# MEDICAL VIROLOGY

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Editors

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## FOREWORD

Over the last few decades Medical Virology has rapidly evolved from a topic reserved to a few scientists to a discipline of general medical interest. This interest is well deserved since viruses as a group are the most common etiological agents involved in human diseases. Successful efforts in controlling viral diseases have resulted, among other things, in the eradication of smallpox and the development of vaccines effective against several viral infections including poliomyelitis, rubella and mumps. However, the challenges still lying ahead are more numerous than our past accomplishments. The purpose of this annual Symposium is to provide a forum where research and development efforts in the diagnostic, clinical and therapeutic areas of Medical Virology are presented and discussed. We hope this dialogue will help to accelerate our understanding of viral infections thus allowing us to control and eradicate this group of diseases.

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# ADVANCES IN THE LABORATORY DIAGNOSIS OF VIRAL INFECTIONS

NATHALIE J. SCHMIDT

## INDICATIONS FOR LABORATORY DIAGNOSIS OF VIRAL INFECTIONS

The laboratory diagnosis of viral infections is assuming increasing importance in clinical medicine. With the development of methods for direct detection of viruses in clinical materials, and for more rapid identification of viral isolates, laboratory assistance in viral diagnosis is frequently sought to aid in patient management.

The prospect for effective antiviral agents has been a strong stimulus for the development of viral diagnostic capabilities, and the marked specificity of some of the antiviral agents, for example, the narrow activity of acyclovir against only some of the human herpesviruses, has emphasized the importance of specific, as well as rapid, identification of the infecting virus.

The diagnosis of viral infections occurring in pregnancy plays an important role in patient care. Examples include the diagnosis of rubella infections in early pregnancy or the determination of rubella immunity status in pregnant women exposed to rubella, the specific diagnosis of genital herpes simplex virus (HSV) infections in pregnant women near to the time of delivery, and the diagnosis of acute or chronic hepatitis B infection in pregnancy.

Management of immunocompromised individuals exposed to herpesvirus infections, and control of cytomegalovirus (CMV) infection in organ transplant recipients or transfused newborns are other examples where rapid and specific viral diagnosis or determination of antibody status are important. Rapid viral diagnosis can also aid in controlling the spread of hospital-acquired infections, such as those caused by HSV, respiratory syncytial virus (RSV), and rotaviruses.

Specific identification of a viral infection can prevent indiscriminate, and potentially harmful, use of antibiotics. Also, specific viral diagnosis may be of prognostic value, as in distinguishing between meningoencephalitis due to HSV and that due to an echovirus.

Identification of sexually transmitted viral infections, such as HSV, CMV and hepatitis B, may aid in controlling the spread of these infections, particularly for agents such as hepatitis B for which effective vaccines are becoming available.

From a public health standpoint, viral diagnosis is essential for the surveillance and control of epidemic diseases such as influenza, poliomyelitis, viral hepatitis, certain arbovirus infections, and highly dangerous diseases such as Lassa fever. Rapid diagnosis in index cases can initiate immunization programs or suitable containment measures.

In the long run, specific viral diagnosis educates physicians to associate particular viruses with certain clinical syndromes. Diagnostic virology has contributed much to clarify the etiology of various central nervous system, respiratory, ocular, gastrointestinal, hepatic and perinatal diseases, as well as certain syndromes occurring in immunosuppressed individuals.

## APPROACHES TO THE LABORATORY DIAGNOSIS OF VIRAL INFECTIONS

The three basic conventional approaches to the laboratory diagnosis of viral infections are (1) direct examination of clinical materials for virus or viral antigen, (2) isolation of virus in laboratory host systems and subsequent identification by immunological methods, and (3) serological diagnosis based upon demonstrating a significant increase in viral antibody over the course of the patient's illness. Methods included under the first category of direct examination permit rapid diagnosis without the need for virus propagation, and this is the area in which there has been the most interest in recent years. In fact, direct methods now permit detection and diagnosis of viral infections caused by agents which can not be propagated in standard laboratory host systems, e.g., the agents of hepatitis A and hepatitis B, rotaviruses, and agents of acute infectious non-bacterial gastroenteritis.

