotopes-labeled ($^{32}$P or $^{35}$S) probes. These require costly instrumentation and pose a biological hazard. Thus, recently, efforts have been made to overcome such problems by developing nucleic acid hybridization techniques which use enzymatic labels in a fashion analogous to immunoassay formats. Methods have been devised which utilize the co-factor biotin as a hapten molecule for the labeling of nucleic acid probes. These methods have proved to be very sensitive in that they exploit the very high affinity of biotin for avidin (a glycoprotein found in egg white) (14). Thus an enzyme coupled with avidin can ultimately detect very small amounts of nucleic acid in the clinical sample (20).

Recently there have been attempts to use such methods with microtiter plates, which are widely available in clinical laboratories. Thus sandwich hybridization assays have been developed in which probe and sample nucleic acid react in solution in a microtiter plate well (42). As the hybridization reactions have a faster rate in liquid phase than in mixed phase on nitrocellulose, the assay performance time can be as short as 3 hours.

Further improvements in labeling techniques and assay formats are necessary to make nucleic acid hybridization techniques applicable to wider clinical settings. As progress is made in the fine tuning of nucleic acid probes, it is likely that such probes will prove to be a versatile tool for the identification of pathogenic microorganisms.

## PERSPECTIVES

Great strides have been made in only a few years in the development of more rapid methods for the diagnosis of infectious diseases. Although it is likely that microscopy and culture will remain a valid standard for the routine observation and identification of microorganisms, new techniques will be increasingly used side by side to detect infectious organisms. They will permit to make important clinical and public health decisions in much shorter time spans, thus decreasing the cost-benefit ratio of their usage.

Immunoassay and nucleic acid hybridization systems, while not widely employed at the present

time, have most of the desirable features of accuracy, rapidity and practicality. Further developments in these assays' efficiency, speed and reagents' long term stability will allow for a marked improvement in the care of patients with infectious diseases. In addition such techniques will allow to collect essential epidemiological data in a number of yet unknown health care situations.

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