## DIRECT EXAMINATION OF SPECIMENS FOR VIRUS

Although direct demonstration of virus in clinical specimens is the most rapid and economical approach to viral diagnosis, it is limited by the small size of viruses, their close association with host materials, and the relatively low concentration of virus at the sites of replication which are accessible for specimen collection.

### Electron Microscopy

For successful detection of viruses by electron microscopy (EM), the agent must be present at concentrations of around $10^6$ or more particles per ml, and the virus must have a distinctive morphology which is readily distinguishable from debris. EM with negative staining is most applicable to detection of herpesviruses and poxviruses in vesicular lesion material, detection of rotaviruses

and adenoviruses in fecal specimens, demonstration of CMV in urine of infected infants under 6 months of age, and detection of hepatitis B antigens in serum. EM can identify major groups of viruses on the basis of their morphology, but can not differentiate between members of the same morphologic group or virus family, for example, between HSV and varicella viruses (Almeida, 1980; Doane, 1980).

Immunological Methods

Direct identification methods for which the specimen is treated with specific viral antibody permit detection and identification of the viral agent in a single step. Sensitivity is increased over that of direct EM because the virus content of the specimen is amplified by the specific antibody, either by aggregation or through a "tag" or label on the antibody. This approach requires sharply specific and high-titered antiserum, and one must have some idea of the agent involved, since it is feasible to test a specimen against only a few appropriate antisera. Also, the method is not generally applicable to viruses in the large groups which contain many distinct serotypes and have no major common antigens, for example, enteroviruses and rhinoviruses.

1. Immunological methods using unlabeled antibodies

a. Immune electron microscopy. For immune electron microscopy (IEM), the specimen is mixed and incubated with viral antibody, and with a known negative serum for a control, negatively stained with phosphotungstic acid, and then observed by EM for the presence of virus-antibody complexes in the portion of the specimen treated with viral antibody. The antibody clumps the virus, concentrates it, and thereby aids in its detection, as well as identification. This approach permitted the initial detection of hepatitis A virus and the infectious non-bacterial gastroenteritis agents, despite the fact that they could not be cultivated.

b. Counterimmunoelectrophoresis. Examination of clinical specimens for viral antigens which react with homologous antisera to produce immunoprecipitates in gels is of limited value because of the high concentration of antigen required to produce visible precipitates. However, counterimmunoelectrophoresis methods have been found to be applicable to direct detection of rotaviruses (Grauballe et al. 1977) and adenoviruses (Mankikar et al. 1979) in stool specimens, and this provides a rapid and inexpensive method for detecting a relatively high proportion of virus-positive specimens.

2. Immunoassays using tagged viral antibodies

Viral antibodies used for direct examination may be tagged with a fluorochrome, a radioisotope, or an enzyme to enhance their ability to detect virus.

a. Immunofluorescence staining. Immunofluorescence (IF) staining is based upon tagging antibody with fluorescein isothiocyanate and demonstrating antibody complexed to virus in the specimen by microscopic examination with ultraviolet illumination. Either the direct, indirect, or anti-complement IF procedure may be used. For direct IF, the viral antibody is tagged with fluorescein and the conjugate is applied to material to be examined for the presence of viral antigen. This method requires a specific antibody conjugate for each viral agent being sought, and a high-titered antiserum for conjugation. However, it has the advantage of greater specificity and freedom from background staining, and requires fewer manipulations and reagents (Emmons and Riggs, 1977).

For indirect IF staining, the specimen is treated with unlabeled viral antiserum, and the reaction of antibodies with virus in the specimen is detected with fluorescein-labeled immune globulins directed against the species of antiserum used in the initial reaction. The indirect method is generally more sensitive than the direct procedure, since the intermediate antiserum increases the surface area available for attachment of the labeled immunoglobulins, and it requires labeled antibodies only against certain animal species, and not against individual viruses. However, indirect staining may be less specific than direct staining, since additional biological reagents are introduced into the reaction system. In addition, indirect staining requires more controls and manipulations.

The anti-complement immunofluorescence (ACIF) method detects virus-antibody complexes through the addition of complement, followed by fluorescein-labeled antibodies to the C3 component. It has high sensitivity for detection of certain herpesvirus antibodies, such as those to the nuclear antigen of Epstein-Barr virus (EBV) and to the early antigens of human CMV. Also, since complement attaches only to virus-antibody complexes, the ACIF procedure avoids the staining of Fc receptors produced by herpesvirus-infected cells, which bind human IgG of all specificities, and may give confusing cytoplasmic staining in indirect IF tests.

IF technics are only beginning to be used to their full potential in the rapid diagnosis of viral infections, largely because of a shortage of satisfactory reagents, standardized procedures, and adequately trained personnel. A few virus laboratories in this country have had long experience in

the routine use of IF technics (Emmons and Riggs, 1977), and the feasibility and reliability of IF staining for rapid viral diagnosis in clinical settings has been well established by Gardner and McQuillin (1980) and other members of the European Group for Rapid Laboratory Viral Diagnosis. These latter workers have collaborated to define, prepare and evaluate viral antisera for use in IF staining, and have stimulated European reference centers and commercial companies to prepare high-quality reagents for viral diagnosis.

Direct demonstration of viral antigens in clinical materials by IF staining can be applied successfully only to specimens which contain large numbers of virus-infected cells that are free from debris or contaminating microorganisms that might give nonspecific fluorescence. Also, adequate sampling (Emmons and Riggs, 1977; Gardner and McQuillin, 1980) of the tissue or cellular exudate is important because of the uneven distribution of viral antigen.

IF staining has been used for demonstration of a variety of respiratory viruses; measles virus and rubella virus in cellular material from nasopharyngeal exudates; for detection of herpesviruses and poxviruses in cells from vesicular lesions, and for demonstration of viral antigens such as those of rabies, HSV, varicella-zoster virus (VZV) and measles viruses directly in tissue specimens.

One noteworthy advantage of IF staining, as compared to virus isolation, is the ability to demonstrate viral antigen, e.g., RSV (Gardner et al. 1970), measles virus (Fulton and Middleton, 1975), and VZV (Drew and Mintz, 1980; Schmidt et al. 1980) in specimens taken late in the course of infection when infectious virus is no longer present, either as a result of complexing with antibody or lability of infectivity. The marked improvement of IF staining over virus isolation for detection of VZV has been demonstrated in studies from our laboratory (Schmidt et al. 1980) and the laboratory of Dr. Lawrence Drew (Drew and Mintz, 1980). All of the specimens in which VZV was detectable were positive by IF, but fewer than 50% of these were positive by virus culture. These results probably reflect both the lability and the strong cell-association of VZV, which make isolation difficult.

Although newer immunoassays for detection of viral antigen which are based upon colorimetric or fluorometric readings may possess certain advantages over IF staining, the IF procedure has the built-in safeguard of showing typical patterns of staining morphology in virus-infected cells, and it is only by seeing stained antigens in intracellular sites that a positive reading can be made.

The demonstration that trypsin treatment can be used to render formalin-fixed, paraffin-embedded tissue sections suitable for IF staining (Rowse-Eagle

et al. 1981; Swoveland and Johnson, 1979) has been an important finding which permits the use of IF staining in situations where fresh or frozen tissue is not available, and also gives better preservation of morphological detail for correlation of antigen distribution with histopathology.

b. Enzyme immunoassays. Enzyme immunoassays (EIA) employ antibodies labeled with an enzyme rather than fluorescein. The labeled antibodies bound to virus, or virus-antibody complexes, are detected by the addition of a substrate upon which the enzyme acts to produce a colored, fluorogenic or luminescent product. Theoretically EIA is more sensitive than IF staining, since the enzyme label has a continuous action on the substrate, producing increasing amounts of reaction product and thus amplifying the initial reaction at the site where virus is present in the specimen.

i) Immunoperoxidase staining. The immunoperoxidase (IP) technic is one type of EIA used for virus detection. This uses horseradish peroxidase as the enzyme label and a substrate which produces an insoluble reaction product. The reaction product at the site of the virus-antibody reaction is detected microscopically, using an ordinary light microscope. The technic has been applied to rapid viral diagnosis in the same manner as IF staining, using either direct or, more commonly, indirect procedures. IP staining is considered to have certain advantages over IF staining. These include the fact that an ordinary light microscope can be used, permanent preparations can be made, and there is the potential for greater sensitivity due to amplification of the reaction by continuous action of the enzyme on the substrate. Undesirable background and nonspecific reactivity can occur in both systems. IF staining is beset with problems of autofluorescence and nonspecific staining, and IP staining is hampered by the fact that certain types of tissues, particularly blood cells, have endogenous peroxidase activity. Various methods have been described for inactivating endogenous peroxidase activity, but some of them may also inactivate viral antigens. The disadvantages of IP staining include the need for a few more manipulations, and the fact that some of the earlier substrates used were mutagenic.

IP staining has been more widely used for rapid identification of viruses isolated in cell cultures than for direct detection of viruses in clinical specimens (Kurstak and Kurstak, 1974). In one comparison of IP with IF methods for detecting respiratory virus antigens in nasopharyngeal secretions, considerable difficulty was encountered with endogenous peroxidase activity in the inflammatory cells in the specimens, and the methods used to eliminate this activity were detrimental to some viral antigens (Gardner et al. 1978). However,

better success has been obtained in applying IP staining to rapid identification of HSV antigens in brain and lesion materials (Benjamin, 1975, 1977; Moseley et al. 1981; Pearson et al. 1979). Also, methods are available for inactivating endogenous peroxidase which are not detrimental to viral antigens (Pearson et al. 1979; Wirahadiredja, 1976), and these should extend the usefulness of IP staining for direct examination of clinical materials.

A recent study by Moseley et al. (1981) showed indirect IP staining to be slightly more sensitive than direct IF for detection of genital HSV infections. Seventy-two percent of 79 patients positive by virus isolation were positive by IP staining, as compared to 65% positive by direct IF. Very similar results were obtained in our laboratory in preliminary studies on a smaller series of patients with genital HSV infections. As in the case of IF staining, positive results must be based upon the demonstration of typical intracellular morphology of the viral antigen.

ii) Enzyme immunoassays employing substrates which give a soluble reaction product. Other EIAs for virus detection employ substrates which give soluble reaction products that are either colored, fluorescent, or luminescent. For most of these systems, viral antibody is coated onto a solid phase, the test material is added, and virus present is bound to the "capture" antibody. After washing, the bound virus is detected through the use of a second viral antiserum, the "detector" antibody, which may be labeled with an enzyme, or unlabeled detector antibody of a species different from that of the capture antibody may be used, followed by labeled antibody directed against the species of the detector antibody.

Fluorogenic and chemiluminescent substrates produce reaction products that can be detected at lower concentrations than can colored reaction products, and test systems employing these substrates have shown greater sensitivity for viral antigen detection than tests using color-producing substrates (Pronovost et al. 1981; Yolken and Stopa, 1979).

Enzyme immunoassays using substrates giving soluble reaction products have been suitable for direct detection of hepatitis viruses (Mathiesen et al. 1978; Wolters et al. 1976), rotaviruses (Yolken and Stopa, 1979; Yolken et al. 1977), and adenoviruses (Johansson et al. 1980) in stool specimens, and have shown some promise for detection of HSV in lesion materials (Pronovost et al. 1981). In the limited extent to which the assays have been applied to direct detection of respiratory viruses, they were only slightly less sensitive than virus isolation (Berg et al. 1980; Chao et al. 1979), and were equally or slightly more sensitive than IF methods for virus detection (Sarkkinen et al. 1981). Success of the